Turun yliopisto
University of Turku

IMPACTS OF WINE YEAST FERMENTATION ON THE CHEMICAL COMPOSITION OF THE BLACKCURRANT

Master thesis in Technology
University of Turku
Department of Biochemistry
Master's Degree Programme in Food Development
December 2018
Laura Aaltonen

UNIVERSITY OF TURKU
Department of Biochemistry

LAURA AALTONEN: Impact of wine yeast fermentation on the chemical compositions of the blackcurrant

Thesis, 50 p.
Food Development
December 2018

– –

Grapes are the most commonly used raw material in the wine making. If wines are made from other fruits than grapes, they are called fruit wines. Blackcurrant is the second most cultivated berry in Finland. The fermentation of wine is usually made with *Saccharomyce*s yeasts, but use of non-*Saccharomyces* yeasts has been an increasing trend in the past few years. The chemical composition thus the overall quality of the wine depends on the yeast used.

The goal of the study was to investigate the effects of *Saccharomyce*s and non-*Saccharomyces* yeasts on the chemical quality of the blackcurrant wine. Five different yeasts were used to ferment blackcurrant juice: two *S. cerevisiae*, one *S. bayanus*, one *Torulaspora delbrueckii* and two *Metschinikowia* species. *Metschinikowia*-yeasts were sequentially inoculated with two *S. cerevisiae* yeasts. The sugars, organic acids and alcohols in the samples were analysed with GC-FID methods and the flavonols and anthocyanins with HPLC-DAD methods.

Differences were found between the yeasts and the most interesting from sequential inoculations. The sequential inoculation consumed fructose and glucose more effectively from wines than pure fermentation. Galacturonic acid was only detected in the sequential. The *Saccharomyces* yeasts did consume total sugars more effectively than other yeasts. Ethanol concentration was lower in the sequential inoculation samples than in samples fermented with one yeast. *S. cerevisiae* and the *S. bayanus* yeasts had the highest concentrations of ethanol. The total flavonols compound quantity increased during the sequential inoculation fermentations. Different species and strains of yeasts have effect on the chemical quality of the blackcurrant.

Keywords: blackcurrant, fruit wine, fermentation, non-*Saccharomyces* yeast, *Saccharomyces cerevisiae*

Contents

Abbreviations

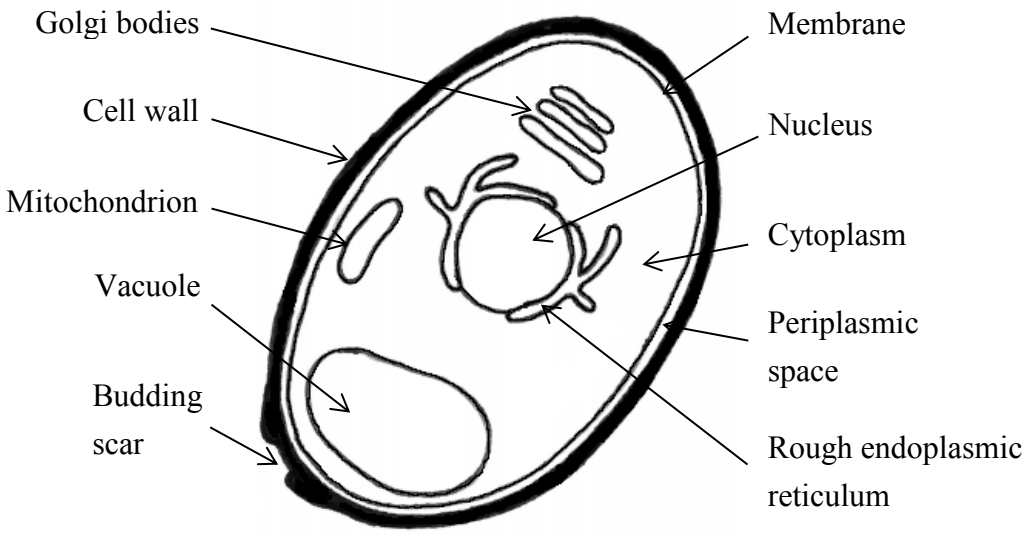
AF	alcoholic fermentation
ADP	adenosine diphosphate
ATP	adenosine triphosphate
FAD	flavin adenine dinucleotide
LAB	lactic acid bacteria
MLF	malolactic fermentation
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide's reduced form
RNA	ribonucleic acid
TCA	tricarboxylic acid

1 Introduction

1.1 Yeasts in winemaking

Earliest evidence of wine making dates back to over 6000 years ago (Jacobson 2006). For thousands of years no added yeasts were used in fermentation and only yeasts attached to the surface of the berries were present. These surface yeasts may contain wide variety of different types of yeasts and strains, so the end products varied largely. To better guarantee the end product, specially cultivated yeasts strains were begun to be used in fermentations. By knowing the fermentation characteristics of the yeast, and thus the end product composition, the variety of different wines grew. (Mateo et al. 2000)

Yeasts are unicellular organisms that belong in the kingdom fungi. Yeasts are one of the simplest eukaryotes. The yeast cell contains cytoplasm with various cell organelles, nucleus containing the chromosomes and as a plant cell both cell wall and cell membrane (Picture 1.). Yeast's cellular membranes play an essential role in the successful alcoholic fermentation and that they also release certain constituents which add to the resulting wine's composition. The yeast cell wall is made of β-glucans and mannoproteins, chitin is only a minute part of its composition. (Ribereau-Gayon et al. 2006a)



Picture 1. Simplified picture of a yeast

The cell wall of *Saccharomyces cerevisiae* has been studied most extensively. The cell wall is made 60 % of glucans which can be divided into fibrous β-1,3 glucan, amorphous β-1,3 glucan and β-1,6 glucan. The fibrous β-1,3 glucan is insoluble in water, acetic acid and alkali; and it is always connected to chitin. The amorphous β-1,3 glucan is insoluble in water but soluble in alkalis, it gives the cell wall its elasticity and acts as an anchor for mannoproteins. The β-1,6 glucan is amorphous and water soluble; it links the different constituents of the cell wall together and is the receptor site for the killer factor. Mannoproteins constitute 25–50 % of cell wall of *S. cerevisiae*. Mannoproteins contain mannose sugar which is linked to the peptide chain by *O*-glycosyl linkages. Chitin is a linear polymer of N-acetylglucosamine and is only found 1–2 % in cell wall; it assures the cell wall integrity. (Ribereau-Gayon et al. 2006a)

Outer layer of cell wall is made of mannoproteins, which are connected to amorphous β-1,3 glucan. The inner layer is made of fibrous β-1,3 glucan and is connected to chitin. The β-1,6 glucan is believed to connect these two layers together. The composition of the cell wall is strongly influenced by nutritive conditions and cell age. The proportion of the glucan increases with respect to the amount of sugar in the culture medium; or in this case the blackcurrant. (Ribereau-Gayon et al. 2006a)

Enzymes are located in the cell wall or periplasmic space. One important enzyme in wine making is invertase (β-fructofuranosidase) which catalyses the hydrolysis of sucrose into glucose and fructose. Invertase is a mannoprotein and it is anchored into β-1,6 glucan of the cell wall. (Ribereau-Gayon et al. 2006a)

The cell membrane controls the exchanges between cell and environment outside it. The membrane is made of lipids and proteins. The lipids are phospholipids and proteins, which have hydrophobic and hydrophilic end. The lipids form a membrane bilayer, where the hydrophobic ends are faced inside. The membrane also contains proteins. Some of the proteins, called integral proteins, are associated in the non-polar region of the membrane bilayer and others are peripheral proteins, which are linked by hydrogen bond to the integral proteins. The membrane also produces cell wall glucan and chitin. (Ribereau-Gayon et al. 2006a)

The sugars and amino acids are actively transported across the membrane by transport protein permeases. The sugars are moved by two different transport systems: high affinity and low affinity system. Low affinity system's activity is essential at the yeast multiplication phase and its activity decreases in the stationary phase. The high affinity system is repressed by high concentration of glucose and the system's activity increases to the end of the fermentation. Glucose is transported the better the more there are sterols and unsaturated phospholipids in the membrane. More glucose is being transported in the stationary and decline phase. That is why aeration influences the successful completion on the alcoholic fermentation during the yeast multiplication phase. The presence of ethanol slows the penetration of glucose into the cell, but it increases the synthesis of membrane phospholipids. (Ribereau-Gayon et al. 2006a)

The cell cytoplasm contains the basic cytoplasmic substance: cytosol. The cytosol contains glycolysis, alcoholic fermentation enzymes and trehalase, which catalyses the hydrolysis of trehalose. Trehalose ensures the yeast viability during the dehydration and rehydration periods by maintaining membrane integrity. Cytoplasmic trehalose concentration decreases during the first stages of fermentation. Glycolysis is explained in the chapter 1.2. The cytoplasm contains also vacuoles which vacuolar proteases autolyse the yeast cell after its death. Autolysis causes the precipitation of lees to the bottom of a fermentation container; and it affects the wine quality. (Ribereau-Gayon et al. 2006a)

Endoplasmic reticulum is linked to the nuclear membrane as well as the cytoplasmic membrane. It is a double membrane system and it addresses the proteins synthesised by attached ribosomes to either vacuole, cytoplasmic membrane or to secretion. The ribosomes can be either free in cytoplasm or attached to the endoplasmic reticulum. The proteins synthesized by free ribosomes stay in cytoplasm. The Golgi apparatus is an extension of endoplasmic reticulum. It is a stack of membrane sacks and vesicles. The vesicles transport the proteins from endoplasmic reticulum to the Golgi apparatus. The Golgi apparatus glycosylates the proteins and then transports them via vesicles to the vacuole or membrane. The vacuole is a single membrane organelle. Depending in the yeasts cellular cycle it may contain more than one vacuole. The vacuole stocks

metabolites before they are used; approximately one quarter of cells amino acids are located inside vacuoles. (Ribereau-Gayon et al. 2006a)

The mitochondria are double membrane organelles which work as respiratory units. The inside of the inner membrane, called the matrix, is where tricarboxylic acid (TCA) cycle takes place. In aerobic conditions *S. cerevisiae* contains about 50 mitochondria, but in anaerobic conditions mitochondria degenerate. High glucose content will prevent the respiration enzymes from working, even in aerobic conditions, and mitochondria degrade. (Ribereau-Gayon et al. 2006a)

Yeasts reproduce either by vegetative multiplication or sexually by forming ascospores. The vegetative multiplication doubles the yeast population in the multiplication phase during fermentation. The round budding scar is left on the surface of the cell wall after multiplication. Sporulation does not normally happen in wine must. In order for the sporulation to happen yeasts must have stopped multiplication by being in an environment, for example, where there are not any fermentable sugars. Yeast forms a sac with a thick cell wall called an ascus and each of these contains four ascospores. The ascii will break in a favorable conditions and continue multiplying. The sporulation of wine yeasts is not driven for, as the spores are often nonviable. (Ribereau-Gayon et al. 2006a)

1.1.1 Killer factors

Certain yeast strains are known as killer strains. These killer strains secrete proteinic toxins to their environment that can kill other sensitive strains. Killer strains can be killer by other toxins. Neutral strains do not produce toxins but are resistant. (Ribereau-Gayon et al. 2006a) These killer factors might have important role in the future to prevent spoilage and there is a potential to completely substitute antimicrobial agents. (Ciani and Comitini 2011)

The killer factor has been discovered in *S. cerevisiae*, but can be found in other yeasts, for example, *Hansenula*, *Pichia*, and *Hanseniaspora*. The killer factor is associated in *S. cerevisiae* with the presence of double-stranded ribonucleic acid (RNA) particles, virus-like particles, in the cytoplasm. There are three kinds of killer activities in

S. cerevisiae which are K1, K2 and K3 toxins. The K1 toxin's pH range is outside of typical must used in wine making and therefore it is inactive. The K2 toxin's pH range is lower than K1's and therefore it is active in the must and wine. K2 strain is also more widespread in the *S. cerevisiae* wine yeasts. (Ribereau-Gayon et al. 2006a) K2 strain is also present in the yeast *Metschnikowia fructicola* (Lallemand Oenology 2018).

The cells are more sensitive to killer effect in their multiplication phase than in the stationary phase. The sensitive cell, exposed to the toxin, manifests serious fundamental alterations which result in the cell's death in about 2 – 3 hours. (Ribereau-Gayon et al. 2006a) *S. cerevisiae*'s killer toxins are only sensitive to *Saccharomyces* strains and those do not affect wild yeasts like *Hanseniaspora* and *Pichia*. The wild yeasts have been the main targets of the antimicrobial agents used in winemaking. The killer factor should be considered only as an additional character for strain selection. (Ciani and Comitini 2011) The killer effect has no effect on humans and animals (Ribereau-Gayon et al. 2006a).

1.1.2 Classification and identification of yeasts

Yeasts are known by two valid names: the teleomorphic name refers to the sexual state producing ascospores and the anamorphic name refers to the asexual state that does not form ascospores. Yeast classification might be difficult, because some yeasts do not sporulate easily and the ability to form ascospores can be lost during long-term storage. Yeast naming has changed during the past decades, so when citing old literature some yeast names might be different. (Jolly et al. 2013) All the yeast names mentioned in this thesis are in their teleomorphic form.

The latest classification of yeasts is based on genetics and molecular taxonomy. Currently over 149 yeast genera is recognised compromising nearly 1500 species (Jolly et al. 2013). *Saccharomyces* yeast's classification has also been due to some changes in the few decades. The *Saccharomyces* yeasts are divided into three groups. The first group is named *Saccharomyces sensu stricto* and it contains the species *S. cerevisiae*, *S. bayanus*, *S. paradoxus* and *S. pastorianus*. The second group is called *Saccharomyces sensu largo* and the third group only contains the species *kluyveri*. Oenolocically only the first group is of interest. (Ribereau-Gayon et al. 2006a)

Saccharomyces yeasts have a large number of different strains that have varying technological properties. Used strain affects the fermentation speed, the nature and quantity of secondary products formed during alcoholic fermentation, and the aromatic characters of the wine. The aromatic character, for example, might be affected by the fermentation or by the autolysis if dead yeast cells. (Ribereau-Gayon et al. 2006a)

The term non-*Saccharomyces* yeast is a loose one and it includes many different yeast species. It is possible to distribute all yeasts used in fermentation, except the *S. cerevisiae* yeasts under this term. (Jolly et al. 2013) This distribution would also include *Saccharomyces bayanus* under non-*Saccharomyces* yeasts, but as *S. bayanus* is part of the genera *Saccharomyces* it is included under the *Saccharomyces* yeasts in this thesis.

