DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

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ABSTRACT

Berries are rich in health-promoting bioactive components and berry consumption may be associated with a lower risk of various chronic diseases. However, only a fraction of the annual yield of berries is exploited and consumed. Development of berry wines may present an approach to increase the utilization of berries. The potential of non-*Saccharomyces* yeasts is increasingly recognized and explored in wine fermentation, whereas little has been done in berry wine production using non-*Saccharomyces* yeasts. Production of berry wines using non-*Saccharomyces* yeasts is a novel biotechnological approach for creating value-added products for the global market. The quality of berry wines is determined by the composition analysis, including the profile and content of both volatile and non-volatile compounds. Systematic research is needed to study the effects of non-*Saccharomyces* yeasts on the chemical composition of berry wines.

In this work, our aim was to study inoculation with different yeast species/strains and fermentation methods on composition of bilberry (*Vaccinium myrtillus* L.) wines. The specific purposes were: 1) to characterize the volatile and/or non-volatile compositions of blue (BB) and white bilberries (WB) juices and their corresponding wines produced by conventional fermentation with *Saccharomyces cerevisiae*; 2) to study the effect of non-*Saccharomyces* yeast and inoculation type on the chemical profiles of BB wines; 3) to compare the dynamic changes in volatile compounds during alcoholic fermentation of BB wines using diverse non-*Saccharomyces* yeasts; 4) to monitor the evolutions of pyranoanthocyanins and their precursor anthocyanin monomers during aging of BB wines fermented with *Saccharomyces* and non-*Saccharomyces* yeasts. Volatile compounds were measured using headspace-solid phase microextraction gas chromatography-mass spectrometry methods. Non-volatile compounds of phenolic compounds were qualitatively and quantitatively analyzed using liquid chromatography coupled with a diode array detector and a mass spectrometer, while ethanol, sugars, and organic acids by gas chromatography-flame ionization detection.

In BB juice, monomeric anthocyanins dominated among the phenolic compounds. Galactosides and glucosides delphinidin and cyanidin were the major anthocyanins among the 15 detected monomeric anthocyanins. Among the 42 nonanthocyanin phenolic compounds detected in BB and WB juices and wines, the levels of most individual compounds in nonpigmented bilberry products were significantly lower than those in pigmented ones. *p*-Coumaroyl monotropeins and quercetin glycosides both were the most predominant compounds in the groups of phenolic acids and flavonols, respectively, whereas the major flavan-3-ols were procyanidin B-type dimer and (−)-epicatechin.

During fermentation with *S. cerevisiae*, the changes in content of nonanthocyanin phenolic compounds depended on the mutation of color. Fermentation significantly elevated the total contents of flavonol (TFO) and phenolic acids (TA) in WB samples. However, TA and TFO in BB juice showed a slight increase and reduction, respectively.

The content of monomeric anthocyanins reduced considerably after fermentation. BB wines produced from pure, sequential, and simultaneous inoculations involving *Schizosaccharomyces pombe* 70572 possessed higher contents of total and most individual monomeric anthocyanins than those with *Torulaspora delbrueckii* and *S. cerevisiae* strains. Yeast fermentation significantly enhanced aroma intensity and volatile complexity of BB juice, particularly the groups of higher alcohols and esters. The contribution to volatile composition of bilberry wines was yeast and inoculation type dependent. Pure fermentations with *T. delbrueckii* and *Metschnikowia pulcherrima* strains were characterized by the high productions of higher alcohols, *Saccharomycodes ludwigii* by esters, and *Zygosaccharomyces bailii* by fatty acids, while fermentations with *S. pombe*, *Hanseniaspora uvarum*, and *Issatchenkia orientalis* strains yielded more off-flavor compounds than that with *S. cerevisiae*. Further improvements of aroma intensity were confirmed in sequential and simultaneous fermentations with *S. cerevisiae* and *T. delbrueckii* or *S. pombe* strains. Moreover, co-fermentation affected volatile profiles of particularly higher alcohols, esters, and carbonyl compounds of BB wines.

In general, fermentation kinetics of non-*Saccharomyces* yeasts was less vigorous than that of *S. cerevisiae* as indicated by low ethanol production or poor sugar consumption. During fermentation, dynamic changes in volatile compounds were determined simultaneously with the development of ethanol concentration. There were also strain-dependent variations with regard to generation and degradation of volatile compounds.

The content of monomeric anthocyanins in BB wines declined significantly during 12 months of aging, and a fraction of the reduction were formed vitisin A-type pyranoanthocyanins (vAPs). Fifteen vAPs were identified in aged bilberry wines. The high generation of pyruvic acid from the metabolism of *S. pombe* strains boosted the formation of vAPs in the wine products. The residual pyruvic acid in fresh bilberry wines consecutively reacted with anthocyanin monomers during aging, and the content of vAPs reached the maximum after 6 months of aging. Sugar moieties in monomeric anthocyanins affected the condensation reactions with pyruvic acid. Pyranoanthocyanins were more stable than their corresponding monomeric anthocyanins, therefore contributing to the stabilization of the color of the berry wines. Methylation in B-ring stabilized the structures of monomeric anthocyanins and pyranoanthocyanins.

SUOMENKIELINEN ABSTRAKTI

Marjoissa on paljon terveydelle edullisia bioaktiivisia yhdisteitä ja marjojen käyttäminen voidaan yhdistää alempaan riskiin saada erilaisia kroonisia tauteja. Vain osa vuotuisesta marjasadosta kuitenkin hyödynnetään. Marjaviinien kehittäminen voi olla yksi mahdollinen tapa lisätä marjojen hyötykäyttöä. *Saccharomyces*-sukuun kuulumattomia hiivoja tarkastellaan ja käytetään kasvavissa määrin viinien valmistuksessa, mutta niiden hyödyntäminen marjaviineissä on ollut vähäistä. *Saccharomyces*-sukuun kuulumattomat hiivat marjaviinien valmistuksessa on uusi bioteknologinen lähestymistapa uusien lisäarvotuotteiden tuomisessa globaaleille markkinoille. Marjaviinien laatu voidaan määrittää haihtuvien ja haihtumattomien yhdisteiden koostumuksen ja pitoisuuksien analyyseilla. Systemaattisia tutkimuksia tarvitaan selvittämään *Saccharomyces*-sukuun kuulumattomien hiivojen vaikutuksesta marjaviinien kemialliseen koostumukseen.

Tämän työn tavoitteena oli tutkia erilaisten hiivalajien, -kantojen ja käymismenetelmien vaikutusta mustikasta (*Vaccinium myrtillus* L.) valmistettujen viinien koostumukseen. Erityisinä tavoitteina oli: 1) karakterisoida sinisen (BB) ja valkoisen (WB) mustikkamehun ja niistä *Saccharomyces cerevisiae* -hiivalla valmistettujen marjaviinien haihtuvien ja haihtumattomien yhdisteiden koostumusta; 2) tarkastella *Saccharomyces*-sukuun kuulumattomien hiivojen ja hiivan ymppäystavan merkitystä mustikkaviinin (BB) kemiallisessa koostumuksessa; 3) vertailla *Saccharomyces*-sukuun kuulumattomilla hiivoilla valmistettujen mustikkaviinien (BB) haihtuvien yhdisteiden dynaamisia muutoksia käymisen aikana; 4) seurata pyranoantosyaniinien ja niiden monomeeristen antosyaniiniesiasteiden muodostumista *Saccharomyces*-sukuun kuuluvilla ja kuulumattomilla hiivoilla valmistettujen mustikkaviinien (BB) kypsymisen aikana. Haihtumattomia yhdisteitä mitattiin viinien ilmatilasta kiinteäfaasimikrouutolla ja kaasukromatografi–massaspektrometri-laitteistolla. Haihtumattomat fenoliset yhdisteet määritettiin laadullisesti ja määrällisesti nestekromatografilla yhdistettynä diodirividetektoriin ja massaspektrometriin, ja puolestaan etanoli, sokerit ja orgaaniset hapot määritettiin kaasukromatografi–liekki-ionisaatiodetektori-laitteistolla.

Sinisessä mustikkamehussa monomeeriset antosyaniinit olivat merkittävin fenolisten yhdisteiden ryhmä. Delfinidiinin ja syanidiinin galaktosidit ja glukosidit olivat pääasiallisimmat antosyaniinit 15:n havaitun yhdisteen joukossa. Sinisistä (BB) ja valkoisista (WB) mustikkamehuista ja -viineistä määritettiin 42 antosyaniineihin kuulumatonta fenolista yhdistettä ja useampien niiden määrät olivat alhaisemmat pigmentoimattomissa tuotteissa kuin pigmentoiduissa. Kversetiinin glykosidit ja *p*-kumaroyylimonotropeiinit olivat

merkittävimmät fenolisten happojen ja flavonolien yhdisteet näytteissä, kun taas prosyaniidien B-tyypin dimeeri ja (–)-epikatekiini olivat pääasiallisimmat flavan-3-oli-yhdisteet. *S. cerevisiae* -hiivakäymisen vaikutus näiden yhdisteiden koostumukseen oli riippuvainen marjan värin mutaatiosta: käyminen lisäsi flavonolien ja fenolisten happojen kokonaismäärää valkoisesta mustikasta valmistetuissa näytteissä. Sinisestä mustikasta valmistetuissa viineissä fenolisten happojen määrä kasvoi hieman, kun taas flavonolien määrä laski.

Monomeeristen antosyaniinien määrä laski merkittävästi käymisen aikana. Mustikkaviineissä (BB), jotka valmistettiin ymppäämällä joko yksin, peräkkäin tai samanaikaisesti *Schizosaccharomyces pombe* -hiivan kantaa 70572, oli enemmän kokonaismäärältään antosyaniineja ja suurinta osaa yksittäisiä monomeerisia antosyaniineja verrattuna *Torulaspora delbrueckii* ja *S. cerevisiae* -hiivakantojen avulla valmistettuihin viineihin. Hiivakäyminen paransi merkitsevästi mustikkamehun aromien intensiteettiä ja haihtuvien yhdisteiden monimutkaisuutta vaikuttamalla erityisesti korkeampien alkoholien ja esterien yhdisteryhmiin. Haihtumattomien yhdisteiden merkitys oli riippuvaista hiivasta ja ymppäysmenetelmästä. Käyminen pelkillä *T. delbrueckii* and *Metschnikowia pulcherrima* -kannoilla sai aikaan korkeampien alkoholien määrän, kun taas *Saccharomycodes ludwigii* -hiivakannalla esterien määrä kasvoi ja *Zygosaccharomyces bailii* -hiivakannalla rasvahappojen määrä erottui muista. *S. pombe*, *Hanseniaspora uvarum* ja *Issatchenkia orientalis* -hiivakannoilla valmistetuissa näytteissä puolestaan esiintyi enemmän haittahajuiksi luokiteltavia yhdisteitä verrattuna *S. cerevisiae* -hiivakantaan. Aromien intensiteetin parantumista havaittiin myös näytteissä, joissa *S. cerevisiae* ja *T. delbrueckii* tai *S. pombe* -hiivakantoja lisättiin peräkkäisesti tai samanaikaisesti. Useamman hiivakannan ymppääminen yhdessä (peräkkäisesti tai samanaikaisesti) vaikutti erityisesti korkeampien alkoholien, esterien ja karbonyyliyhdisteiden profiileihin mustikkaviineissä.

Saccharomyces-sukuun kuulumattomilla hiivoilla käymisen kinetiikka oli yleisesti hillitympi verrattuna *S. cerevisiae* -kantoihin, mikä havaittiin alhaisemmasta etanolin muodostumisesta ja huonommasta sokerien hyödyntämisestä. Haihtuvien yhdisteiden dynaamisia muutoksia tarkasteltiin samanaikaisesti etanolin määrän muodostumisen kanssa käymisen aikana. Näissä muutoksissa havaittiin myös hiivakannasta riippuvia haihtuvien yhdisteiden muodostumisia ja hajoamisia.

Monomeeristen antosyaniinien yhdisteiden määrä mustikkaviineissä (BB) laski merkitsevästi 12 kuukauden kypsymisen aikana. Osa laskusta selittyi vitisiini A -tyypin pyranoantosyaniinien muodostumisella (vAP). Kypsytetyistä mustikkaviineistä havaittiin 15 vAP-yhdistettä. *S. pombe* -hiivakannan korkea palorypälehapon muodostuskyky paransi vAP-yhdisteiden muodostumista. Mustikkaviinien vapaa palorypälehappo reagoi monomeeristen antosyaniinien

kanssa kypsymisen aikana ja vAP-yhdisteiden määrä saavutti huippunsa kuuden kuukauden kypsymisen kohdalla. Pyranoantosyaniinit olivat vakaampia verrattuna vastaaviin monomeerisiin antosyaniineihin vaikuttaen siten viinien värin säilyvyyteen. B-renkaan metylaatio vakautti monomeeristen antosyaniinien ja pyranoantosyaniinien rakenteita.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ANS	anthocyanidin synthase
BB	blue bilberry
CHS	chalcone synthase
cy	cyanidin
DAD	diode array detector
DFR	dihydroflavonol 4-reductase
DGGE	denaturing gradient gel electrophoresis
DHBA	3,4-dihydroxybenzoic acid
DP	degree of polymerization
dp	delphinidin
DPPH·	1,1-diphenyl-2-picrylhydrazyl
DW	dry weight
ESI	electrospray ionization
FGT	flavonoid 3-*O*-glycosyltransferase
F3H	flavanone 3-hydroxylase
FID	flame ionization detector
FT-IR	Fourier transform infrared spectroscopy
FW	fresh weight
GC	gas chromatography
HCA	hydroxycinnamic acid
HPLC	high performance liquid chromatography
HS-SPME	headspace solid-phase microextraction
ITS	internal transcribed spacers
LLE	liquid-liquid extraction
LOD	limits of detection
LOQ	limits of quantitation
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
mv	malvidin
nd	not detected
OAV	odor activity value
OIV	International Organization of Vine and Wine
PAVC	primary aroma volatile compound
PCA	principal component analysis
PCR	polymerase chain reaction
PLS-DA	partial least squares discriminant analysis
pn	peonidin
PR	pure fermentation

pt	petunidin
QTOF	quadrupole time of flight
SAVC	secondary aroma volatile compound
S/N	signal-to-noise ratio
SM	simultaneous fermentation
SPE	solid-phase extraction
SPME	solid-phase microextraction
SQ	sequential fermentation
TA	total phenolic acid content
TACY	total anthocyanin content
TAVC	tertiary aroma volatile compound
TCA	tricarboxylic acid
TDN	1,1,6-trimethyl-1,2-dihydro-napthalene
TFA	total flavan-3-ol content
TFO	total flavonol content
TMACY	total monomeric anthocyanin content
TMS	trimethylsilyl
TPA	total proanthocyanidin content
TPC	total phenolic compound content
UHPLC	ultra-high performance liquid chromatography
UPLC	ultra performance liquid chromatography
UV–Vis	ultraviolet–visible spectrophotometry
vAPs	vitisin-A type pyranoanthocyanins
WB	white bilberry
YAN	yeast assimilable nitrogen

LIST OF ORIGINAL PUBLICATIONS

I. Liu, S., Laaksonen, O., Kortesniemi, M., Kalpio, M., & Yang, B. (**2018**). Chemical composition of bilberry wine fermented with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* and *Schizosaccharomyces pombe*) and *Saccharomyces cerevisiae* in pure, sequential and mixed fermentations. *Food Chemistry*, 266, 262–274.

II. Liu, S., Laaksonen, O., & Yang, B. (**2019**). Volatile composition of bilberry wines fermented with non-*Saccharomyces* and *Saccharomyces* yeasts in pure, sequential and simultaneous inoculations. *Food Microbiology*, 80, 25–39.

III. Liu, S., Laaksonen, O., Yang, W., Zhang, B., & Yang, B. (**2020**). Pyranoanthocyanins in bilberry (*Vaccinium myrtillus* L.) wines fermented with *Schizosaccharomyces pombe* and their evolution during aging. *Food Chemistry*, 305, 125438.

IV. Liu, S., Laaksonen, O., Marsol-Vall, A., Zhu, B., & Yang, B. (**2020**). Comparison of volatile composition between alcoholic bilberry beverages fermented with non-*Saccharomyces* yeasts and dynamic changes in volatile compounds during fermentation. *Journal of Agricultural and Food Chemistry*, 68, 3626–3637.

V. Liu, S., Marsol-Vall, A., Laaksonen, O., Kortesniemi, M., & Yang, B. (**2020**). Characterization and quantification of nonanthocyanin phenolic compounds in white and blue bilberry (*Vaccinium myrtillus*) juices and wines using UHPLC-DAD–ESI-QTOF-MS and UHPLC-DAD. *Journal of Agricultural and Food Chemistry,* 68, 7734–7744.

1 INTRODUCTION

Winemaking, referring to the process of conversion from grape must or juice to wine, is one of the most ancient food processing technologies having a history of thousands of years and is closely linked to the evolution of human civilization.[1,2] Winemaking is a complex biotechnological process with diverse and important metabolites determining the sensory properties of wine. These compounds are concurrently generated or degenerated during the accumulation of ethanol. A well-practiced winemaking technology is one of the critical factors for producing pleasant wines,[3] of which, alcoholic fermentation is an essential process of winemaking and is determined by the presence of different yeasts. In wine industry, optimization of control of alcoholic fermentation during winemaking is an important practice to obtain wine products with high quality. After a long time of exploration in microbiology, *Saccharomyces cerevisiae* has been recognized by enologists by its outstanding and stable fermentation performance and has been widely used for controlling alcoholic fermentation and achieving desirable enological characters.

Non-*Saccharomyces* yeasts, also known as non-conventional yeasts, have been considered as problematic yeasts due to their close association with undesirable spoilage fermentation.[4,5] Nowadays, the role of non-*Saccharomyces* yeasts in alcoholic fermentation has been re-evaluated due to the increasingly reported positive contributions to wine characters through appropriate inoculation approaches and fermentation conditions.[6–9] The group of non-*Saccharomyces* yeasts has far more diverse members than that of *S. cerevisiae*. Therefore, the utilization of non-*Saccharomyces* yeasts in winemaking provides more possibilities of diversified wine characteristics. This opportunely meets the growing demand, in recent years, for novel styles of wines by consumers.

In comparison to grapes, there is a larger collection of species and varieties of nongrape berries. Berries have various health-promoting effects benefiting from the high contents of bioactive compounds, particularly phenolic compounds.[10,11] Berries are usually consumed as fresh or processed into preserves, juices, jams, canned fruits, and jellies to prolong their shelf life and to minimize the postharvest loss. In recent years, the awareness of the nutritional value and health-related properties of berries and the demand for novel berry products by consumers are continuously increasing. These factors have promoted the development and consumption of berry wines.[12,13] Although berry wines do not have a long and prestigious history as grape wines, they are gaining increasing popularity as more attention is being given to the novel range of commercial opportunities and health benefits of berry wines. However, the prevalent studies, at the moment, are mainly focusing on the effect of fermentation using the conventional *S. cerevisiae* on the chemical composition of berries or berry juices.

The application of non-*Saccharomyces* yeasts in berry wine production is very few, close to negligible, unlike the situation in wines. Hence, more studies are needed to investigate the effect of non-*Saccharomyces* yeasts on the characteristics and quality factors of berry wines.

In the literature review part, chemical compounds, including volatile and non-volatile compounds, that have been commonly studied in wines are examined to emphasize their importance in determining wine characters. Due to the extreme scarcity of previous studies on the unitization of non-*Saccharomyces* yeasts in berry wine productions, we have reviewed the previous studies on the effect of non-*Saccharomyces* yeasts on chemical composition of wines during alcoholic fermentation and aging as important references for further investigation on the influences of non-*Saccharomyces* yeast on berry wines. Moreover, opportunities and prospects of the development of berry wines were discussed.

In the experimental part of the doctoral thesis, bilberry (*Vaccinium myrtillus* L.) was chosen as the representative fermentation substrate due to its abundance in the forests particularly in Northern Europe, and its desirable taste and richness of health-beneficial substances. The chemical compositions of juices and wine products produced from pigmented and nonpigmented bilberries with conventional *S. cerevisiae* were investigated. The chemical difference between the final bilberry wines fermented with *S. cerevisiae* and those fermented with diverse non-*Saccharomyces* yeasts was compared. The dynamic change in volatile compounds in bilberry wines during alcoholic fermentation with non-*Saccharomyces* yeasts were studied. Further, the evolution of pyranoanthocyanins and their precursor compounds during aging was monitored. The present study provides novel findings on impact of conventional and non-conventional yeast fermentation on the composition of berry wines. The study produces new insights on the potential of exploiting bilberries in berry wine industry.

2 REVIEW OF THE LITERATURE

2.1 Chemical compounds associated with wine characters

According to a statement in the International Code of Enological Practices issued by International Organization of Vine and Wine (OIV), wine is exclusively defined as the alcoholic beverage with an actual alcohol content higher than 8.5% (*v/v*) resulting from partial or complete alcoholic fermentation of grape or grape must/juice. There are abundant complex steps throughout the process from grape cultivation to wine product acquisition. The characters of wine are determined by grape quality, winemaking practice, and/or aging technique. Color, taste, mouthfeel, and aroma are four key indicators for evaluating wine quality.[14,15]

Color is one of the most easily recognizable organoleptic characteristics among the four indicators. The color of wine generally is the first attribute perceived by consumers and, consequently, significantly affects consumers' acceptance.[16,17] The perception and evaluation of taste, aroma, and mouthfeel of wine are affected by the change of color, to some extent.[18] The combination of chemical compounds perceived by receptors located in the taste buds constitutes the taste character of wine. Theoretically, the terms sweetness, sourness, bitterness, saltiness, and umami all are the branches of taste. However, generally, only sweetness, sourness, and bitterness could be perceived in wine.[19] Mouthfeel is a tactile sensation perceived by receptors in the mouth. Pungency, irritation, and astringency are among the most important chemesthetic sensations responsible for wine characters. Aroma character of a wine is determined by volatile compounds, which are received by the olfactory receptors situated in the nasal cavity.[20]

2.1.1 Sugars, glycerol, and organic acids

Sugars are constantly accumulated in the form of glucose and fructose through the conversion of sucrose during the ripening of grape. Glucose and fructose are the primary nutrient sources of yeast for ethanol production during alcoholic fermentation. Sucrose, whether natural or added, is firstly split into glucose and fructose by enzymes during fermentation. Theoretically, more than 90% of sugars in grapes could be consumed by the yeast with powerful fermentation capacity under an appropriate fermentation condition.[21] Residual sugars in wines are the main contributors of sweetness. According to the labeling standards of wines issued by the OIV in 2015, the wines with residual sugars < 4 g/L are defined as dry wines, between 4 and 12 g/L are medium-dry wines, between 12 and 45 g/L are semi-sweet wines, and > 45 g/L are sweet wines. The acceptability of wine sweetness by consumers is changeable over time. For example, sweet

wines, particularly the Champagne wine with sugar content > 100 g/L, were popular in the nineteenth century, whereas consumers prefer the wines with sugar content < 10 g/L in modern times.[22] Although the intensity of sweetness of wines is determined by the content of residual sugars, other taste and tactile sensations, such as sourness, bitterness, and astringency, may affect the taste of sweetness through mixture suppression and vice versa.[23]

Glycerol is the main sugar alcohol detected in wines, which usually is the most abundant byproduct of alcohol fermentation after water and ethanol. Previous studies have demonstrated positive influences of glycerol on taste and mouthfeel, such as enhancement of sweetness, body, and softness at a concentration higher than its reported sensorial threshold of 5.2 g/L.[24–26] Moreover, glycerol also increases viscosity of wines.[26,27] Generally, the concentration of glycerol in wine could reach 5–11 g/L after alcoholic fermentation, depending on yeast inoculated.[3]

There are two categories of organic acid involving volatile acids and fixed acids, the latter referring to nonvolatile acids. In this section, the term organic acids was used to represent only fixed acids. The relationship between volatile acids and wine characters is presented in section 2.1.2.3.

The group of organic acids is a crucial component building the overall organoleptic properties of wines. Besides the organic acids extracted from grapes, the acids formed from yeast metabolism during fermentation also take a big proportion in wines.[28] More than 100 organic acids have been detected in wines,[3] of which succinic, citric, malic, lactic, and tartaric acids account for more than 90% of total organic acids.[15,29] Organic acids mainly contribute sourness to wines.[19] However, some organic acids, such as lactic, citric, malic, and tartaric acids, simultaneously introduce astringent perception in a low pH solution; lactic acid was likely more astringent than citric and malic acids at pH 3.5–4.5.[30] The perceptions of saltiness and bitterness from succinic acid, which is a main carboxylic acid produced by yeast metabolism during alcoholic fermentation, have been reported in a previous study.[29]

2.1.2 Phenolic compounds

Phenolic compounds are a large and complex but ubiquitous group of secondary metabolites of plants. Phenolic acids, flavonols, and flavan-3-ols are the major groups of phenolic compounds in red and white grapes, whereas anthocyanins and their derivatives are exclusive in red grapes.[31] The total phenolic content in red grapes are generally higher than those in white grapes due to the high amount of anthocyanins and anthocyanin-related compounds in red grape skins. On the one hand, the pigmentation of various plant organs, especially flower and berry skin by anthocyanins facilitates seed dispersal and pollination by attracting

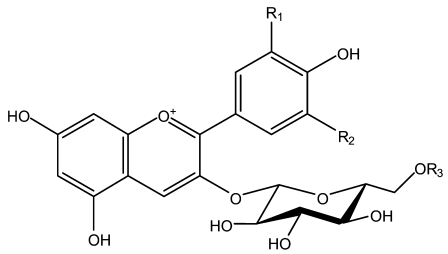
herbivorous animals and insects. Protecting plants from UV damages is another important function of anthocyanins.[32] On the other hand, the formation of other groups of phenolic compounds is also associated with defense response. For example, the astringency flavor of proanthocyanidins could prevent predation from herbivores.[33]

Phenolic compounds are grouped into flavonoids and non-flavonoids based on their differences in chemical structure. Of which flavonols, flavan-3-ols, and anthocyanins are classified into flavonoids due to the contained C6-C3-C6 skeleton, while phenolic acids into non-flavonoids.

Phenolic compounds play important roles in determining wine characteristics and qualities, i.e. color, taste, and mouthfeel. Phenolic compounds in wines comprise those originated from grapes and vine stalks, formed during fermentation, and/or generated during aging process and extracted from oak barrel used for aging and maturation. The influences of different groups of phenolic compounds, i.e. anthocyanins, phenolic acids, flavonols, and flavan-3-ols, on wine characters are reviewed in this section.

2.1.2.1 Anthocyanins

Anthocyanins are anthocyanidins (aglycones) glycosylated in the heterocyclic ring C (**Figure 1**). Totally 23 aglycones with different hydroxylation and methylation pattern in the rings have been found in nature. However, the aglycones of approximately 95% anthocyanins are cyanidin, peonidin, malvidin, delphinidin, petunidin, and pelargonidin.[34] In *Vitis vinifera* fruits, which accounts for more than 95% of all wine grapes over the world, and their wine products, the major anthocyanins consist of glucosides of these six aglycones.[14,35] Some acylated anthocyanins have also been detected in *V. vinifera* grapes and wines, such as 3-*O*-acetylglucosides, 3-*O*-*p*-coumaroylglucosides, and 3-*O*-caffeoylglucosides of anthocyanidins, to name a few (**Figure 1**).[14,36] Besides, 3,5-*O*-diglucosides of anthocyanidins and their acylated derivatives exclusively exist in non-*V. vinifera* and hybrid grapes, such as *V. labrusca*, *V. riparia*, *V. rupestris*, and *V. rotundifolia*.[35,37–39] The presence of acetyl group in anthocyanins increases their resistance to water attack.



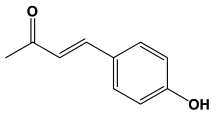
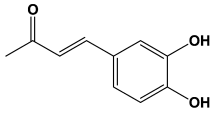
Anthocyanins	R_1	R_2	R_3
cyanidin 3-*O*-glucoside	OH	H	H
peonidin 3-*O*-glucoside	OCH_3	H	H
malvidin 3-*O*-glucoside	OCH_3	OCH_3	H
delphinidin 3-*O*-glucoside	OH	OH	H
petunidin 3-*O*-glucoside	OH	OCH_3	H
pelargonidin 3-*O*-glucoside	H	H	H
anthocyanin 3-*O*-acetylglucoside			
anthocyanin 3-*O*-*p*-coumaroylglucoside			
anthocyanin 3-*O*-*p*-caffeoylglucoside			

Figure 1. Structures of main anthocyanins detected in *V. vinifera* grapes and wines.

Anthocyanins are responsible for the color of red wines, whereas the color varies with the hydroxylation and methylation patterns in B ring. An increase in the number of hydroxyl groups in B ring intensifies bluish color of wines, whereas a shift toward purple has been observed in the anthocyanins with a high degree of methylation.[37] Therefore, malvidin 3-*O*-glucoside and its derivatives generally are among the anthocyanins with the highest intensity of redness in red wines.

In wine matrices, there is a dynamic equilibrium among different monomeric anthocyanin forms, including red flavylium cation, blue-violet quinoidal base, and colorless carbinol pseudobase and yellow chalcone (**Figure 2**).[29] The mutual transformation and equilibrium of the four forms of anthocyanin monomers is pH-dependent. At a low pH (pH < 2), the dominant anthocyanin form is red

flavylium cation, whereas a rapid increase of hydration at the C-2 position generates more carbinol pseudobases from flavylium cations at pH 3–6.[34] The reported pKa of the flavylium-pseudobase equilibrium is 2.7, hence approximately 90% of the anthocyanins are present as colorless types at a typical wine pH (≈3.5).[40]

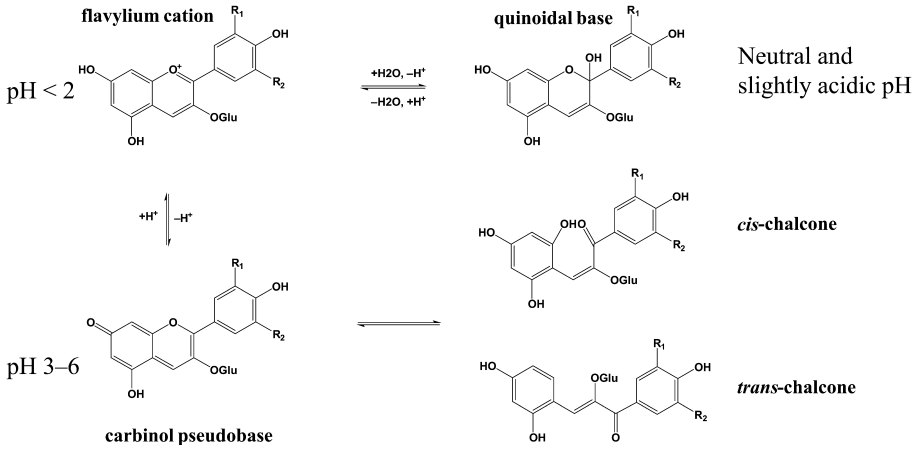


Figure 2. Anthocyanin equilibrium in wines depending on pH.

