BIODIVERSITY OF YEASTS OF LOCAL WINEGRAPES IN TÜRKİYE AND GENOTYPING OF *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

BIODIVERSITY OF YEASTS OF LOCAL WINEGRAPES IN TÜRKİYE AND GENOTYPING OF *SACCHAROMYCES CEREVISIAE*

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Grape must and wine harbors a wide diversity of yeast species responsible for spontaneous alcoholic fermentation and wine aroma. Therefore, the impact of indigenous species and terroir effect on aromatic properties of traditional Turkish wine made from Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit grape varieties collected from three different regions (Ankara, Elazığ and Cappadocia) were investigated, using gas chromatography-mass spectrometry (GC-MS). A total of 56 compounds were identified and quantified in wines from four red *Vitis vinifera* grape varieties grown in Türkiye. Among the quantitated volatile compounds, different volatiles were found to significantly contribute their flavor notes to the overall aroma of Turkish wines. The native non-*Saccharomyces* and *Saccharomyces* yeast diversity in these traditional wine samples made from four red and one white grape varieties at different stages were detected by using sixteen specific oligonucleotide primers in real-time polymerase chain reaction (PCR) analysis. *Saccharomyces cerevisiae* and three predominant non-*Saccharomyces* yeasts *Hanseniaspora uvarum*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* were also quantified by quantitative real-time PCR methods. Biodiversity determined with the use of real-time PCR analysis were compared with DNA sequencing result of internal transcribed spacer

(ITS) region (ITS1–5.8S rRNA– ITS2) and/or D1/D2 domain of the 26S rRNA gene of the isolates. Moreover, the random amplified polymorphic DNA (RAPD) PCR using one Operon primer (OPA-11) and the mini and microsatellite primed (MSP)-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(ATG)_5$ were also applied to investigate the intraspecific genetic diversity between the 46 autochthonous *S. cerevisiae* strains isolated from five different must and wine at different stages. The result indicated that RAPD-PCR and MSP-PCR technique were applicable to the determination of intraspecific genetic diversity between the 46 indigenous *S. cerevisiae* strains. In addition, karyotyping analyses were carried out for selected *S. cerevisiae* strains by pulsed-field gel electrophoresis (PFGE) method which distinguished ten different chromosomal patterns between *S. cerevisiae* strains.

Keywords: *Saccharomyces cerevisiae*, GC-MS, real-time PCR, RAPD/MSP-PCR, PFGE.

ÖZ

TÜRKİYE'DEKİ YEREL ÜZÜM MAYALARININ BİYOÇEŞİTLİLİĞİ VE *SACHAROMYCES CEREVISIAE*'NİN GENOTİPLENMESİ

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Üzüm şırası ve şarap, kendiliğinden alkol fermentasyonu ve şarap aromasından sorumlu çok çeşitli maya türü barındırır. Yerli türlerin Türkiye'nin üç farklı bölgesinden toplanan Kalecik Karası, Boğazkere, Öküzgözü ve Dimrit üzümlerinden yapılan geleneksel şarabın aromatik özellikleri üzerindeki etkisi, gaz kromatografisi-kütle spektrometresi (GC-MS) kullanılarak araştırılmıştır. Türkiye'de yetişen dört *Vitis vinifera* üzüm çeşidinden elde edilen şaraplarda toplam 56 bileşik miktarları tespit edilmiştir. Kantitasyonu yapılan uçucu bileşikler arasında, farklı uçucuların Türkiye'deki şarapların genel aromasına tat notalarına önemli ölçüde katkıda bulunduğu bulunmuştur. Dört kırmızı ve bir de beyaz üzüm çeşidinden yapılan bu geleneksel şarapların, farklı üretim aşamalarında alınan numunelerinde, doğal non-*Saccharomyces* ve *Saccahromyces* maya çeşitliliği 16 spesifik oligonkleotit primer kullanımı ile tespit edilmiştir. *Saccharomyces cerevisiae* ve üç baskın non-*Saccharomyces* maya, *Hanseniaspora uvarum*, *Lachancea termotolerans* ve *Torulaspora delbrueckii* için qPCR miktar tayini yöntemi kullanılarak miktarlar belirlenmiştir. Gerçek zamanlı PCR yöntemi kullanılarak belirlenen biyoçeşitlilik

DNA dizileme sonucu ile karşılaştırılmıştır. Bir Operon primeri (OPA-11) kullanılarak rastgele amplifiye edilmiş polimorfik DNA (RAPD) PCR ve M13, $(GTG)_5$, $(ATG)_5$ primerleri kullanılarak mıkrosatellit primer kullanımlı (MSP)-PCR parmak izi tekniği ile beş farklı üzüm şırası ve şaraptan farklı aşamalarda izole edilen 46 *S. cerevisiae* suşu arasında ki intraspesifik genetik çeşitlilik araştırılmıştır. Bu çalışmanın sonuçları, her iki tekniğin de 46 yerli *S. cerevisiae* suşu arasında, tür içi genetik çeşitliliğin tanımlanmasında etkili ve uygulanabilir olduğunu göstermiştir. Ayrıca seçilmiş *S. cerevisiae* suşları için PFGE yöntemi ile karyotipleme analizleri yapılmıştır. *S. cerevisiae* suşlarında farklı kromozom modelleri ayırt edilmiştir.

Anahtar Kelimeler: *Saccharomyces cerevisiae*, GC-MS, Gerçek zamanlı PCR, RAPD/MSP-PCR, PFGE.

To my mother and sisters, who always support me in all aspects of my life

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LIST OF ABBREVIATIONS

K: Kalecik Karası grape variety

B: Boğazkere grape variety

O: Öküzgözü grape variety

D: Dimrit grape variety

E: Emir grape variety

CM: Cold maceration

NM: Maceration

YPD: Yeast extract peptone dextrose

PCR: Polymerase chain reaction

qPCR: Quantitative real-time polymerase chain reaction

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

PBS: Phosphate buffered saline

Ct: Cycle threshold

NTC: No-template control

E: Efficiency

GC-MS: Gas chromatography-mass spectrometry

MS: Mass spectrometry

FID: Flame ionization detector

OAV: Odor activity values

PCA: Principal component analysis

RAPD-PCR: Random amplified polymorphic DNA polymerase chain reaction

MSP-PCR: Mini and microsatellite primed polymerase chain

PFGE: Pulsed-field gel electrophoresis

CHEF: Contour-clamped homogenous electric field

UPGMA: Unweighted pair group method with arithmetical average

NTSYS: Numerical taxonomy system of multivariate program

kb: Kilobase pairs

bp: Base pair

M: Mega base pairs

β-ME: β-mercaptoethanol

EDTA: Ethylenediaminetetraacetic acid

Tris base: Tris (hydroxymethyl) aminomethane

TBE: Tris borate EDTA

LMP: Low-melting-point

CHAPTER 1

INTRODUCTION

1.1 History of Winemaking and Grapevines

The evidence of winemaking was found in the Neolithic era by biomolecular archaeologists (McGovern et al., 2017). Chemical analyses of ancient organic compounds absorbed into the pottery fabrics from sites in Georgia in the South Caucasus region provided the earliest biomolecular evidence for grape wine and viniculture from the Near East, at ca. 6000–5000 BC (McGovern et al., 2017). It was seen that wine production, which was of great importance in the history of Mesopotamia, had become a different situation with the understanding of boutique winemaking especially since the early 2000s.

The genus of *Vitis* (grapevine) which contained 80 species is classified into two subgenera; *Muscadinia* and *Euvitis* (OIV, 2017). The *Muscadinia* subgenus is comprised of three species, including *Vitis rotundifolia* Michx. var. *popenoei* (Central America), *Vitis rotundifolia* Michx. var. *munsoniana* (Florida), and *Vitis rotundifolia* Michx. var. *rotundifolia* (southeast USA).

Most cultivated grapevines belong to the subgenus *Euvitis* which including three groups as following: The first group is the American group comprised of more than 20 species, such as *V. riparia*, *V. berlandieri*, and *V. rupestris*. The second group is East Asia group consisted of around 55 species, which are currently considered to be of limited importance to viticulture. The third one is the Eurasian group included one single species, *Vitis vinifera* L., which accounts for most of the world's *Vitis* varieties. *Vitis vinifera* is also made up two subspecies: the first one is *sylvestris*, which relating

to the wild form of the vine, and the second subspecies is *vinifera*, referring to the cultivated form (OIV, 2017).

The spread of *Vitis vinifera* varieties of grapevine and winemaking which originated from Asia and distributed to other parts of the world were depicted in Figure 1-1 (P. J. Chambers & Pretorius, 2010).

Figure 1-1 History of *Vitis vinifera* varieties of grapevine and winemaking from Asia (origin) to other parts of the world (P. J. Chambers & Pretorius, 2010).

According to the usage area, grapes are classified into three types as fresh grapes or table grapes, dried grapes and wine grapes. In the world, 10000 grapevine varieties are known from which 13 varieties such as Kyoho, Syrah, Cabernet Sauvignon, Merlot, Tempranillo, Red Globe, Garnacha Tinta (Grenache Noir), and Pinot Noir (Blauer Burgunder) which were black colored varieties and Sultanina, Airen, Chardonnay, Sauvignon Blanc and Trebbiano Toscano which are white varieties covered more than one-third of the world's vineyard area (OIV, 2017).

Türkiye has one of the five biggest grape production area in the world with different national grape varieties (OIV, 2012). However, the ratio of grape used for Turkish winemaking is 5% (Levent & Demir, 2020). The remainder produced in Türkiye is used in the manufacture of must, molasses (pekmez), solid molasse (Bulama), dried berry pulp (Pestil or Bastık), raisin and other products (Uysal et al., 2021). The main viticulture regions in Türkiye are Marmara and Thrace, Aegean, Central Anatolia, South East and East Anatolia. The main red and white grape varieties of Türkiye also summarized in Table 1-1.

Table 1-1 The main red and white grape varieties, their origins and provinces in Türkiye

Regions	**Provinces**	**Red grapes**	**White grapes**
Southeast and East Anatolia	Diyarbakır Elazığ Malatya	Boğazkere Öküzgözü Horozkarası Sergikarası	Dökülgen Kabarcık
Central Anatolia	Ankara Amasya Nevşehir Tokat	Kalecik Karası Ankara Siyahı Dimrit Papazkarası	Kalecik Beyazı Hasandede Beyazı Kabarcık Narince Emir
Aegean	Aydın Denizli İzmir Manisa	Foça Karası Çalkarası	Muscat of Bornova Sultaniye Beylerce
Marmara and Thrace	Bursa Çanakkale Edirne Kırklareli Tekirdağ	Papazkarası Adakarası Karasakız Karalahna	Yapıncak Beylerce Vasilaki

According to the Gumus and Gumus survey (2008), the most widely used Turkish grape varieties to produce wine were Boğazkere, Öküzgözü, Kalecik Karası, and Çalkarası for red winemaking and Sultaniye, Emir, and Narince for white winemaking (Gumus & Gumus, 2008).

Wine has traditionally been produced by natural fermentation caused by the growth of yeasts derived from the grapes. Winemaking or vinification, starts with the selection of grapes, continues with the processing and the fermentation and ends with bottling of the finished wine (Philipp et al., 2021).

Wine production is traditionally done with naturally transmitted microorganisms, and the type, components and microorganisms in traditional wine production vary according to the type of grape varieties possessing different chemical components (Benito et al., 2019). For example, Boğazkere grapes contain a high amount of tannins, while Öküzgözü grapes are softer as a result of less tannins (therefore, it is often preferred to use in the form of couplings). Moreover, in conventional production, culture does not consist of a single microorganism and different species involved in different stages of the fermentation process affected the alcohol level, hardness and aroma of traditional wine (Bagheri et al., 2016). The transformation of grape must into wine is a complex microbiological process involving yeasts and lactic acid bacteria, though only yeasts of the genus *Saccharomyces* (principally *Saccharomyces cerevisiae*) are responsible for alcoholic fermentation. As mentioned above, wine has traditionally been produced by natural fermentation caused by the growth of yeasts derived from the grapes. The composition of the microflora on the surface of the grape is affected by a variety of factors, including temperature, rainfall, and other climatic variables, the ripeness of the crop, the use of fungicides, physical damage caused by fungi, or insects and the grape variety (Chen et al., 2022; Zhu et al., 2016). Although *S. cerevisiae* is only found at low levels on grapes, it multiplies rapidly and displaces other microorganisms present in the grape must. As a result of its ability to tolerate high concentrations of alcohol and to thrive at higher temperatures than other yeasts, *S. cerevisiae* comes to dominate the fermentation environment (Castillo et al., 2020). Although *S. cerevisiae* is the most common

species in wine fermentations and has been the subject of most of the studies performed to date, other species belonging to the *Saccharomyces sensu stricto* complex, due to their phylogenetic proximity to *S. cerevisiae*, may also be present during alcoholic fermentation and even become the predominant species. For instance, *S. bayanus* predominates in wines from regions with a continental climate and *S. paradoxus* has been reported to predominate in Croatian wines (Cocolin et al., 2004; Redžepović et al., 2002). Furthermore, although *Saccharomyces* species form the majority of the flora resident in the winery, non-*Saccharomyces* yeast species of the genera *Metschnikowia*, *Lachancea*, *Torulaspora*, *Candida*, *Hanseniaspora*, *Dekkera*, *Pichia*, *Schizosaccharomyce*s and *Zygosaccharomyces* have also been isolated in the winery environment and in finished wines (Gschaedler, 2017; Y. Zhao et al., 2021).

In addition, *S. cerevisiae* is predominant in the advanced stages of fermentation in wines produced by the traditional method because of its alcohol resistance. *S. cerevisiae* strains are different in terms of fermentation performance and play an important role in the final flavor and quality of wine (Cocolin et al., 2004; Kontogiannatos et al., 2021). On the other hand, in recent years there is an increasing trend for using autochthonous cultures since commercial cultures would not provide desirable characteristics such as flavor complexity which is present in spontaneously fermented wines and the wines produced by commercial cultures are too standardized and ordinary (Bagheri et al., 2018; Philipp et al., 2021).

1.2 Wine Flavor and Aroma

Flavor, the most important characteristic of wine, is the overall interaction of chemical components with the sense of smell and taste (sensory perception) which result in the alcoholic strength, fizziness, acidity, sourness, bitterness, sweetness and astringency of wine (Lambrechts & Pretorius, 2019; Rapp & Mandery, 1986). Whereas flavor referred to the effects of both odor (volatile compounds) and taste (nonvolatile), aroma is only associated with volatile compounds (odor), while the

wine bouquet depend on the more complex flavor bouquet which produce during fermentation and ageing process (Rapp & Mandery, 1986).

The wine aroma was composed of more than 1000 aroma constituents (Zhu et al., 2016). The diversity of aromatic compounds in wine was enormous and varied in concentration from mg/L to ng/L (González-Barreiro et al., 2015; Zhu et al., 2016).

Wine flavor were divided into four classes as following;

The first one was the varietal or primary aroma which depended on the grape variety.

The second one was pre-fermentative aroma which formed during grape processing like extraction of must.

The third one was the fermentative aroma that made by various yeast and bacteria species during alcoholic and malolactic fermentations.

The last one was post-fermentative aroma which was caused by the transformations that occurred during the wine aging process via physicochemical and enzymatic actions in the bottle or in wood (Lambrechts & Pretorius, 2019; Rapp & Mandery, 1986; Zhu et al., 2016).

The main families of aroma compounds were terpenoids (monoterpenoids, sesquiterpenoids, and C13 norisoprenoids), proanthocyanidins (tannins), organic acids and different precursors of aromatic esters, aldehydes, and thiols (González-Barreiro et al., 2015; Perestrelo et al., 2019; Rapp & Mandery, 1986).

The origin, odor description and properties of the chemical families of aroma compounds in wines were listed in Table 1-2 as adapted from González-Barreiro et al. (2015).

Table 1-2 The origin, odor and properties of the chemical groups of aroma compounds in wines

Volatile classes	Subtypes	Origin and aroma descriptions
Pyrazines		- Originate in grapes - Vegetal characters: bell pepper, chili, bean, carrot, potato, peanut, roasted barley
Terpenes - Formed from isoprene units - Monoterpenes (C10) and higher terpenes (>C10)	Monoterpenes: - Hydrocarbons - Alcohols - Aldehydes - Ketones - Esters: free or bound (as glycosides)	- Generally, originate in grapes - Can be produced by some yeasts and molds (but not *Saccharomyces*) - Only free terpenes can be detected sensorially - Fruity/floral aromas
	Higher terpenes (includes naphthalene derivatives)	- Originate in the plants - Fruity and fuel-like characters
Shikimic acid derivatives		- Produced by aromatic amino acid metabolism - Originate in the plants, microbes, and oak barrels
Lactones	Oxygen-containing 5- or 6-member cyclic compounds	- Originate in grapes, microbes, and oak barrels - Typical characters: candy floss, generic sweet stuff, generic fruit, coconut, butter
Esters	- Alcohol: ethanol or alcohol from degradation of amino acids, purine, and pyrimidine - Acid: acetic acid or acid from degradation of amino acids or biosynthesis of fatty acids	In general: - Short chain: fruity, floral - Long chain: perfume, soap - Lower concentrations: fruity, floral - Higher concentrations: perfume Specific examples: - Ethyl acetate: nail polish remover - Ethyl laurate: soap - Isoamyl acetate: banana - Phenethyl acetate: rose oil
Higher alcohols (fusel oils)	From amino acid degradation or biosynthesis	- Made mostly by microbes, can be made by plants
Acids		- From the plant or microbes - Sourness - Other characters: rancid (butyric acid) or pungent (acetic acid)
Phenolic compounds	Flavonoids and non-flavonoids	- Produced by plant -Can be converted into vinyl phenols by microbes (spoilage characters) -Bitterness, astringency

Table 1-2 Continued

Sulfur-containing compounds	Sulfides	- Hydrogen sulfide: rotten egg - Dimethyl sulfide: cabbage, canned corn - Dimethyl disulfide: clam
	Thiols	- Methanethiol: rubber - Ethanethiol: onion, rubber, skunk
	Sulfoxides	- Dimethyl sulfoxide: plastic, rubber hose
	Thio alcohols	- Mercaptoethanol: barnyard - Thiomethylbutanol: garlic, chive - Methionol: raw potato, soy

Terpenes categorized as varietal compounds were widely studied in *Vitis vinifera* grapes (Arcari et al., 2017; Callejon et al., 2010; Duan et al., 2018; Perestrelo et al., 2019; X.-C. Wang et al., 2017) and could be occurred as alcohols, ketones, aldehydes, hydrocarbons, and esters (Table 1-2). These compounds were mainly responsible for floral and fruity (citric) aromas, although some possessed resin-like aroma (p-cimene, α-terpinene, farnesol and myrcene).

C13-Norisoprenoids were a volatile class obtained from grape carotenoids and chemically possessed two major forms megastigmane which consist of β-damascenone, 3-oxo-α-ionol, β-ionone, or 3-hydroxy-β-damascone and non-megastigmane such as (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene and 1,1,6-trimethyl-1,2-dihydronaphthalene (González-Barreiro et al., 2015; Rapp & Mandery, 1986). Even though these aroma compounds were only found at trace quantity, most norisoprenoids had a very low sensory thresholds such as 200 ng/L for *β*-damascenone and 700 ng/L for β-ionone. Therefore, these compounds were contributed to the overall aroma of different wine varieties like Chenin Blanc, Sauvignon Blanc, Cabernet Sauvignon, Chardonnay, Semillon, and Syrah (Lambrechts & Pretorius, 2019; Rapp & Mandery, 1986).

Methoxypyrazines were an aroma class detected in grape and wine that produced through amino acid metabolism and contributed to the vegetal characteristics of wine like 2-*sec*-butyl-3-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine

(IBMP), and 3-isopropyl-2-methoxypyrazine (IPMP) (González-Barreiro et al., 2015).

Sulfur-containing compounds were another aroma group found in wine that subdivided into sulfides, thiols, sulfoxides, and thio alcohols (González-Barreiro et al., 2015; Lambrechts & Pretorius, 2019). The most important sulfur-containing compounds in wine was hydrogen sulfide with rotten egg aroma. As mentioned in Table 1-2, the other sulfur-containing compounds were dimethyl disulfide (clam aroma), dimethyl sulfide (cabbage, canned corn aroma), ethanethiol (onion, rubber aroma), methanethiol (rubber aroma), dimethyl sulfoxide (plastic, rubber hose aroma), mercaptoethanol (barnyard aroma), thiomethylbutanol (garlic, chive aroma) and methionol (raw potato, soy aroma).

Lactones with candy floss, generic fruit, coconut, butter flavor, **acids** with sourness aroma and **phenolic compounds** (flavonoids and non-flavonoids) with bitterness, astringency flavor were also the other aroma classes found in wine (Table 1-2).

One of the possible reasons for the protective effect of wine in cardiovascular diseases was related to the high content of polyphenols (mainly flavonoids, resveratrol), which have significant antioxidant activity (Di Lorenzo et al., 2017).

1.3 Yeasts Present in Wine and Their Influence on Wine Aroma Compounds

The yeasts association with human society was begun many years ago. The fermented foods productions like leavened bread, wine and beer were an old practice that have been applied for many years without any microbiology knowledge. Evidence to produce fermented beverages by yeasts, specially *S. cerevisiae*, returns back as far as 6000 before Christ, that was recorded by some archaeological sites in Egypt, China, Türkiye and Iran (P. J. Chambers & Pretorius, 2010). However, the intended addition and commercial usage of yeasts initiated only at the end of the 19th century after isolation and identification by Louis Pasteur in 1860.

Yeasts (kingdom Fungi) belong to a group of eukaryotic unicellular microorganisms and act in nature as degraders of organic macromolecules and saprotrophs with the sexual state (teleomorph) and the asexual state (anamorph) (Tofalo & Suzzi, 2016). The yeast associated in winemaking can be divided into two groups, non-*Saccharomyces* and *Saccharomyces* yeasts.

The composition of the wine regarding the volatile products of alcoholic fermentation depended not only on fermentation conditions but also on the inoculated or indigenous yeast strain or species. The major part of the wine aroma produced during fermentation carried out by various yeast strains or species (Gschaedler, 2017; Philipp et al., 2021; Rapp & Mandery, 1986). Spontaneous grape must fermentation was naturally initiated by indigenous non-*Saccharomyces* yeast species of the genera *Metschnikowia*, *Lachancea*, *Torulaspora*, *Candida*, *Hanseniaspora*, *Dekkera*, *Pichia*, *Schizosaccharomyce*s and *Zygosaccharomyces* (such as species of *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Candida zemplinina*, and *Hanseniaspora uvarum*) which also known as low fermentation yeasts (Gschaedler, 2017). After passing two or three days, species of *Saccharomyces*, particularly *S. cerevisiae* which were high-fermentation species, grew and dominated fermentation due to their resistance to the high volumes of ethanol and the biodiversity of indigenous non-*Saccharomyces* yeast species decreased because of the toxic effect of the ethanol and the temperature effect (P. J. Chambers & Pretorius, 2010). Though non-*Saccharomyces* yeast species only develop during the first few days of fermentation, these yeasts were able to produce diverse aroma compounds with higher concentrations that significantly affected the final wine quality (S. Benito, 2018a; Gschaedler, 2017; Morata et al., 2019; Padilla et al., 2016; Vicente et al., 2020). In addition, indigenous non-*Saccharomyces* yeasts can influence both the primary and secondary aroma through the production of enzymes and metabolites, respectively (Padilla et al., 2016). During fermentation, many compounds were also made and metabolized by yeast species contributing to the final flavor of wine. Figure 1-2 represented the aroma

compounds formation by wine yeast (Lambrechts & Pretorius, 2019; Swiegers et al., 2005).

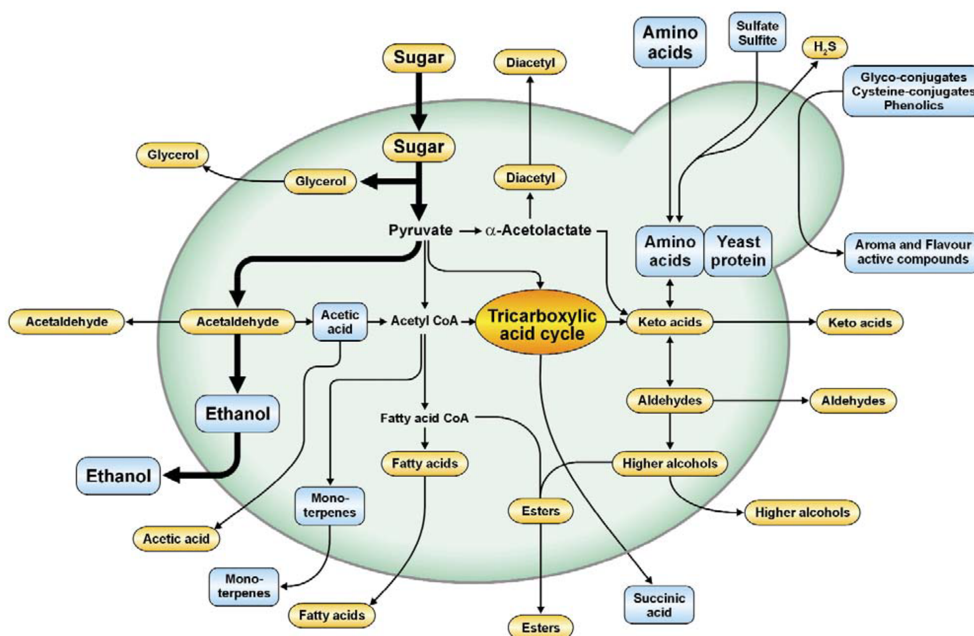


Figure 1-2 Demonstration of the aroma compounds formation by wine yeast (Swiegers et al., 2005).

As depicted in Figure 1-2, the first role of wine yeast was to convert grape sugars to ethanol, carbon dioxide and other sensorially important metabolites without the off flavors' production. The second role was to modify grape-derived components such as cysteine- and glyco-conjugates in order to increase the wines' varietal character (Swiegers et al., 2005). Alcohols, acids, carbonyl compounds, ethyl esters, acetate esters, sulfur compounds, phenols, and monoterpenoids were the main aroma compounds formed by wine yeast (Figure 1-2). The most important flavor compounds in wine made from neutral grape varieties were those arising from the fermentation process, which include mainly ethyl esters, acetate esters, higher alcohol, fatty acids, and aldehydes (Perestrelo et al., 2020). Ethyl esters of hexanoic, octanoic, or decanoic acids and isoamyl or isobutyl acetates were often considered to give wine much of its characteristics (Perestrelo et al., 2020; Swiegers et al., 2005).

Moreover, the most identified chemical families in the wines made from different grape varieties in the world were alcohols, esters, and acids (Arcari et al., 2017; Castillo et al., 2020; Cheng et al., 2015; Perestrelo et al., 2020; Philipp et al., 2021; Rapp & Mandery, 1986; Welke et al., 2014; P. Zhao et al., 2017). Cheng et al (2015) study showed the similar result (high quantities of alcohols, esters, and acids) in aroma compound composition of Chardonnay, Cabernet sauvignon, Italian and Merlot wines evaluated by headspace solid phase microextraction **(**HS-SPME) and gas chromatography-mass spectrometry (GC-MS) in China (Cheng et al., 2015). Duan et al., (2018) reported almost the same aromatic compound in aromatic composition of wine made with *Vitisvinifera L.cv* Cabernet Sauvignon grapes inoculated by different commercial yeasts in China as well (Duan et al., 2018). In another study, a total of 77 compounds were identified by applying HS-SPME followed by GC-MS analysis, among them higher alcohols, esters, and fatty acids were the major chemical families in spontaneously fermented wines made from Sercial, Malvasia de São Jorge, Bastardo, Malvasia Cândida, Verdelho, Boal, Terrantez (white grapes) and Tinta Negra (red grapes) varieties in the Demarcated Region of Madeira, Portugal (Castillo et al., 2020). The Philipp et al, (2021) research found more than 40 esters, as well as higher alcohols and acids in the inoculated and the spontaneously fermented wines made from Gruüner Veltliner, Pinot noir, and Zweigelt grape varieties in Austria (Philipp et al., 2021). However, Callejon et al (2010) reported acetals were the most abundant volatile compounds in all organic red wines made from Merlot grape variety by inoculation of different selected indigenous and commercial *S. cerevisiae* strains, followed by alcohols without ethanol in Spain (Callejon et al., 2010).

In Türkiye, the aroma composition of two white wines namely Narince (Bayram & Kayalar, 2018; Selli et al., 2006), Muscat of Bornova (Y. Karaoğlan & Cabaroğlu, 2020) and three red wines namely Öküzgözü (Şen, 2021), Kalecik Karası (Celik et al., 2019), Çalkarası rosé wine (Darici et al., 2014) were only investigated.

Şen et al (2021) investigated the aroma compounds of Öküzgözü rose wine which inoculated by commercial yeasts (Zymaflore X5 and NBY17). Higher alcohols and

esters were found the main predominant aroma classes in Öküzgözü rose wine (Şen, 2021).

Celik et al, (2019) investigated the impact of malolactic fermentation on the volatile composition of Turkish Kalecik Karası red wines. According to their research, malolactic fermentation was increased the total volatile compounds in both the spontaneously fermented wine and the inoculated wines. Diethyl succinate, ethyl lactate, and γ-butyrolactone content also increased in all Kalecik Karası wines (Celik et al., 2019). This study also reported 3-ethoxy-1-propanol (fruity aroma), 2-phenylethanol (phenylethyl alcohol), and methionol (cooked vegetable, raw potato aroma) in the volatile composition of Turkish Kalecik Karası red wines (Celik et al., 2019).

Darici et al. (2014) evaluated the aroma composition of a Çalkarası rosé wine by gas chromatography-olfactometry (GC-O), sensory evaluation analysis, and four quantitative methods. The obtained result showed that Çalkarası rosé wine had fresh fruit, floral aroma due to high level of 2-phenylethyl and isoamyl acetates. According to their research, 28 aroma compounds were able to contributed their notes to Çalkarası rosé wine (Darici et al., 2014).

According to the studied literatures, the aroma compounds of the four Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines produced traditionally have not been reported, yet. Therefore, the aim of this study was firstly to compare the aroma composition and concentration in these wines which grape varieties grown in three geographically separated viticultural zones (Ankara, Elazığ, Elazığ, and Cappadocia, respectively) in Türkiye and secondly, to investigate the terroir effect on the quality of the Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines for the first time.

1.3.1 Non-*Saccharomyces* Yeasts, Their Properties and Effect on Wine Aroma Profiles

1.3.1.1 *Hanseniaspora*

The genus *Hanseniaspora* (anamorph *Kloeckera*) consists of ten species; *H. uvarum*, *H. guilliermondii*, *H. vineae*, *H. opuntiae*, *H. meyeri*, *H. clermontiae*, *H. osmophila*, *H. occidentalis*, *H. valbyensis*, and *H. thailandica* (Borren & Tian, 2020). *Hanseniaspora* species are the most abundant apiculate yeast species isolated from grapes or wines and have an important function at the beginning stage of fermentation, making aroma compounds and enzymes that increase the diversity of wine flavor and color (Borren & Tian, 2020; Martin et al., 2018).

The asexual reproduction is determined by budding that is bipolar, and blastoconidia are created in basipetal succession. Pseudohyphae can be formed but are rarely well-developed. Colonies are smooth and have the color of cream. Through asci formation sexual reproduction are performed. Glucose can be fermented by *Hanseniaspora* species and nitrate is not assimilated. Pantothenate and myo-inositol are needed for the growth of species (Cadez & Smith, 2011).

The main properties of *Hanseniaspora* species in winemaking is to rise the acetate esters concentration (fruity aroma), terpenes, higher alcohols and sulfur-containing compounds (Martin et al., 2018). *Hanseniaspora* species demonstrate β-glucosidase, β-xylosidase, protease, and glycolytic activities as well (Padilla et al., 2016).

Among the non-*Saccharomyces* yeast species that contributes to the organoleptic quality of wines, *H. uvarum* strains possess the highest enzymatic activity like β-glucosidase (Hu et al., 2018). It was reported that inoculation of grape must with specific *H. uvarum* strain generated the largest concentrations of isobutyl acetate and isoamyl acetate, giving strawberry and banana aromas to the final wine (Borren & Tian, 2020). However, another research showed that excessive *H. uvarum* yeast and *S. cerevisiae* in sequential inoculation increased the concentration of acetate esters

and some volatile phenols triggered nail polish-like aroma in the final wines (Hu et al., 2018). Co-inoculation of some *H. uvarum* strains with *S. cerevisiae* may have the ability to make high amounts of ethyl acetate and acetic acid in final wine that contributed to volatile acidity (wine fault) causing the wine ethereal or vinegar aroma and is considered to rise the aroma complexity of wine, somehow, at lower concentrations (Borren & Tian, 2020; Tristezza et al., 2016).

The second most abundant *Hanseniaspora* species at the beginning of grape must fermentation is *H. guilliermondii* that has been reported to intensify the concentration of acetate esters (2-phenylethyl acetate) contributing to the honey, rose, flowery odors, isoamyl acetate, and hexyl acetate in final wine (Tristezza et al., 2016). *H. guilliermondii* strains were displayed to generate heavy sulfur-containing compounds (trans-2-methyltetrahydrothiophen-3-ol and 3-mercapto-1-propanol) as well during fermentation, contributed to spoiled aromas (Borren & Tian, 2020). Therefore, *H. guilliermondii* like *H. uvarum* can affect the complexity of wines both positively and negatively depending on the concentration of produced compounds.

H. vineae is another *Hanseniaspora* species studied well by researchers, although it is not generally abundant in wild ferments. *H. vineae* displays better fermentative characteristics than other *Hanseniaspora* species due to improved fermentation kinetics and having greater tolerance to ethanol (10%) that allows *H. vineae* yeast to continue sugars fermentation until the end stages of the ferment, causing it to possess a better influence on the final wine aroma (Lleixà et al., 2016). Sauvignon Blanc wines co-fermented with *S. cerevisiae* and *H. vineae* showed a raise in the acetate esters concentration (2-phenylethyl acetate) give fruity and flowery aromas to wine like *H. guilliermondii* strains (Martin et al., 2018). The result of Albillo white wines fermentation using *S. cerevisiae* and sequential inoculation with *H. vineae* showed a significant impact of *H. vineae* fermentation on the contents of terpenes (linalool, geraniol, β-citronellol, α-terpineol) and some volatile phenols with a spicy aroma (Del Fresno et al., 2021).This study confirmed that *H. vineae* affect wine structure and palatability by releasing many cell wall polysaccharides during fermentation and also increase the fruitiness, freshness and floral notes in non-aromatic white varieties

(Del Fresno et al., 2021). The Valera et al. (2020) showed that *H. vineae* and a genetically closely related species, *H. osmophila,* act similarly in respect to fermentation kinetics, ethanol tolerance, and glycolytic activities (Valera et al., 2020).

It was reported that different *Hanseniaspora* species such as *H. guilliermondii*, *H. vineae* and *H. opuntiae* can affect the color of the red wines and the polyphenolic composition due to the increased anthocyanin derived compounds (Martin et al., 2018). The color improvements depend on the *Hanseniaspora* species that have ability to form vitisin (A, B) and malvidin-3-O-glucoside-4-vinylguaiacoland (Medina et al., 2018).

Finally, these set of mentioned properties (enzymatic activity, fermentative capacity, aromatic compound production, and ability to increase the color of wines) makes *Hanseniaspora* species specially *H. uvarum, H. guilliermondii*, *H. opuntiae*, and *H. vineae* suitable unconventional yeasts to use in commercial winemaking.

1.3.1.2 *Lachancea*

According to Lachance and Kurtzman (2011), *Lachancea* genus consists of six species as following: *L. cidri* (formerly *Zygosaccharomyces cidri)*, *L. fermentati* (*Zygosaccharomyces fermentati*), *L. kluyveri* (*Saccharomyces kluyveri*), *L. waltii* (*Kluyveromyces waltii*), *L. meyersii*, and *L. thermotolerans* (*Kluyveromyces thermotolerans/Zygosaccharomyces thermotolerans*). Following the introduction of the *Lachancea* genus (Lachance & Kurtzman, 2011) several other species including *L. dasiensis*, *L. lanzarotensis*, *L. fantastica*, *L. mirantina, L. nothofagi*, and *L. quebecensis* were subsequently identified and placed in this genus (Porter et al., 2019).

The *Lachancea* genus reproduces asexually by multilateral budding. Pseudohyphae are occasionally formed but true hyphae are never created. Through ascus formation sexual reproduction are performed (S. Benito, 2018b). *Lachancea* ferments glucose

vigorously. Although nitrate is not assimilated by *Lachancea* species, ethylamine is used as a sole nitrogen source (Lachance & Kurtzman, 2011).

L. thermotolerans is the most common non-*Saccharomyces* yeast species from *Lachancea* genus used in winemaking and mostly found in grapes and natural spontaneous wine fermentations. It is morphologically globous or ellipsoidal and forms creamy colonies with buttery texture(Á. Benito et al., 2019).

L. thermotolerans strains were able to acidify grape musts and wines because of their unique ability to reduce pH through production of lactic acid during fermentative metabolism (Á. Benito et al., 2019). Lactic acid production was reported to raise the titratable acidity by up to about 9 g/L compared with the *S. cerevisiae* control (Vilela, 2018).

A reduction in pH from about pH 4 to pH 3.5 was also reported in low-acid grape juice fermentation using *L. thermotolerans*. The reduction of pH also increases the color of red wine by raising the color intensity of anthocyanins like the flavylium ion (Vilela, 2018). *L. thermotolerans* yeast can be applied as an antifungal agent by spraying on grapes to increase wine quality (Ponsone et al., 2016).

Ethyl carbamate, biogenic amines, and ochratoxin A (OTA) are the main microbiological food safety concerns for wines, musts, and grape juice, the amount of these toxins especially OTA which is carcinogen to humans often exceed the legal limits. Some *L. thermotolerans* strains have been reported to act as biological control agents to prevent OTA through inhibition of OTA accumulation by preventing the ochratoxigenic fungi growth such as *Aspergillus carbonarius* and *A. niger* (Ponsone et al., 2016).

Finally, *L. thermotolerans* is one of the most common non-*Saccharomyces* yeast influenced on wine quality features such as increasing the positive aroma compounds (ethyl isobutyrate or terpenes) and lactic acid production, a decrease in acetaldehyde and acetic acid concentrations, raising in grape anthocyanins, color, glycerol, pyruvic

acid, polysaccharides and sensory perception (Á. Benito et al., 2019; Porter et al., 2019).

1.3.1.3 *Torulaspora*

According to the Kurtzman (2011), the genus *Torulaspora* consists of six species: *T. delbrueckii*, *T. globose*, *T. franciscae*, *T. maleeae*, *T. pretoriensis*, and *T. microellipsoides* (Kurtzman, 2011).The genus *Torulaspora* has ability to grow in maltose, 2-keto-D-gluconate, galactose, melezitose, ethanol, and 10% NaCl/5% glucose. Among these species, *T. delbrueckii* displays higher growth in ethanol and 2-keto-D-gluconate (Kurtzman, 2011).

The colonies of *Torulaspora* species are spherical to ellipsoidal that divided by multilateral budding (asexual reproduction). *Torulaspora* species are able to make pseudohyphae but not real hyphae. Sexual reproduction occurs through asci that have one to four rough or smooth spherical ascospores. *Torulaspora* species can ferment glucose and other sugars, cannot use nitrate (S. Benito, 2018a; Kurtzman, 2011).

Torulaspora species are commonly found in fermented drinks, grapes, must, wine, and beer due to its moderate fermentation character. Among these species, *T. delbrueckii* is the most studied of the non-*Saccharomyces* species in winemaking (Á. Benito et al., 2019; S. Benito, 2018a; Borren & Tian, 2020).

T. delbrueckii strain normally produces acetic acid in low concentrations and reduces the volatile acidity concentration in wines compared to *S. cerevisiae* that positively influence the wine quality (Á. Benito et al., 2019). *T. delbrueckii* also produces wines with high glycerol content and lower ethanol concentrations than traditional fermentations that solve problems coming from climatic change, like highly alcoholic wines due to high sugar concentrations in grape musts (Puertas et al., 2017).

Several studies demonstrated that *T. delbrueckii* enhances fermentation quality features like odor profiles related to thiols, terpenes, specific fruity esters, low

acetaldehyde production, or mouthfeel properties related to production of polysaccharides or mannoproteins that positively affect sensory perception (Á. Benito et al., 2019; S. Benito, 2018a; P. J. Chambers et al., 2015; Puertas et al., 2017). Succinic acid and linalool are other metabolites generated by particular strains of *T. delbrueckii* which improve varietal aroma of Muscat type wines (P. J. Chambers et al., 2015). In addition, the main concern is that in most cases *T. delbrueckii* has not ability to finish a regular fermentation of wine and has to be applied with a compatible *S. cerevisiae* strain (S. Benito, 2018a).

1.3.1.4 *Candida*

The genus *Candida* is phylogenetically heterogeneous and contained 314 species and the type species *C. vulgaris* (syn. *C. tropicalis*) that belongs to the order *Saccharomycetales* of the phylum *Ascomycota* (Lachance et al., 2011). The diversity of the genus *Candida* is reported by many unique species with respect to microscopic morphology, colony structure, assimilation, and fermentation properties. The colonies of *Candida* are globose, cylindroidal, ellipsoidal or elongated that have the color of cream to yellowish, grow fast, and mature in three days. Asexual reproduction is done by holo-blastic budding and pseudohyphae can be formed. The members of the genus *Candida* do not reproduce sexually (García et al., 2018; Lachance et al., 2011). Most of the members of this genus is mesophilic, developing well at temperatures of 25–30°C, with extremes of below 0°C and up to 50°C. They may ferment sugars like glucose, utilize the nitrate, and make films and pellicles on the liquid media surfaces. Extracellular starch-like compounds are not made. Some species of the genus *Candida* use the inositol and the urease is not normally made (García et al., 2018; Lachance et al., 2011).

Proteolytic, pectinolytic, glycosidasic, lipolytic and urease activities, osmotolerance, resistance to high/low temperatures, tolerance toward ethanol, and production of secondary metabolites, esters, organic acids, higher alcohols, ketones, aldehydes, diacetyl, acids, glycerol, biotin, xylitol, nicotinic acid, and d-β-hydroxyisobutyric

acid are basic properties of *Candida* species useful in the commercial food processing (Borren & Tian, 2020; García et al., 2018; Lachance et al., 2011) and wine (Sidari et al., 2020; Speranza & Bevilacqua, 2017; Tufariello et al., 2020).

Candida species/strains are isolated in high amount from the vineyard and grapes must, although normally do not have ability to fix nitrogen or grow under an anaerobic conditions like wine fermentation environment (Borren & Tian, 2020). *C. zemplinina*, referred to in the literature by its asexual anamorph *Starmerella bacillaris*, is an exception to this. *C. zemplinina* was previously named as *C. stellata* until many strains were reclassified in 2011 by Kurtzman as separate species (Kurtzman et al., 2011)

Several studies showed that *C. zemplinina* (*Starmerella bacillaris*) produce many extracellular enzymes, like glycosidases, glucanases, and pectinases, which enhance the wine fruity odors through cleaving isoprenoids and terpenes (Á. Benito et al., 2019; Padilla et al., 2016; Russo et al., 2020). *C. zemplinina* also increase glycerol (sweet taste) and ethyl acetate at desirable concentrations that improve the mouthfeel of wine and overall aromatic complexity of wine. *C. zemplinina* also demonstrates other important properties, such as growth at low temperatures and high concentrations of sugars and low levels production of acetaldehyde and acetic acid from consumed sugars (Russo et al., 2020; Tufariello et al., 2020). Due to its superior fermentative abilities, ethanol tolerance which is more than 10% (v/v) and extremely fructophilic character, *C. zemplinina* has been discovered to be the most abundant yeast in the middle and at the end of fermentation (at lower abundance) and have an important function in the wine industry. When *C. zemplinina* (fructophilic yeast) co-inoculated or inoculated sequentially with *S. cerevisiae* (glucophilic yeast), fermentation kinetics were increased with both yeasts using their preferred sugar (Borren & Tian, 2020; García et al., 2018).

1.3.1.5 *Metschnikowia*

The genus *Metschnikowia* is described as a large ascomycetous clade that currently consists of 81 species (Kurtzman et al., 2018; Lachance, 2016; Vicente et al., 2020). Asexual reproduction is determined by multilateral budding. The budding cells are spherical to ellipsoid or can be cylindroid, pyriform, or lunate. The sexual life cycle includes the elongate asci formation having only one to two spores that are needle shaped without a whip-like appendage (Lachance, 2016).

The most species of *Metschnikowia* are able to utilize glucose, sucrose, fructose, galactose, and maltose as carbon sources but display weak or inexistent development in lactose, inulin, raffinose, and starch. Nitrate is never used as a nitrogen sources, but lysine and ethylamine usually are, although lysine-deficient strains seem in some species (Lachance, 2016). Most species are moderately fermentative but rarely grow at ethanol concentrations of 6% or above (Lachance, 2011).

Some *Metschnikowia* species shows a moderate fermentation power, different enzymatic activities including color precursors and aromatic, and antimicrobial activity against spoilage fungi and yeasts, causing these species are used to enhance wine quality and aromatic complexity (Á. Benito et al., 2019; Piombo et al., 2018; Vicente et al., 2020). The mentioned characteristics have mainly been reported from studies on *Metschnikowia pulcherrima* wine strains (Morata et al., 2019). However, *M. viticola* and *M. fructicola* have also been found in vineyard or wine-related environments and studied for winemaking purposes (Piombo et al., 2018; Vicente et al., 2020).

M. pulcherrima (anamorph *Candida pulcherrima*) is semi-fermentative yeast in indigenous ferments and isolated from grapes, wines, flowers, nectars, fruits, and tree sap (Morata et al., 2019). It shows a relatively low fermentative ability, compared to other non-*Saccharomyces* yeast species, with a slow reduction of nitrogen reported and less CO_2 generated during fermentation (Vicente et al., 2020). Most strains of *M. pulcherrima* survive only until around 4-5% ethanol

concentrations, limiting its function as a single inoculum for wine fermentation so it is preferred to use in sequential fermentations with more fermentative *S. cerevisiae* yeast strains (Borren & Tian, 2020; Vicente et al., 2020).

Due to the more expression of extracellular hydrolytic enzymes, *M. pulcherrima* can show various enzymes activities such as β-glucosidase, glucanase, pectinase, protease, cellulase, lipase, sulphite reductase and β-lyase (Morata et al., 2019). It displays high proteolytic activity (breaking down proteins into amino acids) that being the substrates needed in the ethyl esters formation, as well as used by *S. cerevisiae* as a source of nutrients (Morata et al., 2019). Several studies also reported that wines sequentially fermented with *S. cerevisiae* and *M. pulcherrima* cultures obtain the higher varietal fruit aroma and sensory scores compared to a pure *S. cerevisiae* culture, because of high β-glucosidase activity of *M. pulcherrima*, which allows for the cleaving of thiols and free terpenes, giving a floral aroma to the final wines (Á. Benito et al., 2019; Marsit et al., 2016; Morata et al., 2019; Vicente et al., 2020). Moreover, other compounds that increase under sequential fermentation are 2-phenylethanol, isoamyl alcohol, methionol, isobutanol, total esters and glycerol. On the other hand, those that generally reduce are benzylic alcohol and 3-methyl-1-butanol (Vicente et al., 2020).

Several studies have reported that *M. pulcherrima* and *M. fructicola* show strong biocontrol activity against some yeast genera such as *Brettanomyces/Dekkera*, *Pichia*, *Hanseniaspora*, and *Candida* that are considered detrimental in winemaking processes and several fungi such as *Botrytis cinerea*, *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., and *Alternaria* sp. that cause grape or must spoilage. The production of the iron sequestering brown-red insoluble pigment pulcherrimin (antimicrobial compound) is responsible for the growth inhibition of yeast and fungi which need iron for their development, while having a low or no effect on *S. cerevisiae* performance (Borren & Tian, 2020; Kurtzman et al., 2018; Morata et al., 2019; Piombo et al., 2018; Vicente et al., 2020). In addition, *M. fructicola* produces chitinase enzymes in the presence of fungal pathogens that may assist in biocontrol activities (Kurtzman et al., 2018; Lachance, 2016; Piombo et al., 2018).

1.3.1.6 *Schizosaccharomyces pombe*

Schizosaccharomyces pombe is a fission yeast and belongs to the phylum *Ascomycota* and subphylum of *Taphrinomycotina* (*Archiascomycotina*) that is evolutionarily remote from budding yeast (Sipiczki, 2000). Asexual reproduction is done by binary fission via the formation of a wall at the center of the cell, being a main difference with *S. cerevisiae*. It is also teleomorph species having four spherical spores per ascus in a linear organization. The colonies of *S. pombe* has a rod-shaped structure. *S. pombe* cells do not assimilate nitrates and they do not have β-glucosidase enzyme needed for breaking down arbutin (Callejo et al., 2017).

S. pombe is able to utilize glucose, fructose, sucrose and maltose and shows a high fermentative power, depending on the strain, 10–13% (v/v) in ethanol under anaerobiosis and 13–15% with slight aeration (Borren & Tian, 2020; Callejo et al., 2017). This species can completely ferment a high sugar grape juice like *S. cerevisiae* strains, despite the other non-*Saccharomyces* yeast species that need to be used in combination with fermentative *S. cerevisiae* strains (Á. Benito et al., 2019; Borren & Tian, 2020). The other technological advantages are the resistance to low pH and high level of sulfur dioxide (Borren & Tian, 2020).

Moreover, *S. pombe* strain has unique oenological interest because of its ability to decrease the total wine acidity through degradation of malic acid (malic acid deacidification) via maloalcoholic fermentation (MAF) with yielding main products of ethanol and CO_2 without producing lactic acid as lactic bacteria does via malolactic fermentation (Á. Benito et al., 2018, 2019; Borren & Tian, 2020). Additionally, *S. pombe* is used to increase food quality or food safety of modern winemaking and brewing industry via high autolytic polysaccharides release, reduction of gluconic acid, reduction of urea levels in musts and beer reducing the risks of ethyl carbamate formation, production of pyruvic acid that related to the pyranoanthocyanin pigments (vitisin A) formation improved red wine color, and cleaning lactic bacteria subtracts while inhibiting biogenic amines formation (Á. Benito et al., 2018; Callejo et al., 2017). On the other hand, one of the main problems

of using *S. pombe* in winemaking industry is related to high levels of acetic acid production (vinegar aroma), which can be solved with the combined use with *S. cerevisiae*, *T. delbrueckii* and *L. thermotolerans* strains (Á. Benito et al., 2018, 2019). *S. pombe* was also reported to be applied in other industries different from grape wine production like fermentation for apple wine, palm wine, sparkling wine, bilberry, and beer (Á. Benito et al., 2019).

1.3.2 ***Saccharomyces*** **Yeasts, Their Properties and Effect on Wine Aroma Profiles**

1.3.2.1 ***Saccharomyces***

The genus *Saccharomyces* belongs to the family *Saccharomycetaceae*, the order *Saccharomycetales* of the phylum *Ascomycota* and kingdom of Fungi. The vegetative cells of *Saccharomyces* species are oval, cylindrical or round, and the asexual reproduction is through multilateral budding. The yeasts are mostly diploid or sometimes of higher ploidy. The main physiological property of the species is their ability for fermentation of sugars to generate CO_2 and ethanol (anaerobic or semi anaerobic). These sugars consist of D- fructose, D-glucose, D-maltose, and D-mannose. Although, most *Saccharomyces* strains have ability to grow on D-galactose in anaerobic or aerobic conditions, none of the strains assimilates pentose, alditols, lactose, and citrate as carbon sources, or utilizes nitrate as a nitrogen source. In yeast fermentation media, nitrogen has an anabolic function for the functional and structural proteins biosynthesis like enzymes and nucleic acids, and a catabolic function in the aroma/flavor production (Walker & Stewart, 2016). *S. cerevisiae* is not able to fix atmospheric nitrogen so need a readily assimilable inorganic nitrogen supplement such as ammonium salts or organic nitrogen like amino acids for growth and fermentative metabolism. Pectin and starch (polysaccharides) are used by some *S. cerevisiae* strains exceptionally (Walker & Stewart, 2016).

Regarding oxygen requirements, although *S. cerevisiae* was known as a facultative anaerobe, this species has not ability to grow in strictly anaerobic environments due to requirement of oxygen which is a growth factor for biosynthesis of sterol (ergosterol) and membrane fatty acid (oleic acid). *S. cerevisiae* is auxotrophic for ergosterol and oleic acid in anaerobic environments, so some fatty acids, oxygen and sterol growth factors can be added to the growing medium for effective alcoholic fermentations (Walker & Stewart, 2016).

The *Saccharomyces* genus is currently building of eight species, namely; *S. cerevisiae*, *S. mikatae*, *S. paradoxus*, *S. kudriavzevii*, *S. jurei*, *S. eubayanus*, *S. uvarum*, *S. arboricola* and two natural hybrids *S. pastorianus* and *S. bayanus* (Alsammar & Delneri, 2020). Some of these species are parents of natural hybrids that either created spontaneously in the nature without the human manipulations or in habitats formed by humans in industrial/laboratorial environments (Alsammar & Delneri, 2020). Among *Saccharomyces* species, hybrids are common in industrial fermentation environments used in wine making and beer fermentation processes. *S. pastorianus* is the most famous industrial hybrid, creating from the cross between *S. eubayanus* (syn. *S. carlsbergensis*) and *S. cerevisiae*. *S. pastorianus* has been applied for centuries in beer fermentation and is used for bottom-fermented beer (lager) production, that performed at low temperatures 5–14°C, in contrast to the top-fermented beers (ale) that carried out at higher temperatures 15–24°C by using *S. cerevisiae* (Alsammar & Delneri, 2020).

S. bayanus is also the result of multiple hybridization between three pure yeast species, *S. uvarum*, *S. cerevisiae*, and *S. eubayanus* used in winemaking and cider fermentation, beer brewing and distilled beverages (Alsammar & Delneri, 2020).

S. eubayanus and *S. bayanus* are the only species of the genus that able to grow in medium without vitamins. Maximum growth temperature differentiates *S. eubayanus*, *S. pastorianus*, and *S. bayanus*, which have not ability to grow at temperatures higher than 35°C, from *S. paradoxus* and *S. cerevisiae* that able to grow at 37°C, and sometimes up to 40–42°C. An active fructose transport system is present

in the *S. pastorianus*, *S. eubayanus*, and *S. bayanus* while fructose uptake is decreased in *S. paradoxus* and *S. cerevisiae*. *S. paradoxus* is differentiated from *S. cerevisiae* with respect to the assimilation of D-mannitol and fermentation of maltose by *S. cerevisiae* (Walker & Stewart, 2016).

S. cerevisiae grows better under slightly acidic conditions with an optimum pH between 4.5 and 6.5. Most strains of *S. cerevisiae* are able to grow at different temperatures (5-40°C). Optimum temperature for maximum growth rate depends on *S. cerevisiae* strains, usually in the region of 25–35°C. The growth of *S. cerevisiae* is inhibited by some organic acids (benzoic and sorbic acid) because of cell membrane permeability dysfunction. Other acids demonstrated to prevent the growth are ferulic acid (50–250 ppm), p-coumaric acid (100–250 ppm) and natural preventing substances, such as tuberine, xylitol, and the antioxidants tertiary butylhydroquinone, butylated hydroxyanisole (Walker & Stewart, 2016). *S. cerevisiae* is the most studied species and the most applied in the wine fermentations due to its fast growth, satisfactory fermentative capacity, and easy adaptation. They have tolerance to SO_2 concentrations at which normally most non-*Saccharomyces* yeasts do not survive.

Finally, *S. cerevisiae* strain has oenological interest due to influence on biochemical reactions including: 1) glycosidases activity which release C13-nor-isoprenoids, terpenes, and aromatic phenols, 2) producing volatile thiols from non-volatile ones which give a desirable passion fruit, grapefruit, citrus aromas into final wine, 3) the bio-absorption of grape compounds like metabolites by its cell's wall glucan polysaccharides and mannoproteins 4) the extraction of the anthocyanin pigments from the grape skins (Kontogiannatos et al., 2021). All mentioned characteristics differ among the *S. cerevisiae* strains and required to be analyzed when selecting yeasts for starter culture development.

1.4 Methods for Identification and Characterization of Wine Yeasts

A crucial factor in the study of non-*Saccharomyces* and *Saccharomyces* yeast biodiversity was the methods used to identify the yeast community present in must and wine samples. Non-*Saccharomyces* and *Saccharomyces* yeast diversity could be found using culture dependent methods in which yeasts were cultured on selective or non-selective media to isolate colonies prior to identification by physiological or molecular techniques, and culture independent methods in which DNA/RNA was directly extracted from samples for downstream analysis (Sumby et al., 2021).

1.4.1 Morphological and Physiological Techniques

Morphological identification of yeast was caried out after colony isolation and growth on specific media. The colonies were analyzed in respect to color, texture, surface, and elevation (Kurtzman et al., 2011).The morphology of yeast cells could be detected by microscopy. In addition, traditional physiological and biochemical experiments consisted of different carbohydrates (galactose, glucose, lactose, maltose, raffinose, saccharose, and trehalose) fermentation or assimilation, growth on specific nitrogen and carbon sources, evaluating vitamin requirements, splitting of arbutin, lipase activity, acid production from glucose, and many others (Kurtzman et al., 2011).