Non-*Saccharomyces* yeasts are divided into three groups based on their properties: aerobic yeasts, apiculate yeasts with low fermentative activity; and yeasts with fermentative metabolism. The last group includes the yeasts *Torulaspora debrueckii*, *Metchinikowia pulherrima* and *Metchinikowia fructicola*. (Jolly et al. 2013)

The use of both *Saccharomyces* yeast and non-*Saccharomyces* yeast in fermentation might have some positive interactions and give rise to new technologies.

1.1.3 Yeasts used in this thesis

Two *Saccharomyces cerevisiae* yeasts and one *Saccharomyces bayanus* yeast were used in this thesis. The other, non-*Saccharomyces*, yeasts used were *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. All the yeasts can be commercially bought and are developed mainly for grapes.

Lalvin® W 15 is a *Saccharomyces cerevisiae* var. *cerevisiae* yeast which has been isolated in 1991 from Müller Thurgau must at the Viticulture Research Station in Wädenswil, Switzerland. It produces high levels of glycerol and succinic acid during fermentation. It has a short adaptation phase and quite high alcohol tolerance. The

technical data sheet does not mention the active killer factor. (Lallemand Oenology 2018)

Lalvin® ICV-K1 (V1116) is a *Saccharomyces cerevisiae* var. *cerevisiae* yeast, which has been isolated in 1972 by Pierre Barre of the INRA Montpellier. It has high resistance to difficult fermentation conditions. It has really short adaptation phase and high alcohol tolerance. The active killer factor is K2. (Lallemand Brewing 2018)

Level™ Biodiva™ is a *Torulaspora delbrueckii* yeast. It has a moderate adaptation phase and the alcohol tolerance is not quite high. It has a very good compatibility with malolactic fermentation, which should lower malic acid concentration. Biodiva™ should be used in sequential inoculation with recommended *Saccharomyces cerevisiae* strains. (Lallemand Oenology 2018)

Level™ Flavia™ is a *Metschnikowia pulcherrima* yeast selected from nature by Universidad de Santiago de Chile. It has a narrow optimal fermentation temperature limit and the alcohol tolerance is low. Flavia™ does not produce alcohol during fermentation, so sequential inoculation after 24 hours with *Saccharomyces cerevisiae* is recommended. (Lallemand Oenology 2018)

IOC Gaïa™ is a *Metschnikowia fructicola* yeast selected by The Institut Français de la Vigne et du Vin in France. It has really weak tolerance to alcohol and after sequential inoculation the yeast dies off. The active killer factor is K2. Gaïa™ does not produce alcohol during fermentation, so sequential inoculation after 24 hours with *Saccharomyces cerevisiae* is recommended. (Lallemand Oenology 2018)

Condessa is a *Saccharomyces bayanus* yeast strain which is made in Sweden and imported by Viinitalo Melkko Oy. *Saccharomyces bayanus* yeasts are normally used as sparkling wine yeasts. (Henrik Mikael Solin, Viinitalo Melkko Oy, personal communication 5.6.2018) No technical datasheet or other information was available for Condessa yeast.

1.2 Glycolysis and alcoholic fermentation

Glycolysis (Figure 1.) is carried out in the cytosol of the yeast cell and in it glucose transforms into pyruvate. First step of glycolysis converts glucose into fructose 1,6-biphosphate which requires two adenosine triphosphate (ATP) molecules. There are three stages total in this conversion: first glucose is phosphorylated into glucose 6-phosphate, second the glucose 6-phosphate is isomerised into fructose 6-phosphate and third the fructose 6-phosphate is phosphorylated into fructose 1,6-biphosphate. The reactions are catalysed by three enzymes: hexokinase, phosphoglucoisomerase and phosphoglucokinase, respectively. (Ribereau-Gayon et al. 2006a)

In the second step fructose 1,6-biphosphate is cleaved into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, the reaction is catalysed by aldolase. There is equilibrium between these two compounds, but as glyceraldehyde 3-phosphate is used in the next stage of glycolysis the conversion of dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, by catalyser triose phosphate isomerase, is quite rapid. That way one molecule of glucose leads to the formation of two molecules of glyceraldehyde-3-phosphate. (Ribereau-Gayon et al. 2006a)

The third step forms 3-phosphoglyserate and ATP through two stages. The first stage is the formation of 1,3-biphosphoglycerate from glyceraldehyde-3-phosphate, catalysed by glyceraldehyde 3-phosphate dehydrogenase. During this reaction the nicotinamide adenine dinucleotide (NAD^+) works as a cofactor and accepts two electrons and a hydrogen atom and nicotinamide adenine dinucleotide's reduced form (NADH) is formed. The second stage is the transfer of phosphoryl group from 1,3-biphosphoglycerate to adenosine diphosphate (ADP), by catalyser phosphoglyceratekinase, after which 3-phosphoglyserate and ATP are formed. (Ribereau-Gayon et al. 2006a)

The last step transforms 3-phosphoglyserate to pyruvate and ATP by three stages. In the first stage the 3-phosphoglyserate is converted into 2-phosphoglyserate by catalyser phosphoglyceromutase. During the second stage the 2-phosphoglyserate is dehydrated into phosphoenolpyruvate, catalysed by enolase. Phosphoenolpyruvate has a high potential for transferring phosphoryl group and in the third stage the pyruvate kinase

catalyses a reaction where ADP is phosphorylated forming ATP and pyruvate. Total of four molecules of ATP is formed, but two ATP's are used immediately to start the glycolysis again. (Ribereau-Gayon et al. 2006a)

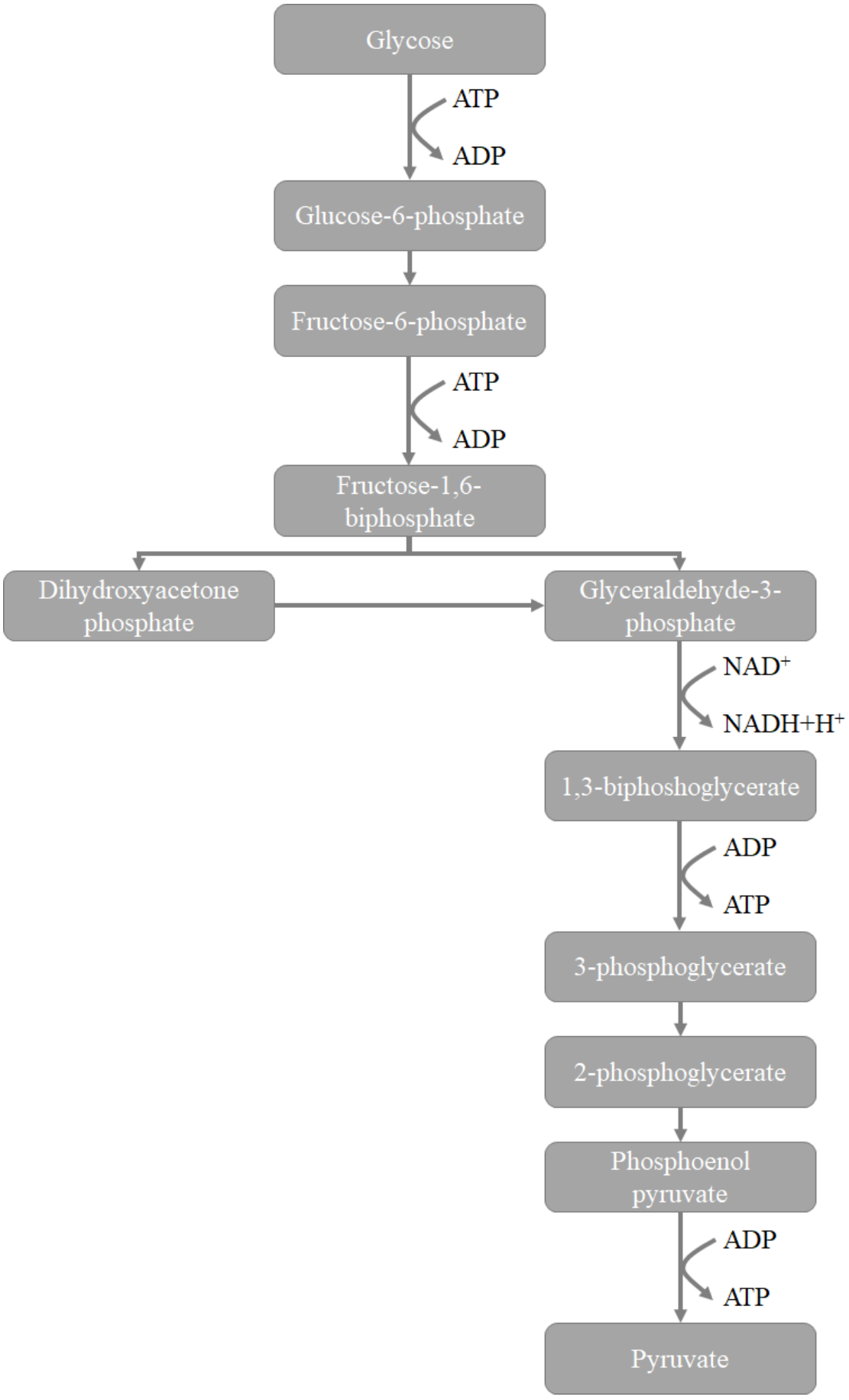


Figure 1. Glycolysis pathway

After the glycolysis it is possible to be followed by alcoholic fermentation (AF) (Figure 2.), glyceropyruvic fermentation or respiration, depending on the conditions. The first step decarboxylates pyruvic acid into acetaldehyde and it is catalysed by pyruvate decarboxylase. Acetaldehyde works as an electron acceptor, and with alcohol dehydrogenase as catalyst, NADH reduces it into ethanol. The formation of NAD^+ in this reaction allows the glycolysis to continue by the forming of the 1,3-biphosphoglycerate. (Ribereau-Gayon et al. 2006a) Must needs to contain approximately 18 g/l of sugars to produce 1 volume-% of ethanol during alcoholic fermentation (Ribereau-Gayon et al. 2006b).

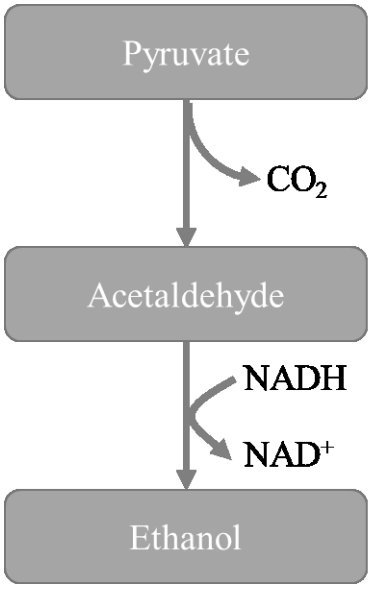


Figure 2. Alcoholic fermentation pathway

There is a competition between AF and respiration. When AF takes place the respiratory is inhibited. High glucose content leads to that *S. cerevisiae* only metabolises sugars by the fermentative pathway; respiration, even in the presence of oxygen, is impossible. The glycose content must be at least 9 grams per litre for the respiration to stop. If aeration takes place the AF happens only in high glucose and fructose concentrations. (Ribereau-Gayon et al. 2006a)

Saccharomyces cerevisiae yeasts were for decades the most common yeasts to be used in the field of fermentation. *Saccharomyces* spp. yeasts and especially *Saccharomyces cerevisiae* were used because they combined all the desired characteristics what were

aspired from yeast; those characteristics included high ethanol production and resistance as well the absence of toxin production (Steensels and Verstrepen 2014; Gschaedler 2017). *S. bayanus* does not ferment galactose and has stronger resistance to ethanol than *S. cerevisiae* (Ribereau-Gayon et al. 2006a). The use of *Saccharomyces* spp. yeasts has reduced the complexity of the wines by limiting the microbial diversity and that way the characteristics of the wine (Steensels and Verstrepen 2014; Gschaedler 2017).

The use of non-*Saccharomyces* yeasts has been in trend in the recent years (Gschaedler 2017). Usually these yeasts are used as fermentation starters. Non-*Saccharomyces* strains lose their culturability during AF; there are numerous factors affecting that, but are usually related to the environmental changes. Those environmental changes can be nitrogen limitation, low oxygen availability and inhibition of increased alcohol. (Wang et al. 2016) *T. delbrueckii* is less tolerant to low oxygen levels than other non-*Saccharomyces* yeasts, and this lead to its death during AF. (Jolly et al. 2013)

The environmental growth conditions should be monitored tightly during the studies of how different yeasts interact with each other.