With the aim of sterilization and preservation, the addition of sulfur dioxide (SO_2) before and after alcoholic fermentation is a common practice in enology. However, this changes the equilibrium of anthocyanins due to the bleaching reaction of sulfite and converts flavylium cations to form their colorless sulfite adducts.[14]

During winemaking process from alcoholic fermentation to aging or maturation, on the one hand, most of the monomeric anthocyanins extracted from grapes convert to more complex and stable pigments *via* the reactions of copigmentation, cycloaddition, and polymerization. On the other hand, most of the rest disappear resulting from degradation, oxidation, and/or precipitation.[17,41,42]

In aqueous solution, the persistent nucleophilic attack from water on the monomeric anthocyanins with red flavylium cation form converts them to colorless hydrated conformations. However, the copigmentation reaction between anthocyanins flavylium cation and colorless copigments, such as flavonoids, phenolic acids, organic acids, and amino acids, through van der Waals interactions could form complexes with a π–π stacking sandwich configuration to stabilize the color exhibition of anthocyanins.[43–45] In young wine, copigmentation contributes 30–50% of wine color.[45] However, in general, the fraction of copigmented anthocyanins decreases significantly during wine aging. Meanwhile, the fraction of polymerized anthocyanins shows a significant

increase.[46] These changes consequently alter the color attributes of wines from red to brown or orange. There is a theory considering that copigmented anthocyanins are intermediates between monomeric and polymerized anthocyanins.[47] Therefore, the elevation of the fraction of copigmented anthocyanins in wines by adding copigments has been considered as an effective approach to stabilize anthocyanins and color during wine aging.[48–50]

Copigmented anthocyanins are more stable and contribute more to the stabilization of color of wines than those monomeric ones in the same condition. However, the formation of these anthocyanin complexes in wines is greatly influenced by various factors, such as pH, temperature, ethanol content, and the concentration of copigments. The greatest magnitude of copigmentation was observed at pH 3.0–3.3.[48,51] The destruction of copigmented complexes deteriorates when the temperature higher than 20 °C,[52] which facilitates the enological practices of cold fermentation and storage. In general, anthocyanins have no effect on wine flavors of aroma, taste, and mouthfeel, while the stack of flavonoids originated from wines in copigmentation complexes reduces their contributions to bitterness and/or astringency of wines, to some extent.[45,53]

Acetaldehyde, pyruvic acid, oxalacetic acid, acetoacetic acid, 3-hydroxy-2-butanone (acetoin), acetone, and 4-vinylphenol are important byproducts from yeast fermentation. Furthermore, they participate in the generation of pyranoanthocyanins *via* a nucleophilic cycloaddition reaction at the C4 position and a hydroxyl group at C5 monomeric anthocyanins, forming an extra pyranic ring (**Figure 3**).[54,55] This reaction starts from alcoholic fermentation and continues during aging. Among the diverse pyranoanthocyanins detected in young and aged red wines, vitisins A and B usually are the dominant ones.[56] These two vitisins particularly refer to the compounds condensed between one malvidin 3-*O*-glucoside and a molecule of pyruvic acid and acetaldehyde, respectively (**Figure 3**).[57] However, according to previous studies, the formation of vitisins A and B showed antagonistic kinetics due to the competition for anthocyanin monomers between pyruvic acid and acetaldehyde.[56] The content of vitisin A in red wine generally is higher than that of vitisin B.[57,58]

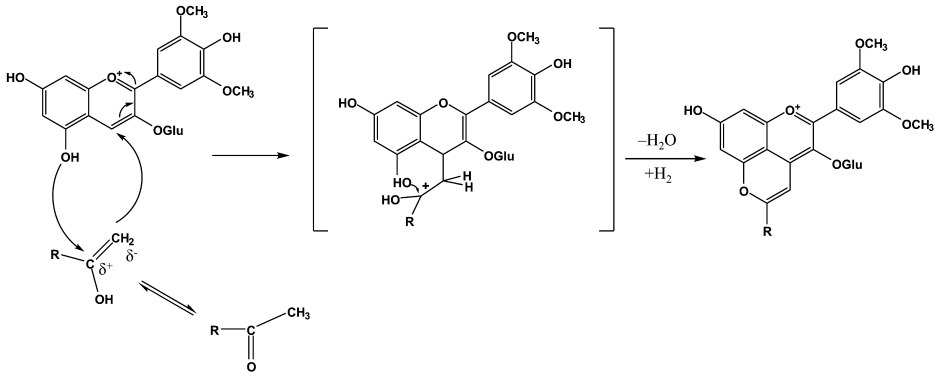


Figure 3. Formation mechanism of vitisins A and B in wines.

Apart from vitisins, the pyranoanthocyanin pigments including pinotins (also known as hydroxyphenyl-pyranoanthocyanins), pyranoanthocyanin-flavanols (flavanyl-pyranoanthocyanins or vinylflavanol-pyranoanthocyanins), methyl-pyranoanthocyanins, portisins (flavanyl/phenyl-vinylpyranoanthocyanins), oxovitisins (pyranone-anthocyanin), and pyranoanthocyanin dimers have also been isolated and identified in red wines (**Figure 4**).[36,57,59]

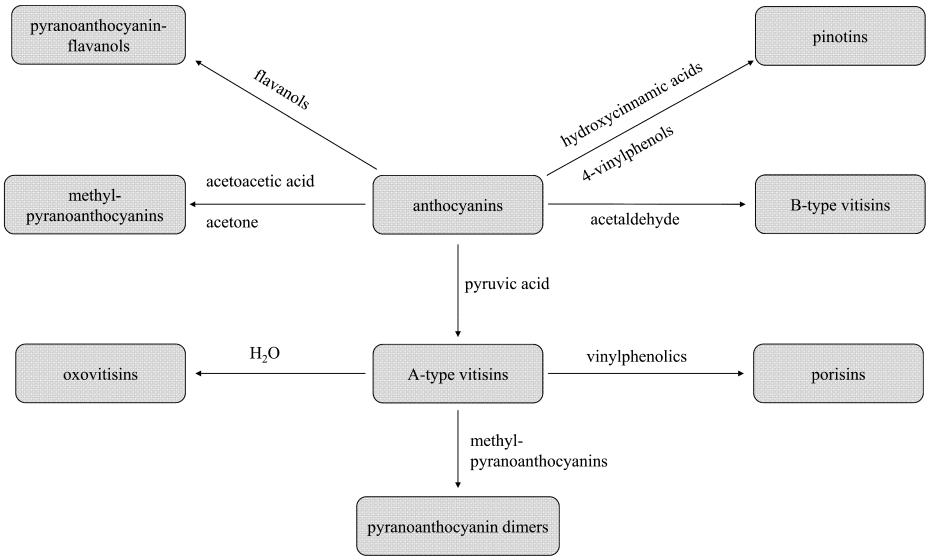


Figure 4. Formation diagrams of common pyranoanthocyanins in red wines.

The formation of pyranoanthocyanins improves the structural stability of anthocyanins against pH change, oxidative degradation, and SO_2 bleaching. Vitisin A was reported to resist bleaching of SO_2 up to a concentration of 250 mg/L and a half of vitisin B is retained at SO_2 concentration of 200 mg/L,

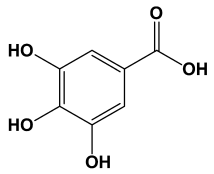
whereas 80% of malvidin 3-*O*-glucoside is bleached at SO_2 concentration of only 80 mg/L.[15] Moreover, vitisins contribute to a high percentage of color in a solution with pH value up to near neutrality, whilst malvidin 3-*O*-glucoside does not confer much color in the pH range 4–6 due to the loss of flavylium structure.[54] Therefore, to improve the stabilization of wine color, additional supplementation of pyruvic acid and/or acetaldehyde could be considered as an option.[60]

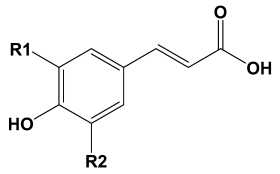
Besides the occurrence of copigmentation and cycloaddition reactions on anthocyanin monomers, polymerization between anthocyanins and flavan-3-ols forms polymeric anthocyanins with the highest stabilization among pigments. In general, the proportion of polymeric anthocyanins increases significantly during wine aging. Approximately 25% anthocyanins are polymerized in young red wine, whereas the proportion elevates to approximately 40% after one year of aging. Additionally, all the pigments will be polymerized when a wine submitted to a long-term of aging.[61–63] Anthocyanin polymerization browns the color of wines. Apart from their impact on color, the high degree of polymerization with anthocyanins reduces the perceptions of astringency and bitterness contributed by flavan-3-ols.[63]

2.1.2.2 Phenolic acids

There are two different groups of phenolic acids, including hydroxybenzoic and hydroxycinnamic acids, in grapes and wines (**Figure 5**). Gallic acid is the major hydroxybenzoic acid detected in wines, whereas it is undetectable in their corresponding grapes. Gallic acid in wines is released from the hydrolysis of gallate esters of tannins. Moreover, the extraction from oak and hydrolysis of oak hydrolyzable tannins increase the content of gallic acid in aged wines.[15,64] Hydroxybenzoic acids are important contributors to bitterness and puckering astringency in wines.[65]

gallic acid





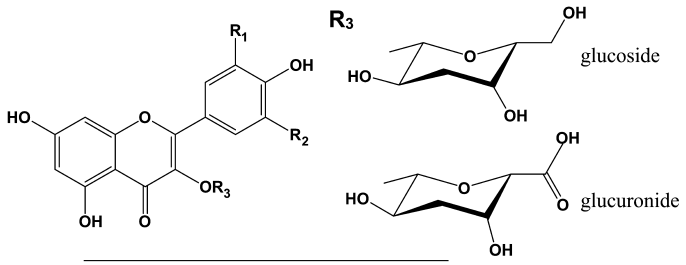
Hydroxycinnamic acids	R_1	R_2
p-coumaric acid	H	H
caffeic acid	H	OH
ferulic acid	H	OCH_3

Figure 5. Structures of common phenolic acids found in wines.

The common hydroxycinnamic acids in wines are *p*-coumaric acid, caffeic acid, and ferulic acid (**Figure 5**). These acids participate in the stabilization of anthocyanins through copigmentation.[14] Likewise, hydroxycinnamic acids are associated with bitterness and astringency characters of wine.[65] However, Vèrette et al. found that hydroxycinnamic acids did not play a direct role in determining the sensory feature of white wines due to the low contents compared to their sensory thresholds.[66]

2.1.2.3 Flavonols

The common flavonols existed in grapes are glycosylated flavonols. Six flavonols, including quercetin, myricetin, laricitrin, kaempferol, isorhamnetin, and syringetin, mainly as 3-*O*-glucoside and 3-*O*-glucuronide, have been detected in grapes (**Figure 6**). However, generally, 3-*O*-glycosides of isorhamnetin, laricitrin, myricetin, and syringetin are almost specific to red grapes.[3] The dominant flavonols in white grape varieties are quercetin and kaempferol derivatives, whereas, occasionally, isorhamnetin- and myricetin-glycosides could be found in trace amounts in some of these varieties.[3] The synthesis of flavonols in grapes is strongly enhanced by sunlight exposure because of the upregulation of genes encoding for flavonol synthase.[67] In comparison with the grapes growing in shade, up to ten times higher of flavonols are found in those cultivated under a sun-exposure condition.[15]



Flavonols	R_1	R_2
quercetin	OH	H
myricetin	OH	OH
laricitrin	OCH_3	OH
kaempferol	H	H
isorhamnetin	OCH_3	H
syringetin	OCH_3	OCH_3

Figure 6. Structures of common flavonols found in wines.

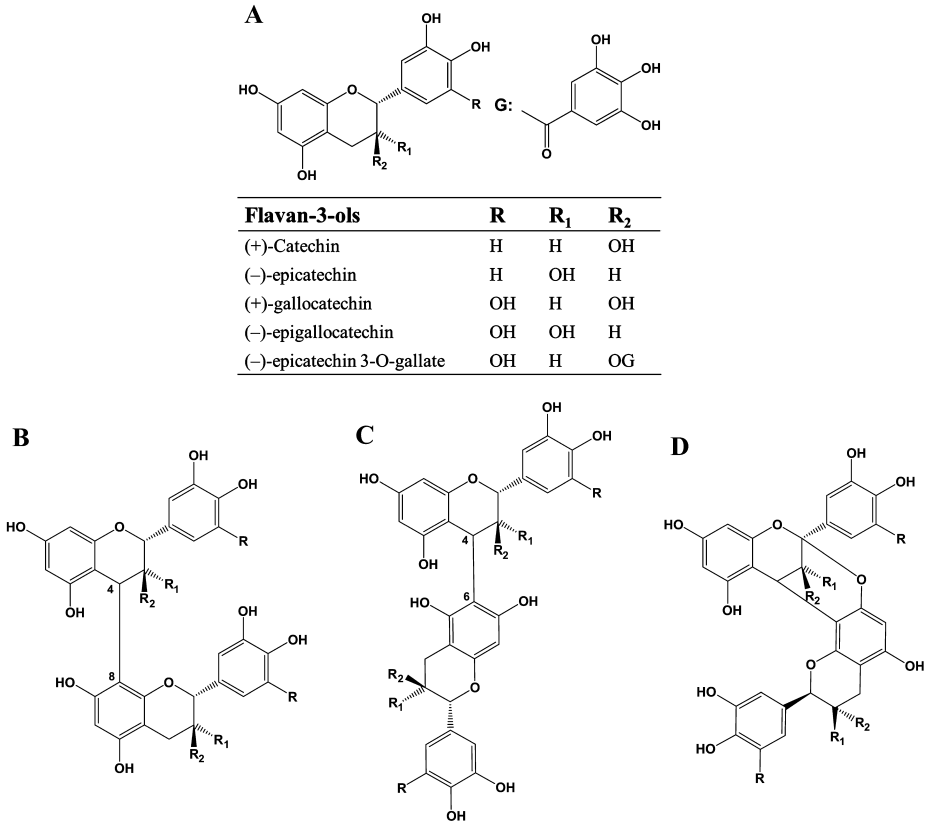
Flavonols in wines are generally originated from grape skins during maceration. The contents of flavonols in white wines are usually lower than those in red ones.[15] During wine fermentation and aging, a fraction of glycosylated

flavonols are hydrolyzed to flavonol aglycones. This leads to a haze or precipitation due to the low solubility of aglycones in aqueous ethanol solution. It is generally accepted that flavonols contribute bitterness to wine, while the contribution of flavonol glycosides to "velvety astringency" has also been observed in red wines.[65] The perception "velvety astringency" is different from the "puckering astringency". The former affects tactile receptors directly, whereas the latter affects indirectly on human sensorium through protein precipitation by condensed tannins.[68]

2.1.2.4 Flavan-3-ols

Flavan-3-ols in grapes and wines are divided into three classes as monomeric, oligomeric, and polymeric flavan-3-ols. **Figure 7A** shows five common monomeric flavan-3-ols detected in grapes and wines, of which (+)-catechin and its isomer (–)-epicatechin are the major ones. The contents of these two compounds are in the ranges of 16–43 and 10–65 mg/L in red wines, respectively,[15] whereas average levels of only 10 and 5 mg/L, respectively, were found in white wines.[69]

Flavan-3-ol oligomers and polymers, also known as proanthocyanidins or condensed tannins, are formed from biochemical condensation of flavan-3-ol units. Proanthocyanidins are crucially important constituents in determining the chemical profiles of wines, accounting for approximately 25–50% of total phenolic content in a typical wine.[70] A-type and B-type proanthocyanidins are the two subclasses of proanthocyanidins in wines. B-type proanthocyanidins are condensed by constitutive flavanol units through C4-C8 or C4-C6 linkages (**Figure 7B, C**). There is an additional linkage between C2-O-C7 or C2-O-C5 in A-type proanthocyanidins (**Figure 7D**). The proanthocyanidins formed by polymerization of catechin and epicatechin are named as procyanidins, while those formed by gallocatechin and epigallocatechin as prodelphinidins. In general, procyanidins in wines are originated from grape seeds and skins, whereas prodelphinidins are extracted from grape skins.[14]



Flavan-3-ols	R	R_1	R_2
(+)-Catechin	H	H	OH
(–)-epicatechin	H	OH	H
(+)-gallocatechin	OH	H	OH
(–)-epigallocatechin	OH	OH	H
(–)-epicatechin 3-O-gallate	OH	H	OG

Figure 7. Structures of common flavanol monomers and proanthocyanidins found in wines.

The content and composition of flavan-3-ols determine the bitterness and astringency attributes of wines.[19] Generally, the contribution of bitterness is mainly from flavan-3-ol monomers, while the intensity significantly decreases with the increase of degree of polymerization (DP). Nevertheless, the increase of DP and the presence of galloyl groups enhance astringency.[71] During aging, hydrolysis and phloroglucinolysis of tannins yield flavan-3-ols of low DP values resulting in the weakening of astringent intensity and the enhancement of bitterness. Moreover, the participation of flavan-3-ols in the formation of polymeric anthocyanins during fermentation and aging reduces their contribution to astringency and bitterness.[63]

Certain wines need to be aged in oak barrels for a period to improve wine quality and complexity. During this process, ellagitannins, the major hydrolyzable tannins, are extracted from wood. Ellagitannins affect wine bitterness and astringency properties, as well as color *via* the formation of anthocyanin-ellagitannins protecting anthocyanins from oxidation.[72,73]

2.1.3 Volatile compounds

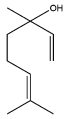
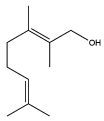
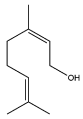
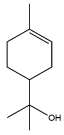
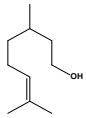
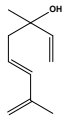
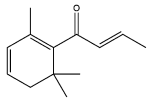
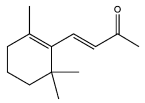
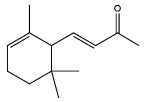
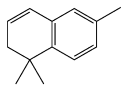
Aroma is one of the key characteristics reflecting wine quality and style, which is determined by a variety of volatile compounds *via* cumulative effect. More than 800 volatile compounds, mainly including alcohols, esters, aldehydes, ketones, acids, terpenes, phenols, and sulfur compounds, have been found in grapes and wines at a varying concentration from ng/L to mg/L.[3,20,74] The total content of volatile compounds in wine is approximately 0.8–1.2 g/L.[20] However, only a small number of these compounds with concentrations higher than their odor thresholds contribute to the overall aroma of wines.[75] Volatile compounds are dynamically generated and degenerated throughout the growth and development of grape and along with the production of wine. Volatile compounds in wines can be further classified into primary (PAVCs), secondary (SAVCs), and tertiary aroma volatile compounds (TAVCs) based on their formation patterns.

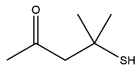
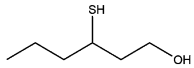
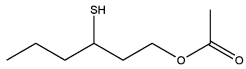
2.1.3.1 Primary aroma volatile compounds

Primary or varietal aroma volatile compounds in wines refer to the free form compounds originated directly from grapes and those derived from precursors present in grapes.[76] Their concentrations and compositions in grapes and wines are highly dependent on the viticultural variables, conditions and practices, for instance, cultivar, climate, soil, water, and vineyard management.[77]

Although the principal volatile compounds contributing to wine characters are those formed during fermentation by yeast metabolism and/or derived from the aging process, PAVCs also play a key role in determining the overall flavor of wines. Monoterpenes are among the most extensively studied PAVCs in *V. vinifera* grapes. In particular, monoterpenes are prevalent in Muscat and Riesling grapes and are responsible for the *floral* and *fruity* odors.[20,76] Therefore, monoterpenes are usually used as markers for the classification of grape variety. Amongst the approximately 50 monoterpenes detected in wines, linalool, geraniol, nerol, α-terpineol, citronellol, and hotrienol are among the representatives of free monoterpenes (**Table 1**).[78] These compounds are biosynthesized from the precursor mevalonate, which is synthesized from acetyl-CoA.[79] The concentration of free monoterpenes is varying in different part of grapes. For example, the concentrations of geraniol and nerol in grape skins are higher than those in fruits.[80]

Table 1. Structures, odor descriptors, thresholds, and concentration ranges of common monoterpenes, C13-norisoprenoids, and polyfunctional thiols in wines

Compound	***Structure***	***Odor descriptor***	***Odor threshold (µg/L)***	***Concentration range in wines (µg/L)***
Monoterpenes				
linalool		floral, fruity	15	white wines: nd–307; red wines: nd–16.4
geraniol		rose, geranium	30	white wines: nd–221; red wines: nd–44.4
nerol		citrus, floral	300	white wines: 16.6–49; red wines: nd–100.3
α-terpineol		floral, wood	250	white wines: nd–123.8; red wines: nd–33
citronellol		green lemon	100	white wines: nd–31.4; red wines: nd–5.5
hotrienol		citrus, fruity	110	Riesling wines: 2.8–116.6
C13-norisoprenoids				
β-damascenone		apple, rose, honey	0.05	white wines: nd–9.4; red wines: 0.29–4.7
β-ionone		seaweed, violet, flower, raspberry	0.09	white wines: 0.11; red wines: 0.032–0.9
α-ionone		sweet fruit	2.6	white wines: nd–123.8; red wines: 0.017–0.54
1,1,6-trimethyl-1,2-dihydro-napthalene		kerosene	2	non-Riesling wines: < 6.4 Riesling wines: < 50

Compound	*Structure*	*Odor descriptor*	*Odor threshold (μg/L)*	*Concentration range in wines (μg/L)*
Polyfunctional thiols				
4-mercapto-4-methylpentan-2-one		blackcurrant, box-tree, broom, passion fruit	3×10^{-3}	white wines: nd–0.088 red blends: 0.005–0.054
3-mercaptohexan-1-ol		grapefruit, passionfruit	60×10^{-3}	white wines: 0.026–18.7 red blends: 0.678–11.5
3-mercaptohexyl acetate		passionfruit, box tree	4×10^{-3}	white wines: nd–2.51 red blends: 0.0046–0.154

The data of odor descriptor, threshold, and concentration range in wines are summarized based on data reported in the literature [15,78,81–83].

Besides free monoterpenes, odorless glycosidically bound monoterpenes are also prevalent in grapes. The common glycoside moieties are glucoside, rhamnoside, arabinoside, and apioside.[84] Although the ratios of bound to free monoterpenes are varying depending on grape variety, glycosylated monoterpenes are generally dominant compared to the free forms. For example, approximately 90% of monoterpenes are glycosidically bound in Muscat grapes.[85] During grape processing and fermentation, odorless monoterpene glycosides convert to aroma-contributing free monoterpenes through enzymatic hydrolysis in an acidic fermentation condition. The conversion is approximately 22–28% during fermentation, whereas only approximately 5% was observed in nonfermented samples during the same time duration.[86] However, in model wine solution, free monoterpenes are directly synthesized from yeast metabolism instead of resulting from conversion of their precursors when monoterpene glycosides are absent.[87]

C13-norisoprenoids are the second group PAVCs detected in wines. They are produced from the oxidation of grape carotenoids.[79] C13-norisoprenoids play important roles in determining wine aroma due to their low odor thresholds (**Table 1**). Similar to monoterpenes, C13-norisoprenoid compounds in grapes are mainly present as glycosidic conjugates. They are converted to their corresponding free forms by the action of glycosidase during grape crushing and fermentation.[31] β-Damascenone, β-ionone, α-ionone, and 1,1,6-trimethyl-1,2-dihydro-napthalene (TDN) are among the commonly studied norisoprenoids in wines. β-Damascenone is a *fruity* (*cooked apple* and *tropical fruit*) odor contributor. Moreover, low concentration of this compound enhances *fruity*

aroma of esters *via* synergistic effect, while suppressing *green* odor contributed by methoxypyrazines.[15,88] Ionones, including α-ionone (*fruity* and *floral*) and β-ionone (*violet* and *raspberry*), are important compounds contributing to wine aroma. The concentration of β-ionone in wines was generally higher than its odor threshold, however, opposite was found for its isomer α-ionone.[88] TDN has a special *kerosene* aroma and has been found in several wine varieties, particularly in Riesling wines.[76]

Polyfunctional thiols are the third set of PAVCs released from their non-volatile bound precursors in grapes. During fermentation, thiols are produced from odorless cysteine conjugates *via* carbon-sulfur β-lyase enzymes in yeasts.[75] Polyfunctional thiols differ from other sulfur compounds in wines due to the presence of additional functional groups containing oxygen.[15] 4-Mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercaptohexyl acetate (3MHA) are among the most important varietal thiols conferring desirable *citrus* and *tropical fruit* odors to wines (**Table 1**).

2.1.3.2 Secondary aroma volatile compounds

SAVCs are those metabolites produced by yeasts during alcoholic fermentation. Besides ethanol, other minor but organoleptically important metabolites, such as higher alcohols, esters, volatile acids, aldehydes, and ketones are released from yeast fermentation (**Figure 8**). Generally, fermentation-derived volatiles accounting for the largest fraction of the total aroma compounds of wines. In this section, the relationship between wine aroma characters and these groups of yeast metabolites are highlighted.

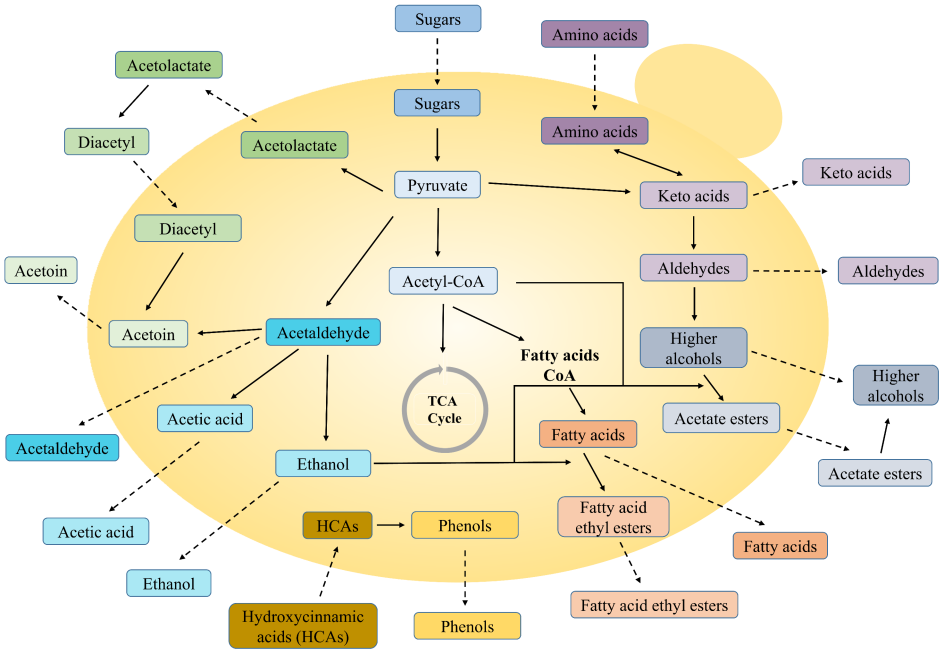


Figure 8. Overview of production of volatile compounds by yeast during fermentation of wines (adapted and redrawn from [31,89]).

Ethanol is indisputably the principal volatile compound produced by yeasts. Ethanol concentration in wines could be up to 14–15% under a standard fermentation condition. The impacts of ethanol on wine sensorial characters are multiple, but could be simply subclassified into direct and indirect approaches.[29] Bitterness, pungency, sweetness, and viscosity are the major and direct flavor properties of ethanol in wines. Ethanol also indirectly affect wine flavor through influencing perceptions of other compounds. For instance, ethanol decreases the intensity of astringency provided by tannins by a masking effect or by increasing their solubility.[15] During wine fermentation and aging, the reactions between ethanol and fatty acids or aldehydes produce esters or acetals, respectively, further changing the sensorial properties of wines.[90,91]

The family of alcohols with more than two carbon atoms are named as higher or fusel alcohols. Higher alcohols are biosynthesized parallel to ethanol production from two pathways: the anabolic pathway from sugars metabolism and the catabolic (Ehrlich) pathway from amino acid metabolism. The formation of intermediate keto acids is a vital step in both pathways (**Figure 8**). However, the substantial presence of amino acids in fermentation matrices inhibits the performance of anabolic pathway and favors the catabolic production of higher alcohols from amino acids.[92] On the basis of the structural difference, higher alcohols are classified into two categories of aliphatic and aromatic alcohols. 2-Methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol are commonly

found aliphatic alcohols, while 2-phenylethanol and 4-(2-hydroxyethyl)phenol (tyrosol) represent major aromatic alcohols in wine. Higher alcohols have both positive and negative impacts on wine quality. For example, aliphatic alcohols generally give undesirable odors to wines, such as *alcohol*, *nail polish*, *medicinal*, and *pungent*. Oppositely, aromatic alcohols are pleasant odor contributors, for example 2-phenylethanol and tyrosol accord *honey* and *rose* aromas to wines.[93,94] Besides these two categories of higher alcohols, sulfur alcohols, for example, methionol, also negatively influence wine flavor (**Table 2**).[29]

Higher alcohols approximately account for 50% of total concentration of aroma compounds (exclude ethanol).[29] Generally, higher alcohols at a total concentration of less than 300 mg/L are considered to contribute to the aroma complexity of wines, while levels exceeding this limit can cause undesirable sensorial sensations.[77] The concentration of higher alcohols is closely correlated to the concentration of their amino acid precursors (often referred as yeast assimilable nitrogen, YAN) in grapes.[95] The formation of higher alcohols from anabolic pathway predominates over those formed from the catabolism of amino acids in media with low YAN.[96] Furthermore, species and strains of yeast, ethanol concentration, and fermentation practice, such as fermentation temperature, aeration, level of suspended solids, and skin contact time, also influence significantly on the concentration of higher alcohols. According to previous reports, high fermentation temperature, presence of oxygen and suspended solids favored the formation of higher alcohols during fermentation.[29,97] During aging, the concentration of higher alcohols is dynamically changing due to the reaction with organic acids to synthesize acetate esters and the hydrolysis of acetate esters (**Figure 8**).[29,98,99]

Although some esters are accumulated during grape ripening, the accumulation levels generally are negligible. For example, the total ester concentrations in Cabernet Sauvignon and Riesling grapes are lower than 1 mg/kg and only methyl hexanoate and (*Z*)-3-hexenyl butanoate were detectable.[100] Among the more than 160 esters detected in wines to date, the majority of them are produced by yeasts during fermentation.[29] Hence, esters are also classified as secondary volatile compounds.