1.4.2 Molecular Techniques

Molecular techniques were used to identify non-*Saccharomyces* and *Saccharomyces* yeast biodiversity after isolation and growth in specific media. Many different techniques have been used for this purpose that were classified as:

a) methods for species identification such as sequencing of ribosomal DNA (rDNA), restriction analysis of ribosomal DNA (rDNA), real-time polymerase chain reaction

(PCR), quantitative real-time PCR (qPCR), PCR-denaturing gradient gel electrophoresis (DGGE), and multilocus sequence typing (MLST),

b) methods for differentiation between strains including hybridization techniques, pulsed-field gel electrophoresis (PFGE) of chromosomes, restriction analysis of mitochondrial DNA, amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD)-PCR, PCR analysis of repetitive genomic DNA (microsatellites and minisatellites), and amplification of delta (δ) sequences (Fernández-Espinar et al., 2011; Tofalo et al., 2013).

1.4.2.1 Real-Time Polymerase Chain Reaction and Quantitative Real-Time PCR method

The identification of non-*Saccharomyces* and *Saccharomyces* yeast biodiversity by using culture-dependent methods were time-consuming and viable but non-culturable (VBNC) yeast species were not able to detect. Therefore, to achieve an accurate study of biodiversity in the complex grape must and wines environment, it was necessary to use culture-independent methods, which allow the identification and quantification of the different yeasts present during cold maceration, maceration and alcoholic fermentation regardless of their culturability by direct DNA extraction from samples. Real-time PCR and qPCR were the most applied and specific methods in the detection and quantification of the yeast biodiversity in grapes, must and wines samples (Díaz et al., 2013; García et al., 2017; Hierro et al., 2006; Lleixà et al., 2018; Pfaffl, 2019; Phister et al., 2007; C. Wang, Esteve-Zarzoso, et al., 2015; X. Wang et al., 2020; Zott et al., 2010).

In this technique, the amplified products are monitored during each PCR cycle using fluorescence-based chemistries. It is based on the detection and quantification of a signal generated by a fluorescent donor dye. The signal is in direct proportion to the quantity of PCR product in the reaction. The process is carried out in a thermocycler coupled to a detector that can acquire and quantify the signal emitted by the donor

in each sample at the end of each cycle. The data obtained are represented as an amplification curve with the point at which the intensity of the signal from the donor becomes greater than the background noise indicated. This is known as the threshold cycle (Ct) and it is inversely proportional to the number of copies of the target sequence in the sample (DNA or cells). Consequently, it can be used to assess the starting quantity of target DNA with a high degree of accuracy over a wide range of concentrations. The fluorescent signal may be derived from intercalating agents or probes. The intercalating agent SYBR green or EVA green binds to double-stranded DNA, leading to an increase in fluorescence with increasing amounts of PCR product (Figure 1-3).

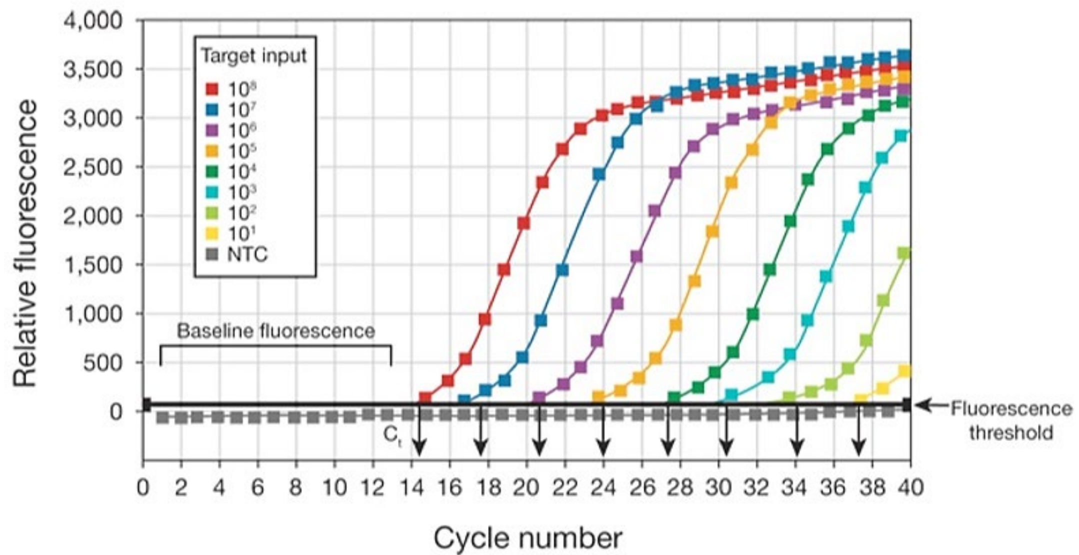


Figure 1-3 A schematic representation of the amplification plots of ten-fold serial dilution series of target

1.4.2.1.1 Quantification Strategies in qPCR

There are two primary strategies used to quantify the amount of template in a qPCR reaction. The first one is absolute quantification and the second one is relative (comparative) quantification (Pfaffl, 2019) as represented in Figure 1-4 . Absolute quantification uses a standard curve from which the concentration of template in the sample is interpolated. The standard curve which generated from serial dilution of a

known concentration of genomic DNA or a cloned target DNA (plasmid) is constructed in parallel to the amplification of samples. Comparing the Ct value obtained for each sample to the standard curve allows calculation of the amount of starting template DNA in the sample. Relative (comparative) quantification is used when comparing the expression of a gene in different strains or in different growth stages of a fungus. In this case, reverse transcription qPCR is used to determine the amount of mRNA of the target gene relative to the amount of mRNA of a calibrator gene, which in most cases is a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, actin, translocation elongation factor 1-α) which is expressed in constant levels (Pfaffl, 2019, 2020). In real-time PCR, two types of chemistries are applied including nonspecific chemistry which relies on DNA intercalating dyes like SYBR green and probe-based chemistry in which TaqMan probe used.

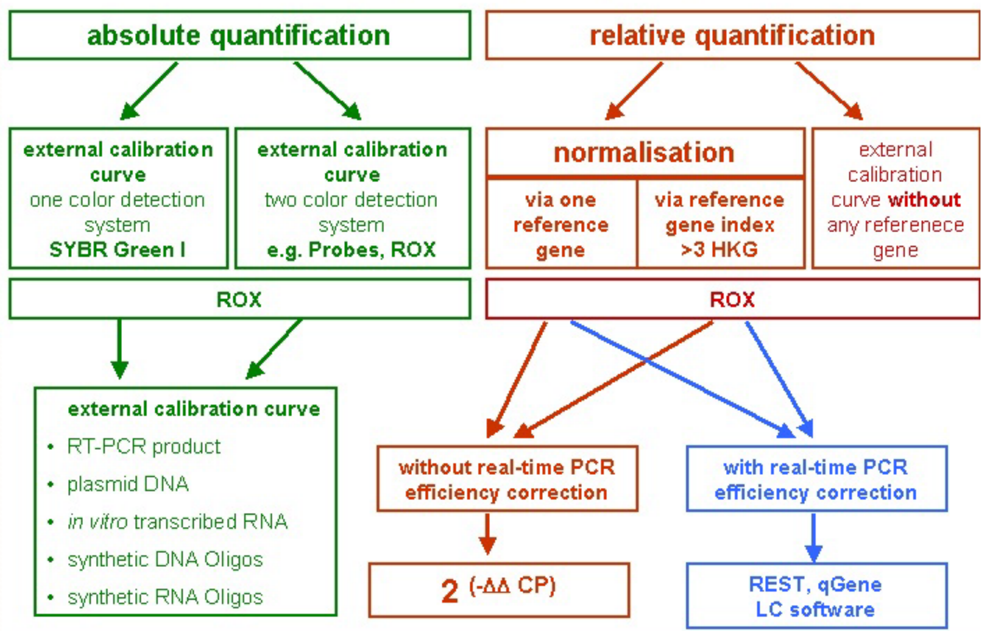


Figure 1-4 Quantification strategies in quantitative real-time PCR (Pfaffl, 2019).

The main advantages of qPCR are the low detection level, often as low as one cell per mL, the speed by which assays are performed, and the ability to quantify yeasts present in alcoholic fermentation (Postollec et al., 2011; C. Wang, Esteve-Zarzoso, et al., 2015). As a result, this method has been applied in many aspects of oenology

and fermentation-related microorganism. Detection of *Brettanomyces bruxellensis* (*Dekkera bruxellensis*) spoilage yeasts was one of the major applications (Tofalo et al., 2012). It was also used to detect the global yeast population (Hierro et al., 2006). Quantification of the predominant non-*Saccharomyces* yeast such as *L. thermotolerans* by Garcia et al. (2017), *T. delbrueckii* by Zott et al. (2010), Diaz et al. (2013), Garcia et al. (2017) and Lleixà et al. (2018), *H. uvarum,* by Hierro et al. (2007) and C. Wang et al. (2015), and for *S. cerevisiae* by Martorell et al. (2005), Hierro et al. (2007), Zott et al. (2010), Diaz et al. (2013), C. Wang et al. (2015), Garcia et al. (2017) and Wang et al. (2020) were also made.

Although Çelik et al. (2017) reported the yeast diversity in Narince which was a native white grape variety in Tokat (Türkiye) by using PCR-RFLP analysis of the 5.8 ITS rRNA region and sequencing of the D1/D2 domains of the 26S gene (Çelik et al., 2017), and Nurgel et al (2005) reported the yeast diversity in Kalecik Karasi which was a native red grape variety of Türkiye by using morphological and physiological techniques (Nurgel et al., 2005), there is no study that used real-time PCR and qPCR methods to detect and quantify yeasts in must and traditional wine made from local grape varieties in Türkiye. Therefore, one of the main molecular techniques selected to use in this study was real-time PCR and qPCR to detect and quantify non-*Saccharomyces* and *Saccharomyces* yeast biodiversity in important local winegrape producing region in Türkiye.

1.4.2.2 Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

The accurate differentiation of all members of the *Saccharomyces sensu stricto* complex (Alsammar & Delneri, 2020) specially *Saccharomyces cerevisiae*, is an important topic because of the practical significance of these species on wine fermentation (Eldarov & Mardanov, 2020; Ramírez-Castrillón et al., 2014), beer brewing (Larroque et al., 2021), bakery (Korhola et al., 2019; Lahue et al., 2020), and dairy products (Andrighetto et al., 2000).

Many molecular techniques have been developed to discriminate between different *S. cerevisiae* strains. Among them, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and the mini and microsatellite primed (MSP)-PCR fingerprinting technique have been widely applied in the literatures. However, there is a lack of study which propose and validate a standardized protocol for the RAPD-PCR and the mini and microsatellite primed (MSP)-PCR fingerprinting technique to discriminate the autochthonous *S. cerevisiae* strains isolated form local winegrape producing region in Türkiye. Therefore, in this study these methodologies were used for mentioned purposes.

Random amplified polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation, polymorphisms in genetic mapping, phylogenetic relationships, taxonomy and biodiversity in strain communities (Williams et al., 1990; Baleiras-Couto et al., 1996; Pfliegler et al., 2014; Kállai et al., 2019). The technique was improved independently by two different laboratories (Welsh & McClelland, 1990; Williams et al., 1990) and called as RAPD and arbitrary primed PCR (AP-PCR), respectively. A single arbitrary and short primer (usually 8–12 nucleotides) is used in the PCR reaction, which anneals randomly at multiple sites on the genomic DNA, resulting in the amplification of many discrete DNA products. These amplified fragments are then migrated on agarose gel and difference in the band patterns are detected (Panigrahi et al., 2019). The polymorphisms between individuals arise from sequence differences in the primer binding sites and are visible as the presence or absence of a particular RAPD band (Figure 1-5). The interpretation and comparing of banding patterns are complicated by visual observation so multiple software tools with different criteria such as Advanced Quantifier, Dolphin 1D, EzQuant, Gel plugin ImageJ, Gel-Pro Analyzer, Gel-Quant, GelComparII, GelQuant Pro, ImageLab, ImageQuant, Molecular imaging software, and PyElph are available to simplify this task as well as to remove the possible suggestibility derived of the human eye and to represent the results as dendrograms (Heras et al., 2016). Workflow to analyze DNA fingerprint images consist of preprocessing of image,

detection of lanes, detection of banding patterns, normalization, fingerprint comparison, and dendrogram generation (Heras et al., 2016).

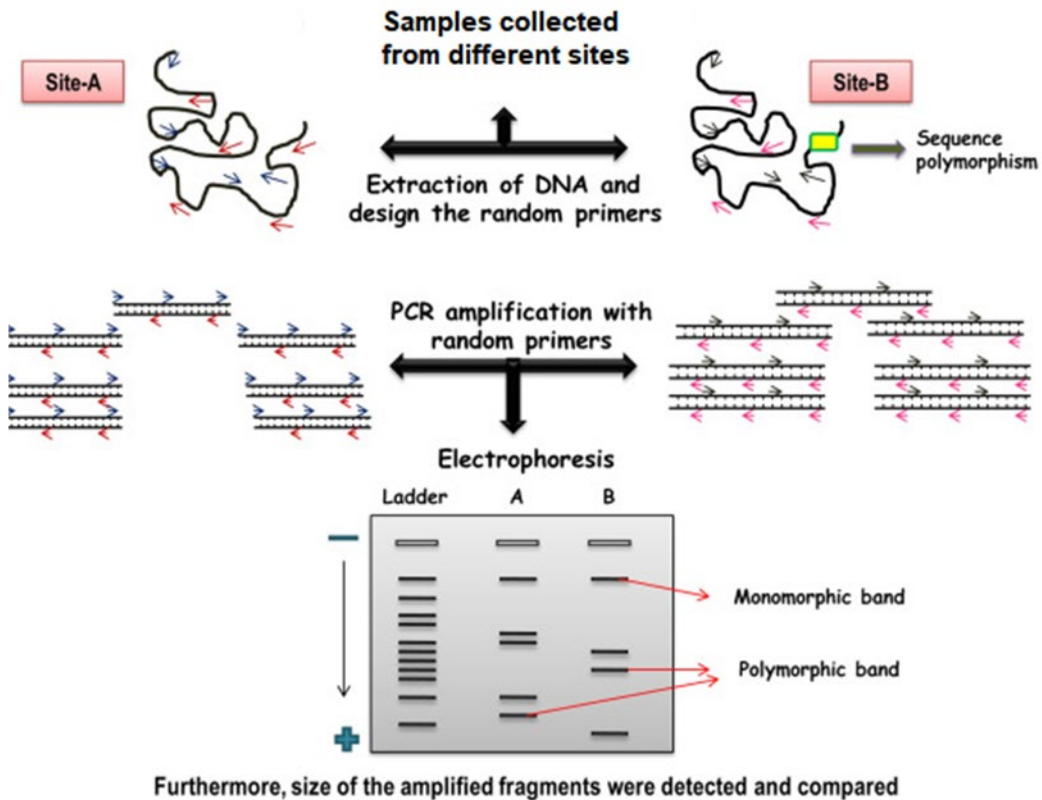


Figure 1-5 Schematic illustration of random amplified polymorphic DNA analysis (Panigrahi et al., 2018).

RAPD-PCR is a laboratory-dependent molecular technique and requires carefully developed laboratory protocols to be reproducible since the quantity and quality of template DNA, concentrations of PCR components (specially $MgCl_2$ concentration), and the thermal cycling conditions (annealing temperature) highly influence the PCR product. Therefore, the PCR reaction conditions and components require to be evaluated to obtain the most discriminating patterns between species or strains (Williams et al., 1990; Kállai et al., 2019).

1.4.2.3 Mini and Microsatellite Primed Polymerase Chain Reaction (MSP-PCR) Fingerprinting

Two categories of repetitive regions exist in the genome of various species: interspersed repeats and tandem repeats (TRs). Interspersed repeats are remnants of transposable elements distributed throughout the genome (the more predominant type of repeat). On the other side, tandem repeats (satellite DNA) are repetitive DNA, which exist directly adjacent, or in tandem, to one another (Gemayel et al., 2012). TRs are further divided into two subcategories: minisatellites and microsatellites. Minisatellites are TRs with unit length (repeated sequence of DNA) larger than 10 nucleotides (≥10 nucleotides). Microsatellites (Simple Sequence Repeats (SSR), Short Tandem Repeats (STR), Simple Sequence Length Polymorphisms (SSLP)) are short TRs with unit length between 1 to 10 nucleotides flanked by highly conserved sequences (Gemayel et al., 2012) and distributed through the whole genome of Eukaryotes (Couto et al., 1996; Oliveira et al., 2006; Pfliegler et al., 2014) and Prokaryotes (Gur-Arie et al., 2000). Microsatellites can be arranged in a simple way (G. K. Chambers & MacAvoy, 2000); for illustration, they consist of several repeats of one or more nucleotides: $(N_1N_2...N_x)_n$, or they may have a more complicated structure; two or more adjoining repeats of motifs: $(CA)_n(GT)_n$, or $(dC\text{-}dA)_n.(dG\text{-}dT)_n$. Mini and microsatellites together are also classified as Variable Number of Tandem Repeats (VNTR) DNA.

Hypervariability of minisatellites and microsatellites loci made researchers to apply MSP-PCR as an ideal technique for genetic identification of closely related individuals, genotyping, mapping, phylogenetic studies, taxonomy and biodiversity studies in strain communities (Williams et al., 1990; Baleiras-Couto et al., 1996; Pfliegler et al., 2014; Kállai et al., 2019).

Moreover, many molecular techniques have been developed to discriminate between different yeast species. Among them, RAPD-PCR using Operon primers and the MSP-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(GAC)_5$, and $(GACA)_4$ have been widely applied in the literature.

For example, RAPD analysis using Operon primers (Operon Technologies, Alameda, USA) were frequently used to discriminate different yeast species. Discrimination of the 15 *S. cerevisiae* strains isolated from wine and beer was carried out by RAPD analysis with primer OPA-11(Couto et al., 1996). According to the authors, different pattern types generated with primer OPA-11 showed heterogeneity among strains of the species *S. cerevisiae*. Thompson and Latorre, (1999) used 29 decaprimers randomly selected from Operon primer kits A, B, and G to analyze *Botrytis cinerea* isolated from table grapes and other host plants in Chile. Banding patterns of RAPD analysis with OPA-4 and OPA-11 primers distinguished isolates of *B. cinerea* from other epiphyte fungi found on table grapes, including *Aspergillus niger*, *Alternaria alternata*, *Cladosporium herbarum*, *Rhizopus stolonifera***,** *Epiccocum nigrum*, *Penicillium* sp., and yeasts such as *Rhodotorula glutinis*, *Cryptococcus laurentii*, and *S. cerevisiae* (Thompson & Latorre, 1999). RAPD banding patterns generated with six decamer primers (OPA-03, OPA-07, OPA-08, OPA-09, OPA-10, OPA-11) also showed the best differentiation between the most common strains of the *Saccharomyces sensu stricto* complex including 19 *S. cerevisiae*, 23 *S. bayanus*, 10 *S. paradoxus*, and 6 *Saccharomyces pastorianus* (Fernández-Espinar et al., 2011). In addition, nine decamer primers (OPA-2, OPA-3, OPA-7, OPA-8, OPA-9, OPA-10, OPA-11, OPA-15, and OPA-16) from Operon Technologies were applied to analyze the yeast microbiota present in a manufacturing plant of candied fruits and nougats (Martorell, Fernández-Espinar, et al., 2005). According to the fingerprinting result, RAPD analysis with OPA-10 and OPA-15 was the best procedure for the characterization in *Zygosaccharomyces bailii* strains, and RAPD with OPA-3 for *Zygosaccharomyces rouxii* strains involved in the spoilage of candied fruits (Martorell, Fernández-Espinar, et al., 2005). Wild yeast species from 17 different fruits (black grapes, green grapes, raisins, cherry, dates, pomegranate, etc.) were also analyzed by arbitrary RAPD primers namely OPA-12, OPB-09 and OPC-06 (Kumar Lathar et al., 2010). According to Lathar et al., (2010) results, amplicon fingerprints obtained by RAPD assay were allowed discrimination among the seventeen isolated yeast species. Moreover, the heterozygosity of the

meiotic segregants from two *S. cerevisiae* (ALKO 743 and ALKO 3460) were assessed using Operon primers (Korhola et al., 2019). The author reported that RAPD analysis with OPA-01, OPA-04, OPA-09, and OPA-11 enabled the distinction between two *S. cerevisiae* (44 segregants of ALKO 743 and 17 of ALKO 3460).

The primer M13 (minisatellite primer from wild-type M13 phage genome) was applied to discriminate *Saccharomyces* species like *S. cerevisiae* (Cocolin et al., 2004; Korhola et al., 2019; Lieckfeldt et al., 1993; Orlić et al., 2010; Santos et al., 2007; Šuranská et al., 2016; Torriani et al., 1999), and strains of non-*Saccharomyces* species such as natural wine strains of *Hanseniaspora* (Bujdosó et al., 2001b; Cadez et al., 2002; Guaragnella et al., 2020), *Candida zemplinina* (Pfliegler et al., 2014), *Candida zeylanoides*, *Rhodotorula mucilaginosa*, *Yarrowia lipolytica*, and *Debaryomyces hansenii* (Andrade et al., 2006). Most of these studies applied MSP-PCR fingerprinting using primer M13 reported the best differentiation between the *Saccharomyces* and non-*Saccharomyces* yeast strains. Šuranská et al. (2016) reported MSP-PCR fingerprinting techniques using primer M13 were able to group the species members of *Saccharomyces* genus isolated from berries and spontaneously fermented musts. Various authors reconfirmed that MSP-PCR using M13 primer was clearly differentiated the *Saccharomyces cerevisiae* strains isolated from different wineries, must, grapes (Cocolin et al., 2004; Orlić et al., 2010; Urso et al., 2008). The M13 primer also gave distinctive patterns that permitted a clear discrimination of the *S .cerevisiae*, *Kluyveromyces marxianus* (anamorph *Candida kefyr*), *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Torulaspora delbrueckii* species isolated from cheeses and dairy products (Andrighetto et al., 2000).

The microsatellite primer $(GTG)_5$ was frequently used to differentiate species of the genus *Saccharomyces* (Capece et al., 2016; Couto et al., 1996; Korhola et al., 2019; Lieckfeldt et al., 1993; Orlić et al., 2010; Ramírez-Castrillón et al., 2014; Santos et al., 2007; Torriani et al., 1999), characterize strains of non-*Saccharomyces* yeast such as *Candida zemplinina* (Pfliegler et al., 2014), *Hanseniaspora* (Cadez et al.,

2002) and study yeast diversity. Most of these studies applied MSP-PCR fingerprinting as a preliminary clustering step for the choice of representative strains to be sequenced. According to the results of these studies, the $(GTG)_5$ primer showed the best discrimination between the *Saccharomyces* and non-*Saccharomyces* yeast strains. The $(GTG)_5$ primer was successfully used to differentiate wine isolates of *S. cerevisiae* (Capece et al., 2012, 2016; Couto et al., 1996; Kállai et al., 2019; Lieckfeldt et al., 1993; Orlić et al., 2010).

According to the reviewed literatures, the microsatellite $(ATG)_5$ primer was rarely used to discriminate different yeast species. Although Cadez et al. (2002) reported that MSP-PCR fingerprinting using $(ATG)_5$ primer produced useful patterns to allow verification of the strain identities of *Hanseniaspora* and *Kloeckera* genus, De Benedictis et al. (2011) results indicated that the primers $(ATG)_5$, $(GTG)_5$, OPA-1, OPA-13 and RM13 generated a similar profile for almost all the *Hanseniaspora uvarum* strains isolated from a spontaneous "Negroamaro" grape must fermentation. Furthermore, there is no study related to the differentiation of the *S. cerevisiae* strains isolated from different wineries, must, and grapes applying the microsatellite $(ATG)_5$ primer in MSP-PCR fingerprinting assay.

These primers OPA-11, M13, $(GTG)_5$ and $(ATG)_5$ have not yet been evaluated for differentiating of the *S. cerevisiae* strains present in grapes, must, and wine in Türkiye. Therefore, the objective of this study was to propose and validate a standardized protocol for the RAPD-PCR using one Operon primer (OPA-11) and the mini and microsatellite primed (MSP)-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(ATG)_5$ to discriminate the autochthonous *S. cerevisiae* strains isolated form Kalecik Karası, Öküzgözü, Boğazkere, Dimrit, and Emir samples (grape, must, wine) and clustering of isolates belonging to the same species.

1.4.2.4 Pulsed-Field Gel Electrophoresis (PFGE)

Wine associated strains show a large diversity in the number and size of chromosomes that can be monitored by pulsed-field gel electrophoresis (PFGE) analysis. PFGE is a method that resolves the intact chromosome-sized DNA molecules into an agarose gel. Identifying of chromosome number and sizes in a cell is referred to as karyotyping. This technique was first described by Schwartz and Cantor (1984) and is still one of the most effective tools to differentiate not only wine yeast species but also wine associated strains and study the biogeography of yeasts in nature as well (du Plessis et al., 2017; Hicks et al., 2018; Kállai et al., 2019; Naumov et al., 2000). single yeast colony is grown in YPD broth medium and mixed with melted agarose to make an agar plug. The yeast cells are lysed in the agar matrix by using specific buffer containing lyticase as an enzyme. Then, the prepared plugs are cut and transferred to an agarose gel. The agarose gel containing yeast cell plugs is subjected to the alternating application of two electric fields in the specific buffer that allows for the resolving of large DNA fragments into the gel (Schwartz & Cantor, 1984). PFGE is also applied to identify the wine related bacterial strains but for doing that the chromosome in the agar plug is digested with specific restriction enzymes which referred as restriction endonuclease analysis PFGE (REA-PFGE). Both karyotyping of yeasts and REA-PFGE were mostly used to identify yeast and bacteria isolated from wine (du Plessis et al., 2017).

The PFGE technique uses variations in several parameters including the variation of pulse time, switch intervals, the electrical field, running temperature, running buffer, concentration of the agarose gel, and the orientation of the field (Birren et al., 1989). There are different types of PFGE in order to separate the chromosomal yeast DNA including; 1. field-inversion gel electrophoresis (FIGE), 2. transverse-alternating field electrophoresis (TAFE), 3. contour-clamped homogenous electric field (CHEF), 4. orthogonal-field alternation gel electrophoresis (OFAGE), 5. rotating gel electrophoresis (RGE), 6. programmable autonomously-controlled electrodes (PACE), and 7. pulsed-homogeneous orthogonal field gel electrophoresis (PHOGE)

(Hacio & Basim, 2001). CHFE is the preferred apparatus (Figure 1-6) in the recent researches and has proved to give straight migrating lanes, good reproducibility, and high resolution over a wide range of sizes (Cai et al., 2014; du Plessis et al., 2017; Hicks et al., 2018).

Figure 1-6 Contour-clamped homogenous electric field

This method of karyotype analysis provides a high level of discrimination between yeasts of the *Saccharomyces* species as well as intra specifically between *S. cerevisiae* strains isolated from different wine-growing regions. This technique has ability to discover polymorphisms in the electrophoretic chromosomal banding patterns of the *S. cerevisiae* strains (Carle & Olson, 1985; Cocolin et al., 2004; Kállai et al., 2019; Pereira et al., 2010; Schütz & Gafner, 1993). Longo and Vezinhet (1993) applied electrophoretic karyotyping for two *S. cerevisiae* strains (a haploid laboratory strain and a diploid enological strain derived from a champagne vineyard) to understand the origin of chromosomal polymorphism between *S. cerevisiae* strains. They showed that the karyotype of the haploid strain was very stable while the diploid strain underwent frequent modifications due to its heterozygotic structure that allows the occurrence of different sizes for homologous chromosomes. These authors concluded that the chromosome length polymorphisms observed among *S. cerevisiae* strains were the result of chromosomal rearrangements (Longo & Vezinhet, 1993).

Polymorphism in the chromosome size of *S. cerevisiae* strains caused variations in chromosomes mobility which could be used to reveal the differences between strains (Pataro et al., 2000). Pataro et al. (2000) analyzed the chromosome polymorphism applying two hybridization probes. The obtained result indicated that

polymorphisms were related to the chromosomic rearrangements which happen during the yeasts growth in the fermentation process (Pataro et al., 2000).

Another study evaluated the genetic polymorphisms of 100 strains of the commercial *S. cerevisiae* isolated from spontaneous fermentations of grape must in the Vinho Verde wine region of Portugal. This study reported a high percentage of chromosomal size variations specially in chromosomes III and VI of *S. cerevisiae* Zymaflore VL1 strain which could be related to the adaptive mechanisms to the changing of the environmental conditions (Schuller et al., 2007). Another study also reported a high level of polymorphism (8 different chromosomal banding patterns) in the electrophoretic karyotyping of nine *S. cerevisiae* strains isolated from Sangiovese grapes of Chianti area (Sebastiani et al., 2004). Some authors demonstrated different karyotypes in Chinese *S. cerevisiae* strains isolated form different geographical and ecological origins (Cai et al., 2014), *S. cerevisiae* strains isolated from the spontaneously fermented sugarcane *aguardente* (alcoholic beverage) in Brazil (Pataro et al., 2000), and *S. cerevisiae* strains isolated from Tokaj (Hungary) wines (Kállai et al., 2019).

The karyotypes of the Turkish *S. cerevisiae* strains isolated from different grape must and wine, to our knowledge, have not been explored yet. Therefore, in this study, the electrophoretic karyotyping of the *S. cerevisiae* strains isolated from different wine-growing regions will be studied to discover the genetic polymorphisms of the Turkish strains.

1.5 The Objective of the Research

The specific aims of this research can be summarized as following:

1. To produce traditional red and white wines (lab-scale) by spontaneous fermentation of indigenous yeast species present in five different grape musts of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir which grown in

three geographically separated viticultural zones (Ankara, Elazığ, and Cappadocia) in Türkiye.

2. To discover the ‘microbial terroir’ (microbial biogeography) of the Ankara, Elazığ, and Cappadocia regions in these uninoculated wines.

3. To reveal the effect of indigenous yeasts and grape varieties on volatiles composition and aromatic attributes to Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wine samples by GC-FID and GC-MS methods.

4. To detect biodiversity of autochthonous non-*Saccharomyces* and *Saccharomyces* yeasts from grapes, musts and wines of local vine-growing regions in Türkiye (Cappadocia, Elazığ and Ankara) by real-time PCR analysis and validate a standardized protocol for real-time PCR.

5. To compare the results of the non-*Saccharomyces* or *Saccharomyces* yeasts colony isolation and identification by internal transcribed spacer (ITS) region (ITS1–5.8S rRNA– ITS2) and/or D1/D2 domain of the 26S rRNA gene sequencing and the detection results obtained by real-time PCR method.

6. To quantify the native non-*Saccharomyces* and *Saccharomyces* yeast species in traditional wine made from local grape varieties in Türkiye, collected from Cappadocia (Emir, Dimrit grapes), Elazığ (Öküzgözü, Boğazkere grapes), Ankara-Kalecik (Kalecik Karası grape) regions by quantitative real-time PCR molecular tool.

7. To propose and validate a standardized protocol for the RAPD-PCR using one Operon primer (OPA-11) and for the mini and microsatellite primed (MSP)-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(ATG)_5$ in order to discriminate the autochthonous *Saccharomyces cerevisiae* strains

isolated form Kalecik Karası, Öküzgözü, Boğazkere, Dimrit, and Emir samples (grape, must, wine) and clustering of isolates belonging to the same species.

8. To propose an optimum protocol for the electrophoretic karyotyping of the *S. cerevisiae* strains isolated from different wine-growing regions in Türkiye by using PFGE method and to discover the genetic polymorphisms in these strains.

9. To use Turkish *S. cerevisiae* strains as a starter culture in global wine production after characterization and classification indigenous *S. cerevisiae* strains at genotypic level by RAPD-PCR, MSP-PCR and PFGE molecular methods.

CHAPTER 2

MATERIAL AND METHODS

Material and methods used in this study were as following;

2.1 Traditional (Spontaneous) Winemaking

2.1.1 Grape Varieties Used and Their Properties

Forty kg of healthy grapes of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit (red grape varieties) and Emir (white grape variety) were harvested from three different regions Ankara, Elazığ, Elazığ, Cappadocia and Cappadocia, respectively in 2017 vintage (Figure 2-1).

Figure 2-1 Kalecik Karası, Boğazkere, Öküzgözü, Dimrit (red) and Emir (white) grape varieties (from left to wright) collected from three different regions in Türkiye.

The collected grapes, sources, dates and ripening index for each variety were listed in Table 1.

Table 2-1The characteristic of grape varieties used in this study

Grape variety	City (source)	Country	Collected date	Brix degree
Kalecik Karası	Kalecik -Ankara	Türkiye	September 2017	22
Boğazkere	Elazığ	Türkiye	October 2017	21
Öküzgözü	Elazığ	Türkiye	October 2017	26
Dimrit	Cappadocia- Nevşehir	Türkiye	September 2017	22
Emir	Cappadocia- Nevşehir	Türkiye	September 2017	18

The collected grape varieties were transported to the Starter Culture Laboratory designed for wine production at the department of food engineering, Middle East Technical University, Ankara, Türkiye.

2.1.2 Destemming and Crushing

After harvesting and transporting the grape varieties to the winery laboratory, Kalecik Karası, Boğazkere, Öküzgözü, Dimrit (red) and Emir (white) grapes were separated from the stems and crushed gently by hands. At this stage, the grape seeds should not be damaged because of tannins present in seeds might impart the bitterness to the final wine. The obtained musts from each grape varieties were separated into three glass jars for two parallel traditional and one commercial (as a control) wine production. Brix, specific gravity, pH and temperature of the all must were measured by refractometer, hydrometer, pH meter and thermometer, respectively (C. Wang, García-Fernández, et al., 2015).

2.1.3 Maceration Process

After crushing, cold maceration and maceration were applied for red grape varieties (Kalecik Karası, Boğazkere, Öküzgözü, Dimrit). For cold maceration, the musts of red grapes were kept at 4°C for 4 days in the refrigerator, and the specific gravities were measured every day by a hydrometer. At the end of the 4th day (4.CM), the specific gravities were measured and red grape musts were undergone into the

maceration stage at 20°C for 4-6 days in Starter Culture Laboratory designed for the winemaking. During this process, the temperature was adjusted at 20°C by help of an air conditioner. In both stages of the maceration, the musts were mixed twice a day in order to get oxygen and prevent mold formation.

2.1.4 Fermentation Process

The maceration stage was terminated, when the specific gravity of the four red grapes reached between 1.010 and 1.020. Then, the must was squeezed with the help of cheesecloth (Hyma et al., 2011). After removing the pulp from musts, free flow musts were put into the clean glass jars (8 L) and the lids of the glass jars were closed with an airlock to prevent the entering of oxygen. After that, alcoholic fermentation started in an anaerobic environment and continued at 25°C until specific gravity reaches 0.990 in the hydrometer which refers to 15% (v/v) and 12% (w/w) alcohol content (Bakker & Clarke, 2011). The ends of the fermentation time were determined when the specific gravity amounts were reached 0.990. Traditional wine production was achieved by spontaneous fermentation of indigenous yeast community present in grape must without dried yeast inoculation. A commercial wine was made with inoculation of commercial *Saccharomyces cerevisiae* dried yeast (Chr. Hansen) according to the manufacturer's procedures as a control, after adding 30 mg/L potassium metabisulphite to the grape musts (C. Wang, García-Fernández, et al., 2015). The red winemaking procedures were performed in duplicate.

In respect to Emir which was a white grape variety, the winemaking procedure was slightly different from red winemaking procedures. After pressing with the help of cheesecloth, the specific gravity and pH of Emir must was measured. Then, the sedimentation was removed by syphoning (after passing 24 h at 15°C). After that, Emir must was poured into the glass jars (8 L) which lids closed with airlocks to start alcoholic fermentation at 18°C in an anaerobic environment (Çelik et al., 2017). All fermentations were done spontaneously without dried yeast inoculation and in duplicate. Also, a commercial wine was made with inoculation of commercial *S.*

cerevisiae dried yeast (Zymoflore VL1 Vin Blanc, Laffort) according to the manufacturer's procedures as a control.

2.1.5 Clarification and Bottling

Clarification was carried out by syphoning 3 times to separate the wine from sediments. After the clarification, the wines were bottled into 750 mL wine bottles and cork caps were placed.

The schematic representation of the red winemaking process was shown in Figure 2-2.

Figure 2-2 Red winemaking procedures; 1. Grape must, 2. Cold maceration, 3. Maceration, 4. Fermentation and 5. Bottling.

In summary, the red winemaking process made from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit grape varieties included harvesting and crushing of grapes, cold maceration at +4°C for 4 days, maceration at 20°C for 4-6 days, pressing (specific gravity should be between 1010 and 1020), alcoholic fermentation at 25°C, end of

alcoholic fermentation (specific gravity should be reached 0.990), clarification (3 times) and bottling (Figure 2-3)

The white winemaking process made from Emir grape variety consist of harvesting the grapes, pressing and removal of sedimentation by syphoning (after passing 24 h. at 15°C), alcoholic fermentation (at 18°C), clarification/filtration (3 times) and bottling (Figure 2-3).

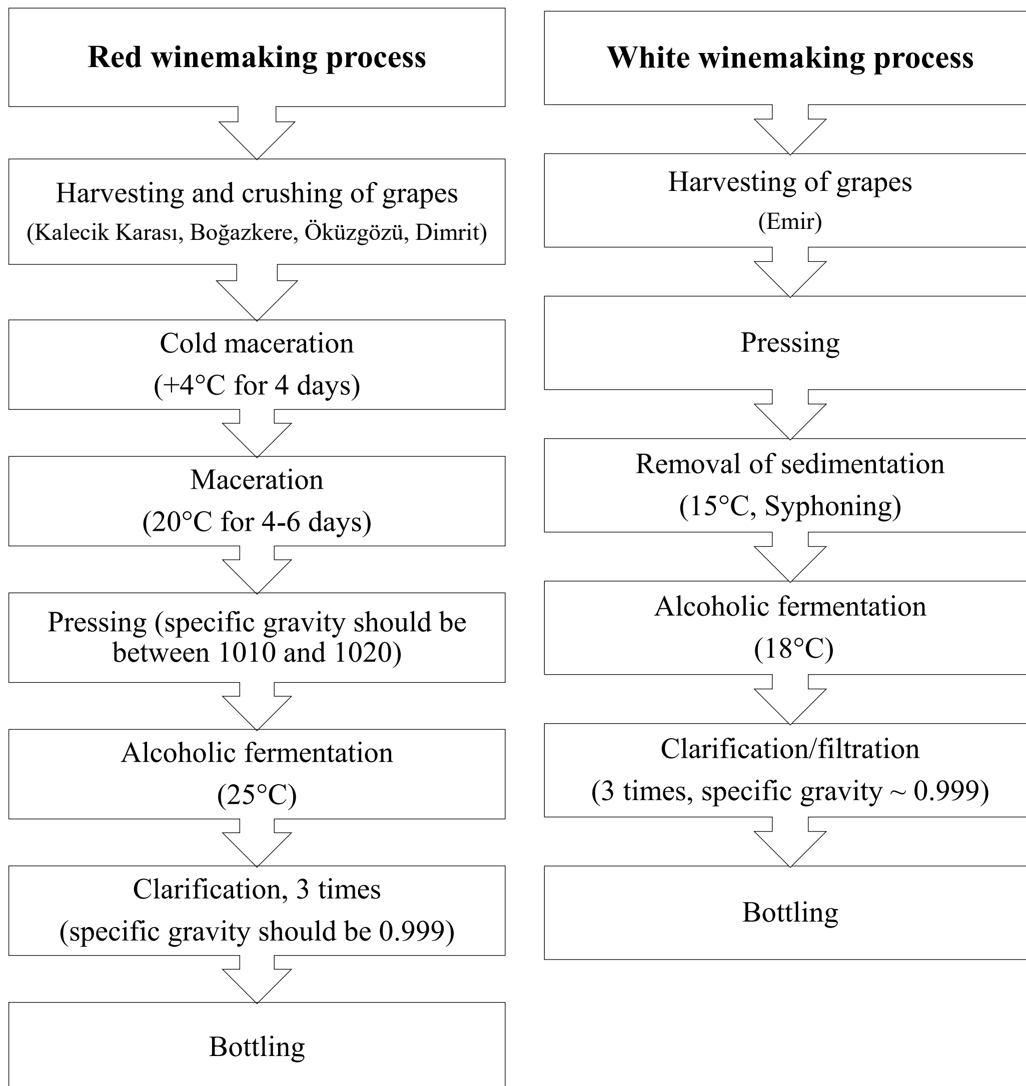


Figure 2-3 Flowchart of red and white wine production from five different Turkish grape varieties

2.1.6 Sampling of Grape Must and Wine

200 mL samples of each Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit grape varieties were taken at different time periods: beginning of the cold maceration time (0.CM), end of the cold maceration time (4.CM), end of the maceration time (4.M/6.M) and end of fermentation time (F).

In respect to Emir which was a white grape variety, 200 mL of the samples were taken at the beginning of fermentation (0. Week) and the end of the first, second, third, and fourth week (1, 2, 3, and 4 W) since there was no maceration stage in the production of white wine and the fermentation process started as soon as grapes were squeezed directly.

2.2 Wine Composition Analysis

2.2.1 Measurement of Alcohol, pH, Volatile Acidity, Total Acidity, and Reducing Sugar

Alcohol, pH, volatile acidity, total acidity, and reducing sugar amounts of produced wines from five different grape varieties were assessed using WineScan™ Auto Equipment (Foss, Type 79067, Denmark) at Kavaklıdere winery company. The measurements were carried out by putting 10 mL of wine samples into the dedicated tubes of analyzer and placed in the instrument's sample chamber. The instrument's probe was immersed into the tubes sequentially, and the analysis were performed for each wine sample. The obtained results were evaluated by the Foss Integrator software (Figure 2-4).

Figure 2-4 WineScan™ Auto Equipment (Foss)

2.2.2 Measurement of Total and Free Sulfur Dioxide

The total and free amount of sulfur dioxide (SO_2) were assessed by Foss equipment composed of FIAstar 5000 analyzer and sampler 5027 at Kavaklıdere winery company (Figure 2-5).

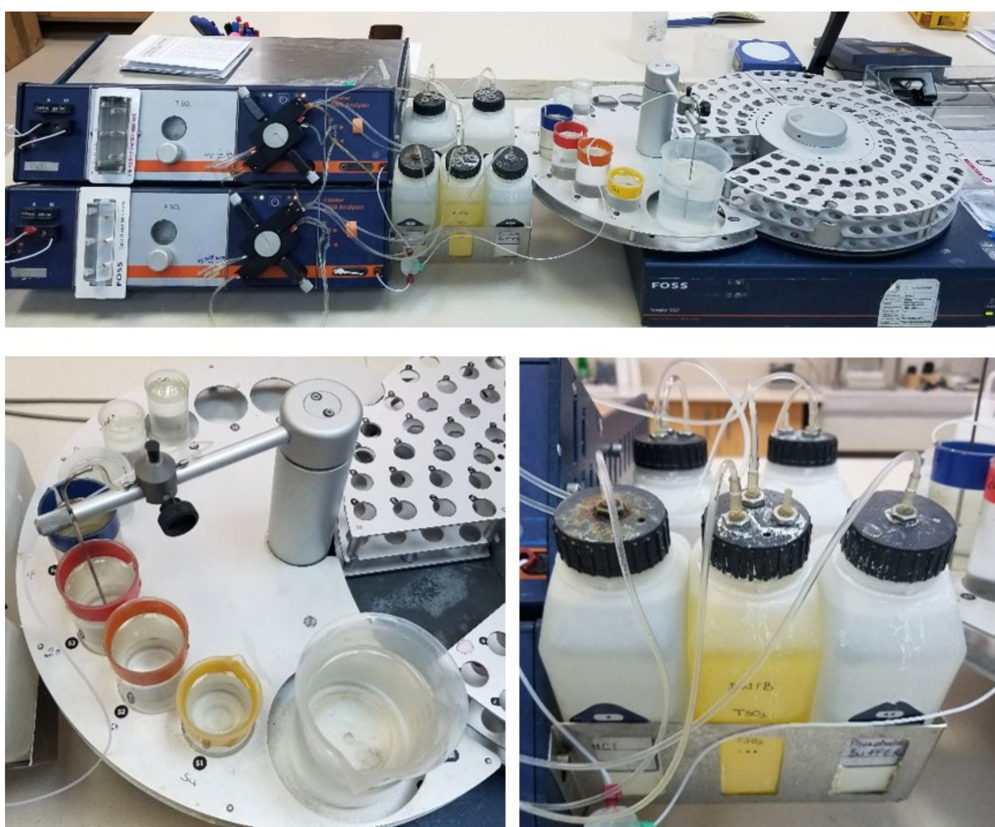


Figure 2-5 FIAstar 5000 analyzer, Foss sampler and five standard solution containers

The instrument composed of five containers for dinitro benzoic acid (DNTB), distilled water, diluted hydrochloric acid (HCl) solution, and two specific buffer

solutions. Standard SO_2 solution was made by mixing of sodium disulfide (0.74 g) and 99% ethanol (50 mL). Distilled water was added in mixture to obtain a total volume of 500 mL. 10, 20, 40, 80, 140, and 200% SO_2 solutions were made by taking 1, 2, 4, 8, 14, and 20 mL of standard solution, respectively, and distilled water was added in mixtures to obtain a total volume of 100 mL. The wine samples and tubes which poured by standard solutions were placed in the sample chamber of the Foss instrument. In order to measure free SO_2 content, the tubes containing 140 and 200% standard solutions were removed from the instrument. After calibration and standard curve construction, the results were given as mg/L by the Sofia software.

2.2.3 Volatile Compounds Analysis

2.2.3.1 Volatile Compounds Extraction of the Wine Samples

The aroma compounds of the four red wines produced from Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit varieties grown in three different regions (Ankara, Elazığ, Elazığ, and Cappadocia, respectively) were obtained by liquid-liquid extraction method (Figure 2-6).

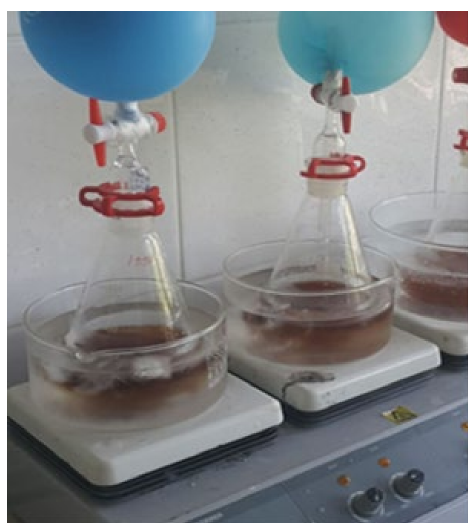


Figure 2-6 Liquid–liquid extraction of volatile compounds of the four red wine samples

Extraction was performed by mixing 45 mL of wine sample with 40 µg of 4-nonanol (as an internal standard) and 50 mL of high purity dichloromethane solvent in a 500 mL Erlenmeyer flask. The mixture was stirred at 4-5°C for 45 min under nitrogen gas. Then, the content was centrifuged at 0°C for 15 min at 5500 rpm. After centrifugation, the water found in the dichloromethane and aromatic extract was dehydrated by using anhydrous sodium sulfate. This organic extract was concentrated in a Vigreux distillation column to 1 mL at 40°C and then to 0.5 mL under a nitrogen gas. The whole process was repeated for 3 times. For determination of aroma substances, the aromatic extracts were directly injected into Gas Chromatography (GC) with Flame Ionization Detector (FID), and Gas Chromatography–Mass Spectrometry GC-MS systems (Selli et al., 2008). Aroma compound extractions were performed in Laboratories of Prof. Dr. Serkan Selli at Çukurova University.

2.2.3.2 Identification and Quantification of Volatile Compounds by GC-FID and GC-MS

The gas chromatography (GC) system was composed of an Agilent 6890N chromatograph equipped with flame ionization detector (FID) and an Agilent 5975B VL mass selective detector (MSD) mass spectrometer (MS) (Agilent Technologies, Wilmington, DE, USA). This system allowed us to simultaneously obtain an FID signal for the quantification, an MS signal for the identification of volatile compounds. Volatile compounds were separated on DB-Wax capillary column (60 m length × 0.25 mm inside diameter × 0.4 µm thickness). Injector and FID detectors temperatures were set at 220 and 250°C, respectively. The temperature of the DB-Wax column was adjusted at 60°C for 3 min. The DB-Wax column temperature was increased from 60 to 220 °C at a rate of 2°C/min and then raised to 245°C at a rate of 3°C/min with a final hold at 245°C for 20 min. The flow rate of helium gas (as the carrier gas) was fixed at 1.5 mL/min. A total of 3 µL of extract was injected into the device.

For identification of the aroma compounds, mass spectrometer (MS) with Agilent 5975B VL MSD (Agilent Technologies, Wilmington, DE, USA) combined with GC was used. Injector type and temperature program of the mass selective detector possessed the same conditions as GC. The speed of helium used as carrier gas was at 1.5 mL/min. Compounds were scanned at a rate of 29–350 mass/load (m/e) at 1 second intervals with an ionization energy of 70 electron volt (eV), an ion source and a quadrupole temperature of 250 and 120 °C, respectively.

The volatile compounds identifications (peaks identification) were carried out by comparing the mass spectrum of the nonstandard compounds with the mass spectra of the commercial spectral database (Wiley 9.0, NIST-11, and Flavor.2L). For confirmation, standard aroma compounds were injected into the GC-MS system under the same conditions. The linear retention index values were calculated according to the *n*-alkane series (Vandendool & Kratz, 1963).

The concentration of volatile compounds was determined from the internal standard method (area of 4-nonanol) according to the following formula (Selli et al., 2008);

$$C_i = (A_i/A_{st}) \times C_{st} \times RF \times HF$$

Where C_i was the concentration of the compound, A_i was peak area of the compound, A_{st} was peak area of the internal standard, C_{st} was the concentration of the internal standard (40 µg/50 mL), RF was response factor, and HF was calculation factor which was a factor to convert sample quantity to L.

2.2.3.3 Odor Activity Values

The contribution of each volatile compound to wine aroma was assessed qualitatively by its odor description and quantitatively by its odor activity values (OAVs). OAVs were calculated using the following equation (Duan et al., 2018);

$$OAV = c/t$$

where c was the total concentration (µg/L) of each compound in the wine samples, and t was the odor threshold value (µg/L) of the compound in water/ethanol solution (Duan et al., 2018). Threshold values of odor were taken from research studies available in the literature (references were shown in Table 3-5).

2.2.3.4 Statistical Analysis

The mean values were measured from at least 3 repetitions which represented as the mean ± standard deviation of replicates. Data of volatile compounds were analyzed by one-way ANOVA and Tukey's Range Test for statistically different values at a significance level of $P \leq 0.05$. Statistical analyses were carried out using the Minitab statistical software (Minitab Inc., version 21, Pennsylvania, USA).

Moreover, principal component analysis (PCA) was carried out to distinguish the aroma compositional similarity of four red wine samples by applying all detected aroma compounds with OAV bigger than 1 (OAV>1) as variables. PCA analysis was performed by XLSTAT (2022) statistical and data analysis program (Addinsoft, New York, USA).

2.2.3.5 Sensory Analysis of Wine Samples

Descriptive Analysis (DA) was used for spontaneously fermented red wines produced from Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit grape varieties by six sensory assessors in conference room of food engineering department of METU, Türkiye (Lesschaeve & Noble, 2010). All panelists were experienced in the sensory analysis. For the analysis, 50 mL wine samples were poured into standard wine-tasting glasses and randomly coded. Wine samples were tasted by experts and scored from zero (week) to five (strong) in paper questionnaire according to nine criteria as following; color, aroma, sweetness, bitterness, fullness, acidity, astringency, final astringency, and overall impression. According to the obtained results, sensory

profiles were demonstrated in the spider chart (radar chart) by applying Excel Office 365 program.

2.3 Real-Time Polymerase Chain Reaction and Quantitative Real-Time PCR Assays

2.3.1 DNA Extraction for Real-Time PCR and qPCR

In order to carry out real-time PCR or qPCR analysis, two different DNA extraction methods were applied. The first one was genomic DNA isolation from 10 mL fresh must and wine of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir. The second one was genomic DNA isolation from four pure reference culture of yeast strains *Hanseniaspora uvarum, Lachancea thermotolerans*, *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* bought from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, German collection of microorganisms and cell cultures GmbH, Germany) to perform quantification of these species in five different must and wine by qPCR.

Genomic DNA was extracted from 10 mL wine or must samples employing the GeneMATRIX Yeast Genomic DNA Purification Kit (EUR_x, Poland), with a modified procedure in respect to the manufacturer's protocol. 10 mL wine or must samples were centrifuged at 10,000 rpm for 20 min at room temperature. The supernatants were discarded, and the cell pellets were washed with 1 mL 1X phosphate buffered saline (PBS) solution. 1X PBS was prepared by dissolving 8 g of sodium chloride (137 mM NaCl), 0.2 g of potassium chloride (2.7 mM KCl), 1.44 g of sodium hydrogen phosphate (10 mM Na_2HPO_4) and 0.24 g of potassium dihydrogen phosphate (1.8 mM KH_2PO_4) in 800 mL of distilled H_2O. The pH was adjusted to 7.4 with 1 N hydrochloric acid. The total volume was completed to 1 L with additional distilled H_2O. Solution was sterilized by autoclaving at 121°C for 20 min. After a first washing step, the pellets were resuspended in 300 µL buffer Lyse BG containing β-mercaptoethanol (β-ME). Lysis mixtures were centrifuged at

10,000 rpm for 1 min and the supernatants were discarded. The pellets were resuspended again in 250 µL buffer Lyse BG containing β-ME and mixed completely by pipetting. Then, lyticase (2 mg/mL) and RNase A (10 mg/mL) were added to the resuspended cell pellets and incubated at 30°C for 45 min. 10 µL Proteinase K (20 mg/mL) were added to the resuspended cell pellets and mixed by several-fold inverting for 3 sec. The samples were incubated at 55°C for 45 min. 350 µL buffer Sol BG were added to the mixtures and incubated at 55°C for 5 min. After vortexing for 15 sec, the lysates were centrifuged for 2 min at maximum speed. The clear supernatants were transferred to DNA binding spin-columns containing 30 µL of activation Buffer BG and centrifuged at 10,000 rpm for 1 min. The spin-columns were removed and the flow-through were discarded. After two washing steps with 450 µL of Wash BGX buffer, the spin-columns were centrifuged again for 1 min at 10,000 rpm to remove Wash BGX buffer completely. DNA was then eluted in 100 µL of Elution buffer (10 mM Tris-HCl, pH 8.5) preheated heated to 70°C. The qualities of extracted DNAs were assessed from the A_{260}/A_{280} ratios using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

The isolation of DNA from four pure reference culture of yeast strains *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* were described in part 2-3-4.

2.3.2 Specific Primers Used for Detection of Yeast Species

Primers specific for the 16 predominant non-*Saccharomyces* and *Saccharomyces* yeasts (*Candida glabrata*, *Candida zemplinina* (*Starmerella bacillaris*), *Candida zeylanoides*, *Hanseniaspora* spp., *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Lachancea thermotolerans*, *Metschnikowia* spp., *Metschnikowia pulcherrima*, *Pichia fermentans*, *Pichia kluyveri*, *Rhodotorula mucilaginosa*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus* (*Pichia anomala*), *Saccharomyces* spp, *Saccharomyces cerevisiae*) found in the winery and in fermentation processes were used to anneal within the 26/28S ribosomal DNA region and the ITS region (Díaz et al., 2013; García et al., 2017; X. Wang et al., 2020; Zott et al., 2010) (Table 2-2).

The specificity of primers was verified by Zott et al. (2010), Diaz et al. (2013), García et al. (2017), and Wang et al. (2020).