1.2.1 Alcohols and organic acids

Plants contain pectins and also an enzyme called pectin methylesterase. Pectin methylesterase is quite a thermostable enzyme, but heat does decrease its enzymatic activity. (Dorokhov et al. 2015) Pectins are also present in yeasts (Belda et al. 2016). Methanol is formed from pectins by enzymic hydrolysis, catalyzed by pectin methylesterase, of the methoxyl groups during fermentation (Ribereau-Gayon et al. 2006b). Methanol is toxic even in small amounts: a dose of about 1 g/kg is lethal. Its excessive formation by the pectin methylesterase is therefore not desired. (Dorokhov et al. 2015)

Fusel alcohols are higher alcohols and have more than two carbon atoms; those are, for example, 1-propanol (propan-1-ol), isobutanol (2-methylpropan-1-ol) and isoamyl alcohol (3-methylbutan-1-ol). The desired concentration of fusel alcohols, for aromatic properties, in wine is below 300 mg/L (Mateo et al. 2000). Fusel alcohols are formed from sugars or amino acid precursors by yeast (Ribereau-Gayon et al. 2006b). The

amino acid is decarboxylated into aldehyde which is then reduced to alcohol; this is known as Ehrlich reaction. Examples of amino acid precursors are valine and leucine which are precursors for isobutanol and isoamyl alcohol, respectively. Isoamyl alcohol can also be formed from α-ketoisocaproate, which is an intermediary product in the synthesis of leucine. As all fusel alcohols do not have an amino acid precursor, like 1-butanol and 1-propanol, they are formed during AF from pyruvate. The precursor of 1-propanol, α-ketobutyrate, is derived from pyruvate and acetyl coenzyme A. Yeasts produce higher concentrations of fusel alcohols if the fermentation happens in high pH, elevated fermentation temperature or aeration. (Ribereau-Gayon et al. 2006a) The fusel alcohol content of wine varies greatly and the species of yeast affects it (Ribereau-Gayon et al. 2006b). It is not clear why yeasts produce fusel alcohols, it might be a detoxification process, a mean to regulate metabolism of amino acids or just using up all sugars. (Ribereau-Gayon et al. 2006a)

Glyceropyruvic fermentation (Figure 3.) takes place in the must together, but not at the same time, with AF. Glyceropyruvic fermentation, in the presence of sulphite, produces glycerol, carbon dioxide and acetaldehyde. At the start of the fermentation the yeasts used in inoculation have little decarboxylase and alcohol dehydrogenase expressed, because the yeasts have grown in the presence of oxygen. This causes the oxidation of NADH to involve dihydroxyacetone and not acetaldehyde, as in AF. In glyceropyruvic fermentation pathway dihydroxyacetone phosphate accepts the electron formed in formation 1,3-biphosphoglycerate and becomes glycerol 3-phosphate, the reaction is catalysed by glycerol 3-phosphate deghydrogenase. Glycerol 3-phosphate is then dephosphorylated into glycerol, catalysed by glycerol 3-phosphate phosphatase. The pathway forms also pyruvate and secondary products. The secondary products are pyruvate derivatives and can be, for example, succinic acid and α-ketoglutaric acid. Because the glyceropyruvic fermentation is usually present in the beginning of the fermentation wines contain some amount of glycerol. (Ribereau-Gayon et al. 2006a)

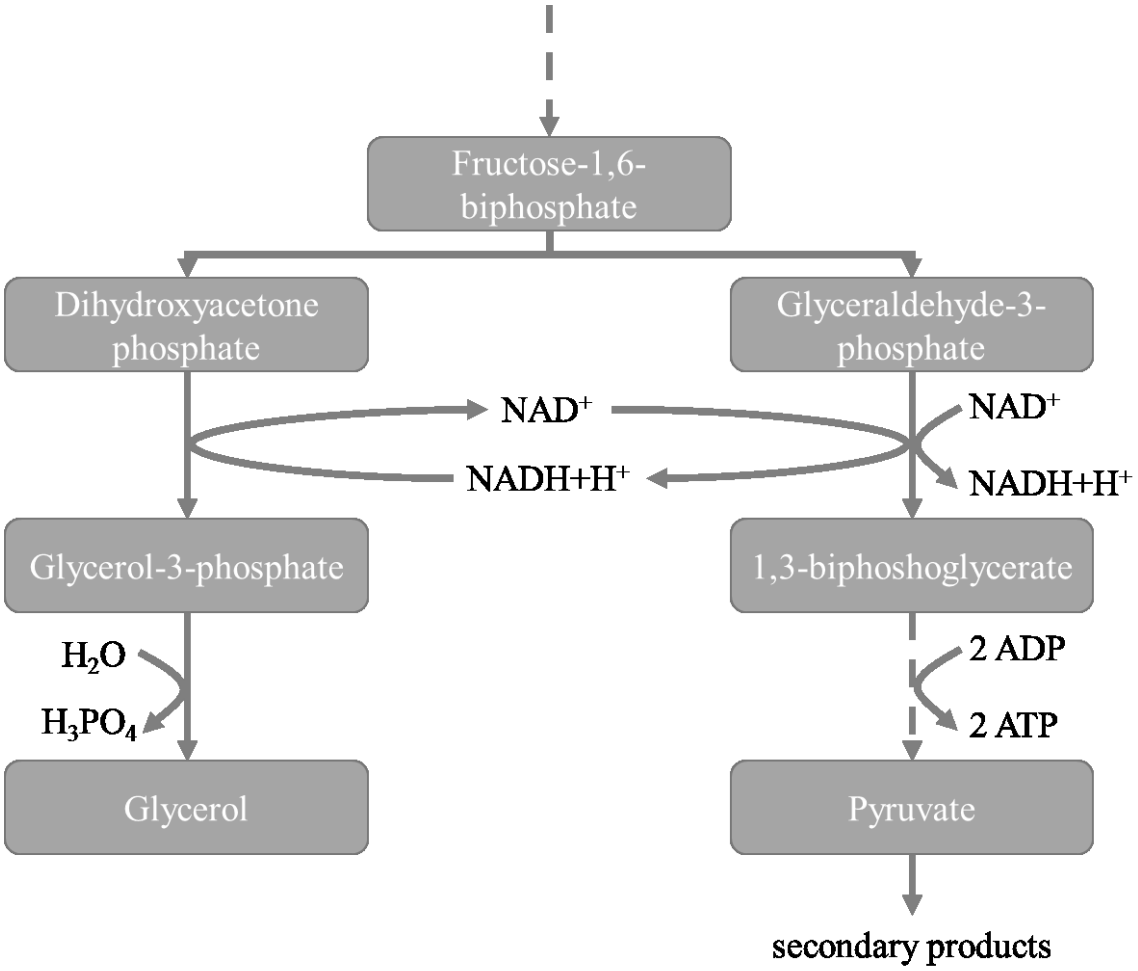


Figure 3. Glyceropyruvic fermentation pathway

The formation of glycerol prevents the use of pyruvate in the AF pathway. The pyruvate is carboxylated into oxaloacetate, by the enzyme pyruvate carboxylase, and is used in the tricarboxylic acid (TCA) cycle. (Ribereau-Gayon et al. 2006a) During anaerobic conditions succinic acid in mainly formed by three pathways; oxidative, reductive and reverse one from glutamate, all of these pathways take some part in the TCA cycle. (Figure 4.) (Coulter et al. 2004)

The complete TCA cycle takes place in aerobic conditions, where it follows up right after glycolysis (Coulter et al. 2004). In anaerobic conditions the oxaloacetate can continue in the TCA cycle the reductive or oxidative pathway. The oxidative pathway of TCA cycle requires the activity of the enzyme succinodehydrogenase to continue the cycle from succinate to fumarate. In aqueous solutions succinic acid is usually in its succinate form. The enzyme can only be activated by coenzyme flavin adenine dinucleotide (FAD), which only works in aerobic conditions and as the conditions are

anaerobic, the TCA cycle cannot be completed and is interrupted in the succinate. It is also possible that the oxidative pathway is interrupted at the α-ketoglutarate, because of the low activity of the α-ketoglutarate hydrogenase in anaerobic conditions. The reductive pathway is therefore believed to be way oxaloacetate continues reactions. The reductive pathway does not however maintain the NAD^+/NADH redox balance and because of it, might only be in a minor part in succinic acid production. The third pathway is the reverse one to form succinic acid during AF from glutamate. Glutamate is converted to α-ketoglutarate, which then continues the oxidative pathway. (Ribereau-Gayon et al. 2006a) Succinic acid is thus formed as a by-product during fermentation and it usually accounts to over 90 % of total organic acids produced (Laminkara 1997; Ribereau-Gayon et al. 2006b).

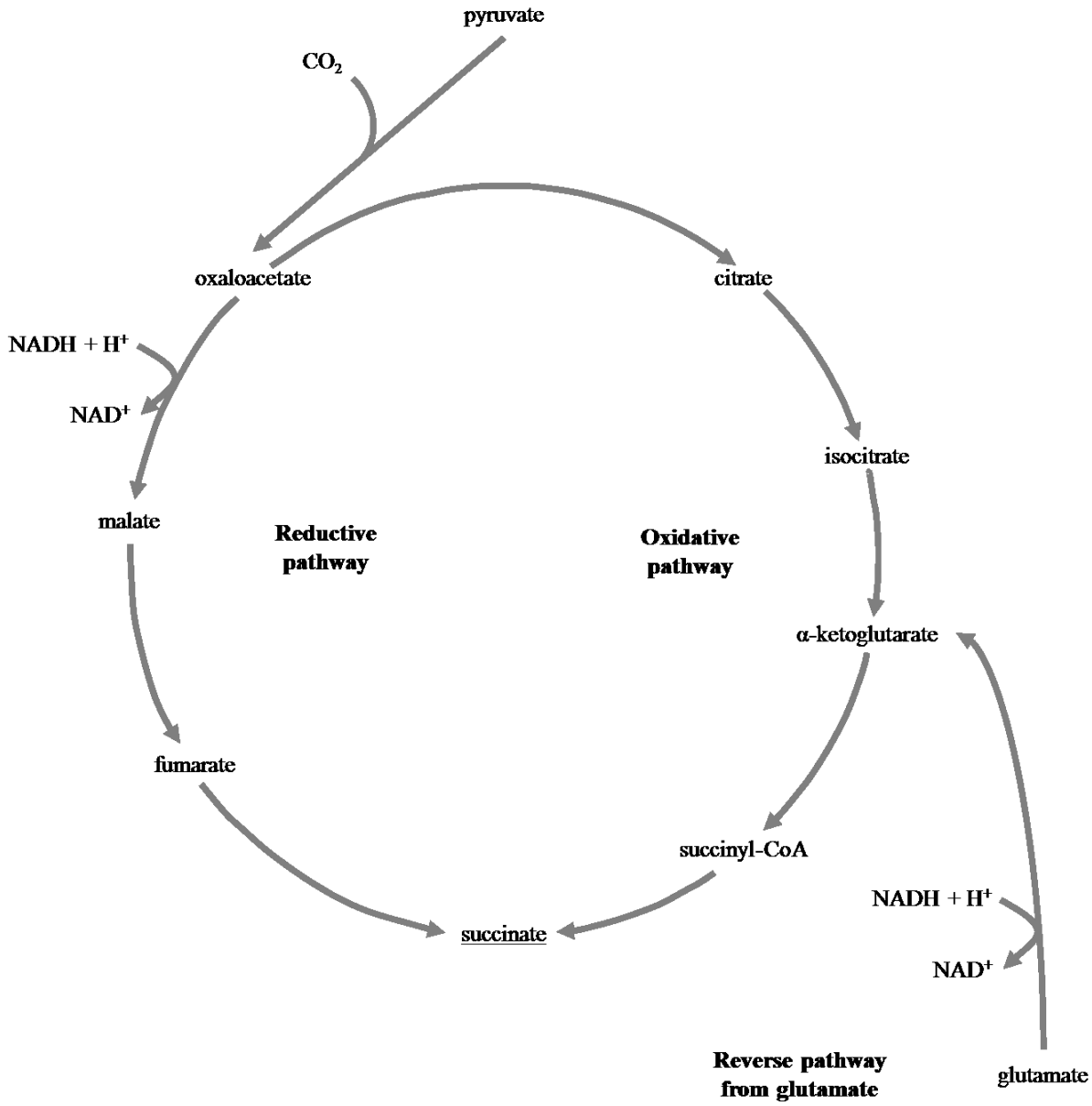


Figure 4. Succinic acid formation during anaerobic fermentation

After AF wine can undergo secondary fermentation known as malolactic fermentation (MLF) (Figure 5). It is the decarboxylation of L-malic acid to L-lactic acid and carbon dioxide. Lactic acid bacteria (LAB) are needed for this reaction, most commonly *Oenococcus oeni*. (Styger et al. 2011) LAB might cause the malic acid concentration to lower during fermentation, which increases the acetic acid concentration (Jacobson 2006). Lactic acid might be formed by other by-products formed by other non-*Saccharomyces* yeasts. Non-*Saccharomyces* yeasts can prevent the growth of LAB through depleting essential nutrient and forming toxic metabolites; that way preventing

the MLF. (Jolly et al. 2013) Although MLF is called fermentation, it does not produce energy, and thus the name is misleading (Jacobson 2006).

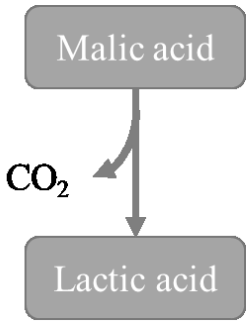


Figure 5. Malolactic fermentation pathway

Small amounts of acetic acid might be formed from citric acid during the malolactic bacteria decomposition (Jacobson 2006; Ribereau-Gayon et al. 2006a), The more there are sugars in the must the more acetic acid the yeast produces. *S. cerevisiae* forms more acetic acid if must has too low or high pH and the temperature is higher than 25 °C during the yeast multiplication phase. (Ribereau-Gayon et al. 2006a) Glycerol concentration also affects favorably to acetic acid production (Ribereau-Gayon et al. 2006a; Jolly et al. 2013). A high amount of acetic acid is not favorable in wines, as it decreases wine quality (Ciani and Comitini 2011).

S. cerevisiae degrades malic acid in the must during AF. The amount degraded varies between different strains. With the help of malic enzyme malic acid is decarboxylated into pyruvic acid. This reaction requires the use of NAD^+. (Ribereau-Gayon et al. 2006a)

1.2.2 Sugars and sugar derivatives

Sugars are more commonly called carbohydrates. Yeasts use fermentable sugars, for example, glucose, and fructose, as nutrients. Glucose and fructose are readily fermentable and also sucrose after hydrolysis into glucose and fructose. Sucrose usually is hydrolysed completely into glucose and fructose by the invertase enzyme of yeast, and thus it should not be present in wine after fermentation. (Ribereau-Gayon et al. 2006b)

Trehalose is a nonreducing sugar, which means it cannot donate electrons, containing two glucose units (Gibney et al 2015). Trehalose can be found in wines as it is produced by yeast autolysis at the end of the fermentation (Ribereau-Gayon et al. 2006b).

Glycosides are sugar derivatives that are formed when non-carbohydrate, known as aglycone, reacts with sugar. Glycosides are, for example, phenolic compounds that can be divided into different categories, one of which is important for this thesis, flavonoids. (Ribereau-Gayon et al. 2006b)

Yellow pigmented flavonoids are molecules where two benzene cycles are bonded with oxygenated heterocycle. The two important flavonoid groups are flavones and flavonols, which are derived from the nucleus of 2-phenyl chromone. In wines also flavan-3-oles are present. Flavonoids may be present in wines in their aglycone forms, as the fermentation may cause the hydrolysis of glycoside. (Ribereau-Gayon et al. 2006b)

Blue and red pigmented anthocyanins are molecules where two benzene cycles are bonded with unsaturated cationic oxygenated heterocycle, which is derived from the nucleus of 2-phenyl-benzopyrylium. Anthocyanins are present in wine in their glycoside form. Monomeric anthocyanins typical to the fruit are not very stable so their concentration lowers the longer the wine is in storage. (Ribereau-Gayon et al. 2006b)

Pectinase enzymes are present during fermentation as endogenous from plants and, yeasts or exogenous in the juice pressing. Pectinase enzymes free the phenolic compounds from the must. Sequential inoculation might increase the pectinase activity and thus the total concentration of phenolic compounds. (Belda et al. 2016)

1.3 Blackcurrant

Blackcurrant (*Ribes nigrum L.*) is the second most cultivated berry in Finland both in cultivation area and production volume. Blackcurrant is somewhat used in production of alcoholic beverages, but mostly it is used in juice industry. (VTT Technical Research Centre of Finland Ltd 2007)

The commercial blackcurrant used in this work contains, according to the nutrition information, 7.8 grams of sugars. (Pakkasmarja Oy 2018) According to the national food composition database in Finland blackcurrant contains 8.6 grams of sugars, from which sucrose is 0.6 grams, fructose 4.4 grams and glucose 3.5 grams. (The National Institute for Health and Welfare 2018)

Organic acids are also present in blackcurrant. The most common organic acid is citric acid with over 50 % of the total organic acid concentration. Malic acid is the second most common organic acid in blackcurrants and tartaric acid is more common than in many other berries. The other organic acids (shikimic and fumaric acid) are not so common and take up to 3 % of the total organic acids. In total blackcurrants' have a high concentration of organic acids compared to other berries. (Mikulic-Petkovsek et al. 2012)

Blackcurrants contain phenolic compounds, for example, anthocyanins and flavonols. Both anthocyanins and flavonols have been found positive effects on the human health (Mazzoni et al. 2016). Synthetisation of both anthocyanins and flavonols are done via the phenylpropanoid pathway as secondary metabolites during development of plants. The highest concentration is found in the peel of the berries. (Mäkilä et al. 2016) Blackcurrants have a high concentration of anthocyanins, compared to other common berries (Borges et al. 2010). The storage conditions as well the time and area of the cultivation impact on the level of anthocyanin and flavonol concentrations (Borges et al. 2010; Mäkilä et al. 2016).