Based on the difference of formation pathway, esters are mainly grouped into two categories of acetate esters and fatty acid ethyl esters. Acetate esters are formed from esterification of alcohols and acetic acid, of which the alcohols are either ethanol or higher alcohols (**Figure 8**). Ethyl acetate, 3-methylbutyl acetate (isoamyl acetate), and 2-phenylethyl acetate are among the most important acetate esters.[93,101] Fatty acid ethyl esters are synthesized from ethanol and fatty acids derived from lipid metabolism (**Figure 8**). Ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate are representatives of this ester group.[93,102]

Esters are critically important participants in determining wine aroma. They generally endow the wine with pleasant aromas, such as *fruity* and *floral* notes (**Table 2**).[89,103] However, ethyl acetate was reported to contribute to an undesirable odor of *varnish* at a high concentration (> 150 mg/L).[29] The formations of esters during fermentation are affected by multiple factors. For instance, fermentation at a low temperature (approximately 10 °C) enhances the synthesis of some acetate esters, whereas a relatively high temperature increases the production of ethyl octanoate, ethyl decanoate, and phenethyl acetate.[104] Moreover, low SO_2 and juice clarification elevate the formation and retention of esters.[29] The inoculated yeasts also play a key role in determining the accumulation of esters due to the participation of various enzymes in yeasts, such as alcohol acetyltransferases and dehydrogenases.[79] Aging conditions, including storage temperature and pH, significantly influence the concentration of esters in wines. For example, the levels of many acetate esters, such as isobutyl acetate, 2-phenylethyl acetate, *cis*-3-hexenyl acetate, 3-methylbutyl acetate, and hexyl acetate, in Colombard and Sauvignon Blanc wines decrease remarkably during bottle aging at a high temperature and low pH, whereas ethyl esters stay roughly constant during aging.[105–107]

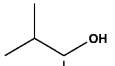
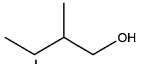
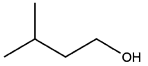
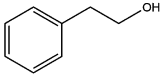
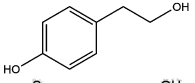
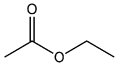
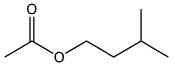
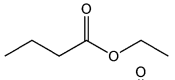
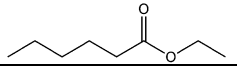
In general, the concentration of volatile acids in wines is 500–1000 mg/L accounting for 10–15% of total acid content.[31] Acetic acid usually is the dominant one constituting approximately 90% of total volatile acids.[78] Acetic acid is formed from the oxidation of acetaldehyde during yeast fermentation (**Figure 8**). Excessive amount (> 0.8 g/L) of this compound is detrimental to wine quality imparting *vinegar-like* aroma. However, it contributes to wine aroma complexity in a concentration range of 0.2–0.7 g/L.[31] The concentration of acetic acid in wines is largely dependent on yeast and fermentation procedure. High content of nitrogen supplement (ammonium) results in the increase of acetic acid in wines.[108] The rest volatile acids in wines, such as propanoic, 2-methylpropanoic, pentanoic, hexanoic, and octanoic acids, are formed from fatty acid metabolism (**Figure 8**). Fatty acids usually contribute with unpleasant odors, like *rancid*, *butter*, and *cheese*, to wine aroma (**Table 2**). Under typical fermentation conditions, the formation of fatty acids start from yeast growth phase and peaks at the end of this period. Thereafter, the concentration of fatty acids reduces during the stationary phase once most of sugars are consumed.[15] The presence of unsaturated fatty acids in grape must stimulates yeast growth in an anaerobic condition and reduces the production rate of short-chain fatty acids.[103] Fatty acids are the precursors for the synthesis of fatty acid ethyl esters during fermentation, hence the concentration of the latter group in wines is related to the generation and degeneration of the former. Aging practice also affects the concentration of fatty acids. For example, wine aging on lees was reported to increase the level of long-chain volatile fatty acids.[109]

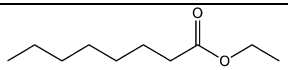
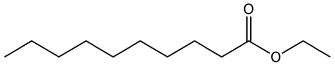
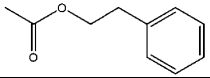
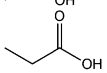
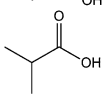
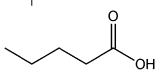
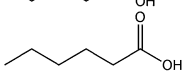
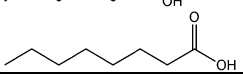
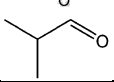
Aldehydes and ketones are the two principal classes of carbonyl compounds found in wines. The carbonyl functional group is located on the terminal carbon of aldehydes, whereas on the internal carbon of ketones. Aldehydes are the precursors for the formation of alcohols (**Figure 8**). Acetaldehyde is the major aldehyde accounting usually for more than 90% of total aldehyde content in wines. Acetaldehyde is an intermediate during the production of ethanol, acetic acid, and acetoin from pyruvate catalyzed by pyruvate decarboxylase (**Figure 8**). Acetaldehyde reaches its highest concentration at the early stage of fermentation and reduces to a low level by the end of fermentation. The concentration of acetaldehyde varies among different wine styles. Generally, Sherry wines contain the highest level of acetaldehyde, followed by white wines and red wines, in the decreasing order.[102] The odor descriptors of acetaldehyde vary with the concentration in wines. At low concentration, it endows a *fruity* odor to wines, whereas at high levels, it is reminiscent of irritating odors of *pungent* and *rotten apple* (**Table 2**).[110] The concentration of acetaldehyde in wines is also yeast dependent. Besides this, fermentation conditions such as medium composition, fermentation temperature, nature of insoluble material used to clarify the must, and oxygen and sulfur dioxide contents greatly affect acetaldehyde concentration, as well.[31,111] Ethanol can transform back to acetaldehyde through oxidation (Fenton reaction) when wines are exposed to air, particularly during wine aging.[102]

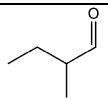
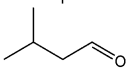
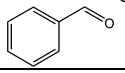
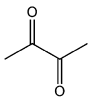
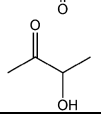
Some other aldehydes, such as 2-methylpropanal, benzaldehyde, and 2,3-methylbutanal, are detected in many wines, particularly in aged port and Sherry wines.[112] These aldehydes occasionally influence wine sensory features depending on the concentrations and their odor thresholds (**Table 2**).

2,3-Butanedione (diacetyl) and acetoin are the two major ketones in wines. 2,3-Butanedione donates wines a *nutty* or *toasty* odor when at a low concentration, whereas contributes *buttery* or *lactic* off-flavor at much above its odor threshold (0.1 mg/L) (**Table 2**). Although 2,3-butanedione is usually originated from metabolic fermentation, yeast biosynthesizes 2,3-butanedione at the concentration of 0.2–0.3 mg/L.[113] Oxygen exposure, fermentation temperature, and sulfur dioxide level are the factors influencing the accumulation of 2,3-butanedione. The majority of 2,3-butanedione is subsequently metabolized to acetoin and subsequently to 2,3-butanediol during fermentation. Acetoin is considered as an organoleptic defect due particularly to its undesirable odor. It imparts a strong *buttery* or *cream* odor to wines at a concentration above its odor threshold (150 mg/L). However, the production of acetoin in topical wines generally does not reach such a high level.[3]

Table 2. Structures, precursors, odor descriptors, odor thresholds, and concentration ranges of major secondary aroma volatile compounds in wines

Compound	*Structure*	*Odor descriptor*	*Odor threshold (mg/L)*	*Concentration range in wines (mg/L)*
Higher alcohols				
2-methyl-1-propanol		alcohol, nail polish	40	9–174
2-methyl-1-butanol		nail polish, malt	1.2	16–31
3-methyl-1-butanol		nail polish, alcohol	30	6–490
2-phenylethanol		rose, honey	10	4–197
4-(2-hydroxyethyl)phenol		rose, honey		20–30
methionol		boiled potato, cauliflower	1	nd–5
Esters				
ethyl acetate		pineapple, fruity, pungent, varnish	7.5	22.5–63.5
3-methylbutyl acetate		banana, fruity, sweet	0.03	0.1–3.4
ethyl butanoate		floral, fruity	0.02	0.01–1.8
ethyl hexanoate		fruity, green apple, banana, brandy, wine-like	0.05	0.03–3.4

Compound	*Structure*	*Odor descriptor*	*Odor threshold (mg/L)*	*Concentration range in wines (mg/L)*
ethyl octanoate		apple	0.02	0.05–3.8
ethyl decanoate		floral, soap	0.2	nd–2.1
2-phenylethyl acetate		floral	0.25	nd–18.5
Volatile acids				
acetic acid		volatile acidity, vinegar	0.7	0.1–1.2
propanoic acid		pungent, rancid	8.1	nd–100
2-methylpropanoic acid		rancid, butter, cheese	30	0.4–2
pentanoic acid		cheese	3	nd–1.8
hexanoic acid		cheese, sweaty	0.42	0.8–4
octanoic acid		rancid, fatty	0.5	0.6–5
Aldehydes				
acetaldehyde		fruity, rotten apple, pungent	110	nd–211
2-methylpropanal		banana, melon, varnish, cheese	0.006	0.001–0.2

Compound	*Structure*	*Odor descriptor*	*Odor threshold (mg/L)*	*Concentration range in wines (mg/L)*
2-methylbutanal		green grass, fruity	0.016	0.003–0.1
3-methylbutanal		malt, unripe banana, apple, cheese	0.004	0.04–0.25
benzaldehyde		roasted, almond	2	0.01–0.76
Ketones				
2,3-butanedione		buttery, nutty, toasty, lactic	0.1	0.005–7.5
3-hydroxy-2-butanone		buttery, cream	150	0.1–60

The data of odor descriptor, threshold, and concentration range in wines are summarized from the literature [15,31,94,102,114–120].

2.1.3.3 Tertiary aroma volatile compounds

Aging or maturation is a common practice in wine industry to improve wines' sensory characteristics, particularly to weaken the mouthfeels of astringency and bitterness of young wines. Traditionally, red wines are subjected to this process. However, nowadays, this step is also employed on white and rose wines. The frequently used containers for wine aging are oak barrels and bottles.

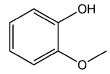
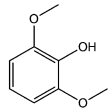
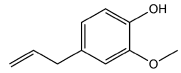
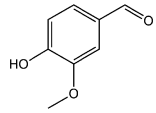
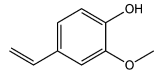
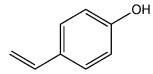
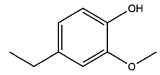
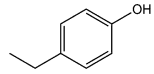
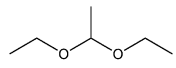
During aging, wine aroma linked to grape variety and fermentation are gradually lost, while TAVCs developed from PAVCs and SAVCs or extracted from oak wood keep increasing with time. Volatile phenols are among the most important TAVCs extracted from wood or derived from their precursors (hydroxycinnamic acid, HCA) by microbiological process (**Figure 8**).[121,122] Volatile phenols found in wines at the concentration ranging from levels of μg/L to mg/L (**Table 3**).[15] Guaiacol, syringol, vanillin, and eugenol are the major volatile phenols extracted from toasted oak wood, formed through the thermal degradation of lignin. The extraction process of most of these phenols is almost complete after 6 months of aging.[3] In general, more volatile phenols are extracted into the wines aged in medium toasted oak wood compared to those in light and high toasted woods.[123] These compounds are reported to positively contribute to wine aroma when present at appropriate concentrations.[15]

4-Vinylphenol, 4-vinylguaiacol, 4-ethylphenol, and 4-ethylguaiacol are the four main volatile phenols originating from their HCA precursors catalyzed by hydroxycinnamate decarboxylases and vinylphenol reductase. Specifically, first, *p*-coumaric and ferulic acids are decarboxylated to 4-vinylphenol and 4-vinylguaiacol, respectively. Afterwards, 4-vinylphenol and 4-vinylguaiacol are converted to 4-ethylphenol and 4-ethylguaiacol, respectively, by vinylphenol reductase. However, it is worth noting that the reduction reaction almost occurs only during fermentation of the wines contaminated by *Brettanomyces*/*Dekkera* spp. since vinylphenol reductase is found almost exclusively in these spoilage strains.[3] Nevertheless, for the wines without contamination, these two ethyl compounds are much more likely extracted from oak barrel during aging.[124] These four phenols are known for their contribution to off-flavor of wine organoleptic characters (**Table 3**). The amount of these four compounds in wines is proportional to the abundance of the population of *Brettanomyces*/*Dekkera*. Alcohol content and aging temperature also affect the level of volatile phenols in wines. Moreover, wine aged on the lees was reported to adsorb a certain amount of phenols.[125] However, the quantities of 4-ethylguaiacol and 4-ethylphenol showed a significant increase when wine aging performed in oak wood.[126] Additionally, wine aged in used American oak barrels extracted these two phenols more than those in new barrels.[127]

During aging, acetal compounds are formed from one aldehyde molecule and two alcohols. Although more than 20 acetals have been isolated from wines,

most of them are documented as odorless compounds with the exception of 1,1-diethoxyethane forming from the reaction between ethanol and acetaldehyde. 1,1-Diethoxyethane is known to impart *cake* and *fruity* aroma to wine.[3] Wine aging significantly induces the accumulation of acetals, and their concentrations are related to aging conditions.[128] For example, the wines treated with high pressure after two months of bottle aging had a higher content of acetals than those unpressurized samples.[90] Wine with higher acidity is reported to favor the formation of acetals during aging.[124] Bottle aging of wines with oxygen treatment increased the concentration of acetaldehyde and subsequently increased the content of acetals.[128]

Table 3. Structures, odor descriptors, odor thresholds, and concentration ranges of major tertiary aroma volatile compounds in wines

Compound	***Structure***	***Odor descriptor***	***Odor threshold (μg/L)***	***Concentration range in aged wines (μg/L)***
Phenols				
guaiacol		smoke, sweet, medicine	910	5.8–21
syringol		smoke, medicine	57	68–488
eugenol		clove, honey	5	<1–87
vanillin		vanilla	200	40–679
4-vinylguaiacol		clove, curry	40	1.4–710
4-vinylphenol		pharmaceutical	20	40–450
4-ethylguaiacol		spice, clove	33	<1–432
4-ethylphenol		phenol, spice	440	118–3696
Acetals				
1,1-diethoxyethane		cake, fruity	1400	500–70000

The data of odor descriptor, threshold, and concentration range in wines are summarized from the literature [31,81,82,94,119,129].

2.2 Yeasts and winemaking

Yeasts are an important element in winemaking. In this section, yeast classification and identification, as well as alcoholic fermentation with conventional *S. cerevisiae* are briefly discussed, whereas emphasis was placed on the influence of alcoholic fermentation with non-*Saccharomyces* yeasts on compounds in wine associated with wine characters.

2.2.1 Yeast classification and identification

Yeasts are eukaryotic unicellular microorganisms, including two phyla of Ascomycota and Basidiomycota.[5] According to the current taxonomic and phylogenetic studies, there are approximately 1500 yeast species classified into 149 genera in nature.[5,102,130] Of which, more than 40 yeast genus and 100 species have been isolated from winemaking ecosystem.[101,131] Eight species constitute the taxon of *Saccharomyces,* including *S. arboricolus*, *S. cerevisiae*, *S. eubayanus*, *S. jurei*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, and *S. uvarum*. However, apart from the dominant *S. cerevisiae*, only *S. uvarum* has been isolated in winemaking environment. *S. kudriavzevii* has been reported in wine production in the hybrid form of *S. cerevisiae* × *S. kudriavzevii*.[102,132]

The joint term of non-*Saccharomyces* yeasts, which is used to differentiate from *Saccharomyces* yeasts, generally comprises all the yeasts isolated from winemaking-related environment other than *Saccharomyces* yeasts. The wide variety of yeasts found in winemaking environment and the similarity of cell morphology and colony of a wide range of yeasts make the identification of yeasts difficult using only conventional methods such as methods based on morphological study. Moreover, the drawbacks of slowness, laboriousness, and requirement for accurate and skillful laboratory experience limit the wide utilization of those traditional phenotypic identification methods. However, along with the development of molecular biological techniques, increasing advanced technologies have been utilized in the field of yeast identification.[133] For instance, the biochemical method of polyacrylamide gel electrophoresis (PAGE) has been used to distinguish strains in the same species or the closely related species based on proteins. The technique of Fourier transform infrared spectroscopy (FT-IR) could identify genus, species, and even strain levels through the measurement of difference on absorption wavelengths of yeasts under infrared light.[134] Another rapid, high reliability and high-throughput biochemical technique of matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) has been extensively used for classifying and identifying yeasts based on the identification of specific protein patterns.[135]

Genome analysis provides accurate results in yeast identification. As the direct detection using gel electrophoresis, indirect methods based on DNA

hybridization, and polymerase chain reaction (PCR) based approaches, involving the combination of PCR with denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative PCR (q-PCR) have been applied in the identification of non-*Saccharomyces* yeasts in wines.[136–138] Additionally, sequencing of rDNA, particularly at the internal transcribed spacers 1 (ITS1) and ITS2 regions and D1/D2 domains of 26S rDNA regions, is among the most widely used techniques in the taxonomy of yeasts.[139,140]

2.2.2 *Saccharomyces cerevisiae* in winemaking

Alcoholic fermentation is a complex microbiological process that converts sugars in grape musts or juices to ethanol and CO_2. In brief, one sugar molecule is firstly converted into two pyruvate molecules through glycolysis. Subsequently, acetaldehyde is produced from pyruvate *via* decarboxylation and reduced into ethanol (**Figure 9**). Glycerol and numerous volatile compounds, such as higher alcohols, esters, and volatile acids, as well as many other secondary byproducts, are simultaneously generated during this process. Pyruvate is further dehydrogenated into acetyl-CoA to participate in the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle and Krebs cycle) to produce organic acids (**Figure 9**). These acids are important contributors to wine sensorial characters. These processes involve a series of enzymatic reactions and energy transformations.

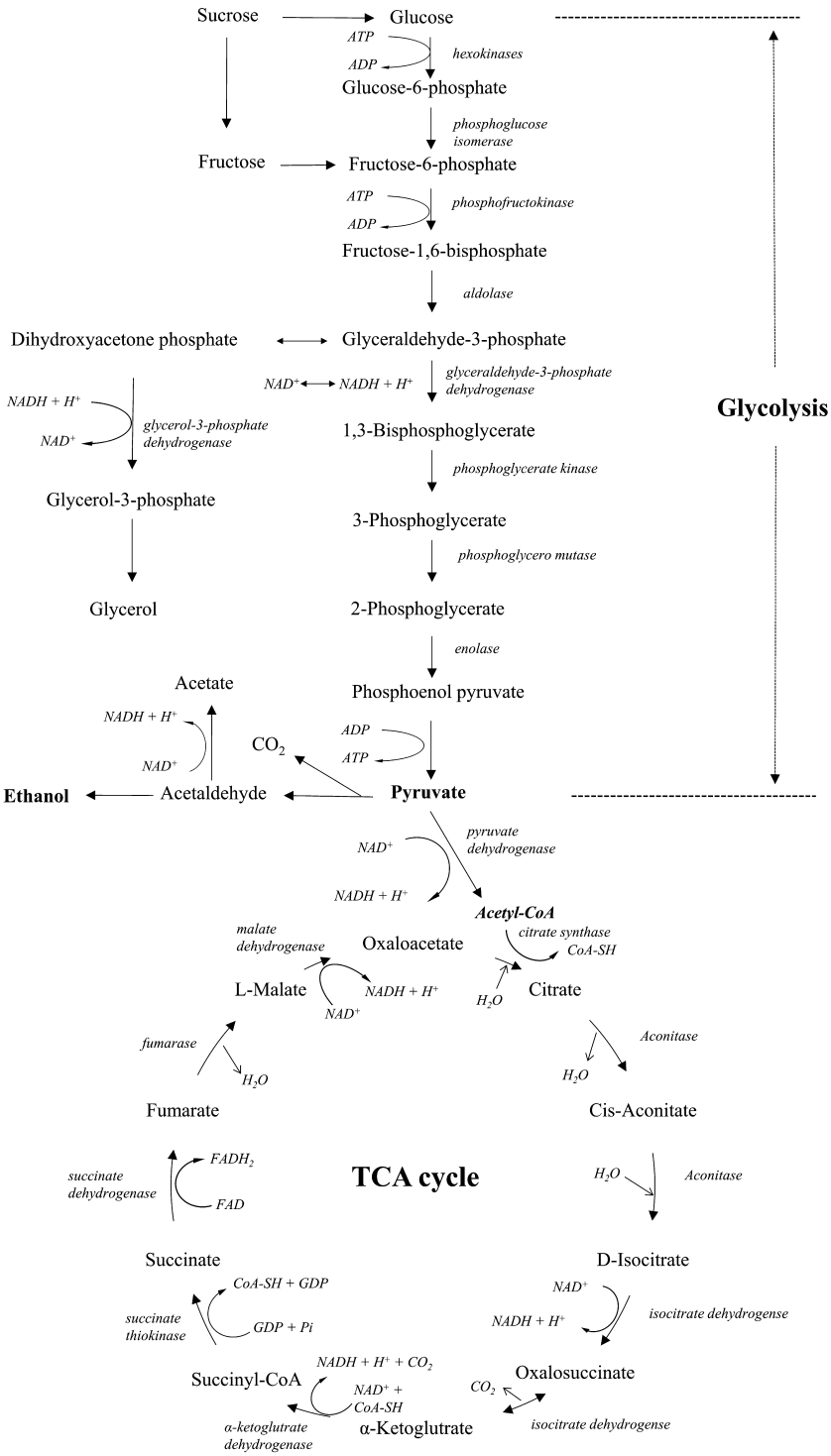


Figure 9. Sugar metabolism during alcoholic fermentation (adapted and redrawn from [6,99,119]).

Yeasts are the protagonists during alcoholic fermentation and play crucial roles in determining the quality of wines. In winery, apart from the production of wines with high quality, guarantee of the standardization of wine products and the controllability of fermentation process is also of equal importance. Spontaneous fermentation without yeast inoculation has been employed in winemaking for centuries. However, the complexity of microorganisms in spontaneous fermentation results in the uncertainty of quality of final wine products. After a long time of scientific and technological improvements in microbiology, commercial active dry yeasts belonging to *S. cerevisiae* were developed and distributed for winemaking in the 1960s, which is considered as one of the most important innovations in winemaking revolutionizing the wine industry.[9,102] The extensive application of commercial yeasts improves the efficiency of fermentation process and standardizes final products. High fermentative capacity, fast fermentation rate, high tolerance to harsh conditions, and low risks of spoilage fermentation are the main enological aptitudes of *S. cerevisiae* in comparison with other yeasts.[141]

2.2.3 Non-*Saccharomyces* yeasts in winemaking

Apart from the aforementioned advantages of using *S. cerevisiae* in winemaking, it is undeniable that the loss of distinctive features from the participation of non-*Saccharomyces* yeasts is among the biggest disadvantages of fermentation with only *S. cerevisiae*. In the past, non-*Saccharomyces* yeasts were considered as problematic strains in fermentation due to their weak fermentation capacities and the production of unknown and complicated aroma compounds that impacts the overall organoleptic quality of wines.[8,9] Therefore, previously, pasteurization of must and addition of SO_2 are the routine procedures to remove non-*Saccharomyces* yeasts from the winemaking environment. However, the preconception about non-*Saccharomyces* yeasts is changing due to increasing studies that have found the positive impacts of non-*Saccharomyces* yeasts on wine quality, especially on flavor complexity. Nevertheless, in comparison with *S. cerevisiae*, the poor fermentation capacity of almost all non-*Saccharomyces* yeasts and the concurrently more or less unpleasant compounds produced by inoculation with a pure non-*Saccharomyces* yeast are often observed. Hence, fermentation with co-cultures (sequential and simultaneous inoculations) non-*Saccharomyces* yeasts with *S. cerevisiae* is a commonly used practice to complete fermentation and is also an optimal way to retain the positive impacts and mitigate or eliminate the negative influences of non-*Saccharomyces* yeasts on wine quality.

In the following section, the contributions of selected non-*Saccharomyces* yeasts on wine quality are reviewed, focusing on *Torulaspora delbrueckii*,

Schizosaccharomyces pombe, *Pachysolen tannophilus*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Zygosaccharomyces bailii*, *Lachancea thermotolerans*, *Issatchenkia orientalis*, and *Saccharomycodes ludwigii*. These non-*Saccharomyces* yeasts are gaining attention in the studies related to winemaking in recent years. The impacts of sequential and simultaneous inoculation of *Saccharomyces* and non-*Saccharomyces* yeasts on wine composition are also reviewed when data are available (**Table 4**).

Table 4. Advantages and disadvantages of non-*Saccharomyces* yeasts in pure inoculation, and/or sequential and simultaneous inoculations with *Saccharomyces cerevisiae* on chemical composition of wines

Yeast	***Inoculation type***	***Advantage***	***Disadvantage***
Torulaspora delbrueckii	pure	↑ glycerol, fruity esters, anthocyanins, vitisin A ↓ acetic acid, acetaldehyde, ethyl acetate, acetoin, and hydrogen sulfide	uncompleted fermentation ↓ vitisin B
	sequential	↑ ethanol, ethyl esters, overall perception, anthocyanins, overall perception ↓ fatty acids, higher alcohols	
	simultaneous	↑ ethanol, ethyl esters, glycerol ↓ acetaldehyde, higher alcohols	
Schizosaccharomyces pombe	pure	↑ vitisin A, chromatic stability, glycerol ↓ malic acid, lactic acid, gluconic acid	↑ acetaldehyde, acetoin
	sequential	↑ vitisins, glycerol ↓ malic acid, acetaldehyde	↓ vitisins, glycerol
	simultaneous	↑ vitisins, glycerol ↓ malic acid, acetaldehyde	↓ vitisins, glycerol
Metschnikowia pulcherrima	pure	↑ terpenes, anthocyanin, color, polysaccharides, antimicrobial activity ↓ acetic acid, 3-mercaptohexyl acetate, fatty acids	uncompleted fermentation ↑ higher alcohols ↓ esters
	sequential	↑ ethanol, acetate esters, glycerol	
	simultaneous	↑ ethanol, acetate esters	
Hanseniaspora uvarum	pure	↑ monoterpenes, 2-phenylethyl acetate, 3-methylbutyl acetate ↓ acetaldehyde, fatty acids, higher alcohols	uncompleted fermentation ↑ acetic acid, ethyl acetate, acetoin, and sulfur compounds

Yeast	*Inoculation type*	*Advantage*	*Disadvantage*
Hanseniaspora uvarum	sequential	↑ ethanol ↓ acetic acid, ethyl acetate, acetoin, and sulfur compounds, higher alcohols, fatty acids, and acetaldehyde	↑ higher alcohols, fatty acids, and acetaldehyde
	simultaneous	↑ ethanol ↓ acetic acid, ethyl acetate, acetoin, and sulfur compounds, higher alcohols, fatty acids, and acetaldehyde	↑ higher alcohols, fatty acids, and acetaldehyde
Zygosaccharomyces bailii	pure	↑ fructophilic activity, tolerance to harsh conditions, polysaccharides ↓ malic acid, acetic acid, acetaldehyde	↑ sediment, cloudiness, turbidity, hydrogen sulfide, methanol, acetoin, and ethyl acetate
	sequential	↑ esters, anthocyanins, color	
	simultaneous	↑ ethyl esters	↑ ethyl acetate
Lachancea thermotolerans	pure	↑ lactic acid, color intensity, anthocyanins, ethyl esters, 2-phenylethanol ↓ acetic acid, acetaldehyde, fatty acids, acetoin, higher alcohols	uncompleted fermentation ↓ glycerol
	sequential	↑ ethanol, lactic acid, color intensity, anthocyanins, glycerol ↓ acetic acid, higher alcohols	↑ acetic acid
	simultaneous	↑ ethanol, lactic acid, color intensity, anthocyanins, glycerol ↓ acetic acid, higher alcohols	↑ acetic acid
Issatchenkia orientalis	pure	↓ malic acid, acetaldehyde, higher alcohols, acetate esters, fatty acids, 1-propanol, 2-butanol, 3-methyl-1-butanol,2,3-butanediol,	↑ methanol, acetoin, ethyl acetate ↓ glycerol, 2-phenylethanol

Yeast	*Inoculation type*	*Advantage*	*Disadvantage*
Saccharomycodes ludwigii	pure	↑ limpidity and effervescence (sparkling wine), polysaccharides, glycerol ↓ 2,3-butanediol, acetic acid	↑ resistance to SO_2 and ethanol, ethyl acetate, acetic acid, acetoin, 2-methyl-propanol, 1-pentanol, 3-methyl-1-butanol, acetaldehyde
	sequential	↓ethyl acetate, acetaldehyde, 2-methyl-1-propanol, 3-methyl-1-butanol	
	simultaneous	↓ ethyl acetate, acetaldehyde, 2-methyl-1-propanol, 3-methyl-1-butanol	
Pachysolen tannophilus	pure	↑ xylose consumption ↓ malic acid	

The content changes of compounds with underlines refer to the comparison between co-inoculations (sequential and simultaneous inoculations) and pure inoculation with corresponding non-*Saccharomyces* yeast. Otherwise, referring to the comparison between co-inoculations and pure inoculation with *S. cerevisiae*.

2.2.3.1 Torulaspora delbrueckii

T. delbrueckii is one of the most important non-*Saccharomyces* yeasts that has been widely used in winemaking. Moreover, it is the first non-*Saccharomyces* yeast that has been commercialized and utilized at industrial level.[142] It is characterized by the relatively high fermentation ability and low production of off-flavor compounds compared to other non-*Saccharomyces* yeasts. For example, fermentation with pure *T. delbrueckii* was reported to significantly reduce acetic acid production even in the medium with high content of sugars (hyperosmotic medium).[143,144] In comparison with *S. cerevisiae*, lower productions of undesirable volatile compounds, such as acetaldehyde, ethyl acetate, acetoin, and hydrogen sulfide, are the positive contributions of *T. delbrueckii* to wine quality.[145–148] *T. delbrueckii* could also improve wine sensorial properties through increasing the levels of some desirable compounds, such as glycerol and fruity esters.[142,149,150]

Despite the aforementioned positive impacts of *T. delbrueckii* on wine quality, it is not recommended to produce wine using *T. delbrueckii* alone since *T. delbrueckii* cannot independently complete typical alcoholic fermentation due to its maximum ethanol production of about 9–10%.[142] For this reason, inoculating *T. delbrueckii* sequentially or simultaneously with *S. cerevisiae* is a commonly used approach to overcome this problem. Several previous studies have found that sequential and simultaneous fermentations with *T. delbrueckii* and *S. cerevisiae* significantly increased ethanol production of 2.7–6.2% (*v/v*) compared to the fermentation with pure *T. delbrueckii*.[143,151] These processes also significantly modified the productions of flavor compounds. For example, sequential and simultaneous fermentations significantly increased the content of ethyl esters (exclude ethyl acetate) by approximately 7–10 times compared to that with pure *T. delbrueckii*.[151] Similar results were obtained in numerous other studies.[142,145,150,152] The concentrations of acetaldehyde in *T. delbrueckii*/*S. cerevisiae* sequential and simultaneous fermentations were 21 mg/L and 11–27 mg/L less than those in pure *T. delbrueckii* fermentation, respectively.[143] In comparison with the control fermentation with pure *S. cerevisiae*, the co-fermentation involving *T. delbrueckii* remarkably reduced the contents of fatty acids and higher alcohols.[153–155] Moreover, through the performance of sensory evaluation, Loira et al., concluded that the overall perception of the wines produced by sequential fermentations was better than that of wines produced by single-culture fermentation with *S. cerevisiae*.[152]

Fermentations involving *T. delbrueckii* also impact the contents of anthocyanins and anthocyanin-derived compounds in wines. Specifically, the Pinotage wine fermented with pure *T. delbrueckii* had approximately 50 mg/L higher total anthocyanin content than those fermented with pure *S. cerevisiae*.[156] While a significant increase by approximately 46 mg/L in total anthocyanin

content was detected in sequential fermentation using the two yeasts in comparison with pure *S. cerevisiae* fermentation.[157] This phenomenon could be explained by the less anthocyanin adsorption in *T. delbrueckii* cell walls.[158] In comparison with fermentation with pure *S. cerevisiae*, fermentation with pure *T. delbrueckii* was reported to enhance the formation of vitisin A due to its higher production of pyruvic acid,[145] whereas the vitisin B content showed significant reduction resulting from the less production of acetaldehyde from *T. delbrueckii*.[142] Consistent results have also been detected in sequential fermentation with *T. delbrueckii* and *S. cerevisiae*.[152,159] The development of anthocyanin profiles further modifies the color attributes of wines.[158] Fermentation involving *T. delbrueckii* was reported to alter the profiles of other phenolic compounds, i.e. flavonols, flavan-3-ols, and phenolic acids, and further affected the mouthfeel and taste of the final wines.[160,161]

2.2.3.2 Schizosaccharomyces pombe

S. pombe is drawing increasing attention in winemaking due mainly to its outstanding deacidification capacity compared to other yeast species.[162] In general, the degradation degree of malic acid by inoculation with *S. pombe* varies from 75% to 100% during fermentation.[163] The reduction of malic acid content is of significance to weaken green apple sourness, acidity, and puckering astringency of wines. With regard to the wines with a high content of malic acid, the performance of malolactic fermentation with *Oenococcus oeni* is the common procedure to transform malic acid to lactic acid.[164] However, numerous studies have found that some degenerations occurred during malolactic fermentation are detrimental to wine quality, such as the declines in anthocyanins and color intensity and promotion of the formation of biogenic amines.[165–167] Therefore, carrying out alcoholic and malolactic fermentations in parallel with inoculation of *S. pombe* may circumvent the problem, to some extent.[168,169] Simultaneously, due to the final products of malic acid conversion from *S. pombe* are ethanol and CO_2, fermentation with *S. pombe* avoids the high accumulation of lactic acid.[5,167] This characteristic of *S. pombe* has been noticed by the OIV and approved the regulation of "Deacidification by *Schizosaccharomyces*" in 2013 (Code OENO 1/03).