Table 2-2 Specific primers used for real-time PCR analysis

Yeast Species	Primer Name	Primer Sequence (5′ to 3′)	Annealing Temperature (°C)	Reference
Candida glabrata	CG-5fw	GAGGGTGTCAGTTCTTTGT	56	(Díaz et al., 2013)
	GC-3bw	GTGAGCTGCGAGAGTC		
Candida zemplinina	CZ-2	CTTGGGTGTCGAAAGGCG	62	(Zott et al., 2010)
	CAST	CAATATGCGTTCAAAAATTCAAT		
Candida zeylanoides	CZ-5fw	CGATGAGATGCCCAATTCCA	58	(Díaz et al., 2013)
	CZ-3bw	GAAGGGAACGCAAAATACCAA		
Hanseniaspora spp.	Hauf 2L	CCCTTTGCCTAAGGTACG	62	(Zott et al., 2010)
	Hauf 2R	CGCTGTTCTCGCTGTGATG		
Hanseniaspora uvarum	HU-5fw	GGCGAGGGATACCTTTTCTCTG	59	(Díaz et al., 2013)
	HU-3bw	GAGGCGAGTGCATGCAA		
Issatchenkia orientalis	ISA 1	GTTTGAGCGTCGTTTCCATC	62	(Zott et al., 2010)
	ISA 2	AGCTCCGACGCTCTTTACAC		
Lachancea thermotolerans	LTH2-F	CGCTCCTTGTGGGTGGGGAT	60	(García et al., 2017)
	LTH2-R	CTGGGCTATAACGCTTCTCC		
Metschnikowia spp.	Mt8F	TTCCTCACCCTCGTAAGACTACC	66	(X. Wang et al., 2020)
	Mt8R	CGGACCCAAATCTCTTCAAATT		
Metschnikowia pulcherrima	MPL3	CTCTCAAACCTCCGGTTTG	60	(Zott et al., 2010)
	MPR3	GATATGCTTAAGTTCAGCGGG		
Pichia fermentans	PF-5fw	TTGCCTATGCTCTGAGGCC	61	(Díaz et al., 2013)
	PF-3bw	TCCATGTCGGGCGCAAT		
Pichia kluyveri	PK-5fw	AGTCTCGGGTTAGACGT	55	(Díaz et al., 2013)
	PK-3bw	GCTTTTCATCTTTCCTTCACA		
Rhodotorula mucilaginosa	RM-5fw	GCGCTTTGTGATACATTTTC	54	(Díaz et al., 2013)
	RM-3bw	CCATTATCCATCCCGGAAAA		
Saccharomyces spp.	SC 1	GAAAACTCCACAGTGTGTTG	63	(Zott et al., 2010)
	SC 2	GCTTAAGTGCGCGGTCTTG		
Saccharomyces cerevisiae	SC-5fw	AGGAGTGCGGTTCTTTCTAAAG	59	(Díaz et al., 2013)
	SC-3bw	TGAAATGCGAGATTCCCCCA		
Torulaspora delbrueckii	Tods L2	CAAAGTCATCCAAGCCAGC	63	(Zott et al., 2010)
	Tods R2	TTCTCAAACAATCATGTTTGGTAG		
Wickerhamomyces anomalus	PA-5fw	ACGTCATAGAGGGTGAGAAT	57	(Díaz et al., 2013)
	PA-3bw	AAACACCAAGTCTGATCTAATG		

2.3.3 Real-Time PCR Reaction

Real-time PCR reactions were carried out in a 25 µL reaction mixture that consisted of 1X HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis BioDyne, Estonia), 100 nM of each forward and reverse primers, 2 µL of template DNA. Ultra-pure water was added to obtain a total volume of 25 µL. Each reaction mixture was loaded in polypropylene optical 96-well plates on an ABI 7500 real-time PCR System (Applied Biosystems, USA). Each reaction was made in duplicate. The real-time PCR program was adjusted to each primer set (Table 2-2). A no-template control (NTC) and a positive control were used for each primer pair. The mixture was heated to 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 54–66°C (depending on the primer pairs) for 1 min, and extension at 72°C for 30 s. For each well, the SYBR green fluorescent light emission was recorded in real time by generating overlapping spectra in the wavelength range of 500–660 nm and data were analyzed with the dedicated software (SDS version 1.3.1, Applied Biosystems).

2.3.4 Melting Curve Analysis

Melting curve analysis was carried out for each assay by monitoring fluorescence continuously between 54°C and 95°C with 0.5°C increments for cells. Change in fluorescence was plotted over temperature to determine at what point denaturation of PCR amplicons occurs. A single peak showed that a single PCR product was amplified in each reaction (Martorell, Querol, et al., 2005).

2.3.5 Absolute Quantification and Standard Curves Construction for qPCR Analysis

For absolute quantification an external standard was needed. Therefore, a sample with known copy number of genomic DNA was diluted to create a standard dilution

series for a standard curve. The cycle threshold (Ct) of unknown samples and of the external standard dilution series were compared and used to estimate the amount of the unknown samples. Furthermore, the amplification efficiency of samples and the amplification efficiency of standards were identical. Finally, the same reaction conditions were applied to standards as well as unknown samples. Therefore, the Ct values of an external standard were determined together with those of the samples in each assay (Pfaffl, 2019; Phister et al., 2007).

In order to quantify the amount of target non-*Saccharomyces* and *Saccharomyces* yeasts in unknown must and wine of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir samples, external calibration curves were prepared to reproduce highly specific and reliable data. For standard curve construction, four reference yeast strains *H. uvarum, L. thermotolerans, T. delbrueckii* and *S. cerevisiae* bought from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) were firstly reactivated by the following procedure:

The glass ampoule of DSMZ strains from the secondary packaging were removed and the tip of the ampoule were heated in a flame. By placing two or three drops of water onto the hot tip, the glasses were cracked. The insulation material was removed with forceps and the inner vial was taken out. Then, 0.5 mL of yeast extract peptone glucose (YPD) broth composed of 10 g/L yeast extract, 10 g/L peptone (Lab M Limited, UK), and 20 g/L glucose (Sigma-Aldrich, USA) were added. The plug was replaced and allowed the pellet to rehydrate for up to 30 minutes. The content was gently mixed with an inoculation loop. After mixing the content, about half of the whole amount was transferred to a test tube with 5 mL of the YPD liquid medium, the other half was also streaked onto a YPD agar plate prepared by mixing 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, and 20 g/L agar (Sigma-Aldrich, USA), pH adjusted to 4.5 using orthophosphoric acid. Finally, broth and agar cultures were incubated at 25-30°C for 48-72 h (depending on strain).

After reactivation of four reference yeast strains, a fresh cultures of *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* grown in YPD medium, were

centrifuged for 15 min at 8000 rpm. The supernatants were discarded and the pellets were resuspended in sterile peptone buffer. After repeating the washing step for two more times, the pellets were resuspended in 2 mL of filter-sterilized red grape juice. These cultures were then serially diluted in filter-sterilized red grape juice (previously filtered through a 0.2-µm filter). 100 µL of each 10-fold dilutions were plated on YPD medium and incubated for 48-72 h at 25°C to obtain colony forming units per milliliter at each dilution. DNA was also isolated from 2 mL of each dilution using a GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (EUR_x, Poland) with a modified procedure in respect to the manufacturer's protocol. This DNA was then used in qPCR reactions.

Four reference strains *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* were serially diluted in red wine and used for the construction of the standard curves. Standard curves for quantification of unknown samples and determination of amplification efficiency were generated by plotting the Ct values of the qPCR reactions performed on DNA from these dilution series against the log input cells per mL (Hierro et al., 2007). All standard curves represent at least two independent experiments. The amplification efficiency (E) was calculated using the slope of the regression line in the standard curve. Efficiency and percentage of efficiency was calculated by the following formula;

$$E = 10^{-1/slope}$$

$$E\ (\%) = (E-1) \times 100$$

A slope close to -3.32 indicated optimal 100% PCR amplification efficiency (Pfaffl, 2019).

2.4 Random Amplified Polymorphic DNA and Mini, Microsatellite Primed Polymerase Chain Reaction Analyses

2.4.1 Yeast Strains

The 46 indigenous *Saccharomyces cerevisiae* strains were subjected to RAPD-PCR and MSP-PCR analysis to investigate the intraspecific genetic diversity between the strains isolated from five different grape must and wine (Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir) at variable stages and clustering of isolates belonging to the same species. The *S. cerevisiae* strains studied, their designations, sources, and the accession numbers of the rDNA sequences were listed in Table 2-3.

2.4.2 Isolation of Genomic DNA for RAPD-PCR and MSP-PCR Assay

The 46 *S. cerevisiae* strains were grown in YPD broth containing 10 g/L yeast extract, 10 g/L peptone (Lab M Limited, UK), and 20 g/L glucose (Sigma-Aldrich, USA) for 36-48 h at 30°C. Genomic DNA was extracted from the 46 pure cultures of *S. cerevisiae* strains employing the GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (EUR_x, Poland), with a modified procedure in respect to the manufacturer's protocol as described in 2-3-1 part. DNA was eluted in 100 µL of Elution buffer (10 mM Tris-HCl, pH 8.5) preheated heated to 70°C. The qualities of the extracted DNAs were analyzed on agarose gels (1% w/v) and assessing the A260/A280 ratio using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

Table 2-3 *Saccharomyces cerevisiae* strains studied by RAPD-PCR, MSP-PCR and PFGE analyses, their designations, sources, and the accession numbers of the rDNA sequences

Yeast strain	Strain designation		Source				Accession numbers	
	ID in the DNA-Sequencing	ID in the laboratory collection	Grape must/ wine	City	Country	Isolated date	ITS1-5.8S-ITS2	D1/D2
S. cerevisiae	B2	BA 0.CM NS2	Boğazkere	Elazığ	Türkiye	2017	MN796556	-
S. cerevisiae	B3	BA 0.CM NS3	Boğazkere	Elazığ	Türkiye	2017	MN796557	-
S. cerevisiae	B5	BA 0.CM NS5	Boğazkere	Elazığ	Türkiye	2017	MN796559	-
S. cerevisiae	B6	BA 0.CM NS6	Boğazkere	Elazığ	Türkiye	2017	MN796560	-
S. cerevisiae	B14	BB 0.CM NS6	Boğazkere	Elazığ	Türkiye	2017	MN796568	-
S. cerevisiae	B22	BA 4.CM NS6	Boğazkere	Elazığ	Türkiye	2017	MN796573	-
*S. cerevisiae***	B35	BA 6.NM NS2	Boğazkere	Elazığ	Türkiye	2017	MN796585	MN817291
S. cerevisiae	B36	BB 4.CM S10	Boğazkere	Elazığ	Türkiye	2017	-	MN817292
S. cerevisiae	D16	DA 4.NM S1	Dimrit	Cappadocia- Nevşehir	Türkiye	2017	-	MN817298
S. cerevisiae	D17	DA 4.CM S3	Dimrit	Cappadocia- Nevşehir	Türkiye	2017	-	MN817299
S. cerevisiae	D18	DA 4.CM S1	Dimrit	Cappadocia- Nevşehir	Türkiye	2017	-	MN817300
S. cerevisiae	D19	DA 4.CM S8	Dimrit	Cappadocia- Nevşehir	Türkiye	2017	-	MN817301
S. cerevisiae	K44	KB 4.CM S5	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796462	-
S. cerevisiae	K45	KA 2.NM S6	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796463	-
S. cerevisiae	K46	KB 2.NM S4	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796464	-
*S. cerevisiae***	K48	KA 4.NM S8	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796466	MN817275
S. cerevisiae	K49	KB 4.NM S2	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796467	-
S. cerevisiae	K50	KB 4.NM S10	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	MN796468	-
S. cerevisiae	K71	KA 32.NM S2	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	-	MN817279
S. cerevisiae	K72	KA 4.NM S2	Kalecik Karası	Kalecik -Ankara	Türkiye	2017	-	MN817280
S. cerevisiae	O10	OB 4.CM NS5	Öküzgözü	Elazığ	Türkiye	2017	MN796528	-
S. cerevisiae	O12	OA 0.CM NS3	Öküzgözü	Elazığ	Türkiye	2017	MN796530	-

Table 2-3 Continued

S. cerevisiae	O24	OA 4.CM NS5	Öküzgözü	Elazığ	Türkiye	2017	MN796542	-
S. cerevisiae	O39	OB 4.CM S1	Öküzgözü	Elazığ	Türkiye	2017	-	MN817286
S. cerevisiae	O40	OB 4.CM S2	Öküzgözü	Elazığ	Türkiye	2017	-	MN817287
S. cerevisiae	E1	EA 0.W NS1	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796483	-
S. cerevisiae	E2	EA 0.W NS2	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796484	-
S. cerevisiae	E4	EA 0.W NS4	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796485	-
S. cerevisiae	E5	EA 0.W NS5	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796486	-
S. cerevisiae	E6	EB 0.W NS1	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796487	-
S. cerevisiae	E15	EA 1.W NS5	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796495	-
S. cerevisiae	E16	EA 1.W NS6	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796496	-
S. cerevisiae	E20	EA 1.W NS10	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796499	-
S. cerevisiae	E21	EB 1.W NS1	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796500	-
S. cerevisiae	E22	EB 1.W NS2	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796501	-
S. cerevisiae	E23	EB1.W NS3	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796502	-
S. cerevisiae	E27	EB 1.W NS7	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796505	-
S. cerevisiae	E29	EB 1.W NS9	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796506	-
S. cerevisiae	E31	EA 2.W NS1	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796508	-
S. cerevisiae	E38	EA 0.W NS9	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796513	-
S. cerevisiae	E45	EB 3.W S5	Emir	Cappadocia- Nevşehir	Türkiye	2017	MN796487	MN817281
S. cerevisiae	S13*	S13*	Kalecik Karası	Kalecik -Ankara	Türkiye	2016	-	-
S. cerevisiae	S15*	S15*	Kalecik Karası	Kalecik -Ankara	Türkiye	2016	-	-
S. cerevisiae	S16*	S16*	Kalecik Karası	Kalecik -Ankara	Türkiye	2016	-	-
S. cerevisiae	S18*	S18*	House wine starter culture, 38409LM, Germany				-	-
S. cerevisiae	CH	CH	MERIT™ Chr. Hansen, Hoersholm, Denmark				-	-

B, Boğazkere grape; D, Dimrit grape; K, Kalecik Karası grape; O, Öküzgözü grape; E, Emir grape varieties. A, A parallel; B, B parallel; CM, cold maceration; NM, maceration; W, week. 0.CM, 0. day of cold maceration; 4.CM, 4th day of cold maceration; 2.NM, 2nd day of maceration; 4.NM, 4th day of maceration; 6.NM, 6th day of maceration; 0.W, 0. week; 1.W, 1st week; 2.W, 2nd week; 3.W, 3th week.

CH, reference strain of *S. cerevisiae* (Christian Hansen, MERIT™); *, *S. cerevisiae* strains isolated by Çağri Çavdaroğlu (2017); **, Identified as *S. cf. cerevisiae* according to ITS region and *S. cerevisiae* according to D1/D2 sequencing result (Aktuna,2019).

2.4.3 Primers Used in RAPD-PCR and MSP-PCR Analyses

RAPD analysis was carried out with one random 10-mer primer (5'-CAATCGCCGT-3') OPA-11 (Couto et al., 1996; Korhola et al., 2019). MSP-PCR fingerprinting was performed using one minisatellite primer M13 (5′-GAGGGTGGCGGTTCT-3′) derived from the bacteriophage M13 core sequence (Lieckfeldt et al., 1993; Pfliegler et al., 2014; Yang et al., 2020), and two microsatellite primers $(GTG)_5$ and $(ATG)_5$ as shown in Table 2-4 (De Benedictis et al., 2011; Kállai et al., 2019; Lieckfeldt et al., 1993).

Table 2-4 One 10-mer primer, one minisatellite and two microsatellite primers used in RAPD-PCR and MSP-PCR

Primer	Sequence (5' to 3')	Annealing T	Application	Reference
OPA-11	CAATCGCCGT	32	RAPD-PCR	(Couto et al., 1996; Korhola et al., 2019).
M13	GAGGGTGGCGGTTCT	52	MSP-PCR	(Lieckfeldt et al., 1993; Yang et al., 2020)
$(GTG)_5$	GTGGTGGTGGTGGTG	52	MSP-PCR	(Kállai et al., 2019; Lieckfeldt et al., 1993)
$(ATG)_5$	ATGATGATGATGATG	38	MSP-PCR	(De Benedictis et al., 2011)

T, temperature (°C).

2.4.4 RAPD-PCR and MSP-PCR Reaction

Different experiment conditions were used to optimize the reproducibility of the RAPD and MSP-PCR Fingerprinting assay (Williams et al., 1990), in which concentrations of template DNA, primer (0.1-0.3 µM), dATP, dCTP, dGTP and dTTP (100-200 µM), $MgCl_2$ concentration (1.5-3 mM) and 5 U/µL FIREPol DNA polymerase (Solis BioDyne, Estonia) were varied to determine which conditions produced the strongest and most reproducible patterns. The optimized PCR reaction mixture was contained 50 ng of template DNA, 0.3 µM of the primer for both RAPD-PCR and MSP-PCR (OPA-11, M13, $(GTG)_5$, $(ATG)_5$), 200 µM of each dNTPs, 2.5 mM MgCl2, 1 U of FIREPol DNA polymerase, 1X reaction Buffer B (0.8 M Tris-

HCl, 0.2 M (NH4)2SO4, 0.2% w/v Tween-20) without Mg^{2+} (Solis BioDyne, Estonia), and sterile nuclease free water in 25 µL of final volume.

The amplification reactions were carried out in a MJ Mini thermal cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 repetitions of 94°C for 1 min, 1 min at 32°C for primer OPA-11, at 52°C for primer M13 and $(GTG)_5$, at 38°C for primer $(ATG)_5$, ramp to 72°C with 0.6 °C/s, at 72°C for 1 min. Final extension was carried out at 72°C for 2 min and subsequently cooled to 4°C. The 46 *S. cerevisiae* strains were subjected to RAPD-PCR and MSP-PCR analysis with optimized PCR reaction conditions at least twice. A negative control reaction (with no DNA template) was used. RAPD and MSP-PCR products were separated by electrophoresis through 2% (w/v) agarose gels (Sigma, USA) in 1×TBE at 100 V for 75 min. Gels were stained with ethidium bromide (0.5 mg/mL) for 30-40 min and visualized under ultraviolet light in Gel Doc XR imaging system using Quantity One software (Bio-Rad, USA). The molecular sizes of DNA fragments were obtained with comparison with a 100 base pair molecular marker. The 100 bp DNA ladder contained 13 discrete DNA fragments ranging from 100 bp to 3000 bp (Solis BioDyne, Estonia).

2.4.5 Statistical Analysis

Gels containing the RAPD-PCR profiles obtained with OPA-11 primer and MSP-PCR fingerprinting with M13, $(GTG)_5$, $(ATG)_5$ primers of the *S. cerevisiae* isolates were normalized, using the Solis BioDyne 100 bp DNA molecular ladder, loaded into each gel. Only strong and clearly reproducible RAPD and MSP bands were scored as present (1) or absent (0) for each of the primer-strain combinations. The variable similarity matrix was created using Dice coefficient by the Numerical Taxonomy System of multivariate program (NTSYS-pc) version pc2.1 (Rohlf, 2000). Dendrogram for the 46 *S. cerevisiae* strains were then generated from RAPD-PCR and MSP-PCR's combination data using Unweighted Pair Group Method with Arithmetical average (UPGMA) analysis.

2.5 Pulsed-Field Gel Electrophoresis

2.5.1 Yeast Strains

The 46 indigenous *S. cerevisiae* strains were subjected to PFGE analysis to investigate the intraspecific genetic diversity between the strains isolated from five different grape must and wine (Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir) at variable stages and clustering of isolates belonging to the same species. The *S. cerevisiae* strains studied, their designations, sources, and the accession numbers of the rDNA sequences were listed before in Table 2-3.

2.5.2 Materials and Buffers Used in PFGE Analysis

The detailed of material, buffer and stock solutions used in PFGE experiments were listed as following:

- Sterile deionized water
- Distilled water
- Yeast extract peptone glucose broth (YPD; 10 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose)
- YPD agar (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose and 20 g/L agar, pH adjusted to 4.5 using orthophosphoric acid).
- Ethylenediaminetetraacetic acid (EDTA)
- Tris (hydroxymethyl) aminomethane (Tris base)
- Boric acid (BH_3O_3)
- Sodium hydroxide (NaOH)
- Sodium phosphate (0.01 M)

- N-lauroylsarcosine sodium ($C_{15}H_{28}NNaO_3$)
- 50% Glycerol
- Proteinase K: 20 mg/mL solution in sterile deionized water was prepared and stored at −20°C.
- Lyticase (50 KU)
- 9% beta-mercaptoethanol
- 10X Tris-Borate-EDTA (TBE) stock solution: 108 g tris base, 55 g boric acid, 7.5 g EDTA and 1 L deionized water was mixed thoroughly and autoclaved at 121°C for 15 min.
- 1X TBE buffer: 50 mL 10X TBE and 450 mL sterile deionized water was mixed.
- 1 M tris stock solution: 121.14 g of tris base was dissolved in 800 mL of H_2O. pH was adjusted to the desired value by adding concentrated HCl. The solution allowed to cool at room temperature before making final adjustments to the pH. The volume of the solution was adjusted to 1 L with H_2O. The solution was autoclaved at 121°C for 15 min.
- 1 M EDTA, pH 7.5; 0.5 M EDTA, pH 9.0; 0.05 M EDTA, pH 7.5.
- LET buffer: 0.5 M EDTA, 0.01 M Tris, pH 7.5.
- NDS buffer: 0.5 M EDTA, 0.01 M Tris, pH 7.5, 1% (w/v) N-lauroylsarcosine sodium.
- Low-melting-point (LMP) agarose (2% in 1X TBE) for DNA plug preparation
- 1% LMP agarose for sealing agarose preparation
- 1% LMP agarose for agarose gel preparation

- Running buffer: 110 mL of 10X TBE buffer and 2090 mL deionized water was mixed.
- 10 mg/mL thiourea solution: 0.5 g thiourea and 50 mL sterile deionized water was mixed.
- 0.5 µg/mL ethidium bromide solution (stock solution 1 mg/mL).
- Contour-clamped homogeneous electric field (CHEF) DNA Size Marker (*S. cerevisiae*, Bio-Rad) (Figure 2-7).

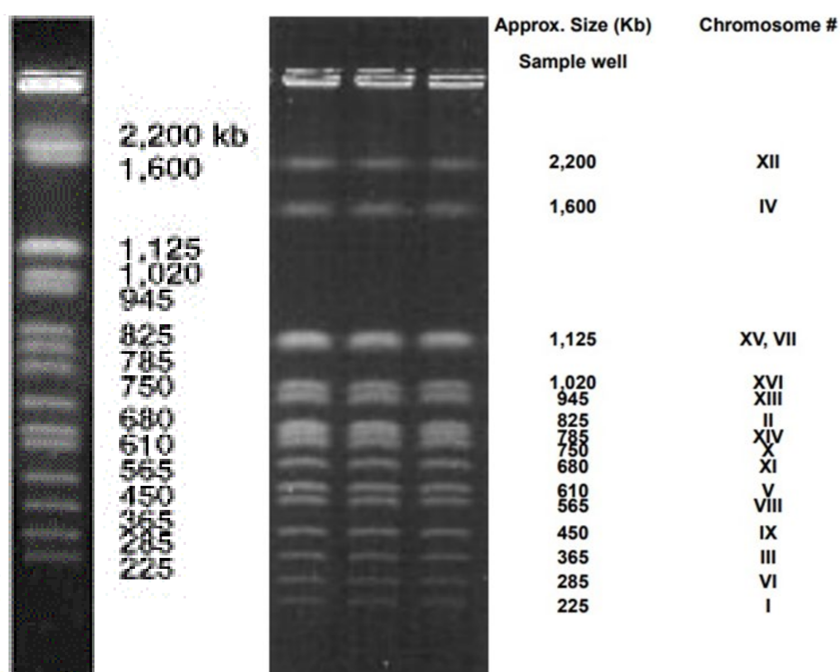


Figure 2-7 CHEF *S. cerevisiae* chromosomal DNA Size Marker (catalog number; 170-3605) Strain YNN295 with approximate DNA size of 225-2,200 kilobase pairs (Bio-Rad).

2.5.3 PFGE Procedure

Six mL of a 48-36-h old *S. cerevisiae* grown in YPD broth were centrifuged at 8000 rpm for 5 min and the pellet was resuspended in 300 µL of 0.05 M EDTA, pH 8. 100 µL of sodium phosphate 0.01 M, glycerol 50% [v/v], containing 1 mg/mL of lyticase (Sigma) was added and incubated at 45°C for 20 min. 250-300 µL of 2% LMP

agarose was gently added in the tubes and the plug modules were poured. After solidification at room temperature, the agarose blocks containing the *S. cerevisiae* cells were removed from the modules and mixed with LET buffer, containing 9% [v/v] mercaptoethanol (Sigma), overnight at 37°C. Plugs were washed three times for 15 min each with 0.05 M EDTA, pH 8, at room temperature. Plugs were subjected to a proteinase-K treatment (1 mg/mL, Sigma) in NDS buffer and incubated 12-18 h at 50°C. Plugs were washed three times for 15 min each. 5 mL of fresh 0.5 M EDTA (pH 9.0) was added to the plugs to store the plugs at 4°C for months. PFGE was performed using the CHEF system (Bio-Rad). After optimization of the PFGE analysis, agarose gels (1%) were loaded with sliced pieces of *S. cerevisiae* plugs and runs were performed for 24 h at 14°C in running buffer, using a voltage of 6 V/cm, and an initial and final switch of 60 and 120 s, respectively. After finishing the run, gels were stained in 1X TBE containing 0.5 µg/mL ethidium bromide for 45 min. Agarose gels were transferred into distilled water for de-staining for 2 h. The gels were photographed under the UV light (Cocolin et al., 2004; Hicks et al., 2018; Schwartz & Cantor, 1984). CHEF 225-2,200 kilobase *S. cerevisiae* chromosomal DNA Size Marker (catalog number; 170-3605, Bio-Rad) with approximate DNA size of 225, 285, 365, 450, 565, 610, 680, 750, 785, 825, 945, 1,020, 1,125, 1,600, and 2,200 kb pairs was used (Figure 2-7).

2.5.4 Statistical Analysis

PFGE gels containing different chromosomal patterns of *S. cerevisiae* strains were normalized, using Bio-Rad *S. cerevisiae* chromosomal DNA Size Marker, loaded into each gel. The variable similarity matrix was created using Dice coefficient by the Numerical Taxonomy System of multivariate program (NTSYS-pc) version pc2.1 (Rohlf, 2000). Dendrogram for the 46 *S. cerevisiae* strains were then generated from PFGE data using Unweighted Pair Group Method with Arithmetical average (UPGMA) analysis.

CHAPTER 3

RESULT AND DISCUSSION

In this study, workflow and all designed experiments were summarized in the following flowchart (Figure 3-1).

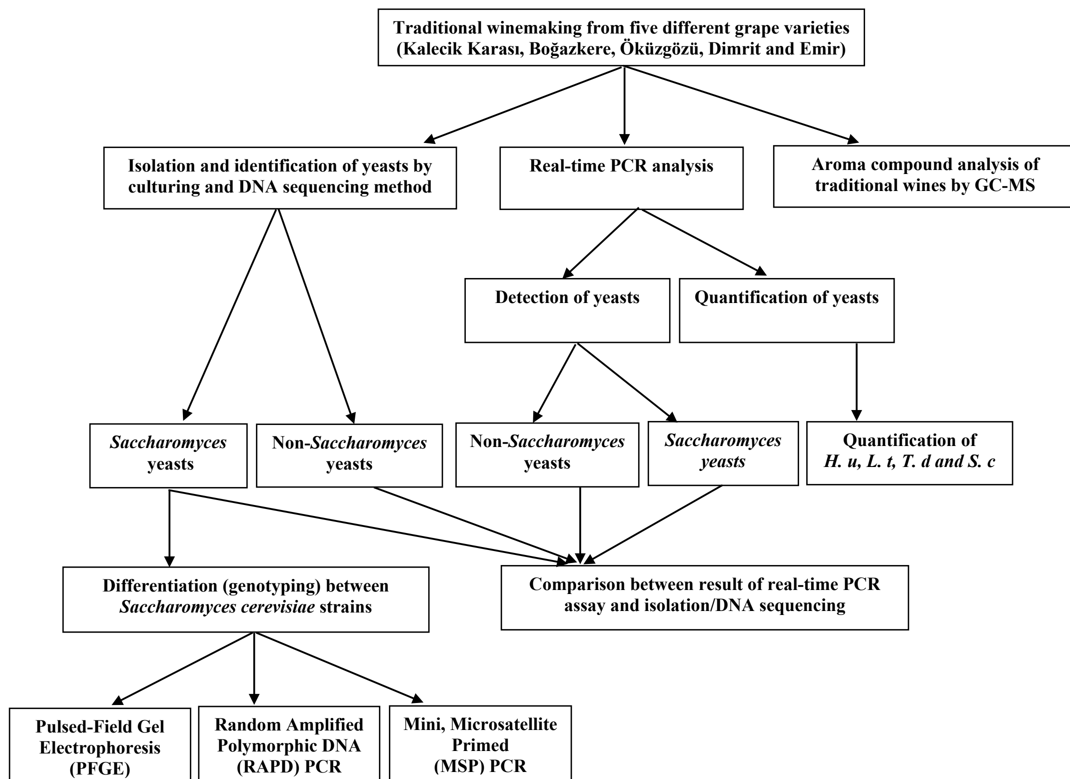


Figure 3-1 Workflow of the designed experiments in this study

The dissertation can be divided into six different section that, as a whole, complement each other.

First of all, traditional wine production was achieved by spontaneous fermentation of indigenous yeast community present in five different Turkish grape musts of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir.

Secondly, the effect of indigenous yeasts and terroir in these traditional wine samples which grape varieties grown in three geographically separated viticultural zones (Ankara, Elazığ, and Cappadocia) were investigated by GC-FID and GC-MS.

Thirdly, the biodiversity of non-*Saccharomyces* and *Saccharomyces* yeasts in these traditional wine samples were detected by real-time PCR assay.

Fourthly, qPCR was used to quantify *Saccharomyces cerevisiae* and three predominant non-*Saccharomyces* yeasts *Hanseniaspora uvarum, Lachancea thermotolerans* and *Torulaspora delbrueckii*.

Fifthly, biodiversity determined with the use of real-time PCR analysis were compared with DNA sequencing result (Aktuna, 2019) of internal transcribed spacer (ITS) region (ITS1–5.8S rRNA– ITS2) and/or D1/D2 domain of the 26S rRNA gene of the isolates.

Finally, the intraspecific genetic diversity and genotyping between the 46 autochthonous *S. cerevisiae* strains isolated from five different grape must and wine at different stages and can be used as starter cultures for wine production were revealed by applying three different molecular methods as RAPD-PCR, MSP-PCR and PFGE.

3.1 Wine Composition Analysis

Traditional Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir winemaking was achieved by spontaneous fermentation of indigenous non-*Saccharomyces* and *Saccharomyces* yeasts present in these grape musts. Alcohol (ethanol, v/v %), pH, volatile acidity, total acidity, reducing sugar, total sulfur dioxide (SO_2) contents of these five wines at the end of fermentation (<0.999) were measured using WineScan™ Auto Equipment (Foss, Type 79067, Denmark) and Foss FIAstar 5000 analyzer at Kavaklıdere winery company. The normalized results were given in the Table 3-1.

Table 3-1 The enological analysis result of the spontaneously fermented Turkish wines

Wine samples	Ethanol (v/v %)	pH	Volatile acidity (g/L acetic acid)	Total acidity (g/L tartaric acid)	Reducing sugar (g/L)	Total SO_2 (mg/L)
Kalecik Karası	12.9	3.62	0.91	3.8	3.5	75
Boğazkere	12.3	3.49	0.19	4.3	4.5	106
Öküzgözü	12	3.17	0.39	4.9	1	71
Dimrit	12.3	3.45	0.16	4.6	4.0	75
Emir	11.5	3.11	0.11	5.01	1.1	119

The alcohol concentration of all wines made from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, (red) and Emir (white) grape varieties were between 11.5 and 12.9 (v/v %) which were within the acceptable limit of the Turkish, European and U.S wine standards (Table 3-2).

According to the wine standards, pH value of wines should be between 3 and 3.5 (Table 3-2). In this study, pH of all wines except Kalecik Karası (3,62) were within the standard limits. The pH value depended on various factors such as the grape varieties, the brix amount at harvesting time, soil moisture, season, and mineral composition during ripening. Thereafter, several factors like overripening of grapes, long growing periods due to cool weather, and lack of early precipitation could cause the wine pH value to be in the range of 3.5-4.0 (Boulton et al., 1999).

Moreover, the contents of total sulfur dioxide (mg/L), volatile acidity (g/L acetic acid) and total acidity (g/L tartaric acid) were also in the standard limits. If the acetic concentration was lower than 0.72 g/L, its effect was not seen in sensory analysis. However, if its content raised to 0.90 g/L, a bitterness could be sensed. In Kalecik Karası wine samples the volatile acidity as acetic acid was measured 0.91 g/L (Table 3-1).

In respect to the reducing sugar content of wine, the wine with a sugar concentration of less than 4 g/L were classified as a dry wine (Turkish and European wine standards, Table 3-2). Reducing sugar amount of Kalecik Karası, Öküzgözü, Dimrit, and Emir wines were assessed less than 4 g/L so these wines classified as dry wines. However, only Boğazkere wine were grouped in medium-dry wine category due to its reducing sugar amount which was 4.5 g/L (Table 3-1).

Table 3-2 Turkish, European and U.S. wine standards

Compounds	**Turkish food codex**	**European standard**	**U.S. standard**
Alcohol (v/v %)	9< and <15	<15	<14
pH	3< and <3.5	3< and <3.5	3< and <3.5
Total sulfur dioxide (mg/L)	red wine: <150 white wine: <200	red wine: <150 white wine: <200	<350 ppm
Volatile acidity (g/L acetic acid)	red wine: <1.2 white wine: <1.08	red wine: <1.2 white wine: <1.08	red wine: <1.4 white wine: <1.2
Total acidity (g/L tartaric acid)	>3.5	>3	>3
Reducing sugar (g/L)	dry wine: <4 and <9[*] medium-dry wine: 4< and <12 medium-sweet wine: 12< and <45 sweet wine: 45<	dry wine: <4 and <9[**] medium-dry wine: <12, or 18[***] medium-sweet wine: <45 sweet wine: >45	-

[*], when total acidity as tartaric acid was <2 g/L sugar amount; [**], when total acidity was not >2 g/L below sugar amount; [***], when total acidity was not >10 g/L below amount of sugar.

In summary, the enological analysis results of these traditional wines were compared with Turkish, European and U.S wine standards (Table 3-2). The obtained results were within the acceptable limit of all standards. Hence, these wines were acceptable products for further analyses or usage.

In addition to the traditional wines made of indigenous non-*Saccharomyces* and *Saccharomyces* yeasts in the five grape varieties, commercial wines made from Kalecik Karası (red) and Emir (white) grape as references were also reported in detail by Aktuna (2019).

3.2 Volatile Compounds Analysis of Wines

3.2.1 Identification and Quantification of Volatile Compounds

The Peaks of the volatile compounds identified in spontaneously fermented red wines made from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit grape varieties grown in three different regions (Ankara, Elazığ, Elazığ, Cappadocia, respectively) were shown in the following four gas chromatography-mass spectrometry (GC-MS) chromatograms (Figures 3-2, 3-3, 3-4, 3-5). Aroma compound analysis for white grape variety (Emir) in traditionally fermented wine, and wine inoculated with different strains were compared and reported by Aktuna (2019). Therefore, traditionally fermented Emir wine was not included in this section.

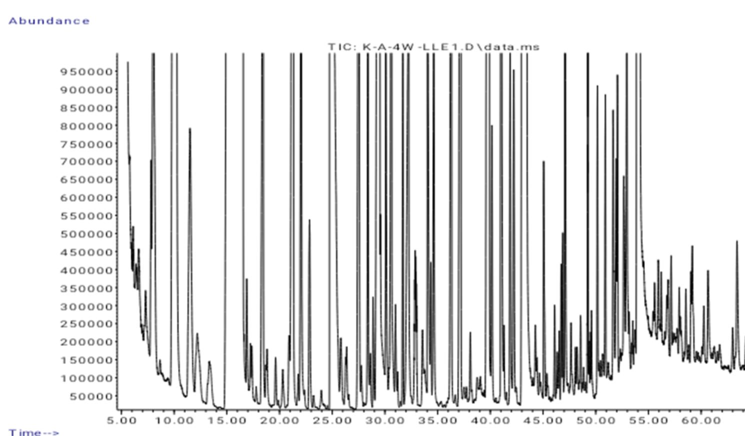


Figure 3-2 GC-MS chromatogram of Kalecik Karası wine

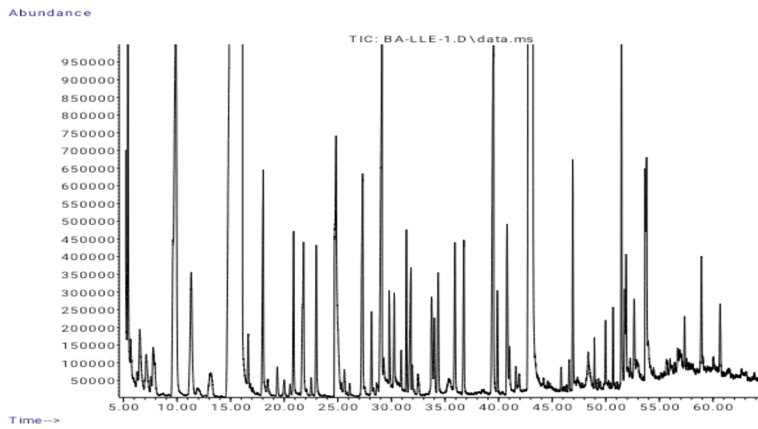


Figure 3-3 GC-MS chromatogram of Boğazkere wine

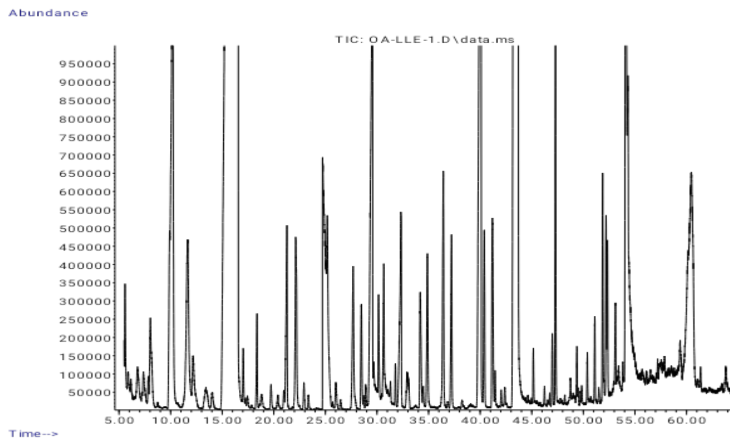


Figure 3-4 GC-MS chromatogram of Öküzgözü wine

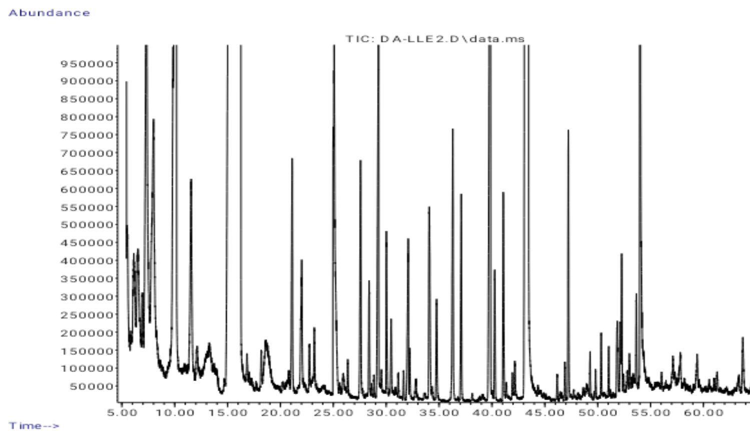


Figure 3-5 GC-MS chromatogram of Dimrit wine

The volatile compounds identified in four traditionally fermented red swines, concentrations (µg/L) and linear retention index values on the DB-Wax column for these compounds were shown in Table 3-3. Mean values (µg/L) of the GC analyses of triplicate extractions and standard deviations were calculated. A total of 56 compounds were identified and quantified in traditional wines made from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit grape varieties grown in three different regions; Ankara, Elazığ, Elazığ, and Cappadocia, respectively (Table 3-3).

Table 3-3 Concentration and aromatic parameters of volatile compounds in four different traditionally fermented Turkish wines

No	Compound	LRI[1]	Concentration (µg/L)[2]			
	Alcohols		**Kalecik Karası**	**Boğazkere**	**Öküzgözü**	**Dimrit**
1	1-Propanol	1031	2765.34 ± 103.52^{A}	416.50 ± 51.48^{C}	1129.25 ± 171.71^{BC}	1537.46 ± 784.45^{B}
2	Isobutyl alcohol	1098	12600.61 ± 474.10^{B}	14372.37 ± 811.56^{B}	15759.77 ± 2347.30^{B}	20595.28 ± 1297.96^{A}
3	1-Butanol	1151	479.13 ± 7.32^{A}	265.51 ± 17.55^{B}	318.34 ± 62.41^{AB}	361.95 ± 134.40^{AB}
4	Isoamyl alcohol	1236	113586.80 ± 3887.80^{B}	191703.66 ± 11550.14^{A}	175316.85 ± 21403.99^{A}	195318.93 ± 30127.23^{A}
5	3-Methyl-1-pentanol	1341	61.43 ± 4.53^{B}	165.03 ± 7.61^{A}	155.13 ± 16.99^{A}	184.26 ± 63.87^{A}
6	3-Ethoxy-1-propanol	1389	227.37 ± 9.25^{A}	95.97 ± 6.13^{B}	184.26 ± 31.37^{AB}	208.86 ± 63.69^{A}
7	Methionol	1721	712.09 ± 26.68^{AB}	356.27 ± 16.37^{B}	993.81 ± 85.43^{A}	813.39 ± 263.58^{A}
8	Benzyl alcohol	1853	255.13 ± 8.93^{A}	168.53 ± 18.45^{B}	116.59 ± 24.81^{C}	118.06 ± 7.11^{C}
9	2-Methyl-2-buten-1-ol	1332	ND	216.78 ± 8.43^{A}	187.30 ± 34.62^{A}	170.69 ± 23.40^{A}
10	Phenylethyl alcohol	1916	30431.02 ± 637.41^{A}	60522.15 ± 2616.55^{A}	61564.93 ± 8292.97^{A}	186075.24 ± 2326.96^{A}
11	3-Methyl-3-buten-1-ol	1250	134.04 ± 1.18	ND	ND	ND
12	1-Pentanol	1262	82.61 ± 6.48	ND	ND	ND
13	2,3-Butanediol	1517	5195.34 ± 209.66^{B}	1047.60 ± 80.30^{D}	6508.73 ± 401.42^{A}	2948.01 ± 480.88^{C}
14	1-Heptanol	1421	51.87 ± 4.81^{B}	116.83 ± 13.27^{AB}	92.96 ± 9.05^{B}	175.64 ± 56.37^{A}
15	(Z)-3-Hexen-1-ol	1394	45.11 ± 2.68^{C}	766.47 ± 80.9^{A}	193.47 ± 121.24^{BC}	359.67 ± 61.20^{B}
16	(E)-3-Hexen-1-ol	1384	39.99 ± 3.17^{C}	201.18 ± 2.48^{A}	88.61 ± 9.70^{B}	ND
17	2-(Methylthio) ethanol	1497	62.38 ± 2.90	ND	ND	ND
18	1.2-Propanediol	1583	103.07 ± 5.38	ND	ND	ND
19	3-Penten-2-ol	1160	266.97 ± 7.10^{B}	345.20 ± 13.37^{B}	506.95 ± 128.98^{B}	1470.74 ± 681.98^{A}
20	2-Hexanol	1313	33.77 ± 2.93^{B}	269.58 ± 18.52^{A}	244.83 ± 72.76^{A}	207.28 ± 24.38^{A}
21	1-Hexanol	1347	ND	456.55 ± 38.58^{B}	1411.42 ± 146.54^{A}	593.71 ± 61.33^{B}
22	2-Octanol	-	ND	2344.66 ± 1316.00^{A}	2159.79 ± 1092.65^{A}	2340.28 ± 2063.23^{A}
	Total alcohols		167134.07	273830.84	266932.99	413479.45

Table 3-3 Continued

	Acetates					
23	Isoamyl acetate	1132	807.86 ± 59.84^{B}	1627.56 ± 58.44^{AB}	2444.88 ± 435.56^{A}	1819.43 ± 35.05^{AB}
24	Phenethyl acetate	1827	226.46 ± 14.20^{B}	222.56 ± 15.32^{B}	617.40 ± 114.08^{A}	286.57 ± 6.51^{B}
	Total acetates		1034.32	1850.12	3062.28	2106
	Esters					
25	Ethyl lactate	1363	2059.53 ± 125.75^{A}	609.51 ± 24.12^{B}	1037.43 ± 172.58^{B}	1401.57 ± 602.73^{AB}
26	Ethyl octanoate	1412	150.67 ± 6.73^{A}	161.21 ± 4.47^{A}	173.87 ± 46.73^{A}	155.52 ± 11.42^{A}
27	Ethyl-3-hydroxybutyrate	1472	245.64 ± 24.47^{BC}	175.46 ± 10^{C}	366.87 ± 65.81^{AB}	396.22 ± 61.46^{A}
28	Diethyl succinate	1701	259.38 ± 19.83^{A}	265.75 ± 18.38^{A}	370.06 ± 106.24^{A}	223.18 ± 46.50^{A}
29	Ethyl 4-hydroxybutanoate	1819	3684.69 ± 129.87^{BC}	2279.25 ± 138.25^{C}	7850.77 ± 1115.02^{A}	4362.58 ± 572.37^{B}
30	Monoethyl succinate	2350	2107.06 ± 177.58^{B}	54.13 ± 3.23^{C}	3522.21 ± 563.20^{A}	1444.17 ± 131.51^{B}
31	Ethyl-3-hydroxypropionate	1587	65.18 ± 5.02	ND	ND	ND
32	Ethyl 2-hydroxy-3-phenylpropanoate	2223	135.75 ± 11.14^{A}	113.30 ± 5.95^{A}	ND	174.82 ± 61.29^{A}
33	Methyl 4-hydroxybutanoate	1802	56.39 ± 1.68	ND	ND	ND
34	Ethyl hexanoate	1241	249.93 ± 23.04^{C}	498.31 ± 36.59^{A}	462.10 ± 29.72^{A}	367.28 ± 36.40^{B}
35	Diethyl dl-malate	2053	36.58 ± 2.27	ND	ND	ND
	Total esters		9050.8	4156.92	13783.31	8525.34
	Aldehydes					
36	Nonanal	1658	922.22 ± 0.00	922.22 ± 0.00	922.22 ± 0.00	922.22 ± 0.00
	Total aldehydes		922.22	922.22	922.22	922.22
	Ketones					
37	Acetoin	1291	1292.28 ± 26.83^{A}	964.05 ± 29.37^{B}	566.97 ± 76.85^{C}	357.65 ± 105.05^{D}
	Total ketones		1292.28	964.05	566.97	357.65
	Acids					
38	Butanoic acid	1604	161.70 ± 12.85^{B}	141.25 ± 10.44^{B}	462.64 ± 148.80^{A}	172.25 ± 53.95^{B}
39	Hexanoic acid	1832	556.21 ± 50.67^{B}	313.45 ± 28.59^{B}	1195.88 ± 304.74^{A}	594.27 ± 212.02^{B}

Table 3-3 Continued

40	Octanoic acid	2106	433.89 ± 35.17^{B}	282.36 ± 29.00^{B}	1235.58 ± 291.21^{A}	737.46 ± 226.05^{B}
41	Propanoic acid	1508	124.89 ± 5.71^{B}	60.42 ± 5.22^{B}	159.31 ± 36.20^{A}	157.05 ± 40.51^{A}
42	Nonanoic acid	2157	22.81 ± 0.73^{C}	106.30 ± 6.09^{AB}	165.50 ± 52.89^{A}	63.24 ± 17.58^{BC}
43	Pentanoic acid	1689	463.38 ± 43.73	ND	ND	ND
44	Isobutyric acid	1579	389.59 ± 29.94^{B}	354.43 ± 206.06^{B}	717.02 ± 84.93^{A}	688.80 ± 8.81^{A}
45	Heptanoic acid	1934	74.43 ± 1.94	ND	ND	ND
46	Acetic acid	1403	3414.62 ± 56.48^{A}	212.67 ± 8.20^{C}	1927.60 ± 214.68^{B}	1533.07 ± 333.13^{B}
47	Isovaleric acid	2717	ND	270.69 ± 24.84^{B}	804.24 ± 139.80^{A}	1011.86 ± 98.97^{A}
	Total acids		5641.52	1741.57	6667.77	4958
	Lactones					
48	γ-butyrolactone	1592	1175.99 ± 30.32^{A}	1355.98 ± 109.79^{A}	1865.80 ± 69.91^{A}	1537.02 ± 138.29^{A}
49	Pantolactone	2034	77.34 ± 3.87^{B}	106.10 ± 15.08^{AB}	202.44 ± 76.43^{A}	105.87 ± 7.40^{AB}
	Total lactones		1253.33	1462.08	2068.24	1642.89
	Phenols					
50	2-Methoxy-4-vinylphenol	2168	153.11 ± 7.83^{A}	101.68 ± 8.50^{A}	128.42 ± 14.54^{A}	191.53 ± 24.93^{A}
51	4-Vinyl-phenol	2334	100.22 ± 4.13	ND	ND	ND
	Total phenols		253.33	101.68	128.42	191.53
	Other Compounds					
52	Tyrosol	2965	2217.50 ± 33.10	ND	ND	ND
53	Soleron	2096	97.06 ± 8.48^{B}	135.36 ± 12.69^{AB}	204.48 ± 28.95^{AB}	249.07 ± 105.49^{A}
54	Guaiacol	1840	94.69 ± 4.78^{A}	164.87 ± 10.68^{A}	164.47 ± 56.66^{A}	129.75 ± 57.69^{A}
55	Geraniol	1844	25.89 ± 1.60	ND	ND	ND
56	Syringol	2854	190.71 ± 0.51^{BC}	143.90 ± 10.93^{C}	318.72 ± 68.09^{A}	251.10 ± 26.25^{AB}
	Total other compounds		2625.85	444.13	687.67	629.92
	TOTAL		189207.72	285473.61	294819.87	432813

[1]LRI, linear retention index calculated on a DB-Wax capillary column; [2], Concentration data were the means of three repetitions ± standard deviation in μg/L; ND, not detected. Different superscripts letters indicated statistical differences according to ANOVA by Tukey's Range test ($p \leq 0.05$).

The identified aroma compounds were grouped by chemical families as follows: alcohols, acetates, esters, aldehydes, ketones, acids, lactones, phenols and other compounds. In general, considering the total concentrations of chemical families identified in the red wines made from four varieties grown in three different regions in Türkiye, the major chemical families found were alcohols, esters, and acids. Acetates, lactones, ketones, aldehydes, other compounds, and phenols were identified as minor compounds (Table 3-4).

Table 3-4 The total concentrations (mg/L) of each chemical family in wines of four varieties grown in three different regions in Türkiye

Chemical groups	**Total concentration (mg/L)**			
	Kalecik Karası	**Boğazkere**	**Öküzgözü**	**Dimrit**
Total alcohols	167.13 ± 5.41	273.83 ± 16.67	266.93 ± 34.45	413.48 ± 38.52
Total acetates	1.03 ± 0.07	1.85 ± 0.07	3.06 ± 0.55	2.11 ± 0.04
Total esters	9.05 ± 0.53	4.16 ± 0.24	13.78 ± 2.10	8.53 ± 1.52
Total aldehydes	0.92 ± 0.00	0.92 ± 0.00	0.92 ± 0.00	0.92 ± 0.00
Total ketones	1.29 ± 0.03	0.96 ± 0.03	0.57 ± 0.08	0.36 ± 0.11
Total acids	5.64 ± 0.24	1.74 ± 0.32	6.67 ± 1.27	4.96 ± 0.99
Total lactones	1.25 ± 0.03	1.46 ± 0.12	2.07 ± 0.15	1.64 ± 0.15
Total phenols	0.25 ± 0.01	0.10 ± 0.01	0.13 ± 0.01	0.19 ± 0.02
Total other compounds	2.63 ± 0.05	0.44 ± 0.03	0.69 ± 0.15	0.63 ± 0.19

According to the quantitative data (Table 3-3 and 3-4), the Kalecik Karası wine had 52 volatile compounds and its total concentrations were 189.21 mg/L, including 19 alcohols (167.13 mg/L), 2 acetates (1.03 mg/L), 11 esters (9.05 mg/L), 1 aldehyde (0.92 mg/L), 1 ketone (1.29 mg/L), 9 acids (5.64 mg/L), 2 lactones (1.25 mg/L), 2 phenols (0.25 mg/L) and 5 other compounds (2.63 mg/L).

The Boğazkere wine had 44 volatile compounds with concentration of 285.47 mg/L in total, which included 18 alcohols (273.83 mg/L), 2 acetates (1.85 mg/L), 8 esters (4.16 mg/L), 1 aldehyde (0.92 mg/L), 1 ketone (0.96 mg/L), 8 acids (1.74 mg/L), 2 lactones (1.46 mg/L), 1 phenol (0.10 mg/L) and 3 other compounds (0.44 mg/L).

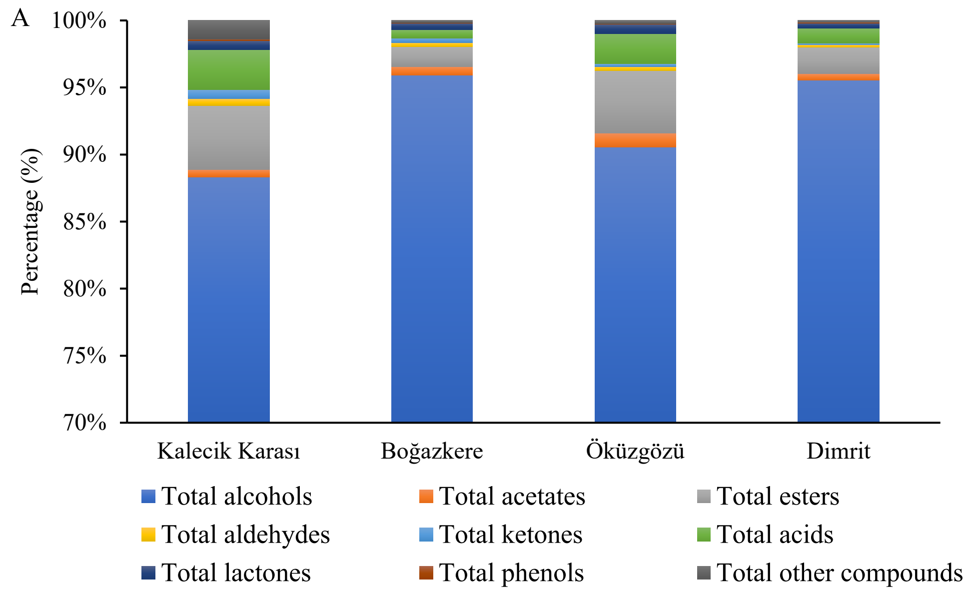
The Öküzgözü wine had 43 volatile compounds and its total concentrations were 294.82 mg/L, including 18 alcohols (266.93 mg/L), 2 acetates (3.06 mg/L), 7 esters (13.78 mg/L), 1 aldehyde (0.92 mg/L), 1 ketone (0.57 mg/L), 8 acids (6.67 mg/L), 2 lactones (2.07 mg/L), 1 phenol (0.13 mg/L) and 3 other compounds (0.69 mg/L).

The Dimrit wine had 43 volatile compounds and its total concentrations were 432.81 mg/L including 17 alcohols (413.48 mg/L), 2 acetates (2.11 mg/L), 8 esters (8.53 mg/L), 1 aldehyde (0.92 mg/L), 1 ketone (0.36 mg/L), 8 acids (4.96 mg/L), 2 lactones (1.64 mg/L), 1 phenol (0.19 mg/L) and 3 other compounds (0.63 mg/L).

Figure 3-6 also demonstrated the contribution of the quantified chemical groups to the volatile profiles of Kalecik Karası, Boğazkere, Öküzgözü and Dimrit wines as percentage in two different graphs in respect to the grape varieties (Figure 3-6 A) and total chemical families (Figure 3-6 B).

As mentioned before, the major chemical families found were alcohols, esters, and acids. Cheng et al (2015) study also obtained the similar result in aroma compound composition of Chardonnay, Cabernet sauvignon, Italian and Merlot wines in China (Cheng et al., 2015). However, Callejon et al (2010) reported acetals were the most abundant volatile compounds in all organic red wines made from Merlot grape variety by inoculation of different selected indigenous and commercial *S. cerevisiae* strains, followed by alcohols without ethanol (Callejon et al., 2010).

Various aroma compounds were commonly detected in wines made from different grape varieties in the world that could be derived from grapes, yeast strain during the fermentation, and the winemaking process (Arcari et al., 2017; Castillo et al., 2020; Cheng et al., 2015; Perestrelo et al., 2020; P. Zhao et al., 2017). Notably, Celik et al, (2019) investigated the impact of malolactic fermentation on the volatile composition of Turkish Kalecik Karası red wines. According to their research, malolactic fermentation was increased the total volatile compounds in both the spontaneously fermented wine and the inoculated wines. Diethyl succinate, ethyl lactate, and γ-butyrolactone content also increased in all Kalecik Karası wines (Celik et al., 2019).



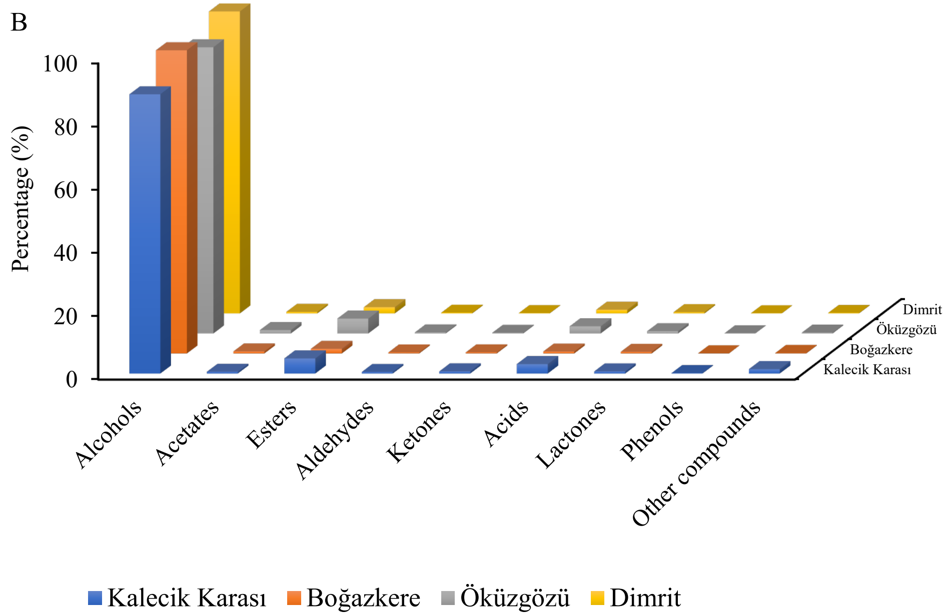


Figure 3-6 Contribution (%) of the quantified chemical groups to the volatile profiles of Kalecik Karası, Boğazkere, Öküzgözü and Dimrit wines demonstrated in two different graphs. X-axis represented the percentage and Y-axis represented the grape varieties (A) and total chemical families (B).