1.4 Aim of the study

Aim of the study was to investigate how yeast fermentation impacts to the chemical characteristics of blackcurrant wine. Especially the sequential inoculations and those results were important.

Fruit wine is wine made from berries or fruits, other than grapes. All kinds of eatable berries and fruits can be used. Berries have a greatly different chemical composition

compared to grapes, which affects the fermentation characteristics of wine yeasts. (Saarela et al. 2010)

As the use of *Saccharomyces cerevisiae* yeasts grew more popular over the years, there also rose a new demand to other styles of wines. These wines were believed to be archived by using both non-*Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* yeasts together. This mixed fermentation is also known as sequential inoculation, where *Saccharomyces cerevisiae* yeast is added after non-*Saccharomyces cerevisiae* yeast to the fermentable must. The time after *Saccharomyces cerevisiae* yeast is added depends on the yeast used. The end products may vary largely due to the interactions between different yeasts. Sequential inoculation might give more favourable end results and reduce negative metabolic activities. (Ciani and Comitini 2009)

Both *Saccharomyces cerevisiae* yeasts and also non-*Saccharomyces cerevisiae* yeasts were to be used for fermentation in this work. Total of six different yeasts were chosen for fermentation; three *Saccharomyces* yeast and three non-*Saccharomyces cerevisiae* yeasts. Two of the non-*Saccharomyces* yeasts were meant to use in sequential inoculation with *Saccharomyces* yeast, those were sequentially inoculated with *Saccharomyces cerevisiae* yeast after 24 hours of fermentation. The mixed fermentation can have very different results than the usual single yeast fermentations. Condessa yeast was chosen because it is regular market yeast in Finland and thus its results are applicable to Finnish consumer. The other yeasts were chosen because the fermentation characteristics were familiar, as those yeasts had been used in our department before.

The following analyses are carried on of all the blackcurrant wines: gas chromatographic analysis of ethanol and fusel alcohols, HPLC-DAD analysis of anthocyanins and flavonols and hydroxycinnamic acid conjugates, as well as the gas chromatographic analysis of sugars and acids.

2 Materials and Methods

2.1 Cold pressing and fermentation of the blackcurrant juice

Commercial blackcurrant berries (100 % kotimaista mustaherukka, Pakkasmarja Ltd, Suonenjoki, Finland) were purchased from a local supermarket. All the berries were from the same batch (01052019). The berries were thawed in small amounts on a microwave two times for 1.5 minutes at 350 W. Thawed berries were cold pressed, without enzyme, to juice at a pressure of 140 kg/cm^3 (Mäkilä et al. 2016). The juice was pasteurised in 50 mL glass bottles in boiling water, after 30 seconds, when the temperature of the juice was approximately 97 °C, the bottles were cooled down in an ice bath. The temperature was monitored during all of the pasteurisations.

100 mL of the cooled, pasteurised juice was weighted on a scale (Mettler Toledo, Columbus, OH, USA) and the amount of measuring weight's worth, approximately 104 g, of juice was added into each of the fermentation bottles. 0.75 g of the yeast was rehydrated with 0.9 g of Go-Ferm (Lallemand, Montreal, QC, Canada) in a 20 mL of water which temperature was according to each yeast's technical data sheet. The used yeasts were: W 15 (Lalvin®, Montreal, QC, Canada), ICV-K1 (Lalvin®, Montreal, QC, Canada), Biodiva™ (Level™, Edwardstown, Australia), Flavia™ (Level™, Edwardstown, Australia), Gaïa™ (IOC, Edwardstown, Australia), and Condessa (Sweden, imported by Viinitalo Melkko).

After the rehydration time, which was unique to each yeast, 0.66 mL of yeast broth was added into each fermentation bottle. All the fermentations were made in batches of three. The fermentation bottles were stored in a box where the bottles were in total darkness and the box was in room temperature. The temperature inside and outside the box was monitored during the first fermentations.

Brix was measured with refractometer (Hanna Instruments, Woonsocket, RI, USA) before and after pasteurisations and it was monitored during the fermentation. Two different *Metschnikowia* yeast batches were fermented for a 24 hour period before they were stopped with a yeast stopper (Viinitalo Melkko, Lahti, Finland). Sequential inoculation was done with the two *Metschnikowia* yeasts and two *S. cerevisiae* yeasts.

All the wines, except the ones with only *Metschnikowia* yeast, were fermented to at least 9 °Bx before adding the yeast stopper (Viinitalo Melkko, Lahti, Finland). After the fermentation the excess yeast was removed by centrifugation and the wines were stored in – 80 °C.

Total of 11 different samples were prepared: Biodiva, Condessa, W15, ICV-K1, Gaïa, Flavia, Gaïa + W15, Flavia + W15, Flavia + ICV-K1 and samples taken before and after pasteurisation.

2.2 Gas chromatographic analysis of sugars and acids as trimethyl silyl derivatives

Sugars and acids were analysed from the blackcurrant wine samples. Internal standards used were, for sugars, xylitol (Sigma, Saint Louis, MO, USA) and, for acids, tartatic acid (Sigma, Saint Louis, MO, USA). The standards used for calculating correction factors were: malic acid (Fluka, Morris Plains, NJ, USA), citric acid (VWR Chemicals, Radnor, PA, USA), quinic acid (Sigma-Aldrich, Saint Louis, MO, USA), ascorbic acid (VWR, Radnor, PA, USA), tartaric acid (Sigma, Saint Louis, MO, USA), fumaric acid (Sigma-Aldrich, Saint Louis, MO, USA), succinic acid (Merck, USA), shikimic acid (Sigma, Saint Louis, MO, USA), galacturonic acid (California corporation for biochemical research, Los Angeles, CA, USA), fructose (Merck, Kenilworth, NJ, USA), glucose (Merck, Kenilworth, NJ, USA), pyruvic acid (Aldrich, Saint Louis, MO, USA), lactic acid (Sigma, Saint Louis, MO, USA), and sucrose (J.T. Baker, Radnor, PA, USA). The concentrations of all standards were approximately 0.5 g / 100 mL

Samples were prepared by diluting 0.25 mL of sample with 4.25 mL of MQ-water and adding 0.25 mL of both internal standards. The dilutions were then filtered with 0.20 µm RC-filters (VWR, Radnor, PA, USA). 300 µL of filtrate was then evaporated in an autosampler bottle in a heat block at 50 °C under nitrogen flow for 60 minutes. The next day 500 µL of TriSil® (Pierce, Rockford, IL, USA) reagent was added to all of the autosampler bottles, shaken vigorously for 5 minutes, and the bottles were incubated in heat block at 60 °C for 30 minutes. The samples were cooled down to room temperature before analysis. The analysis was done according to the chromatographic conditions in Table 1. Three parallel samples were made from each fermentation.

Table 1. Chromatographic conditions

Instrument			
GC-FID	Shimadzu GC-2010Plus with Autoinjector AOC-20i / Autosampler AOC 20s, flame ionization detector and LabSolutions software (Shimadzu corporation, Kyoto, Japan)		
Column	SPB™-1 (30 m x 0.25 mm ID, liquid film 0.25 µm Supelco, Bellefonte, PA, USA)		
Analytical conditions			
Carrier gas	Helium, flow rate 1.9 mL/min		
Injection temperature	210 °C		
Injection mode	split/splitless, split ratio 15:1		
Injection volume	1 µL		
Column temperature (initial)	150 °C		
Temperature 150 °C	Hold	2 min	Rate 4.0 °C/min
210 °C		0 min	40.0 °C/min
275 °C		5 min	
Total analysis time	28.625 min		
Detector temperature	290 °C		

2.3 Gas chromatographic analysis of ethanol and fusel alcohols

Ethanol and fusel alcohols were analysed from the blackcurrant wine samples. Internal standard used was *n*-Butanol (Riedel-de Haën, Morris Plains, NJ, USA). The standards used for calculating correction factor were: 10 % ethanol (Altia, Helsinki, Finland), 1 % methanol (Sigma-Aldrich, Saint Louis, MO, USA), 1 % *n*-Propanol (J.T. Baker, Radnor, PA, USA), 1 % isopropyl alcohol (VWR Chemicals, Radnor, PA, USA), 1 % isoamyl alcohol (Sigma-Aldrich, Saint Louis, MO, USA), 1 % acetic acid (Sigma-Aldrich, Saint Louis, MO, USA), 1 % *n*-Pentanol (Merck, Kenilworth, NJ, USA), 1 % isobutanol (Merck, Kenilworth, NJ, USA), 1 % acetaldehyde (Fluka AG, Buchs, Switzerland), and 1 % glycerol (Fisher Bioreagents, Hampton, NH, USA).

Samples were prepared by filtering the wine with 0.45 µL RC-filter (VWR, Radnor, PA, USA). 190 µL of the sample and 10 µL of internal standard were added into the

autosampler bottle. Three parallel samples were made from each fermentation. Standards were prepared by adding 200 µL of standard and 200 µL of undiluted *n*-Butanol (Riedel-de Haën, Morris Plains, NJ, USA). The analysis was done according to the chromatographic conditions in Table 2.

No alcoholic fermentation takes place in the exclusively *Metschnikowia* yeasts batches, so no ethanol and fusel alcohols were measured from fermentation samples before sequential inoculation.

Table 2. Chromatographic conditions

Instrument	
GC-FID	Shimadzu GC-2010Plus with Autoinjector AOC-20i / Autosampler AOC 20s, flame ionization detector and LabSolutions software (Shimadzu corporation, Kyoto, Japan)
Column	HP Innowax (30 m, id 0.25 mm, d_f 0.25 µm, Hewlett-Packard, CA, USA)
Analytical conditions	
Carrier gas	Helium, flow rate 42.00 mL/min
Injection temperature	220 °C
Injection mode	split/splitless, split ratio 1:25
Injection volume	0.2 µL
Column temperature (initial)	40 °C
Temperature 40 °C	Hold 8 min
240 °C	0 min 10.0 °C/min
240 °C	2 min
Total analysis time	30 min
Detector temperature	280 °C

2.4 HPLC-DAD analysis of flavonols and hydroxycinnamic acid conjugates

Samples were prepared by weighting approximately 2 g of wine into a plastic tube. 5 ml of ethyl acetate was added and the tube was vortexed for 1.5 minutes. The tube was centrifuged for 5 minutes in 1000 g. The upper phase was transferred into a boiling flask. Extraction phases were repeated three times. The extract was evaporated gently with a rotary evaporator (Heidolph, Schwabach, Germany) in a 35 °C water bath. After evaporation the extract was diluted with 1 mL of methanol (Sigma-Aldrich, Saint Louis, MO, USA) and it was filtered 0.45 µm PTFE-filter (VWR, Radnor, PA, USA) into an autosampler bottle. Three parallel samples were made from each fermentation.

Quercetin-3-*O*-glucoside (Extrasynthese, Genay, France) standards were prepared. Total of five different dilutions were made. The first standard was done by diluting the stock standard 1:1 to methanol. The second standard was prepared by diluting first standard 1:1 to methanol. All the remaining three standards were prepared by diluting every time the previous standard 1:1 to methanol. All dilutions were made parallel three times and every parallel dilution was analysed two times. The analyses were done according to the chromatographic conditions and gradient program in Table 3 and 4. Flavonols are monitored at 360 nm.

Table 3. Chromatographic conditions

HPLC-DAD	Shimadzu Nexera with two LC-30AD solvent delivery units, SIL-30AC autosampler, CTO-20AC column oven, SPD-M20A diode array detector, CBM-20A controller, and LCsolution software. (Shimadzu corporation, Kyoto, Japan)
Column	Aeris Peptide XB-C18 (3.6 µm, 150 x 4.6 mm; Phenomenex, Torrance, CA, USA)
Flow rate	1 mL/min
Mobile phases	Solvent A: 0.1 % formic acid in MQ-water Solvent B: 0.1 % formic acid in acetonitrile
Injection volume	10 µL
Oven temperature	30 °C
Run time	45 min

Table 4. The gradient program

Time	Action	Value (%)
0	Pump B Conc.	2
15	Pump B Conc.	18
20	Pump B Conc.	18
30	Pump B Conc.	20
35	Pump B Conc.	60
40	Pump B Conc.	2
45	Stop	2

2.5 HPLC-DAD analysis of anthocyanins

Samples were prepared by weighting approximately 0.5 grams of wine into a microcentrifuge tube and diluting it with same amount of methanol (Sigma-Aldrich, Saint Louis, MO, USA) and hydrochloric acid (J.T. Baker, Radnor, PA, USA) solution (99:1). The tube was centrifuged for 1 minute in 4000 g. The upper phase was filtered

with 0.45 µm PTFE-filter (VWR, Radnor, PA, USA) into an autosampler bottle. Three parallel samples were made from each fermentation.

Cyanidin-3-*O*-glucoside (Extrasynthese, Genay, France) standards were prepared. Total of five different dilutions were made. The first standard was done by diluting the stock standard 1:1 to methanol-hydrochloric acid solution (MeOH/HCl, 99:1) and the remaining standards were prepared by diluting every time the previous standard 1:1 with MeOH/HCl. All dilutions were made parallel three times and every parallel dilution was analysed two times. The analyses were done according to the chromatographic conditions and gradient program in Table 5 and 6. Anthocyanins are monitored at 520 nm.