Gluconic acid is considered as an indicator of rottenness level of harvested grapes and *Botrytis* infection of wines. The presence of this compound was reported to cause microbiological instability and high bondable SO_2.[29,92,170] A protocol has been developed to reduce gluconic acid content in grape musts by inoculating *S. pombe*.[170] As a result, approximately 70% gluconic acid was consumed after inoculation with *S. pombe* without negative effects on the analytical or sensory quality of the resulting wines.

Additionally, in comparison with the fermentation with *S. cerevisiae*, high production of pyruvic acid and, consequently, high formation of vitisin A and its derivatives were detected in the fermentation with *S. pombe* in numerous studies,[154,158,162,171] which improves the chromatic stability of wines, particularly during a long-term aging.

High synthesis of glycerol during fermentation is another microbiologic characteristic of *S. pombe*. The improvement of glycerol production from fermentation with *S. pombe* is strain-dependent varying from 14% to 42% higher than those with *S. cerevisiae*.[171,172]

However, the OIV has also reminded that the undesirable impacts on wine flavor are not negligible. High productions of unpleasant compounds of acetaldehyde and acetoin in the fermentations with *S. pombe* compared to those with *S. cerevisiae* were demonstrated in winemaking.[5,169,173] Therefore, with the aim to limit or eliminate the negative impacts of *S. pombe* on wine quality, the strain of *S. pombe* usually is inoculated together with *S. cerevisiae*. Del Fresno et al. have detected that almost all malic acid was consumed in the wines fermented with either pure *S. pombe* or mixed inoculants, and, meanwhile, the concentration of acetaldehyde in the fermentations with sequential and simultaneous yeasts showed significantly decrease.[174] Similar results were also obtained in other studies.[154,175] Although mixed fermentations resulted in decreases in the contents of vitisins and glycerol, their levels are still higher than those obtained with fermentation using pure *S. cerevisiae*.[154,162,175]

2.2.3.3 Metschnikowia pulcherrima

M. pulcherrima is another non-*Saccharomyces* yeast than *T. delbrueckii* that has been commercialized. *M. pulcherrima* is getting popularity in both laboratorial and industrial levels of wine production in recent years. Numerous studies have reported the positive impacts of *M. pulcherrima* on the volatile compositions of wines. For instance, significantly lower productions of acetic acid, a polyfunctional thiol of 3-mercaptohexyl acetate, and fatty acids, including octanoic and decanoic acids, were detected in Sauvignon Blanc wine produced with pure fermentation with *M. pulcherrima* than that produced with pure *S. cerevisiae*. However, the generation of geraniol is the contrary.[176] The increase in the content of geraniol was also verified in synthetic grape juice fermentation.[177] The high production of terpenes in the fermentation with *M. pulcherrima* is associated with the high activity of β-glucosidase in the extracellular of this strain.[5]

Fermentation with *M. pulcherrima* has been reported to affect phenolic composition and color of wine. Tempranillo wine produced by *M. pulcherrima* showed 37% higher anthocyanin content, especially non-acylated anthocyanins and malvidin 3-*O*-acetylglucoside, than that by *S. cerevisiae*.[178] Due to the high

polygalacturonase activity of this strain, *M. pulcherrima* has been proposed as a positive yeast for enhancing wine color.[179] Moreover, the high release of polysaccharides has been detected in the fermentation with *M. pulcherrima*.[180] Antimicrobial activity is another typical characteristic related to *M. pulcherrima*. *M. pulcherrima* possesses a broad and effective antimicrobial action on undesired wild spoilage yeasts, such as *Brettanomyces*/*Dekkera* but had no influence on the growth of *S. cerevisiae*.[181]

The reports on the fermentation capacity of *M. pulcherrima* are of significant difference varying ethanol production from < 4 to approximately 10% (*v/v*).[159,176,180,182] This may be due to the different distinct biotypes within *M. pulcherrima* species. Moreover, *M. pulcherrima* is reported as a strong producer of higher alcohols but a weak producer of esters.[159,183] Therefore, *M. pulcherrima* is usually inoculated together with *S. cerevisiae*. As expected, the ethanol production in co-fermentation of *M. pulcherrima* and *S. cerevisiae* significantly increased to approximately 14%.[178,183] Furthermore, increasing production of ester, particularly acetate esters, was detected in the Muscat d'Alexandrie and Chardonnay wines produced by sequential inoculation.[182,184] Consistent results were also obtained in Vidal blanc icewine and Merlot wine produced by simultaneous fermentations.[183,185] The content of glycerol increased significantly in the sequential fermentation with *M. pulcherrima*/*S. cerevisiae* compared to the pure fermentation with *S. cerevisiae*.[186]

The reports on the effects of co-fermentation on higher alcohol content are controversial. Several studies documented that the content of higher alcohols showed a significant decrease after sequential fermentation in Verdicchio and Verdejo white wines and Vidal blanc icewine,[177,185,187] whereas some studies reported that the concentration of higher alcohols increased rather than decreased in Sauvignon Blanc and Muscat d'Alexandrie wines,[182,183] indicating that the difference of matrix, fermentation condition, and yeast biotype all influence the production of higher alcohols in fermentation involving *M. pulcherrima*.

2.2.3.4 Hanseniaspora uvarum (Kloeckera apiculate)

H. uvarum is one of the major yeast strains present in the early stage of spontaneous fermentation, thereafter it disappears during fermentation and shows an extremely low viable count value at the end of fermentation due to its low ethanol tolerance or to other toxic compounds besides ethanol.[188] *H. uvarum* is viewed by most researchers as a detrimental yeast due to its extremely poor fermentation ability and high productions of undesirable flavor compounds, such as acetic acid, ethyl acetate, acetoin, and sulfur compounds.[102,189–191] Ciani et al. surveyed the ethanol production of 14 *H. uvarum* strains and found that all these strains produced ethanol less than to a final concentration of 6% after fermentation.[192] Romano et al. compared the production of aroma compounds

between 52 strains of *S. cerevisiae* and 59 strains of *H. uvarum* and found that the average level of acetic acid in the wines fermented with *H. uvarum* strains was approximately 2 g/L, whereas less than 0.6 g/L of acetic acid was detected in wines produced by *S. cerevisiae* strains.[190] Moreover, there have been about 9 and 3 times, respectively, higher ethyl acetate and acetoin productions in the white wine fermented with pure *H. uvarum* than that fermented with *S. cerevisiae* monoculture in the laboratory scale fermentation.[192] While approximately 2 and 8 times higher these two compounds were previously found in red wines produced in pilot scale.[190] The high amount of volatile sulphur compounds in the wine fermented with *H. uvarum* was reflected in acetic acid-3-(methylthio)propyl ester, *trans*-2-methyltetrahydro-thiophen-3-ol, and 2-mercaptoethanol.[193] Therefore, enologists have warned that cautious consideration is needed when using *H. uvarum* in winemaking.[5]

Some positive impacts on wine flavor associated with *H. uvarum* have been found in several studies. For example, in comparison with the wine produced with *S. cerevisiae*, more *floral* and *fruity* contributors of 2-phenylethyl acetate and 3-methylbutyl acetate,[193] while approximately 15 mg/L lower acetaldehyde were detected in the wines fermented with *H. uvarum*.[194] Some previous studies found that the total fatty acid (sum of hexanoic, octanoic, and decanoic acids)[195] and total higher alcohol contents[193] in *H. uvarum* wines were almost 3.5- and 4-fold, respectively, lower than those in *S. cerevisiae* wines, which is beneficial for weakening the detrimental *alcohol*, *nail polish*, *rancid*, and/or *fatty* odors in wines. Fermentation with *H. uvarum* is also characterized by the high release of monoterpenes due to the high β-glucosidase activity hydrolyzing glycoconjugated monoterpenes precursors.[196]

Sequential and simultaneous fermentations of *H. uvarum* and *S. cerevisiae* significantly increased ethanol production, although the final content of ethanol is still 0.9–1.6% lower than the wine produced with *S. cerevisiae* alone.[193,197] This is a common characteristic of non-*Saccharomyces* yeasts that consumes more sugars for the biosynthesis of yeast biomass or the formation of byproducts.[192,198] In specific terms, *H. uvarum* requires more than 19 g of sugars to produce 1% (*v/v*) of ethanol,[199] whereas *S. cerevisiae* generally only needs 17.5 g.[21] The concentrations of acetic acid, ethyl acetate, acetoin, and sulfur compounds unsurprisingly showed significant reduction after co-fermentation. Although sequential and simultaneous fermentations led to concurrently increase in the concentrations of higher alcohols, fatty acids, and acetaldehyde to some extent due to the neutralizing effect of *S. cerevisiae*, their concentrations were still lower than the corresponding levels in wines fermented with pure *S. cerevisiae*.[191,193]

2.2.3.5 Zygosaccharomyces bailii

Highly clarified must, excessively low or high fermentation temperature, lack of nitrogen sources, and high sugar content may cause stuck alcoholic fermentation.[119] *Z. bailii* has been used as an effective species to restart stuck fermentation in the German wine industries starting from 2007.[119] This is primarily due to the fructophilic activity and the high tolerances of this species to osmotic stress, low pH, high concentration of preservatives, such as organic acids, high level of ethanol, and heat.[102,200]

The presence of *Z. bailii* is often related to the visual faults of sediment, cloudiness or turbidity in dry wines.[13,102] *Z. bailii* is also characterized by the high production of polysaccharides, which is beneficial for improving wine flavor and mouthfeel qualities by increasing the perceptions of viscosity and fullness on the palate.[5] Similar to *S. pombe* strains, *Z. bailii* also shows a strong deacidification of malic acid (40–100%) during fermentation.[13,201] Additionally, the deacidification is enhanced by the acetic acid metabolism, 28–62% initial acetic acid being consumed by *Z. bailii* under aerobic conditions.[202] Due to the distinctive metabolism of *Z. bailii* during fermentation, corresponding *Z. bailii* wines differ from those fermented with conventional *S. cerevisiae*. With regard to the off-flavor compounds, wines produced by pure inoculation of *Z. bailii* had significantly higher concentrations of hydrogen sulfide (H_2S), methanol, acetoin, and ethyl acetate than those fermented with *S. cerevisiae*, whilst significantly low concentration of acetaldehyde was found in *Z. bailii* wines.[8,203–205]

Simultaneous inoculation of *S. cerevisiae* and *Z. bailii* at inoculum ratio of 1:1 was reported to favor the production of ethyl esters, including ethyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, whereas excessive dominance (1:100 or 1:1000) of *Z. bailii* promoted the production of ethyl acetate to a level above the critical level (150 mg/L) contributing to undesirable odor.[203] However, a significantly different result was observed in another study reporting that the concentration of ethyl acetate in the fermentation with co-inoculums did not show statistical difference compared to the control fermentation with *S. cerevisiae* monoculture, even increased the inoculum ratio to 1:10000.[206] Canonico et al. compared the impact of sequential inoculations of *M. pulcherrima*, *T. delbrueckii,* and *Z. bailii* with *S. cerevisiae* on wine volatile composition under aeration condition and found that only *Z. bailii*/*S. cerevisiae* slightly increased ester production.[186] Furthermore, sequential fermentation of *Z. bailii* and *S. cerevisiae* was reported to reduce approximately ethanol content (by 2%, *v/v*)[207] and increase anthocyanin content by 36% thus improving wine color[178] compared to pure fermentation with *S. cerevisiae*. The increase of anthocyanins may be due to the release of pectinase enzymes during maceration or difference in anthocyanin adsorption in yeast cell walls.[179,208]

2.2.3.6 *Lachancea thermotolerans*

Over the past few decades, global warming results in a serious decrease of acidity of grapes, particularly the viticultural region located in the tropical and temperate zones. This further lowers the overall acidity and changes the sugar/acid balance of their corresponding wines. Addition of food-grade acids, particularly tartaric acid, is a common practice to solve this problem in the wineries under this circumstance. However, this procedure of acid addition leads to some other problems, such as the precipitation of tartaric acid and potassium, to break the chemical stability of final wines.[205] For this reason, microbiological acidification is gaining acceptance in wine industry. *L. thermotolerans* is specifically isolated and commercialized for this purpose. The improvement of acidity by inoculation of *L. thermotolerans* results from the conversion of sugars to lactic acid. Kapsopoulou et al. reported that fermentation with *L. thermotolerans* monoculture produced approximately 9.6 g/L higher content of lactic acid than that with *S. cerevisiae*.[209] Gobbi et al. found that the lactic acid production difference between *L. thermotolerans* and *S. cerevisiae* is 3.26 mg/L (3.42 vs 0.16 mg/L), which led to a 2.27 g/L higher of total acidity and a 0.13 lower of pH.[210]

However, excessively high amount of lactic acid is harmful to wine flavor and *L. thermotolerans* has intermediate fermentative capacity (7–8% *v/v* in ethanol),[159] therefore it is necessary to combine *L. thermotolerans* with *S. cerevisiae* to mitigate the acidification and to complete alcoholic fermentation. As expected, the ethanol production in co-fermentations reached the same level as that in control fermentation with *S. cerevisiae*.[211] The production of lactic acid in sequential fermentation showed a moderate increase by 0.6–5.1 g/L compared to the control, while an increase by 0.18–0.65 mg/L was observed in simultaneous fermentation.[210,211]

The acidification by the participation of *L. thermotolerans* in fermentation contributes to the quality improvement of the wines produced in warm climates, giving roundness and balanced acidity to the wines and further improving their freshness. Moreover, due to the pH reduction, the coloration of anthocyanin molecules in wine matrix increases. Consequently, the color intensity of the wine produced with yeasts involving *L. thermotolerans* was reported to have an approximately 10% increase.[205] Additionally, the anthocyanins content in the wine from fermentation involving *L. thermotolerans* further increases due to its lower adsorption capacity of anthocyanins compared to *S. cerevisiae*.[212] Hranilovic et al. reported an approximately 7% higher anthocyanin content in sequential fermentation compared to that in the control fermentation with *S. cerevisiae*,[213] while an even greater increase (approximately 22%) has also been reported.[157]

Aside from the improvement of freshness and color, aroma complexity is also modified when *L. thermotolerans* is involved. A 230 mg/L lower concentration of acetic acid was previously reported in the fermentation with *L. thermotolerans* monoculture than that in the pure *S. cerevisiae* fermentation.[209] Although sequential and simultaneous inoculations enhanced the release of acetic acid, the levels of acetic acid were still 20–60 mg/L lower than the control with pure *S. cerevisiae* fermentation.[211] However, Del Fresno et al. reported a contrary result suggesting that an approximately 140 mg/L higher acetic acid was produced in sequential inoculation,[174] while Santiago Benito et al. found no difference between these two methods of fermentation.[214]

Mixed fermentation with these two cultures significantly increased the production of glycerol with up to 1.85 g/L higher than that in control with pure *S. cerevisiae*.[174,210,215,216] However, pure *L. thermotolerans* fermentation produced a significantly lower concentration of glycerol compared to the control.[209] These results indicate that *L. thermotolerans* possesses a great capacity to produce glycerol, whereas the incomplete consumption of sugars may hinder the release of glycerol.

L. thermotolerans is also characterized by the low productions of aldehydes, particularly acetaldehyde, fatty acids, and acetoin[159,205,216,217] and the high productions of ethyl esters and 2-phenylethanol.[205,214] The conclusion on the production of higher alcohols in the fermentation involving *L. thermotolerans* is controversial. With this regard, Gobbi et al. reported that the content of higher alcohols in the fermentation with pure *L. thermotolerans* was significantly lower than the corresponding levels in the control, of which the biggest difference was observed in 2-methyl-1-propanol and 3-methyl-1-butanol.[210] Although sequential and simultaneous fermentations significantly elevated their levels, the amounts were still significantly lower than the control. Benito et al. suggested that 3-methyl-butanol decreased significantly in simultaneous inoculation, whereas the 2-methyl-1-propanol concentration in sequential and simultaneous fermentations and 3-methyl-butanol in simultaneous fermentation did not show significant changes.[218] On the contrary, Del Fresno et al. detected a 20 mg/L increase in higher alcohol content in sequential fermentation, reflected mainly in the concentrations of 2-methyl-1-propanol, 1-propanol, and 1-butanol.[174] Consistent results were also obtained previously in sequential fermentations and simultaneous fermentation.[159,215,216] These discrepancies could be explained by the great biodiversity in *L. thermotolerans* strains in terms of the capacity for higher alcohol production (up to 40%).[168]

2.2.3.7 Issatchenkia orientalis (Pichia kudiavzevii)

I. orientalis is another non-*Saccharomyces* yeast which possesses powerful malic acid degradation capacity. Mónaco et al. isolated an *I. orientalis* strain in

Patagonia showing a 38% malic acid degradation in microvinification, while the level in control *S. cerevisiae* was 22%.[219] The deacidification of *I. orientalis* increased pH by approximately 0.2–0.3 unit. Fermentation with pure *S. cerevisiae* showed a 3.3-fold higher malic acid concentration than that with simultaneous inoculation of *I. orientalis*, a yeast isolated from Korean grape wine pomace, and *S. cerevisiae* at inoculum ratio of 1:1 (*v/v*).[220] Moreover, the wine produced at this fermentation condition significantly reduced the concentrations of acetaldehyde, 1-propanol, 2-butanol, and 3-methyl-1-butanol but significantly increased the concentration of methanol and, further got the highest scores in flavor, taste, and color aspects by sensory evaluation.[220] The improvement of color and the lower accumulations of acetaldehyde, 2-butanol, and 3-methyl-1-butanol as well as the higher production of methanol were verified in the pure fermentation with *I. orientalis* in comparison with the fermentation with *S. cerevisiae*.[221] In the same study, *I. orientalis* was also profiled by the low productions of glycerol, 2,3-butanediol, and 2-phenylethanol. Cordero-Bueso et al. compared the composition of total 23 volatile compounds between the wines produced by pure *S. cerevisiae* and *I. orientalis* and found that the concentrations of acetaldehyde, higher alcohols, acetate esters, and fatty acids were significantly lower, whereas the acetoin content is significantly higher in the latter sample.[148]

Excessively high generation of ethyl acetate is a key characteristic of *I. orientalis* as indicated by 220–730 mg/L production of this compound in winemaking involving *I. orientalis*, whereas the amount in typical wines is 10–100 mg/L.[222]

2.2.3.8 Saccharomycodes ludwigii

S. ludwigii is previously considered as one of the contaminative and problematic non-*Saccharomyces* yeasts during winemaking due to its capacity to produce a high amount of unpleasant metabolites, the resistance to high concentration of SO_2 and ethanol (up to 12%, *v/v*), as well as the ubiquity in winemaking environment ranging from the surfaces of grape and fermentation equipment to cellar. *S. ludwigii* is difficult to be fully eradicated from wine matrix *via* the remedial addition of SO_2 at the end of fermentation.[8,192,223,224] High ethyl acetate production in the range from 160 to 560 mg/L was reported in a previous study investigating 11 strains of *S. ludwigii*. Contrastively, only 30–40 mg/L of ethyl acetate was produced from 3 strains of *S. cerevisiae*.[8] The result was verified by another study suggesting an average of approximately 300 mg/L of ethyl acetate was produced from 25 strains of *S. ludwigii* but approximately 50 mg/L from 127 strains of *S. cerevisiae*.[190]

Strains of *S. ludwigii* have also been reported to be characterized by the high productions of acetic acid, acetoin, 2-methyl-propanol, 1-pentanol, and 3-

methyl-1-butanol from a study on the production of secondary metabolites during wine fermentation with 19 strains of *S. ludwigii*.[225] An accordant result was obtained by Ciani and Maccarelli comparing the enological properties of 27 strains of *S. ludwigii* and 50 strains of *S. cerevisiae*.[192] The result suggested that the average concentrations of acetoin, ethyl acetate, and acetaldehyde were 310, 289, and 88 mg/L, respectively, among the strains of *S. ludwigii*, 56, 35, and 63 mg/L for the *S. cerevisiae* strains. However, the acetic acid production is slightly lower among the former strains than the latter.

The high production of off-flavor compounds significantly reduces the potential use of *S. ludwigii* in enology. However, the strains of *S. ludwigii* also have several positive contributions to wine quality. For example, red sparkling wine second-fermented with *S. ludwigii* presented higher limpidity and effervescence than that with *S. cerevisiae*.[173] Additionally, high releases of polysaccharides[8,226] and glycerol but low production of 2,3-butanediol[190,192] were found to be the fermentation characteristics of *S. ludwigii*.

The negative impacts of *S. ludwigii* on wine organoleptic quality have been modulated to a certain extent through co-inoculation with *S. cerevisiae* as the ethyl acetate concentration was reduced from 543 mg/L in pure *S. ludwigii* fermentation to 99 mg/L in simultaneous fermentation of *S. ludwigii* and *S. cerevisiae* at inoculum ratio 1:1.[8] Moreover, the concentrations of acetaldehyde (29 vs 31 mg/L), 2-methyl-1-propanol (89 vs 69 mg/L), and 3-methyl-1-butanol (126 vs 113 mg/L) in the co-fermentation reduced to the levels close to the fermentation with pure *S. cerevisiae*. These were also achieved by regulating fermentation conditions as lower concentrations of acetaldehyde, acetoin, ethyl acetate, and higher alcohols were yielded in the wine fermented at 25 °C than that at 15 °C.[223]

2.2.3.9 Pachysolen tannophilus

P. tannophilus is the first non-*Saccharomyces* yeast found to produce significant amounts of ethanol from xylose with the conversion ratio of 1 g xylose/0.41 g ethanol.[227,228] The reproduction of *P. tannophilus* is aerobic, hence oxygen condition is a key factor determining the conversion process from xylose to ethanol.[229] This process is also influenced by the nutrition level, ethanol content, temperature, and pH in matrix.[228,230] Besides xylose, glucose, mannose, galactose, and glycerol are the carbon sources of *P. tannophilus* for producing ethanol as well.[229,231]

The deacidification was reported in the fermentation with *P. tannophilus* resulting from the consumption of malic acid.[232] The fermentation capacity of *P. tannophilus* is poor consuming only 47.7% sugars in a synthetic grape juice (glucose 75 g/L, fructose 75 g/L, tartaric acid 3 g/L, pH 3.5).[204] Therefore, sequential or simultaneous inoculation with *S. cerevisiae* is needed to complete

alcoholic fermentation. However, to the best of the knowledge of the author, there are still no reports on the effect of co-inoculation of *S. cerevisiae* and *P. tannophilus* on the chemical composition of wine.

2.3 Nongrape berry wine production

In botanical terminology, “berry” is the fleshy fruit comprising seeds (pips) and pulp (pericarp) produced from the ovary of a single flower. The seeds are usually embedded in the fleshy interior of the ovary, and the edible pericarp are developed from the outer layer of the ovary wall. The pericarp is divided into three layers: exocarp, mesocarp, and endocarp (**Figure 10**).[233,234] In common usage, the term “berry” is defined as the small edible fruit often characterized as being juicy, rounded, brightly colored, sweet, or sour, without a stone or pit according to the Merriam-Webster Dictionary.

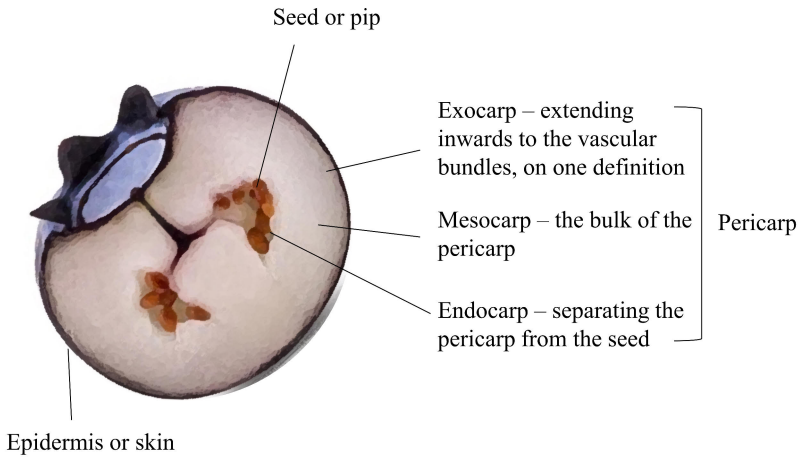


Figure 10. Anatomical diagram of typical berry, blueberry as an example.

Due to difference between the everyday and botanical uses of the term "berry" there are three different categories of berries. The first one includes the fruits that are berries under both definitions, such as the most typical grapes (*Vitis* spp.), the *Vaccinium* species including blueberry (*V. corymbosum*), bilberry (*V. myrtillus*), cranberry (*V. macrocarpon*), lingonberry (*V. vitis-idaea*), and huckleberry (*V. ovatum*), and the *Ribes* species including black (*R. nigrum*) and red and white currants (*R. rubrum*) and gooseberry (*R. uva-crispa*), as well as some commonly eatable fruits, such as goji berry (*Lycium barbarum*) and elderberry (*Sambucus nigra*). Berries in the second category are those fruits that are botanically berries but not commonly known as berries, such as banana (*Musa* spp.), orange (*Citrus* × *sinensis*), lemon (*Citrus limon*), persimmon (*Diospyros kaki*), avocado (*Persea americana*), and even including those are

usually considered as vegetables, such as tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), and peppers (*Capsicum* spp.). Third category covers those fruits that are commonly called berries but actually are not berries in the strict sense of botanical terminology, such as blackberry (*Rubus allegheniensis*), strawberry (*Fragaria* × *ananassa*), and black raspberry (*Rubus coreanus*). They are aggregate fruits containing seeds from different ovaries of a single flower. Moreover, the typical multiple fruits of black mulberry (*Morus nigra*) and drupe of cherry (*Prunus avium*) and lychee (*Litchi chinensis*) also belong to the third category.[11,13,235]

Unlike alcoholic beverages made from grape or grape must/juice through yeast fermentation, which are exclusively defined using the term "wine". There is a lack of a consistent standard of the definition of alcoholic beverages produced from berries other than grapes. However, it is generally accepted to use the nomenclature of "berry wine" to define this type of products, for example, bilberry wine or cranberry wine refers to fermented alcoholic beverages produced from juice or must of bilberries and cranberries, respectively.[13] Furthermore, in the field of berry wine production, the "berry" in the term "berry wine" generally follows the common usage of "berry" instead of strictly following its botanical terminology. For instance, the "wines" produced from blackberry and strawberry are conventionally named as blackberry wine and strawberry wine, respectively.[236] On the contrary, the wines produced from the "bigger" botanical berries, such as banana, mango, orange, and persimmon, are usually denominated as "fruit wines", which is a broader term also covering "berry wines". Unless otherwise specified, the term "berry" applied in the text below refers to the nongrape berry species.

2.3.1 Health-promoting compounds in berries

Proper quality of berries is an important element for making berry wines with premium quality. Berries are often considered as the functional foods due to their high content of bioactivity compounds, such as vitamins, minerals, and particularly phenolic compounds.