In order to analyze the differences in the four red wines made from Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit varieties grown in three different regions (Ankara, Elazığ, Elazığ, Cappadocia, respectively), a comparison of the subtotal of each chemical family between the wines was individually made (Figure 3-7). To assess the difference between each volatile compound for the tested four red wine samples, a Tukey test (α = 0.05%) was also applied. Both significant and not significant differences were observed between the samples as shown in Table 3-3.

In addition, all statistical analyses carried out for wine aroma compounds were given at Appendix A.

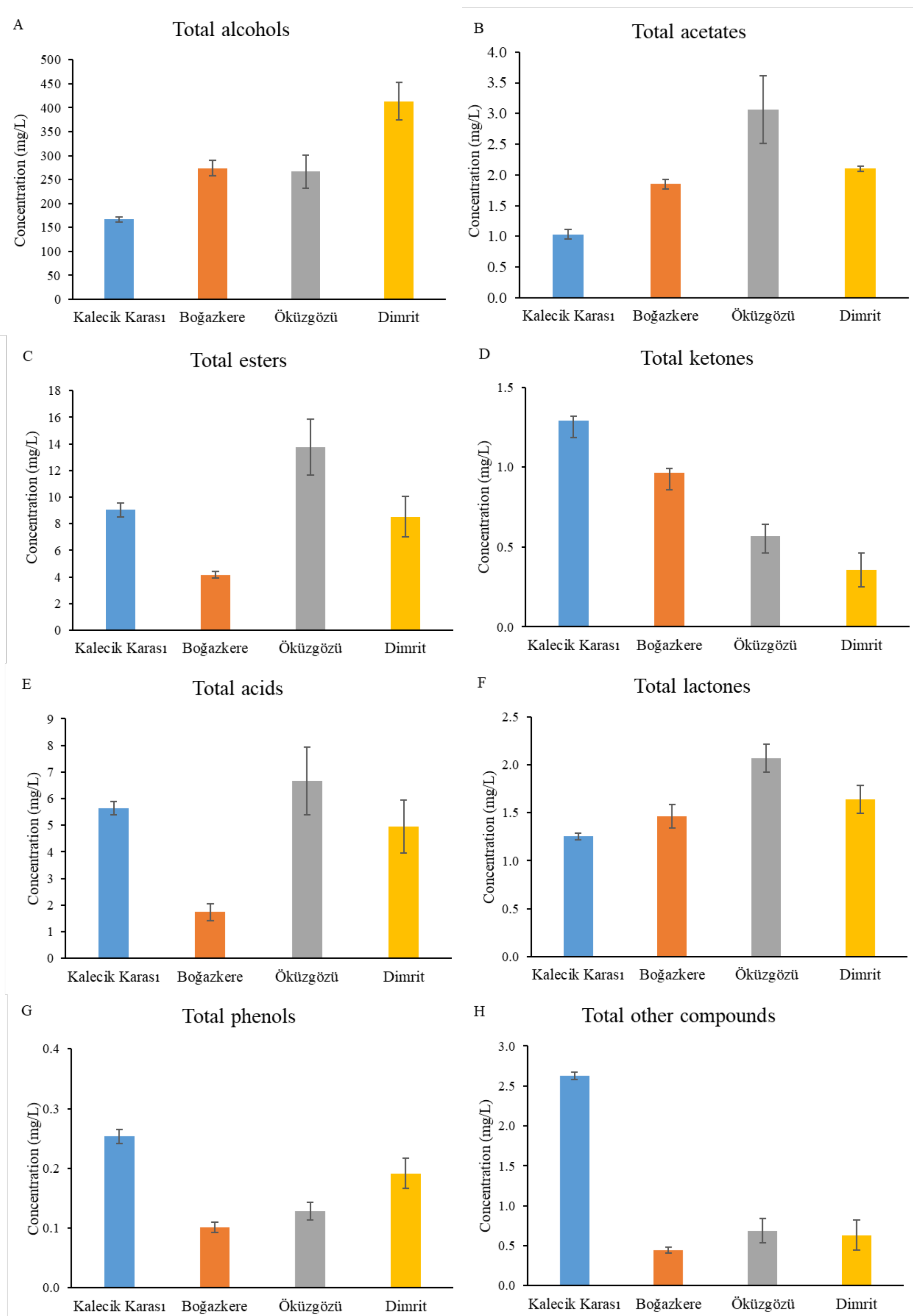


Figure 3-7 Total concentrations of aroma compounds: A, alcohols; B, acetates; C, esters; D, ketones; E, acids; F, lactones; G, phenols; H, other compounds in four different red wines made from Kalecik Karası, Boğazkere, Öküzgözü and Dimrit grape varieties. Bars represented the standard deviation of three repetitions.

Alcohols were found to be the most dominant compounds in all four traditional Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines, because they accounted for the largest proportion of the total volatiles (Table 3-4). These compounds produced during alcoholic fermentation through yeast metabolism via the anabolic pathway (glucose converted into alcohols) and the catabolic pathway (amino acid metabolism, Ehrlich mechanism) which play an important role in the flavor of wines (Duan et al., 2018; Lambrechts & Pretorius, 2019). Among the alcohols, isoamyl alcohol showed the highest concentration in all four traditional red wine as following 195.32, 191.70, 175.32 and 113.59 mg/L in Dimrit, Boğazkere, Öküzgözü and Kalecik Karası wine samples, respectively. The Kalecik Karası wine showed significantly lower level of isoamyl alcohol. Another alcohol present at a very high concentration was 2-phenylethanol (phenylethyl alcohol) in all four spontaneously fermented red wines with the highest concentration of 186.08 mg/L in Dimrit wine, following 61.56, 60.52, and 30.43 mg/L in Öküzgözü, Boğazkere, and Kalecik Karası wines, respectively. In addition, our data showed no clear differences in the concentrations of phenylethyl alcohol between the four wines. While 3-methyl-3-buten-1-ol, 1-pentanol, 2-(methylthio) ethanol, and 1, 2-propanediol were only detected in Kalecik Karası wine which grape variety harvested from Ankara-Kalecik region, 2-methyl-2-buten-1-ol, 2-octanol (not significant), and 1-hexanol (significant) were found in Boğazkere, Öküzgözü, and Dimrit wines which grape varieties harvested from Elazığ, and Cappadocia regions in Türkiye. Moreover, (E)-3-hexen-1-ol was found in all wines except for Dimrit wine produced from grapes grown in Cappadocia with a significant difference in the content. The concentrations of other minor alcohols were also shown in Table 3-3. At concentrations below 300 mg/L, higher alcohols contributed to the desirable complexity of wine; when their concentrations exceed 400 mg/L higher alcohols were regarded as a negative quality factor (Cheng et al., 2015; Rapp & Mandery, 1986). In our study, the total concentration of alcohols in three spontaneously fermented Boğazkere, Öküzgözü and Kalecik Karası wines were below 300 mg/L contributing to the desirable

complexity of aroma except in Dimrit wine with the concentration of 413.48 mg/L (Figure 3-7).

After alcohols, esters were the second volatile with a high quantity in all four traditional red wines. These compounds are a significant group of aromatic compounds produced by various yeast species during fermentation of wine through two pathways. The first one is enzyme-free formation of esters by interaction between an acid and an alcohol. The second one is the enzymatic reaction (Lambrechts & Pretorius, 2019). Different yeast strains were found to release important enzymes that initiated the esters formation in wines during alcoholic fermentation (Bagheri et al., 2018; Callejon et al., 2010; Duan et al., 2018). In our study, ethyl 4-hydroxybutanoate showed the highest concentration in all four traditional red wines with significant differences as following 7.85, 4.36, 3.68 and 2.28 mg/L in Öküzgözü, Dimrit, Kalecik Karası and Boğazkere wines, respectively (Table 3-3). Although ethyl lactate, ethyl octanoate, ethyl-3-hydroxybutyrate, ethyl hexanoate, diethyl succinate, and monoethyl succinate were identified in all wines, ethyl-3-hydroxypropionate, methyl 4-hydroxybutanoate and diethyl dl-malate were only found in Kalecik Karası wine made from grapes grown in Ankara-Kalecik region in Türkiye. Moreover, ethyl 2-hydroxy-3-phenylpropanoate was detected in Kalecik Karası, Boğazkere, and Dimrit wines except in Öküzgözü wine produced from grapes grown in Elazığ without significant differences in the content (Table 3-3). In addition, the total highest concentration of esters was measured in Öküzgözü as 13.78 mg/L, next in Kalecik Karası (9.05 mg/L), Dimrit (8.53 mg/L) and finally Boğazkere (4.16 mg/L) wines (Figure 3-7). The total ester concentrations varied according to different factors, mainly grape varieties, maceration or fermentation temperatures, and indigenous or inoculated yeast strains (Philipp et al., 2021; Rapp & Mandery, 1986). The Philipp et al, (2021) research found 43 different ester compounds in the inoculated and the spontaneously fermented wines made from Gruüner Veltliner, Pinot noir, and Zweigelt grape varieties in Austria (Philipp et al., 2021). Furthermore, 49 esters categorized as ethyl esters, acetate esters, fatty acid

ethyl esters, and other esters were identified in the volatile composition of wine made with *Vitis vinifera L.cv* Cabernet Sauvignon grapes in China (Duan et al., 2018).

In our study, two acetate esters isoamyl acetate and phenethyl acetate were detected in all four traditional Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines. Isoamyl acetate and phenethyl acetate concentrations were significantly higher in Öküzgözü wine produced from grapes grown in Elazığ. These compounds were responsible for the desirably fruity of wine produced by the enzymatic esterification reaction between acetic acid and the corresponding higher alcohol (Castillo et al., 2020).

The third volatile with a high quantity in all four traditional red wines was acids (Table 3-3). Normally acids were produced by different yeast and bacteria species through metabolism of fatty acids (Lambrechts & Pretorius, 2019). In our study, acetic acid was the highest volatile acid in Kalecik Karası, Öküzgözü, and Dimrit wines with the concentrations of 3.41, 1.93 and 1.53 mg/L, respectively. Acetic acid was also found in Boğazkere wine with a concentration of 0.21 mg/L, however this acid did not have the highest content in acid group of Boğazkere wine. According to the literatures (Lambrechts & Pretorius, 2019), more than 90% of the volatile acid of the word wines contained acetic acid. Butanoic acid, hexanoic acid, octanoic acid, propanoic acid, nonanoic acid, and isobutyric acid were detected in all four traditional red wines with significant differences in content. Additionally, the concentration of these acids was significantly higher only in Öküzgözü wine made from grapes grown in Elazığ. While pentanoic acid and heptanoic acid were only identified in Kalecik Karası wine, isovaleric acid was found in Boğazkere, Öküzgözü, and Dimrit wine with a significant different in concentration. Duan et al., (2018) reported almost the same acids in aromatic composition of wine made with *Vitis vinifera L.cv* Cabernet Sauvignon grapes inoculated by different commercial yeasts in China. In another study, seven different acids were found in volatile composition of spontaneously fermented wines made from Sercial, Malvasia de São Jorge, Bastardo, Malvasia Cândida, Verdelho, Boal, Terrantez (white grapes) and Tinta Negra (red grapes) varieties in the Demarcated Region of Madeira, Portugal

(Castillo et al., 2020). Castillo et al, (2020) also reported acetic acid was the largest volatile fatty acid in wines similar to our obtained result. Moreover, the total highest concentration of acids was measured in Öküzgözü as 6.67 mg/L, following Kalecik Karası (5.64 mg/L), Dimrit (4.96 mg/L) and Boğazkere (1.74 mg/L) wines (Figure 3-7).Some studies reported that fatty acids could be affected the complexity of wine aroma both positively (4-10 mg/L) and negatively (above 20 mg/L) which depended on the concentration of acids in wines (Duan et al., 2018; Lambrechts & Pretorius, 2019). In our study, all identified acids showed concentrations below 4 mg/L (Table 3-3), revealing these acids may not significantly influence on the complexity of wine aroma.

Carbonyl compounds composed of aldehydes and ketones were found in all four red wines (Table 3-3). Nonanal was the only aldehyde which detected in Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines with the same concentration (0.92 mg/L). In ketones group, only acetoin was found in Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines with significant differences in concentration as following 1.29, 0.96, 0.57, and 0.36 mg/L, respectively (Figure 3-7). Carbonyl compounds (aldehydes and ketones) may be produced by decarboxylation of acids carried by different yeast species, such as α-keto acids including α- ketobutiric, α-ketolatic, α-ketoisocaproic, α-ketoisovaleric acids and others (Welke et al., 2014). Duan et al., (2018) reported nonanal and acetoin as carbonyl compounds in addition to 5 other aldehydes and one ketone in aromatic composition of wine made with *Vitis vinifera L.cv* Cabernet Sauvignon grapes inoculated by different commercial yeasts in China (Duan et al., 2018). On the other hand, Castillo et al, (2020) study revealed different carbonyl compounds except nonanal and acetoin in carbonyl composition of spontaneously fermented wines made from Sercial, Malvasia de São Jorge, Bastardo, Malvasia Candida, Verdelho, Boal, Terrantez (white grapes) and Tinta Negra (red grapes) varieties in the Madeira, Portugal (Castillo et al., 2020).

In lactone chemical family, γ-butyrolactone (not significant) and pantolactone (significant) were found in all Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines. The total highest concentration of lactones was measured in Öküzgözü as 2.07

mg/L, next in Dimrit (1.64 mg/L), followed Boğazkere (1.46 mg/L) and Kalecik Karası (1.25 mg/L) wines (Figure 3-7).

In phenol group, 2-methoxy-4-vinylphenol was identified in all wines. Our quantitative data showed no clear differences in the concentrations of 2-methoxy-4-vinylphenol between four wines (Table 3-3). The other detected phenol was 4-vinyl-phenol (0.1 mg/L) which only detected in Kalecik Karası wine produced from grapes grown in Ankara-Kalecik region. The total highest concentration of phenols was measured in Kalecik Karası as 0.25 mg/L, next in Dimrit (0.19 mg/L), followed Boğazkere (0.13 mg/L) and Öküzgözü (0.10 mg/L) wines (Figure 3-7).

In other compounds group, tyrosol and geraniol were only found in Kalecik Karası wine while soleron, syringol (significant), and guaiacol (not significant) were identified in Boğazkere, Öküzgözü, and Dimrit wines. The total highest concentration of other compounds was measured in Kalecik Karası as 2.63 mg/L, next in Öküzgözü (0.69 mg/L), followed Dimrit (0.63 mg/L) and Boğazkere (0.44 mg/L) wines (Figure 3-7).

These differences between aroma compounds of four Turkish red wines could be related to the topographical, climatic environmental conditions, microbial and agro-pedological that affected grape and wine composition and quality which referred to “terroir” (Jackson & Lombard, 1993). Cheng et al (2015) study showed that different shoot positions in China affected on the quality and aroma compounds of Chardonnay, Cabernet sauvignon, Italian and Merlot wines (Cheng et al., 2015). Another study revealed that the volatile production and the wine quality were affected by yeast-yeast interactions that meant non-*Saccharomyces* species supported or inhibited the growth of other *Saccharomyces* or non-*Saccharomyces* species in the complex consortium (Bagheri et al., 2018). Interestingly in Austria, Philipp et al (2021) revealed that inoculated wines with single active dry yeast strains inhibited the diversity of the chemical compounds and also decreased aroma complexity which could be related to the suppression of the regional wine microbial ecosystems responsible for spontaneous fermentations (Philipp et al., 2021).

According to the obtained result, spontaneously fermented grape juice is becoming a more common in global wine production (Philipp et al., 2021).

3.2.2 Calculation of Odor Activity Values

In order to identify the most important wine odorants of spontaneously fermented wines, the aroma index (odor activity value, OAV) was calculated for all the identified chemical species. Volatiles with OAV > 1 were commonly considered the compounds able to contribute to wine aroma and also perceived by the human nose (Arcari et al., 2017). Those aroma active compounds with OAV between 0.1 and 1 might also contribute to the formation of wine aroma due to the interaction effect in the wine matrix (Table 3-5).

Among the 52, 44, 43, 43 quantitated compounds in the Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines, 12, 12, 12, 13 compounds reached concentrations higher than their odor threshold (OAV> 1), respectively that meant these volatile compounds contributed their flavor notes to the overall aroma in the four Turkish wines as shown in Table 3-5.

Table 3-5 Odor activity value of aroma compounds (OAV>1), odor threshold and odor description in four Turkish wines

No	Compound	LRI*	Odor Threshold (μg/L)	Odor description**	Odor activity value			
					Kalecik Karası	Boğazkere	Öküzgözü	Dimrit
	Alcohols							
1	Isoamyl alcohol	1236	60000	Whiskey, nail polish (1)	1.89	3.20	2.92	3.25
2	3-Ethoxy-1-propanol	1389	100	Fruity (2, 4)	2.27	1	1.84	2.09
3	Methionol	1721	500	Cooked vegetable (4), raw potato, garlic (2)	1.42	<1	1.99	1.63
4	Phenylethyl alcohol	1916	10000	Rose, pollen, perfume, honey (1, 3)	3.04	6.05	6.16	18.60
5	(*Z*)-3-Hexen-1-ol	1394	400	Green, cypress (1)	<1	1.92	<1	1
	Acetates							
6	Isoamyl acetate	1132	30	Banana, fruity, sweet (1)	26.93	54.25	81.50	60.65
7	Phenethyl acetate	1827	250	Pleasant, floral (1), rose (2)	1	1	2.47	1.15
	Esters							
8	Ethyl octanoate	1412	5	Fruity, pineapple, floral (1), sweet, fresh (2)	30.13	32.24	34.77	31.10
9	Ethyl hexanoate	1241	5	Flowery, fruity (1), ripe banana (2)	49.99	99.66	92.42	73.46
	Aldehydes							
10	Nonanal	1658	2.8	Citrusy, floral (4)	329.36	329.36	329.36	329.36
	Acids							
11	Isovaleric acid	2717	30	Rancid, cheese, floral (2)	ND	9.02	26.81	33.73
	Phenols							
12	2-Methoxy-4-vinylphenol	2168	40	Spices, curry (4)	3.83	2.54	3.21	4.79
	Other Compounds							
13	Guaiacol	1840	10	Smoke, sweet, medicine (4)	9.47	16.49	16.45	12.98
14	Geraniol	1844	20	Roses, geranium (3)	1.29	ND	ND	ND

*, LRI, linear retention index calculated on a DB-Wax capillary column; ND, not detected.
**, Numbers in parenthesis represented references as: 1, (X.-C. Wang et al., 2017); 2, (Celik et al., 2019); 3, (Arcari et al., 2017); 4, (Welke et al., 2014).

Among alcohols, isoamyl alcohol (whiskey, nail polish aroma), 3-ethoxy-1-propanol (fruity aroma), methionol or 3-methylthio-1-propanol (cooked vegetable, raw potato aroma), phenylethyl alcohol (rose, pollen, perfume aroma) were found to contribute their flavor notes to the overall aroma in the all four Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wine samples due to their high OAV values. However, (*Z*)-3-Hexen-1-ol compound responsible for the green, cypress flavor (X.-C. Wang et al., 2017) was only contributed to Boğazkere wine aroma (Table 3-5). As mentioned before, these compounds produced during alcoholic fermentation through yeast metabolism via the anabolic pathway (glucose converted into alcohols) and the catabolic pathway (amino acid metabolism, Ehrlich mechanism) which play an important role in the flavor of wines (Duan et al., 2018; Lambrechts & Pretorius, 2019). It was also reported that production of higher alcohols was related to the *S. cerevisiae* strains which carried out the fermentation process (Capozzi et al., 2015; Castillo et al., 2020). Among alcohols, isoamyl alcohol was responsible of fragrant component of alcohols and represented the most present in wine (Capozzi et al., 2015). Celik et al, (2019) also reported 3-ethoxy-1-propanol (fruity aroma) and methionol (cooked vegetable, raw potato aroma) in the volatile composition of Turkish Kalecik Karası red wines (Celik et al., 2019). Moreover, 2-phenylethanol (phenylethyl alcohol) biosynthesized from phenylalanine was associated with aromatic descriptors of roses and found in volatile composition of different wines (Arcari et al., 2017; Castillo et al., 2020; Celik et al., 2019; Duan et al., 2018; X.-C. Wang et al., 2017).

In acetates categories, isoamyl acetate reported to exhibit fruity, banana and sweet scents (Wang et al., 2017) was found in all Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines. Phenethyl acetate (rose, floral) was also found to provide flavor notes to the overall aroma in all Kalecik Karası, Boğazkere, Öküzgözü, Dimrit wines due to their high OAV (Table 3-5). These compounds were responsible for the desirably fruity of wine produced by the enzymatic esterification reaction between acetic acid and the corresponding higher alcohol (Castillo et al., 2020). Various

studies reported these two acetate esters were contributed their flavor notes to the overall wine aroma (Castillo et al., 2020; Celik et al., 2019; X.-C. Wang et al., 2017).

In esters groups, ethyl octanoate responsible for fruity, pineapple, floral aroma and ethyl hexanoate responsible for flowery and fruity aroma were detected in all Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines. These two ethyl esters were found to contribute their flavor notes to the overall aroma in all four Turkish red wine samples due to their high OAV values (Table 3-5). These esters gave a fruity and floral flavor to the wine overall aroma in different countries as well (Arcari et al., 2017; Cheng et al., 2015; Duan et al., 2018; X.-C. Wang et al., 2017). Duan et al., (2018) reported ethyl octanoate and ethyl hexanoate as fatty acid ethyl esters compounds in aromatic composition of wine made with *Vitis vinifera L.cv* Cabernet Sauvignon grapes inoculated by different commercial yeasts in China (Duan et al., 2018).

In carbonyl compounds, only one aldehyde (nonanal) was detected in all red wines which due to its OAV value could be incorporated into the Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines overall aroma (Table 3-5). Cai et al. (2014) showed that carbonyl compounds were able to indirectly contribute their flavor notes to the overall aroma in wine through a synergetic effect, although their concentration in wine was relatively low (Cai et al., 2014). In our study, all Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines showed the same concentrations of nonanal at the end of fermentation, indicating that these wine samples might had citrusy (Welke et al., 2014) or green (Duan et al., 2018) flavors. Duan et al. (2018) study was reported nonanal could give a unpleasant flavor to wines (Duan et al., 2018), oppose to Welke et al., (2014) research that reported this aldehyde contributed to a positive citrusy and floral aroma in wine (Welke et al., 2014).

In acids categories, only isovaleric acid was identified in Boğazkere, Öküzgözü, Dimrit wines not in Kalecik Karası wine. This acid gave a cheese and floral flavor (Celik et al., 2019) to the Boğazkere, Öküzgözü and Dimrit wines overall aroma because of their OAV value (Table 3-5). Although Celik et al., (2019) study reported

Turkish Kalecik Karası red wines possessed isovaleric acid in the volatile composition, this acid was not found in the volatile composition of Kalecik Karası wine in our study.

In phenols groups, 2-methoxy-4-vinylphenol (4-vinylguaiacol) was detected in all Kalecik Karası, Boğazkere, Öküzgözü, Dimrit wines (Table 3-5), which contributed to spices, curry flavor (Welke et al., 2014) in these wines (OAV>1).

In other compounds groups, guaiacol was detected in all Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines and gave smoke, sweet flavor (Welke et al., 2014) to these wines because of OAV>1 . However, Geraniol responsible for roses, geranium aroma (Arcari et al., 2017) was identified only in Kalecik Karası wine that its flavor notes contributed to the overall aroma in Kalecik Karası wine (Table 3-5).

3.2.3 Principal Component Analysis

Principal component analysis (PCA) was carried out to investigate the aroma compositional similarity of Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wine samples by applying all detected aroma compounds with OIV bigger than 1 (OAV>1) as variables (Figure 3-8), since these aroma compounds were the main volatiles that contributed to the overall aroma in these four red wines. According to the PCA results, two different principal components (PC) were identified, and these two PCs revealed 81.34 % of the total variance (factor one; F1: 51.34 %, factor two; F2: 29.99 %). Figure 3-8 (A) displayed the projection of the variables with regard to the single factor (PC1 or PC2) on the PC1 × PC2 factor plane. Figure 3-8 (B) showed two different groups in the score plot. Concerning the score plot, the first group was Boğazkere, Öküzgözü, and Dimrit wines produced from grapes grown in Elazığ, Elazığ, and Cappadocia, respectively. These wines were located at the positive position in the PC1. Kalecik Karası wine was characterized in the second group which made from grapes grown in Ankara-Kalecik region in Türkiye and was positioned on the negative scale of the PC1. As shown in Figure 3-8, the first group

was positively correlated with phenylethyl alcohol, isovaleric acid, phenethyl acetate, isoamyl acetate, ethyl octanoate, isoamyl alcohol, guaiacol, ethyl hexanoate and (z)-3-hexen-1-ol with regard to PC1 which played important roles in the aromatic characteristic of the Boğazkere, Öküzgözü, and Dimrit wines, whereas the second group was negatively associated with methionol, 2-methoxy-4-vinylphenol, 3-ethoxy-1-propanol and geraniol with respect to PC1. Regarding the four red wines, PCA demonstrated that grape varieties had a strong effect on the aroma compounds.

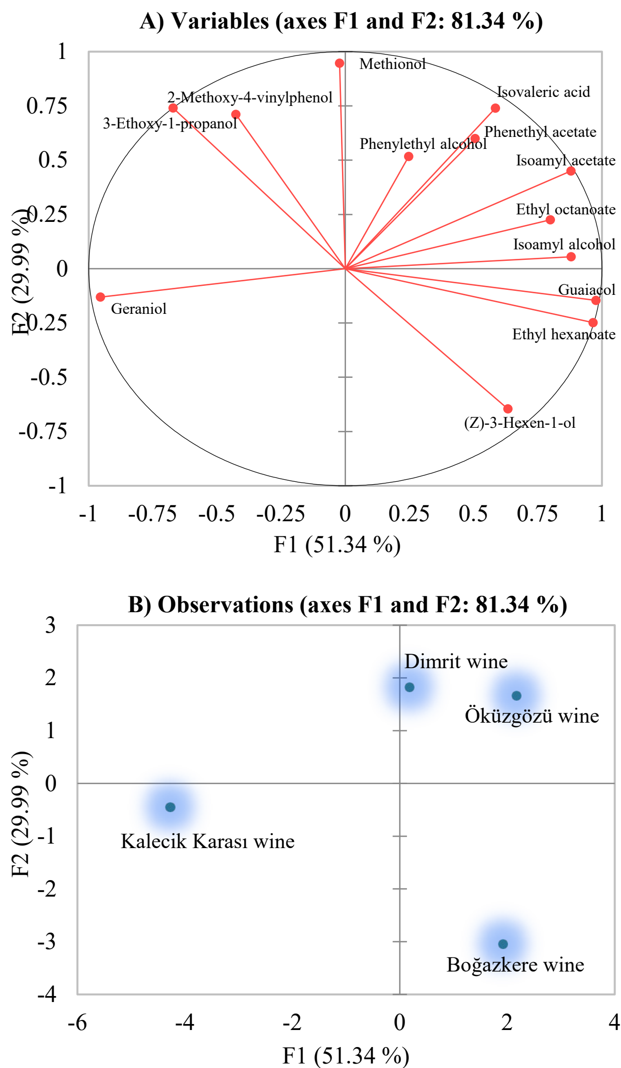


Figure 3-8 Principal component analysis of aroma compounds with OAV value above 1 in the wines made from four red grape varieties. A, Projection of the quantified aroma compounds with OAV>1 (variables) on the factor plane (PC1 × PC2). B, Score plot for the two principal components representing these four red wines in Türkiye.

3.2.4 Sensory Analysis of Wine Samples

The result of the sensory analysis for four red wines carried out by six expert sensory assessors were shown in spider (radar) chart (Figure 3-9).

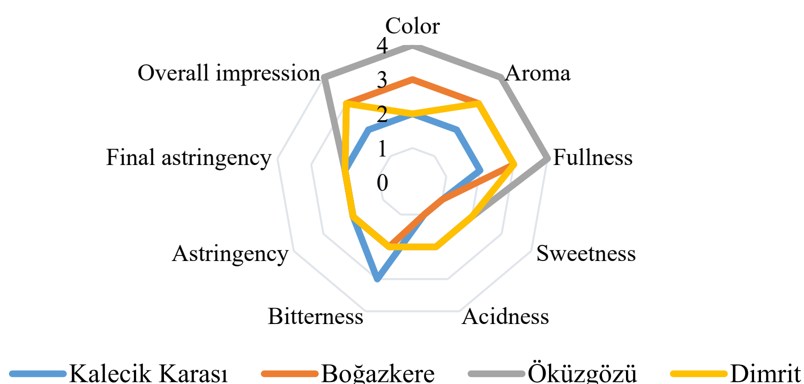


Figure 3-9 Spider chart for sensory analyses of Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines.

As represented in Figure 3-9, the Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines were assessed from zero (week) to five (strong) by the degustators according to nine criteria as following; color, aroma, sweetness, bitterness, fullness, acidity, astringency, final astringency, and overall impression. The obtained result indicated that Öküzgözü wine was more desirable based on color, aroma, fullness, and overall impression and got the highest score in these criteria. The aroma and fullness of Kalecik Karası wine were evaluated lower than other wines while the bitterness of this wine was assessed higher. The sweetness and acidness of Öküzgözü, Dimrit wines were found higher than the Kalecik Karası, and Boğazkere wines. All wines were assessed the same in terms of astringency, and final astringency. In terms of overall impression, Öküzgözü wine obtained the highest score and Kalecik Karası wine get the lowest sensory score. Moreover, Boğazkere and Dimrit wines were in good quality and got the same score. Therefore, it is worth analyzing the yeast population of these traditional wines. PCR techniques were carried out to determine the yeast diversity.

3.3 Real-Time PCR and Quantitative PCR (qPCR) Analyses

3.3.1 Biodiversity Detection of non-*Saccharomyces* and *Saccharomyces* Yeast Species in Spontaneously Fermented Wines

Non-*Saccharomyces* and *Saccharomyces* yeast species were detected in fresh grape must, during cold maceration, maceration and alcoholic fermentation of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir samples by using 16 primers as mentioned in Table 2-2 which were namely *Candida glabrata*, *Candida zemplinina* (*Starmerella bacillaris*), *Candida zeylanoides*, *Hanseniaspora* spp., *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Lachancea thermotolerans*, *Metschnikowia* spp., *Metschnikowia pulcherrima*, *Pichia fermentans*, *Pichia kluyveri*, *Rhodotorula mucilaginosa*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus* (*Pichia anomala*), *Saccharomyces* spp., and *Saccharomyces cerevisiae* (Díaz et al., 2013; García et al., 2017; X. Wang et al., 2020; Zott et al., 2010).

Amplification plots were created by plotting the fluorescent signal from each sample against cycle number which represented the accumulation of the target products over the duration of the real-time PCR experiment. Amplification plots of all must/wine samples of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir were depicted in the Appendices B, C, D, E and F, respectively. In all amplification plots, the green straight line represented the threshold line. No amplification was observed in no template controls (NTC).

The specificity of a real-time PCR assay was determined by the primers and reaction conditions used. The specificity of the real-time PCR reactions was also confirmed by running melting curve analysis. Melting curve analysis was carried out for each assay by monitoring fluorescence continuously between 54°C and 95°C with 0.5°C increments for cells. Change in fluorescence was plotted over temperature to determine at what point denaturation of PCR amplicons occurs. A single peak showed that a single PCR product was amplified in each reaction (Martorell, Querol,

et al., 2005). Melting curve analyses of the target non-*Saccharomyces* and *Saccharomyces* yeast products of the must/wine of the Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir samples were represented in Appendices G and H.

The detection results obtained by real-time PCR method were shown in Table 3-6 and 3-7. Non-*Saccharomyces* and *Saccharomyces* yeast species detected in fresh grape must, during cold maceration, and maceration (0.CM, 0 day of cold maceration; 4.CM, fourth day of cold maceration; 2.M, second day of maceration; 4.M, fourth day of maceration; 6.M, sixth day of maceration) of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit samples (Table 3-6) and Emir samples during 0.W (0. week), 1.W (1st week), 2.W (2nd week), 3.W (3th week) and 4.W (4th week) of fermentation (Table 3-7) were as following;

Table 3-6 Non-*Saccharomyces* and *Saccharomyces* yeasts detection in Kalecik Karası, Boğazkere, Öküzgözü and Dimrit samples by real-time PCR

	Kalecik Karası				**Boğazkere**			**Öküzgözü**			**Dimrit**		
	Sampling (Day)												
Yeast species	**0. CM**	**4. CM**	**2. M**	**4. M**	**0. CM**	**4. CM**	**6. M**	**0. CM**	**4. CM**	**6. M**	**0. CM**	**4. CM**	**4. M**
Candida glabrata	-	-	+	+	+	+	+	-	-	-	+	+	+
Candida zemplinina	+	+	+	+	-	-	-	-	-	-	+	+	+
Candida zeylanoides	-	-	-	-	-	-	-	-	-	-	-	-	-
Hanseniaspora spp.	+	+	+	+	+	+	+	+	+	+	+	+	+
Hanseniaspora uvarum	+	+	+	+	+	+	+	+	+	+	+	+	+
Issatchenkia orientalis	+	+	+	+	+	+	+	+	+	+	+	+	+
Lachancea thermotolerans	+	+	+	+	-	-	-	+	+	+	-	-	-
Metschnikowia spp.	+	+	+	+	+	+	+	-	-	-	+	+	+
Metschnikowia pulcherrima	-	-	-	-	-	-	-	-	-	-	-	-	-
Pichia fermentans	-	-	-	-	+	+	+	+	+	+	+	+	+
Pichia kluyveri	+	+	+	+	-	-	-	-	-	-	-	-	-
Rhodotorula mucilaginosa	+	+	+	+	-	-	-	-	-	-	-	-	-
Saccharomyces spp.	+	+	+	+	+	+	+	+	+	+	+	+	+
Saccharomyces cerevisiae	+	+	+	+	+	+	+	+	+	+	+	+	+
Torulaspora delbrueckii	+	+	+	+	-	-	-	-	-	-	-	-	-
Wickerhamomyces anomalus	+	+	+	+	-	-	-	-	-	-	-	-	-

0.CM, 0. day of cold maceration; 4.CM, 4th day of cold maceration; 2.M, 2nd day of maceration; 4.M, 4th day of maceration; 6.M, 6th day of maceration; Positive (+), detected; Negative (-), not detected.

Table 3-7 Non-*Saccharomyces* and *Saccharomyces* yeasts detection of Emir must and wine samples by real-time PCR

Yeast species	Emir				
	Sampling (Week)				
	0.W	**1.W**	**2.W**	**3.W**	**4.W**
Candida glabrata	-	-	-	-	-
Candida zemplinina	-	-	-	-	-
Candida zeylanoides	-	-	-	-	-
Hanseniaspora spp.	+	+	+	+	+
Hanseniaspora uvarum	+	+	+	+	+
Issatchenkia orientalis	+	+	+	+	+
Lachancea thermotolerans	-	-	-	-	-
Metschnikowia spp.	-	-	-	-	-
Metschnikowia pulcherrima	-	-	-	-	-
Pichia fermentans	-	-	-	-	-
Pichia kluyveri	-	-	-	-	-
Rhodotorula mucilaginosa	-	-	-	-	-
Saccharomyces spp.	+	+	+	+	+
Saccharomyces cerevisiae	+	+	+	+	+
Torulaspora delbrueckii	+	+	+	-	-
Wickerhamomyces anomalus	+	+	+	+	+

W, week of alcoholic fermentation; Positive (+), detected; Negative (-), not detected.

Hanseniaspora spp., *H. uvarum*, *I. orientalis, Saccharomyces spp*., and *S. cerevisiae* were detected in all four red Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit grape musts/wines samples of 0 day of cold maceration, 4th day of cold maceration, 2th, 4th and 6th day of maceration while *C. zeylanoides* and *M. pulcherrima* were not detected in these samples (Table 3-6).

Although *P. kluyveri*, *R. mucilaginosa*, *T. delbrueckii* and *W. anomalus* (*P. anomala*) were only observed in Kalecik Karası samples in 0 and 4th day of cold maceration, 2nd and 4th day of maceration, *P. fermentans* was not detected.

Moreover, *Metschnikowia* spp. and *C. glabrata* were found in three of samples (Kalecik Karası, Boğazkere and Dimrit) but not detected in Öküzgözü must/wine

samples. Also, *C. glabrata* was only detected in 2nd and 4th day of maceration of Kalecik Karası samples, not detected during cold maceration.

In addition, *C. zemplinina* (*Starmerella bacillaris*) was seen in two samples of Kalecik Karası and Dimrit during cold maceration, and maceration while *L. thermotolerans* was only found in Kalecik Karası and Öküzgözü must/wine samples during cold maceration, and maceration times (Table 3-6).

In regard to Emir samples (white grape variety), *Hanseniaspora* spp., *H. uvarum*, *I. orientalis, Saccharomyces spp.*, *S. cerevisiae* and *W. anomalus (P. anomala)* were detected during 0, fist, second, third, and fourth weeks of fermentation except for *T. delbrueckii* which only detected during 0.W, 1.W, and 2.W of fermentation. Furthermore, *C. glabrata, C. zemplinina*, *C. zeylanoides*, *L. thermotolerans*, *Metschnikowia spp., M. pulcherrima*, *P. fermentans*, *P. kluyveri* and *R. mucilaginosa* were not found in Emir grape must and wine samples (Table 3-7).

According to the reviewed literatures, real-time PCR assay was rarely used to only detect target non-*Saccharomyces* and *Saccharomyces* yeast species in grape must and wine samples (Díaz et al., 2013; García et al., 2017; Hierro et al., 2006; Lleixà et al., 2018; Phister et al., 2007; C. Wang, Esteve-Zarzoso, et al., 2015; X. Wang et al., 2020; Zott et al., 2010), these studies generally used quantitative real-time PCR method (instead of real-time PCR) to quantify target yeasts in grape must and wine samples. Therefore, in qPCR part of this study, all these papers were discussed.

3.3.2 Comparative Detection of Non-*Saccharomyces* and *Saccharomyces* Yeast Species in Grape Must or wine Samples

Target non-*Saccharomyces* and *Saccharomyces* yeast species from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir samples were determined and detected (Table 3-6 and 3-7) by real-time PCR using specific oligonucleotide primers (Table 2-2). The must and wine samples obtained at various stages were analyzed in parallel for both real-time PCR detection and isolation of strains. The detection results

obtained by real-time PCR method were compared with the results of colony isolation and identification by internal transcribed spacer (ITS) region (ITS1–5.8S rRNA– ITS2) and/or D1/D2 domain of the 26S rRNA gene sequencing (Table 3-8).

Table 3-8 Comparative detection of non-*Saccharomyces* and *Saccharomyces* yeast species in Turkish grape must samples by real-time PCR and isolation/DNA sequencing

	Grape variety									
Yeast species	**Kalecik Karası**		**Boğazkere**		**Dimrit**		**Öküzgözü**		**Emir**	
	RT-PCR	Isolation/ DNA sequencing	RT-PCR	Isolation/ DNA sequencing	RT-PCR	Isolation/ DNA sequencing	RT-PCR	Isolation/ DNA sequencing	RT-PCR	Isolation/ DNA sequencing
C. glabrata	+	-	+	-	+	-	-	-	-	-
C. zemplinina	+	-	-	-	+	+	-	-	-	-
C. zeylanoides	-	-	-	-	-	-	-	-	-	-
Hanseniaspora spp.	+	+	+	-	+	-	+	+	+	+
H. uvarum	+	+	+	-	+	-	+	-	+	+
I. orientalis	+	-	+	-	+	-	+	-	+	-
L. thermotolerans	+	-	-	-	-	-	+	+	-	-
Metschnikowia spp.	+	+	+	+	+	+	-	-	-	-
M. pulcherrima	-	-	-	-	-	-	-	-	-	-
P fermentans	-	-	+	-	+	-	+	-	-	-
P. kluyveri	+	-	-	-	-	-	-	-	-	-
R. mucilaginosa	+	+	-	-	-	-	-	-	-	-
Saccharomyces spp.	+	+	+	+	+	+	+	+	+	+
S. cerevisiae	+	+	+	+	+	+	+	+	+	+
T. delbrueckii	+	-	-	-	-	-	-	-	+	-
W. anomalus	+	+	-	-	-	-	-	+	+	+

RT-PCR, real-time polymerase chain reaction; Positive (+), detected; Negative (-), not detected.

204 non-*Saccharomyces* and 265 *Saccharomyces* yeast species were isolated at different cold maceration, maceration and fermentation times from five different grape must/wines by using selective growth media (Aktuna, 2019). Then, selected yeasts (104 non-*Saccharomyces* and 77 *Saccharomyces* yeasts) were identified by ITS region and/or D1/D2 domain of the 26S rRNA gene sequencing. Almost all non-*Saccharomyces* yeasts of red grape samples were isolated in cold maceration (CM) and maceration (M) stages. According to ITS region and/or D1/D2 domain sequencing results, 9 non-*Saccharomyces* species belonging to 7 genera and 1

Saccharomyces species were identified; *C. zemplinina (Starmerella bacillaris)*, *Hanseniaspora* spp. (*H. uvarum*, *H. opuntiae*, *H. guilliermondii*), *L. thermotolerans*, *Metschnikowia* spp., *R. mucilaginosa*, *Solicoccozyma aeria*, *W. anomalus* (*Pichia anomala*) and *S. cerevisiae* (Aktuna, 2019).

Isolation/sequencing results were in agreement with the results of real-time PCR in respect to *C. zemplinina*, *Hanseniaspora* spp., *H. uvarum*, *R. mucilaginosa*, *W. anomalus*, *L. thermotolerans*, *Metschnikowia* spp., *Saccharomyces* spp. and *S. cerevisiae* detection. Moreover, *C. zeylanoides* was not detected neither by real-time PCR nor isolation/sequencing method. However, several species such as *C. glabrata*, *I. orientalis*, *P. fermentans*, *P. kluyveri*, *T. delbrueckii* were only detected by real-time PCR. This was an expected result due to the presence of viable but non-culturable microorganisms in grape must or wine samples and low detection level of cells (dead and viable) in real time PCR. Monoazide dyes (propidium monoazide bromide and ethidium monoazide bromide) were not used to eliminate the DNA from dead cells (Andorrà et al., 2010; Navarro et al., 2020) in order to reveal the diversity of non-*Saccharomyces* and *Saccharomyces* yeast (both dead and live cells) in the complex yeast community in Turkish grape must and wine (García et al., 2017). Zot et al (2010) study reported that only 1% of the dead cells were detected in microbial ecosystem of grape must and wine (Zott et al., 2010).

Species members belonging to *Metschnikowia* genus were detected in 3 wine varieties (Kalecik Karası, Boğazkere, Dimrit) by real-time PCR (Table 3-8) using genus specific primers designed by Wang et al. (2020). In a similar manner, members of these *Metschnikowia* spp. were also isolated and identified at genus level by isolation/sequencing results. In none of the grape samples, *M. pulcherrima* was detected by real-time PCR using primers designed by Zott et al. (2010). In fact, Zott et al. (2010), in their study, also reported that specific primers they designed for *M. pulcherrima* indicated low efficiency (51%) of amplification specificity in standard curve development for quantification. Similarly, Ženišová et al. (2014) also reported that detection of their *M. pulcherrima* isolates was not achieved by primers MPL3, MPR3 (designed by Zott et al., 2010) using real-time PCR. Moreover, the results

have indicated that the *Metschnikowia* spp. detected in Kalecik Karası, Boğazkere, and Dimrit must samples were most probably not *M. pulcherrima* but another species in those three samples since *M. pulcherrima* was not isolated.

Zott et al., (2010) also found similar result in respect to other non-*Saccharomyces* species, especially *H. uvarum*, *C. zemplinina* (*Starmerella bacillaris*), *T. delbrueckii* and *I. orientalis* at the end of the cold maceration and early stage of fermentation process by real-time PCR method (Zott et al., 2010).

Study on yeast biodiversity and the effect of yeast species on wine quality has lately become an important issue in wine microbiology. In this study, it was shown that real-time PCR was useful for tracking the presence of target yeast species. The results of real-time PCR detection were in agreement with those of isolation and identification with DNA sequencing. Real-time PCR allows a reliable and sensitive detection of low yeast cells. Thus, more diversity of yeasts was obtained according to the results of real-time PCR.

3.3.3 Absolute Quantification and Standard Curves Construction for qPCR Analysis

According to the literature reviews and our preliminary experiments, non-*Saccharomyces* species, which can positively affect the aroma or taste of wine and can be used as starter cultures, were selected for quantification in this section (S. Benito, 2018a, 2018b; Borren & Tian, 2020; Garofalo et al., 2018; Lambrechts & Pretorius, 2019; Puertas et al., 2017). *H. uvarum, L. thermotolerans, T. delbrueckii* as non-*Saccharomyces* species and *S. cerevisiae* were selected for quantification in Kalecik Karası, Boğazkere, Öküzgözü, Dimrit grape must and wine samples.

In order to quantify the amount of these target Non-*Saccharomyces* and *Saccharomyces* yeasts in unknown must and wine samples, external calibration curves were prepared to reproduce highly specific and reliable data. For standard curve construction, four reference yeast strains *H. uvarum, L. thermotolerans, T.*

delbrueckii and *S. cerevisiae* from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) were used. These four different strains *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* were serially diluted in red wine and used for the construction of the standard curves. Standard curves were created by plotting the cycle threshold (C_T) values of the qPCRs performed on DNA of dilution series of yeast cells against the colony forming unit (CFU) per mL (Figure 3-10, 3-11, 3-12, 3-13).

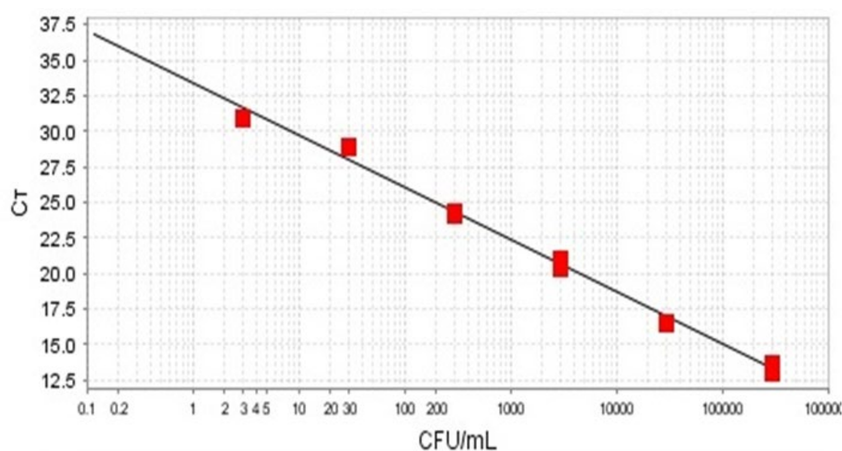


Figure 3-10 Standard curve constructed for quantification of abundance of *H. uvarum* in must or wine samples

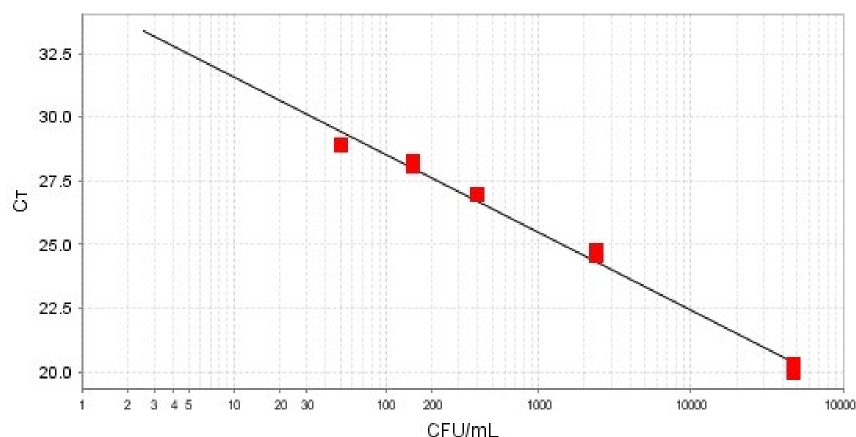


Figure 3-11 Standard curve constructed for quantification of abundance of *L. thermotolerans* in must or wine samples

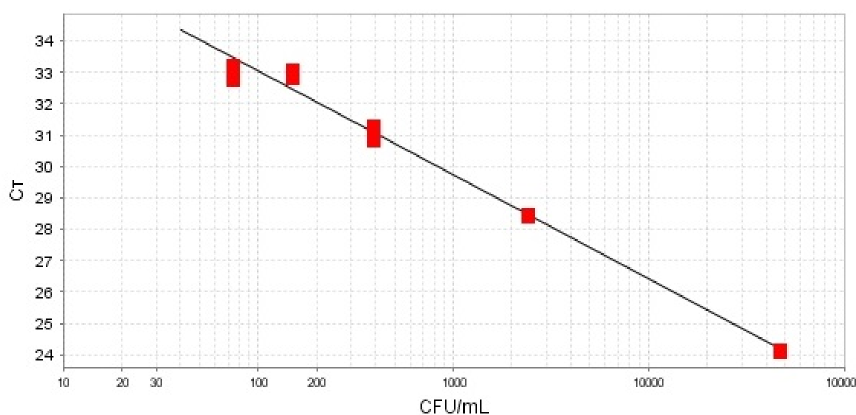


Figure 3-12 standard curve constructed for quantification of abundance of *T. delbrueckii* in must or wine samples

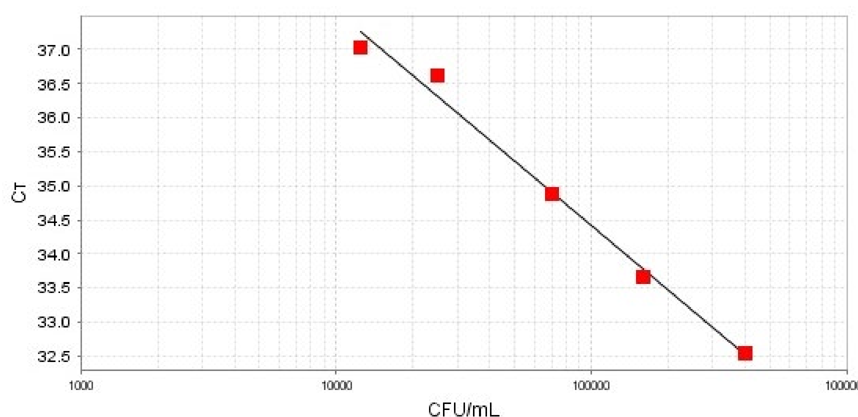


Figure 3-13 Standard curve constructed for quantification of abundance of *S. cerevisiae* in must or wine samples

The amplification efficiency (E) was calculated using the slope of the regression line in the standard curve. Efficiency and percentage of efficiency were estimated by the formula $E = 10^{-1/slope}$ and E (%) = (E-1) × 100 (Pfaffl, 2019). A slope close to -3.32 indicated optimal 100% PCR amplification efficiency. Correlation coefficients (r^2), slope, intersection and efficiency (%) of standard curves obtained from serial dilutions of yeast cells of *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* were shown in Table 3-9. The obtained data were mean ± standard deviation of triplicate qPCR amplifications.

Table 3-9 Correlation coefficients (r^2), slope, intersection and percentage of efficiency of standard curves conducted with four reference yeast species

Yeast strain	**Correlation coefficients**	**Slope**	**Intersection**	**Efficiency (%)**
Hanseniaspora uvarum	0.993 ± 0.00	-3.659 ± 0.05	33.330 ± 0.41	87.6 ± 8.90
Lachancea thermotolerans	0.991 ± 0.00	-3.043 ± 0.04	34.568 ± 0.61	113.1 ± 3.72
Torulaspora delbrueckii	0.990 ± 0.00	-3.315 ± 0.01	39.652 ± 0.05	100.2 ± 0.45
Saccharomyces cerevisiae	0.989 ± 0.00	-3.145 ± 0.08	50.134 ± 0.61	107.9 ± 5.29

Data were mean ± standard deviation of triplicate qPCR amplifications. Efficiency was estimated by the formula $E= 10^{-1/slope}$ and E (%) = (E-1) × 100.

The result of quantification of *S. cerevisiae* and three predominant non-*Saccharomyces* yeasts *H. uvarum, L. thermotolerans*, and *T. delbrueckii* present in wine/must samples of Kalecik Karası, Boğazkere, Öküzgözü and Dimrit, during cold maceration (CM), maceration (M) and alcoholic fermentation by qPCR were given in Table 3-10, 3-11, 3-12, 3-13, respectively.

Table 3-10 Quantification of four yeast populations in Kalecik Karası samples during cold maceration, maceration and alcoholic fermentation by qPCR

	Kalecik Karası						
Yeast species	**Sampling (Day)**						
	0.CM	**2.CM**	**4.CM**	**2.M**	**4.M**	**14.F**	**28.F**
H. uvarum	$9.1\times10^1\pm 1.6\times10^2$	$7.5\times10^1\pm 5.8\times10^1$	$2.6\times10^1\pm 0.1\times10^1$	$8.7\times10^3\pm 4.4\times10^1$	$3.3\times10^3\pm 1.2\times10^2$	$1.2\times10^2\pm 0.9\times10^1$	$5.4\times10^1\pm 0.8\times10^1$
L. thermotolerans	$1.2\times10^1\pm 0.1\times10^1$	$1.2\times10^1\pm 0.5\times10^1$	$2.6\times10^1\pm 0.4\times10^1$	$3.8\times10^1\pm 1\times10^1$	$0.3\times10^1\pm 0.1\times10^1$	ND	ND
T. delbrueckii	$3.2\times10^1\pm 0.4\times10^1$	$9.5\times10^1\pm 1\times10^1$	$1.4\times10^2\pm 1.9\times10^1$	$6\times10^2\pm 5.9\times10^1$	$1.4\times10^2\pm 2.4\times10^1$	$0.3\times10^1\pm 0.1\times10^1$	ND
S. cerevisiae	$6.9\times10^2\pm 3.3\times10^1$	$6\times10^2\pm 1\times10^2$	$6\times10^2\pm 9.6\times10^1$	$9.8\times10^3\pm 6.5\times10^1$	$8.6\times10^3\pm 1.6\times10^1$	$8.2\times10^2\pm 6.7\times10^1$	$7.7\times10^2\pm 1.4\times10^1$

0.CM, 0. day of cold maceration; 2.CM, 2nd day of cold maceration; 4.CM, 4th day of cold maceration; 2.M, 2nd day of maceration; 4.M, 4th day of maceration; 14.F, 14th day of fermentation; 28.F, 28th day of fermentation. ND, not detectable. Values were the numbers of CFU per milliliter ± standard deviations.

Table 3-11 Quantification of two yeast populations in Boğazkere samples during cold maceration, and maceration by qPCR

	Boğazkere		
Yeast species	**Sampling (Day)**		
	0.CM	**4.CM**	**6.M**
H. uvarum	$3.1\times10^1 \pm 0.4\times10^1$	$3.8\times10^1 \pm 1\times10^1$	$2.2\times10^1 \pm 1.6\times10^1$
S. cerevisiae	$1.7\times10^2 \pm 0.8\times10^1$	$1.6\times10^2 \pm 1.2\times10^1$	$1.9\times10^2 \pm 2.7\times10^1$

0.CM, 0. day of cold maceration; 4.CM, 4th day of cold maceration; 6.M, 6th day of maceration.
Values were the numbers of CFU per milliliter ± standard deviations.

Table 3-12 Quantification of three yeast populations in Öküzgözü samples during cold maceration, and maceration by qPCR

Yeast species	Öküzgözü		
	Sampling (Day)		
	0.CM	4.CM	6.M
H. uvarum	$3.8\times10^1 \pm 0.1\times10^1$	$2.6\times10^1 \pm 0.2\times10^1$	$1.9\times10^1 \pm 0.2\times10^1$
L. thermotolerans	$2.8\times10^1 \pm 0.1\times10^1$	$4.8\times10^1 \pm 0$	$8.8\times10^1 \pm 0$
S. cerevisiae	$9.8\times10^1 \pm 1\times10^1$	$1.7\times10^2 \pm 2.4\times10^1$	$3.8\times10^2 \pm 1.1\times10^2$

0.CM, 0. day of cold maceration; 4.CM, 4th day of cold maceration; 6.M, 6th day of maceration.
Values were the numbers of CFU per milliliter ± standard deviations.