Table 5. Chromatographic conditions

HPLC-DAD	Shimadzu Nexera with two LC-30AD solvent delivery units, SIL-30AC autosampler, CTO-20AC column oven, SPD-M20A diode array detector, CBM-20A controller, and LCsolution software. (Shimadzu corporation, Kyoto, Japan)
Column	Aeris Peptide XB-C18 (3.6 µm, 150 x 4.6 mm; Phenomenex, Torrance, CA, USA)
Flow rate	1 mL/min
Mobile phases	Solvent A: 5 % formic acid in MQ-water Solvent B: 5 % formic acid in acetonitrile
Injection volume	10 µL
Oven temperature	36 °C
Run time	35 min

Table 6. The gradient program

Time	Action	Value (%)
0	Pump B Conc.	5
10	Pump B Conc.	10
15	Pump B Conc.	15
20	Pump B Conc.	40
30	Pump B Conc.	90
35	Pump B Conc.	5
35	Stop	5

3 Results

3.1 Monitoring the fermentation

Brix was monitored during all of the fermentations; it was also measured before and after the pasteurisations (Table 7). The brix measurements were then carried on during fermentation until the brix was 9 °Bx or lower, after that the fermentation was stopped. The results of brix measurements during fermentations are shown in Table 8.

Temperature was monitored during all of the five pasteurisations; the results of the measurements are seen in Figure 6. The figure presents only the temperatures measured from the pasteurisation bottles during the pasteurisations. Temperature was also monitored during the first fermentation. There was no difference in the temperature inside the box used for the fermentations or the room temperature.

Table 7. The average brix measurement results from before and after pasteurisation.

	1st batch (°Bx)	2nd batch (°Bx)	3rd batch (°Bx)	4th batch (°Bx)
Before pasteurisation	15.7	15.2	14.7	14.9
After pasteurisation	14.8	15.5	14.3	15.2

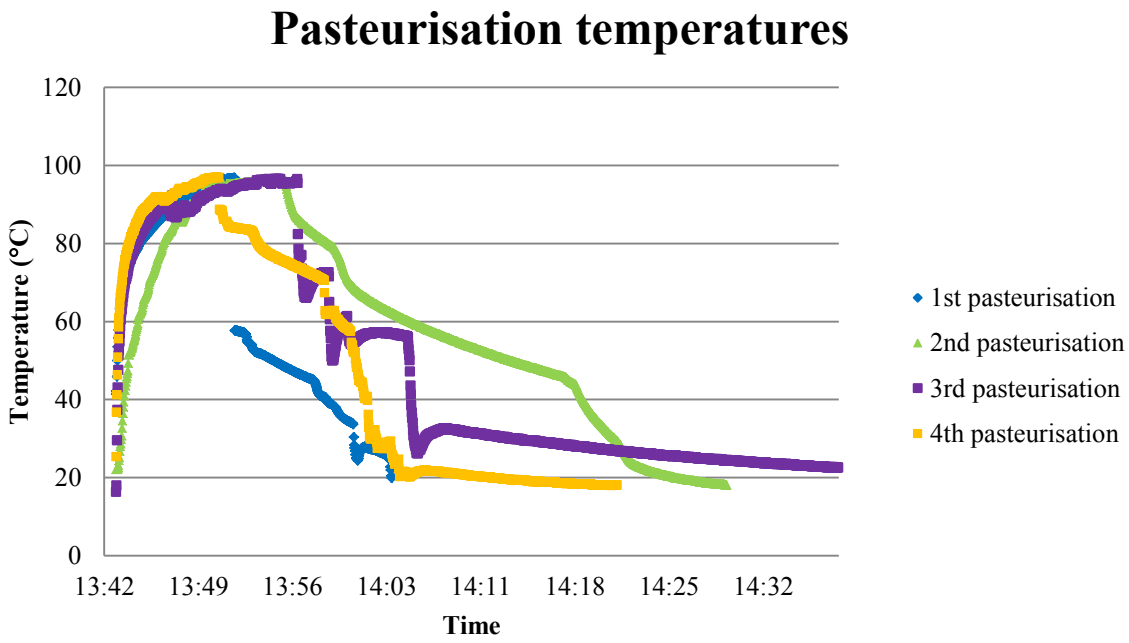


Figure 6. Pasteurisation temperatures, measured from pasteurisation bottles.

Table 8. The average brix measurements from fermentation samples. The standard deviation is calculated from three parallel samples.

Day	Biodiva * (°Bx)	Condessa * (°Bx)	W15 * (°Bx)	ICV-K1 * (°Bx)	Flavia + W15 ** (°Bx)	GAÏA + W15 *** (°Bx)	Flavia + ICV-K1 **** (°Bx)
1	14.0 ± 0.2	14.2 ± 0.2	13.2 ± 0.3	14.0 ± 0.4	14.4 ± 0.1	14.4 ± 0.3	14.2 ± 0.3
2			11.2 ± 0.6		13.2 ± 0.1	13.6 ± 0.0	13.7 ± 0.1
3						11.7 ± 0.2	12.5 ± 0.3
4						10.5 ± 0.5	10.0 ± 0.2
5	9.2 ± 0.2	13.2 ± 0.4	8.6 ± 0.6	8.5 ± 0.2	9.0 ± 0.2	9.2 ± 0.3	9.0 ± 0.2
6	8.7 ± 0.4		8.3 ± 0.1	8.3 ± 0.1		9.0 ± 0.1	9.1 ± 0.1
7	8.3 ± 0.1	10.1 ± 0.6	8.3 ± 0.1	8.1 ± 0.1			
8	8.2 ± 0.0	9.2 ± 0.3	8.1 ± 0.1	8.1 ± 0.1			
9		8.4 ± 0.5					
11		8.0 ± 0.1					

* fermented in the first batch, ** fermented in the second batch, *** fermented in the third batch, **** fermented in the fourth batch

Brix was also measured from Flavia and GAÏA samples before sequential inoculation, after 24 hours, which results were 13.9 ± 0.1 and 14.7 ± 0.2 respectively. The fermentation was stopped immediately after Brix measurement.

3.2 Sugars and organic acids

The content of sugars and acids in each wine was calculated according to the equation below, where A = area, C = concentration and k = correction factor (unique for each analyte), standard stands for internal standard used:

$$\frac{A_{Analyte}}{C_{analyte}} = k * \frac{A_{standard}}{C_{standard}}$$

The correction factor for each analyte was calculated using the equation:

$$k = \frac{A_{analyte} * C_{standard}}{A_{standard} * C_{analyte}}$$

$$k_{sucrose} = \frac{688537 * 0.51282}{947877.5 * 0.51579} = 0.72222$$

The result for one fermentation of Biodiva yeast was calculated using the equation:

$$C_A = \frac{A_{analyte} * C_{standard}}{k * A_{standard}}$$

$$C_{A(sucrose)} = \frac{40026{,}66 * 0{,}51282}{0.72222 * 233100.7} = 0.1219\ g/100mL$$

Total results are calculated from three parallel averages and all dilutions were taken into account during calculations. The results for sugars and acids are seen in Table 9 and 10. The standard deviation is calculated from three parallel averages.

Table 9. The acid results in fermentation samples and in samples before and after pasteurisation.

	Sucrose (g/100 mL)	Fructose (g/100 mL)	Glucose (g/100 mL)
Before pasteurisation	0.9644 ± 0.2794	3.4231 ± 0.5509	3.1488 ± 0.4133
After pasteurisation	0.7107 ± 0.2749	3.6694 ± 0.5928	3.3653 ± 0.3119
Biodiva	0.1062 ± 0.0137	0.0972 ± 0.1278	0.0821 ± 0.1016
Condessa	0.0162 ± 0.0012	0.1849 ± 0.0114	0.1008 ± 0.0035
W15	0.2763 ± 0.0170	0.0112 ± 0.0004	0.0168 ± 0.0008
ICV-K1	0.0566 ± 0.0317	0.0084 ± 0.0018	0.0149 ± 0.0034
GAÏA	0.5204 ± 0.0136	3.4454 ± 0.0518	3.0635 ± 0.0392
Flavia	0.6003 ± 0.0463	3.4512 ± 0.2492	3.2807 ± 0.2992
GAÏA + W15	0.3479 ± 0.0370	nd	nd
Flavia + W15	0.3909 ± 0.0353	nd	nd
Flavia + ICV-K1	0.3898 ± 0.0166	nd	nd

nd, not detected

Table 10. The acid results in fermented samples and in samples before and after pasteurisation.

	Succinic acid (g/100 mL)	Malic acid (g/100 mL)	Shikimic acid (g/100 mL)	Citric acid (g/100 mL)	Quinic acid (g/100 mL)	Ascorbic acid (g/100 mL)	Galacturonic acid (g/100 mL)
Before pasteurisation	nd	0.3072 ± 0.0551	0.0388 ± 0.0048	3.1229 ± 0.4124	0.0427 ± 0.0053	0.0877 ± 0.0097	nd
After pasteurisation	nd	0.3126 ± 0.0462	0.0395 ± 0.0033	3.1753 ± 0.2830	0.0448 ± 0.0030	0.0876 ± 0.0108	nd
Biodiva	0.0770 ± 0.0029	0.2783 ± 0.0037	0.0332 ± 0.0014	3.3236 ± 0.0304	0.0240 ± 0.0008	0.0915 ± 0.0017	nd
Condessa	0.1043 ± 0.0256	0.2934 ± 0.0725	0.0399 ± 0.0097	3.5647 ± 0.8989	0.0245 ± 0.0210	0.0922 ± 0.0251	nd
W15	0.0694 ± 0.0003	0.2791 ± 0.0034	0.0361 ± 0.0003	3.2104 ± 0.0816	0.0230 ± 0.0003	0.0860 ± 0.0032	nd
ICV-K1	0.0681 ± 0.0036	0.2815 ± 0.0070	0.0364 ± 0.0011	3.2694 ± 0.1547	0.0236 ± 0.0007	0.0881 ± 0.0060	nd
GAÏA	nd	0.2954 ± 0.0065	0.0392 ± 0.0006	3.0904 ± 0.0903	0.0394 ± 0.0008	0.0994 ± 0.0011	nd
Flavia	nd	0.3089 ± 0.0207	0.0409 ± 0.0020	3.3140 ± 0.0995	0.0461 ± 0.0020	0.0938 ± 0.0109	nd
GAÏA + W15	0.0465 ± 0.0020	0.2577 ± 0.0091	0.0372 ± 0.0013	3.0308 ± 0.2063	0.0233 ± 0.0009	0.0956 ± 0.0040	0.0176 ± 0.0019
Flavia + W15	0.0485 ± 0.0014	0.2832 ± 0.0120	0.0405 ± 0.0018	3.5104 ± 0.2247	0.0254 ± 0.0013	0.0992 ± 0.0052	0.0256 ± 0.0016
Flavia + ICV-K1	0.0478 ± 0.0099	0.2803 ± 0.0078	0.0360 ± 0.0011	3.2127 ± 0.0923	0.0226 ± 0.0008	0.0855 ± 0.0032	0.0192 ± 0.0018

nd, not detected

Trehalose was detected, but not quantified in GAÏA + W15 and Flavia + W15 fermentations. No pyruvic acid or lactic acid were detected in any of the samples.

3.3 Alcohols

The content of alcohols in each fermentation was calculated according to the equation below, where A = area, C = concentration and k = correction factor (unique for each analyte), standard stands for internal standard used:

$$\frac{A_{Analyte}}{C_{analyte}} = k * \frac{A_{standard}}{C_{standard}}$$

The correction factor for each analyte was calculated using the equation:

$$k = \frac{A_{analyte} * C_{standard}}{A_{standard} * C_{analyte}}$$

$$k_{methanol} = \frac{140465 * 0.995}{282021 * 0.999} = 0.4961$$

The result for one fermentation of Biodiva was calculated using the equation:

$$C_A = \frac{A_{analyte} * C_{standard}}{k * A_{standard}}$$

$$C_{A(methanol)} = \frac{51544.33 * 0.4961}{0.72222 * 3246433} = 0.1592\ mL/100mL$$

Total results are calculated from three parallel averages and all dilutions were taken into account. The results for ethanol and fusel alcohol quantities are seen in Table 11. The standard deviation was calculated from three parallel averages.

Table 11. The alcohol results in alcoholic fermentation samples.

Yeast	Ethanol (mL/100 mL)	Methanol (mL/100 mL)	Acetaldehyde (mL/100 mL)	Acetic acid (mL/100 mL)	1-Propanol (mL/100 mL)	Isobutanol (mL/100 mL)	Isoamyl alcohol (mL/100 mL)
Biodiva	4.0488 ± 0.3711	0.1065 ± 0.0458	0.0005 ± 0.0002	0.0083 ± 0.0015	0.0021 ± 0.0003	0.0086 ± 0.0005	0.0073 ± 0.0008
Condessa	3.9252 ± 0.0340	0.0993 ± 0.0006	0.0023 ± 0.0007	0.0013 ± 0.0002	0.0021 ± 0.0008	0.0094 ± 0.0001	0.0090 ± 0.0002
W15	3.8925 ± 0.1966	0.0292 ± 0.0193	0.0018 ± 0.0006	0.0152 ± 0.0024	0.0033 ± 0.0003	0.0077 ± 0.0001	0.0063 ± 0.0003
ICV-K1	4.4954 ± 0.1918	0.9235 ± 1.4460	0.0008 ± 0.0004	0.0150 ± 0.0025	0.0026 ± 0.0001	0.0101 ± 0.0008	0.0085 ± 0.0001
GAÏA + W15	3.7749 ± 0.3778	0.0147 ± 0.0005	0.0019 ± 0.0002	0.0134 ± 0.0013	0.0029 ± 0.0003	0.0085 ± 0.0007	0.0055 ± 0.0007
Flavia + W15	3.6727 ± 0.1952	0.0143 ± 0.0023	0.0018 ± 0.0005	0.0155 ± 0.0009	0.0024 ± 0.0003	0.0077 ± 0.0003	0.0046 ± 0.0002
Flavia + ICV-K1	3.4878 ± 0.1960	0.0848 ± 0.0124	0.0006 ± 0.0002	0.0122 ± 0.0010	0.0017 ± 0.0001	0.0089 ± 0.0003	0.0059 ± 0.0002

No *n*-Pentanol, isopropyl alcohol or glycerol were detected in any of the samples.

3.4 Flavonols

The content of flavonols in each sample was calculated as a quercetin glucoside equivalent using the standard curve (Figure 7). Example HPLC-DAD chromatogram for yeast W15 is seen in Figure 8.