2.3.1.1 Vitamins and minerals

Vitamins mainly help to boost the immune system and reduce inflammation of humans. They also are considered to possess a certain therapeutic effect on chronic diseases such as heart disease and diabetes.[237] Vitamin C (ascorbic acid) is among the most typical vitamins that originated from berries. Humans cannot synthesize vitamin C because of the absence of L-gulonolactone oxidase enzyme. Berries are a rich sources of vitamin C. Blackcurrants contain vitamin C at levels

up to 150 mg/100 g fresh weight (FW), followed by strawberries (up to 85 mg/100 g and raspberries (up to 32 mg/100 g).[10,238,239]

Berries are also rich sources of essential minerals, such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), sodium (Na), and aluminum (Al). Minerals are recognized to play important roles for human health participating in a range of physiological processes such as the development of bones and teeth and strengthening of muscles. They also participate in a series of physiological and biochemical processes in humans, including influencing water and electrolyte balance, metabolic catalysis, oxygen binding, and hormone functions.[237,240] Nile and Park summarized the reported content of minerals in six species of berries and found that blackcurrants are characterized by the highest contents of Ca, Fe, P, and K, blackberries by Mg and Mn, and cranberries and raspberries by Na and Zn.[237] High concentrations of Ca, K, and particularly barium (Ba) were previously detected in strawberries, as well.[240]

2.3.1.2 Phenolic compounds

Numerous epidemiological studies have indicated that consumption of berries rich in phenolic compounds is associated with reduced incidence of numerous diseases and disorders, such as cardiovascular disease, cancer, inflammatory, hepatotoxicity, oxidative stress, and cataract.[10,237,241–248]

Generally, the phenolic profile of different species of berries varies significantly. Even berries of the same species show a significant difference in phenolic composition depending on subspecies and varieties, growth environment, harvesting time, and storage condition. For example, among the different species of the genus *Vaccinium* of bilberry, blueberry, cranberry, and lingonberry, bilberry usually has the highest level of anthocyanins, despite the diverse methodological procedures in the extraction and analysis of phenolic compounds applied in different studies (**Table 5**). This results from the high amount of anthocyanins existing not only in the skin but also in the pulp of bilberry. With a content of total anthocyanins of 1402 mg/100 g fresh weight, bilberry is considered as one of the best natural food sources of anthocyanins (**Table 5**).[249] Fifteen monomeric anthocyanins consisting of five anthocyanidins (cyanidin, peonidin, delphinidin, petunidin, and malvidin) glycosylated different sugar moieties (glucose, galactose, and arabinose) have been detected in bilberry, of which cyanidin- and delphinidin-glycosides are the major compounds.[11,250] High content of anthocyanins is also the characteristic of the phenolic profile of blackcurrant and blackberry (**Table 5**). Cyanidin-glycosides are the major anthocyanins in both of these berry species, rutinose and glucose being the dominant sugar moieties in blackberry and blackberry, respectively. Additionally, delphinidin 3-*O*-glucoside and delphinidin 3-*O*-rutinoside are also

dominating anthocyanins in blackcurrant.[11] The anthocyanin profile of strawberry is significantly different from other berries listed in **Table 5** with pelargonidin 3-*O*-glucoside as the most abundant anthocyanin, accounting for 60–95% of total anthocyanin content.[251]

As mentioned in section 2.1.2.1, the biosynthesis of anthocyanins in berries is to facilitate seed dispersal by attracting herbivorous animals. Anthocyanins accumulate *via* phenylpropanoid/flavonoid pathway throughout berry ripening process (**Figure 11**). Berry mutants with white skin and/or pulp, resulting from mutation in structural and/or regulatory genes of the anthocyanin synthesis pathway, are rarely found in nature. However, a species of white bilberry with albinism was recently discovered in Finnish and Slovenian forests.[252–254] The albino appearance of the bilberry mutant results from the low expression of structural genes, particularly chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-*O*-glycosyltransferase (FGT) (**Figure 11**), and the strongly down-regulation of *VuMYBPA1* and *VuMYBC2* transcription factors.[252,253]

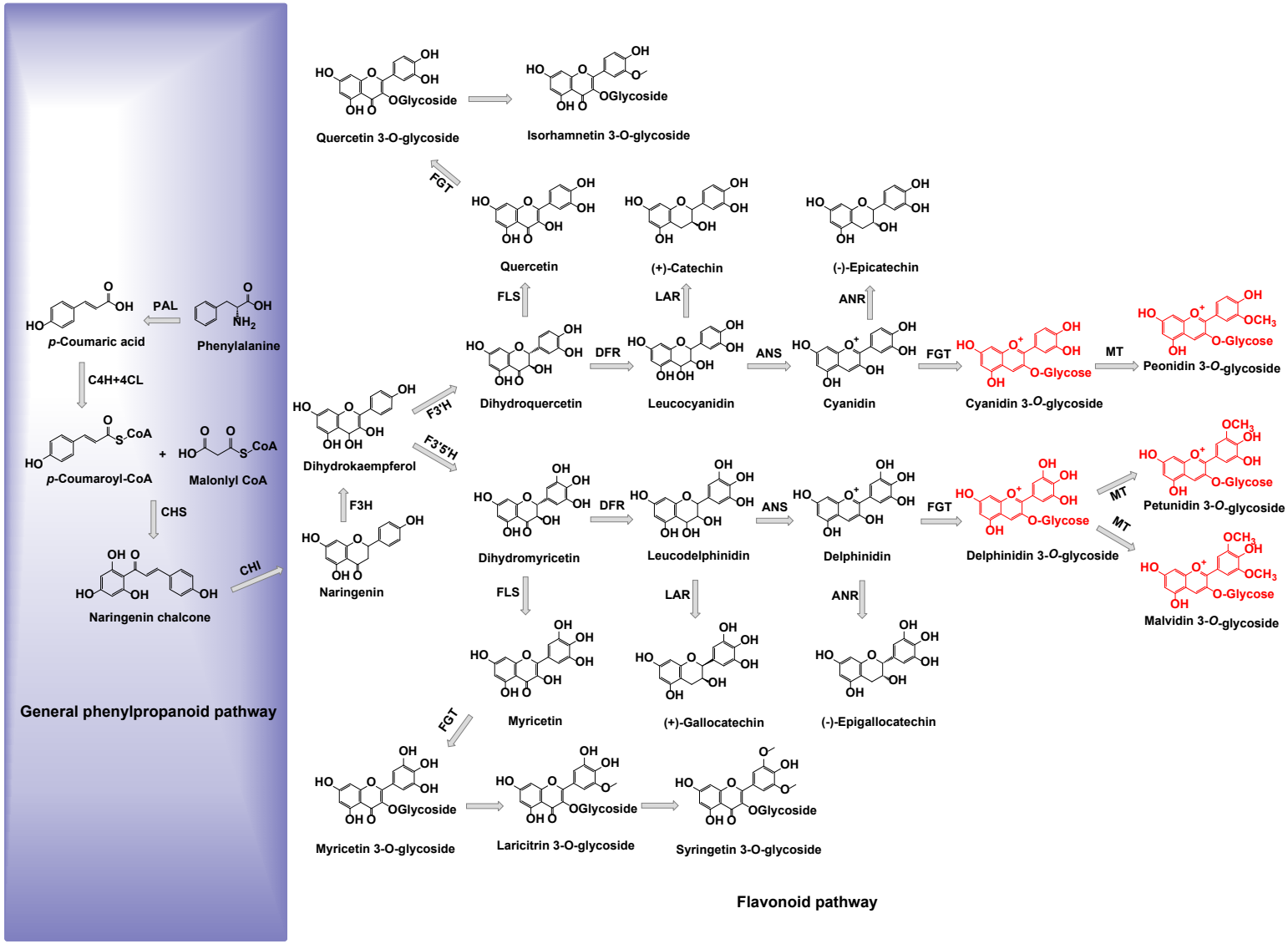

p-Coumaric acid
PAL
Phenylalanine
C4H+4CL
p-Coumaroyl-CoA
Malonyl CoA
CHS
Naringenin chalcone
CHI
General phenylpropanoid pathway
Naringenin
F3H
Dihydrokaempferol
F3'H
F3'5'H
Dihydroquercetin
Dihydromyricetin
DFR
Leucocyanidin
Leucodelphinidin
ANS
Cyanidin
Delphinidin
FGT
Cyanidin 3-O-glycoside
Delphinidin 3-O-glycoside
MT
Peonidin 3-O-glycoside
Petunidin 3-O-glycoside
Malvidin 3-O-glycoside
FLS
Quercetin
Myricetin
LAR
(+)-Catechin
(+)-Gallocatechin
ANR
(-)-Epicatechin
(-)-Epigallocatechin
Quercetin 3-O-glycoside
Isorhamnetin 3-O-glycoside
Myricetin 3-O-glycoside
Laricitrin 3-O-glycoside
Syringetin 3-O-glycoside
Flavonoid pathway

Figure 11. Simplified flavonoid biosynthesis pathway, with emphasis on the flavonoids found in *Vaccinium myrtillus*.[252,253] Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FGT, flavonoid 3-O-glycosyltransferase; MT, methyltransferase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. Figure reprinted from the original publication **V** with permission from American Chemistry Society.

Table 5 shows also the contents of nonanthocyanin compounds in various berry species reported in previous studies. The content and composition of phenolic acids play an important role in determining the diversity of phenolic profile of berries. Bilberries contain a high amount of phenolic acids and the composition of phenolic acids depends on the growing region. 3-*O*-Caffeoylquinic acid (chlorogenic acid) was reported as the dominant hydroxycinnamic acid in the bilberries growing in Finland and Slovenia, accounting for approximately 43% and 85% of the total phenolic acid content (TA) in bilberries of the two origins, respectively.[254,255] However, gallic acid derivatives were found as the major phenolic acids (87% of TA) in the bilberry grown in Macedonia.[256] High concentration of chlorogenic acid has also been detected in berries of other *Vaccinium* species, including blueberries and cranberries, whereas it was undetectable in bog bilberries.[257] *p*-Coumaric acid was the major hydroxycinnamic acid reported in lingonberries.[258] In a previous study analyzing the distribution of phenolic compounds in 18 Nordic berry species, chokeberry and sweet rowanberry possessed the highest contents of caffeic and ferulic acids.[258] Ellagic acid is the hydroxybenzoic acid derivative dominating in raspberries and strawberries representing 88% and 51% of TA, respectively.[259]

Table 5. The content ranges of total phenolic compounds (TPC), total anthocyanins (TACY), total phenolic acids (TA), total flavonols (TFO), total flavan-3-ols (TFA), and total proanthocyanidins (TPA) of various berry species reported in previous studies

Berry	***TPC***	***TACY***	***TA***	***TFO***	***TFA***	***TPA***	***Reference***
bilberry *(Vaccinium myrtillus)*	461–1124 mg/100 g FW	376–1402 mg/100 g FW	96–143 mg/100 g FW	4–17 mg/100 g FW	24 mg/100 g FW	13 mg/100 g FW	254,256,260
	2690–3470 mg/100 g DW	2230–5190 mg/100 g DW	390 mg/100 g DW	96–449 mg/100 g DW			261,262
blackberry *(Rubus allegheniensis)*	417–595 mg/100 g FW	13–484 mg/100 g FW	37–39 mg/100 g FW	275–312 mg/100 g FW			263,264
blackcurrant *(Ribes nigrum)*	222–401 mg/100 g FW	207–384 mg/100 g FW	4–7 mg/100 g FW	9–11 mg/100 g FW			265
	598–2798 mg/100 g DW	530–2700 mg/100 g DW	8–39 mg/100 g DW	18–60 mg/100 g DW	10–23 mg/100 g DW	275–623 mg/kg DW	266,267
black mulberry *(Morus nigra)*	164–2977 mg/100 g FW	3–18 mmol/100 g FW					268–270
blueberry *(Vaccinium corymbosum)*	264–844 mg/100 g FW	189–2762 mg/100 g FW	12 mg/100 g FW	5.6 mg/100 g FW	64–133 mg/100 g FW	83–120 mg/100 g FW	257,271–274
cherry *(Prunus avium)*	85–162 mg/ 100 g FW	25–94 mg/ 100 g FW					275
chokeberry *(Aronia melanocarpa)*		307–1480 mg/100 g FW	184 mg/100 g FW	71 mg/100 g FW		113–664 mg/100 g FW	274,276
	2080 mg/100 g DW	280–1950 mg/100 g DW	600 mg/100 g DW	101 mg/100 g DW		3992–5182 mg/100 g DW	11,276
cranberry *(Vaccinium macrocarpon)*	224–624 mg/100 g FW	40–207 mg/100 g FW				132–278 mg/100 g FW	257,277

Berry	*TPC*	*TACY*	*TA*	*TFO*	*TFA*	*TPA*	*Reference*
elderberries *(Sambucus nigra)*	1092–1374 mg/100 g FW			45–57 mg/100 g FW			[278–280]
lingonberry *(Vaccinium vitis-idaea)*	582–760 mg/ 100 g FW	35–130 mg/ 100 g FW		522–647 µmol /100 g FW	25 mg/100 g FW		[258,281]
raspberry *(Rubus coreanus)*	126–359 mg/100 g FW	31–43 mg/100 g FW 321–3651 mg/100 g DW				240 mg/100 g FW	[11,274,282,283] [284]
redcurrant *(Ribes rubrum)*	67–153 mg/100 g FW 615–1268 mg/100 g DW	7–19 mg/100 g FW 32–111 mg/100 g DW	0.3 mg/ 100 g FW	0.04 mg/100 g FW 0.5–1.5 mg/100 g DW		7.6–25.6 mg/kg DW	[285,286] [267,287]
sea buckthorn *(Hippophaë rhamnoides)*				23–250 mg/100 g FW		23–135 mg/100 g FW 340–1941 mg/100g DW	[274,288] [289–291]
strawberry *(Fragaria × ananassa)*	57–225 mg/100 g FW	8–80 mg/100 g FW		0.7–6.7 mg/100 g FW	11–45 mg/100 g FW	9–186 mg/100 g FW	[11,251,274]

Flavonol composition varies extensively in different berry species. Flavonols generally present in berries as flavonol glycosides. The six aglycones detected in grapes, mentioned in section 2.1.2.3, can also be detected in different berries. While the sugar moieties include glucose, galactose, rutinose, glucuronic acid, xylose, arabinose, and rhamnose, as well as some as both furanoside and pyranoside, such as arabinofuranoside and arabinopyranoside.[278] High contents of flavonols have been reported in sea buckthorns, crowberries, cranberries, blackcurrants, and redcurrants originating from Finland, accounting for 87%, 82%, 67%, 52%, and 44% of total phenolic contents, respectively.[259] Mikulic-Petkovsek et al., studied the distribution of flavonols in 28 wild and cultivated berry species, suggesting that elderberries contained the highest content of total flavonols (45–57 mg/100 g FW).[278] In the same study, high contents of TFO (>20 mg/100 g FW) were detected in chokeberries, blackberries, cranberries, rowanberries, and blackcurrants as well, whereas strawberries and white currants contained the lowest levels. Among the flavonols detected in these berry species, glycosylated quercetins represented the highest proportion (46–100%) of TFO, whereas isorhamnetin glycosides (50–62%) prevailed in wild strawberries and gooseberries and sea buckthorn, and 49–66% flavonols presented in currant species are kaempferol glycosides. Myricetin glycosides were only detected in chokeberries, rowanberries, and the berries of *Vaccinium* such as bilberries and blueberries.

Berries contain a certain amount of flavan-3-ols as monomers and condensed polymers known as proanthocyanidins (PAs) (**Table 5**). With regard to the monomeric types in berries, (+)-catechin and (–)-epicatechin are the two primary flavan-3-ols.[292] In bilberries, the dominant flavan-3-ol monomer generally is (–)-epicatechin accounting for about 98% of total flavan-3-ol monomer content.[260] However, besides these two flavan-3-ols, gallocatechin was found in bilberries at a high level (3.5 mg/100 g FW) in a previous study.[254] In the same study, more than 10 mg/100 g FW of procyanidin dimer and termer were detected in the samples. Määttä-Riihinen et al. compared the content of catechins in four *Vaccinium* berry species and demonstrated that (+)-catechin dominated in lingonberries and cranberries and (–)-epicatechin in bilberries and bog bilberries.[293] Moreover, the content of these two compounds in cranberries was higher than that in the other three species. In a previous study analyzing the contents of flavan-3-ols and procyanidins in blackberries, blueberries, cherries, gooseberries, cranberries (red and black forms), raspberries, and strawberries, blackberries contained the highest level of (–)-epicatechin, followed by raspberries, cherries, and blueberries. In contrast, strawberries and cherries were characterized by the high content of (+)-catechin. In comparison to other berry species, higher concentrations of B-type procyanidins were detected in blackberries and raspberries, while cranberries contained the highest level of A-

type procyanidins.[292] Sea buckthorn is a berry species of interest in recent years due to the high content of PA. Yang et al. compared the concentration of PA in the wild sea buckthorns grown in Finland, China, and Canada and found that genetic background and growth location affected TPA with the highest value of TPA found in the sea buckthorn berry samples collected from northern Finland.[289]

2.3.2 Opportunities of berry wine production

Although the history of berry wines is not as long or as prestigious as that of grape wines, more attention is being given to the new commercial opportunities and health benefits of berry wines. Over the past years, berry wines are gaining grounds in the alcoholic beverage industry, particularly in Europe, China, Japan, America, and Brazil.[13] Multiple factors have contributed to the popularity of berry wines globally.

Firstly, the cost is low for industrial transformation. The technological establishment of berry wine production is a major contributing factor to the introduction of berry wines commercially. A great advantage of berry wine production is that the manufacturing facilities and technologies used for the production of berry wines are similar to or even identical as those for the production of wines. Therefore, as a good basis of technologies has already been set up, the industrial transformation from wine production to berry wine production requires only minimal effort and investment.

Secondly, there are abundant species and varieties of cultivated and wild berry crops available with unique characteristics of color, flavor, and nutritional value. Taking Finland as an example, there are approximately 50 varieties of wild berries growing in the Finnish forests, of which 37 are edible (https://www.arktisetaromit.fi/en/berries/). Generally, all the edible berries could be fermented to berry wines after some modulation of practice and pretreatment of raw materials. It could lead to a range of wine products with varying characteristics available to meet the diversified needs of consumers. This opportunely meets the increasing demand for novel and unique wine products by the market.

Thirdly, the harvest period and shelf life of berries are short as generally only a couple of months are the optimal time for picking and for consumption of fresh berries. Moreover, the cultivation areas for some popular cultivated berries are expanding with the purpose of economic benefits, thus causing oversupply. Therefore, apart from the great portion of harvested berries are consumed freshly, preservation and processing of berries into preserves, juice, jams, canned fruits, and jellies are the common ways to prolong the shelf life of berries, so that

they can be consumed months or years round and transported safely to consumers all over the world, not only those living near the growing region. However, the actual transformation is extremely low compared to the colossal yield of berries on the earth. For example, only 5−8% of the total bilberry yield in Nordic countries (>500 million kg/year) is exploited annually.[294,295] Even worse, nearly 35–40% of this portion is lost due to the lack of proper postharvest management and processing facilities,[296] which is a considerable economic loss to the orchardists or farmers. Furthermore, the common berries products may not increase much the revenue due to their relatively low added values. Processing of the berries to wine products could increase the added value as well as minimize postharvest losses.

Fourthly, the increasing trend of low alcoholic beverages is an important factor contributing to potential growth in berry wines. In 2007, the World Cancer Research Fund International (WCRF) stated a reduction in the risk of breast and bowel cancer by 7% due to the decreases in alcohol content from 14.2% to 10%.[297] Hence, a global strategy to reduce the harmful use of alcohol was carried out by the WHO since 2010.[298] Grapes contain a high level of fermentable sugars to generate typically 12−13% around ethanol after fermentation. The content of sugar in most of berries in nature is lower than that in grapes (**Table 6**), resulting in the production of beverages with low alcohol. Although the addition of sugars to the berries with extremely low content of sugars is a common practice for making berry wines with proper alcohol level, the production of alcohol can be controlled by adjusting the amount of added sugars.

Table 6. Sugar and acid contents in various berry species detected in previous studies (g/kg fresh berries)

Berry	***Sugar***	***Acid***	***Reference***
bilberry	46.8	10.2	[255]
blackcurrant	6.6–22.2	1.4–6.6	[266]
cherry	120–224	54–100	[299]
chokeberry	130–176	15	[276]
mulberry	18–76	86.5	[270,300]
raspberry	45−50	16–23	[300,301]
sea buckthorn	3–72	24–54	[302]

The fifth factor is the abundance of health-promoting compounds in berries. As have discussed in section 2.2.1, berries contain a high content of health-benefit compounds. These bioactivity compounds in berries transform into their wine products after fermentation. In a previous study on the phenolic content and antioxidant activity of blackberry and blueberry wines, blueberry (n = 12) and

blackberry wines (n = 10) both had high levels of TPC as 1086 and 1265 mg/L, respectively and blackberry wines had the highest antioxidant activity.[303] The high values of antioxidant activity and total phenolic content were determined in bilberry, blackberry, and black mulberry wines, as well.[304] The phenolic compositions of blackberry, cherry, raspberry, blackcurrant, and strawberry wines were spectrophotometric analyzed in a previous study.[305] The former four wine products all possessed TPC more than 1500 mg/L, while cherry and blackcurrant wines contained the highest levels of anthocyanins. Sixteen red and two white nongrape wines produced from the berries originated from Finland were compared to the control grape wines on flavonol composition by Vuorinen et al.[12] The contents of flavonols in red berry wines are comparable to those in red grape wines. It is widely acknowledged that excessive consumption of alcohol products is strongly negative on human and public health. However, some beneficial effects of moderate drinking have been reported. For example, light-to-moderate consumption of alcohol was reported to associate with cardioprotective effect and lower coronary heart disease incidence and mortality, and the lowest risk was found at 20 g/day.[306–308] Moreover, lowering the risk of type 2 diabetes and reducing cognitive function losses related to moderate drinking was suggested in several studies.[309,310] However, the author must point out here that no pattern of drinking is entirely risk-free and consumers should be aware that a range of health risks are balanced against the benefits they might derive from drinking.

2.3.3 *Saccharomyces cerevisiae* in berry wine production

Because of the short history of scientific research on berry wines, the prevalent studies, at the moment, are mainly focusing on the effect of fermentation using the conventional *S. cerevisiae* on the chemical composition of berry juices. Some previous reports on typical berry wines produced from alcoholic fermentation with *S. cerevisiae* were introduced in this section. Special focus was placed on the evolution of chemical composition of berry wines during alcoholic fermentation.

Blueberry wines. The changes of phenolic and volatile compounds during alcoholic fermentation of blueberry wines made from two different southern highbush cultivars (Misty and O'Neal) were previously studied.[311] At the beginning of the fermentation, the total contents of monomeric anthocyanins, phenolics, and flavonoids increased rapidly in the 1–4 days due to the extraction of these compounds from the berry skins. Their contents in wines dropped quickly in the later stage of fermentation. The total monomeric anthocyanins in final blueberry wines reduced toward the levels significantly lower than those

found in blueberry juices, whereas the total phenolic and flavonoid contents were approximately two times higher than those in juices. Volatile profile of the blueberry wines changed significantly during fermentation as indicated by the accumulation of higher alcohols and esters, the degeneration of C6 compounds, and the extraction and hydrolysis of terpenoids precursors.

Significant increase in total phenolic content after fermentation was also detected in rabbiteye blueberry wines.[312] The antioxidant activity was simultaneously increased. Oppositely, in this study, the total anthocyanin content in final blueberry wine was two times higher than those in bilberry juice. This was probably due to the differences in cultivar and fermentation technology.

Strawberry wines. The total anthocyanin concentration in strawberry puree decreased by 19% after alcoholic fermentation mostly due to reduction in pelargonidin 3-*O*-glucoside, which is the dominant individual anthocyanin accounting for 70% of total anthocyanins. On the other hand, the acylated anthocyanins and pyranoanthocyanins suffered much fewer losses than other anthocyanins due to the stabilization effects from acylation and cycloaddition.[313] Alcoholic fermentation led to significant increases in concentrations of homovanillic acid and *p*-hydroxybenzoic acid, while a significant decrease in galloyl bis-HHDP-glucose was observed.[314]

Song et al. investigated the evolution of 78 volatile compounds during alcoholic fermentation of strawberry wine.[315] The lowest total amount of aroma compounds (89 mg/L) was found at the early stage of fermentation, while the content peaked at the end of fermentation (901 mg/L), especially those of higher alcohols and esters (808 mg/L and 75 mg/L, respectively). Specifically, the concentration of the dominant higher alcohols of 2,3-butanediol, 3-methyl-1-butanol, 2-phenylethanol, 2-methyl-1-propanol, and 1-octanol, and esters of ethyl acetate, increased tens to hundreds times. However, the concentrations of aldehydes and ketones did not show a consistent changing trend during fermentation, and the concentration of terpenes increased as the maceration progressed and declined after separation of the skin residue from strawberry wine, ending at a concentration, which is 62% higher than the level in strawberry fruit.

Mulberry wines. The changes in phenolic compounds, color, and antioxidant activity of mulberry wine during alcoholic fermentation were studied.[316] Total phenolics and total flavonoids increased rapidly from day 0 to 3, but the changes from day 3 to 10 were not obvious. During fermentation, total anthocyanins and two major anthocyanin monomers, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside, increased first followed by a decrease. Specifically, 3-*O*-glucoside and 3-*O*-rutinoside of cyanidins reached their maximum at day 1 and 2,

respectively; thereafter, cyanidin 3-*O*-glucoside decreased rapidly, whereas cyanidin 3-*O*-rutinoside was more stable. The color parameters changed significantly from day 0 to 2 and showed unobvious changes from day 2 to 10. Similar changes were observed in the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) and reducing power.

A significant decrease in total anthocyanins was also previously reported in another study investigating the influence of alcoholic fermentation on antioxidant activity and phenolic levels from mulberries.[317] Approximately 50% and 34% of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside decreased in mulberry juice after fermentation due to decomposition. However, hydroxycinnamic acid derivatives increased their concentration during fermentation, while total flavonols were not affected by the fermentation process resulting from the progressive conversion of glycosylated flavonols to their corresponding flavonol aglycones. A positive correlation (R = 0.6229) was observed between the antioxidant activity and the total flavonol content during fermentation.

Raspberry wines. In a previous study analyzing the changes in physicochemical properties and key compounds of three types of Korean black raspberry wines fermented from juice (type-1), juice with pulp (type-2), and juice with pulp and seed (type-3), the color intensity of type-1 sample was significantly weakened with a 50% decrease in anthocyanin content, whereas the color intensity and total anthocyanins was strengthened in the other two types by supplementation with pulp and/or seed.[318] Citric acid was the major organic acid (approximately 90%) in all the juice and fermented samples. Total organic acids and amino acid decreased in content by 14–20% and 53–91%, respectively after fermentation. The total volatile compound contents in the three raspberry wines were approximately 4–6 times higher than that in juice, and nine new compounds of particularly higher alcohols and esters were formed after fermentation. In comparison with other samples, the type-3 raspberry wine contained the highest contents of anthocyanin, polyphenols, proanthocyanin, amino acid, as well as the greatest antioxidant activity.

Similar results were also reported by Cho et al., the total phenolic content gradually increased and total anthocyanin content slightly increased during raspberry wine production with whole raspberry fruit.[319] The significant increase in the contents of gallic acid (GA) and 3,4-dihydroxybenzoic acid (DHBA) during fermentation increased the DPPH· radical-scavenging activity of raspberry wines. The rats administered with raspberry wine concentrate showed a significantly higher antioxidant activity in their blood plasma than those administered with raspberry fruit extracts.

2.3.4 Non-*Saccharomyces* yeasts in berry wine production

Trials of non-*Saccharomyces* yeasts in berry wine productions are being carried out due to increasing positive reports on wine quality of non-*Saccharomyces* yeasts in winemaking. However, the published information on the berry wines fermented with non-*Saccharomyces* yeasts is still far more limited compared to those in wines.

Non-*Saccharomyces* yeasts of *T. delbrueckii* and *M. pulcherrima* have been tested in pure and sequential inoculations with *S. cerevisiae* in the production of cherry wines.[320] Fermentation with monoculture of non-*Saccharomyces* yeasts needed two times longer time than that with *S. cerevisiae* to complete alcoholic fermentation. However, the combination of *Saccharomyces* and non-*Saccharomyces* yeasts significantly accelerated fermentation rates. Fermentation involving *T. delbrueckii* lowered the yield of acetic acid. For the volatile compounds determined, pure fermentation with non-*Saccharomyces* yeasts generated the lowest volatile intensities and multistarter fermentations increased the values, particularly the *M. pulcherrima*/*S. cerevisiae* combination significantly boosted the production of higher alcohols, ester, acids, and terpenes. Whilst the sequential fermentation of *T. delbrueckii*/*S. cerevisiae* significantly increased the releases of fruity esters and higher alcohols but decreased the production of acids. Sensory evaluation revealed that the cherry wine produced by the former yeast combination reinforced *sweet*, *green*, and *fatty* notes, while that by the latter one enhanced *fruity* odor but reduced *green* note. Later the same authors investigated the effect of sequential and simultaneous inoculations of *T. delbrueckii* and *S. cerevisiae* on the organoleptic quality of cherry wines.[25] They found that simultaneous inoculation inhibited the growth of *T. delbrueckii* ascribing to the competition for nitrogen by *S. cerevisiae*. In comparison with the fermentation with *S. cerevisiae* monoculture, the sequential and simultaneous fermentations obviously increased the yields of aroma compounds and showed similar values in the total amount of volatile components. However, the cherry wine produced from sequential fermentation possessed more higher alcohols, esters, and varietal compounds, mainly represented by 2-methyl-1-propanol, 3-methyl-1-butanol, ethyl 3-methylbutanoate, ethyl hexanoate, ethyl hex-3-enoate, ethyl octanoate, β-phenylethanol, and linalool than that from simultaneous fermentation. Furthermore, the former cherry wine obtained the highest score after sensory evolution, mainly due to the higher intensity in *fruity* and *floral* senses. These assays evidenced the positive impact of co-culture fermentations involving non-*Saccharomyces* yeasts in improving the overall sensory intensity and enhancing the overall aromatic complexity.

The impacts of non-*Saccharomyces* yeasts on the chemical composition, particularly volatile profile, of lychee wines have been assessed. Chen et al. used

three non-*Saccharomyces* yeasts, namely *T. delbrueckii*, *Williopsis saturnus*, and *Kluyveromyces lactis*, to ferment lychee juices with pure culture.[321] They detected that *T. delbrueckii* had the fastest fermentation rate and the highest sugar consumption leading to ethanol content of 7.6% (*v/v*). Moreover, fermentation with this strain generated higher concentrations of 3-methyl-1-butanol, 2-phenylethyl alcohol, ethyl octanoate, and ethyl decanoate and retained high odor activity values (OAVs) of lychee aroma-character compounds *cis*-rose oxide and linalool. *W. saturnus* consumed the lowest amount of sugars resulting in very low content of ethanol (0.8%, *v/v*), while this strain consumed the highest amount of nitrogen. Fermentation with *K. lactis* was characterized by the moderate level of ethanol production (3.4%, *v/v*) and the highest OAVs of geraniol and citronellol. Both *W. saturnus* and *K. lactis* over-produced ethyl acetate to reach concentrations of 880 and 323 mg/L, respectively.

The effect of simultaneous and sequential fermentations with *T. delbrueckii* and *S. cerevisiae* on volatile and non-volatile compositions of lychee wines was previously studied.[322] *T. delbrueckii* monoculture had a better ability to retain the odor-active terpenes and terpenoids derived from lychee fruits. The simultaneous fermentation had a similar aroma characteristic to that of the fermentation with pure *S. cerevisiae*. The lychee wine fermented with sequential inoculation is richer in higher alcohols (3-methyl-1-butanol and 2-phenylethyl alcohol) and esters (ethyl octanoate, ethyl decanoate, ethyl hexanoate, and 2-phenylethyl acetate) compared to that with *T. delbrueckii* monoculture.

T. delbrueckii was also applied in longan (*Dimocarpus longan*) wine production as monoculture and co-cultures with *S. cerevisiae*.[323] Of which, sequential and simultaneous inoculations significantly increased the production of 3-methyl-1-butanol and 2-methyl-1-propanol compared to mono-inoculation of *T. delbrueckii*. At the same time, simultaneous cultures produced the highest contents of total volatile compounds, 2-phenylethanol, and total esters mainly due to the higher productions of ethyl hexanoate, ethyl dodecanoate, ethyl heptanoate, and ethyl benzoate than pure and sequential fermentations. The longan wine fermented with simultaneous inoculation achieved a noticeable intensity of *floral* and *fruity* aromas.

2.3.5 Aging process in berry wine production

It is well known that almost all wine products could be enjoyed directly by consumers after alcoholic fermentation. Sometimes, it is also necessary to subject the products to an aging process to modify their organoleptic characteristics, such as astringency, bitterness, and color stability, thus to improve the overall quality of final products.[14] Among the parameters, wine

color is determined by anthocyanins. Therefore, the impact of aging process on the anthocyanin profile of berry wines is a subject worth studying.

After 12 weeks of aging of bilberry wines, a significant loss of 72.6–97.6% in total anthocyanin content was reported.[324] The loss rates of anthocyanidin arabinosides and galactosides were faster than the corresponding glucosides, and anthocyanidin-glucosides were almost the only monomeric anthocyanins detected after 12 weeks of aging. At the same time, the concentrations of pigmented polymers and pyranoanthocyanins increased significantly during storage and peaked after 6–9 weeks and 12 weeks of aging, respectively. Moreover, the formation of pyranoanthocyanins in bilberry wines proceeded faster than commercial red wines as the first vitisin A-type pyranoanthocyanin in bilberry wine was already detected during the third week of aging.