Table 3-13 Quantification of two yeast populations in Dimrit samples during cold maceration, and maceration by qPCR

Yeast species	Dimrit		
	Sampling (Day)		
	0.CM	4.CM	4.M
H. uvarum	$9.2\times10^1 \pm 0.8\times10^1$	$1.8\times10^2 \pm 4.1\times10^1$	$3\times10^2 \pm 1.6\times10^1$
S. cerevisiae	$1.5\times10^2 \pm 0.9\times10^1$	$1.6\times10^2 \pm 0.4\times10^1$	$5\times10^2 \pm 6.7\times10^1$

0.CM, 0. day of cold maceration; 4.CM, 4th day of cold maceration; 4.M, 4th day of maceration.
Values were the numbers of CFU per milliliter ± standard deviations.

The quantification of non-*Saccharomyces* yeasts was done according to standard curves. According to the quantification result, the amount of *T. delbrueckii* yeast showed an increasing in Kalecik Karası must, and a decrease was observed in the later stages of fermentation during wine formation. Except for Kalecik Karası, *T. delbrueckii* yeast was not found in wines made from other grape varieties. *L. thermotolerans* was detected in small amounts in Kalecik Karası and Öküzgözü grape varieties and not detected in Boğazkere and Dimrit grape varieties. *H. uvarum* was detected in small amounts in all grape varieties. The amount of *H. uvarum* decreased during cold maceration, increased during maceration and a slow decrease again during fermentation. In *S. cerevisiae* yeast, the highest number was obtained on the second day of maceration in Kalecik Karası samples, after which no recorded changes were observed in the number. However, this amount was not observed to decrease. In Öküzgözü, Boğazkere and Dimrit grape varieties approximately similar results were obtained (Figure 3-14). Similar results have been reported in the

literatures (Andorrà et al., 2010; Díaz et al., 2013; Hierro et al., 2006; Martorell, Querol, et al., 2005).

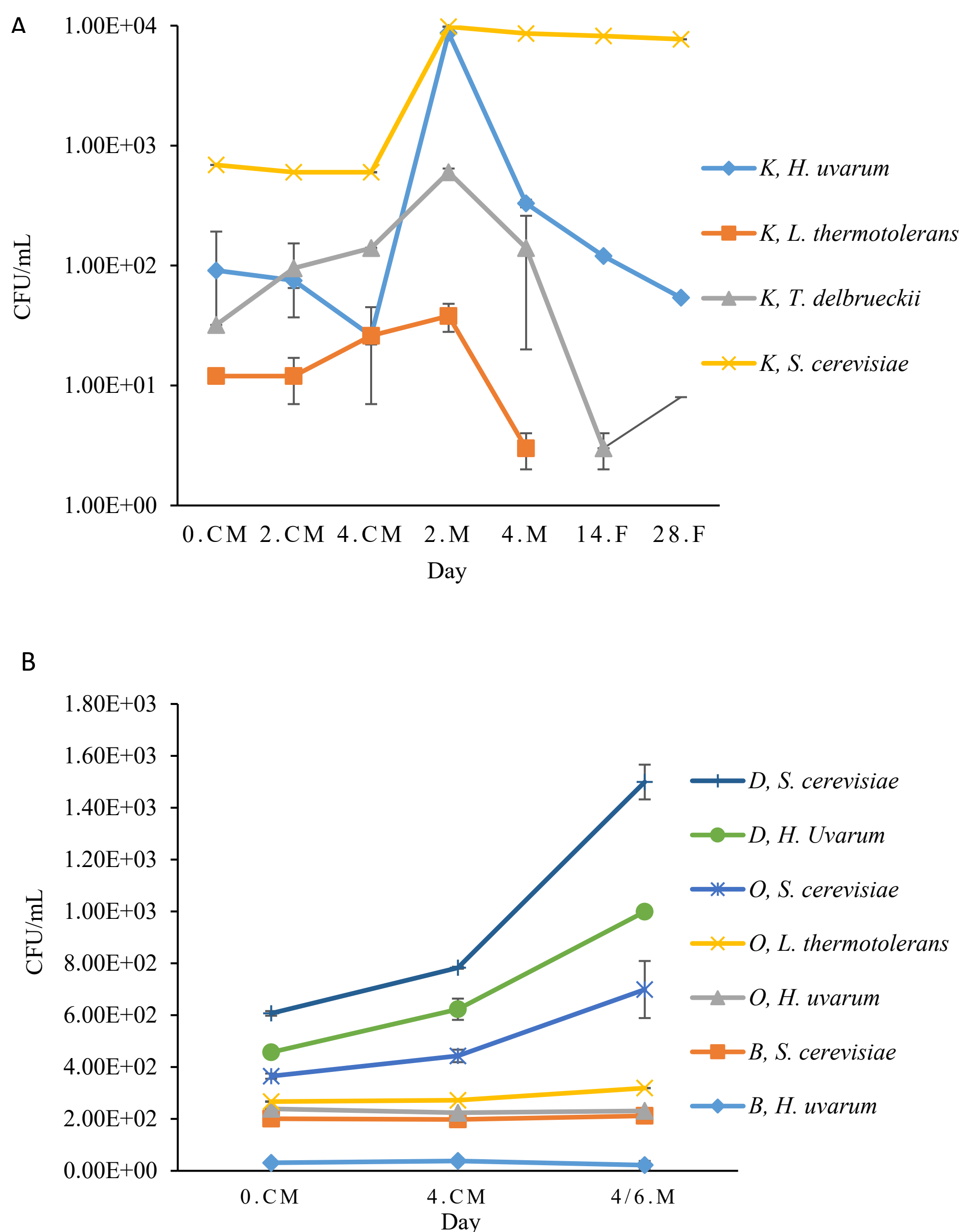


Figure 3-14 A) Quantification of *H. uvarum, L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* yeast populations in Kalecik Karası samples during 0.CM, 0. day of cold maceration; 2.CM, 2nd day of cold maceration; 4.CM, 4th day of cold maceration; 2.M, 2nd day of maceration; 4.M, 4th day of maceration; 14.F, 14th day of fermentation; 28.F, 28th day of fermentation. B) Quantification of *H. uvarum, L. thermotolerans*, and *S. cerevisiae* yeast populations in Boğazkere, Öküzgözü and Dimrit samples during 0.CM, 4.CM, and 4/6.M. Bars represented the standard deviation of three repetitions.

Quantification studies in the literatures have generally been published for a single organism in wine because of the sensitivity of the assay. For example, for *Oenococcus oeni* strain in wine (Pinzani et al., 2004), for *Hanseniaspora* species (Phister et al., 2007) in wine and must, for total yeast population in wine (Hierro et al., 2006), and for *S. cerevisiae* in wine (Martorell, Querol, et al., 2005).

Quantification for ***L. thermotolerans*** by Garcia et al. (2017), for ***T. delbrueckii*** by Zott et al. (2010), Diaz et al. (2013), Garcia et al. (2017) and Lleixà et al. (2018), for ***H. uvarum**,* by Hierro et al. (2007) and C. Wang et al. (2015), and for ***S. cerevisiae*** by Martorell et al. (2005), Hierro et al. (2007), Zott et al. (2010), Diaz et al. (2013), C. Wang et al. (2015), Garcia et al. (2017) and Wang et al. (2020) were also made.

In this study, successful results were obtained in the quantification of selected yeasts with qPCR assay. It has been concluded that the amount of strain desired to be followed by this method can be determined successfully during maceration and fermentation periods.

3.4 Genetic Identification of *Saccharomyces Cerevisiae* Strains

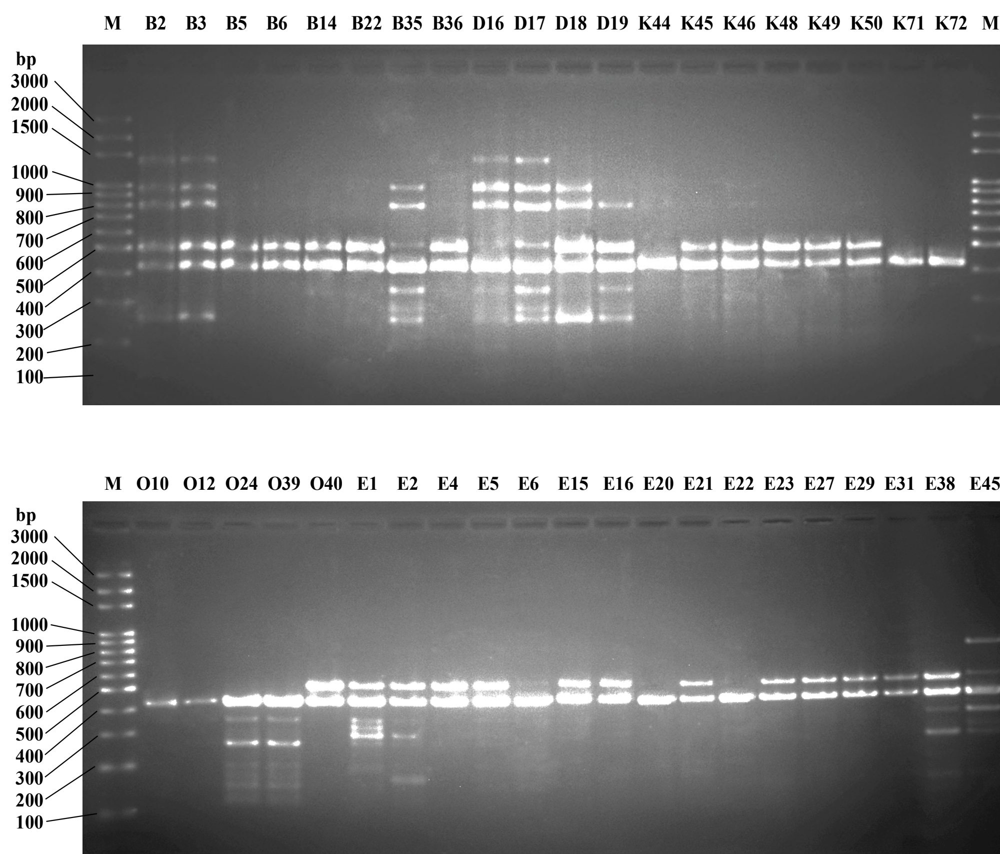
The accurate differentiation of all members of the *Saccharomyces sensu stricto* complex specially *S. cerevisiae*, is an important topic because of the practical significance of these species on wine fermentation (Eldarov & Mardanov, 2020; Ramírez-Castrillón et al., 2014). *S. cerevisiae* strains is most widely used as starter cultures for wine production. Therefore, strains of this yeast species were selected for further study.

The 46 indigenous *S. cerevisiae* strains were isolated and subjected to the RAPD-PCR using one Operon primer (OPA-11) and MSP-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(ATG)_5$ to investigate the genetic diversification of the strains isolated from five different grape must and wine at variable stages and clustering of isolates belonging to the same species. The *S. cerevisiae* strains studied, their designations, sources, and the accession numbers of the rDNA sequences were

mentioned before in Table 2-3. The isolates were preliminary identified as *S. cerevisiae* on the basis of sequence analysis of their internal transcribed spacer (ITS) or D1/D2 domain.

3.4.1 Random Amplified Polymorphic DNA Polymerase Chain Reaction with the Decamer Primer OPA-11

RAPD-PCR band patterns of 46 *S. cerevisiae* strains generated with 10-mer primer OPA-11 were shown in Figure 3-15.



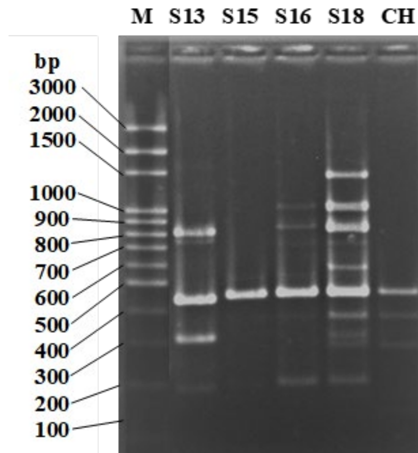


Figure 3-15 RAPD-PCR band patterns of 46 strains of *S. cerevisiae* generated with 10-mer primer OPA-11. B, Boğazkere; D, Dimrit; K, Kalecik Karası; O, Öküzgözü; E, Emir grape varieties; CH, *S. cerevisiae* reference (MERIT Chr. Hansen); M, 100 bp DNA size marker.

One to ten PCR products were obtained with the RAPD-PCR reaction using primer OPA-11 and the size of amplified fragments varied from 150 bp to 1400 bp. A PCR fragment with the size of 450 bp was obtained for all strains except for the S13 strain. Lines with the sizes of 330 bp 430 bp were typical only of the E1 and S13 strains, respectively. In turn, a product with the size of 600 bp was typical only of the S18 strain. The S18 strain also showed the highest number of PCR products by using primer OPA-11 (Table 3-14). Either absence or presence of the amplified DNA fragments enabled the division of the analyzed strains into seventeen groups as shown in Table 3. The first included strains B5, B6, B14, B22, B36, K45, K46, K48, K49, K50, O40, E4, E5, E15, E16, E21, E23, E27, E29, and E31, the second strains K44, K71, K72, O10, O12, E6, E20, E22, and S15, the third strains B2, B3, the fourth strains O24, O39, and the fifth to seventeenth strains B35, D16, D17, D18, D19, E1, E2, E38, E45, S13, S16, S18, and CH, respectively (Table 3-14). A high degree of heterogeneity was obtained with primer OPA-11 in differentiation of the 46 indigenous strains of *S. cerevisiae* isolated from five different grape must and wine in this study.

Table 3-14 Seventeen different band patterns of *S. cerevisiae* strains generated by RAPD-PCR with primer OPA-11

***S. cerevisiae* strains**	**Approximate band patterns (bp)**	**PCR product**	**Pattern type**
B5, B6, B14, B22, B36, K45, K46, K48, K49, K50, O40, E4, E5, E15, E16, E21, E23, E27, E29, E31	500, 450	2	I
K44, K71, K72, O10, O12, E6, E20, E22, S15	450	1	II
B2, B3	1400, 950, 800, 500, 450, 270	6	III
O24, O39	450, 350, 270, 200, 150	5	IV
B35	950, 800, 450, 350, 280, 270	6	V
D16	1400, 950, 800, 450	4	VI
D17	1400, 950, 800, 500, 450, 350, 280, 270	8	VII
D18	950, 800, 500, 450, 270	5	VIII
D19	800, 500, 450, 350, 270	5	IX
E1	500, 450, 350, 330, 300	5	X
E2	500, 450, 300, 150	4	XI
E38	500, 450, 350, 270	4	XII
E45	800, 500, 450, 350, 280, 270	6	XIII
S13	800, 750, 430, 300, 200	5	XIV
S16	1000, 800, 750, 450, 200	5	XV
S18	1400, 1000, 800, 750, 600, 450, 400, 300, 280, 200	10	XVI
CH	450, 400, 280	3	XVII

RAPD-PCR analysis using Operon primers (Operon Technologies, Alameda, USA) were frequently used to discriminate different yeast species. Discrimination of the 15 *Saccharomyces cerevisiae* strains isolated from wine and beer was carried out by RAPD analysis with primer OPA-11 (Baleiras Couto et al., 1996). According to the authors, different pattern types generated with primer OPA-11 showed heterogeneity among strains of the species *S. cerevisiae*.

RAPD banding patterns generated with six decamer primers (OPA-03, OPA-07, OPA-08, OPA-09, OPA-10, OPA-11) also showed the best differentiation between the most common strains of the *Saccharomyces sensu stricto* complex including 19

S. cerevisiae, 23 *S. bayanus*, 10 *S. paradoxus*, and 6 *S. pastorianus* (Fernández-Espinar et al., 2003).

In addition, nine decamer primers (OPA-2, OPA-3, OPA-7, OPA-8, OPA-9, OPA-10, OPA-11, OPA-15, and OPA-16) were applied to analyze the yeast microbiota present in a manufacturing plant of candied fruits and nougats (Martorell, Fernández-Espinar, et al., 2005). According to the Martorell et al. (2005) fingerprinting results, RAPD analysis with OPA primers was the best procedure for the characterization of spoilage yeast strains.

Moreover, the heterozygosity of the meiotic segregants from two *S. cerevisiae* (ALKO 743 and ALKO 3460) were assessed using Operon primers (Korhola et al., 2019). The author reported that RAPD analysis with OPA-01, OPA-04, OPA-09, and OPA-11 enabled the distinction between two *S. cerevisiae* (44 segregants of ALKO 743 and 17 of ALKO 3460).

A high degree of heterogeneity was also obtained with primer OPA-11 in differentiation of the 46 indigenous strains of *S. cerevisiae* isolated from five different grape must and wine in this study. A comparison of the above-mentioned results with those obtained in our study reconfirmed high usability of the Operon primers used for discrimination of the *S. cerevisiae* strains isolated from wine.

3.4.2 Minisatellite Primed Polymerase Chain Reaction Fingerprinting with the Primer M13

MSP-PCR fingerprints of 46 strains of *S. cerevisiae* amplified with the minisatellite primer M13 were shown in Figure 3-16.

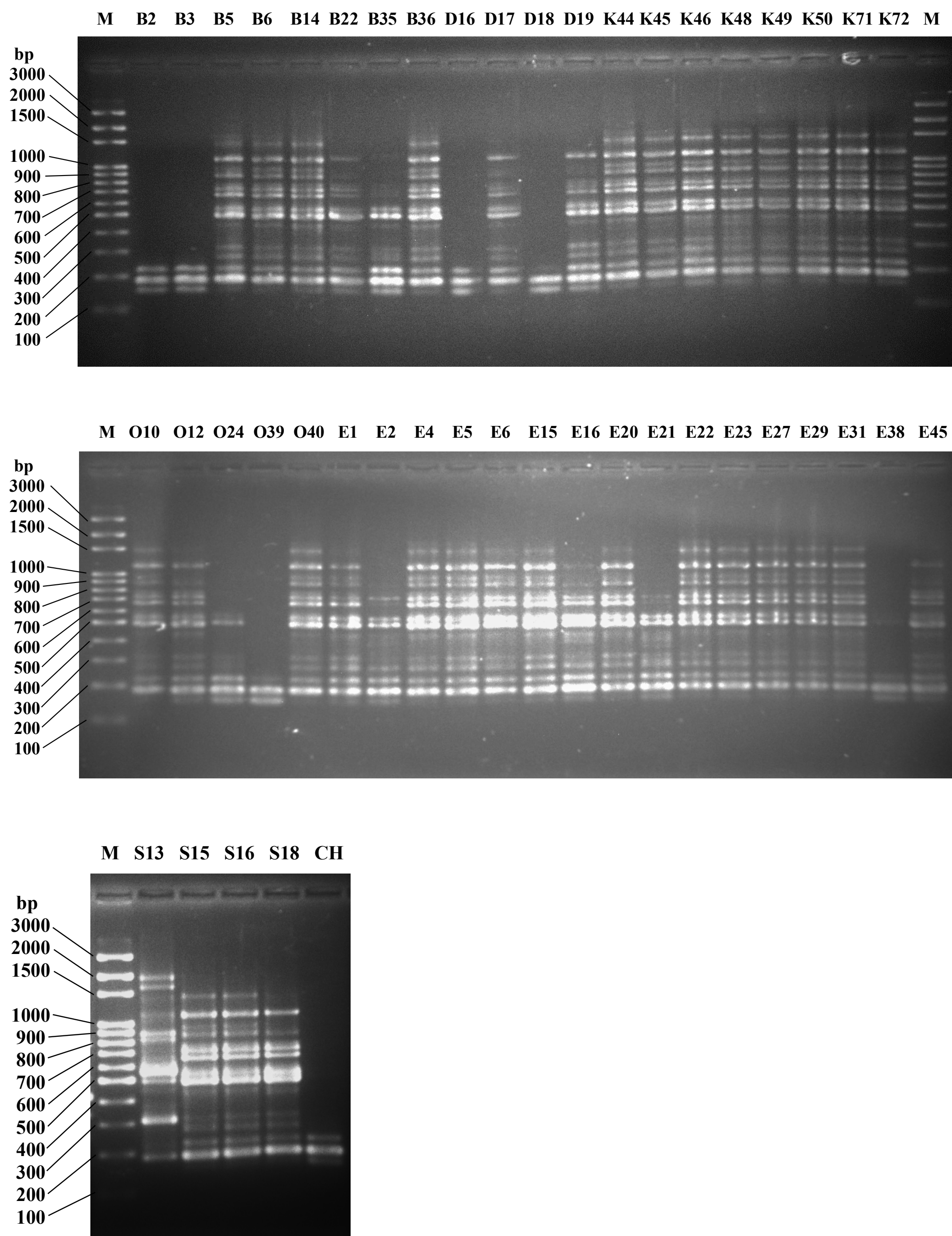


Figure 3-16 MSP-PCR fingerprints of 46 strains of *S. cerevisiae* amplified with the minisatellite primer M13. B, Boğazkere; D, Dimrit; K, Kalecik Karası; O, Öküzgözü; E, Emir grape varieties; CH, *S. cerevisiae* reference (MERIT Chr. Hansen); M, 100 bp DNA size marker.

Two to twelve PCR products were amplified with the MSP-PCR reaction using the minisatellite primer M13 and the size of amplified fragments ranged from 170 to

2000 bp. The highest number of amplicons by using primer M13 was twelve for B5, B6, B14, B36, K44, K45, K46, K48, K49, K50, K71, K72, O10, O40, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, S15, and S16 strains, while the lowest number was two amplicons for D18 and O39 strains (Table 3-15). A PCR fragment with the size of 190 bp was obtained for all strains. A product with the size of 220 bp was amplified for all strains except for the D18, O39 and S13 strains. Lines with the sizes of 2000, 1750, and 850 bp were typical only of the S13 strain (Figure 3-16). The different fingerprinting profiles obtained using primer M13 allowed the discrimination of 46 *S. cerevisiae* strains into 12 groups as summarized in Table 3-15.

Table 3-15 Twelve different fingerprinting of *S. cerevisiae* strains generated by MSP-PCR using primer M13

***S. cerevisiae* strains**	**Approximate band patterns (bp)**	**PCR product**	**Pattern type**
B5, B6, B14, B36, K44, K45, K46, K48, K49, K50, K71, K72, O10, O40, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, S15, S16	1500, 1250, 990, 900, 700, 650, 550, 500, 320, 280, 220, 190	12	I
B2, B3, D16, E38, CH	220, 190, 170	3	II
B22, D17	1250, 700, 650, 550, 500, 320, 280, 220, 190	9	III
E2, E16, E21	700, 650, 550, 500, 320, 280, 220, 190, 170	9	IV
B35	550, 500, 320, 280, 220, 190, 170	7	V
D18, O39	190, 170	2	VI
D19	1250, 700, 650, 550, 500, 320, 280, 220, 190, 170	10	VII
O12, E45	1250, 900, 700, 650, 550, 500, 320, 280, 220, 190, 170	11	VIII
O24	550, 500, 320, 280, 220, 190, 170	7	IX
E1	1250, 990, 900, 700, 650, 550, 500, 320, 280, 220, 190	11	X
S13	2000, 1750, 1250, 900, 850, 550, 500, 320, 190	9	XI
S18	1250, 900, 700, 650, 550, 500, 320, 280, 220, 190	10	XII

The primer M13 was used to discriminate *Saccharomyces* species like *S. cerevisiae* (Cocolin et al., 2004; Korhola et al., 2019; Lieckfeldt et al., 1993; Orlić et al., 2010; Santos et al., 2007; Šuranská et al., 2016; Torriani et al., 1999), and strains of non-

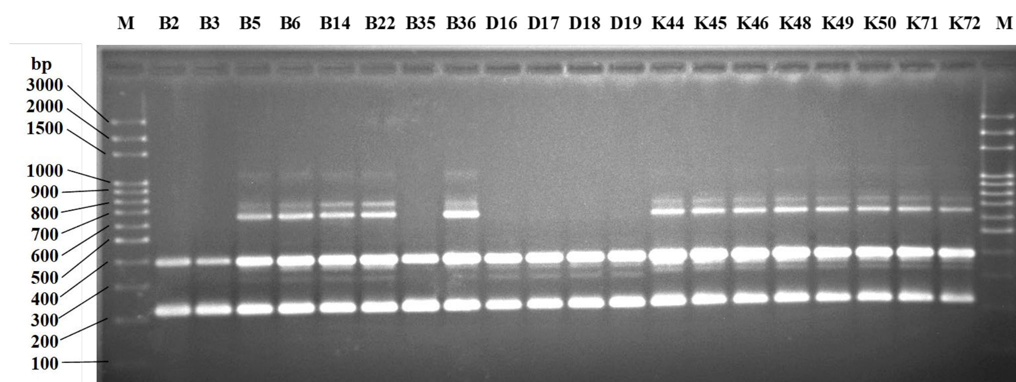
Saccharomyces species such as natural wine strains of *Hanseniaspora* (Bujdosó et al., 2001a; Cadez et al., 2002; Guaragnella et al., 2020), *C. zemplinina* (Pfliegler et al., 2014), *C. zeylanoides*, *R. mucilaginosa*, *Yarrowia lipolytica*, and *Debaryomyces hansenii* (Andrade et al., 2006). Most of these studies applied MSP-PCR fingerprinting using primer M13 reported the best differentiation between the *Saccharomyces* and non-*Saccharomyces* yeast strains.

Šuranská et al. (2016) reported MSP-PCR fingerprinting techniques using primer M13 were able to group the species members of *Saccharomyces* genus isolated from berries and spontaneously fermented musts. Various authors reconfirmed that MSP-PCR using M13 primer was clearly differentiated the *S. cerevisiae* strains isolated from different wineries, must, grapes (Cocolin et al., 2004; Orlić et al., 2010; Urso et al., 2008).

In this study, MSP-PCR fingerprinting using primer M13 allowed the discrimination of the 46 *S. cerevisiae* strains into 12 groups isolated from five different grape must and wine as demonstrated in Table 3-15.

3.4.3 Microsatellite Primed Polymerase Chain Reaction Fingerprinting with the Primer $(GTG)_5$

The following electrophoretic picture was obtained using the microsatellite primer $(GTG)_5$ in MSP-PCR assay for discrimination of 46 strains of *S. cerevisiae* (Figure 3-17).



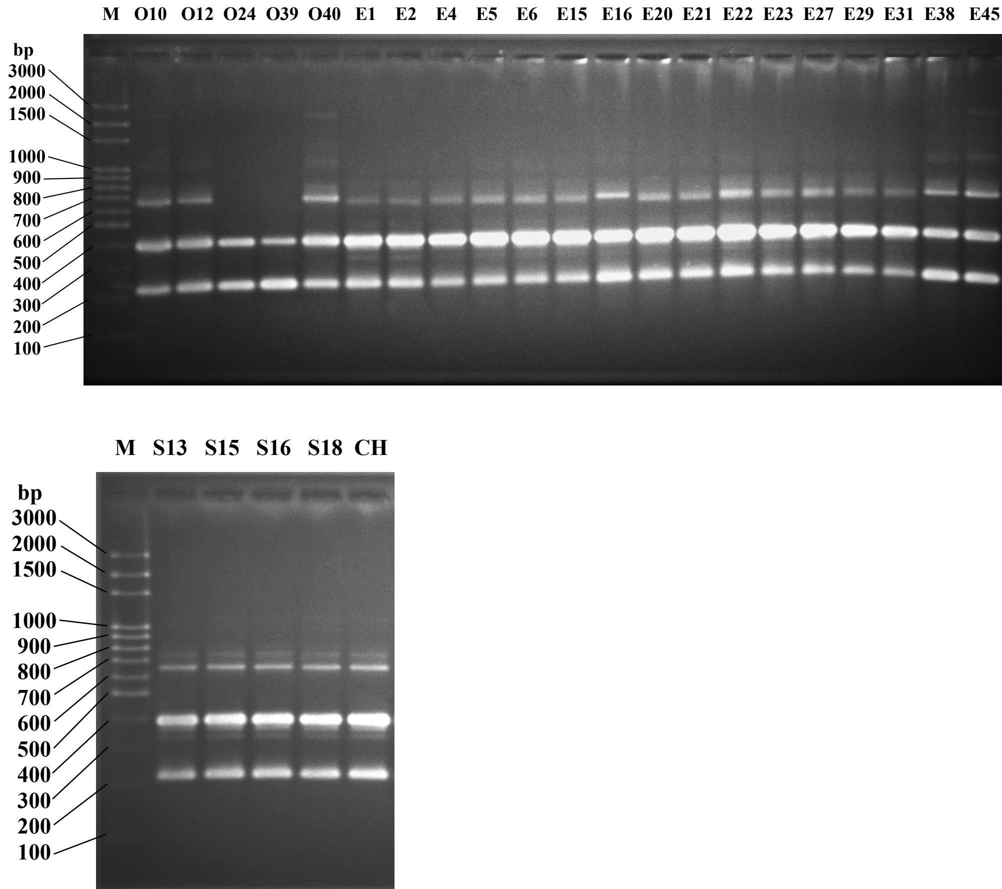


Figure 3-17 MSP-PCR fingerprints of 46 strains of *S. cerevisiae* amplified with the microsatellite primer $(GTG)_5$. B, Boğazkere; D, Dimrit; K, Kalecik Karası; O, Öküzgözü; E, Emir grape varieties; CH, *S. cerevisiae* reference (MERIT Chr. Hansen); M, 100 bp DNA size marker.

Only two and four PCR products were generated with the MSP-PCR reaction using the microsatellite primer $(GTG)_5$. Low-specific products were sometimes obtained with the primer $(GTG)_5$, and they were not analyzed further. The different fingerprinting profiles obtained using primer $(GTG)_5$ allowed the clustering of 46 *S. cerevisiae* strains into two groups. The first group included B5, B6, B14, B22, B36, K44, K45, K46, K48, K49, K50, K71, K72, O10, O12, O40, E1, E2, E4, E5, E6, E15, E16, E20, E21, E22, E23, E27, E29, E31, E38, E45, S13, S15, S16, S18, and CH strains with the amplified fragment sizes of 750, 650, 400, 230 bp, and the second B2, B3, B35, D16, D17, D18, D19, O24, O39 strains with the amplicon sizes of 400 bp and 230 bp (Table 3-16).

Table 3-16 Two different fingerprinting of *S. cerevisiae* strains amplified by MSP-PCR using primer $(GTG)_5$

***S. cerevisiae* strains**	**Approximate band patterns (bp)**	**PCR product**	**Pattern type**
B5, B6, B14, B22, B36, K44, K45, K46, K48, K49, K50, K71, K72, O10, O12, O40, E1, E2, E4, E5, E6, E15, E16, E20, E21, E22, E23, E27, E29, E31, E38, E45, S13, S15, S16, S18, CH	750, 650, 400, 230	4	I
B2, B3, B35, D16, D17, D18, D19, O24, O39	400, 230	2	II

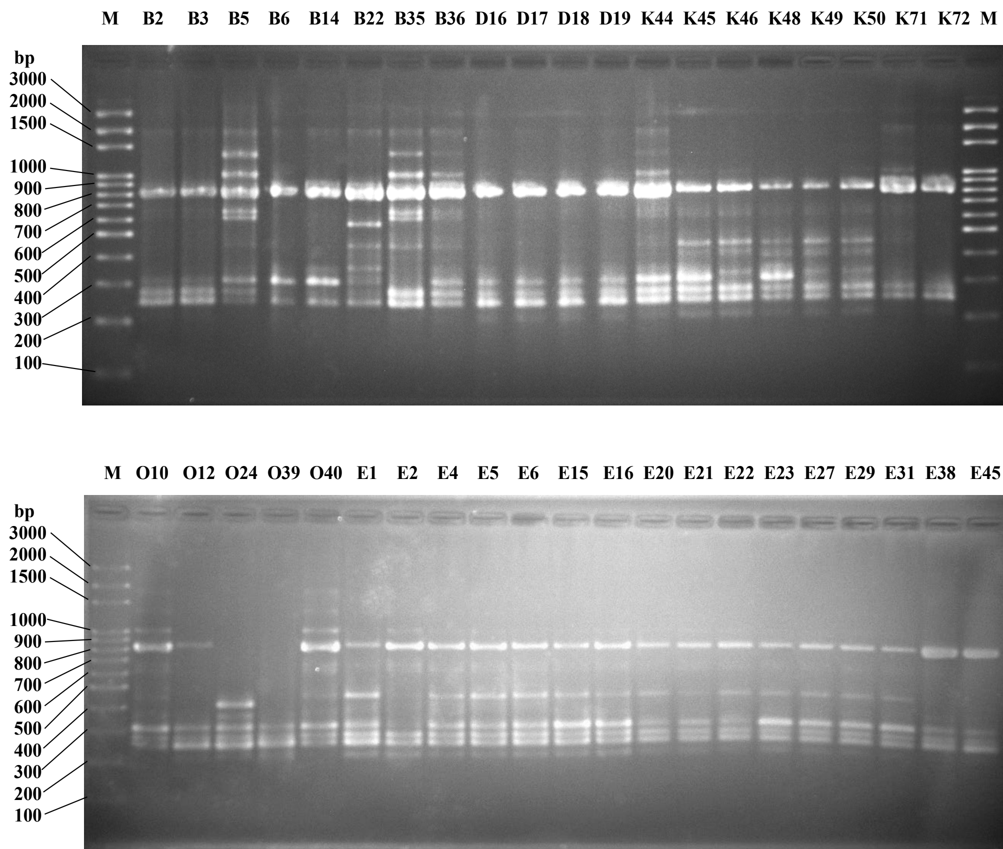
In contrast to the observation of Lieckfeldt et al. (1993), Baleiras-Couto et al. (1995), Orlić et al. (2010), Capece et al. (2016), and Kállai et al. (2019) which successfully used the primer $(GTG)_5$ for differentiation of brew and wine isolates of *S. cerevisiae*, the obtained results in this study indicated that low usability of the primer $(GTG)_5$ for differentiation of Turkish grape must and wine's *S. cerevisiae* strains. However, it is important to note that this study was applied *S. cerevisiae* isolates different from those analyzed by Lieckfeldt et al. (1993), Baleiras-Couto et al. (1995), Orlić et al. (2010), Capece et al. (2016), and Kállai et al. (2019).

The microsatellite primer $(GTG)_5$ was frequently used to differentiate species of the genus *Saccharomyces* (Capece et al., 2016; Lieckfeldt et al., 1993; Orlić et al., 2010; Ramírez-Castrillón et al., 2014; Santos et al., 2007; Torriani et al., 1999), characterize strains of non-*Saccharomyces* yeast such as *Candida zemplinina* (Pfliegler et al., 2014), and *Hanseniaspora* (Cadez et al., 2002; Guaragnella et al., 2020). Most of these studies applied MSP-PCR fingerprinting as a preliminary clustering step for the choice of representative strains to be sequenced. According to the results of these studies, the $(GTG)_5$ primer showed the best discrimination between the *Saccharomyces* and non-*Saccharomyces* yeast strains.

However, the primer $(GTG)_5$ did not demonstrate high discrimination capacity toward the analyzed strains which constitute two separate groups in terms of an electrophoretic profile in this study.

3.4.4 Microsatellite Primed Polymerase Chain Reaction Fingerprinting with the Primer $(ATG)_5$

The following electrophoretic pictures were obtained using the microsatellite primer $(ATG)_5$ in MSP-PCR assay for discrimination of 46 strains of *S. cerevisiae* (Figure 3-18).



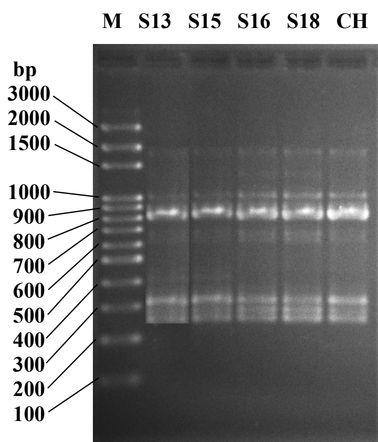


Figure 3-18 MSP-PCR fingerprints of 46 strains of *S. cerevisiae* amplified with the microsatellite primer $(ATG)_5$. B, Boğazkere; D, Dimrit; K, Kalecik Karası; O, Öküzgözü; E, Emir grape varieties; CH, *S. cerevisiae* reference (MERIT Chr. Hansen); M, 100 bp DNA size marker.

Three to ten amplicons were obtained with the MSP-PCR reaction using the microsatellite primer $(ATG)_5$ and the size of amplified fragments ranged from 250 to 2000 bp (Table 3-17).

The highest number of amplicons by using primer $(ATG)_5$ was ten for the B5 strain, while the lowest number was three amplicons for B6, B14 (800, 310, 250 bp), K71, K72 (800, 280, 250 bp), and O39 (310, 280, 250 bp). A PCR fragment with the size of 250 bp was obtained for all strains. A product with the size of 800 bp was amplified for all strains except for O24 and O39 strains. The line with the size of 550 bp was typical only of the B22 strain. The band pattern with the size of 350 bp was only obtained in the case of B22 and O24 strains (Figure 3-18 and Table 3-17).

The different fingerprinting profiles obtained using primer $(ATG)_5$ allowed the discrimination of 46 *S. cerevisiae* strains into 13 groups as summarized in Table 3-17.

Table 3-17 Thirteen different fingerprinting of *S. cerevisiae* strains amplified by MSP-PCR using primer $(ATG)_5$

***S. cerevisiae* strains**	**Approximate band patterns (bp)**	**PCR product**	**Pattern type**
B2, B3, D16, D17, D18, D19, O12, E38, E45	800, 310, 280, 250	4	I
B5	2000, 1250, 1000, 800, 650, 600, 450, 310, 280, 250	10	II
B6, B14	800, 310, 250	3	III
B22	800, 550, 450, 350, 310, 250	6	IV
B35	2000, 1250, 1000, 800, 650, 600, 450, 280, 250	9	V
B36, K44, O10, O40, S13, S15, S16, S18, CH	2000,1250, 1000, 800, 310, 280, 250	7	VI
K45, K46, K48, K49, K50	800, 450, 400, 310, 280, 250	6	VII
K71, K72	800, 280, 250	3	VIII
O24	400, 350, 310, 280, 250	5	IX
O39	310, 280, 250	3	X
E1	800, 450, 310, 280, 250	5	XI
E2	1000, 800, 280, 250	4	XII
E4, E5, E6, E15, E16, E20, E21, E22, E23, E27, E29, E31	800, 450, 310, 280, 250	5	XIII

Although the microsatellite $(ATG)_5$ primer was rarely used to discriminate different yeast species (Cadez et al., 2002)(De Benedictis et al., 2011), in this study MSP-PCR using primer $(ATG)_5$ was clearly differentiated the Turkish 46 *S. cerevisiae* strains isolated from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir samples (grape, must, wine) and effectively clustered them into 13 groups for the first time as demonstrated in Table 3-17.

A single RAPD-PCR or MSP-PCR reaction was not sufficient to separate all the strains. However, strains which appear identical in the amplification with one primer can be separated in reaction with a different primer (Torriani et al., 1999). Therefore, a combined analysis of RAPD-PCR profiles obtained using one arbitrary primer OPA-11 and MSP-PCR fingerprinting technique using primers as M13, $(GTG)_5$, $(ATG)_5$ was applied to discriminate the 46 *S. cerevisiae* strains (Table 3-18).

Table 3-18 The heatmap of similarity coefficient (%) among the 46 indigenous *S. cerevisiae* strains based on combined RAPD and MSP-PCR

SCs	B2	B3	B5	B6	B14	B22	B35	B36	D16	D17	D18	D19	K44	K45	K46	K48	K49	K50	K71	K72	O10	O12	O24	O39	O40	E1	E2	E4	E5	E6	E15	E16	E20	E21	E22	E23	E27	E29	E31	E38	E45	S13	S15	S16	S18	CH
B2	100																																													
B3	100	100																																												
B5	47	47	100																																											
B6	50	50	86	100																																										
B14	50	50	86	100	100																																									
B22	50	50	78	86	86	100																																								
B35	62	62	69	49	49	53	100																																							
B36	50	50	94	91	91	78	61	100																																						
D16	93	93	44	47	47	47	59	47	100																																					
D17	74	74	67	73	73	73	72	71	67	100																																				
D18	93	93	44	47	47	47	59	47	85	67	100																																			
D19	72	72	69	76	76	76	71	74	65	91	71	100																																		
K44	46	46	92	89	89	76	63	98	49	68	43	71	100																																	
K45	51	51	88	93	93	84	54	90	49	72	49	76	88	100																																
K46	51	51	88	93	93	84	54	90	49	72	49	76	88	100	100																															
K48	51	51	88	93	93	84	54	90	49	72	49	76	88	100	100	100																														
K49	51	51	88	93	93	84	54	90	49	72	49	76	88	100	100	100	100																													
K50	51	51	88	93	93	84	54	90	49	72	49	76	88	100	100	100	100	100																												
K71	46	46	83	93	93	78	55	89	48	70	42	73	91	91	91	91	91	91	100																											
K72	46	46	83	93	93	78	55	89	48	70	42	73	91	91	91	91	91	91	100	100																										
O10	46	46	92	89	89	76	63	98	49	68	43	71	100	88	88	88	88	88	91	91	100																									
O12	57	57	79	88	88	83	59	84	61	74	55	83	86	86	86	86	86	86	90	90	86	100																								
O24	59	59	51	55	55	60	65	55	56	67	56	75	56	60	60	60	60	60	56	56	56	67	100																							
O39	67	67	35	36	36	36	50	38	64	51	72	61	39	39	39	39	39	39	38	38	39	50	77	100																						
O40	50	50	94	91	91	78	61	100	47	71	47	74	98	90	90	90	90	90	89	89	98	84	55	38	100																					
E1	50	50	83	87	87	83	57	84	47	75	47	78	82	90	90	90	90	90	84	84	82	84	59	43	84	100																				
E2	56	56	73	76	76	76	62	78	53	68	53	76	76	76	76	76	76	76	78	78	76	83	65	48	78	78	100																			
E4	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100																		
E5	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100																	
E6	49	49	88	93	93	84	57	89	51	71	46	74	91	96	96	96	96	96	95	95	91	90	59	41	89	89	74	98	98	100																
E15	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100	98	100															
E16	63	63	79	83	83	88	64	80	61	74	61	83	77	86	86	86	86	86	80	80	77	90	67	50	80	84	88	88	88	86	88	100														
E20	49	49	88	93	93	84	57	89	51	71	46	74	91	96	96	96	96	96	95	95	91	90	59	41	89	89	74	98	98	100	98	86	100													
E21	63	63	79	83	83	88	64	80	61	74	61	83	77	86	86	86	86	86	80	80	77	90	67	50	80	84	88	88	88	86	88	100	86	100												
E22	49	49	88	93	93	84	57	89	51	71	46	74	91	96	96	96	96	96	95	95	91	90	59	41	89	89	74	98	98	100	98	86	100	86	100											
E23	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100	98	100	88	98	88	98	100										
E27	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100	98	100	88	98	88	98	100	100									
E29	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100	98	100	88	98	88	98	100	100	100								
E31	53	53	90	95	95	86	55	92	50	74	50	77	89	98	98	98	98	98	93	93	89	88	57	40	92	92	77	100	100	98	100	88	98	88	98	100	100	100	100							
E38	80	80	56	61	61	61	56	60	71	63	79	72	56	62	62	62	62	62	57	57	56	69	65	74	60	65	67	63	63	59	63	74	59	74	59	63	63	63	63	100						
E45	65	65	75	83	83	78	69	80	58	88	63	91	78	82	82	82	82	82	80	80	78	89	68	54	80	84	78	83	83	81	83	84	81	84	81	83	83	83	83	75	100					
S13	40	40	64	57	57	52	53	68	42	50	42	52	69	57	57	57	57	57	58	58	69	62	45	38	68	60	57	58	58	60	58	53	60	53	60	58	58	58	58	45	60	100				
S15	46	46	92	89	89	76	63	98	49	68	43	71	100	88	88	88	88	88	91	91	100	86	56	39	98	82	76	89	89	91	89	77	91	77	91	89	89	89	89	56	78	69	100			
S16	47	47	86	82	82	69	62	91	49	67	44	69	92	81	81	81	81	81	83	83	92	79	55	40	91	75	69	82	82	84	82	71	84	71	84	82	82	82	82	51	75	75	92	100		
S18	48	48	75	69	69	65	62	79	50	70	41	65	80	69	69	69	69	69	71	71	80	75	52	37	79	71	69	70	70	72	70	67	72	67	72	70	70	70	70	48	75	75	80	88	100	
CH	63	63	62	53	53	53	63	67	67	50	60	53	68	54	54	54	54	54	54	54	68	65	50	55	67	52	63	55	55	56	55	65	56	65	56	55	55	55	55	75	62	57	68	62	67	100

Different colors represented the level of genetic similarity among the strains ranged between 35% (dark red colored, lowest) and 100% (green colored, highest)

The level of genetic similarity among the 46 strains ranged between 35% and 100% as shown in the heatmap of similarity matrix Table 3-18. The highest genetic similarity 100% was observed among the following strains combination;

(B2-B3),

(B36-O40),

(K44-O10-S15),

(B6-B14),

(K45-K46-K48-K49-K50),

(E4-E31-E29-E23-E27-E15-E5),

(E6-E20-E22),

(K71-K72), and (E16-E21), while the lowest genetic similarity 35% was seen between strains O39 and B35.

Moreover, the similarity of the 46 *S. cerevisiae* strains, estimated by the Dice's coefficient, was shown in the UPGMA dendrogram of Figure 3-19. After cluster analysis, five groups and nine single-strain clusters were identified using a coefficient of discrimination of 88%. A good differentiation of the 46 *S. cerevisiae* strains isolated from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir grape varieties collected from three different regions (Ankara, Elazığ and Cappadocia) was observed (Figure 3-19).

Cluster I grouped isolates from Boğazkere (B2, B3) and Dimrit (D16, D18) grape varieties.

Cluster II grouped isolates from Öküzgözü (O10, O40), Boğazkere (B5, B36) and Kalecik Karası grape varieties (K44) in 2017 and (S15, S16) 2016 vintage.

Cluster III classified isolates from Boğazkere (B6, B14), Kalecik Karası (K45, K46, K48, K49, K50, K71, K72) and Emir (E4, E31, E29, E23, E27, E15, E5, E6, E20, E22, E1) grape varieties.

Cluster IV was formed by three strains from which two (E16, E21) were from Emir and one (O12) from Öküzgözü grape varieties.

Cluster V was contained in two Dimrit (D17, D19) and one Emir (E45) isolates.

Nine strains, namely E38, CH, O24, O39, B35, B22, E2, S13, and S18, did not share any similarity with other isolates and formed a single-strain cluster as shown in Figure 3-19.

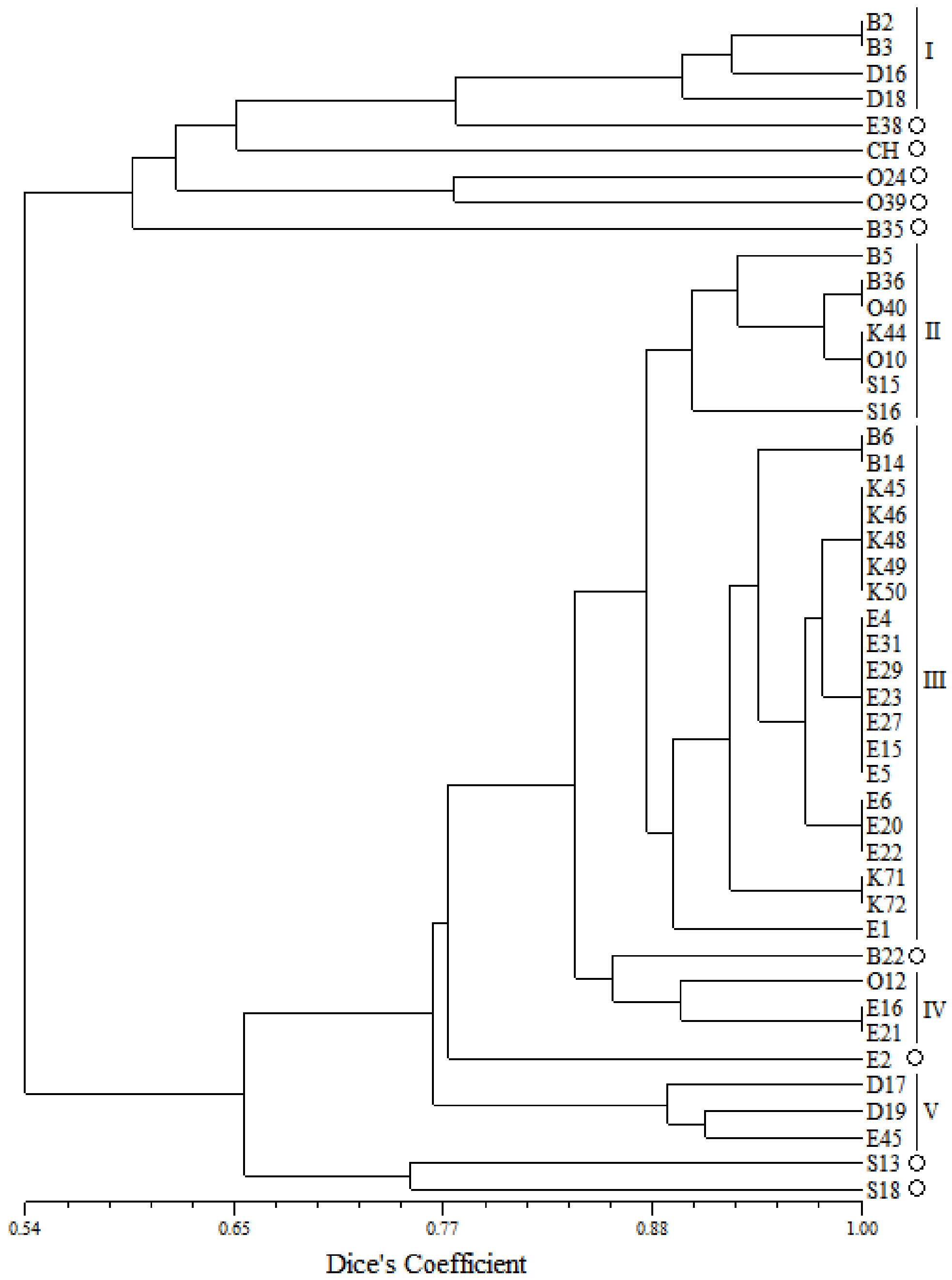


Figure 3-19 Dendrogram for the 46 indigenous *S. cerevisiae* strains constructed from RAPD-PCR and MSP-PCR's combination data using UPGMA and similarity matrices.

3.4.5 Karyotyping of *Saccharomyces Cerevisiae* Strains

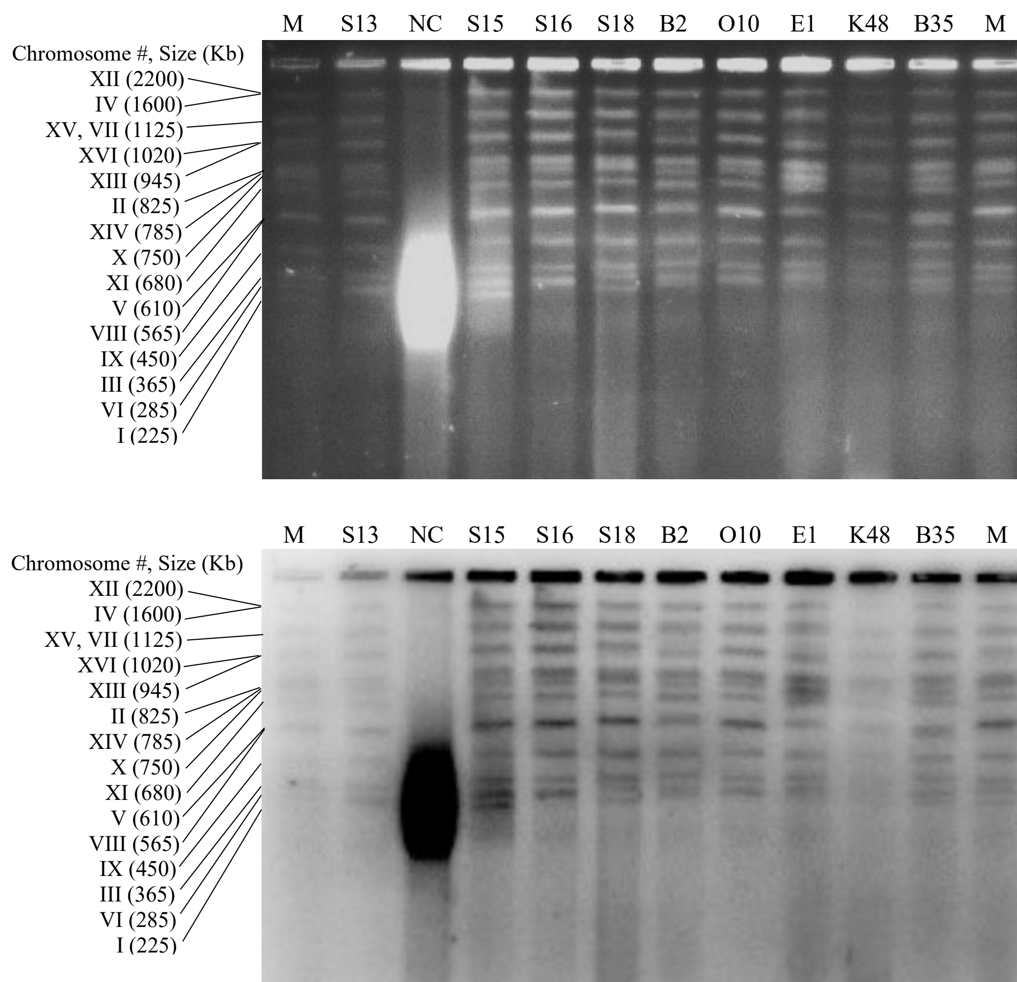
As mentioned in previous part, the 46 indigenous *S. cerevisiae* strains were subjected to the RAPD-PCR and MSP-PCR fingerprinting technique to investigate the genetic diversities (similarity or dissimilarity) of the strains isolated from five different grape must and wine at variable stages. In this section, karyotyping of the selected *S. cerevisiae* strains were carried out by PFGE method to identify the chromosome number and sizes of the 46 *S. cerevisiae* strains for discovering the genetic polymorphisms of the strains.

The *S. cerevisiae* strains studied, their designations, sources, and the accession numbers of the rDNA sequences were mentioned before in Table 2-3. The isolates were preliminary identified as *S. cerevisiae* on the basis of sequence analysis of their internal transcribed spacer (ITS) or D1/D2 domain.

3.4.5.1 Optimization of the PFGE Assay

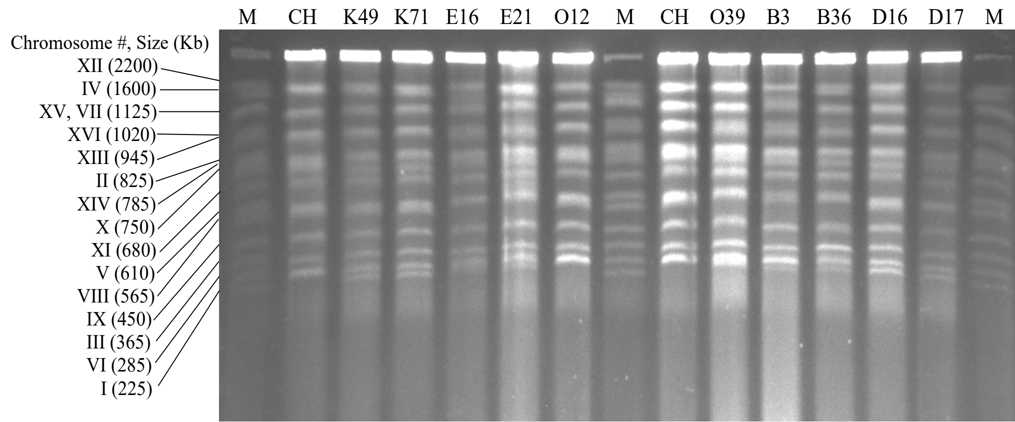
Wine associated *S. cerevisiae* strains show a large diversity in the number and size of chromosomes that can be monitored by PFGE analysis. Many factors, such as voltage, switch interval, running time, agarose concentration of the gel, running temperature, running buffer, and angle of the alternating electric field, were known to affect DNA migration in PFGE gels (Birren et al., 1989; Schwartz & Cantor, 1984). Among these factors, the switch interval, voltage and running time significantly affected DNA migration and were controlled. A low voltage and long switch interval were required to separate large DNA molecules. The voltage and switch interval had an inverse relationship for effective separation of a certain size of chromosomes. Firstly, we systematically changed the switch interval and voltage in order to determine the optimal conditions for separating *S. cerevisiae* chromosomes. After that, the running time was changed (22, 24, 27 h was tested). Agarose gels (1%, which concentration also optimized, data not shown) were loaded with sliced pieces of *S. cerevisiae* plugs and runs were performed for 22, 24, and 27

h at 14°C in running buffer, using a voltage of 4.5 V/cm, and an initial and final switch of 60 and 145 second, respectively (Figures 3-20, 3-21, 3-22).