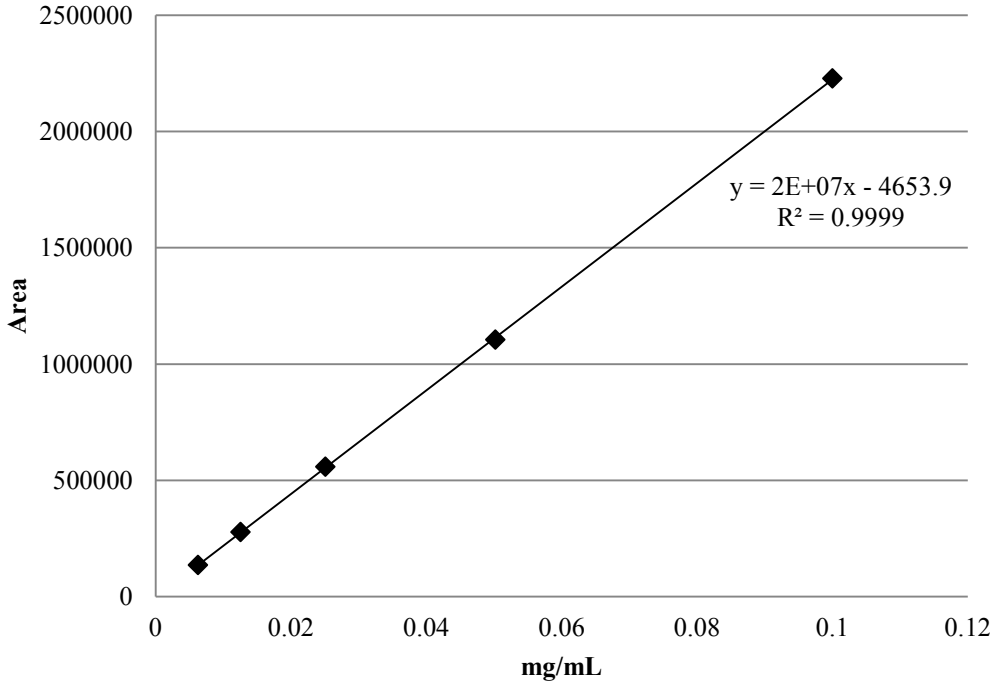


Figure 7. Standard curve of quercetin-3-*O*-glucoside with the formula and R^2 correlation coefficient.

The result for one fermentation of Biodiva was calculated using the equation from standard curve; the mass of the sample was taken into account:

$$x = \frac{\frac{(y-b)}{a}}{sample\ mass} \times 100$$

$$x_{myricetin} = \frac{\frac{27794 - (-4653.9)}{2E+07}}{1.5659} \times 100 = 0.0010\ mg/100\ g$$

Total results are calculated from three parallel averages.

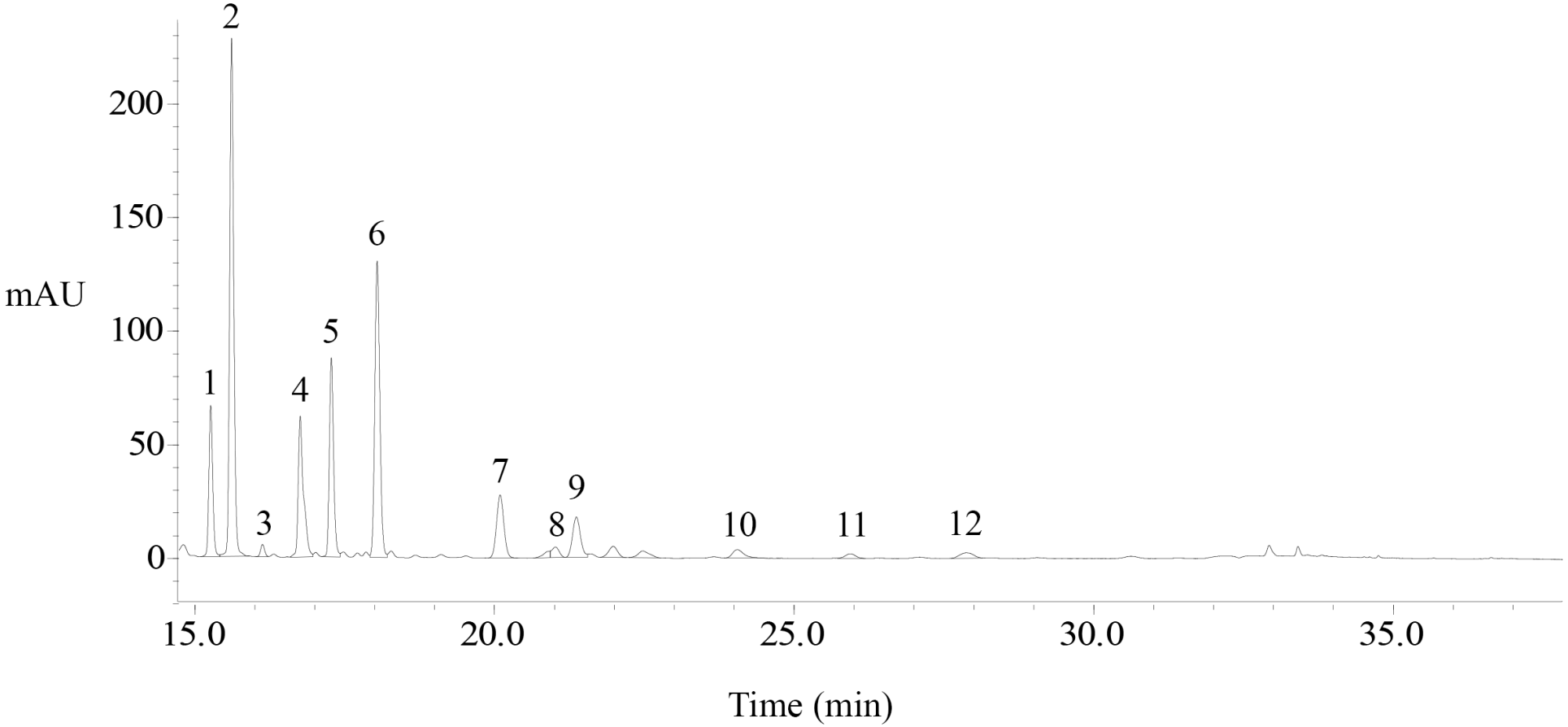


Figure 8. HPLC-DAD chromatogram of identified flavonol compounds of yeast W15. The numbering of the peaks corresponds to Table 12.

Table 12. Identified flavonol compounds.

Nr	Compound	λ_{max} (nm)	t_r (min)	literature
1	myricetin-3-*O*-rutinoside	355	15.268 - 0.010	a,b
2	myricetin-3-*O*-glucoside	256, 354	15.616 - 0.011	a,b
3	myricetin-3-*O*-arabinoside	346	16.132 - 0.010	a
4	aureusidin-3-*O*-glucoside	255, 355	16.761 - 0.010	a
5	quercetin-3-*O*-rutinoside	216, 352	17.283 - 0.012	a,b
6	quercetin-3-*O*-glucoside	255, 352	18.047 - 0.015	a,b
7	kaempferol-3-*O*-rutinoside	256, 349	20.100 - 0.021	a,b
8	quercetin-3-*O*-malonylglucoside	347	21.025 - 0.022	a,b
9	isorhamnetin-3-*O*-rutinoside	365, 344	21.373 - 0.027	a
10	isorhamnetin-3-*O*-glucoside	369	24.050 - 0.032	a
11		346	25.945 - 0.035	
12	myricetin	355	27.883 - 0.043	a

a Mäkilä *et al.* (2016), b Borges *et al.* (2003)
The numbering corresponds to Figure 8.

The identified flavonols compounds are seen in Table 12, total of 14 different flavonol compounds were identified from the fermentations. The concentrations of flavonol compounds of the fermentations are seen in Table 13.

Table 13. The concentration of flavonols in the fermentations.

Nr	Biodiva (mg/100 g)	Condessa (mg/100 g)	W15 (mg/100 g)	ICV-K1 (mg/100 g)	Gaïa (mg/100 g)	Gaïa + W15 (mg/100 g)	Flavia (mg/100 g)	Flavia + W15 (mg/100 g)	Flavia + ICV-K1 (mg/100 g)	Before pasteurisation (mg/100 g)	After pasteurisation (mg/100 g)
1	0.8470 ± 0.0878	0.8734 ± 0.1224	0.8018 ± 0.0372	0.8808 ± 0.0255	0.5579 ± 0.0191	0.9046 ± 0.0598	0.6707 ± 0.0378	0.8625 ± 0.0338	0.8563 ± 0.0571	0.3845 ± 0.0374	0.3342 ± 0.0736
2	2.7766 ± 0.2899	3.0281 ± 0.2416	2.7972 ± 0.0099	3.0845 ± 0.1440	2.8668 ± 0.1052	3.9775 ± 0.3933	3.9458 ± 0.1859	3.7250 ± 0.1912	3.1997 ± 0.3024	2.2968 ± 0.0747	2.3079 ± 0.1842
3	0.0748 ± 0.0079	0.0806 ± 0.0079	0.0751 ± 0.0007	0.0813 ± 0.0041	0.0757 ± 0.0028	0.0940 ± 0.0096	0.1067 ± 0.0052	0.0902 ± 0.0042	0.0855 ± 0.0064	0.0807 ± 0.0010	0.0766 ± 0.0025
4	0.9650 ± 0.1116	1.0811 ± 0.0850	0.9863 ± 0.0054	1.0812 ± 0.0478	1.1297 ± 0.0403	1.4816 ± 0.1428	1.5560 ± 0.0865	1.4111 ± 0.0730	1.2447 ± 0.1162	0.9652 ± 0.0266	0.9806 ± 0.0647
5	1.2059 ± 0.1396	1.2916 ± 0.1547	1.1435 ± 0.0284	1.2550 ± 0.0283	1.0732 ± 0.0368	1.3551 ± 0.0974	1.3185 ± 0.0486	1.2932 ± 0.0601	1.3056 ± 0.0893	0.9005 ± 0.0600	0.8163 ± 0.1373
6	1.8803 ± 0.1258	2.0486 ± 0.1565	1.9262 ± 0.0147	2.0880 ± 0.0842	2.1230 ± 0.0720	2.6766 ± 0.2182	2.2429 ± 1.1720	2.5570 ± 0.1127	2.4634 ± 0.1791	2.0256 ± 0.0197	2.0991 ± 0.0239
7	0.6129 ± 0.0755	0.6708 ± 0.0504	0.6270 ± 0.0151	0.6941 ± 0.0361	0.6723 ± 0.0271	0.8868 ± 0.0921	2.2429 ± 0.4962	0.8536 ± 0.0554	0.8172 ± 0.0717	0.6751 ± 0.0121	0.6930 ± 0.0245
8	0.1223 ± 0.0135	0.1292 ± 0.0119	0.1190 ± 0.0034	0.1304 ± 0.0016	0.1107 ± 0.0036	0.1617 ± 0.0146	0.3410 ± 0.3537	0.1506 ± 0.0109	0.1375 ± 0.0110	0.0837 ± 0.0097	0.0768 ± 0.0149
9	0.4081 ± 0.0210	0.4590 ± 0.0553	0.4178 ± 0.0101	0.4461 ± 0.0201	0.6059 ± 0.0173	0.6554 ± 0.0528	0.6054 ± 0.3877	0.6296 ± 0.0311	0.5704 ± 0.0385	0.5734 ± 0.0039	0.5971 ± 0.0048

Nr	Biodiva (mg/100 g)	Condessa (mg/100 g)	W15 (mg/100 g)	ICV-K1 (mg/100 g)	Gaïa (mg/100 g)	Gaïa + W15 (mg/100 g)	Flavia (mg/100 g)	Flavia + W15 (mg/100 g)	Flavia + ICV-K1 (mg/100 g)	Before pasteurisation (mg/100 g)	After pasteurisation (mg/100 g)
10	0.1341 ± 0.0238	0.1591 ± 0.0276	0.1437 ± 0.0063	0.1513 ± 0.0196	0.2164 ± 0.0054	0.2761 ± 0.0365	0.2591 ± 0.1301	0.2463 ± 0.0188	0.1678 ± 0.0231	0.1263 ± 0.0007	0.1882 ± 0.0140
11	0.0721 ± 0.0103	0.0786 ± 0.0073	0.0722 ± 0.0028	0.0805 ± 0.0048	0.0846 ± 0.0041	0.1058 ± 0.0122	0.1395 ± 0.0308	0.1026 ± 0.0068	0.0985 ± 0.0096	0.0916 ± 0.0023	0.0969 ± 0.0012
12	0.1108 ± 0.0149	0.1205 ± 0.0097	0.1133 ± 0.0045	0.1246 ± 0.0094	0.1344 ± 0.0060	0.1718 ± 0.0212	0.1939 ± 0.0030	0.1650 ± 0.0121	0.1437 ± 0.0143	0.1334 ± 0.0015	0.1381 ± 0.0012

nd, not detected

The standard deviation is calculated from three parallel averages. The numbering corresponds to Figure 8.

3.5 Anthocyanins

The content of anthocyanins in the samples was calculated as a cyanidin-3-*O*-glycoside equivalent using the standard curve (Figure 9). Example HPLC-DAD chromatogram for yeast W15 is seen in Figure 10.

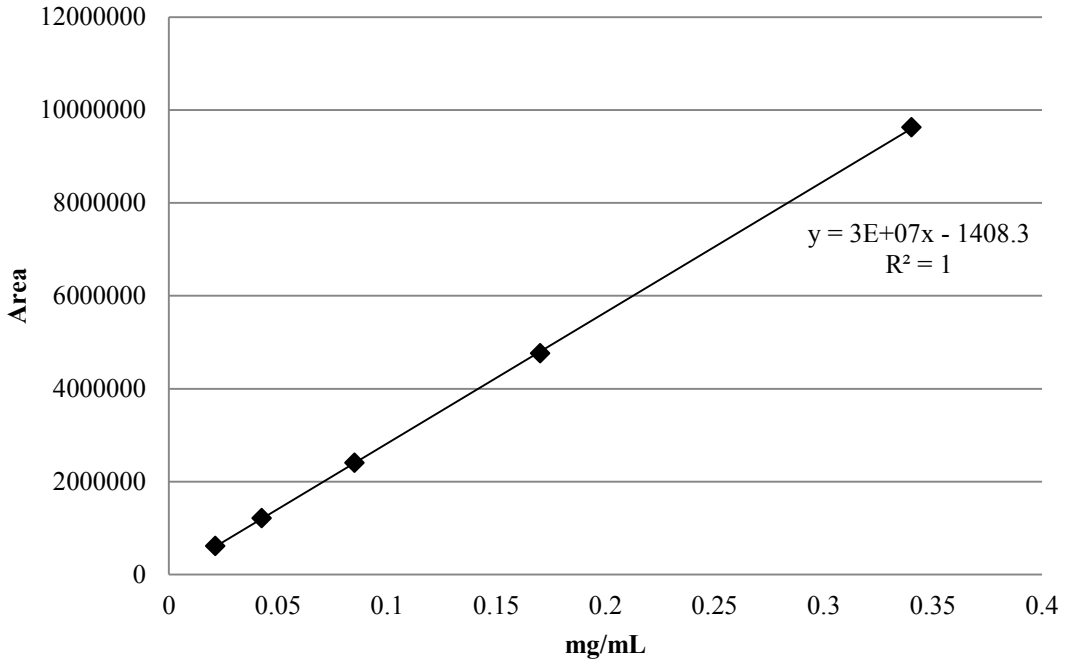


Figure 9. Standard curve of cyanidin-3-*O*-glucoside with the formula and R^2 correlation coefficient.