Red raspberry wines aged for 6 months in darkness showed a significant degradation of anthocyanin pigments, resulting in a total loss of at least 50%.[325] Cyanidin 3-*O*-glucoside was the most unstable anthocyanin, disappearing completely even after fermentation; as major anthocyanin, cyanidin 3-*O*-sophoroside was the most stable pigment with the highest retention during aging. Later the same authors studied the effects of aging on anthocyanin composition of blackberry wines using a similar method.[326] The loss of anthocyanins was 85–100% after 6 months of aging, while the concentration of an acylated cyanidin derivative showed a proportional increase.

The effect of 6 months of bottle aging on the anthocyanin composition and chromatic characteristics of bog bilberry wines was previously investigated.[327] A decline of 22–31% of total anthocyanins due to a dramatic decrease in the contents of delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, and malvidin 3-*O*-arabinoside was detected. Aging of bog bilberry wine weakened color intensity with a dramatic change in color hue from initial red-purple up to final red-brick nuances.

Changes in anthocyanin copigmentation and color attributes of bog bilberry wine during 6 months of aging were recently studied by the same authors.[328] Tannic acid and gallic acid extracted from Chinese gallnut were added to bog bilberry wines as copigments to stabilize anthocyanins and color. Copigment addition significantly retained redness while alleviated the increase in the yellow shade and lightness. Compared to the control bog bilberry wine aged for 6 months without copigments addition, the samples treated with copigments had 1.4–1.8 times higher total anthocyanin content. The percentages of copigmented and polymeric anthocyanins in copigment added bog bilberry wines were higher and lower than those in the control, respectively. The bog bilberry wines treated with a high dosage of gallic acid had the highest value of redness and the highest percent of copigmented anthocyanins, but the lowest percentage of polymeric anthocyanins.

Color, anthocyanins copigmentation and polymerization, and antioxidant capacity of mulberry wine aged for 1, 3, and 12 months were previously investigated.[329] Monomeric anthocyanins dominated in young mulberry wine, whereas copigmented and polymeric anthocyanins presented extremely low percentages. However, the proportions of non-monomeric anthocyanins increased significantly along with the dramatic decrease of monomeric anthocyanins. The DPPH· radical scavenging ability increased after storage and was highly correlated with the polymeric anthocyanin content ($R = 0.98$). Over aging time, color density and redness reduced significantly, but brightness and blueness showed significantly increase, resulting in the color changed from red to brown.

With regard to blueberry wine, 16 months of aging significantly reduced the concentrations of organic acids, including citric, tartaric, malic, and succinic acids, and a high fraction of volatile compounds, such as acetaldehyde, acetoin, esters, higher alcohols, and terpenic compounds. However, the typical tertiary volatile compounds, such as 4-vinylguaiacol and eugenol, showed significant increases in content during aging.[330]

2.4 Concluding remarks

The character of wine is greatly determined by the quality of grape variety and the winemaking technology applied. The composition of the secondary metabolites formed during vine growth is among a critically important factor determining grape quality. Alcoholic fermentation is an essential element during the production of wine and is determined by the participation of yeast. Aging is sometimes a process of importance to improve the organoleptic characters of wines. During alcoholic fermentation, the initial metabolites originated from grapes keep evolving, such as the primary aroma compounds of glycosidically bound monoterpenes, C13-norisoprenoids, and polyfunctional thiols, while the phenolic compounds of monomeric anthocyanins, flavonols, and flavan-3-ols are degraded. At the same time, new compounds are formed including copigmented and polymeric anthocyanins, pyranoanthocyanins, and large numbers of secondary volatile compounds, such as higher alcohols, esters, volatile acids, aldehydes, and aldehydes. During aging process, these aforementioned compounds continuously change in composition and tertiary volatile compounds, such as volatile phenols and acetals, are accumulated.

In wine industry, the yeast species *S. cerevisiae* is considered as the most appropriate strains for alcoholic fermentation to produce wine with desirable organoleptic characters, but non-*Saccharomyces* yeasts were usually regarded as problematic yeasts by enologists. However, recently, winemakers have re-

evaluated the role of non-*Saccharomyces* yeasts during alcoholic fermentation and started to use them in laboratory-scale fermentation and even in industrial level due to their increasingly reported positive contributions to wine quality.

Berries are rich in bioactive compounds beneficial for human health. Berries also have unique flavors. However, due to the short harvest period and shelf life of berries, new value-added products are necessary to improve the availability and quality of berry products. Therefore, production of berry wines could be an important approach for berry processing. Due to the relatively short history of the development, berry wines are generally produced by fermentation with commercial *S. cerevisiae* strains. Potential of non-*Saccharomyces* yeasts in berry wine production has not been well explored. The outcomes obtained from the studies of winemaking provide important references indicating potentials of application of non-*Saccharomyces* yeasts in berry wine productions. For example, inoculation involving the strain of *S. pombe*, Z. *bailii*, *I. orientalis*, or *P. tannophilus* may provide a microbiological alternative of deacidification for the berries with high acidity. On the other hand, the capacity of *L. thermotolerans* to convert sugars to lactic acids and *P. tannophilus* to convert xylose to ethanol could be used as means to improve the overall acidity and reduce the residual sugars in fermentation of berries with a low acidity or a high xylose content. Moreover, inoculation involving *T. delbrueckii*, *M. pulcherrima*, *S. ludwigii*, or *L. thermotolerans* could be applied to enhance the accumulation of some desirable compounds, such as glycerol, terpenes, and fruity esters, and fermentation involving *H. uvarum* to reduce the content of compounds causing unpleasant sensory properties, such as fatty acids. Furthermore, the color of berry wines may be stabilized by inoculating *T. delbrueckii*, *S. pombe*, *M. pulcherrima*, or *I. orientalis*. So far, only a limited number of studies have been reported on application of non-*Saccharomyces* yeasts in berry wine production. The findings of these studies strongly indicate the potential of these non-conventional yeasts in berry wine production. Systematic and in-depth studies are needed to understand the impact of non-*Saccharomyces* yeasts on the chemistry and quality of berry wines during fermentation and aging.

3 AIMS OF THE STUDY

The investigation of non-*Saccharomyces* yeasts in berry wine productions, especially of bilberry wine, is lacking at this moment. The overall aim of the research was to study the effects of non-*Saccharomyces* yeasts on the chemical profile of bilberry wines during alcoholic fermentation and aging processes with a special focus on volatile and non-volatile compounds.

The specific aims of the individual studies were to:

1) characterize and quantify volatile and/or non-volatile compounds in blue and white bilberry juices and wines (**I, II, V**);

2) compare the chemical compositions of final bilberry wines fermented with diverse non-*Saccharomyces* yeasts in pure inoculation as well as in sequential and simultaneous fermentations with *S. cerevisiae* against conventional fermentation with *S. cerevisiae* (**I**, **II**);

3) monitor and compare the fermentation kinetics and the dynamic changes in volatile compounds in bilberry wines during alcoholic fermentation with different non-*Saccharomyces* yeasts (**IV**);

4) investigate the evolution of pyranoanthocyanins and their precursor monomeric anthocyanins during aging of bilberry wines fermented with different yeasts (**III**).

4 MATERIALS AND METHODS

4.1 Berry juice preparation

The wild pigmented bilberries (hereafter referred colored or blue bilberry (BB)) used in this research were purchased as frozen from local supermarkets in Turku, Finland. They were harvested throughout Finland and pooled by Arctic International Oy (Sotkamo, Finland) at the harvesting season in 2016 (studies **I–III**) and 2017 (studies **IV** and **V**). The wild white bilberries (WB) were collected from several locations in forests in Nagu, Finland in 2017 (study **V**).

The bilberries were stored at –20 °C until processing and analysis. **Figure 12** shows the overall scheme of juice preparation. In the first three studies, the initial °Brix values of bilberry juices were adjusted to 20.0 by adding sucrose, while the values were adjusted to 14.0 in study **IV** due to several non-*Saccharomyces* yeasts employed in this study were sensitive to hyperosmotic condition. In order to compare the phenolic composition of the juice and wines produced from blue bilberries in study **IV** with the products prepared from white bilberry, the °Brix value in study **V** was adjusted to 14.0, as well.

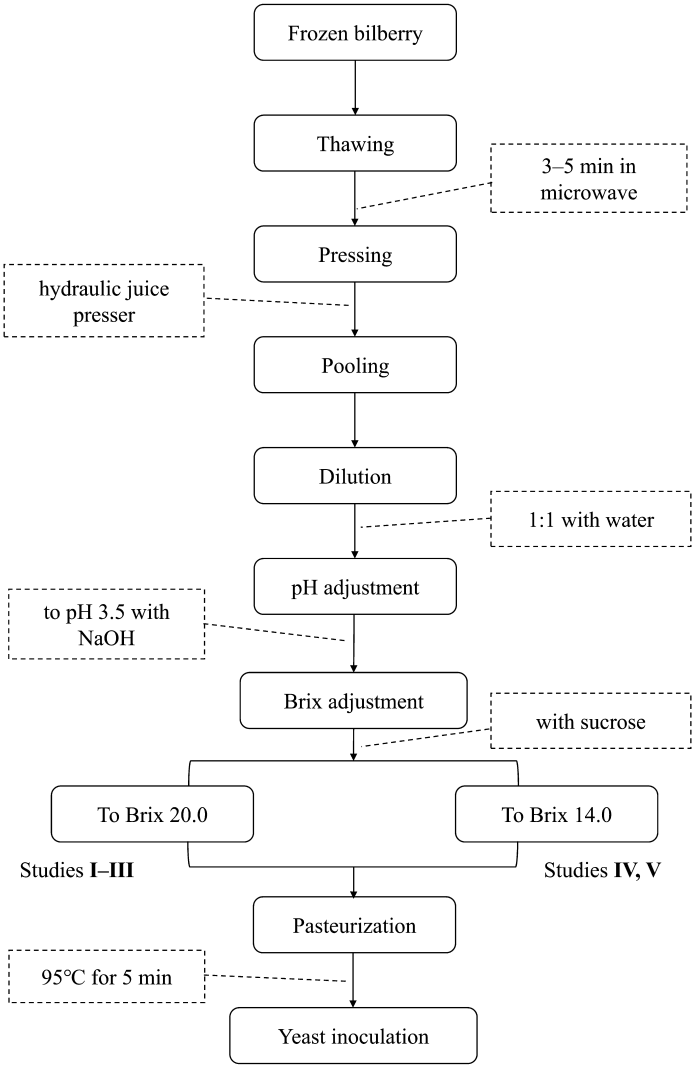


Figure 12. Overall scheme of bilberry juice preparation before fermentation.

4.2 Yeast strains and culture condition

Cultures of *Saccharomyces cerevisiae* Lalvin V1116 (SC1116) and *Torulaspora delbrueckii* 291 (TD291) were purchased from Lallemand Inc. (Montreal, Canada). Strains of *T. delbrueckii* 70526 (TD70526), *Schizosaccharomyces pombe* 70572 (SP70572), *S. pombe* 3796 (SP3796), *Saccharomycodes ludwigii* 3447 (SL3447), *Metschnikowia pulcherrima* 70321 (MP70321), *Lachancea thermotolerans* 3434 (LT3434), *Issatchenkia orientalis* 3433 (IO3433), *Hanseniaspora uvarum* 26650 (HU26650), *Pachysolen tannophilus* 70352 (PT70352), and *Zygosaccharomyces bailii* 70492 (ZB70492) were purchased

from DSMZ Institute (Braunschweig, Germany). The proliferation of these yeasts was carried out in sterilized YPD medium or YM medium at 25 ℃ for 48 h with 150 rpm shaking.

4.3 Laboratory-scale fermentation and aging treatment

Prior to inoculation, the yeast cell population was determined by the spread plate technique in YPD or YM agars. The yeast cells in broths were centrifuged at 4500 × *g* for 10 min and washed three times with 0.9% sterile sodium chloride solution, after which the yeast pellets were collected and resuspended in the same medium before being used for fermentation of bilberry juice.

Three fermentation types were conducted in studies **I**–**III**: pure fermentations (PR) by inoculation with a single *S. cerevisiae* (SC11116) or each of the non-*Saccharomyces* yeast strain (TD291, TD70526, SP3796, or SP70572); sequential fermentation (SQ) by inoculation with a non-*Saccharomyces* yeast as the starter strain, followed by inoculation with the *S. cerevisiae* to complete the fermentation; and simultaneous fermentation (SM) by co-inoculation with *S. cerevisiae* and a non-*Saccharomyces* yeast at the same time. In study **IV**, ten yeasts of SC1116, TD291, SP70572, SL3447, MP70321, LT3434, IO3433, HU26650, PT70352, and ZB70492 were studied in pure fermentation. While only SC1116 strain was used in study **V**. Each of the cultures were inoculated at the cell counts of 10^7 CFU/mL. Laboratory-scale fermentations were conducted in Duran bottles at 25 ℃ in darkness. The production of CO_2 during fermentation was released from air valves or via unscrewing caps of bottles regularly. During fermentation, °Brix value and bottle weight loss were regularly monitored till the completion of fermentation. In study **IV,** investigating the dynamic changes in volatile compounds during fermentation, the fermented samples were successively taken every 3 days. A series of bottles of juices (total 12 bottles) was inoculated for each yeast strain to avoid the possible impact of volume reduction caused by repeated sampling. After fermentation, all the bilberry wines were centrifuged to remove yeast cells and solids and the supernatants were kept at −80 °C until analysis.

In order to monitor the evolution of pyranoanthocyanins and their precursor anthocyanin monomers during aging (study **III**), the bilberry wines were stored at +6 °C in darkness and successively taken for analysis after 1, 6 and 12 months of aging.

4.4 Physicochemical characteristics of berry juices and wines

Individual sugars and organic acids in bilberry juices and wines were analyzed as trimethylsilyl (TMS) derivatives by gas chromatography equipped with a flame ionization detector (GC-FID, Shimadzu, Japan) and an SPB-1 column (30 m × 0.25 mm i.d., 0.25 μm, Supelco, Bellefonte, PA) (studies **I–III**). Identification was performed by comparing retention times between analytes and reference compounds. Quantification was carried out using sorbitol as an internal standard for sugars, and tartaric acid for organic acids.[331]

Ethanol and glycerol were measured by the same model GC-FID as described for sugars and acids but equipped with an HP-INNOWAX column (30 m × 0.25 mm i.d., 0.25 μm, Hewlett- Packard, Avondale, PA) (studies **I**, **II**, **and IV**). The compounds were identified by comparing the retention times to their responding standards and were quantified using calibration curves constructed by external standards with different concentrations.

4.5 Qualitative and quantitative analyses of phenolic compounds

4.5.1 Liquid chromatographic analysis

The separation of phenolic compounds, including anthocyanins and anthocyanin derivatives in studies **I** and **III** and nonanthocyanin phenolic compounds (phenolic acids, flavonols, and flavan-3-ols) in study **V**, were carried out with liquid chromatography (LC) systems coupled with diode array detectors (DAD) and a reverse phase XB-C18 column (150 × 4.60 mm, 3.6 μm, Phenomenex, Torrance, CA). Water and acetonitrile, both containing 5% (*v/v*) formic acid, were used as mobile phases A and B, respectively.[332] Anthocyanin-related compounds, phenolic acids, flavonols, flavan-3-ols were recorded at 520 nm, 320 nm, 350 nm, and 280 nm, respectively. Quantification of phenolic compounds was conducted using external standard methods.

4.5.2 Liquid chromatography–mass spectrometric analysis

The Waters Acquity ultra performance liquid chromatography system (UPLC) equipped with a Waters 2996 DAD detector and a Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA) and an electrospray interface was used for the qualitative analysis of anthocyanins and pyranoanthocyanins in studies **I** and **III**.

In study **I**, the identification of anthocyanins was performed in positive ESI mode. The capillary, cone voltage, and extractor voltage were set at 3.25 kV, 30 V, and 2.5 V, respectively. Mass spectra were scanned in the *m/z* 250–1000. In the tandem mass spectrometric analysis, the capillary, cone voltage, and extractor voltage were set at 0.8 kV, 20 V, and 2 V, respectively. The collision energy was 20 eV. The source and desolvation temperatures were 120 °C and 500 °C, respectively. The desolvation and cone gas flow were 899 L/h and 97 L/h, respectively.

In study **III**, some modifications were made based on study **I** as capillary was improved to 3.5 kV, cone voltage to 35 V, and extractor voltage to 3 V. Anthocyanins and pyranoanthocyanins were identified by comparing retention times, UV–Vis spectra, and mass spectra in UPLC–MS/MS to their corresponding standards, when available. Otherwise, tentative identification was performed by comparing these parameters with the data in the literature. To further identify vitisin A-type pyranocyanidin pigments, the synthesis of these compounds was performed using their corresponding monomeric anthocyanins and pyruvic acid in bilberry model wine.

The qualitative analysis of nonanthocyanin phenolic compounds in study **V** was carried out using the Bruker Elute UHPLC systems coupled with an Ultra-High Resolution Impact II Qq-Time-of-Flight mass spectrometer (QTOF-MS, Bruker Daltonik GmbH, Bremen, Germany) and an ESI source in both positive and negative ionization modes in the range of *m/z* 20 to 1000. The ESI parameters of end plate offset and drying gas (N_2) flow were set at 500 V and 12.0 L/min for both positive and negative ionization, while capillary voltage, nebulizer gas (N_2) pressure, and drying gas temperature were set at 4.5 kV, 4.8 bar, and 350 °C, respectively for positive ion mode and 3.5 kV, 4.0 bar, and 300 °C, respectively for negative ion mode. Before each set of injection, sodium formate (10 mM) was continually introduced to the six-port valve from a direct infusion syringe pump at the flow rate of 180 µL/min in high-precision calibration (HPC) mode for high-accuracy mass calibration. For confirmation of elemental compositions of phenolic compounds, the mass error (ppm) was calculated as the difference between the measured mass and the theoretical mass of a given molecular formula, expressed as:

$$\text{mass error} = \frac{m_{measured} - m_{theoretical}}{m_{theoretical}} \times 10^6$$

4.6 Determination of volatile compounds

The volatile compounds in the bilberry juices and wines were determined using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME–GC–MS) (studies **II** and **IV**).

In study **II**, the extraction of volatile compounds was carried out with a 2 cm DVB/CAR/PDMS fiber (50/30 μm, Supelco, Bellefonte, PA). The extracted volatile compounds were analyzed in a Trace 1310 gas chromatography coupled with a TSQ8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A DB-WAX polar capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, Folsom, CA) and an SPB-624 mid-polarity capillary column (60 m × 0.25 mm i.d. × 1.4 μm film thickness, Supelco, Bellefonte, PA) were used to separate volatile compounds. Helium was used as the carrier gas. Mass spectra were recorded in electron impact (EI) mode at 70 eV with a scan range *m/z* 33−300. The volatile compounds were identified by matching the obtained mass spectra with the standard NIST library and comparing the retention indices (RIs) with those reported in the literature and the NIST database (https://webbook.nist.gov/chemistry/). Several compounds were further confirmed by comparing the RIs and mass spectra with those of authentic standards. Individual compounds separated with the DB-WAX column was semi-quantified with the aid of the internal standard (4-methyl-2-pentanol, 802 μg/mL in methanol) by comparing their base peak areas.[217,333]

The condition of HS-SPME–GC–MS in study **IV** was the same as that in study **II**. Ascribed to the outstanding performance of DB-WAX in the separation of volatile compounds in bilberry products demonstrated in study **II,** only DB-WAX was used in study **IV**. However, with the aim to determine volatile compounds more authentic, most of the detected volatiles in study **IV** were identified and quantitated with the aid of authentic standards. To minimize the interference of ethanol on the extraction of other volatile compounds on fiber coating, the quantitation of volatile compounds was carried out using calibration curves built with their authentic standards from nine different concentrations in synthetic bilberry wine matrices. In the calibration equations y = ax + b,

$$x = \frac{\text{peak area (authentic standard)}}{\text{peak area (internal standard)}}, \; y = \frac{\text{concentration (authentic standard)}}{\text{concentration (internal standard)}}$$

Five standard calibration curves were obtained for an individual volatile compound with ethanol concentration at 2, 4, 6, 8, and 10% (*v/v*), respectively. An appropriate calibration curve was selected, based on the ethanol concentration in bilberry wines, for the quantitation of volatile compounds following the principle of proximity of ethanol content. The compounds without

corresponding standards were quantitated based on the calibration curves obtained from the standards of the same chemical group with similar chemical structures. Limits of detection (LODs) and quantitation (LOQs) for volatile standards were estimated as the concentration of the analytes that provided a signal-to-noise ratio (S/N) of 3 and 10, respectively.

4.7 Statistical analysis

One-way analysis of variance (ANOVA) (studies **I–IV**) and independent-samples *t*-test (study **V**) were employed to determine the content and composition difference of analytes using SPSS or R software. The Bivariate correlations between the reduction in monomeric anthocyanin content and the increase in pyranoanthocyanin content were evaluated with Spearman's rank correlation ($\alpha < 0.05$) using SPSS 25.0 (study **III**). Multivariate models of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were established with Unscrambler X software (studies **I**, **II**, and **IV**).

5 RESULTS AND DISCUSSION

5.1 Chemical compositions of berry juices

5.1.1 Sugars, organic acids, and glycerol (study I)

Five individual sugars were detected in bilberry juice, of which sucrose was the dominant one accounting for approximately 54% of total sugars, followed by glucose (23%) and fructose (23%) (**Figure 13**). The total organic acid content was 6.7 g/L with quinic acid (39%) as the most abundant acid, followed by citric acid (32%) and malic acid (23%). A similar result has been reported in fresh fruit of wild bilberry originated from Finland,[255] suggesting that juice processing, including thawing, pressing, and pasteurization, may not significantly alter the composition of organic acids. As the main byproduct of fermentation, as expected, glycerol was not detected in bilberry juice.

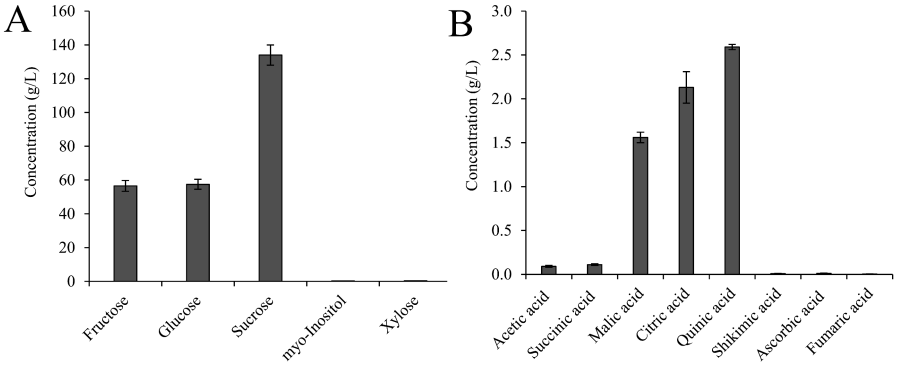


Figure 13. Concentrations of sugars (**A**) and organic acids (**B**) detected in bilberry juice.

5.1.2 Phenolic compounds (studies I and V)

A total of 15 monomeric anthocyanins were detected in BB juice with galactose, glucose, and arabinose as the sugar moieties bound to delphinidin, cyanidin, petunidin, peonidin, and malvidin (**Figure 14**). Galactosides and glucosides of delphinidin and cyanidin were the most abundant anthocyanin monomers accounting for approximately 50% of total monomeric anthocyanins (TMACY) (study **I**). The result is consistent with previous studies.[250,255] The composition of anthocyanin monomers in bilberry and bilberry products is significant different from that in *V. vinifera* and their products as the predominant anthocyanins generally are malvidin-based anthocyanins in the latter.[14,35] These

anthocyanins were absent in the juice of white bilberries (WB) based on peak monitoring at 520 nm using HPLC-DAD and the scanning of characteristic protonated molecular ions and aglycone fragment ions of anthocyanins in QTOF/MS analysis (study **V**). Consistent results were previously reported in white currant cultivars.[334,335]

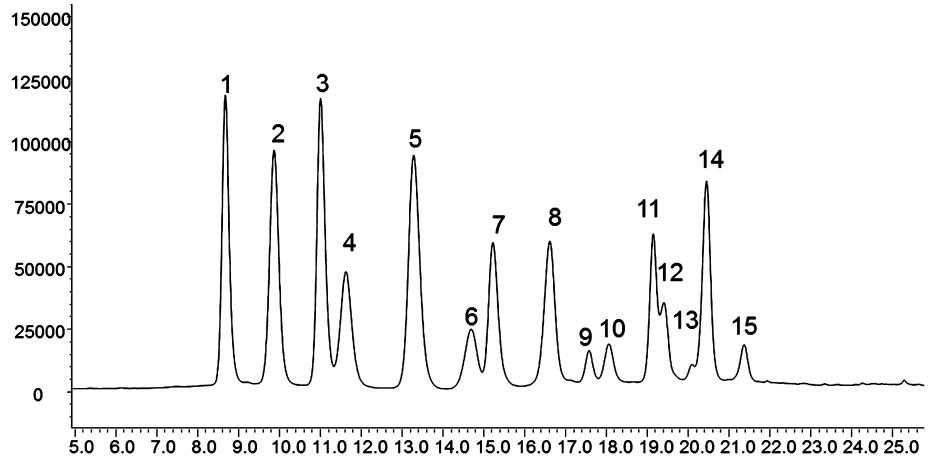


Figure 14. HPLC-DAD chromatogram of monomeric anthocyanins detected in blue bilberry juice. The peaks 1–15 refer to compounds of: 1: delphinidin 3-*O*-galactoside, 2: delphinidin 3-*O*-glucoside, 3: cyanidin 3-*O*-galactoside, 4: delphinidin 3-*O*-arabinoside, 5: cyanidin 3-*O*-glucoside, 6: petunidin 3-*O*-galactoside, 7: cyanidin 3-*O*-arabinoside, 8: petunidin 3-*O*-glucoside, 9: peonidin 3-*O*-galactoside, 10: petunidin 3-*O*-arabinoside, 11: peonidin 3-*O*-glucoside, 12: malvidin 3-*O*-galactoside, 13: peonidin 3-*O*-arabinoside, 14: malvidin 3-*O*-glucoside, 15: malvidin 3-*O*-arabinoside.

Individual nonanthocyanin phenolic compounds in white and blue bilberry juices were analyzed in study **V** (**Table 7**). Phenolic acids were the major polyphenols in both white and blue juices, being 76.9% and 80.5% of the content of total nonanthocyanin phenolics, respectively, followed by flavonols (17.8% and 14.4%, respectively) and flavan-3-ols (5.3% and 5.1%, respectively). The total contents of phenolic acids (126.29 mg/L), flavonols (29.15 mg/L), and flavan-3-ols (8.69 mg/L) in BB juice were significantly higher than those in WB juice (67.34, 12.07, and 4.24 mg/L, respectively). This may be associated with the low expression of the genes encoding the enzymes acting not only on anthocyanin biosynthesis but also on flavonol and flavan-3-ol biosynthesis pathways in white bilberry (**Figure 11**). For example, the low expressions of F3H, DFR, and ANS genes may reduce the accumulations of substrates for the formations of flavonol aglycones and monomeric flavan-3-ols.

p-Coumaroyl monotropeins were identified for the first time in bilberry products through the combination of chromatographic separation and high mass accuracy measurements (mass error < |5| ppm). These compounds dominated among the detected phenolic acids in blue and white juices accounting for 55% and 41% of total phenolic acids, respectively. Among the total 22 detected phenolic acids in the two types of juice, *p*-coumaroylquinic acid was the exclusively detected hydroxycinnamic acid in WB juice, while the dicaffeoylquinic acids were exclusive in BB juice.