M S13 NC S15 S16 S18 B2 O10 E1 K48 B35 M
Chromosome #, Size (Kb)
XII (2200)
IV (1600)
XV, VII (1125)
XVI (1020)
XIII (945)
II (825)
XIV (785)
X (750)
XI (680)
V (610)
VIII (565)
IX (450)
III (365)
VI (285)
I (225)

Figure 3-20 PFGE of *S. cerevisiae* strains isolated from Boğazkere (B), Dimrit (D), Kalecik Karası (K), Öküzgözü (O), and Emir (E) grape varieties at different wine-making stages. NC, negative control (*Metschnikowia chrysoperlae*); M, CHEF DNA Size Marker, *S. cerevisiae* (Bio-Rad). Runs were performed for 22 h at 14°C in running buffer, using a voltage of 4.5 V/cm, and an initial and final switch of 60 and 145 second, respectively.



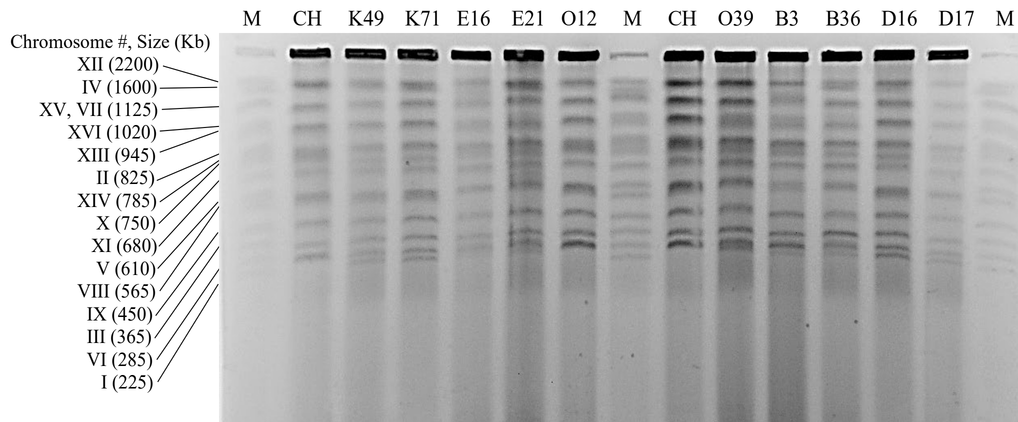
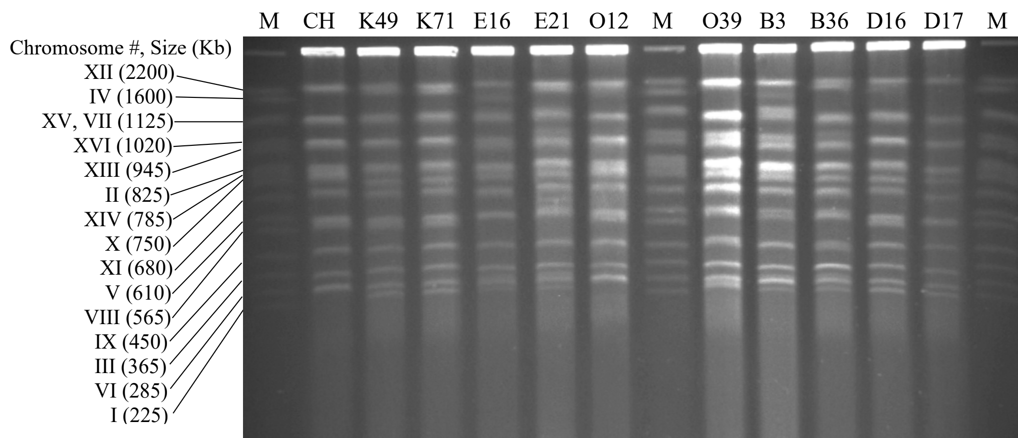


Figure 3-21 PFGE of *S. cerevisiae* strains isolated from Boğazkere (B), Dimrit (D), Kalecik Karası (K), Öküzgözü (O), and Emir (E) grape varieties at different wine-making stages. M, CHEF DNA Size Marker, *S. cerevisiae* (Bio-Rad). Runs were performed for 24 h at 14°C in running buffer, using a voltage of 4.5 V/cm, and an initial and final switch of 60 and 145 second, respectively.



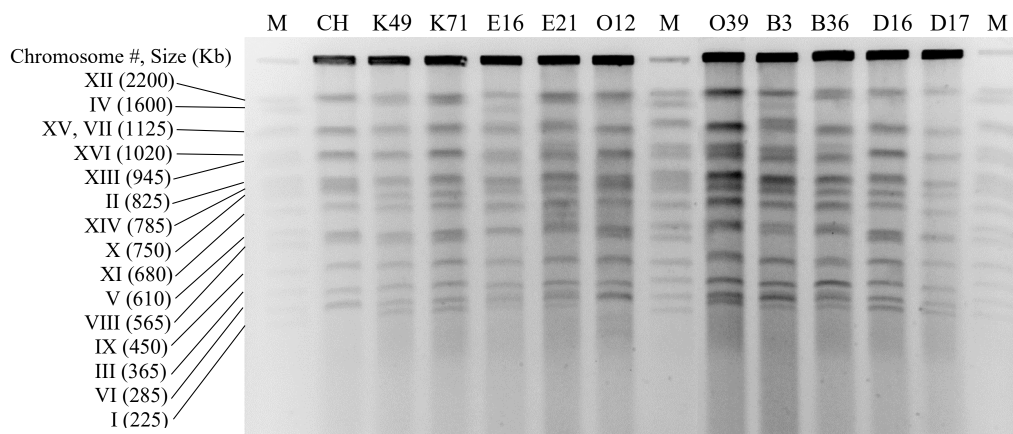
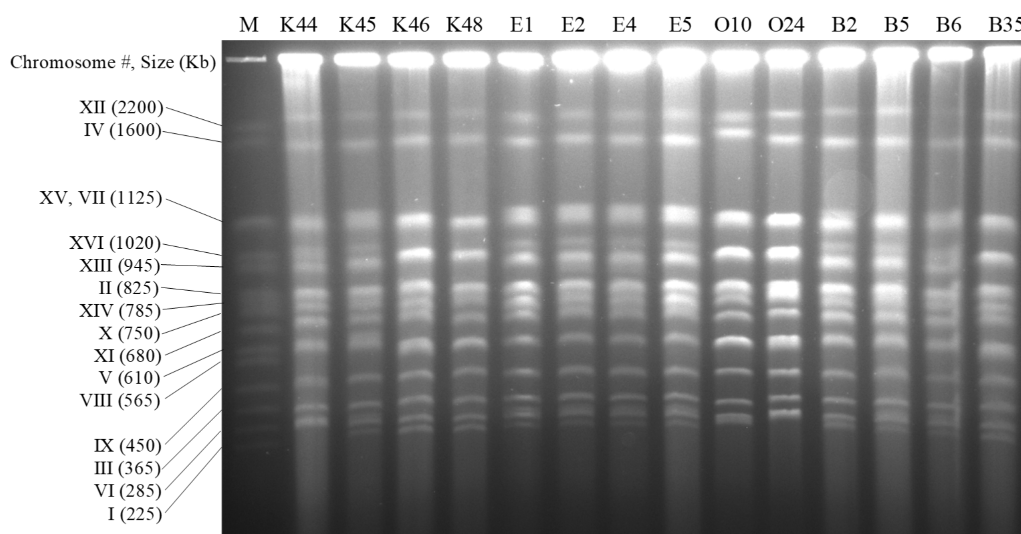
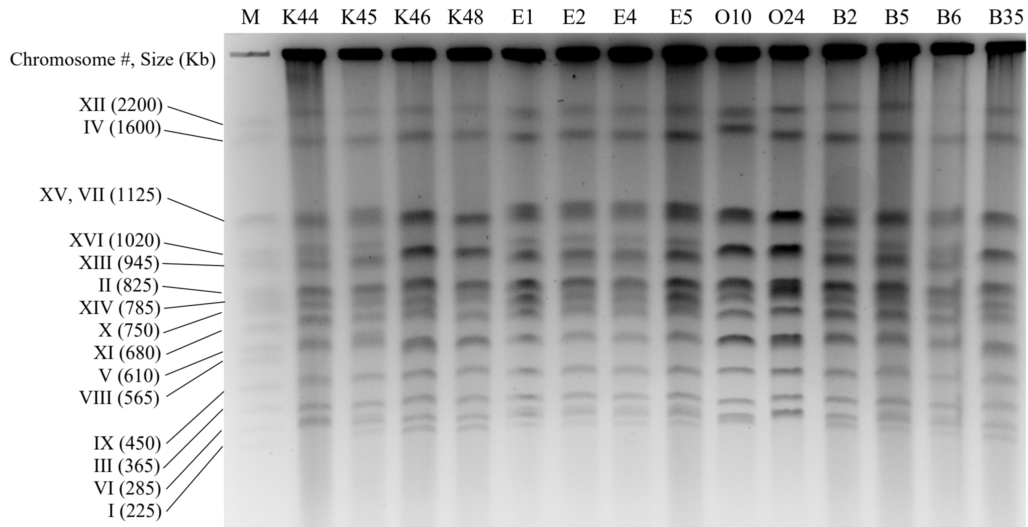
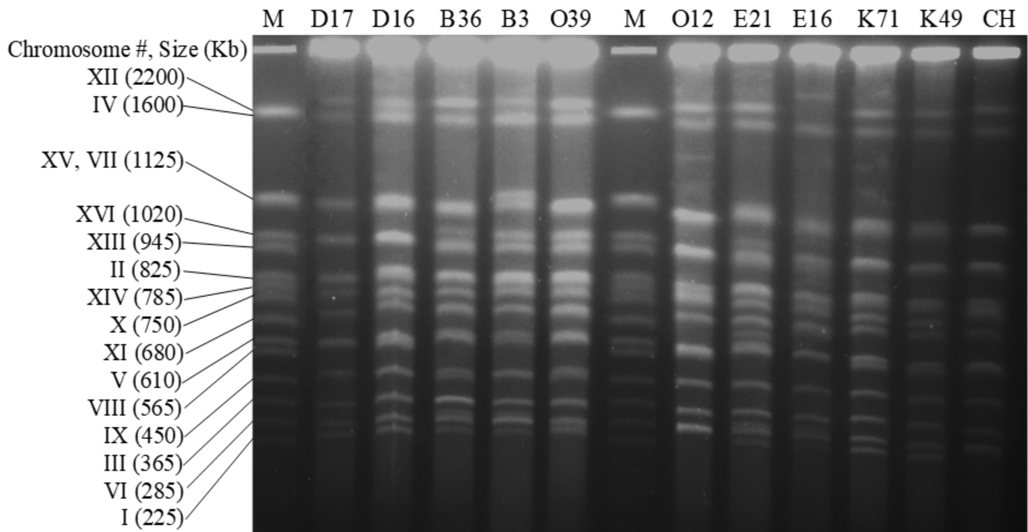


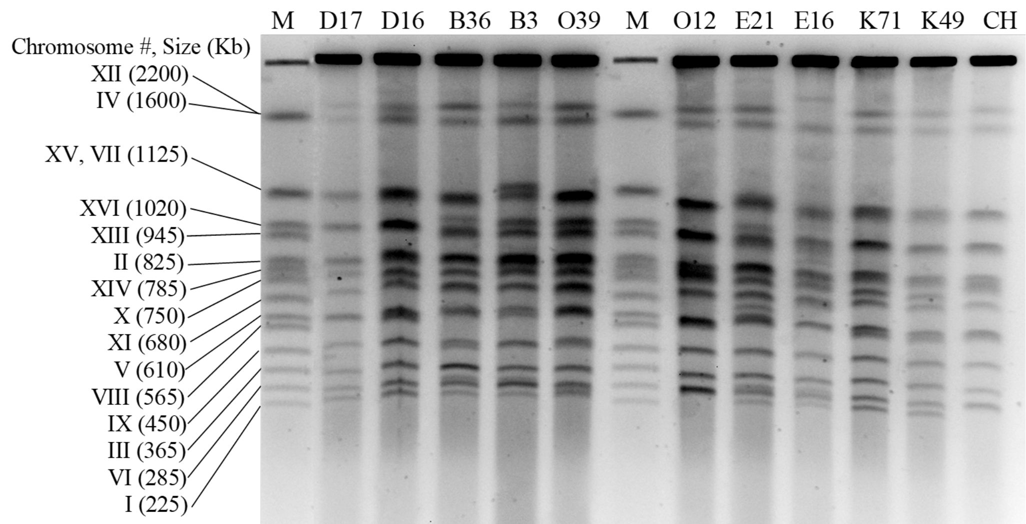
Figure 3-22 PFGE of *S. cerevisiae* strains isolated from Boğazkere (B), Dimrit (D), Kalecik Karası (K), Öküzgözü (O), and Emir (E) grape varieties at different wine-making stages. CH, Commercial *S. cerevisiae* (Christian Hansen, MERIT™); M, CHEF DNA Size Marker, *S. cerevisiae* (Bio-Rad). Runs were performed for 27 h at 14°C in running buffer, using a voltage of 4.5 V/cm, and an initial and final switch of 60 and 145 second, respectively.

Here we investigated the optimum conditions for separating chromosomes of 46 *S. cerevisiae* strains. The best separating conditions for the *S. cerevisiae* chromosomal DNAs isolated from Boğazkere (B), Dimrit (D), Kalecik Karası (K), Öküzgözü (O), and Emir (E) grapes at different wine-making stages was in a 1% Pulsed Field Certified Agarose gel for 24 h at 14°C in running buffer, with 60 second switch interval, angle of 120° and voltages of 6 V/cm (Figures 3-23).




M K44 K45 K46 K48 E1 E2 E4 E5 O10 O24 B2 B5 B6 B35
Chromosome #, Size (Kb)
XII (2200)
IV (1600)
XV, VII (1125)
XVI (1020)
XIII (945)
II (825)
XIV (785)
X (750)
XI (680)
V (610)
VIII (565)
IX (450)
III (365)
VI (285)
I (225)


M D17 D16 B36 B3 O39 M O12 E21 E16 K71 K49 CH
Chromosome #, Size (Kb)
XII (2200)
IV (1600)
XV, VII (1125)
XVI (1020)
XIII (945)
II (825)
XIV (785)
X (750)
XI (680)
V (610)
VIII (565)
IX (450)
III (365)
VI (285)
I (225)


M D17 D16 B36 B3 O39 M O12 E21 E16 K71 K49 CH
Chromosome #, Size (Kb)
XII (2200)
IV (1600)
XV, VII (1125)
XVI (1020)
XIII (945)
II (825)
XIV (785)
X (750)
XI (680)
V (610)
VIII (565)
IX (450)
III (365)
VI (285)
I (225)

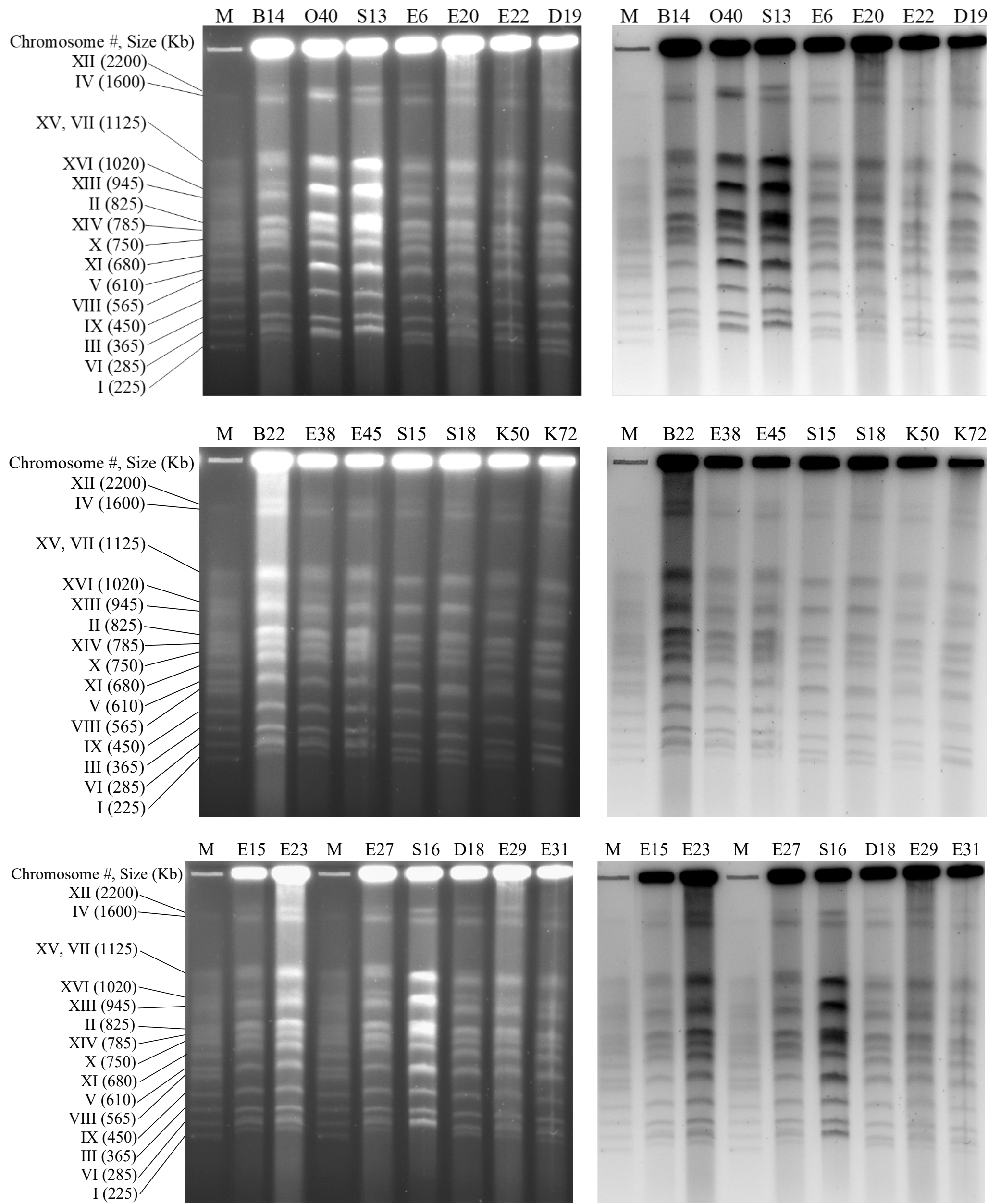


Figure 3-23 PFGE of 46 *S. cerevisiae* strains isolated from Boğazkere (B), Dimrit (D), Kalecik Karası (K), Öküzgözü (O), and Emir (E) grape varieties at different wine-making stages. CH, Commercial *S. cerevisiae* (Christian Hansen, MERIT™); M, CHEF DNA Size Marker, *S. cerevisiae* (Bio-Rad). Runs were performed for 24 h at 14°C in running buffer, using a voltage of 6 V/cm, and an initial and final switch of 60 and 120 second with angle of 120°, respectively.

After statistical analysis and normalization of the obtained results, ten chromosomal banding patterns could be distinguished in the electrophoretic karyotypes of 46 Turkish *S. cerevisiae* strains (Table 3-19).

Table 3-19 Different chromosomal patterns of 46 *S. cerevisiae* strains distinguished by electrophoretic karyotyping

Chromosome patterns	*S. cerevisiae* strains*	Approximate chromosomal DNA size (kb) and number															
		2200 XII	**1600 IV**	**1125 XV**	**1125 VII**	**1020 XVI**	**945 XIII**	**825 II**	**785 XIV**	**750 X**	**680 XI**	**610 V**	**565 VIII**	**450 IX**	**365 III**	**285 VI**	**225 I**
Profile 1	B2, B5, B6, B14, B22, D18, E1, E2, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, E38, E45	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+f
Profile 2	B35, D17, D19, K46, K48, S15, S18	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+
Profile 3	CH, O12, O24, S13, S16	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+s	-
Profile 4	K44, K72	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-
Profile 5	O10, O40	+	>1600	+	-	+	-	+	+	+	+	+	-	+	+	+	+f
Profile 6	D16, K49, K71	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Profile 7	B3, K45, K50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+f
Profile 8	B36, O39	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+f
Profile 9	E21	+	+	+	+	+	+	+	+	+	+	>610,+	-	+	+	+	+f
Profile 10	E16	>2200	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+f

B, Boğazkere; D, Dimrit; K, Kalecik Karası; O, Öküzgözü; E, Emir grape varieties; f, faint; s, sharp.
O10, 1800 kb; E21, 610+640 kb; E16, 2400 kb.
*, The full ID numbering of *S. cerevisiae* strains were listed in Table 2-3.

As shown in Table 3-19 and Figure 3-23, ten chromosomal banding patterns were assessed in the karyotyping of the 46 *S. cerevisiae* strains isolated from Boğazkere, Dimrit, Kalecik Karası, Öküzgözü, and Emir grapes at different wine-making stages as following;

Profile 1:

The largest group consisted of twenty strains isolated from three different grape varieties, two different locations (Elazığ, Cappadocia) and in the same year (2017). This profile was observed in B2, B5, B6, B14, B22, D18, E1, E2, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, E38, E45 isolates. In this profile, 1125 kb band corresponded to two chromosomes XV and VII was resolved into two bands, as opposed to CHEF *S. cerevisiae* chromosomal DNA size marker (Bio-Rad) that resolved into a single band. 1020+945 kb bands (chromosome XVI and XIII) were also observed as two bands like CHEF DNA size marker. In addition, the 565 kb band corresponded to chromosome VIII was not observed. Moreover, the 225 kb band (chromosome I) were resolved into one faint band with about 250 kb in size. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1125+1020+945+825+785+750+680+610+450+365+285+250 (faint) kb.

Profile 2:

This profile was observed in B35, D17, D19, K46, K48, S15, S18 strains isolated from three different grape varieties, three different locations (Elazığ, Cappadocia, Ankara) and in two different years (S15 strain was isolated from Kalecik Karası grape variety in 2016 and the rest of the strains isolated in 2017. In addition, S18 was a house wine starter culture (38409LM) originated from Germany). In this profile, instead of 1020+945 kb bands (chromosome XVI and XIII), a single band was detected in approximately 1000 kb region. In addition, the 565 kb band corresponded to chromosome VIII was not observed but the 225 kb band

(chromosome I) was observed as a discrete band. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1000+825+785+750+680+610+450+365+285+ 225 (kb).

Profile 3:

This profile was observed in CH, O12, O24, S13, S16 strains isolated from two different grape varieties, two different locations (Elazığ, Kalecik-Ankara) and in two different years (O12 and O24 strains isolated from Öküzgözü grape must in 2017 while the S13, S16 strains were isolated from Kalecik Karası grape variety in 2016. In addition, CH was a fermenting *S. cerevisiae* strain bought from Chr. Hansen company (MERIT™, Hoersholm, Denmark) as a reference wine fermentation strain). In this profile, instead of 1020+945 kb bands, a single band was observed in the approximately 1000 kb region. The 565 kb band corresponded to chromosome VIII was not observed as well. While the 285 kb band (chromosome VI) was seen sharp in the ethidium-stained gels the 225 kb band (chromosome I) was not observed. The strains of this profile were different from profile 2 only because of the absence of the 225 kb band. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1000+825+785+750+680+610+450+365+285 (sharp) kb.

Profile 4:

This profile was observed in two strains K44 and K72 isolated from same grape variety, location, and year. In this profile, 1020+945 kb bands were resolved into two bands like CHEF *S. cerevisiae* chromosomal DNA size marker. In addition, the 565 kb band corresponded to chromosome VIII and the 225 kb band corresponded to chromosome I were not observed in the electrophoretic karyotypes of K44 and K72 strains. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1020+945+825+785+750+680+610+450+365+285 (kb).

Profile 5:

This profile was observed in O10 and O40 strains isolated from the same grape variety, location, and year. In this profile, the 1600 kb band corresponded to chromosome IV was observed above around 1800 kb region which was unique only for this profile. In place of 1020+945 kb bands, a single band was observed in the approximately 1000 kb region. In addition, the 565 kb band corresponded to chromosome VIII was not observed. Moreover, the 225 kb band (chromosome I) were resolved into one faint band with approximately 250 kb in size. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1800+1125+1000+825+785+750+680+610+450+365+285+250 (faint) kb.

Profile 6:

This profile was observed in D16, K49, K71 strains isolated from two different grape varieties, two different locations (Cappadocia-Nevşehir, Kalecik-Ankara) and in the same year (2017).

In this profile, instead of 1020+945 kb bands, a single band in the approximately 1000 kb region was observed. In addition, the 565 kb band (chromosome VIII) and the 225 kb band (chromosome I) were monitored in the ethidium-stained gels. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1000+825+785+750+680+610+565+450+365+285+225 kb.

Profile 7:

This profile was observed in B3, K45, K50 strains isolated from two different grape varieties, two different locations (Elazığ, Kalecik-Ankara) and in 2017. In this profile, 1125 kb band corresponded to two chromosomes XV and VII was resolved into two bands, as opposed to CHEF *S. cerevisiae* chromosomal DNA size marker (Bio-Rad) that resolved into a single band. 1020+945 kb bands (chromosome XVI and XIII) were also observed as two bands like CHEF DNA size marker. In addition, the 565 kb band corresponded to chromosome VIII was also observed in ethidium-

stained gels. Moreover, the 225 kb band (chromosome I) were resolved into one faint band with about 250 kb in size. The strains of this profile were differed from profile 1 and 8 due to the resolving of the 565 kb band (chromosome VIII) and the 1125 kb band (chromosome VII), respectively. The strains of this profile (B3, K45, K50) were unique among the other studied *S. cerevisiae* strains since all of the 16 yeast chromosomal DNA were fully resolved. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1125+1020+945+825+785+750+680+610+565+450+365+285+250 (faint) kb

Profile 8:

This profile was observed in B36, O39 strains isolated from the same location (Elazığ), and year (2017) but from two different grape varieties. In this profile, 1020+945 kb bands (chromosome XVI and XIII) were resolved into two bands like CHEF DNA size marker. In addition, the 565 kb band corresponded to chromosome VIII was observed in the electrophoretic karyotype of B36 and O39 strains. Moreover, the 225 kb band (chromosome I) were resolved into one faint band with about 250 kb in size. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1020+945+825+785+750+680+610+565+450+365+285+250 (faint) kb.

Profile 9:

This profile was observed only in the E21 strain isolated from Emir grape must in 2017. In this profile, 1125 kb band was resolved into two bands, as opposed to CHEF *S. cerevisiae* chromosomal DNA size marker (Bio-Rad) that resolved into a single band. 1020+945 kb bands were also observed as two bands like CHEF DNA size marker. In addition to the 610 kb band (chromosome V), a single band with a molecular weight larger than 610 kb (~ 620 kb) was monitored in electrophoretic karyotype of E21 strain. The resolving of an extra band with approximately 620 kb

in size caused E21 strain to form a unique profile. Moreover, the 565 kb band corresponded to chromosome VIII was not observed in ethidium-stained gels, but the 225 kb band was resolved into one faint band with approximately 250 kb in size. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2200+1600+1125+1125+1020+945+825+785+750+680+620+610+450+365+285+ 250 (faint) kb.

Profile 10:

This profile was observed in the E16 strain isolated from Emir grape must in 2017. In place of the 2200 kb band corresponded to the chromosome XII, a single band with a molecular weight larger than 2200 kb (~ 2400 kb) was monitored in electrophoretic karyotype of E16. The absence of the 2200 kb band caused E16 strain to form a unique profile. In this profile, instead of 1020+945 kb bands, a single band was detected in approximately 1000 kb region. The 565 kb band corresponded to chromosome VIII was not observed but the 225 kb band (chromosome I) was resolved into one faint band with about 250 kb in size. In this profile, approximate chromosomal DNA size (kb) were distinguished as following;

2400+1600+1125+1000+825+785+750+680+610+450+365+285+ 250 (faint) kb.

In the electrophoretic karyotyping of all the 46 *S. cerevisiae* strains (Figure 3-23), the 2200 kb band (chromosome XII) was observed above the *S. cerevisiae* chromosomal DNA size marker (Bio-Rad) band, and the 1600 kb band (chromosome IV) was observed lower than the marker band. That was exceptional for O10 and O40 strains (profile 5) due to the resolving of the 1600 kb band into a discrete band with a molecular weight larger than 1600 kb (~ 1800 kb). Although the 1125 kb band (chromosome VII, XV) was resolved into a single band in the *S. cerevisiae* chromosomal DNA size marker (Bio-Rad), two bands were observed in the strains of the profile 1, 7, and 9. Moreover, in the PFGE of the *S. cerevisiae* chromosomal DNA size marker, the 1020+945 kb bands (chromosome XVI and XIII) were

observed as two bands like the strains of the profile 1, 4, 7, 8, 9, and opposed to the rest of the profiles that resolved into a single band with approximately 1000 kb in size. Furthermore, the 565 kb band (chromosome VIII) was observed as a single band in marker like the strains of the profile 6, 7, 8 and this band was not resolved in the rest of strains. While the 225 kb band (chromosome I) was not resolved in PFGE of strains in profile 3 and 4, this band was detected in the rest of profiles.

Moreover, the level of genetic similarity among the 46 strains obtained from PFGE analysis ranged between 80% and 100% calculated in heatmap of similarity matrix using Dice's coefficient (Table 3-20).

The highest genetic similarity 100% was observed among the following strains combination;

(B2, B5, B6, B14, B22, D18, E1, E2, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, E38, E45),

(B35, D17, D19, K46, K48, S15, S18),

(CH, O12, O24, S13, S16),

(K44, K72),

(O10, O40),

(D16, K49, K71),

(B3, K45, K50),

and (B36, O39) colored dark green in the heatmap of similarity matrix, while the lowest genetic similarity 80% was seen among the following strains combination; (E21, D16), (E21, K49) and (E21-K71) which colored dark red in Table 3-20.

The level of genetic similarity among the remained *S. cerevisiae* strains were calculated in similarity matrix using Dice's coefficient (Table 3-20).

Table 3-20 The heatmap of similarity coefficient (%) among the 46 indigenous *S. cerevisiae* strains based on PFGE analysis

SCs	B2	B3	B5	B6	B14	B22	B35	B36	D16	D17	D18	D19	K44	K45	K46	K48	K49	K50	K71	K72	O10	O12	O24	O39	O40	E1	E2	E4	E5	E6	E15	E16	E20	E21	E22	E23	E27	E29	E31	E38	E45	S13	S15	S16	S18	CH
B2	100																																												
B3	97	100																																											
B5	100	97	100																																										
B6	100	97	100	100																																									
B14	100	97	100	100	100																																								
B22	100	97	100	100	100	100																																							
B35	86	83	86	86	86	86	100																																						
B36	93	97	93	93	93	93	86	100																																					
D16	83	87	83	83	83	83	96	90	100																																				
D17	86	83	86	86	86	86	100	86	96	100																																			
D18	100	97	100	100	100	100	86	93	83	86	100																																		
D19	86	83	86	86	86	86	100	86	96	100	86	100																																	
K44	93	90	93	93	93	93	92	93	89	92	93	92	100																																
K45	97	100	97	97	97	97	83	97	87	83	97	83	90	100																															
K46	86	83	86	86	86	86	100	86	96	100	86	100	92	83	100																														
K48	86	83	86	86	86	86	100	86	96	100	86	100	92	83	100	100																													
K49	83	87	83	83	83	83	96	90	100	96	83	96	89	87	96	96	100																												
K50	97	100	97	97	97	97	83	97	87	83	97	83	90	100	83	83	87	100																											
K71	83	87	83	83	83	83	96	90	100	96	83	96	89	87	96	96	100	87	100																										
K72	93	90	93	93	93	93	92	93	89	92	93	92	100	90	92	92	89	90	89	100																									
O10	86	83	86	86	86	86	85	86	81	85	86	85	85	83	85	85	81	83	81	85	100																								
O12	89	86	89	89	89	89	96	89	92	96	89	96	96	86	96	96	92	86	92	96	88	100																							
O24	89	86	89	89	89	89	96	89	92	96	89	96	96	86	96	96	92	86	92	96	88	100	100																						
O39	93	97	93	93	93	93	86	100	90	86	93	86	93	97	86	86	90	97	90	93	86	89	89	100																					
O40	86	83	86	86	86	86	85	86	81	85	86	85	85	83	85	85	81	83	81	85	100	88	88	86	100																				
E1	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100																			
E2	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100																		
E4	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100																	
E5	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100																
E6	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100															
E15	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100														
E16	86	83	86	86	86	86	85	86	81	85	86	85	85	83	85	85	81	83	81	85	85	88	88	86	85	86	86	86	86	86	86	100													
E20	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100												
E21	97	94	97	97	97	97	83	90	80	83	97	83	90	94	83	83	80	94	80	90	83	86	86	90	83	97	97	97	97	97	97	83	97	100											
E22	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100										
E23	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100									
E27	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100	100								
E29	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100	100	100							
E31	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100	100	100	100						
E38	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100	100	100	100	100					
E45	100	97	100	100	100	100	86	93	83	86	100	86	93	97	86	86	83	97	83	93	86	89	89	93	86	100	100	100	100	100	100	86	100	97	100	100	100	100	100	100	100				
S13	89	86	89	89	89	89	96	89	92	96	89	96	96	86	96	96	92	86	92	96	88	100	100	89	88	89	89	89	89	89	89	88	89	86	89	89	89	89	89	89	89	100			
S15	86	83	86	86	86	86	100	86	96	100	86	100	92	83	100	100	96	83	96	92	85	96	96	86	85	86	86	86	86	86	86	85	86	83	86	86	86	86	86	86	86	96	100		
S16	89	86	89	89	89	89	96	89	92	96	89	96	96	86	96	96	92	86	92	96	88	100	100	89	88	89	89	89	89	89	89	88	89	86	89	89	89	89	89	89	89	100	96	100	
S18	86	83	86	86	86	86	100	86	96	100	86	100	92	83	100	100	96	83	96	92	85	96	96	86	85	86	86	86	86	86	86	85	86	83	86	86	86	86	86	86	86	96	100	96	100
CH	89	86	89	89	89	89	96	89	92	96	89	96	96	86	96	96	92	86	92	96	88	100	100	89	88	89	89	89	89	89	89	88	89	86	89	89	89	89	89	89	89	100	96	100	96	100

Different colors represented the level of genetic similarity among the strains ranged between 80% (dark red colored, lowest) and 100% (green colored, highest).

The similarity of the 46 *S. cerevisiae* strains, estimated by the Dice's coefficient, was shown in the UPGMA dendrogram of Figure 3-24.

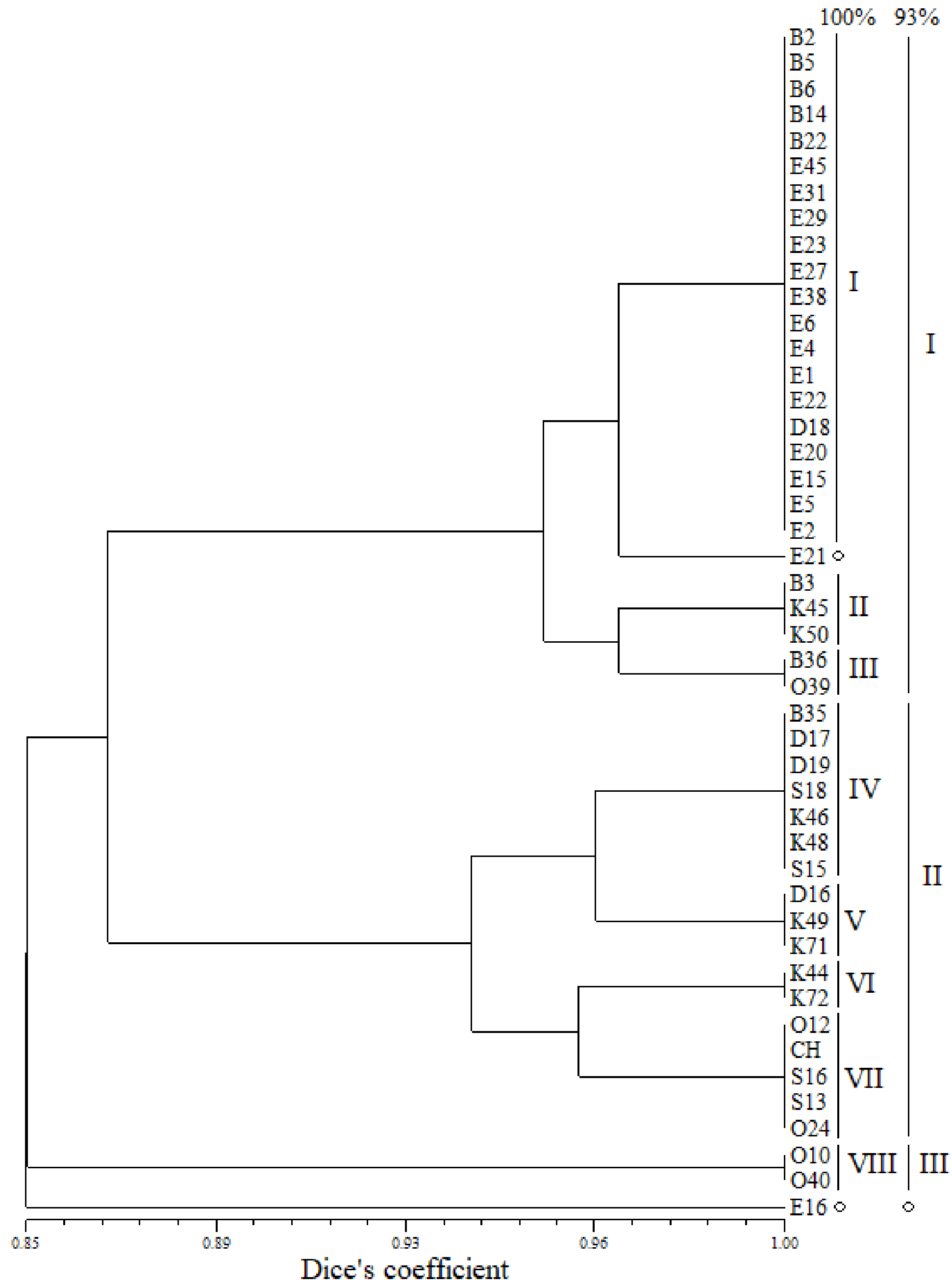


Figure 3-24. Dendrogram for the 46 indigenous *S. cerevisiae* strains constructed from PFGE banding pattern data using UPGMA method and similarity matrices

After cluster analysis, two different coefficients of discrimination of 100% and 93% were applied. By using a coefficient of discrimination of 100%, eight groups and two

single-strain clusters were identified. A good differentiation of the 46 *S. cerevisiae* strains isolated from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir grape varieties collected from three different regions (Ankara, Elazığ and Cappadocia) was observed (Figure 3-24).

Cluster I contained the largest group consisted of twenty strains isolated from three different grape varieties, two different locations (Elazığ, Cappadocia) but in the same year 2017. Cluster I grouped isolates from Boğazkere (B2, B5, B6, B14, B22), Dimrit (D18) and Emir (E1, E2, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31, E38, E45) grape musts and wines.

Cluster II contained strains from Boğazkere (B3) and Kalecik Karası (K45, K50) grape varieties isolated from two different locations Elazığ and Ankara in 2017, respectively.

Cluster III was formed by two strains isolated from two different grape variety Boğazkere (B36) and Öküzgözü (O39) but the same location (Elazığ) and year (2017).

Cluster IV grouped isolates from Boğazkere (B35), Dimrit (D17, D19), Kalecik Karası (K46, K48) isolated in 2017 and (S15, S18) isolated in 2016 from three different locations Elazığ, Cappadocia, and Ankara, respectively.

Cluster V classified strains from two different grape varieties Dimrit (D16) and Kalecik Karası (K49, K71), harvested from two different locations (Cappadocia-Nevşehir, Kalecık-Ankara) but in the same year (2017).

Cluster VI was formed by two strains isolated from one grape variety Kalecik Karası (K44, K72) harvested from Kalecik (Ankara) in 2017.

Cluster VII classified strains from Öküzgözü (O12, O24), Kalecik Karası (S13, S16) and CH isolated from two different locations (Elazığ, Kalecik-Ankara) and in two different years (O12 and O24 strains isolated in 2017 while the S13, S16 strains were isolated in 2016. In addition, CH was a fermenting *S. cerevisiae* strain bought from

Chr. Hansen company (MERIT™, Hoersholm, Denmark) as a reference wine fermentation strain).

Cluster VIII was formed by two strains isolated from one grape variety Öküzgözü (O10, O40) collected from Elazığ in 2017.

Two strains, namely E16 and E21 isolated from Emir grape must in 2017, formed a single-strain cluster by using a coefficient of discrimination of 100% as depicted in UPGMA dendrogram of Figure 3-24.

In addition, by using a coefficient of discrimination of 93%, three groups and one single-strain clusters were identified among the studied *S. cerevisiae* strains (Figure 3-24). Cluster I grouped 26 out of 46 strains consisted of all five grape varieties (red and white grape variety) as following; Boğazkere (B2, B3, B5, B6, B14, B22, B36), Dimrit (D18), Kalecik Karası (K45, K50), Öküzgözü (O39) and Emir (E1, E2, E4, E5, E6, E15, E20, E21, E22, E23, E27, E29, E31, E38, E45) isolated from three different locations (Elazığ, Cappadocia, Ankara) but in the same year 2017.

Cluster II contained 17 strains isolated only from red grape must or wines as following; Boğazkere (B35), Dimrit (D16, D17, D19), Öküzgözü (O12, O24), Kalecik Karası (K44, K46, K48, K49, K71, K72) isolated in 2017 and (S13, S15, S16, S18) isolated in 2016 from three different locations Elazığ, Cappadocia, Ankara and CH (Chr. Hansen, reference wine fermentation strain, Denmark).

Cluster III was formed by two strains isolated from one grape variety Öküzgözü (O10, O40) collected from Elazığ in 2017.

One strains E16 isolated from Emir grape must (white grape variety) in 2017, formed a single-strain cluster by using a coefficient of discrimination of 93% as depicted in UPGMA dendrogram of Figure 3-24.

The electrophoretic karyotyping of the 46 *S. cerevisiae* strains revealed a high polymorphism in the number, size and intensity of the bands. The electrophoretic chromosome patterns analysis showed that the differences between the chromosomal banding patterns.

Although electrophoretic karyotype analysis was reported to be efficient for the differentiation of the *Saccharomyces* species specifically between *S. cerevisiae* strains in the wine industry (Carle & Olson, 1985; Cocolin et al., 2004; Kállai et al., 2019; Pereira et al., 2010; Schütz & Gafner, 1993), some studies were also differentiated species of the genera such as *Candida* (Doi et al., 1992; Hicks et al., 2018), *Lachancea* (Naumova et al., 2007), *Kluyveromyces* (Belloch et al., 1998), *pichia* (Johnston & Mortimer, 1986) and other wine-associated non-*Saccharomyces* yeasts (Johnston & Mortimer, 1986; Raspor et al., 2001) by this method. These studies reported that yeast species varied in their chromosomal makeup and as well as the chromosomal length polymorphisms (CLP) occurred within species.

Polymorphism in the chromosome size of *S. cerevisiae* strains caused variations in chromosomes mobility which could be used to reveal the differences between strains (Pataro et al., 2000). Pataro et al. (2000) analyzed the chromosome polymorphism applying two hybridization probes (YNLO75W and Ade2) which could show significant differences in the chromosomes XIV and XV mobilities. The obtained result indicated that polymorphisms were related to the chromosomic rearrangements which happen during the yeasts growth in the fermentation process. In our studied *S. cerevisiae* strains, the 1125 kb band corresponding to the chromosome VII and XV showed different resolving pattern as well (Figure 3-23). Schuller et al. (2007) evaluated the genetic polymorphisms of 100 strains of the commercial *S. cerevisiae* ZymafloreVL1 isolated from spontaneous fermentations of grape must in the Vinho Verde wine region of Portugal. This study reported a high percentage of chromosomal size variations specially in chromosomes III and VI of *S. cerevisiae* Zymaflore VL1 strain which could be related to the adaptive mechanisms to the changing of the environmental conditions (Schuller et al., 2007). However, we did not recognize any differences in chromosomes III and VI mobilities in the PFGE of our *S. cerevisiae* strains. Interestingly, these chromosomes were fully resolved in all electrophoretic karyotyping of *S. cerevisiae* isolates in this study.

Another study also reported a high level of polymorphism (8 different chromosomal banding patterns) in the electrophoretic karyotyping of nine *S. cerevisiae* strains isolated from Sangiovese grapes of Chianti area (Sebastiani et al., 2004).

Some authors demonstrated different karyotypes in Chinese *S. cerevisiae* strains isolated form different geographical and ecological origins (Q.-M. Wang et al., 2012), *S. cerevisiae* strains isolated from the spontaneously fermented sugarcane *aguardente* (alcoholic beverage) in Brazil (Pataro et al., 2000), and *S. cerevisiae* strains isolated from Tokaj (Hungary) wines more than a century ago (Kállai et al., 2019). Kallai et al. (2019) found no correlation between the karyotypes of *S. cerevisiae* strains and their origin.

The high variability of the Turkish *S. cerevisiae* strains observed by PFGE analysis in this study were in agreement with the findings of Longo and Vezinhet (1993). These authors applied electrophoretic karyotyping for two *S. cerevisiae* strains (a haploid laboratory strain and a diploid enological strain derived from a champagne vineyard) to understand the origin of chromosomal polymorphism between *S. cerevisiae* strains. They showed that the karyotype of the haploid strain was very stable while the diploid strain underwent frequent modifications due to its heterozygotic structure that allows the occurrence of different sizes for homologous chromosomes. Longo and Vezinhet (1993) reported frequent changes at the level of chromosomes I and VI (chromosomal rearrangements). In our studied *S. cerevisiae* strains, the 225 kb band corresponding to chromosome I were not observed in the electrophoretic karyotypes of *S. cerevisiae* strains in profile 3 (CH, O12, O24, S13, S16) and profile 4 (K44, K72) but this band was detected in the karyotypes of profiles 1, 2, 5, 6, 7, 8, 9, and 10 with a variation in size (~ 250 and 225 kb) as described in chromosomal DNA size of each profile (Figure 3-23). Although Longo and Vezinhet (1993) showed frequent changes for the chromosome VI, this chromosome was resolved in the electrophoretic karyotypes of all *S. cerevisiae* isolates with the similar pattern in our study. In addition, these authors showed a unique band corresponded to the chromosome V was able to generate a doublet during mitosis like E21 strain in our study. According to Longo and Vezinhet (1993), the six smallest

chromosomes were not equally susceptible to structural modifications. Chromosome IX was never modified and chromosome III was only rarely modified (Longo & Vezinhet, 1993). As mentioned in our PFGE results, these two chromosomes IX and III were observed in all *S. cerevisiae* strains. Finally, these authors concluded that the chromosome length polymorphisms observed among *S. cerevisiae* strains were the result of chromosomal rearrangements during mitosis, hybridization and meiotic recombination either reciprocal or non-reciprocal between homologous chromosomes of different sizes (Longo & Vezinhet, 1993).

Valero et al. (2007) study reported 104 different chromosomal patterns among 608 *Saccharomyces* strains obtained from three different vineyards in France during 3 years. This study showed that differences in autochthonous wine yeast biodiversity influenced by climatic conditions and age and size of vineyards (Valero et al., 2007).

It is important to note that variation in ploidy and aneuploidy (gain or loss of chromosomes) was common in *S. cerevisiae* strains with different geographical and ecological origins (Peter et al., 2018) which could be related to rapid adaptation of *S. cerevisiae* strains to environmental changes. Peter et al. (2018) reported a total of 342 cases of aneuploidy that frequently observed in chromosomes I, III and IX and affected 19.1% of the examined 1,011 *S. cerevisiae* strains.

Many previous researches were found intermediate to high levels of genetic divergence among indigenous *S. cerevisiae* isolates, which was likely due to geographical attributes, changing of the wine environment, and anthropogenic practices like SO_2 treatment (Chen et al., 2022; Peter et al., 2018).

To summarize, the clustering analysis and the electrophoretic karyotyping of the Turkish *S. cerevisiae* strains which showed 10 different chromosomal patterns among 46 *S. cerevisiae* strains revealed the genetic diversity of indigenous *S. cerevisiae* strains isolated from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit (red) and Emir (white) grapes at different wine-making stages in three geographically separated viticultural zones (Ankara, Elazığ, and Cappadocia) in Türkiye. PFGE showed differences in the karyotypes of the selected *S. cerevisiae* strains, as well as

between strains isolated from the same source. This confirmed the presence of a great biodiversity of yeast strains from grape musts of local vine-growing regions in Türkiye.

3.4.6 RAPD-PCR and MSP-PCR combination versus PFGE method

The comparison between RAPD/MSP-PCR and PFGE data carried out for the 46 indigenous *S. cerevisiae* strains were deduced from Figures 3-19 and 3-24. Overall, PFGE typing resulted in three groups and one single-strain clusters by using a coefficient of discrimination of 93% containing genetically related or identical strains versus five groups and nine single-strain clusters using the RAPD/MSP-PCR typing technique based on a homology of >88% (Table 3-21).

Table 3-21 Cluster classification of the 46 *S. cerevisiae* strains based on RAPD/MSP-PCR and PFGE obtained by the UPGMA method

Cluster No	RAPD/MSP-PCR (88%) *	PFGE (93%) *
I	B2, B3, D16, D18	B2, B3, B5, B6, B14, B22, B36, D18, K45, K50, O39, E1, E2, E4, E5, E6, E15, E20, E21, E22, E23, E27, E29, E31, E38, E45
II	B5, B36, O10, O40, K44, S15, S16	B35, D16, D17, D19, O12, O24, K44, K46, K48, K49, K71, K72, S13, S15, S16, S18, CH
III	B6, B14, K45, K46, K48, K49, K50, K71, K72, E1, E4, E5, E6, E15, E20, E22, E23, E27, E29, E31	O10, O40
IV	E16, E21, O12	E16
V	D17, D19, E45	
VI	E38	
VII	E2	
VIII	O24	
IX	O39	
X	B35	
XI	B22	
XII	CH	
XIII	S13	
XIV	S18	

*, Coefficient of discrimination.

Although these clusters were not identical, for cluster I in PFGE, relatively small numbers of strains did not belong to cluster III on the basis of RAPD/MSP-PCR. In the case of cluster II of PFGE, 3 out of 17 strains were in the same cluster of RAPD/MSP-PCR. The other strains of PFGE cluster II were assorted in RAPD/MSP group I, III, IV, V and single-strain clusters. In the case of cluster III of PFGE which contained two strains, these two strains were grouped in cluster II of RAPD/MSP-PCR. For cluster IV of PFGE which contained single strain, this strain was also belonged to this cluster on the basis of RAPD/MSP-PCR. RAPD/MSP group VI, VII, VIII, IX, X, XI, XII, XIII and XIV comprised 9 strains identified as unique genotypes. We also noticed that some PFGE clusters were resolved by RAPD/MSP-PCR typing. Comparison of PFGE and RAPD data supports the conclusion that PFGE deduced relatedness among strains was corroborated by RAPD/MSP-PCR and vice versa.

Molecular typing techniques need to be carried out using a standard protocol in order to increase inter-laboratory reproducibility (Williams et al., 1990; Kállai et al., 2019). In this study, both methods were validated and standardized to a satisfactory level that revealed an appropriate index of genetic diversity among the *S. cerevisiae* strains tested. However, PFGE method showed the high level of genetic similarities between strains. That meant slight differences were observed in electrophoretic karyotyping (chromosomal patterns) of the Turkish *S. cerevisiae* strains.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

In this study, traditional wine production was achieved by spontaneous fermentation of indigenous yeast species present in five different grape musts of Kalecik Karası, Boğazkere, Öküzgözü, Dimrit, and Emir which grown in three geographically separated viticultural zones (Ankara, Elazığ, and Cappadocia) in Türkiye. Therefore, wines from such uninoculated fermentations were observed to maintain the 'microbial terroir' (microbial biogeography) of the Ankara, Elazığ, and Cappadocia regions in Türkiye.

The effect of indigenous yeasts (terroir) on volatiles composition and aromatic attributes in these wine samples were investigated by GC-FID and GC-MS. A total of 56 volatile compounds were detected. Among the 52, 44, 43, 43 quantitated volatile compounds, 12, 12, 12, 13 volatiles were found to significantly contribute their flavor notes to the overall aroma of Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit wines, respectively. Principal component analysis also demonstrated that grape varieties had a strong effect on the aroma compounds. Concerning the PCA plot, the first group Boğazkere, Öküzgözü, and Dimrit wines produced from grapes grown in Elazığ, Elazığ, and Cappadocia, respectively was positively correlated with phenylethyl alcohol (rose, honey aroma), isovaleric acid (cheese, floral aroma), phenethyl acetate (floral, rose aroma), isoamyl acetate (banana, fruity aroma), ethyl octanoate (fruity, floral aroma), isoamyl alcohol (whiskey aroma), guaiacol (smoke, sweet aroma), ethyl hexanoate (flowery, fruity aroma) and (z)-3-hexen-1-ol (green, cypress aroma) with regard to PC1 which played important roles in the aromatic characteristic of these wines, whereas the second group contained Kalecik Karası wine was negatively associated with methionol (cooked vegetable aroma), 2-methoxy-4-vinylphenol (spices, curry aroma), 3-ethoxy-1-propanol (fruity aroma) and geraniol (roses, geranium aroma) with respect to PC1.

The biodiversity of autochthonous non-*Saccharomyces* and *Saccharomyces* yeasts in must and wine samples made from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit and Emir grape varieties were detected by real-time PCR. 16 Specific oligonucleotide primers for real-time PCR were used to analyze predominant non-*Saccharomyces* (*C. glabrata*, *C. zemplinina*, *C. zeylanoides*, *Hanseniaspora* spp., *H. uvarum*, *I. orientalis*, *L. thermotolerans*, *Metschnikowia* spp., *M. pulcherrima*, *P. fermentans*, *P. kluyveri*, *R. mucilaginosa*, *T. delbrueckii*, *W. anomalus*) and *Saccharomyces* yeasts (*Saccharomyces* spp., *S. cerevisiae*) in fresh grape must, during maceration and alcoholic fermentation. The detection results obtained by real-time PCR method were compared with the results of colony isolation and identification by internal transcribed spacer (ITS) region (ITS1–5.8S rRNA– ITS2) and/or D1/D2 domain of the 26S rRNA gene sequencing. Isolation/sequencing results were in concordant with the results of real-time PCR in respect to *C. zemplinina*, *Hanseniaspora* spp., *H. uvarum*, *R. mucilaginosa*, *W. anomalus*, *L. thermotolerans*, *Metschnikowia* spp., *Saccharomyces* spp. and *S. cerevisiae* detection. Moreover, *C. zeylanoides* was not detected neither by real-time PCR nor isolation/sequencing method. However, several species such as *C. glabrata*, *I. orientalis*, *P. fermentans*, *P. kluyveri*, *T. delbrueckii* were only detected by real-time PCR. This result indicated the usefulness of real-time PCR for tracking the presence of target yeast species in the complex yeast community of Turkish grape must and wine.

Moreover, successful results were obtained in the quantification of selected yeasts *H. uvarum, L. thermotolerans, T. delbrueckii* as non-*Saccharomyces* species and *S. cerevisiae*, which can positively affect the aroma or taste of wine and can be used as starter cultures, with qPCR assay. It has been concluded that the amount of strain desired to be followed by this method can be determined successfully during maceration and fermentation periods. In addition, few molecular techniques have been developed to detect both spoilage and beneficial yeast species populations directly from must or wine. Therefore, the real-time PCR method used in this study would allow winemakers to quickly identify yeast population levels specially spoilage one for making efficient processing decisions to remove spoilage.

This study is the first to systematically analyze the biodiversity and volatile compound of the spontaneously fermented wines in Türkiye.

Furthermore, the analysis of genotyping profiles showed genetic diversity between the investigated strains. The results of the present study indicated that RAPD-PCR and MSP-PCR combination techniques which resulted in five groups and nine single-strain clusters by using a coefficient of discrimination of >88% were applicable to the identification of intraspecific genetic diversity between the 46 indigenous *S. cerevisiae* strains isolated from five different grape must and wine at variable stages and the evaluation of their genetic relationship, giving an important support for the study of yeast populations, the modification of them during wine fermentation processes and using them as starter cultures.

In addition, a high degree of heterogeneity was also obtained with primer OPA-11 in differentiation of the 46 indigenous strains of *S. cerevisiae* which resulted in seventeen different band patterns generated by RAPD-PCR using primer OPA-11 in this study. Therefore, it could be recommended to use as a powerful method to discriminate between *S. cerevisiae* strains in various studies.

The clustering analysis of PFGE profiles resulted in three groups and one single-strain clusters by using a coefficient of discrimination of 93% and the electrophoretic karyotyping of the *S. cerevisiae* strains which showed 10 different chromosomal patterns among 46 *S. cerevisiae* strains also revealed the genetic diversity of indigenous *S. cerevisiae* strains isolated from Kalecik Karası, Boğazkere, Öküzgözü, Dimrit (red) and Emir (white) grapes at different wine-making stages in Türkiye. PFGE showed differences in the karyotypes of the selected *S. cerevisiae* strains, as well as between strains isolated from the same source. This confirmed the presence of a great biodiversity of yeast strains from grape musts of local vine-growing regions in Türkiye. The karyotypes of the Turkish *S. cerevisiae* strains isolated from different grape must and wine have not been explored yet so this dissertation was revealed their genetic polymorphism for the first time.

In this study we were characterized and classified indigenous *S. cerevisiae* strains at genotypic level by RAPD-PCR, MSP-PCR and PFGE so it would be possible to use

these *S. cerevisiae* strains as a starter culture in wine production. It was also recommended to carry out further phenotypic characterization of these yeasts (already done for some of them) to produce regional or global wine inoculated by these strains.