The result for one fermentation of Biodiva was calculated using the equation from standard curve; the dilution factor was also taken into account:

$$x = \frac{\left[\frac{(y-b)}{a}\right] * 2}{dilution\ factor}$$

$$x_{\text{petunidin}-3-O-\text{glucoside}} = \frac{\left[\frac{6810-(-1408.3)}{3E+07}\right] * 2}{0.9970} \times 100 = 0.00027\ mg/100\ g$$

Total results are calculated from three parallel averages.

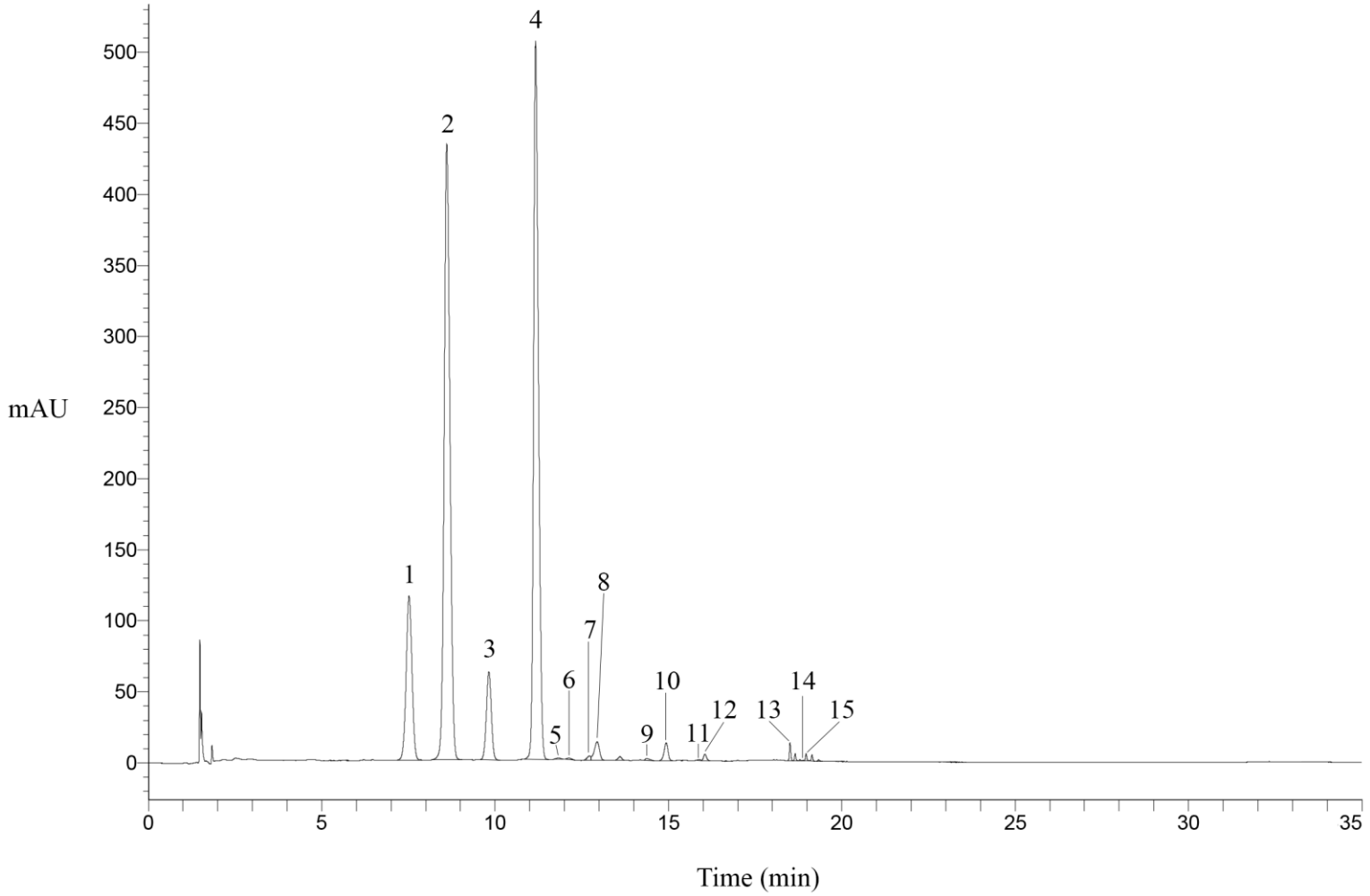


Figure 10. HPLC-DAD chromatogram of identified anthocyanin compounds of yeast W15. The numbering of the peaks corresponds to Table 14.

Table 14. Identified anthocyanins.

Nr	Compound	λ_{max} (nm)	t_r (min)	literature
1	delphinidin-3-*O*-glucoside	523	7.507 ± 0.047	a-c
2	delphinidin-3-*O*-rutinoside	524	8.589 ± 0.056	a-c
3	cyanidin-3-*O*-glucoside	515	9.805 ± 0.053	a-c
4	cyanidin-3-*O*-rutoniside	517	11.146 ± 0.067	a-c
5	petunidin-3-*O*-glucoside	279, 518	11.810 ± 0.048	c
6	cyanidin-3-*O*-arabinoside	277, 518	12.119 ± 0.047	c
7	petunidin-3-*O*-rutinoside	525	12.708 ± 0.081	b,c
8		526	12.936 ± 0.037	
9	pelargonidin-3-*O*-glucoside	278, 517	14.370 ± 0.036	c
10	pelargonidin-3-*O*-rutinoside	517	14.929 ± 0.033	c
11	peonidin-3-*O*-rutinoside	279, 522	15.887 ± 0.024	b,c
12		520	16.040 ± 0.059	
13	malvidin-3-*O*-glucoside *	280, 531	18.507 ± 0.012	b,c
14	delphinidin-3-*O*-(6´´-coumaroylglucoside)	282, 524	18.804 ± 0.144	b,c
15	cyanidin-3-*O*-(6´´-coumaroylglucoside)	282, 521	19.001 ± 0.045	b,c

a Mäkilä *et al.* (2016), b Nielsen *et al.* (2003), c Slimestad and Solheim (2002)

* can also be a malvidin-3-*O*-rutinoside

The numbering corresponds to Figure 10.

The identified anthocyanins are seen in Table 14, total of 15 different anthocyanins compounds were identified from the fermentations. The concentrations of anthocyanin compounds of the fermentations are seen in Table 15.

Table 15. The concentration of anthocyanins in the fermentations.

Nr	Biodiva (mg/100 g)	Condessa (mg/100 g)	W15 (mg/100 g)	ICV± K1 (mg/100 g)	Gaïa (mg/100 g)	Gaïa + W15 (mg/100 g)	Flavia (mg/100 g)	Flavia + W15 (mg/100 g)	Flavia + ICV± K1 (mg/100 g)	Before pasteurisation (mg/100 g)	After pasteurisation (mg/100 g)
1	8.8954 ± 1.6400	8.5531 ± 2.0746	8.2318 ± 0.5386	7.7478 ± 0.8751	9.9703 ± 0.7851	12.402 ± 1.9892	18.067 ± 2.5898	11.7303 ± 1.0468	8.7331 ± 0.7191	13.128 ± 4.9884	12.959 ± 2.1721
2	32.702 ± 5.1866	31.362 ± 6.9201	30.418 ± 1.9396	28.722 ± 3.0815	32.104 ± 2.2620	40.705 ± 5.9623	57.716 ± 8.3208	39.7176 ± 3.1146	31.1837 ± 3.2134	43.428 ± 16.3440	42.842 ± 7.4157
3	4.5522 ± 0.8898	4.3805 ± 1.0795	3.7958 ± 0.2267	3.5515 ± 0.4319	5.6151 ± 0.4168	6.0583 ± 1.0712	10.050 ± 1.3867	5.7789 ± 0.5263	4.0064 ± 0.3654	7.3011 ± 2.7467	7.2951 ± 1.2464
4	32.5243 ± 5.2052	31.100 ± 7.0035	30.282 ± 1.7412	28.700 ± 3.0978	33.1529 ± 2.2679	41.733 ± 6.0378	58.523 ± 8.3899	40.393 ± 3.1351	30.9565 ± 3.0736	43.541 ± 16.3895	43.589 ± 7.6481
5	0.0750 ± 0.0164	0.0723 ± 0.0203	0.0727 ± 0.0078	0.0784 ± 0.0141	0.1111 ± 0.0121	0.1078 ± 0.0175	0.2062 ± 0.0403	0.1143 ± 0.0224	0.0878 ± 0.0144	0.1818 ± 0.1424	0.1801 ± 0.1019
6	0.0914 ± 0.0098	0.0793 ± 0.0296	0.0877 ± 0.0087	0.0930 ± 0.0246	0.1206 ± 0.0174	0.1045 ± 0.0247	0.2056 ± 0.0239	0.1024 ± 0.0180	0.0986 ± 0.0231	0.1876 ± 0.0891	0.1777 ± 0.0537
7	0.1198 ± 0.0310	0.1225 ± 0.0292	0.1717 ± 0.0228	0.1366 ± 0.0183	0.3855 ± 0.0416	0.4399 ± 0.0623	0.5992 ± 0.0493	0.4379 ± 0.0159	0.2308± 0.0065	0.1296 ± 0.0515	0.3700 ± 0.0737
8	0.8672 ± 0.1296	0.8282 ± 0.1802	0.8119 ± 0.0581	0.7647 ± 0.0808	0.8284 ± 0.0567	1.0155 ± 0.1395	1.4766 ± 0.2086	1.0265 ± 0.0763	0.8287± 0.0818	1.1896 ± 0.4437	1.1471 ± 0.1993
9	0.0890 ± 0.0147	0.0941 ± 0.0125	0.0803 ± 0.0052	0.0881 ± 0.0198	0.0790 ± 0.0048	0.1068 ± 0.0481	0.2268 ± 0.0315	0.0989 ± 0.0133	0.0815 ± 0.0092	0.0961 ± 0.0337	0.1042 ± 0.0168

Nr	Biodiva (mg/100 g)	Condessa (mg/100 g)	W15 (mg/100 g)	ICV± K1 (mg/100 g)	Gaïa (mg/100 g)	Gaïa + W15 (mg/100 g)	Flavia (mg/100 g)	Flavia + W15 (mg/100 g)	Flavia + ICV± K1 (mg/100 g)	Before pasteurisation (mg/100 g)	After pasteurisation (mg/100 g)
10	0.6361 ± 0.0889	0.6129 ± 0.1193	0.5986 ± 0.0409	0.5634 ± 0.0559	0.7843 ± 0.3593	0.7843 ± 0.3593	1.1064 ± 0.1543	0.7821 ± 0.0566	0.6047 ± 0.0569	0.8710 ± 0.3236	0.8434 ± 0.1448
11	0.0330 ± 0.0027	0.0493 ± 0.0271	0.0335 ± 0.0007	0.0356 ± 0.0062	0.0352 ± 0.0031	nd	0.0746 *	nd	0.0419 ± 0.0109	0.0704 ± 0.0212	0.0192 *
12	0.0986 ± 0.0305	0.1110 ± 0.0316	0.2145 ± 0.0273	0.1699 ± 0.0174	0.2620 ± 0.0356	0.4408 ± 0.2678	0.4120 ± 0.0201	0.4713 ± 0.0190	0.2604 ± 0.0042	0.0570 ± 0.0198	0.2462 ± 0.0557
13	0.2434 ± 0.0447	0.2340 ± 0.0562	0.2308 ± 0.0135	0.2192 ± 0.0232	0.2642 ± 0.0231	0.3063 ± 0.0468	0.4709 ± 0.0652	0.2986 ± 0.0216	0.2347 ± 0.0202	0.3608 ± 0.1366	0.3594 ± 0.0610
14	0.1061 ± 0.0176	0.1034 ± 0.0212	0.1041 ± 0.0062	0.0964 ± 0.0099	0.1156 ± 0.0108	0.1324 ± 0.0554	0.1991 ± 0.0262	0.1265 ± 0.0088	0.1036 ± 0.0073	0.1617 ± 0.0608	0.1615 ± 0.0277
15	0.0367 ± 0.0099	0.0405 ± 0.0047	0.0957 ± 0.0119	0.0144 ± 0.0006	nd	0.0485 ± 0.0539	0.0253 ± 0.0010	0.0318 ± 0.0035	nd	0.0112 ± 0.0068	0.0160 ± 0.0022

nd, not detected

The standard deviation is calculated from three parallel averages. The numbering corresponds to Figure 10.

* no standard deviation calculated, since was only identified in one sample.

4 Discussion

The blackcurrant used in this study was market variety; it was not possible to identify the cultivar. It is known that different cultivars may have major differences in chemical composition, where also the harvest time affects it. This time it was not possible to identify the blackcurrant used, but it is something that must be taken into account in next studies.

The pasteurisation was done each time at the same water temperature, which was nearly 100 °C at that point. Adding the room temperature juices cooled the water down a little bit before the temperature rose again to the boiling point. Because the pasteurisation was done by hand in boiling water, a quite large error margin is present. As seen in Figure 1 the pasteurisation bottles did not reach the pasteurisation temperature at the same time between different pasteurisations. The cooling down was done in an ice bath, where the whole bottle stand did not fit correctly. It did cause inconsistent cooling which was present especially in the 3rd pasteurisation, where the temperature did not lower steadily. It is assumed that the pasteurisation worked and all the bacteria were destroyed during it.

Temperature stayed the same during the fermentations inside the box. The bottles were only removed from the storage during the brix measurements. The box made sure that some sudden temperature changes in the outside environment did not affect the fermentation bottles and it also helped to ensure that all the fermentations were accomplished the same way.