Table 7. Nonanthocyanin phenolic compounds detected in white (WB) and blue bilberry (BB) juices and wines[a]. Table reprinted from the original publication **V**

Compound	***Content (mg/L)***				***t-test[b]***		
	WB juice	***WB wine***	***BB juice***	***BB wine***	***WB juice vs BB juice***	***WB juice vs WB wine***	***BB juice vs BB wine***
phenolic acids							
5-caffeoylquinic acid	8.12 ± 0.05	11.27 ± 0.17	4.34 ± 0.20	4.38 ± 0.14	***	***	
protocatechuic acid hexoside	0.38 ± 0.01	1.23 ± 0.31	0.90 ± 0.08	0.71 ± 0.02	***	*	**
p-coumaric acid derivative-1	1.11 ± 0.02	1.15 ± 0.04	2.59 ± 0.19	2.65 ± 0.09	***		
p-coumaric acid derivative-2	0.06 ± 0.00	0.04 ± 0.01	0.24 ± 0.08	0.21 ± 0.15	*		
caffeic acid hexoside-1	16.37 ± 0.12	0.16 ± 0.02	22.93 ± 0.64	5.09 ± 0.20	***	***	***
3-caffeoylquinic acid	3.39 ± 0.09	2.71 ± 0.08	6.74 ± 0.23	4.41 ± 0.20	***	***	***
4-caffeoylquinic acid	0.03 ± 0.03	0.06 ± 0.00	0.30 ± 0.05	0.43 ± 0.11	***		
caffeic acid	0.74 ± 0.01	17.21 ± 0.33	0.55 ± 0.13	17.48 ± 0.54		***	***
caffeic acid hexoside-2	2.76 ± 0.06	3.05 ± 0.07	1.95 ± 0.10	2.07 ± 0.26	***	***	
caffeoylquinic acid isomer	0.37 ± 0.02	0.43 ± 0.20	0.32 ± 0.04	0.24 ± 0.03	*		*
p-coumaroylquinic acid	0.07 ± 0.01	0.09 ± 0.03	–	–			
p-coumaric acid	2.28 ± 0.06	3.56 ± 0.13	4.26 ± 0.15	5.57 ± 0.14	***	***	***
dicaffeoylquinic acid-1	–	–	0.56 ± 0.32	0.52 ± 0.17			
dicaffeoylquinic acid-2	–	–	0.99 ± 0.24	1.08 ± 0.13			
caffeic acid derivative hexoside	1.07 ± 0.02	1.12 ± 0.04	0.94 ± 0.11	0.93 ± 0.03			
p-coumaroyl monotropein-1	12.83 ± 0.13	14.65 ± 0.30	10.65 ± 0.66	10.91 ± 0.44	***	***	
p-coumaroyl monotropein-2	14.98 ± 0.18	24.95 ± 0.37	58.82 ± 1.44	53.53 ± 1.57	***	***	**
p-coumaric acid derivative	0.24 ± 0.01	1.28 ± 0.03	0.52 ± 0.03	0.55 ± 0.06	***	***	
p-coumaric acid derivative-a	1.21 ± 0.01	2.10 ± 0.04	4.88 ± 0.11	4.93 ± 0.13	***	***	
p-coumaric acid derivative-b	0.98 ± 0.01	1.54 ± 0.02	1.29 ± 0.14	1.37 ± 0.04	*	***	
p-coumaric acid derivative-c	0.04 ± 0.01	0.31 ± 0.03	0.65 ± 0.03	0.75 ± 0.04	***	***	*
caffeic acid derivative	0.30 ± 0.01	0.70 ± 0.01	1.87 ± 0.06	1.71 ± 0.06	***	***	*
total phenolic acids	67.34 ± 0.86	87.62 ± 2.22	126.29 ± 5.03	119.51 ± 4.55	***	***	*

Compound	Content (mg/L)				t-test[b]		
	WB juice	WB wine	BB juice	BB wine	WB juice vs BB juice	WB juice vs WB wine	BB juice vs BB wine
flavonols							
myricetin 3-*O*-galactoside	0.29 ± 0.01	0.31 ± 0.04	2.36 ± 0.10	2.39 ± 0.07	***		
myricetin 3-*O*-glucoside	–	1.48 ± 0.04	1.94 ± 0.46	4.00 ± 0.69			**
quercetin 3-*O*-galactoside	4.48 ± 0.12	5.01 ± 0.07	8.20 ± 0.34	8.06 ± 0.34	***	***	
quercetin 3-*O*-glucuronide	5.17 ± 0.10	5.76 ± 0.12	6.38 ± 0.18	5.93 ± 0.13	***	***	**
quercetin 3-*O*-glucoside	0.25 ± 0.02	0.33 ± 0.01	1.43 ± 0.10	1.41 ± 0.04	***	***	
laricitrin 3-*O*-galactoside	–	–	0.22 ± 0.02	0.18 ± 0.00			*
laricitrin 3-*O*-glucoside	0.26 ± 0.01	0.31 ± 0.01	2.34 ± 0.05	2.33 ± 0.08	***	**	
quercetin 3-*O*-arabinoside	0.25 ± 0.02	0.26 ± 0.03	0.92 ± 0.06	0.90 ± 0.06	***		
quercetin 3-*O*-xyloside	0.27 ± 0.02	0.36 ± 0.01	0.52 ± 0.03	0.47 ± 0.02	***	**	
myricetin aglycone	0.32 ± 0.01	0.44 ± 0.01	0.60 ± 0.03	0.67 ± 0.03	***	***	**
isorhamnetin 3-*O*-galactoside	–	–	0.46 ± 0.12	0.44 ± 0.12			
syringetin 3-*O*-galactoside	0.23 ± 0.01	0.47 ± 0.01	0.90 ± 0.03	0.84 ± 0.04	***	***	
syringetin 3-*O*-glucoside	0.37 ± 0.00	0.34 ± 0.01	2.28 ± 0.07	2.40 ± 0.06	***		
quercetin aglycone	0.19 ± 0.00	0.31 ± 0.00	0.40 ± 0.02	0.42 ± 0.01	***	***	
syringetin aglycone	–	0.22 ± 0.00	0.21 ± 0.01	0.28 ± 0.02			***
total flavonols	12.07 ± 0.15	15.58 ± 0.20	29.15 ± 0.7	30.72 ± 0.84	***	***	*
flavan-3-ols							
(–)-epigallocatechin	0.21 ± 0.01	0.37 ± 0.25	0.25 ± 0.04	0.31 ± 0.06			
(+)-catechin	–	–	1.48 ± 0.18	1.26 ± 0.02			*
procyanidin B-type dimer	1.86 ± 0.13	1.47 ± 0.36	3.27 ± 0.39	4.05 ± 0.72	**		
(−)-epicatechin	1.96 ± 0.14	0.90 ± 0.41	2.56 ± 0.10	1.63 ± 0.19	***	**	***
procyanidin B-type trimer	0.21 ± 0.02	0.39 ± 0.20	1.13 ± 0.13	0.80 ± 0.08	***		**
total flavan-3-ols	4.24 ± 0.26	3.14 ± 0.36	8.69 ± 0.35	8.04 ± 0.59	***	**	

[a] –: not detected.

[b] Independent-samples *t*-test. *, **, and ***: significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

A total of 15 flavonols were detected in BB juice (**Table 7**), of which myricetin 3-*O*-glucoside, laricitrin 3-*O*-galactoside, isorhamnetin 3-*O*-galactoside, and syringetin aglycone were undetectable in WB juice. Quercetin 3-*O*-galactoside and quercetin 3-*O*-glucuronide were the most abundant flavonols in blue and white bilberry juices accounting for approximately 60% and 80% of total flavonols, respectively. Interestingly, according to the previous studies on the phenolic characteristics of bilberry, myricetin was the only aglycone which can be detected in BB fruit but not in WB mutant.[254,336] The maximum level of this compound reached in fully colored fruit.[252] Therefore, the detection of myricetin aglycone in WB juice and quercetin and syringetin aglycones in BB juice indicated that hydrolysis of glycosylated flavonols is likely to take place during juice processing to yield the corresponding aglycones.

Five flavan-3-ols, including three monomeric flavan-3-ols ((–)-epigallocatechin, (+)-catechin, and (−)-epicatechin) and two oligomeric procyanidins (procyanidin B-type dimer and procyanidin B-type trimer), were quantified in bilberry juices (**Table 7**). (+)-Catechin was the unique individual flavan-3-ol in BB juice. (−)-Epicatechin and procyanidin B-type dimer contributed most to the total flavan-3-ols contents in the two juice varieties. Similar to phenolic acids and flavonols, the contents of most individual flavan-3-ols in BB juice were significantly higher than that in WB juice.

5.1.3 Volatile compounds (study II)

The semi-quantification of volatile compounds in bilberry juice and wines was determined by the DB-WAX column. Totally 28 compounds were detected in bilberry juice, including 10 higher alcohols, 5 esters, 2 monoterpenes, 5 ketones, 4 aldehydes, and 2 benzenes, whereas acetals were undetected. 2-Ethyl-1-hexanol, ethyl acetate, linalool, 4-methyl-2-pentanone, hexanal, and 1,3-di-*tert*-butylbenzene were respectively the most abundant compounds in the above-mentioned groups of volatiles.

5.2 Fermentation kinetics difference between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts (studies I and IV)

The fermentation kinetics, expressed as the evolution of ethanol during the production of bilberry wines fermented with nine non-*Saccharomyces* yeasts and one control *S. cerevisiae* in pure inoculation models, were illustrated in **Figure 15A** (study **IV**).

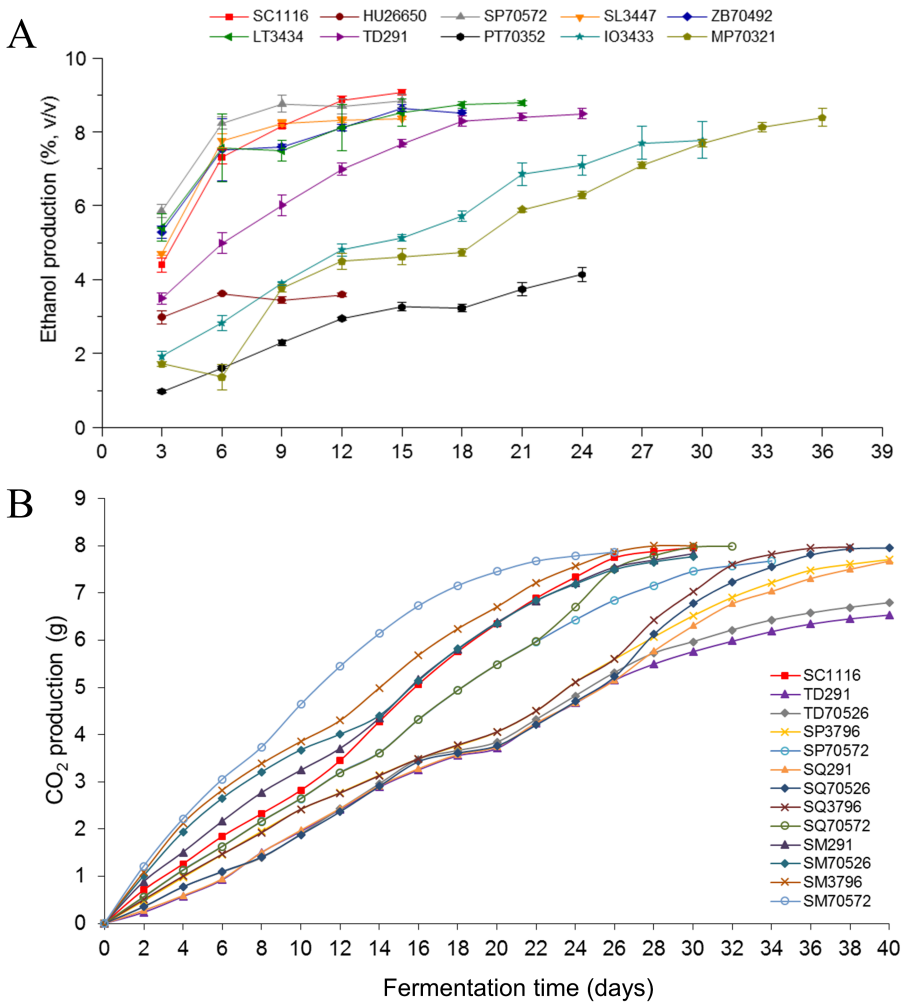


Figure 15. Fermentation kinetics, expressed as ethanol production (**A**) and CO_2 production (**B**), of *Saccharomyces* and non-*Saccharomyces* yeasts during bilberry wine production. The yeasts are *S. cerevisiae* V1116 (SC1116), *H. uvarum* 26650 (HU26650), *S. pombe* 70572 (SP70572), *S. ludwigii* 3447 (SL3447), *Z. bailii* 70492 (ZB70492), *L. thermotolerans* 3434 (LT3434), *T. delbrueckii* 291 (TD291), *P. tannophilus* 70352 (PT70352), *I. orientalis* 3433 (IO3433), and *M. pulcherrima* 70321 (MP70321). SQ and SM refer to sequential and simultaneous inoculations of SC1116 and one non-*Saccharomyces* yeast, respectively. Figures reprinted from the original publications **I** and **IV** with permission from Elsevier and American Chemical Society.

The fermentations inoculated with the strains SC1116, SP70572, SL3447, ZB70492, and LT3434 differed from the fermentations with other strains by the higher production of ethanol or the shorter fermentation duration. Remarkably,

more than 80% of the final ethanol content was generated during the first 6 days of fermentations with these five yeast strains. The fermentation kinetics of fermentation with TD291, IO3433, or MP70321 showed a linear trend with time. The final ethanol productions of the above seven non-*Saccharomyces* yeasts peaked at approximately 8–9%, whereas the values were still lower by 0.2–1.3 degree than that of *S. cerevisiae*. The fermentation capacities PT70352 and HU26650 were obviously poorer than those of the other strains as indicated by the ethanol concentration of 4.1% and 3.6%, respectively, in the fermented products. The poor fermentation ability and low ethanol tolerance of *H. uvarum* and *P. tannophilus* have been verified in wine fermentation.[192,204] The production of fermented beverages with low ethanol content is considered as one of the most important potential applications of non-*Saccharomyces* yeasts due to their poorer capacities for converting sugars to ethanol.

The participation of *S. cerevisiae* in sequential and simultaneous inoculations with *T. delbrueckii* and *S. pombe* strains on fermentation kinetics, monitored as CO_2 production, was reported in studies **I** (**Figure 15B**). In comparison with the fermentations with pure non-*Saccharomyces* yeasts, the combinations of non-*Saccharomyces* yeast and *S. cerevisiae* dramatically expedited the fermentation rates and shortened the fermentation durations, particularly in simultaneous inoculations, due to the high sugar consumption capacity of *S. cerevisiae*.

5.3 Effect of fermentation on chemical compositions of berry juices (studies I, II, and V)

The effect of pure, sequential, and simultaneous fermentations with *S. cerevisiae* (SC1116) and non-*Saccharomyces* yeasts (TD291, TD70526, SP3796, and SP70572) on the change in concentration of sugars, organic acids, glycerol, phenolic compounds, and volatile compounds in bilberry juices was analyzed in studies **I, II,** and **V**.

5.3.1 Sugars, organic acids, and glycerol (study I)

Dry bilberry wines (residual sugar < 1 g/L) were obtained by the inoculations involving *S. cerevisiae* or *S. pombe* strains, whereas a high amount of residual sugars still existed in the bilberry wines fermented with pure inoculation of TD291 (27.5 g/L) or TD70526 (16.5 g/L). The relatively low conversion of sugars to ethanol by *T. delbrueckii* stains has also been reported in winemaking.[142] However, this makes it possible to use *T. delbrueckii* as pure stains for producing semi-sweet and sweet berry wines. Interestingly, fructose was the major residual sugar instead of sucrose and glucose in the pure

fermentations with *T. delbrueckii,* indicating these strains consumed sucrose and glucose in preference to fructose during fermentation. The participation of *S. cerevisiae* in the sequential and simultaneous inoculations with *T. delbrueckii* guaranteed the completion of fermentation.

Alcoholic fermentation significantly affected the content of organic acids, particularly of malic, acetic, lactic, pyruvic, and succinic acids. Fermentations involving *S. pombe* strains consumed almost all the initial malic acid in bilberry juices, followed by *T. delbrueckii* strains (20–49% reduction) and *S. cerevisiae* (16%), indicating that the deacidification by *S. pombe* strains in bilberry matrix is as excellent as that in grape matrix. The reduction of malic acid can reduce the endowed harsh 'green apple sourness', acidity, and puckering astringency.[172] Lactic and pyruvic acids, which are absent in bilberry juice, accumulated remarkably during fermentation. Inoculations involving *T. delbrueckii* and *S. pombe* strains promoted the productions of lactic acid and pyruvic acid, respectively. However, fermentations involving *T. delbrueckii* strains produced bilberry wines with significantly lower acetic acid concentration than the bilberry wines produced by other strains. The results are in agreement with that found in winemaking.[144]

As one of the primary contributions to wine quality, the enhancement of glycerol production from non-*Saccharomyces* yeasts, which has been reported in winemaking,[142,171,172,186] was also verified in this research as 16–65% more of this compound was detected in the bilberry wines produced by pure and sequential inoculations involving *T. delbrueckii* and *S. pombe* strains than that produced by *S. cerevisiae*, particularly the fermentations involving *S. pombe* strains. However, in bilberry wine, simultaneous fermentations significantly reduced the generation of glycerol compared to pure fermentation with *S. cerevisiae*. Although the decrease of glycerol production resulting from simultaneous fermentation involving *T. delbrueckii* and *S. pombe* strains has also been reported in winemaking, its concentration in wines fermented with simultaneous inoculation was still higher than that fermented with *S. cerevisiae*. The difference indicated that the performance of *T. delbrueckii* and *S. pombe* strains in glycerol generation in bilberry wine is somehow different from that in winemaking.

5.3.2 Anthocyanin monomers and pyranoanthocyanins (study I)

Fermentation significantly reduced the total content of monomeric anthocyanins (TMACY) by 9.3–41.5%. TMACY in the bilberry wines fermented involving *S. pombe* 70572 strain, especially the PR70572 and SQ70572 samples, were higher than those fermented with other yeasts. This may have resulted from less adsorption of anthocyanins on the cell walls of this strain.[337] The concentrations

of individual anthocyanins also showed significant degradations, particularly the glucosides of cyanidin and delphinidin, whereas the galactosides and arabinosides of anthocyanidins remained relatively more constant. Malvidin glycosides were more stable than the anthocyanins with other aglycones as indicated by the lower reduction in content during fermentation.

In study **I**, six vitisin A-type pyranoanthocyanins (vAPs), two derived from hexosides of petunidin, one from hexoside of peonidin, and three from glycosides of malvidin, were quantified in bilberry wines. The pyranoanthocyanins with aglycones of delphinidin and cyanidin were also identified in MS analyses, whereas they were not quantified due to the coelution with other major peaks in chromatograms. Since vAPs were formed *via* cycloaddition reaction between anthocyanin monomers and pyruvic acid, which is produced by yeast metabolism during fermentation, thus they unsurprisingly were not detected in bilberry juice. The concentrations of vAPs in bilberry wines produced from pure and sequential fermentations involving *S. pombe* strains were significantly higher than that fermented with *S. cerevisiae* resulting from the higher production of pyruvic acid of the former yeasts. However, the long-time participation (simultaneous inoculation) of *S. cerevisiae* in the fermentation with *S. pombe* strains obviously inhibited the formation of vAPs. The production of vAPs was greatly depended on the concentration of pyruvic acid (correlation coefficient $R = 0.874$, $p < 0.01$).

5.3.3 Nonanthocyanin phenolic compounds (study V)

Compositional changes in nonanthocyanin phenolic compounds in white and blue bilberry juices were also analyzed in study **V** with the impact of alcoholic fermentation with *S. cerevisiae*. The contents of most individual phenolic acids and flavonols in WB juice were increased or remained constant after fermentation (**Table 7**), resulting in an increase of 30% and 29% in TA and TFO, respectively. This may be due to the gradual accumulation of ethanol during fermentation enhanced the extraction of these compounds from the debris of bilberry pulp and skin. The changes in the content of these three phenolic groups resulted in an increase by 27% in TPC (**Figure 16**), and this may consequently enhance bitterness and astringency characters to WB wine.

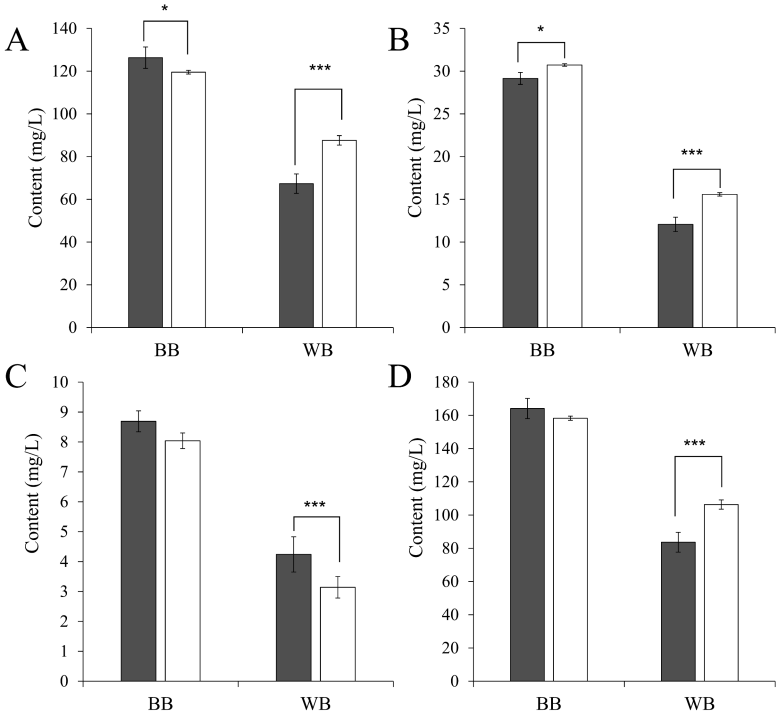


Figure 16. Total contents of phenolic acids (**A**), flavonols (**B**), flavan-3-ols (**C**), and total content of phenolic compounds calculated as sum of the three groups presented in A, B, and C (**D**) in berry juices (gray bars) and wines (white bars). * and *** refer to significant different at $p < 0.05$ and $p < 0.001$, respectively using independent-samples *t*-test.

However, despite the remarkable increases in TFO and TA from WB juice to wine, only a 5% increase of TFO and, conversely, a 5.4% reduction of TA were detected in BB samples. These results may be explained by the participation of phenolic acids and flavonols in chemical reactions with anthocyanins such as co-pigmentation, cycloaddition, and polymerization, which have been discussed in section 2.1.2.1, during the fermentation of BB juice. The reactions involving phenolic acids and flavonols may partly mask their contributions to bitterness and/or astringency of bilberry wines. Interestingly, *p*-coumaroyl monotropeins still dominated among the phenolic acids detected in WB and BB wines accounting for approximately 50% of TAs.

5.3.4 Volatile compounds (study II)

The aroma intensity of bilberry juice was likely enhanced by fermentation as reflected by remarkably higher concentrations of total volatile compounds detected in bilberry wines than that in bilberry juice (**Figure 17**). Higher alcohols

and esters, the two major groups of secondary aroma volatile compounds, were dominant in the fermented samples, being 14–54 times and 6–9 times higher than those in unfermented juice. However, seven compounds, mainly belong to the groups of ketone and aldehyde, were reduced to the levels of undetectable after fermentation. Moreover, fermentation also significantly improved the volatile complexity of bilberry juice, as indicated by more diversified profiles of volatile compounds were associated with bilberry wines (**Figure 18A**).

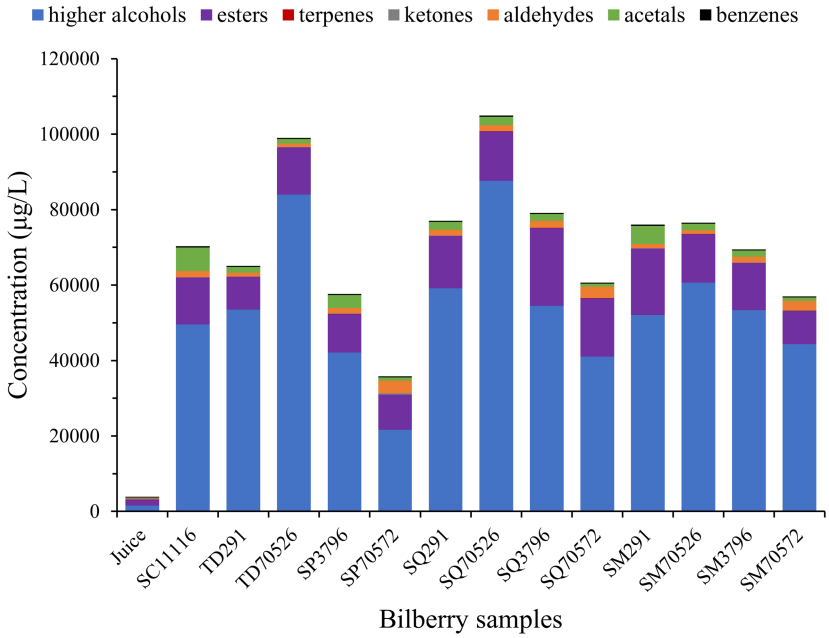
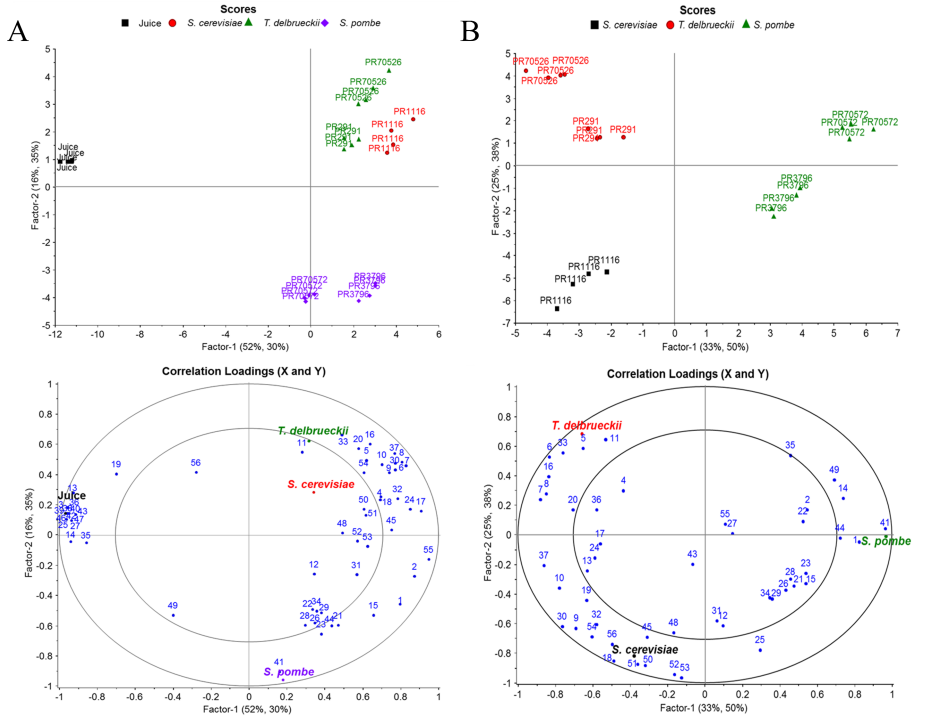


Figure 17. Concentrations of total volatile compounds and seven different groups of volatile compounds in bilberry juice and wines fermented with *T. delbrueckii* (TD291 and TD70526) and *S. pombe* (SP3796 and SP70572) in pure fermentation as well as in sequential (SQ) and simultaneous inoculations (SM) with *S. cerevisiae* V1116 (SC1116).



1	1-hexanol	20	isoamyl acetate	39	4-methyl-2-pentanone
2	(Z)-3-hexen-1-ol	21	ethyl hexanoate	40	4,6-dimethyl-2-heptanone
3	(E)-2-hexen-1-ol	22	isopentyl 3-methylbutyrate	41	acetoin
4	1-propanol	23	ethyl heptanoate	42	6-methyl-5-hepten-2-one
5	2-methyl-1-propanol	24	ethyl lactate	43	2,6,8-trimethyl-4-nonanone
6	1-butanol	25	methyl 2-hydroxy-3-methylbutanoate	44	acetaldehyde
7	2-methyl-1-butanol	26	ethyl caprylate	45	3-methylbutanal
8	3-methyl-1-butanol	27	2,6,8-trimethyl-4-nonanol	46	hexanal
9	4-methyl-1-pentanol	28	methyl decanoate	47	(E)-2-hexenal
10	3-methyl-1-pentanol	29	ethyl caprate	48	nonanal
11	3-ethoxy-1-propanol	30	diethyl succinate	49	benzaldehyde
12	1-heptanol	31	ethyl 9-decenoate	50	1-ethoxy-1-methoxyethane
13	2-ethyl-1-hexanol	32	ethyl 4-hydroxybutanoate	51	1,1-diethoxyethane
14	1-octanol	33	phenethyl acetate	52	2,4,5-trimethyl-1,3-dioxolane
15	threo-2,3-butanediol	34	ethyl dodecanoate	53	2,4-dimethyl-1,3-dioxane
16	2-phenylethanol	35	linalool	54	1-(1-ethoxyethoxy)pentane
17	ethyl acetate	36	α-terpineol	55	1,3,5-trimethylbenzene
18	ethyl 3-methylbutyrate	37	β-citronellol	56	1,3-di-tert-butylbenzene
19	4-methyl-2-pentyl acetate	38	2-pentanone		

Figure 18. PLS-DA models using contents of volatiles as X-data (**A**: n = 56; **B**: n = 49) to explain (**A**) the differences between unfermented juice and pure yeast fermented samples (Y-data; n = 4) and (**B**) the difference between yeast (Y-data; n = 3) in pure fermentation samples. The variable codes in correlation loadings plots refer to those in the bottom table. Figure reprinted from the original publication **II** with permission from Elsevier.

In order to investigate the key variables contributing to the differences that were influenced by yeast species only, one more PLS-DA model was established (**Figure 18B**). Fermentations with *T. delbrueckii* strains were characterized by a higher amount of higher alcohols compared to those with *S. pombe* strains (**Figure 17**), especially the dominant compounds of 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol, which together accounted for approximately 90% of total higher alcohols. Remarkable difference in the production of higher alcohols was also observed between the strains in the same species, for example TD291 and SP70572 produced significantly less alcohols than TD70526 and SP3796 did, respectively. In general, fermentations with *S. pombe* strains produced more off-flavor compounds, such as acetoin and acetaldehyde, than those with *T. delbrueckii* and *S. cerevisiae* strains. However, bilberry wine fermented with SC1116 differed from other bilberry wine samples by the higher levels of acetals.

Figure 19 shows the impact of fermentation type (pure, sequential, and simultaneous) on volatile compositions of bilberry wines produced with strains involving *T. delbrueckii* (**Figure 19A**) and *S. pombe* (**Figure 19B**).

In the PLS-DA models, sequential and simultaneous fermentations were characterized by more variables than pure fermentations indicating that combined inoculation of *S. cerevisiae* and non-*Saccharomyces* yeasts intensified aroma intensity and complexity of bilberry wines. However, the intensification correlated positively with the presence of *S. cerevisiae* during *T. delbrueckii* wine fermentation; in contrast, the correlation was negative in *S. pombe* wines. The combination of *S. cerevisiae* and the two studied species of non-*Saccharomyces* contributed to the increase in content of esters compared to the pure inoculation with non-*Saccharomyces* yeast. While simultaneous fermentations of SC1116/TD70526 and SC1116/TD291 significantly decreased the content of higher alcohols and increased the content of esters, respectively (**Figure 17**). In winemaking, co-fermentation of *S. cerevisiae* and *T. delbrueckii* has been used to reduce the concentration of acetaldehyde (section 2.2.3.1). In contrast, the presence of *S. cerevisiae* in sequential fermentation with *T. delbrueckii* strains in bilberry wine making was found to significantly increase the concentration of acetaldehyde compared to the fermentations with pure *T. delbrueckii* strains. The result indicated that fermentation substrate is a key factor to be taken into account when determining fermentation characters of non-*Saccharomyces* yeasts on volatile profile. Sequential and simultaneous fermentations with *S. pombe* and *S. cerevisiae* significantly increased the total content of higher alcohols but decreased the content of ketones (**Figure 17**). Acetoin (code 41) was the key compound separating the pure fermentation with *S. pombe* strains from the corresponding sequential and simultaneous fermentations. Mixed inoculations of *S. cerevisiae* and *S. pombe* strains

significantly reduced the production of this compound. Moreover, *S. pombe* strains in sequential inoculation type favored the production of the volatile compounds with *fruity* odor, particularly esters.

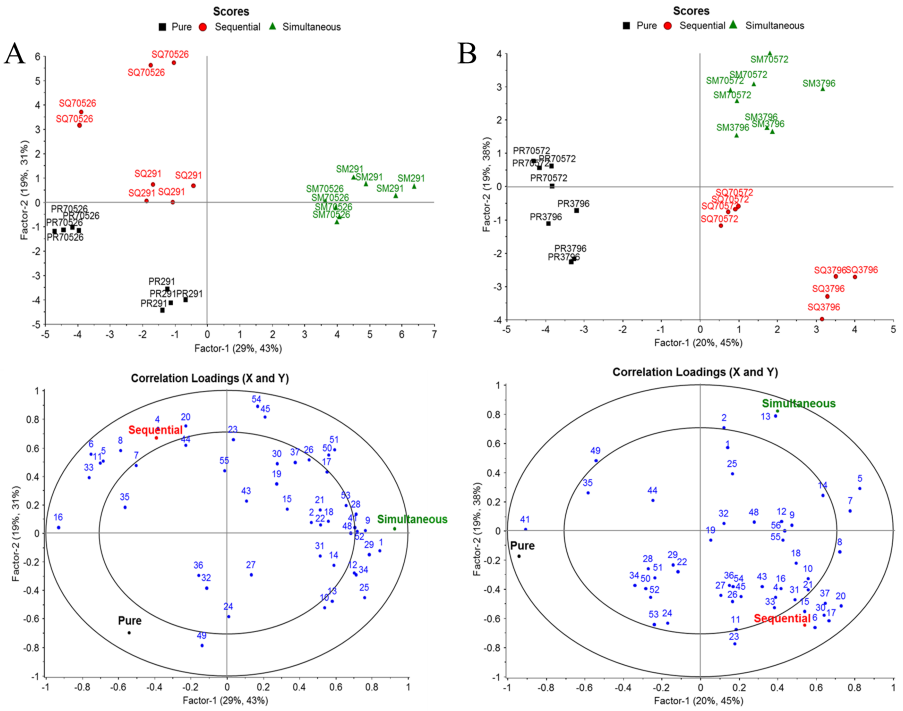


Figure 19. PLS-DA model using contents of volatiles as X-data (n = 49) to explain the differences between three fermentation types (Y-data. N = 3) in **A.** *T. delbrueckii* and **B**. *S. pombe* samples. The variable codes of volatile compounds in correlation loading plots refer to those in Figure 18. Figure reprinted from the original publication **II** with permission from Elsevier.