Finally, there is growing interest among winemakers in using indigenous yeasts that are better adapted to local grape varieties and winemaking condition, thus reflecting the unique "microbial terroir" of a given region.

REFERENCES

Aktuna, I. (2019). Isolation, identification and characterization of non-*Saccharomyces* and *Saccharomyces* yeasts in traditional wines made from five different national grape in Turkey. Middle East Technical University, Graduate School of Natural and Applied Sciences, Biotechnology Department, MSc Thesis.

Alsammar, H., & Delneri, D. (2020). An update on the diversity, ecology and biogeography of the *Saccharomyces* genus. *FEMS Yeast Research*, *20*(3), 1–12. https://doi.org/10.1093/femsyr/foaa013

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J. M., & Mas, A. (2010). Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *International Journal of Food Microbiology*, *144*(2), 257–262. https://doi.org/10.1016/j.ijfoodmicro.2010.10.003

Andrade, M. J., Rodríguez, M., Sánchez, B., Aranda, E., & Córdoba, J. J. (2006). DNA typing methods for differentiation of yeasts related to dry-cured meat products. *International Journal of Food Microbiology*, *107*(1), 48–58. https://doi.org/10.1016/j.ijfoodmicro.2005.08.011

Andrighetto, C., Psomas, E., Tzanetakis, N., Suzzi, G., & Lombardi, A. (2000). Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. *Letters in Applied Microbiology*, *30*(1), 5–9. https://doi.org/10.1046/j.1472-765x.2000.00589.x

Arcari, S. G., Caliari, V., Sganzerla, M., & Godoy, H. T. (2017). Volatile composition of Merlot red wine and its contribution to the aroma: optimization and validation of analytical method. *Talanta*, *174*(June), 752–766. https://doi.org/10.1016/j.talanta.2017.06.074

Bagheri, B., Bauer, F. F., & Setati, M. E. (2016). The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. *South African Journal of Enology and Viticulture*, *36*(2), 243–251. https://doi.org/10.21548/36-2-957

Bagheri, B., Zambelli, P., Vigentini, I., Bauer, F. F., & Setati, M. E. (2018). Investigating the effect of selected non-*Saccharomyces* species on wine ecosystem function and major volatiles. *Frontiers in Bioengineering and Biotechnology*, *6*(NOV), 1–12. https://doi.org/10.3389/fbioe.2018.00169

Bakker, J., & Clarke, R. J. (2011). Wine flavour chemistry. In *Wine Flavour*

Chemistry, Second Edition (Second). Wiley. https://doi.org/10.1002/9781444346022

Baleiras Couto, M. M., Eijsma, B., Hofstra, H., Huis in't Veld, J. H., & van der Vossen, J. M. (1996). Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology*, *62*(1), 41–46. https://doi.org/10.1128/aem.62.1.41-46.1996

Bayram, M., & Kayalar, M. (2018). White wines from Narince grapes: Impact of two different grape provenances on phenolic and volatile composition. *Oeno One*, *52*(2), 81–92. https://doi.org/10.20870/oeno-one.2018.52.2.2114

Belloch, C., Barrio, E., García, M. D., & Querol, A. (1998). Inter- and intraspecific chromosome pattern variation in the yeast genus *Kluyveromyces*. *Yeast (Chichester, England)*, *14*(15), 1341–1354. https://doi.org/10.1002/(SICI)1097-0061(199811)14:15<1341::AID-YEA328>3.0.CO;2-U

Benito, Á., Calderón, F., & Benito, S. (2018). *Schizosaccharomyces pombe Biotechnological Applications in Winemaking* (Vol. 1721, pp. 217–226). Humana Press. https://doi.org/10.1007/978-1-4939-7546-4_19

Benito, Á., Calderón, F., Benito, S., Benito, Calderón, & Benito. (2019). The influence of non-*Saccharomyces* species on wine fermentation quality parameters. *Fermentation*, *5*(3), 54. https://doi.org/10.3390/fermentation5030054

Benito, Calderón, & Benito. (2019). The influence of non-*Saccharomyces* species on wine fermentation quality parameters. *Fermentation*, *5*(3), 54. https://doi.org/10.3390/fermentation5030054

Benito, S. (2018a). The impact of *Torulaspora delbrueckii* yeast in winemaking. *Applied Microbiology and Biotechnology*, *102*(7), 3081–3094. https://doi.org/10.1007/s00253-018-8849-0

Benito, S. (2018b). The impacts of *Lachancea thermotolerans* yeast strains on winemaking. *Applied Microbiology and Biotechnology*, *102*(16), 6775–6790. https://doi.org/10.1007/s00253-018-9117-z

Birren, B. W., Hood, L., & Lai, E. (1989). Pulsed field gel electrophoresis: studies of DNA migration made with the programmable, autonomously-controlled electrode electrophoresis system. *Electrophoresis*, *10*(5–6), 302–309. https://doi.org/10.1002/elps.1150100506

Borren, E., & Tian, B. (2020). The important contribution of non-*Saccharomyces*

yeasts to the aroma complexity of wine: A Review. *Foods*, *10*(1), 13. https://doi.org/10.3390/foods10010013

Bujdosó, G., Egli, C. M., & Henick-Kling, T. (2001a). Characterization of *Hanseniaspora* (*Kloeckera*) strains isolated in Finger Lakes wineries using physiological and molecular techniques. *Food Technology and Biotechnology*, *39*(2), 83–91.

Bujdosó, G., Egli, C. M., & Henick-Kling, T. (2001b). Inter- and intra-specific differentiation of natural wine strains of *Hanseniaspora* (*Kloeckera*) by physiological and molecular methods. *Food Technology and Biotechnology*, *39*(1), 19–28.

Cadez, N., Raspor, P., de Cock, A. W. A. M., Boekhout, T., & Smith, M. T. (2002). Molecular identification and genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*. *FEMS Yeast Research*, *1*(4), 279–289. https://doi.org/10.1111/j.1567-1364.2002.tb00046.x

Cadez, N., & Smith, M. T. (2011). *Hanseniaspora Zikes* (1912). In *The Yeasts* (Vol. 2, Issue 1912, pp. 421–434). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00032-X

Cai, J., Zhu, B.-Q., Wang, Y.-H., Lu, L., Lan, Y.-B., Reeves, M. J., & Duan, C.-Q. (2014). Influence of pre-fermentation cold maceration treatment on aroma compounds of Cabernet Sauvignon wines fermented in different industrial scale fermenters. *Food Chemistry*, *154*, 217–229. https://doi.org/10.1016/j.foodchem.2014.01.003

Callejo, M. J., González, C., & Morata, A. (2017). Use of non-*Saccharomyces* yeasts in bottle fermentation of aged beers. In *Brewing Technology* (Issue August). InTech. https://doi.org/10.5772/intechopen.68793

Callejon, R. M., Clavijo, A., Ortigueira, P., Troncoso, A. M., Paneque, P., & Morales, M. L. (2010). Volatile and sensory profile of organic red wines produced by different selected autochthonous and commercial *Saccharomyces cerevisiae* strains. *Analytica Chimica Acta*, *660*(1–2), 68–75. https://doi.org/10.1016/j.aca.2009.09.040

Capece, A., Granchi, L., Guerrini, S., Mangani, S., Romaniello, R., Vincenzini, M., & Romano, P. (2016). Diversity of *Saccharomyces cerevisiae* strains isolated from two Italian wine-producing regions. *Frontiers in Microbiology*, *7*(JUN), 1–11. https://doi.org/10.3389/fmicb.2016.01018

Capece, A., Romaniello, R., Siesto, G., & Romano, P. (2012). Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneously fermenting

grapes from an Italian “heroic vine-growing area.” *Food Microbiology*, *31*(2), 159–166. https://doi.org/10.1016/j.fm.2012.03.010

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., & Spano, G. (2015). Microbial terroir and food innovation: The case of yeast biodiversity in wine. *Microbiological Research*, *181*, 75–83. https://doi.org/10.1016/j.micres.2015.10.005

Carle, G. F., & Olson, M. V. (1985). An electrophoretic karyotype for yeast. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(11), 3756–3760. https://doi.org/10.1073/pnas.82.11.3756

Castillo, M., Silva, E. da, Câmara, J. S., & Khadem, M. (2020). Molecular Identification and VOMs Characterization of *Saccharomyces cerevisiae* Strains Isolated from Madeira Region Winery Environments. *Processes*, *8*(9), 1058. https://doi.org/10.3390/pr8091058

Celik, Z. D., Cabaroglu, T., & Krieger-Weber, S. (2019). Impact of malolactic fermentation on the volatile composition of Turkish Kalecik karası red wines. *Journal of the Institute of Brewing*, *125*(1), 92–99. https://doi.org/10.1002/jib.540

Çelik, Z. D., Erten, H., Darici, M., & Cabaroğlu, T. (2017). Molecular characterization and technological properties of wine yeasts isolated during spontaneous fermentation of *Vitis vinifera* L.cv. Narince grape must grown in ancient wine making area Tokat, Anatolia. *BIO Web of Conferences*, *9*, 02017. https://doi.org/10.1051/bioconf/20170902017

Chambers, G. K., & MacAvoy, E. S. (2000). Microsatellites: consensus and controversy. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *126*(4), 455–476. https://doi.org/10.1016/S0305-0491(00)00233-9

Chambers, P. J., Borneman, A. R., Varela, C., Cordente, A. G., Bellon, J. R., Tran, T. M. T., Henschke, P. A., & Curtin, C. D. (2015). Ongoing domestication of wine yeast: past, present and future. *Australian Journal of Grape and Wine Research*, *21*, 642–650. https://doi.org/10.1111/ajgw.12190

Chambers, P. J., & Pretorius, I. S. (2010). Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Reports*, *11*(12), 914–920. https://doi.org/10.1038/embor.2010.179

Chen, Y., Jiang, J., Song, Y., Zang, X., Wang, G., Pei, Y., Song, Y., Qin, Y., & Liu, Y. (2022). Yeast diversity during spontaneous fermentations and oenological characterisation of indigenous *Saccharomyces cerevisiae* for potential as wine

starter cultures. *Microorganisms*, *10*(7), 1455. https://doi.org/10.3390/microorganisms10071455

Cheng, G., Liu, Y., Yue, T.-X., & Zhang, Z.-W. (2015). Comparison between aroma compounds in wines from four *Vitis vinifera* grape varieties grown in different shoot positions. *Food Science and Technology (Campinas)*, *35*(2), 237–246. https://doi.org/10.1590/1678-457X.6438

Cocolin, L., Pepe, V., Comitini, F., Comi, G., & Ciani, M. (2004). Enological and genetic traits of isolated from former and modern wineries. *FEMS Yeast Research*, *5*(3), 237–245. https://doi.org/10.1016/j.femsyr.2004.08.005

Couto, M. M. B., Eijsma, B., Hofstra, H., Huis In'T Veld, J. H. J., & Van Der Vossen, J. M. B. M. (1996). Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Applied and Environmental Microbiology*, *62*(1), 41–46. https://doi.org/10.1128/aem.62.1.41-46.1996

Darici, M., Cabaroglu, T., Ferreira, V., & Lopez, R. (2014). Chemical and sensory characterisation of the aroma of Çalkarasi{dotless} rosé wine. *Australian Journal of Grape and Wine Research*, *20*(3), 340–346. https://doi.org/10.1111/ajgw.12095

De Benedictis, M., Bleve, G., Grieco, F., Tristezza, M., Tufariello, M., & Grieco, F. (2011). An optimized procedure for the enological selection of non-*Saccharomyces* starter cultures. *Antonie van Leeuwenhoek*, *99*(2), 189–200. https://doi.org/10.1007/s10482-010-9475-8

Del Fresno, J. M., Escott, C., Loira, I., Carrau, F., Cuerda, R., Schneider, R., Bañuelos, M. A., González, C., Suárez-Lepe, J. A., & Morata, A. (2021). The impact of *Hanseniaspora vineae* fermentation and ageing on lees on the terpenic aromatic profile of white wines of the albillo variety. *International Journal of Molecular Sciences*, *22*(4), 1–14. https://doi.org/10.3390/ijms22042195

Di Lorenzo, C., Badea, M., Colombo, F., Orgiu, F., Frigerio, G., Pastor, R. F., & Restani, P. (2017). Antioxidant activity of wine assessed by different in vitro methods. *BIO Web of Conferences*, *9*, 04008. https://doi.org/10.1051/bioconf/20170904008

Díaz, C., Molina, A. M., Nähring, J., & Fischer, R. (2013). Characterization and Dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *BioMed Research International*, *2013*, 1–13. https://doi.org/10.1155/2013/540465

Doi, M., Homma, M., Chindamporn, A., & Tanaka, K. (1992). Estimation of

chromosome number and size by pulsed-field gel electrophoresis (PFGE) in medically important *Candida* species. *Journal of General Microbiology*, *138*(10), 2243–2251. https://doi.org/10.1099/00221287-138-10-2243

du Plessis, H. W., du Toit, M., Hoff, J. W., Hart, R. S., Ndimba, B. K., & Jolly, N. P. (2017). Characterisation of non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. *South African Journal of Enology & Viticulture*, *38*(2), 46–63. https://doi.org/10.21548/38-1-819

Duan, W.-P., Zhu, B.-Q., Song, R.-R., Zhang, B., Lan, Y.-B., Zhu, X., Duan, C.-Q., & Han, S.-Y. (2018). Volatile composition and aromatic attributes of wine made with *Vitisvinifera L .cv* Cabernet Sauvignon grapes in the Xinjiang region of China: effect of different commercial yeasts. *International Journal of Food Properties*, *21*(1), 1423–1441. https://doi.org/10.1080/10942912.2018.1479860

Eldarov, M. A., & Mardanov, A. V. (2020). Metabolic Engineering of wine strains of *Saccharomyces cerevisiae*. *Genes*, *11*(9), 964. https://doi.org/10.3390/genes11090964

Fernández-Espinar, M. T., Barrio, E., & Querol, A. (2003). Analysis of the genetic variability in the species of the *Saccharomyces sensu stricto* complex. *Yeast*, *20*(14), 1213–1226. https://doi.org/10.1002/yea.1034

Fernández-Espinar, M. T., Llopis, S., Querol, A., & Barrio, E. (2011). Molecular identification and characterization of wine yeasts. In *Molecular Wine Microbiology* (Issue July 2018, pp. 111–141). Elsevier. https://doi.org/10.1016/B978-0-12-375021-1.10005-0

García, M., Esteve-Zarzoso, B., Cabellos, J., & Arroyo, T. (2018). Advances in the Study of *Candida stellata. Fermentation*, *4*(3), 74. https://doi.org/10.3390/fermentation4030074

García, M., Esteve-Zarzoso, B., Crespo, J., Cabellos, J. M., & Arroyo, T. (2017). Yeast monitoring of wine mixed or sequential fermentations made by native strains from D.O. "Vinos de Madrid" using real-time quantitative PCR. *Frontiers in Microbiology*, *8*(DEC), 1–15. https://doi.org/10.3389/fmicb.2017.02520

Garofalo, C., Berbegal, C., Grieco, F., Tufariello, M., Spano, G., & Capozzi, V. (2018). Selection of indigenous yeast strains for the production of sparkling wines from native Apulian grape varieties. *International Journal of Food Microbiology*, *285*(July), 7–17. https://doi.org/10.1016/j.ijfoodmicro.2018.07.004

Gemayel, R., Cho, J., Boeynaems, S., & Verstrepen, K. J. (2012). Beyond junk-variable tandem repeats as facilitators of rapid evolution of regulatory and coding sequences. *Genes*, *3*(3), 461–480. https://doi.org/10.3390/genes3030461

González-Barreiro, C., Rial-Otero, R., Cancho-Grande, B., & Simal-Gándara, J. (2015). Wine aroma compounds in grapes: a critical review. *Critical Reviews in Food Science and Nutrition*, *55*(2), 202–218. https://doi.org/10.1080/10408398.2011.650336

Gschaedler, A. (2017). Contribution of non-conventional yeasts in alcoholic beverages. *Current Opinion in Food Science*, *13*, 73–77. https://doi.org/10.1016/j.cofs.2017.02.004

Guaragnella, N., Chiara, M., Capece, A., Romano, P., Pietrafesa, R., Siesto, G., Manzari, C., & Pesole, G. (2020). Genome sequencing and comparative analysis of three *Hanseniaspora uvarum* indigenous wine strains reveal remarkable biotechnological potential. *Frontiers in Microbiology*, *10*(January), 1–14. https://doi.org/10.3389/fmicb.2019.03133

Gumus, S. G., & Gumus, A. H. (2008). *THE Wine sector in Turkey: Survey on 42 wineries*. Bulgarian Journal of Agricultural Science.

Gur-Arie, R., Cohen, C. J., Eitan, Y., Shelef, L., Hallerman, E. M., & Kashi, Y. (2000). Simple sequence repeats in *Escherichia coli*: abundance, distribution, composition, and polymorphism. *Genome Research*, *10*(1), 62–71. http://www.ncbi.nlm.nih.gov/pubmed/10645951

Hacio, E., & Basim, L. U. (2001). Pulsed-Field Gel Electrophoresis (PFGE) Technique and its use in molecular biology. *Turkish Journal of Biology*, *25*(4), 405–418.

Heras, J., Domínguez, C., Mata, E., Pascual, V., Lozano, C., Torres, C., & Zarazaga, M. (2016). A survey of tools for analysing DNA fingerprints. *Briefings in Bioinformatics*, *17*(6), 903–911. https://doi.org/10.1093/bib/bbv016

Hicks, L., Van der Graaf, C. M., Childress, J., Cook, E., Schmidt, K., Rosenzweig, F., & Kroll, E. (2018). Streamlined preparation of genomic DNA in agarose plugs for pulsed-field gel electrophoresis. *Journal of Biological Methods*, *5*(1), e86. https://doi.org/10.14440/jbm.2018.218

Hierro, N., Esteve-Zarzoso, B., González, Á., Mas, A., & Guillamón, J. M. (2006). Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Applied and Environmental Microbiology*, *72*(11), 7148–7155. https://doi.org/10.1128/AEM.00388-06

Hierro, N., Esteve-Zarzoso, B., Mas, A., & Guillamón, J. M. (2007). Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. *FEMS Yeast Research*, *7*(8), 1340–1349. https://doi.org/10.1111/j.1567-1364.2007.00304.x

Hu, K., Jin, G. J., Xu, Y. H., & Tao, Y. S. (2018). Wine aroma response to different participation of selected *Hanseniaspora uvarum* in mixed fermentation with Saccharomyces cerevisiae. *Food Research International*, *108*(March), 119–127. https://doi.org/10.1016/j.foodres.2018.03.037

Hyma, K. E., Saerens, S. M., Verstrepen, K. J., & Fay, J. C. (2011). Divergence in wine characteristics produced by wild and domesticated strains of *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *11*, 540–551. https://doi.org/10.1111/j.1567-1364.2011.00746.x

Jackson, D. I., & Lombard, P. B. (1993). Environmental and Management Practices Affecting Grape Composition and Wine Quality - A Review. *American Journal of Enology and Viticulture*, *44*(4), 409 LP – 430. http://www.ajevonline.org/content/44/4/409.abstract

Johnston, J. R., & Mortimer, R. K. (1986). Electrophoretic karyotyping of laboratory and commercial strains of *Saccharomyces* and other yeasts. *International Journal of Systematic Bacteriology*, *36*, 569–572. https://doi.org/10.1099/00207713-36-4-569

Kállai, Z., Pfliegler, W. P., Mitercsák, J., Szendei, G., & Sipiczki, M. (2019). Preservation of diversity and oenological properties of wine yeasts during long-term laboratory maintenance: A study of strains of a century-old Tokaj wine yeast collection. *LWT*, *101*(December 2018), 789–798. https://doi.org/10.1016/j.lwt.2018.12.002

Kontogiannatos, D., Troianou, V., Dimopoulou, M., Hatzopoulos, P., & Kotseridis, Y. (2021). Oenological potential of autochthonous *Saccharomyces cerevisiae* yeast strains from the greek varieties of agiorgitiko and moschofilero. *Beverages*, *7*(2). https://doi.org/10.3390/BEVERAGES7020027

Korhola, M., Naumova, E. S., Partti, E., Aittamaa, M., Turakainen, H., & Naumov, G. I. (2019). Exploiting heterozygosity in industrial yeasts to create new and improved baker's yeasts. *Yeast*, *36*(9), 571–587. https://doi.org/10.1002/yea.3428

Kumar Lathar, P., Sharma, A., & Thakur, I. (2010). Isolation and random amplified polymorphic DNA (RAPD) analysis of wild yeast species from 17 different fruits. *Journal of Yeast and Fungal Research*, *1*(8), 146–151. http://www.academicjournals.org/JYFR

Kurtzman, C. P. (2011). Torulaspora. In *The Yeasts* (Vol. 2, Issue 1904, pp. 867–874). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00075-6

Kurtzman, C. P., Fell, J. W., & Boekhout, T. (2011). Definition, classification and nomenclature of the yeasts. In *The Yeasts* (Vol. 1). Elsevier B.V. https://doi.org/10.1016/B978-0-444-52149-1.00001-X

Kurtzman, C. P., Robnett, C. J., Basehoar, E., & Ward, T. J. (2018). Four new species of *Metschnikowia* and the transfer of seven *Candida* species to *Metschnikowia* and *Clavispora* as new combinations. *Antonie van Leeuwenhoek*, *111*(11), 2017–2035. https://doi.org/10.1007/s10482-018-1095-8

Lachance, M.-A. (2011). *Metschnikowia.* In *The Yeasts* (Vol. 2, Issue 1899, pp. 575–620). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00046-X

Lachance, M.-A. (2016). *Metschnikowia* : half tetrads, a regicide and the fountain of youth. *Yeast*, *33*(11), 563–574. https://doi.org/10.1002/yea.3208

Lachance, M.-A., Boekhout, T., Scorzetti, G., Fell, J. W., & Kurtzman, C. P. (2011). *Candida*. In *The Yeasts* (Vol. 2, Issue 1923, pp. 987–1278). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00090-2

Lachance, M.-A., & Kurtzman, C. P. (2011). *Lachancea*. In *The Yeasts* (Vol. 2, Issue 2003, pp. 511–519). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00041-0

Lahue, C., Madden, A. A., Dunn, R. R., & Smukowski Heil, C. (2020). History and domestication of *Saccharomyces cerevisiae* in bread baking. *Frontiers in Genetics*, *11*, 584718. https://doi.org/10.3389/fgene.2020.584718

Lambrechts, M. G., & Pretorius, I. S. (2019). Yeast and its importance to wine aroma - A Review. *South African Journal of Enology & Viticulture*, *21*(1). https://doi.org/10.21548/21-1-3560

Larroque, M. N., Carrau, F., Fariña, L., Boido, E., Dellacassa, E., & Medina, K. (2021). Effect of *Saccharomyces* and non-*Saccharomyces* native yeasts on beer aroma compounds. *International Journal of Food Microbiology*, *337*(July 2020). https://doi.org/10.1016/j.ijfoodmicro.2020.108953

Lesschaeve, I., & Noble, A. C. (2010). Sensory analysis of wine. In *Managing Wine Quality* (pp. 189–217). Elsevier. https://doi.org/10.1533/9781845699284.2.189

Levent, A., & Demir, Z. (2020). The Varieties and characteristics of grapes grown in Southeast Anatolia Region. Güneydoğu Anadolu bölgesinde yetişen üzüm

çeşitleri ve geometrik özellikleri. *Batman University Journal of Life Sciences*, *10*(2), 121–135.

Lieckfeldt, E., Meyer, W., & Börner, T. (1993). Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *Journal of Basic Microbiology*, *33*(6), 413–425. https://doi.org/10.1002/jobm.3620330609

Lleixà, J., Kioroglou, D., Mas, A., & Portillo, M. del C. (2018). Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and *Botrytis* infected grapes. *International Journal of Food Microbiology*, *281*(February), 36–46. https://doi.org/10.1016/j.ijfoodmicro.2018.05.016

Lleixà, J., Martín, V., Portillo, M. del C., Carrau, F., Beltran, G., & Mas, A. (2016). Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Frontiers in Microbiology*, *7*(MAR), 1–12. https://doi.org/10.3389/fmicb.2016.00338

Longo, E., & Vezinhet, F. (1993). Chromosomal rearrangements during vegetative growth of a wild strain of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *59*(1), 322–326. https://doi.org/10.1128/aem.59.1.322-326.1993

Marsit, S., Dequin, S., Kontogiannatos, D., Troianou, V., Dimopoulou, M., Hatzopoulos, P., Kotseridis, Y., Teresa Fernández-Espinar, M., Llopis, S., Querol, A., Barrio, E., Fernandez-Espinar, M. T., Llopis, S., Querol, A., Barrio, E., Belda, I., Ruiz, J., Alastruey-Izquierdo, A., Navascués, E., … Barrio, E. (2016). Unraveling the enzymatic basis of wine “flavorome”: a phylo-functional study of wine related yeast species. *Frontiers in Microbiology*, *7*(2), 1–13. https://doi.org/10.3389/fmicb.2016.00012

Martin, V., Valera, M., Medina, K., Boido, E., & Carrau, F. (2018). Oenological impact of the H*anseniaspora/Kloeckera* yeast genus on wines—A Review. *Fermentation*, *4*(3), 76. https://doi.org/10.3390/fermentation4030076

Martorell, P., Fernández-Espinar, M. T., & Querol, A. (2005). Molecular monitoring of spoilage yeasts during the production of candied fruit nougats to determine food contamination sources. *International Journal of Food Microbiology*, *101*(3), 293–302. https://doi.org/10.1016/j.ijfoodmicro.2004.11.014

Martorell, P., Querol, A., & Fernández-Espinar, M. T. (2005). Rapid Identification and enumeration of *Saccharomyces cerevisiae* cells in wine by real-time PCR. *Applied and Environmental Microbiology*, *71*(11), 6823–6830. https://doi.org/10.1128/AEM.71.11.6823-6830.2005

McGovern, P., Jalabadze, M., Batiuk, S., Callahan, M. P., Smith, K. E., Hall, G. R.,

Kvavadze, E., Maghradze, D., Rusishvili, N., Bouby, L., Failla, O., Cola, G., Mariani, L., Boaretto, E., Bacilieri, R., This, P., Wales, N., & Lordkipanidze, D. (2017). Early Neolithic wine of Georgia in the South Caucasus. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(48), E10309–E10318. https://doi.org/10.1073/pnas.1714728114

Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2018). Effects of non-*Saccharomyces* yeasts on color, anthocyanin, and anthocyanin-derived pigments of Tannat grapes during fermentation. *American Journal of Enology and Viticulture*, *69*(2), 148–156. https://doi.org/10.5344/ajev.2017.17055

Morata, A., Loira, I., Escott, C., del Fresno, J. M., Bañuelos, M. A., & Suárez-Lepe, J. A. (2019). Applications of *Metschnikowia pulcherrima* in wine biotechnology. *Fermentation*, *5*(3), 63. https://doi.org/10.3390/fermentation5030063

Naumov, G. I., Masneuf, I., Naumova, E. S., Aigle, M., & Dubourdieu, D. (2000). Association of *Saccharomyces bayanus* var. *uvarum* with some French wines: genetic analysis of yeast populations. *Research in Microbiology*, *151*(8), 683–691. https://doi.org/10.1016/s0923-2508(00)90131-1

Naumova, E. S., Serpova, E. V., & Naumov, G. I. (2007). Molecular systematics of *Lachancea* yeasts. *Biochemistry. Biokhimiia*, *72*(12), 1356–1362. https://doi.org/10.1134/s0006297907120097

Navarro, Y., Torija, M. J., Mas, A., & Beltran, G. (2020). Viability-PCR allows monitoring yeast population dynamics in mixed fermentations including viable but non-culturable yeasts. *Foods*, *9*(10). https://doi.org/10.3390/foods9101373

Nurgel, C., Erten, H., Canbas, A., Cabaroglu, T., & Selli, S. (2005). Yeast flora during the fermentation of wines made from *Vitis vinifera* L. *cv.* Emir and Kalecik Karasi grown in Anatolia. *World Journal of Microbiology and Biotechnology*, *21*(6–7), 1187–1194. https://doi.org/10.1007/s11274-005-1106-6

OIV. (2017). Distribution of the world's grapevine varieties. In *Focus OIV 2017*. http://www.oiv.int/public/medias/5888/en-distribution-of-the-worlds-grapevine-varieties.pdf

Oliveira, E. J., Pádua, J. G., Zucchi, M. I., Vencovsky, R., & Vieira, M. L. C. (2006). Origin, evolution and genome distribution of microsatellites. *Genetics and Molecular Biology*, *29*(2), 294–307. https://doi.org/10.1590/S1415-47572006000200018

Orlić, S., Vojvoda, T., Babić, K. H., Arroyo-López, F. N., Jeromel, A., Kozina, B.,

Iacumin, L., & Comi, G. (2010). Diversity and oenological characterization of indigenous *Saccharomyces cerevisiae* associated with Žilavka grapes. *World Journal of Microbiology and Biotechnology*, *26*(8), 1483–1489. https://doi.org/10.1007/s11274-010-0323-9

Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, *7*(MAR), 1–20. https://doi.org/10.3389/fmicb.2016.00411

Panigrahi, S., Velraj, P., & Subba Rao, T. (2019). Functional microbial diversity in contaminated environment and application in bioremediation. In *Microbial Diversity in the Genomic Era* (pp. 359–385). Elsevier. https://doi.org/10.1016/B978-0-12-814849-5.00021-6

Pataro, C., Guerra, J. B., Petrillo-Peixoto, M. L., Mendonça-Hagler, L. C., Linardi, V. R., & Rosa, C. A. (2000). Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentation in Brazil. *Journal of Applied Microbiology*, *89*(1), 24–31. https://doi.org/10.1046/j.1365-2672.2000.01092.x

Pereira, G. V., Ramos, C. L., Galvão, C., Souza Dias, E., & Schwan, R. F. (2010). Use of specific PCR primers to identify three important industrial species of *Saccharomyces* genus: *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces pastorianus*. *Letters in Applied Microbiology*, *51*(2), 131–137. https://doi.org/10.1111/j.1472-765X.2010.02868.x

Perestrelo, R., Silva, C., & Câmara, J. S. (2019). Madeira wine volatile profile. A platform to establish Madeira wine aroma descriptors. *Molecules*, *24*(17), 3028. https://doi.org/10.3390/molecules24173028

Perestrelo, R., Silva, C., Gonçalves, C., Castillo, M., & Câmara, J. S. (2020). An approach of the Madeira wine chemistry. *Beverages*, *6*(1), 1–25. https://doi.org/10.3390/beverages6010012

Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergström, A., Sigwalt, A., Barre, B., Freel, K., Llored, A., Cruaud, C., Labadie, K., Aury, J.-M., Istace, B., Lebrigand, K., Barbry, P., Engelen, S., Lemainque, A., Wincker, P., … Schacherer, J. (2018). Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nature*, *556*(7701), 339–344. https://doi.org/10.1038/s41586-018-0030-5

Pfaffl, M. W. (2019). Quantification Strategies in real-time polymerase chain reaction. In *Polymerase Chain Reaction: Theory and Technology*. Caister Academic Press. https://doi.org/10.21775/9781912530243.05

Pfaffl, M. W. (2020). *MIQE and PCR*.

Pfliegler, W. P., Horváth, E., Kállai, Z., & Sipiczki, M. (2014). Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. *Microbiological Research*, *169*(5–6), 402–410. https://doi.org/10.1016/j.micres.2013.09.006

Philipp, C., Bagheri, B., Horacek, M., Eder, P., Bauer, F. F., & Setati, M. E. (2021). Inoculation of grape musts with single strains of *Saccharomyces cerevisiae* yeast reduces the diversity of chemical profiles of wines. *PLOS ONE*, *16*(7), e0254919. https://doi.org/10.1371/journal.pone.0254919

Phister, T. G., Rawsthorne, H., Joseph, C. M. L., & Mills, D. a. (2007). Real-time PCR assay for detection and enumeration of *Hanseniaspora* species from wine and juice. *American Journal of Enology and Viticulture*, *58*(2), 229–233. https://doi.org/10.5344/ajev.2007.58.2.229

Pinzani, P., Bonciani, L., Pazzagli, M., Orlando, C., Guerrini, S., & Granchi, L. (2004). Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Letters in Applied Microbiology*, *38*(2), 118–124. https://doi.org/10.1111/j.1472-765X.2003.01462.x

Piombo, E., Sela, N., Wisniewski, M., Hoffmann, M., Gullino, M. L., Allard, M. W., Levin, E., Spadaro, D., & Droby, S. (2018). Genome sequence, assembly and characterization of two *Metschnikowia fructicola* strains used as biocontrol agents of postharvest diseases. *Frontiers in Microbiology*, *9*(APR), 1–17. https://doi.org/10.3389/fmicb.2018.00593

Ponsone, M. L., Nally, M. C., Chiotta, M. L., Combina, M., Köhl, J., & Chulze, S. N. (2016). Evaluation of the effectiveness of potential biocontrol yeasts against black sur rot and ochratoxin A occurring under greenhouse and field grape production conditions. *Biological Control*, *103*, 78–85. https://doi.org/10.1016/j.biocontrol.2016.07.012

Porter, T. J., Divol, B., & Setati, M. E. (2019). *Lachancea* yeast species: Origin, biochemical characteristics and oenological significance. *Food Research International*, *119*(February), 378–389. https://doi.org/10.1016/j.foodres.2019.02.003

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., & Sohier, D. (2011). Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiology*, *28*(5), 848–861. https://doi.org/10.1016/j.fm.2011.02.008

Puertas, B., Jiménez, M. J., Cantos-Villar, E., Cantoral, J. M., & Rodríguez, M. E.

(2017). Use of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in semi-industrial sequential inoculation to improve quality of Palomino and Chardonnay wines in warm climates. *Journal of Applied Microbiology*, *122*(3), 733–746. https://doi.org/10.1111/jam.13375

Ramírez-Castrillón, M., Mendes, S. D. C., Inostroza-Ponta, M., & Valente, P. (2014). (GTG)5 MSP-PCR fingerprinting as a technique for discrimination of wine associated yeasts? *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0105870

Rapp, A., & Mandery, H. (1986). Wine aroma. *Experientia*, *42*(8), 873–884. https://doi.org/10.1007/BF01941764

Raspor, P., Mozina, S. S., & Cadez, N. (2001). Identification of yeasts from the grape/must/wine system. In *Food Microbiology Protocols* (Vol. 14, Issue July 2014, pp. 243–257). Humana Press. https://doi.org/10.1385/1-59259-029-2:243

Redžepović, S., Orlić, S., Sikora, S., Majdak, A., & Pretorius, I. S. (2002). Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. *Letters in Applied Microbiology*, *35*(4), 305–310. https://doi.org/10.1046/j.1472-765X.2002.01181.x

Rohlf, F. J. (2000). *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System*. Exeter Software Setauket, New York, Applied Biostatistics, Inc.

Russo, P., Tufariello, M., Renna, R., Tristezza, M., Taurino, M., Palombi, L., Capozzi, V., Rizzello, C. G., & Grieco, F. (2020). New insights into the oenological significance of *Candida zemplinina*: Impact of selected autochthonous strains on the volatile profile of Apulian wines. *Microorganisms*, *8*(5), 628. https://doi.org/10.3390/microorganisms8050628

Santos, S. K. B. Dos, Basílio, A. C. M., Brasileiro, B. T. R. V., Simões, D. A., Silva-Filho, E. A. Da, & de Morais, M. (2007). Identification of yeasts within *Saccharomyces sensu stricto* complex by PCR-fingerprinting. *World Journal of Microbiology and Biotechnology*, *23*(11), 1613–1620. https://doi.org/10.1007/s11274-007-9407-6

Schuller, D., Pereira, L., Alves, H., Cambon, B., Dequin, S., & Casal, M. (2007). Genetic characterization of commercial *Saccharomyces cerevisiae* isolates recovered from vineyard environments. *Yeast*, *24*(8), 625–636. https://doi.org/10.1002/yea.1496

Schütz, M., & Gafner, J. (1993). Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *Journal of Applied Bacteriology*, *75*(6), 551–

558. https://doi.org/10.1111/j.1365-2672.1993.tb01594.x

Schwartz, D. C., & Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, *37*(1), 67–75. https://doi.org/10.1016/0092-8674(84)90301-5

Sebastiani, F., Pinzauti, F., Casalone, E., Rosi, I., Fia, G., Polsinelli, M., & Barberio, C. (2004). Biodiversity of *Saccharomyces cerevisiae* strains isolated from Sangiovese grapes of Chianti area. *Annals of Microbiology*, *54*(4), 415–426. http://hdl.handle.net/2158/252232

Selli, S., Canbas, A., Cabaroglu, T., Erten, H., Lepoutre, J.-P., & Gunata, Z. (2006). Effect of skin contact on the free and bound aroma compounds of the white wine of *Vitis vinifera* L. *cv* Narince. *Food Control*, *17*(1), 75–82. https://doi.org/10.1016/j.foodcont.2004.09.005

Selli, S., Canbas, A., Varlet, V., Kelebek, H., Prost, C., & Serot, T. (2008). Characterization of the most odor-active volatiles of orange wine made from a Turkish cv. Kozan (Citrus sinensis L. Osbeck). *Journal of Agricultural and Food Chemistry*, *56*(1), 227–234. https://doi.org/10.1021/jf072231w

Şen, K. (2021). The influence of different commercial yeasts on aroma compounds of rosé wine produced from *cv.* Öküzgözü grape. *Journal of Food Processing and Preservation*, *45*(7), 1–11. https://doi.org/10.1111/jfpp.15610

Sidari, R., Martorana, A., Zappia, C., Mincione, A., & Giuffrè, A. M. (2020). Persistence and effect of a multistrain starter culture on antioxidant and rheological properties of novel wheat sourdoughs and bread. *Foods*, *9*(9), 24–29. https://doi.org/10.3390/foods9091258

Sipiczki, M. (2000). Where does fission yeast sit on the tree of life? *Genome Biology*, *1*(2), REVIEWS1011. https://doi.org/10.1186/gb-2000-1-2-reviews1011

Speranza, B., & Bevilacqua, A. (2017). Starter Cultures in Food Production. In *Starter Cultures in Food Production*. https://doi.org/10.1002/9781118933794

Sumby, K. M., Caliani, N. S., & Jiranek, V. (2021). Yeast diversity in the vineyard: how it is defined, measured and influenced by fungicides. *Australian Journal of Grape and Wine Research*, *27*(2), 169–193. https://doi.org/10.1111/ajgw.12479

Šuranská, H., Vránová, D., & Omelková, J. (2016). Isolation, identification and characterization of regional indigenous S*accharomyces cerevisiae* strains. *Brazilian Journal of Microbiology*, *47*(1), 181–190. https://doi.org/10.1016/j.bjm.2015.11.010

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research*, *11*(2), 139–173. https://doi.org/10.1111/j.1755-0238.2005.tb00285.x

Thompson, J. R., & Latorre, B. A. (1999). Characterization of *Botrytis cinerea* from table grapes in Chile using RAPD-PCR. *Plant Disease*, *83*(12), 1090–1094. https://doi.org/10.1094/PDIS.1999.83.12.1090

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Aguzzi, I., Corsetti, A., & Suzzi, G. (2013). Biogeographical characterization of *Saccharomyces cerevisiae* wine yeast by molecular methods. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00166

Tofalo, R., Schirone, M., Corsetti, A., & Suzzi, G. (2012). Detection of *Brettanomyces* spp. in red wines using real-time PCR. *Journal of Food Science*, *77*(9), M545–M549. https://doi.org/10.1111/j.1750-3841.2012.02871.x

Tofalo, R., & Suzzi, G. (2016). Yeasts. In *Encyclopedia of Food and Health* (pp. 593–599). Elsevier. https://doi.org/10.1016/B978-0-12-384947-2.00762-5

Torriani, S., Zapparoli, G., & Suzzi, G. (1999). Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. Diversity of *Saccharomyces strains* from Amarone wine. *Antonie van Leeuwenhoek*, *75*(3), 207–215. https://doi.org/10.1023/a:1001773916407

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., & Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Frontiers in Microbiology*, *7*(MAY). https://doi.org/10.3389/fmicb.2016.00670

Tufariello, M., Capozzi, V., Spano, G., Cantele, G., Venerito, P., Mita, G., & Grieco, F. (2020). Effect of co-inoculation of *Candida zemplinina*, *Saccharomyces cerevisiae* and *Lactobacillus plantarum* for the industrial production of Negroamaro wine in Apulia (Southern Italy). *Microorganisms*, *8*(5), 726. https://doi.org/10.3390/microorganisms8050726

Urso, R., Rantsiou, K., Dolci, P., Rolle, L., Comi, G., & Cocolin, L. (2008). Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. *FEMS Yeast Research*, *8*(7), 1053–1062. https://doi.org/10.1111/j.1567-1364.2008.00364.x

Uysal, T., Ayaz, A., Akdemir, U., Korkutal, İ., Bahar, E., & Candar, S. (2021).

Viticulture tradition in Turkey. *Tekirdag Viticulture Research Institute*, *1*(1), 39–54. https://doi.org/10.52001/vis.2021.5

Valera, M. J., Boido, E., Dellacassa, E., & Carrau, F. (2020). Comparison of the glycolytic and alcoholic fermentation pathways of *Hanseniaspora vineae* with *Saccharomyces cerevisiae* wine yeasts. *Fermentation*, *6*(3), 78. https://doi.org/10.3390/fermentation6030078

Valero, E., Cambon, B., Schuller, D., Casal, M., & Dequin, S. (2007). Biodiversity of *Saccharomyces* yeast strains from grape berries of wine-producing areas using starter commercial yeasts. *FEMS Yeast Research*, *7*(2), 317–329. https://doi.org/10.1111/j.1567-1364.2006.00161.x

Vandendool, H., & Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography*, *11*(C), 463–471. https://doi.org/10.1016/s0021-9673(01)80947-x

Vicente, J., Ruiz, J., Belda, I., Benito-Vázquez, I., Marquina, D., Calderón, F., Santos, A., & Benito, S. (2020). The genus *Metschnikowia* in enology. *Microorganisms*, *8*(7), 1038. https://doi.org/10.3390/microorganisms8071038

Vilela, A. (2018). *Lachancea thermotolerans*, the non-*Saccharomyces* yeast that reduces the volatile acidity of wines. *Fermentation*, *4*(3), 56. https://doi.org/10.3390/fermentation4030056

Walker, G., & Stewart, G. (2016). *Saccharomyces cerevisiae* in the production of fermented beverages. *Beverages*, *2*(4), 30. https://doi.org/10.3390/beverages2040030

Wang, C., Esteve-Zarzoso, B., Cocolin, L., Mas, A., & Rantsiou, K. (2015). Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. *Food Research International*, *78*, 195–200. https://doi.org/10.1016/j.foodres.2015.10.014

Wang, C., García-Fernández, D., Mas, A., & Esteve-Zarzoso, B. (2015). Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Frontiers in Microbiology*, *6*(OCT), 1–8. https://doi.org/10.3389/fmicb.2015.01156

Wang, Q.-M., Liu, W.-Q., Liti, G., Wang, S.-A., & Bai, F.-Y. (2012). Surprisingly diverged populations of *Saccharomyces cerevisiae* in natural environments remote from human activity. *Molecular Ecology*, *21*(22), 5404–5417. https://doi.org/10.1111/j.1365-294X.2012.05732.x

Wang, X.-C., Li, A.-H., Dizy, M., Ullah, N., Sun, W.-X., & Tao, Y.-S. (2017). Evaluation of aroma enhancement for "Ecolly" dry white wines by mixed inoculation of selected *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae*. *Food Chemistry*, *228*, 550–559. https://doi.org/10.1016/j.foodchem.2017.01.113

Wang, X., Glawe, D. A., Weller, D. M., & Okubara, P. A. (2020). Real-time PCR assays for the quantification of native yeast DNA in grape berry and fermentation extracts. *Journal of Microbiological Methods*, *168*(September 2019), 105794. https://doi.org/10.1016/j.mimet.2019.105794

Welke, J. E., Zanus, M., Lazzarotto, M., & Alcaraz Zini, C. (2014). Quantitative analysis of headspace volatile compounds using comprehensive two-dimensional gas chromatography and their contribution to the aroma of Chardonnay wine. *Food Research International*, *59*, 85–99. https://doi.org/10.1016/j.foodres.2014.02.002

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, *18*(24), 7213–7218. https://doi.org/10.1093/nar/18.24.7213

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, *18*(22), 6531–6535. https://doi.org/10.1093/nar/18.22.6531

Y. Karaoğlan, S., & Cabaroğlu, T. (2020). A comparison of the volatile and phenolic compositions of Muscat of Bornova wines from two different terroirs in the Aegean region of Turkey. *Food Science and Technology*, *40*(4), 844–853. https://doi.org/10.1590/fst.21819

Yang, H., Liu, T., Zhang, G., & He, G. (2020). Intraspecific diversity and fermentative properties of *Saccharomyces cerevisiae* from Chinese traditional sourdough. *LWT*, *124*, 109195. https://doi.org/10.1016/j.lwt.2020.109195

Zhao, P., Gao, J., Qian, M., & Li, H. (2017). Characterization of the key aroma compounds in Chinese syrah wine by gas chromatography-olfactometry-mass spectrometry and aroma reconstitution studies. *Molecules*, *22*(7). https://doi.org/10.3390/molecules22071045

Zhao, Y., Sun, Q., Zhu, S., Du, F., Mao, R., Liu, L., Tian, B., & Zhu, Y. (2021). Biodiversity of non-*Saccharomyces* yeasts associated with spontaneous fermentation of Cabernet Sauvignon wines from Shangri-La wine region, China. *Scientific Reports*, *11*(1), 5150. https://doi.org/10.1038/s41598-021-

83216-x

Zhu, F., Du, B., & Li, J. (2016). Aroma compounds in wine. In *Grape and Wine Biotechnology* (Issue 2016, pp. 267–322). InTech. https://doi.org/10.5772/65102

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., & Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiology*, *27*(5), 559–567. https://doi.org/10.1016/j.fm.2010.01.006

APPENDICES

A. Statistical Analysis Carried Out for Identified Aroma Compounds in Kalecik Karası, Boğazkere, Öküzgözü, and Dimrit Wines

One-way ANOVA: 1-Propanol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	8724569	86.89%	8724569	2908190	17.67	0.001
Error	8	1316426	13.11%	1316426	164553		
Total	11	10040996	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
405.652	86.89%	81.97%	2961959	70.50%

Means

Group	N	Mean	StDev	95% CI
BA	3	416.5	51.5	(-123.6, 956.6)
DA	3	1537	784	(997, 2078)
KA	3	2765.3	103.5	(2225.3, 3305.4)
OA	3	1129.3	171.7	(589.2, 1669.3)

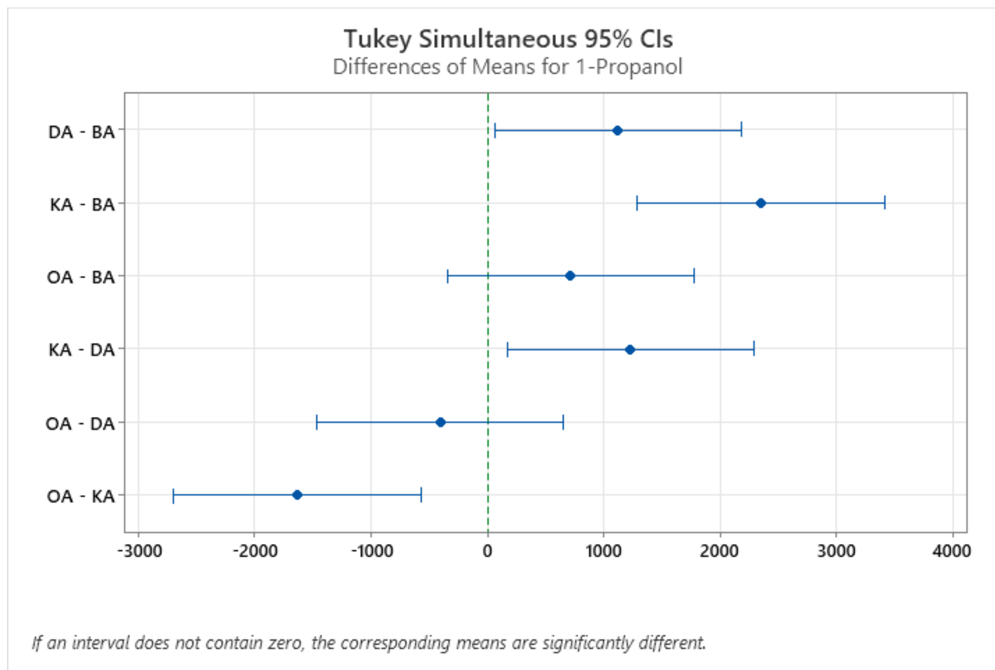
Pooled StDev = 405.652

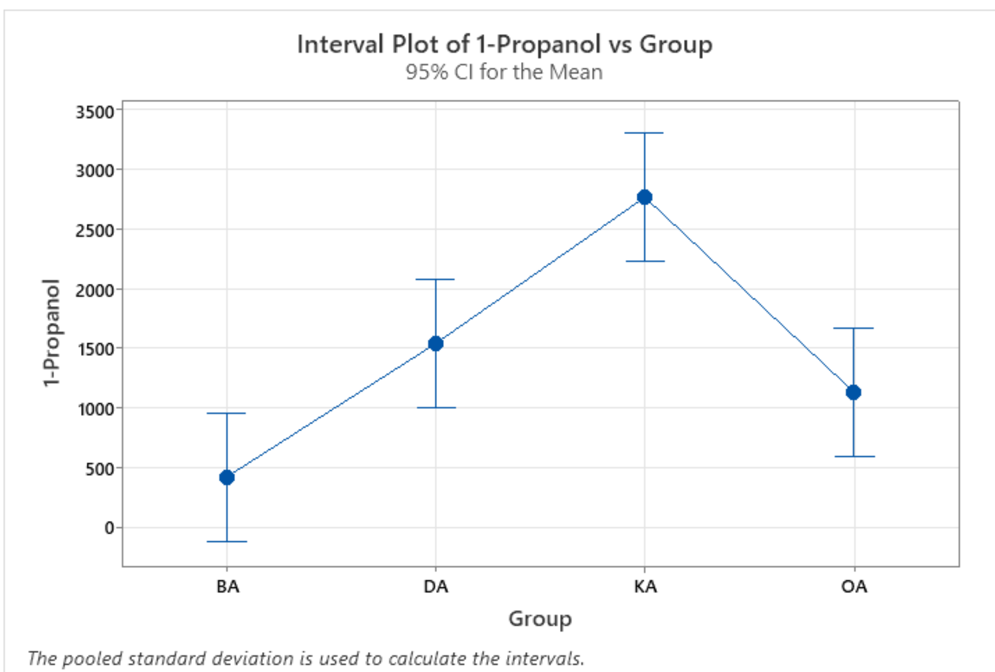
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
KA	3	2765.3	A		
DA	3	1537		B	
OA	3	1129.3		B	C
BA	3	416.5			C

Means that do not share a letter are significantly different.





One-way ANOVA: Isobutyl alcohol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	105799373	86.75%	105799373	35266458	17.46	0.001
Error	8	16155814	13.25%	16155814	2019477		
Total	11	121955186	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
1421.08	86.75%	81.78%	36350581	70.19%

Means

Group	N	Mean	StDev	95% CI
BA	3	14372	812	(12480, 16264)
DA	3	20595	1298	(18703, 22487)
KA	3	12601	474	(10709, 14493)
OA	3	15760	2347	(13868, 17652)

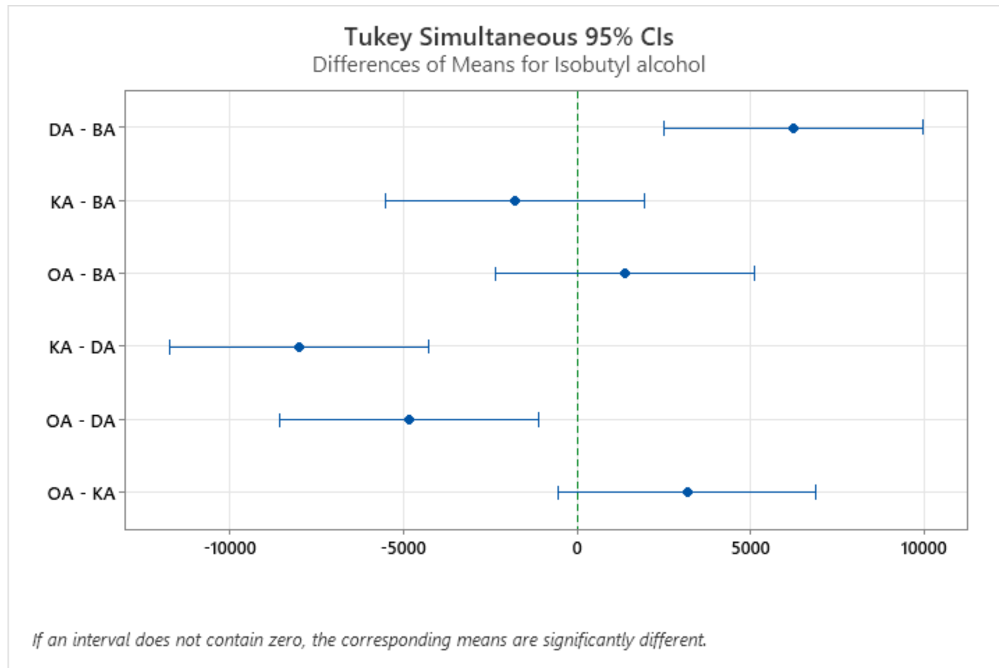
Pooled StDev = 1421.08

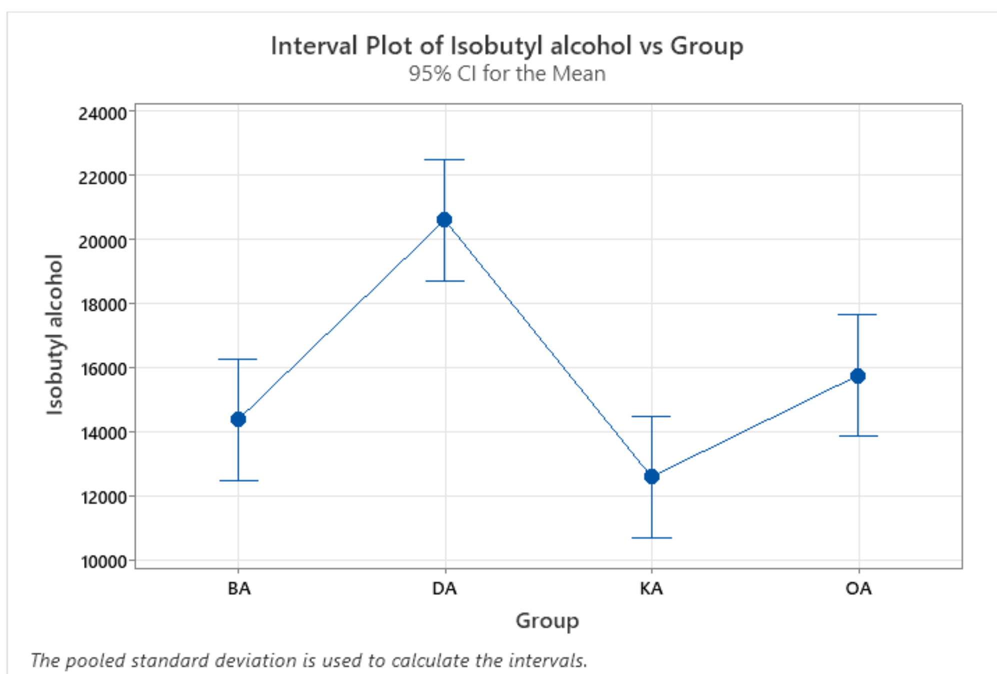
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
DA	3	20595	A
OA	3	15760	B
BA	3	14372	B
KA	3	12601	B

Means that do not share a letter are significantly different.