Blackcurrant must has a higher concentration of fructose and glucose after the pasteurisation and the sucrose concentration has lowered, this would give evidence on an invertase activity after pasteurization (Table 9.). Lewis and Young (2012) reported that the invertase activity usually is a sign that the pasteurisation heat has been insufficient. Is might also be possible that the invertase had activity during the pasteurisation, but not after it.

Flavia and Gaïa fermentations have a higher concentration of fructose and glucose than the blackcurrant must before fermentation. In 24 hours the sucrose concentration has

lowered about 0.1 g/100 mL; this is a result of the invertase activity by yeast. The sequential inoculation did not elevate the invertase activity and the three fermentations made with sequential inoculation have the highest concentration of sucrose. All the other yeasts consumed the sugars evenly. Condessa yeast took the most time to ferment, but it still did not have the lowest total concentration of sugars.

Wang et al. (2016) reported that *M. pulcherrima* has a higher consumption rate of glucose when sequentially inoculated with *S. cerevisiae* yeasts. This is true to the findings presented in this work; all the sequentially inoculated fermentations, with yeast Flavia and also with the *M. fructicola* yeast Gaïa, consumed all the glucose. All fructose was also consumed by the sequentially inoculated fermentations.

Succinic acid was not detected from Flavia or Gaïa fermentations, as there has no alcoholic fermentation taken place. Succinic acid was only detected in the fermentations where alcohol was produced. Jolly et al (2013) has reported that the formation of succinic acid correlates to high ethanol production and tolerance. The ethanol concentration was highest with the ICV-K1 yeast, which did not however produce the largest amount of succinic acid. The succinic acid was produced less in the sequential inoculation fermentations, and there was also less ethanol detected in those samples.

Coulter et al (2004) reported that *S. bayanus* has usually a higher yield of succinic acid produced. Condessa fermentation had the highest concentration of succinic acid, 0.1043 g/100 mL, compared to all the other yeasts; even with the standard deviation taken into account. The ethanol concentration of Condessa yeast was not especially high, but not low either.

Fermentation made with Biodiva yeast should have a lower concentration of malic acid as it has a higher compatibility for MLF. The malic acid concentration of Biodiva is one of the lowest, lower it is only in the Gaïa + W15 sequential inoculation fermentation. The low malic acid concentration usually is a sign that MLF has been present in the fermentation, but not any lactic acid was detected in any of the samples.

Richard and Hilditch (2009) reported that galacturonic acid is the main constituent of pectin and that pectin can be hydrolysed into galacturonic acid by enzymes.

Galacturonic acid was only detected in the sequential inoculation samples. It might be possible that the sequential inoculation did hydrolyse the pectin and turned it into galacturonic acid.

No pyruvic acid was detected in any sample which is a sign that AF was completed in all of the fermentations and also, if glyceropyruvic fermentation had taken place, all pyruvate had turned into secondary products.

Jolly et al. (2013) reported that the use of non-*Saccharomyces* yeasts yields lower ethanol concentration. This is not entirely true to the findings in this thesis. Ethanol concentration of Biodiva sample was as high as the fermentations made with *Saccharomyces* yeasts and the sequential inoculation fermentations produced only a little less ethanol than the fermentation made with only *Saccharomyces* yeasts. These results are not in conclusion with the Jolly et al. (2013) study as no link between non-*Saccharomyces* yeast and low ethanol concentration was found. The ethanol concentration was not measured from only Flavia or Gaïa fermentations, since those two yeasts do not produce any alcohol during the fermentations.

Wang et al. (2016) reported that *T. delbrueckii* strains and *M. pulcherrima* strains have a small decrease in culturability during fermentation. This would also affect the fermentation properties of the yeasts. While the fermentations made with Flavia yeast had the lowest concentrations of ethanol, Biodiva did have one of the highest concentrations of ethanol.

Methanol concentration is lowest with the fermentations made with W15 yeast. The sequential inoculation fermentations have even lower concentration than the single yeast W15 fermentation. None of the samples have toxic methanol concentration. Ribereau-Gayon et al. (2006b) wrote that methanol is formed from pectins. Pectins are present in the must and pectin methylesterase enzyme in yeasts. Because the methanol concentration is not the same in each fermentation the other yeasts must have had a higher enzyme concentration than the W15 yeast.

1-Propanol is formed from pyruvate during AF. It has a low concentration in all of the fermentations and there does not seem to be any difference between the different yeasts.

Isobutanol and isoamyl alcohol are formed from amino acid precursors. There is no big difference between the results for both of these fusel alcohols; only Condessa and ICV-K1 have slightly higher results.

Both flavonols and anthocyanins have been identified using literature. The identification was done comparing retention times and λ_{max} of each compound to compounds presented in literature. If the λ_{max} varied too much from the usual one for both flavonols and anthocyanins the compound was rejected from the results. There are a few compounds that were not able to be identified; the λ_{max} would mean that those are flavonols or anthocyanins, but were not found in any literature.

Slimestad and Solheim (2002) reported that the first four anthocyanins (delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside) are the most abundant ones in blackcurrants. Woodward et al. (2011) reported that the concentration of the four most common anthocyanins are not affected by the juice processing. There is a small decrease in anthocyanins concentration after pasteurisation, but considering the standard deviation limits there might not be a difference at all.

Belda et al (2016) reported that *M. pulcherrima* yeast used in sequential inoculation together with *S. cerevisiae* yeast increases the total phenolic compound concentration compared to the single inoculation with *S. cerevisiae*. All of the sequential inoculations increased total concentration of flavonols, also the one where *M. fructicola* yeast were used. The total concentration of anthocyanins did decrease in both sequential inoculations as well as regular fermentations.

5 Conclusions

The environment, like nitrogen, low oxygen and increased alcohol, has an effect on the yeast fermentability. Also the changes in the culture media, in this case the blackcurrant juice, affect the final product. During this study only temperature was monitored, but must acidity is also one attribute that affects the final results. In the follow up studies acidity, before and during fermentation, should be monitored. All the wines were stored in –70 °C after fermentation; at this point it is not sure how this affects the wine quality and that way the final chemical composition. One follow up study would be to study the difference between different storing conditions.

It is possible that batches have been affected to different amounts of oxygen during fermentation, which would change the final results; as the low oxygen concentration causes some yeasts to die prematurely during AF. The interactions between the yeasts might not be repeated in bigger batches and these results only relate to the sample size made in this thesis. The impact on how much the environment affects these fermentations is not clear. Other follow up study would be to test different batch sizes in different environmental conditions.

It is possible to break down pectins with enzyme. If the pectins are broken down enzymatically during the juice pressing the methanol concentration would only be the result about the pectin activity of the yeasts. This is something that could be taken into account in the next studies.

All in all the study did find differences between the yeasts used and how they affect the chemical characteristics of the blackcurrant. Some yeasts had larger impact and especially the sequential inoculation did give the most interesting results. The sequential inoculation did use up all glucose and fructose and released galacturonic acid from pectins. Blackcurrant does not naturally contain any succinic acid, so the succinic acid concentration was completely the result of yeast activity. As the blackcurrants were ripe, but not outdated, no spontaneous AF had taken place. Therefore all the alcohol concentrations were the result of AF by yeasts. Fermentation seems to increase the concentrations of flavonols, but decreases the total concentration of anthocyanins.

References

Belda, I., Conchillo, L.B., Ruiz, J., Navascués, E., Marquina, D. & Santos, A. (2016) Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking. *Int J Food Microbiol.*, **223**: 1−8.

Borges, G., Degeneve, A., Mullen, W. & Crozier, A. (2010) Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants and cranberries. *J. Agric. Food Chem.*, **58**: 3901−3909.

Ciani, M. & Comitini, F. (2011) Non-*Saccharomyces* yeasts have a promising role in biotechnological approaches to winemaking. *Ann. Microbiol.*, **61**: 25−32.

Coulter, A.D., Godden, P.W. & Pretorius P.S. (2004) Succinic acid − How is it formed, what is its effect on titratable acidity, and what factors influence its concentration in wine? *Australian and New Zealand Wine Industry Journal*, **19**: 16–25.

Dorokhov Y.L., Shindyapina, A.V., Sheshukova, E.V. & Komarova, T.V. (2015) Metabolic methanol: Molecular pathways and physiological roles. *Physiol. Rev.*, **95**: 603–644.

Eaton. Lalvin W 15 technical datasheet. <http://www.eaton.de/EN/ecm/idcplg?IdcService=GET_FILE&allowInterrupt=1&RevisionSelectionMethod=LatestReleased&noSaveAs=0&Rendition=Primary&dDocName=PCT_868237>. (Accessed 10.07.2018).

Gibney, P.A., Schieler, A., Chen., J.C., Raboniwitz, J.D. & Botsstein, D. (2015) Characterizing the in vivo role of trehalose in *Saccharomyces cerevisiae* using the AGT1 transporter. *Proc Natl Acad Sci USA.*, **112**: 6116–6121.

Jacobson, J.L. (2006) Introduction to Wine Laboratory Practices and Procedures, 1st ed., pp. 61–172. Springer Science & Business Media, New York, USA

Gschaedler, A. (2017) Contribution of non-conventional yeasts in alcoholic beverages. *Curr. Opin. Food Sci.*, **13**:73–77.

Jolly, N.P., Varela, C. & Pretorius, I.S. (2014) Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research*, **14**: 215–237.

Lallemand Brewing. Lalvin ICV K1-V1116 technical datasheet. <http://www.lallemandbrewing.com/wp-content/uploads/2017/03/icv-k1_yeast_0.pdf>. (Accessed 10.07.2018).

Lallemand Oenology. Biodiva™ technical datasheet. <http://catalogapp.lallemandwine.com/uploads/yeasts/docs/d441f52d43e8c74d0071debbd7ff415c479e1fdf.pdf>. (Accessed 10.07.2018).

Lallemand Oenology. Flavia™ technical datasheet. <http://catalogapp.lallemandwine.com/uploads/yeasts/docs/fda8d509060cd5c8ca43be62c5c63ff421103fcb.pdf>. (Accessed 10.07.2018).

Lallemand Oenology. Gaïa™ technical datasheet. <http://catalogapp.lallemandwine.com/uploads/yeasts/docs/fb8e1d0b8c8bb51c62288183495020f49c99e0f4.pdf>. (Accessed 10.07.2018).

Lallemand Oenology. Product details for Lalvin W 15. http://www.lallemandwine.com/en/central-europe/products/catalogue/wine-yeasts/44/lalvin-w15/ (Accessed 11.07.2018).

Laminkara, O. (1997) Changes in organic acid composition during fermentation and aging of noble muscadine wine. *J. Agric. Food Chem.*, **45**: 935−937.

Lewis, M.J. & Young, T.W. (2012) Brewing, 2nd ed., pp. 322–325. Kluwer Academic/Plenum Publishers, Dordrecht, Netherlands

Mateo, J.J., Jiménez, M., Pastor, A. & Huerta, T. (2000) Yeast starter cultures affecting wine fermentation and volatiles. *Food Res. Int.*, **34**: 307−314.

Mazzoni, L., Perez-Lopez, P., Giampieri, F., Alvarez-Suarez, J. M., Gasparrini, M., Forbes-Hernandez, T.Y., Quiles, J.L., Mezzettic, B. and Battino, M. (2016) The Genetic aspects of berries: from field to health. *J. Sci. Food Agric.*, **96**: 365–371.

Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., & Veberic, R. (2012) Composition of sugars, organic acids, and total phenolics in 25 wild or cultivated berry species. *J. Food Sci.* **77**: C1064−C1070

Mäkilä, L., Laaksonen, O., Alanne, A-L., Kortesniemi, M., Kallio, H. & Yang B. (2016) Stability of hydroxycinnamic acid derivatives, flavonyl glycosides, and anthosyanins in black currant juice. *J. Agric. Food Chem.*, **64**: 4584−4598.

National Food Composition Database in Finland. Blackcurrant food compoments. https://fineli.fi/fineli/en/elintarvikkeet/444?q=mustaherukka&foodType=ANY&portionUnit=G&portionSize=100&sortByColumn=name&sortOrder=asc&component=2331& (Accecced 19.09.2018)

Nielsen, I.L.F., Haren, G.R., Magnussen, E.L., Dragsted, L.O. & Rasmussen S.E. (2003) Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency. *J. Agric. Food Chem.*, **51**: 5861−5866.

Pakkasmarja Oy. Product details for commercial Blackcurrant. https://www.pakkasmarja.fi/tuotteet/kotimaiset-pakastemarjat/viljellyt-marjat/mustaherukka-200g/ (Accecced 19.09.2018).

Richard, P. & Hilditch, S. (2009) D-Galacturonic acid catabolism in microorganisms and its biotechnological relevance. *Appl Microbiol Biotechnol.*, **82**: 597−604.

Ribereau-Gayon, P., Dubourdieu, D., Doneche, B. & Lonvaud, A. (2006a) Handbook of Enology Volume 1 The Microbiology of wine and vinifications, 2nd ed., pp. 1–76. John Wiley & Sons Ltd, Chichester, UK

Ribereau-Gayon, Y., Glories, P., Maujean, A. & Dubourdieu, D. (2006b) Handbook of Enology Volume 2 The Chemistry of wine stabilization and treatments, 2nd ed., pp. 61–172. John Wiley & Sons Ltd, Chichester, UK

Saarela, A.-M., Hyvönen, P., Määttälä, S. & von Wright, A. (2010) Elintarvikeprosessit, 3rd ed., pp. 217–218. Savonia-ammattikorkeakoulu, Kuopio, Finland

Slimestad R. & Solheim H. (2002) Anthocyanins from black currants (*Ribes nigrum L.*). *J. Agric. Food Chem.*, **50**: 3228−3231.

Steensels, J. & Verstrepen, K.J. (2014) Taming wild yeast: Potential of conventional and nonconventional yeasts in industrial fermentations. *Annu. Rev. Microbiol.* **68**: 61–81

Styger, G., Prior, B. & Bauer F.F. (2011) Wine flavour and aroma. *J. Ind. Microbiol. Biotechnol.*, **38**: 1145−1159

VTT Technical Research Centre of Finland Ltd. Selvitys marjojen ja marjasivuvirtojen hyödyntämispotentiaalista Suomessa. <https://media.sitra.fi/2017/02/27173257/VTTn20marjaselvitys20b-2.pdf> (Accecced 19.09.2018).

Wang, C., Mas, A. & Esteve-Zarzoso, B. (2016) The Interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strains specific. *Front. Microbiol.*, **7**: 1−10.

Woodward G.M, McCarthy, D., Pham-Thanh, D. & Kay, C.D. (2011) Anthocyanins remain stable during commercial blackcurrant juice processing. *J. Food Sci.*, **76**: S408−S414