Overall, on the basis of the results in this study, combined inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts reduced concentration of undesirable volatile compounds caused by pure fermentation with non-*Saccharomyces* yeasts while kept their positive features, to some extent. Specifically, sequential and simultaneous inoculations of *S. pombe* strains with *S. cerevisiae* as well as simultaneous fermentation using *T. delbrueckii* strains and *S. cerevisiae* are the optimal strategies. The results evidenced that, similar to winemaking, co-fermentation of *Saccharomyces* and non-*Saccharomyces* yeasts is also an optimal protocol for bilberry wine production.

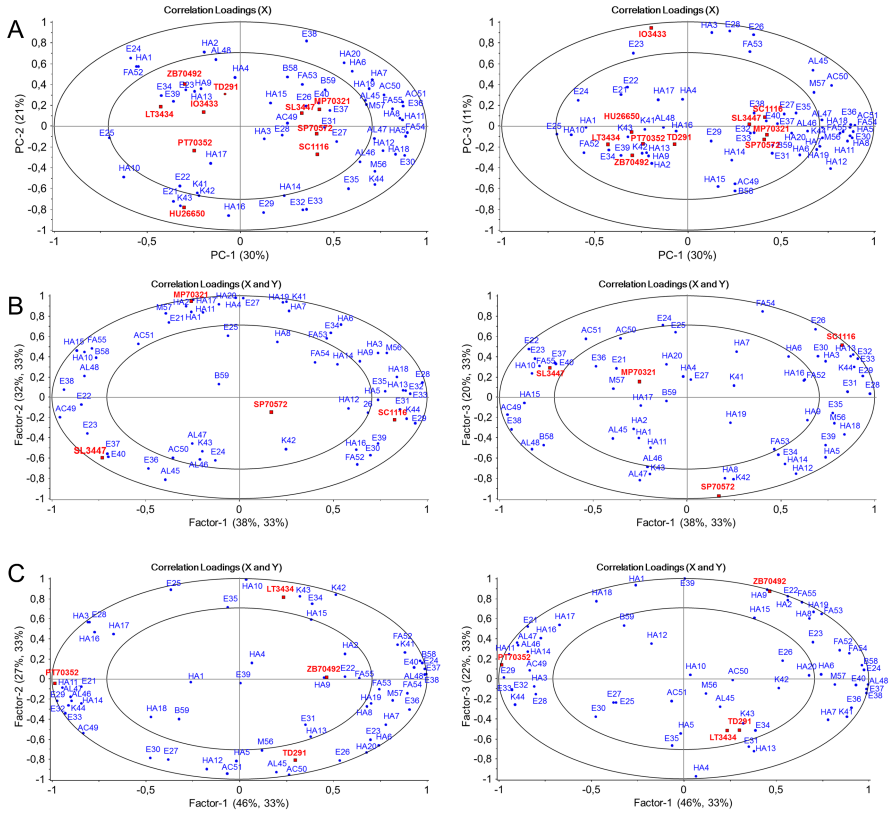
5.4 Dynamic change in volatile compounds during alcoholic fermentation (study IV)

In order to explore the effect of more diverse non-*Saccharomyces* yeasts on volatile compounds in berry wines, nine non-*Saccharomyces* yeasts, which have been employed in winemaking and discussed in section 2.2.3, were used in bilberry wine productions in study **IV**. The strain *S. cerevisiae* V1116 was used as the control. The volatile profiles of the 10 different bilberry wines were characterized and the dynamic change in volatile compounds during fermentation was monitored using HS-SPME–GC−MS.

During the HS-SPME process, an equilibrium is established among liquid sample matrix, gaseous headspace, and the stationary phase of fiber coating. The equilibrium could be expressed as:

$$n = \frac{K_{fs} V_f V_s C_o}{K_{fs} V_f + K_{hs} V_h + V_s}$$

where n is mass of volatile compounds extracted by the fiber coating. K_{fs} and K_{hs} are fiber/sample matrix and headspace/sample matrix partition coefficient, respectively. V_f, V_s, and V_h are fiber coating, sample, and headspace volume, respectively.[338] Therefore, when a HS-SPME method is established, namely an appropriate fiber is selected, the sample and headspace volumes are confirmed, and the HS-SPME conditions are optimized, the amount of a volatile compound adsorbed on the SPME fiber coating is consequently determined by both K_{fs} and K_{hs} and further determined by the chemical composition of sample matrix. Hence, any changes in the sample matrix, such as ionic strength, ethanol concentration, and pH, may affect K_{fs} and K_{hs}.[339] Ethanol is constantly produced and accumulated during bilberry wine fermentation and is the most abundant volatile compound formed. The continuous increase in ethanol concentration during fermentation alters the solubility of analytes in the liquid phase as well as competes for the active sites in the stationary phase with other volatile compounds, which further affects the equilibrium. The impact of ethanol content on the extraction efficiency of other volatile compounds to HS-SPME fiber was demonstrated in this study. Therefore, with the aim to carry out a reliable quantitation of volatile compounds, the quantitative analysis was conducted by a series of calibration curves taking ethanol concentration into account.



HA1	1-propanol	E21	methyl acetate	K41	2,3-butanedione
HA2	2-methyl-1-propanol	E22	ethyl acetate	K42	2,3-pentanedione
HA3	2-pentanol	E23	ethyl propionate	K43	acetoin
HA4	1-butanol	E24	ethyl isobutyrate	K44	6-methyl-5-hepten-2-one
HA5	2-hexanol	E25	isobutyl acetate	AL45	acetaldehyde
HA6	2-methyl-1-butanol	E26	ethyl butanoate	AL46	2-methyl-1-butanal
HA7	3-methyl-1-butanol	E27	ethyl isovalerate	AL47	3-methyl-1-butanal
HA8	isohexanol	E28	isoamyl acetate	AL48	benzaldehyde
HA9	2-heptanol	E29	methyl hexanoate	AC49	1-ethoxy-1-methoxyethane
HA10	3-methyl-2-butanol	E30	ethyl hexanoate	AC50	1,1-diethoxyethane
HA11	3-methyl-1-pentanol	E31	hexyl acetate	AC51	1-(1-ethoxyethoxy)pentane
HA12	1-hexanol	E32	ethyl (*Z*)-3-hexenoate	FA52	isobutanoic acid
HA13	3-ethoxy-1-propanol	E33	ethyl (*E*)-3-hexenoate	FA53	pentanoic acid
HA14	(*Z*)-3-hexen-1-ol	E34	ethyl lactate	FA54	heptanoic acid
HA15	(*E*)-2-hexen-1-ol	E35	methyl 2-hydroxy-3-methylbutanoate	FA55	octanoic acid
HA16	1-heptanol	E36	ethyl octanoate	M56	linalool
HA17	2-ethyl-1-hexanol	E37	ethyl decanoate	M57	α-terpineol
HA18	1-octanol	E38	ethyl 9-decenoate	B58	1,3,5-trimethylbenzene
HA19	methionol	E39	phenethyl acetate	B59	1,3-di-tert-butylbenzene
HA20	2-phenylethanol	E40	ethyl laurate		

Figure 20. PCA and PLS-DA models using contents of volatile compounds (n = 59) to explain the differences between bilberry wines fermented with different yeasts (n = 10 in PCA; n = 4 in PLS-DA). The variable codes in **A**, **B**, and **C** refer to those in the bottom table. Figure reprinted from the original publication **IV** with permission from American Chemical Society.

Multivariate analyses, including PCA and PLS-DA models, were established to investigate the impact of yeasts on the aroma differentiation of bilberry wines (**Figure 20**). The intensities of volatile compounds in the bilberry wines produced with SC1116, SP70572, SL3447, and MP70321 (group 1) were more intense than those with HU26650, IO3433, PT70352, LT3434, ZB70492, and TD291 (group 2), indicating that the overall aroma complexity of the samples in group 1 was higher than those in group 2 (**Figure 20A**). HU26650 and IO3433 were the only two strains producing ethyl acetate higher than 150 mg/L endowing an off-flavor odor to bilberry wines. The excessive high production of ethyl acetate from inoculation with these two stains has also reported in winemaking.[192,222] Fermentations with these two strains were also characterized by increased production of other unpleasant compounds, such as 2,3-butanedione (K41), 2,3-pentanedione (K42), and acetoin (K43) in HU26650 sample; and 2-pentanol (HA3) and pentanoic acid (FA53) in IO3433 sample (**Figure 20A**). Bilberry wines produced with SL3447, MP70321, and SP70572 differed from the control wine fermented with SC1116 by higher content of esters (particular ethyl acetate), higher alcohols, and undesirable compounds, respectively (**Figure 20B**). The fermentation characteristics of *S. ludwigii*, *M. pulcherrima*, and *S. pombe* in volatile profile in bilberry wine fermentation are similar to that in winemaking.[5,8,159,169,173,183] Significant difference in the concentration of aldehydes, ketones, and acetals was found between the bilberry wines produced with LT3434 and TD291. While fermentation with ZB70492 was generally characterized by the high production of fatty acids (**Figure 20C**), which is a new finding compared to that in winemaking.

Figure 21 shows the evolution of 59 volatile compounds during the 10 different fermentations. The evolution pattern of volatile compounds generally is yeast dependent but also follows certain behaviors. The dynamic changes in higher alcohols and esters during fermentation within the same yeast species displayed similar evolution patterns, namely the concentrations of the compounds in these two groups increased constantly and peaked at the middle or later fermentation stage, indicating these main secondary aroma compounds were accumulated as a result of the metabolism of yeasts. However, the significant reduction in the concentration of some higher alcohols occurred during the later stage of fermentation, partly due to the esterification reaction to yield their corresponding esters; on the other hand, the decline of esters may have resulted from the increasing release of cellular esterases along the fermentation. The evolution patterns of carbonyl compounds (aldehydes and ketones) and acetals were similar as accumulated at the early stage of fermentation followed by a significant decrease. The concentrations of fatty acids usually increased gradually for a certain period and then decreased significantly in all the fermentations with the exception of the fermentations with SP70572 and

MP70321, which displayed a gradual increase throughout the fermentation process. Monoterpenes accumulated gradually during all the 10 fermentations and reached the maximum concentrations at the end of fermentation, while the concentrations of benzenes peaked at the middle or middle-end stage and remained at the high level until the completion of fermentation. Since sequential inoculation with *S. cerevisiae* is one of the usually exploited protocol with the aim to mitigate the accumulation of volatile compounds having potentially negative impact while maintaining the volatiles with positive impact on aroma of alcoholic beverages by non-*Saccharomyces* yeasts. This result provides useful information for determining the optimal time point of *S. cerevisiae* inoculation after a non-*Saccharomyces* yeast to obtain fermented beverages with optimum quality.

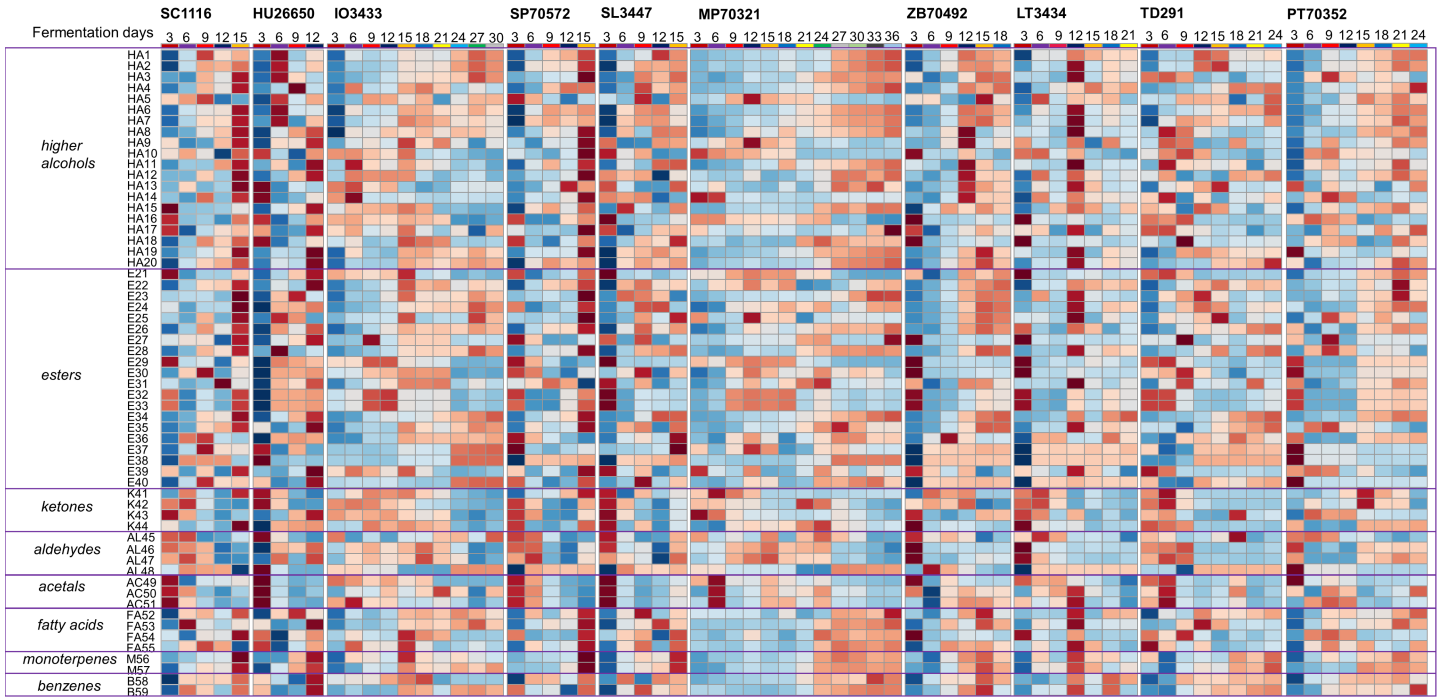


Figure 21. Heatmap visualization of the dynamic change in the concentration (based on normalized concentration) of the detected 59 volatile compounds during bilberry wine productions with 10 different yeasts. Each row on the heatmap represents the normalized concentration of an individual volatile compound (three replicates). Each column represents one fermentation with a particular strain after a particular period. The color scheme from blue to red represents the normalized value from low to high. The variable codes of volatile compounds refer to those in Figure 20. Figure reprinted from the original publication **IV** with permission from American Chemical Society.

5.5 Evolution of monomeric anthocyanins and pyranoanthocyanins in berry wines fermented with *S. pombe* strains during aging (study III)

The high production of pyruvic acid from the inoculation of *S. pombe* strains has been demonstrated in study **I**. Therefore, *S. pombe* strains SP3796 and SP70572 were selected as the distinguishing pyruvic acid producer to transform the natural colorants in bilberry juice to vAPs during the production of bilberry wine in study **III**: the evolutions of anthocyanin monomers and pyranoanthocyanins during one year of aging were monitored in this study.

5.5.1 Monomeric anthocyanins

Total monomeric anthocyanin content decreased significantly during aging, being only 43–47% of initial TMACY remained in 12 months aged bilberry wines (**Figure 22**). A 12–16% reduction in TMACY occurred during the first month of aging, verifying that the instability of anthocyanin monomers. The concentrations of glycosylated delphinidins and cyanidins reduced more than those of glycosylated petunidins, peonidins, and malvidins, indicating that methylation in B-ring stabilized molecular structure of anthocyanins. During the 12 months of aging, bilberry wine produced from fermentation with SC1116 showed the lowest reduction in concentration of total and individual monomeric anthocyanins (data are shown in the Supplementary Table 2 of study **III**), suggesting that metabolism of *S. pombe* strains during fermentation enhanced the transformation of monomeric anthocyanins to other compounds during the aging process.

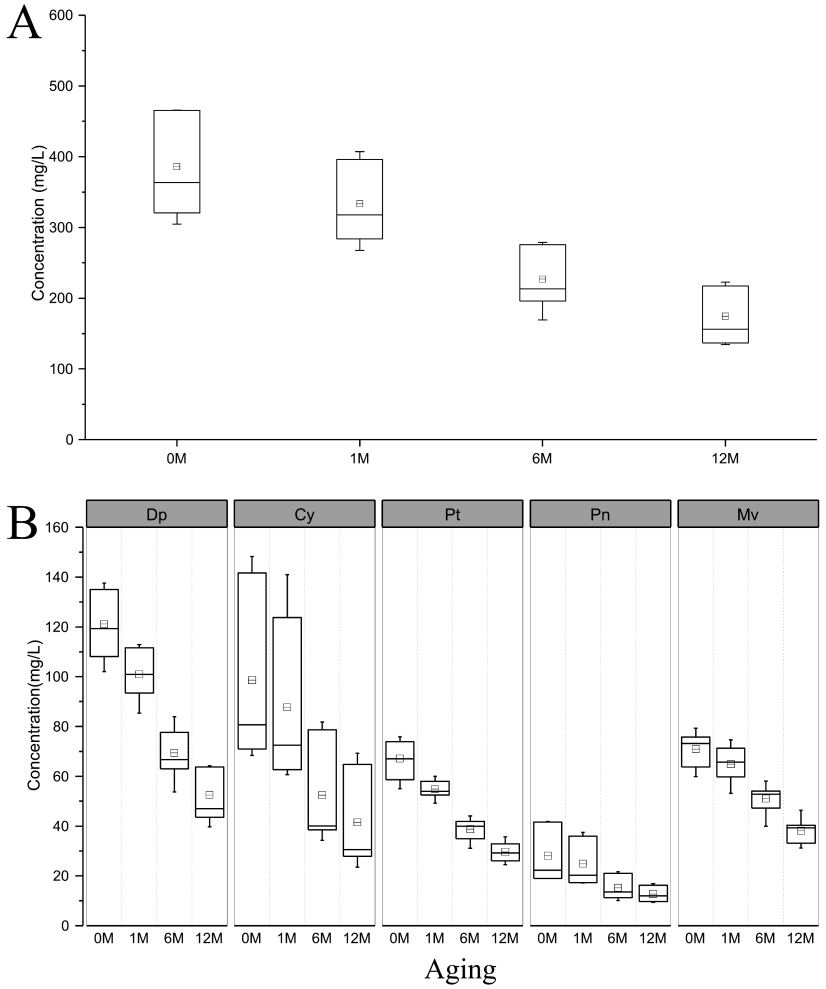


Figure 22. The total concentration of monomeric anthocyanins (**A**) and the distribution of the concentrations of monomeric anthocyanins with delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv) aglycones (**B**) in bilberry wines during different stages of aging. 0M refers to bilberry wine without aging; 1, 6, and 12M refer to 1, 6, and 12 months aged bilberry wines, respectively. Figure reprinted from the original publication **III** with permission from Elsevier.

5.5.2 Pyranoanthocyanins

Because of the improvement of the separation and identification methods of pyranoanthocyanins in study **III**, a total of 15 vitisin A-type pyranoanthocyanins, formed by condensation reaction between pyruvic acid and all the 15 monomeric anthocyanins present in bilberry juice, were determined in bilberry wines

(**Figure 23**). The predomination of malvidin 3-*O*-glucoside and the low level and even absence of common anthocyanins, such as glycosides of delphinidin, peonidin, petunidin, and cyanidin, with common sugar moieties such as galactose and arabinose in *V. vinifera* (section 2.1.2.1) hinder the generation of other vAPs than vitisin A. The detection of 15 vAPs in bilberry wines indicates that cycloaddition with pyruvic acid can occur in all the 15 monomeric anthocyanins.

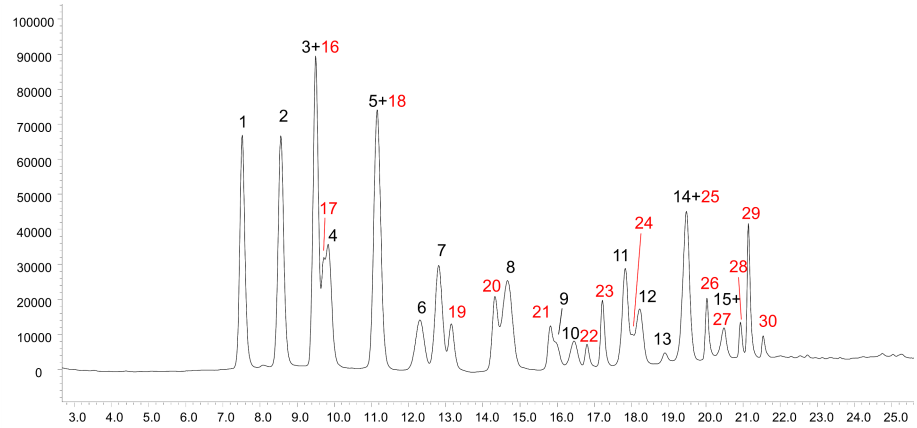


Figure 23. HPLC-DAD chromatograms (detected at 520 nm) of a six months aged bilberry wine fermented by pure *S. pombe* 70752. The peaks 1–15 refer to those in Figure 14. The peaks 16–30 are: 16: delphinidin 3-*O*-galactoside-pyruvic acid, 17: delphinidin 3-*O*-glucoside-pyruvic acid, 18: delphinidin 3-*O*-arabinoside-pyruvic acid, 19: cyanidin 3-*O*-galactoside-pyruvic acid, 20: cyanidin 3-*O*-glucoside-pyruvic acid, 21: cyanidin 3-*O*-arabinoside-pyruvic acid, 22: petunidin 3-*O*-galactoside-pyruvic acid, 23: petunidin 3-*O*-glucoside-pyruvic acid, 24: petunidin 3-*O*-arabinoside-pyruvic acid, 25: peonidin 3-*O*-galactoside-pyruvic acid, 26: peonidin 3-*O*-glucoside-pyruvic acid, 27: peonidin 3-*O*-arabinoside-pyruvic acid, 28: malvidin 3-*O*-galactoside-pyruvic acid, 29: malvidin 3-*O*-glucoside-pyruvic acid, 30: malvidin 3-*O*-arabinoside-pyruvic acid. Figure reprinted from the original publication **III** with permission from Elsevier.

Among the fresh bilberry wines fermented with three different yeasts using three diverse inoculation approaches, the bilberry wines produced from fermentations involving *S. pombe* strains, particularly the strain *S. pombe* 70572, promoted the production of vAPs. The presence of *S. cerevisiae* 1116 in sequential and simultaneous fermentations with *S. pombe* 70572 significantly reduced the generation of vAPs, whereas the concentrations of total vAPs in

these samples were still higher than that in the sample produced with pure fermentation using *S. cerevisiae* (**Figure 24**).

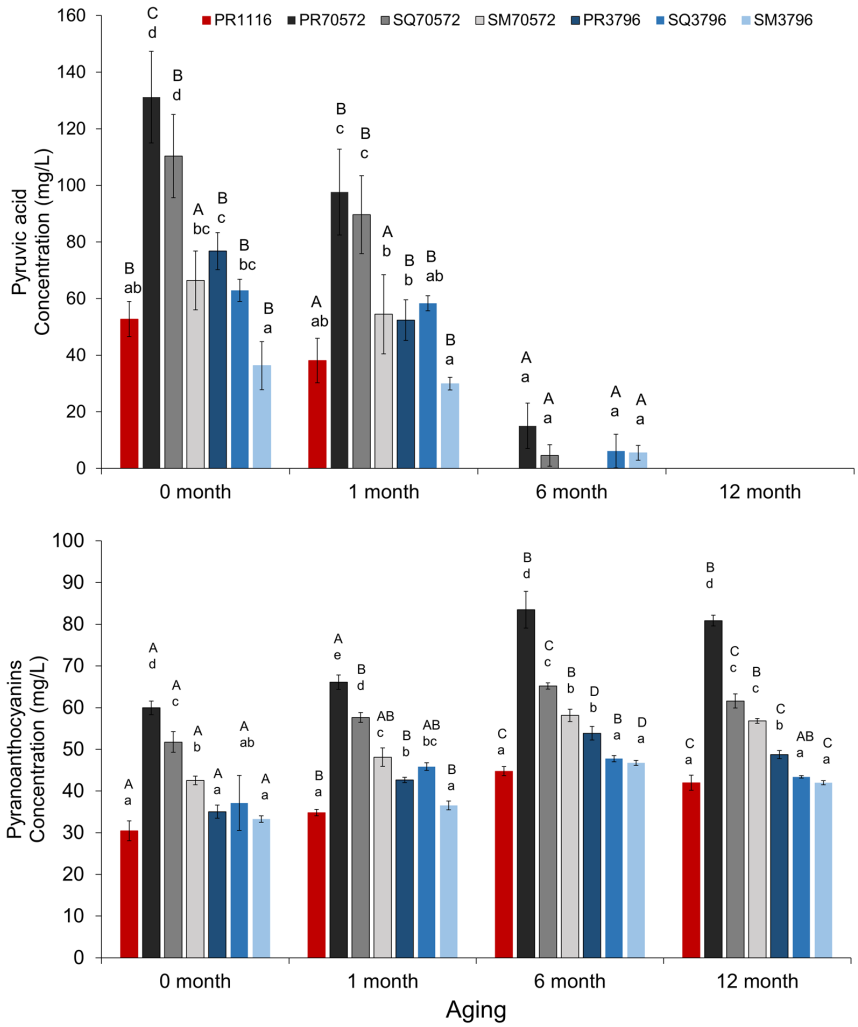


Figure 24. Concentrations of total content of vitisin A-type pyranoanthocyanins in bilberry wines produced by pure (PR), sequential (SQ), and simultaneous (SM) fermentations with *S. pombe* and *S. cerevisiae* during aging. Different uppercase letters in the same color bars represent significant differences ($p < 0.05$) in contents in the same bilberry wine after different aging periods. Different lowercase letters in the same subarea (within each aging time) indicate significant differences ($p < 0.05$) in contents in bilberry wines produced with different fermentation methods at the same aging period. Figure reprinted from the original publication **III** with permission from Elsevier.

The contents of vAPs in bilberry wines increased gradually and peaked after six months of aging, reaching levels being 1.3–1.5 times higher than those in their corresponding non-aged samples. The concentrations of most individual vAPs showed continuous increase during the first six months of aging. The highest level of vAPs was detected in the bilberry wine PR70572 aged for six months. The contents of vAPs derived from galactosides and arabinosides of anthocyanidins were generally lower than of those from anthocyanidin glucosides.

Although the content of total vAPs declined during the latter six months of aging, the reduction (2.2–10.2%) was significantly lower than that observed in monomeric anthocyanins (20.5–25.7%) during this period. Methylation in B-ring stabilized the structure of pyranoanthocyanins as indicated by the higher reduction in concentrations of vAPs formed from anthocyanins with non-methylated aglycones (delphinidin and cyanidin) than those from anthocyanins with methylated aglycones (peonidin, petunidin, and malvidin). In the same aging period, the reduction in content of vAPs with sugar moieties of galactose and arabinose was usually lower than those with glucose, suggesting that sugar moiety impacted on the stability of vAPs.

The generation of vAPs during the first six months of aging was related to the degeneration of their precursors of pyruvic acid ($R = -0.728$) and monomeric anthocyanins ($R = -0.624$) *via* correlation analysis. The stronger relationship between vAPs and pyruvic acid than that of vAPs and monomeric anthocyanins indicated that, during aging, the reduction of pyruvic acid was mainly due to the formation vAPs, but the decrease in anthocyanin monomers was due to cycloaddition reactions on the one hand, and to other concomitant reactions, such as oxidation on monomeric anthocyanins, on the other hand. Moreover, the negative correlations between vAPs with methylated B-rings and their corresponding anthocyanin monomers were stronger than those with non-methylated B-rings. We speculate that the stabilization effect of B-ring methylation on monomeric anthocyanins reducing the oxidative degradation of these compounds during aging. Therefore, more anthocyanin monomers were left for the vAPs formation.

6 SUMMARY AND CONCLUSION

The chemical compositions of juices and wines produced from blue (BB) and white bilberries (WB) were profiled *via* gas chromatography and liquid chromatography analytical methods. The dynamic changes in volatile compounds and anthocyanin-related compounds were analyzed during the processes of fermentation and aging of fermentation products, respectively. Special focus was set on the effect of non-*Saccharomyces* yeasts on the chemical composition of bilberry wines in comparison to the conventional *Saccharomyces cerevisiae*.

The profiles of phenolic compounds, including anthocyanins, phenolic acids, flavonols, and flavan-3-ols, in BB juice and wine, were significantly different from those of corresponding products prepared from WB. Alcoholic fermentation significantly affected the chemical composition of BB juices, and the effects were yeast and inoculation type dependent. Ethanol accumulation during fermentation influenced the quantification of other volatile compounds as well as the monitoring of the dynamic changes in the composition and content of these compounds. During fermentation and aging, the generation of pyranoanthocyanins is closely related to the degeneration of their precursors of monomeric anthocyanins and pyruvic acid. The high production of pyruvic acid from the fermentations involving *Schizosaccharomyces pombe* boosted the synthesis of pyranoanthocyanins.

This research brings novel scientific knowledge on the fermentation of bilberry wines using non-*Saccharomyces* yeasts, also providing new technological information to berry processing companies and berry wine practitioners for the utilization of normal pigmented bilberry and its nonpigmented mutant to produce value-added products. The study on the metabolite profiles of bilberry juices and wines provide new insights and breakthroughs in chemistry and biochemistry of berry wines.

So far, low content of sugars and high acidity has partly hampered the development of berry wine industry. It is difficult to produce berry wines with acceptable quality without any modification of berry materials as fermentation substrates. Moreover, the lack of suitable fermentation techniques is another challenge in berry wine production. Non-*Saccharomyces* yeasts have been applied in the wine industry for improving the quality traits and complexity of wines. In this research, we used bilberry as an example of nongrape berries, to study the impact of fermentation with non-*Saccharomyces* yeasts on compositional profiles of berry wines. This research facilitates a better understanding of the potential of the non-conventional species/strains in berry wine production and their influences on berry wine quality. In view of this in-

depth study, bilberry wine can represent a model system to investigate these variables in other berry materials.

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刘树勋

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APPENDIX: ORIGINAL PUBLICATIONS

I. Reprinted from *Food Chemistry* **2018**, 266, 262–274, with permission from Elsevier Ltd.

II. Reprinted from *Food Microbiology* **2019**, 80, 25–39, with permission from Elsevier Ltd.

III. Reprinted from *Food Chemistry* **2020**, 305, 125438, with permission from Elsevier Ltd.

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DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.
44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.
45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.
55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.
56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.
57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.
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59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.
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