One-way ANOVA: 1-Butanol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	74411	62.50%	74411	24804	4.45	0.041
Error	8	44638	37.50%	44638	5580		
Total	11	119049	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
74.6975	62.50%	48.44%	100435	15.64%

Means

Group	N	Mean	StDev	95% CI
BA	3	265.5	17.6	(166.1, 365.0)
DA	3	361.9	134.4	(262.5, 461.4)
KA	3	479.13	7.32	(379.68, 578.58)
OA	3	318.3	62.4	(218.9, 417.8)

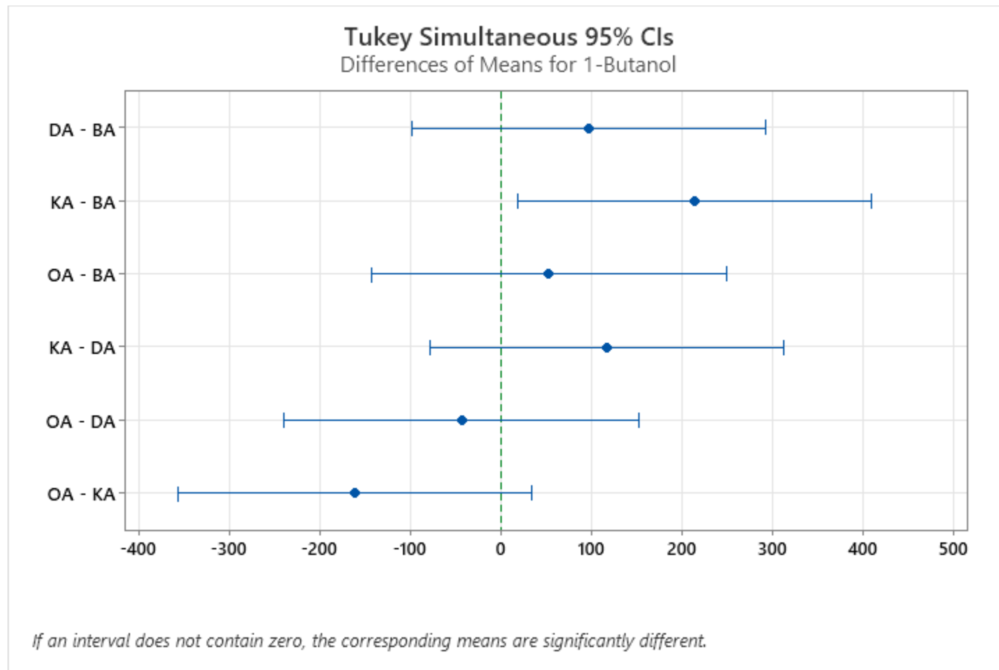
Pooled StDev = 74.6975

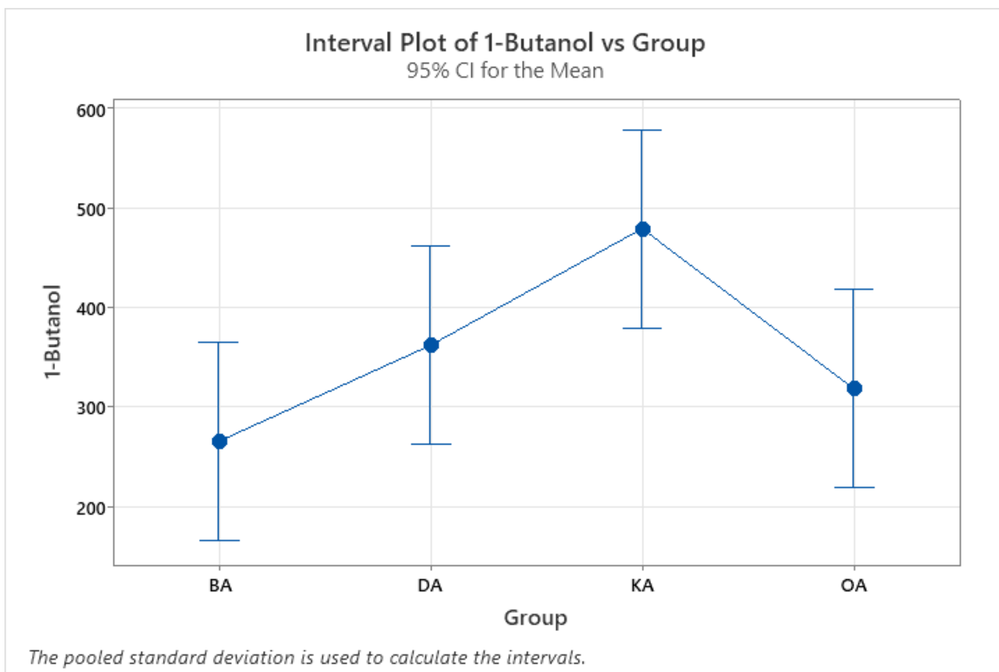
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
KA	3	479.13	A	
DA	3	361.9	A	B
OA	3	318.3	A	B
BA	3	265.5		B

Means that do not share a letter are significantly different.





One-way ANOVA: Isoamyl alcohol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	12955992469	81.05%	12955992469	4318664156	11.41	0.003
Error	8	3028602642	18.95%	3028602642	378575330		
Total	11	15984595112	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
19457.0	81.05%	73.95%	6814355945	57.37%

Means

Group	N	Mean	StDev	95% CI
BA	3	191704	11550	(165799, 217608)
DA	3	195319	30127	(169414, 221223)
KA	3	113587	3888	(87682, 139491)
OA	3	175317	21404	(149412, 201221)

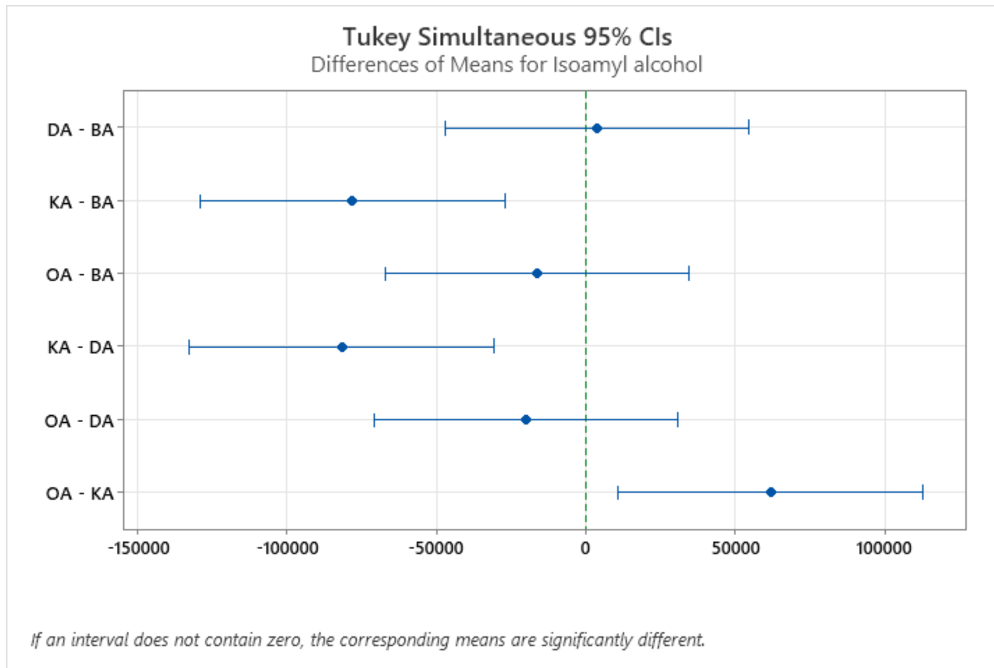
Pooled StDev = 19457.0

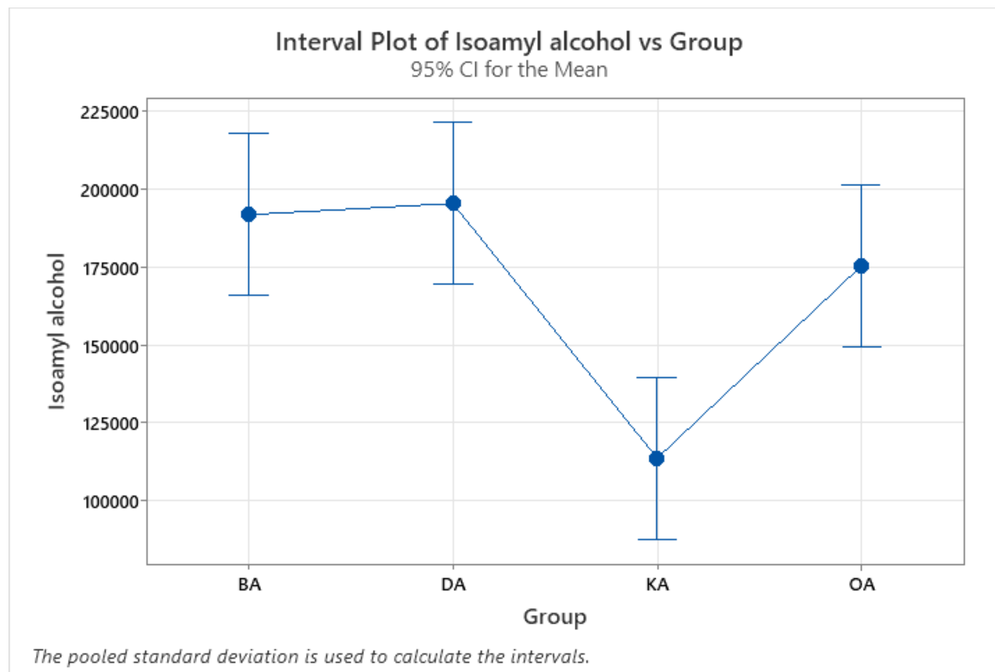
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	195319	A	
BA	3	191704	A	
OA	3	175317	A	
KA	3	113587		B

Means that do not share a letter are significantly different.





One-way ANOVA: 3-Methyl-1-pentanol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	26933	75.18%	26933	8978	8.08	0.008
Error	8	8894	24.82%	8894	1112		
Total	11	35826	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
33.3425	75.18%	65.87%	20011.0	44.14%

Means

Group	N	Mean	StDev	95% CI
BA	3	165.03	7.61	(120.64, 209.42)
DA	3	184.3	63.9	(139.9, 228.7)
KA	3	61.44	4.53	(17.05, 105.83)
OA	3	155.13	16.99	(110.74, 199.52)

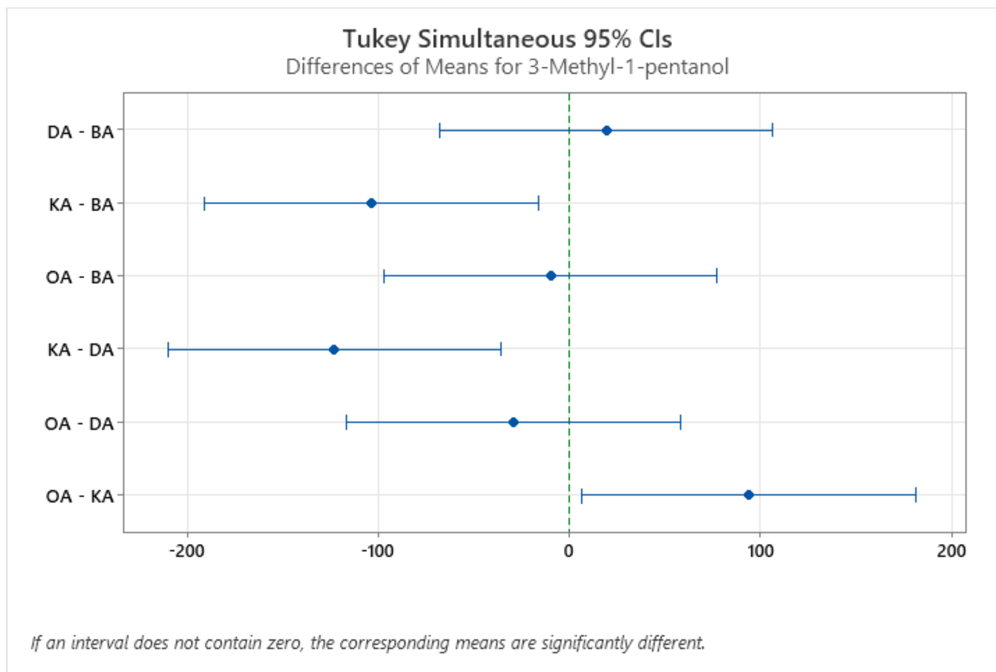
Pooled StDev = 33.3425

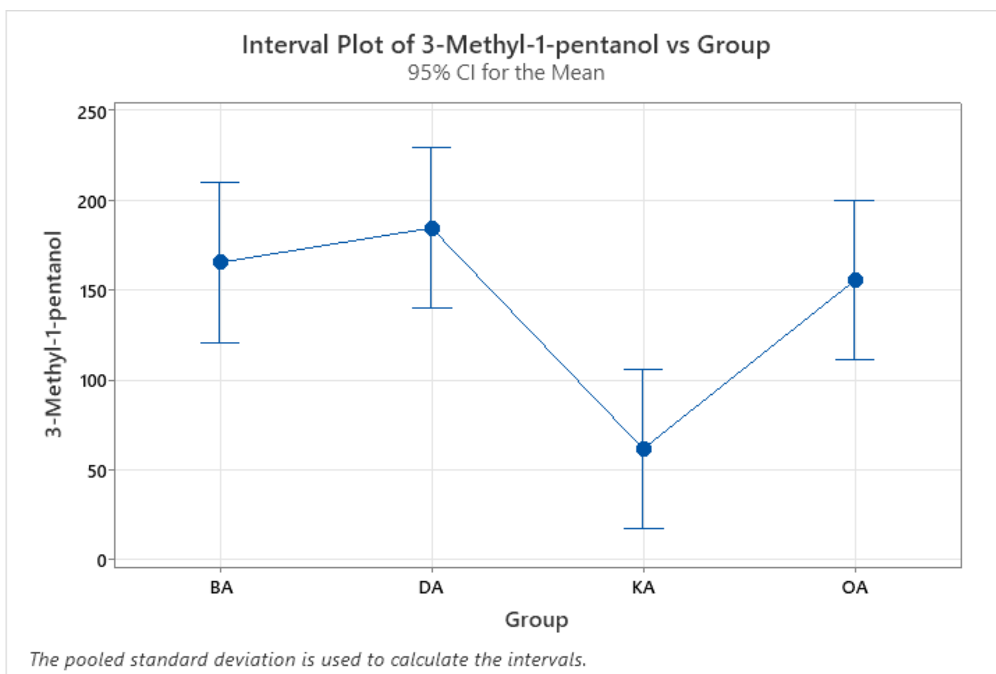
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	184.3	A	
BA	3	165.03	A	
OA	3	155.13	A	
KA	3	61.44		B

Means that do not share a letter are significantly different.





One-way ANOVA: 3-Ethoxy-1-propanol versus Group

Analysis of Variance

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Group	3	30463	74.68%	30463	10154	7.87	0.009
Error	8	10328	25.32%	10328	1291		
Total	11	40791	100.00%				

Model Summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
35.9298	74.68%	65.19%	23237.1	43.03%

Means

Group	N	Mean	StDev	95% CI
BA	3	95.97	6.14	(48.13, 143.80)
DA	3	208.9	63.7	(161.0, 256.7)
KA	3	227.38	9.25	(179.54, 275.21)
OA	3	184.3	31.4	(136.4, 232.1)

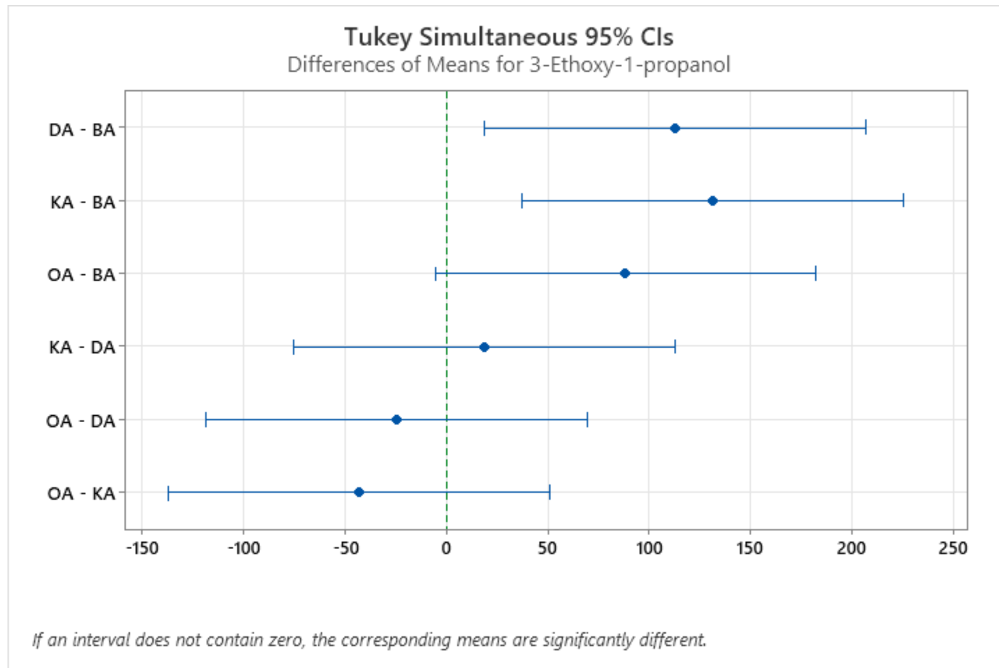
Pooled StDev = 35.9298

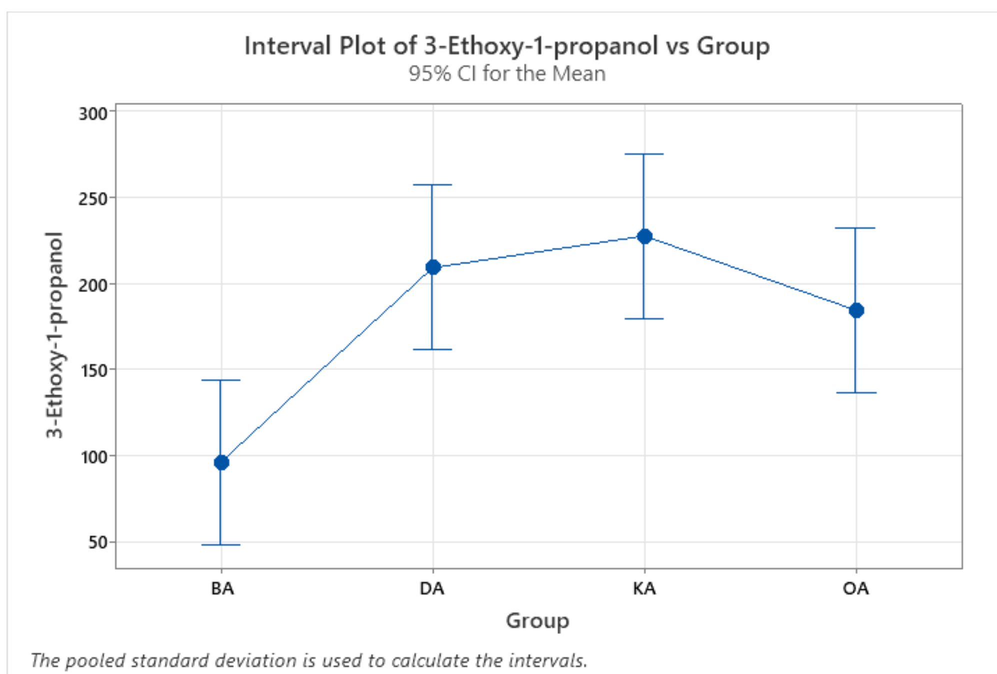
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
KA	3	227.38	A	
DA	3	208.9	A	
OA	3	184.3	A	B
BA	3	95.97		B

Means that do not share a letter are significantly different.





One-way ANOVA: Methionol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	648146	216049	11.11	0.003
Error	8	155511	19439		
Total	11	803658			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
139.423	80.65%	73.39%	56.46%

Means

Group	N	Mean	StDev	95% CI
BA	3	356.27	16.38	(170.65, 541.90)
DA	3	813	264	(628, 999)
KA	3	712.1	26.7	(526.5, 897.7)
OA	3	993.8	85.4	(808.2, 1179.4)

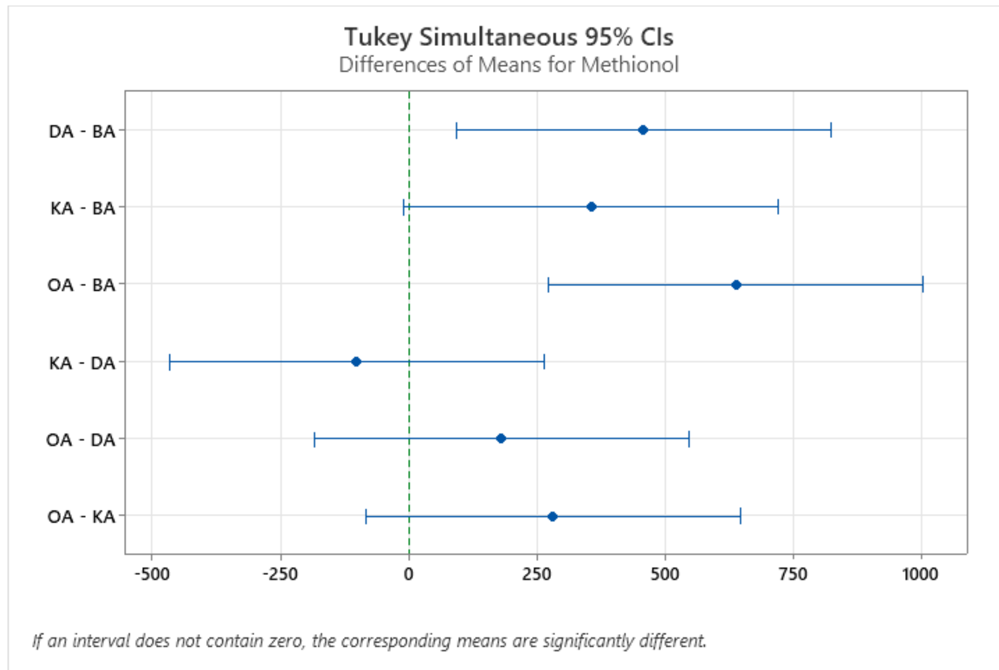
Pooled StDev = 139.423

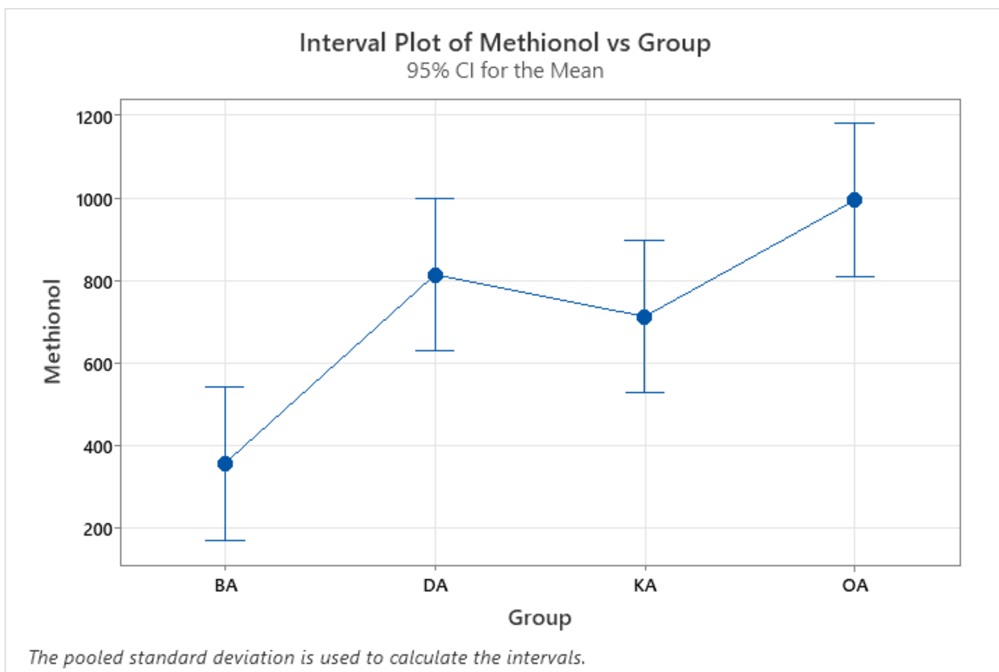
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	993.8	A	
DA	3	813	A	
KA	3	712.1	A	B
BA	3	356.27		B

Means that do not share a letter are significantly different.





One-way ANOVA: Benzyl alcohol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	38049	12682.9	46.69	0.000
Error	8	2173	271.6		
Total	11	40222			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
16.4810	94.60%	92.57%	87.84%

Means

Group	N	Mean	StDev	95% CI
BA	3	168.5	18.5	(146.6, 190.5)
DA	3	118.06	7.11	(96.12, 140.01)
KA	3	255.14	8.93	(233.20, 277.08)
OA	3	116.6	24.8	(94.6, 138.5)

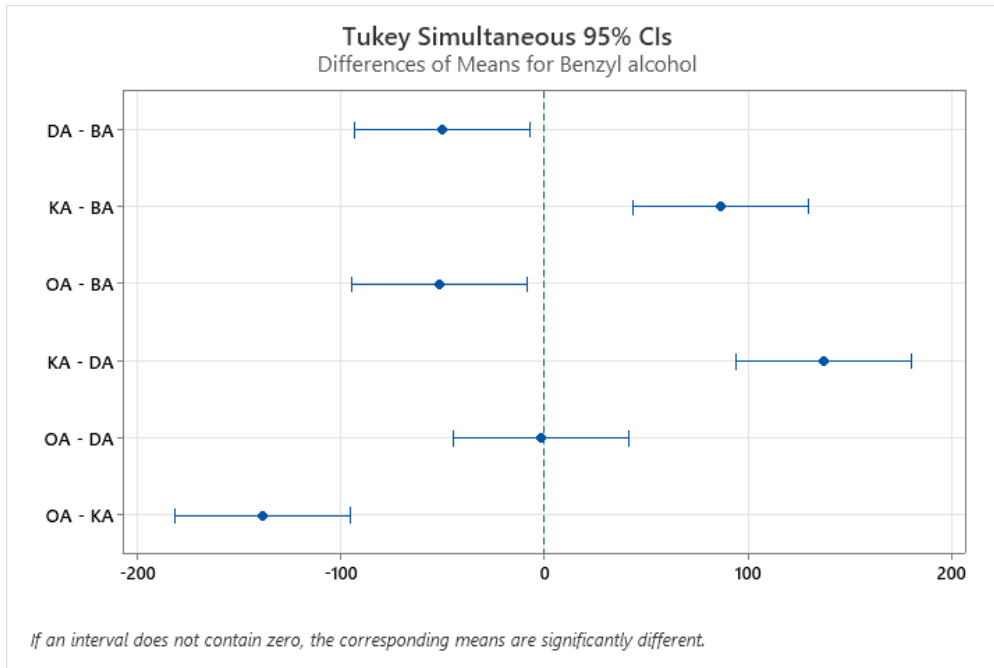
Pooled StDev = 16.4810

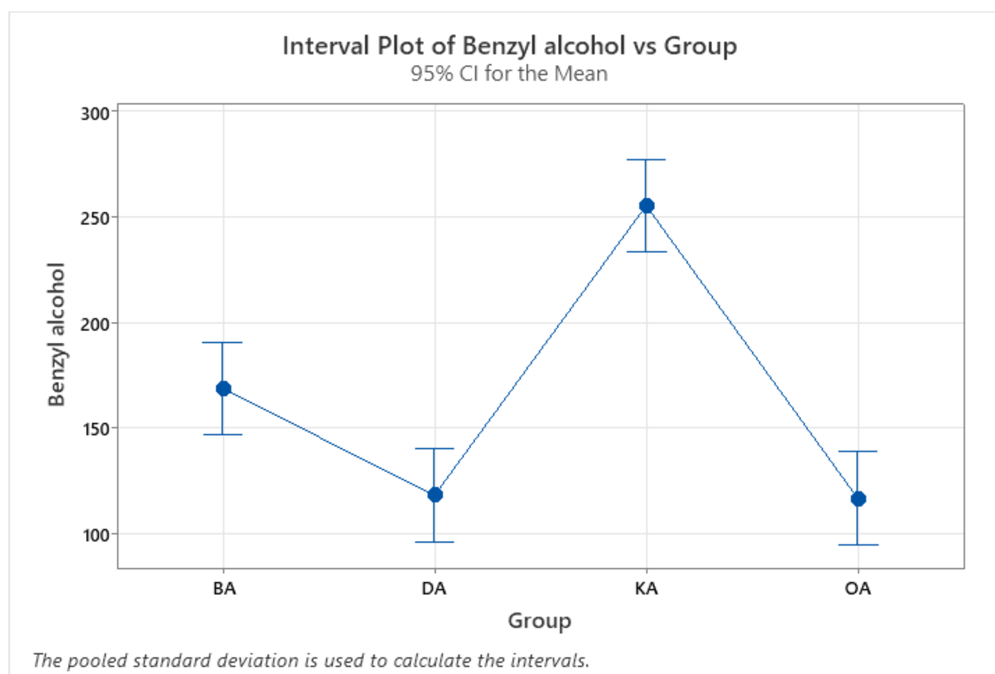
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
KA	3	255.14	A		
BA	3	168.5		B	
DA	3	118.06			C
OA	3	116.6			C

Means that do not share a letter are significantly different.





One-way ANOVA: 2-Methyl-2-buten-1-ol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	3269	1634.7	2.70	0.146
Error	6	3635	605.8		
Total	8	6904			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
24.6126	47.35%	29.81%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	216.78	8.43	(182.01, 251.55)
DA	3	170.7	23.4	(135.9, 205.5)
OA	3	187.3	34.6	(152.5, 222.1)

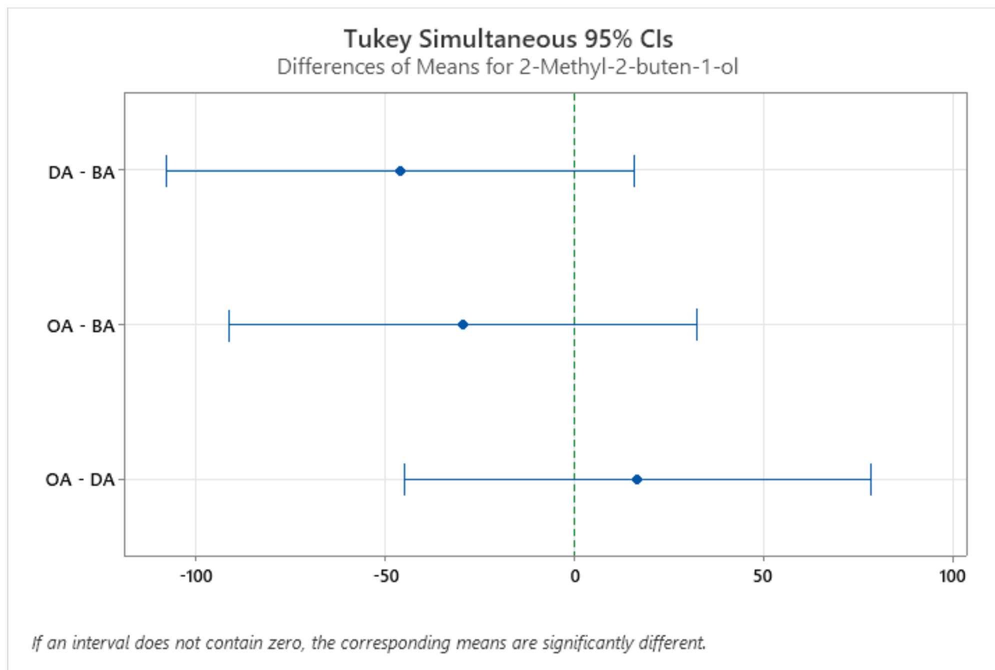
Pooled StDev = 24.6126

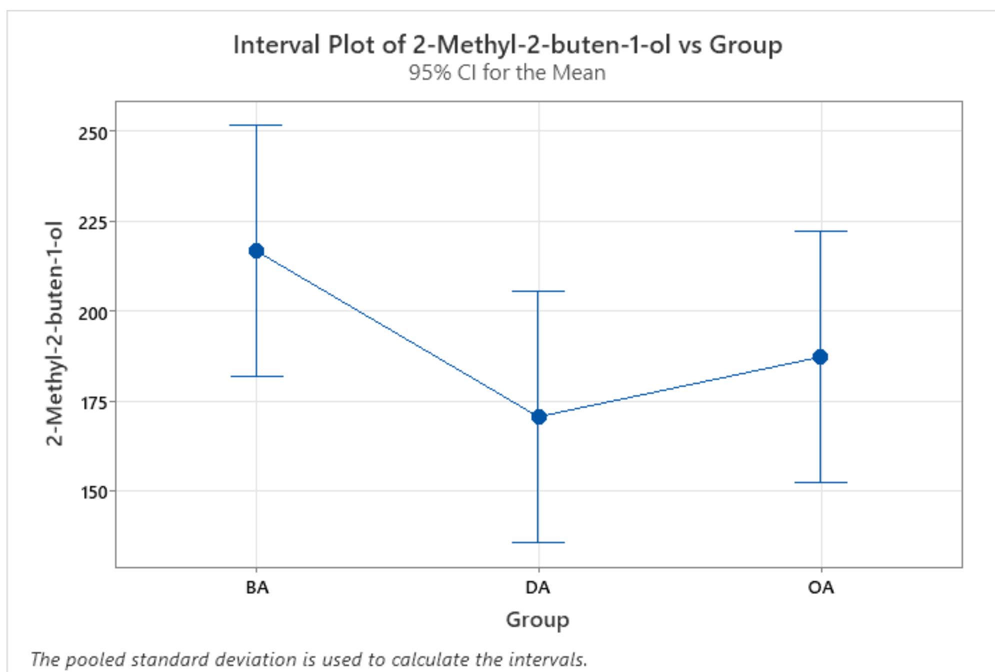
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
BA	3	216.78	A
OA	3	187.3	A
DA	3	170.7	A

Means that do not share a letter are significantly different.





One-way ANOVA: Phenethyl alcohol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	43025554479	14341851493	1.06	0.419
Error	8	1.08363E+11	13545389265		
Total	11	1.51389E+11			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
116385	28.42%	1.58%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	60522	2617	(-94429, 215473)
DA	3	186075	232606	(31124, 341027)
KA	3	30431	637	(-124520, 185382)
OA	3	61565	8293	(-93386, 216516)

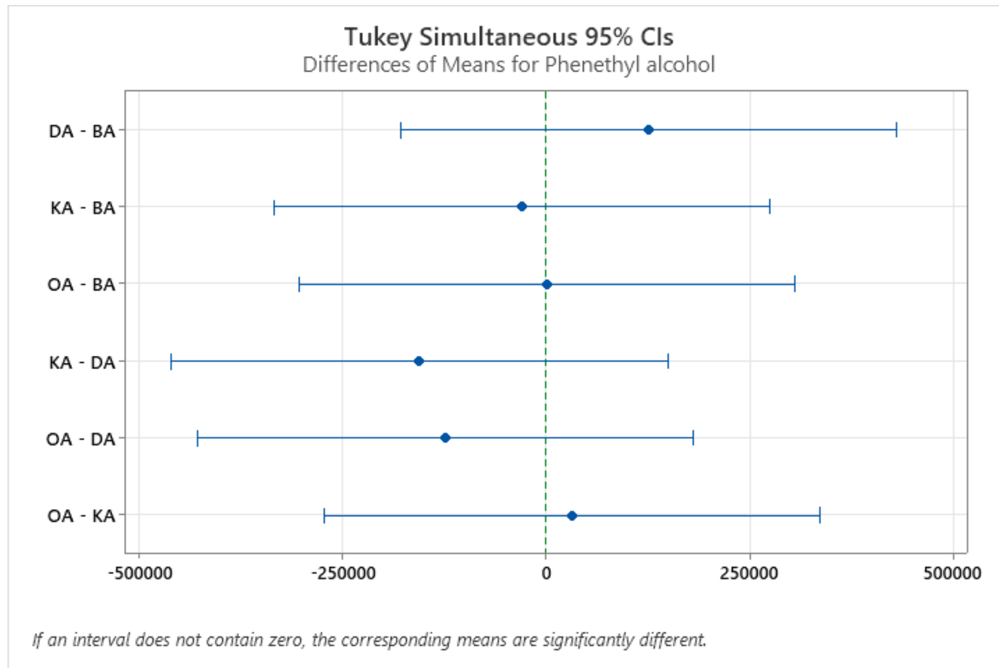
Pooled StDev = 116385

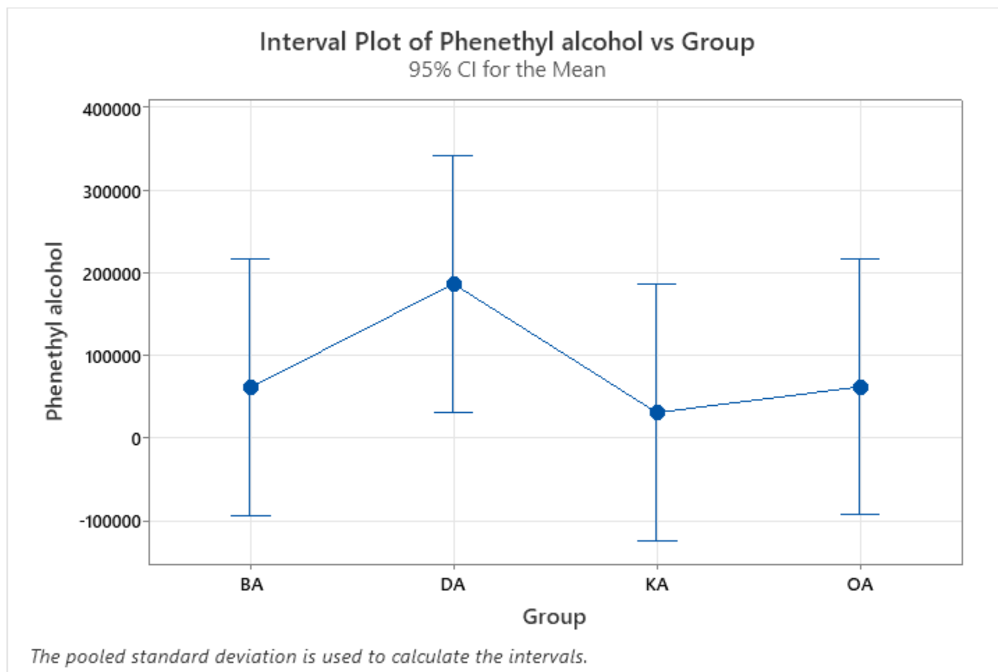
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
DA	3	186075	A
OA	3	61565	A
BA	3	60522	A
KA	3	30431	A

Means that do not share a letter are significantly different.





One-way ANOVA: 2,3-Butanediol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	52570243	17523414	158.30	0.000
Error	8	885587	110698		
Total	11	53455830			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
332.714	98.34%	97.72%	96.27%

Means

Group	N	Mean	StDev	95% CI
BA	3	1047.6	80.3	(604.6, 1490.6)
DA	3	2948	481	(2505, 3391)
KA	3	5195	210	(4752, 5638)
OA	3	6509	401	(6066, 6952)

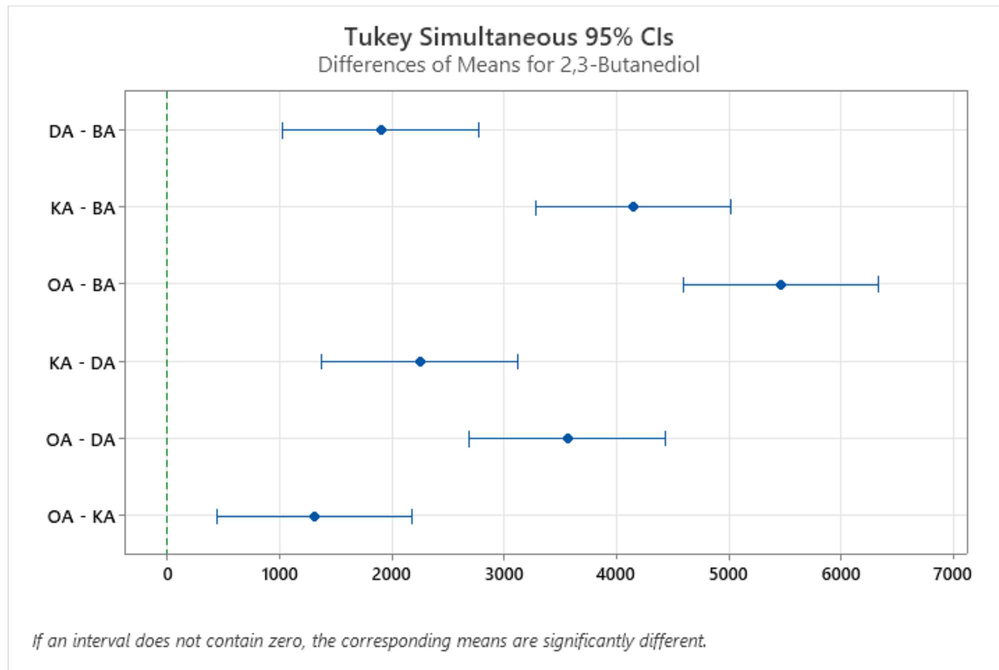
Pooled StDev = 332.714

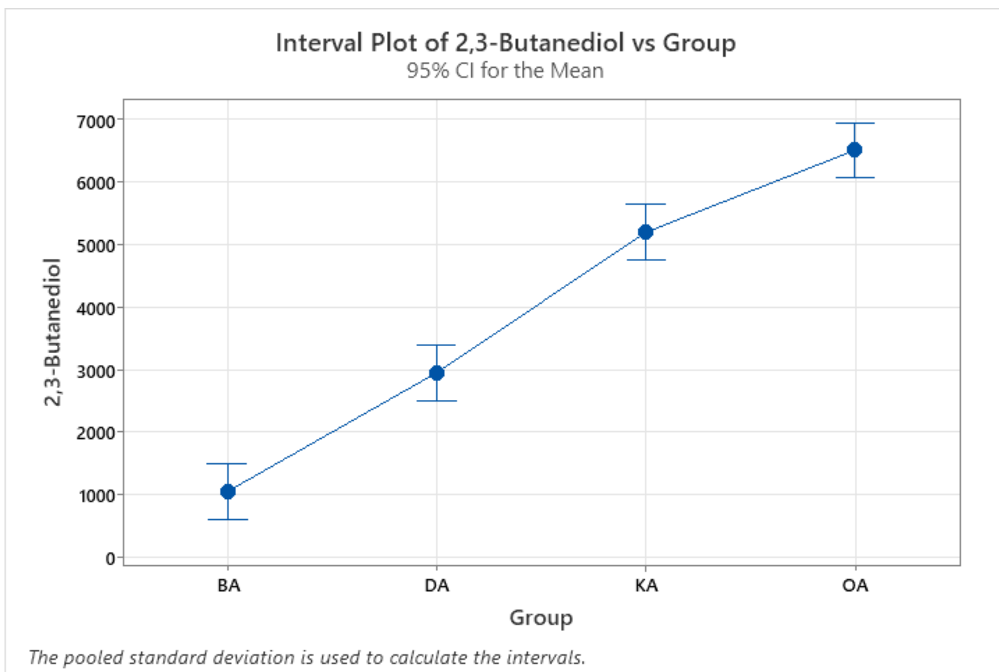
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping			
OA	3	6509	A			
KA	3	5195		B		
DA	3	2948			C	
BA	3	1047.6				D

Means that do not share a letter are significantly different.





One-way ANOVA: 1-Heptanol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	24067	8022.2	9.28	0.006
Error	8	6919	864.9		
Total	11	30986			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
29.4088	77.67%	69.30%	49.76%

Means

Group	N	Mean	StDev	95% CI
BA	3	116.83	13.28	(77.67, 155.98)
DA	3	175.6	56.4	(136.5, 214.8)
KA	3	51.87	4.82	(12.72, 91.02)
OA	3	92.96	9.05	(53.81, 132.12)

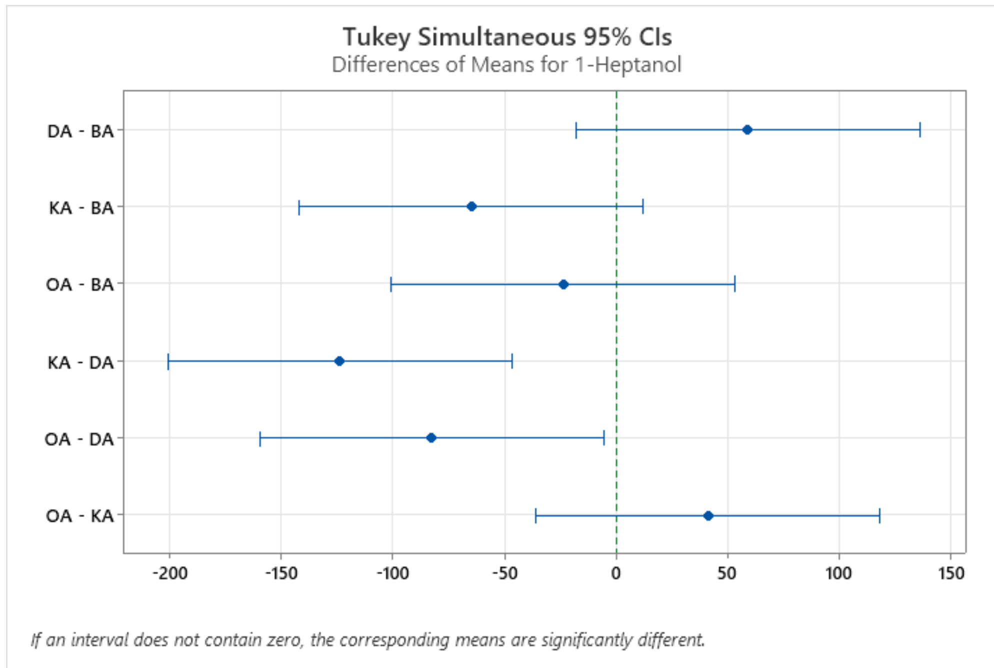
Pooled StDev = 29.4088

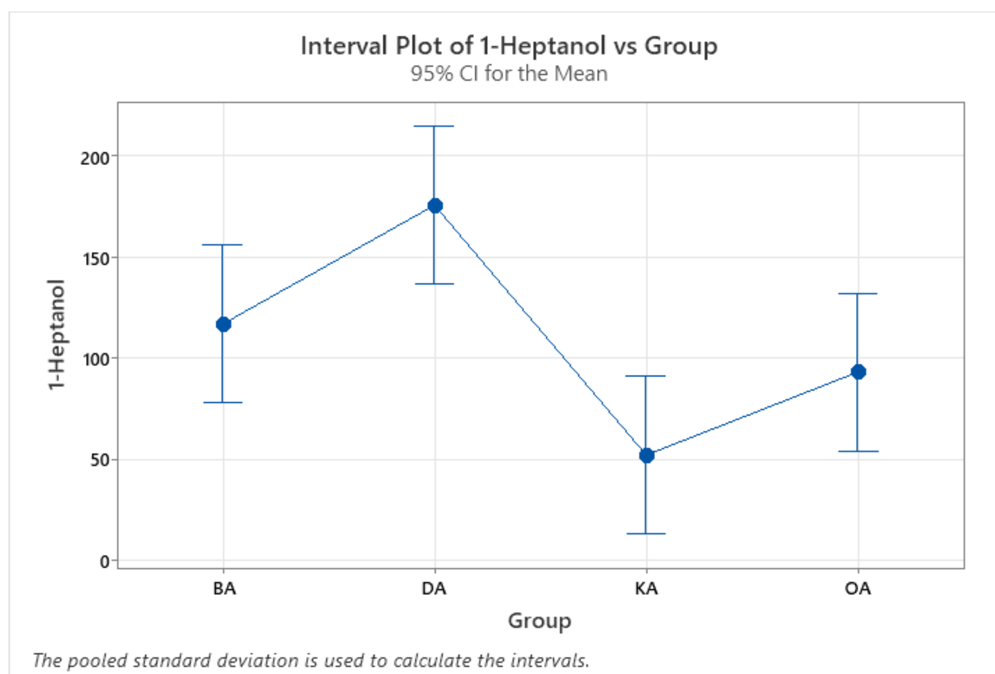
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	175.6	A	
BA	3	116.83	A	B
OA	3	92.96		B
KA	3	51.87		B

Means that do not share a letter are significantly different.





One-way ANOVA: (Z)-3-Hexen-1-ol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	872058	290686	46.51	0.000
Error	8	49995	6249		
Total	11	922053			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
79.0532	94.58%	92.54%	87.80%

Means

Group	N	Mean	StDev	95% CI
BA	3	766.5	80.9	(661.2, 871.7)
DA	3	359.7	61.2	(254.4, 464.9)
KA	3	45.12	2.69	(-60.13, 150.37)
OA	3	193.5	121.2	(88.2, 298.7)

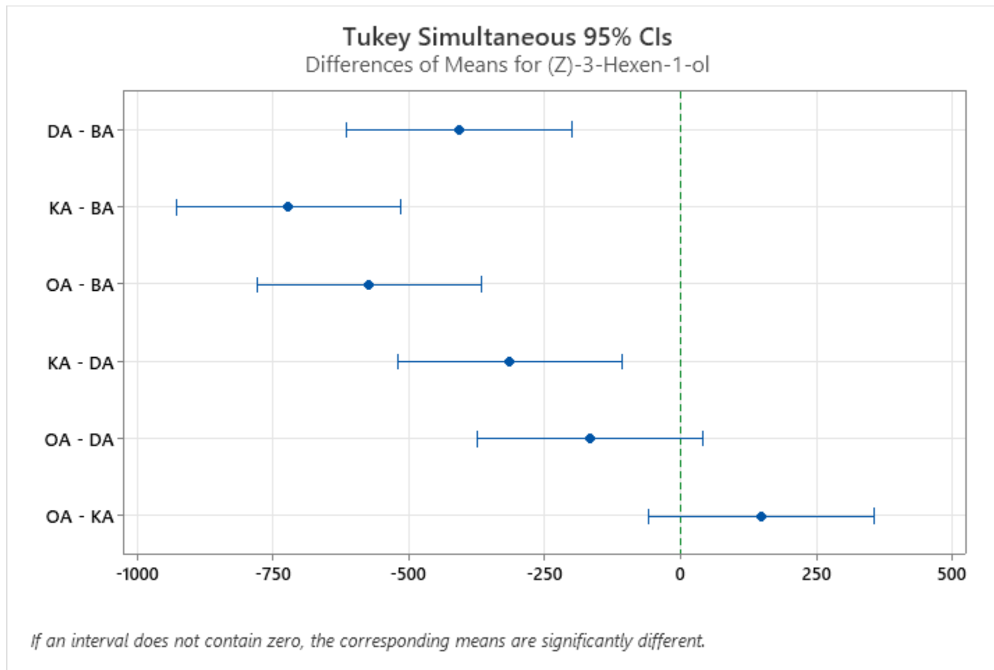
Pooled StDev = 79.0532

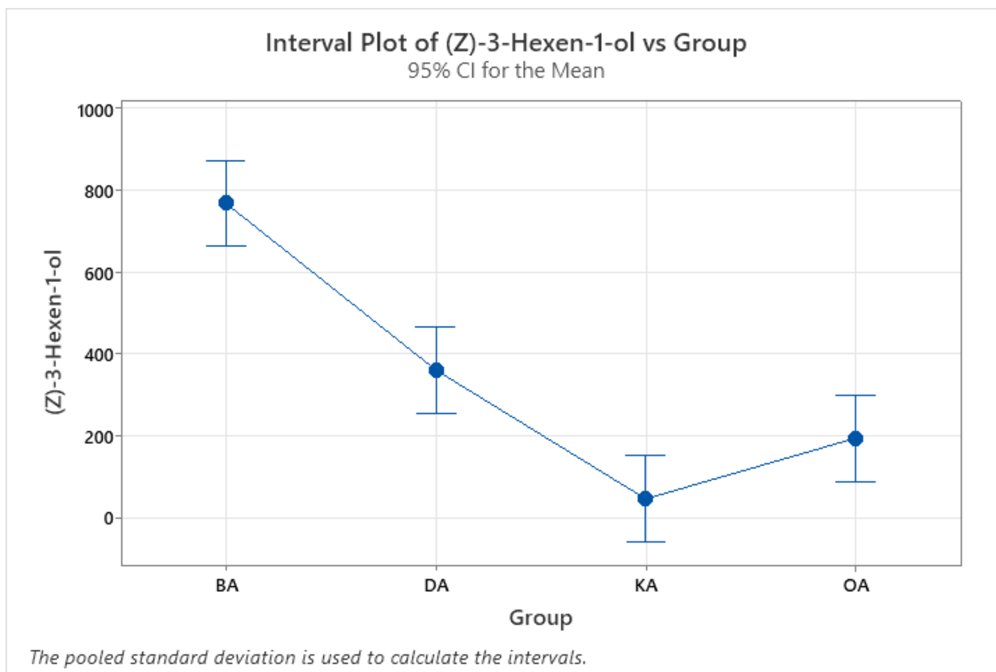
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
BA	3	766.5	A		
DA	3	359.7		B	
OA	3	193.5		B	C
KA	3	45.12			C

Means that do not share a letter are significantly different.





One-way ANOVA: (E)-3-Hexen-1-ol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	41019.7	20509.8	557.23	0.000
Error	6	220.8	36.8		
Total	8	41240.5			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
6.06685	99.46%	99.29%	98.80%

Means

Group	N	Mean	StDev	95% CI
BA	3	201.18	2.48	(192.61, 209.76)
KA	3	39.99	3.17	(31.42, 48.56)
OA	3	88.61	9.70	(80.04, 97.18)

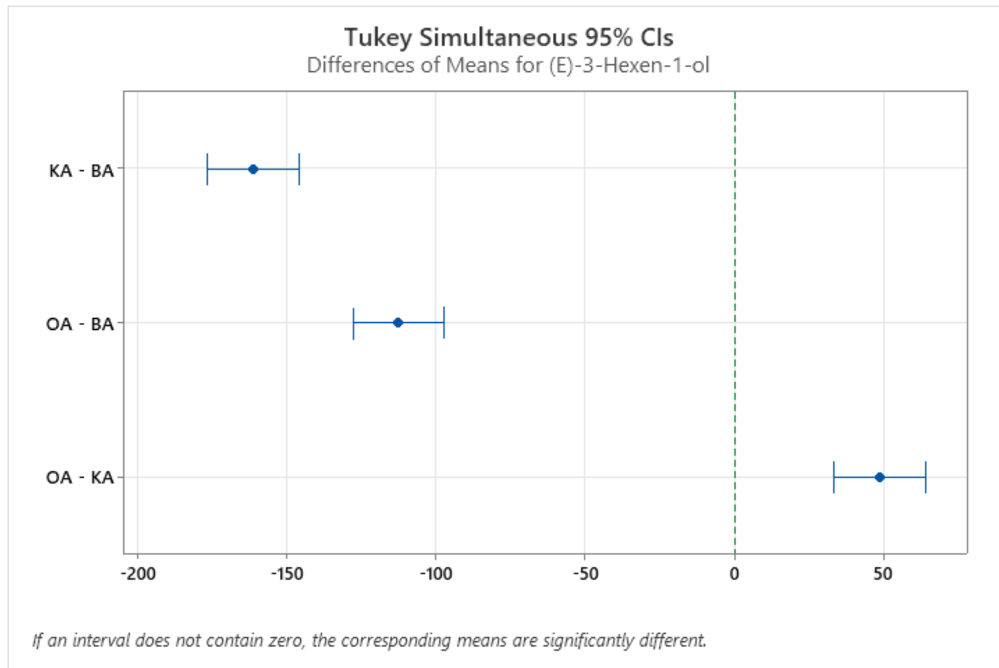
Pooled StDev = 6.06685

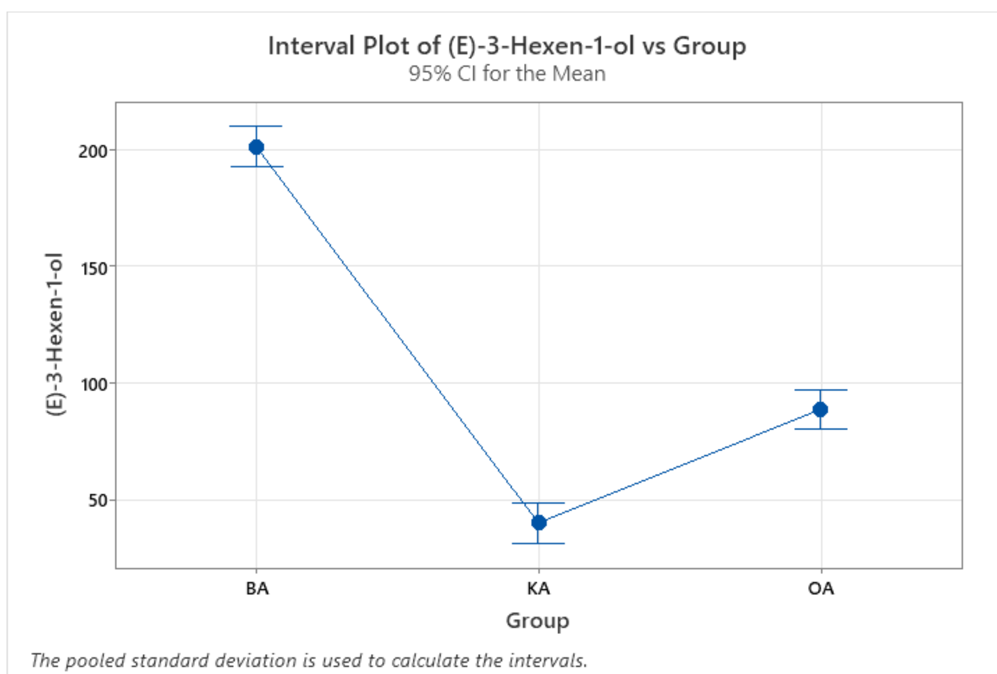
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
BA	3	201.18	A		
OA	3	88.61		B	
KA	3	39.99			C

Means that do not share a letter are significantly different.





One-way ANOVA: 3-Penten-2-ol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	2801002	933667	7.75	0.009
Error	8	963909	120489		
Total	11	3764912			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
347.115	74.40%	64.80%	42.39%

Means

Group	N	Mean	StDev	95% CI
BA	3	345.20	13.38	(-116.94, 807.33)
DA	3	1471	682	(1009, 1933)
KA	3	266.98	7.10	(-195.16, 729.12)
OA	3	506.9	129.0	(44.8, 969.1)

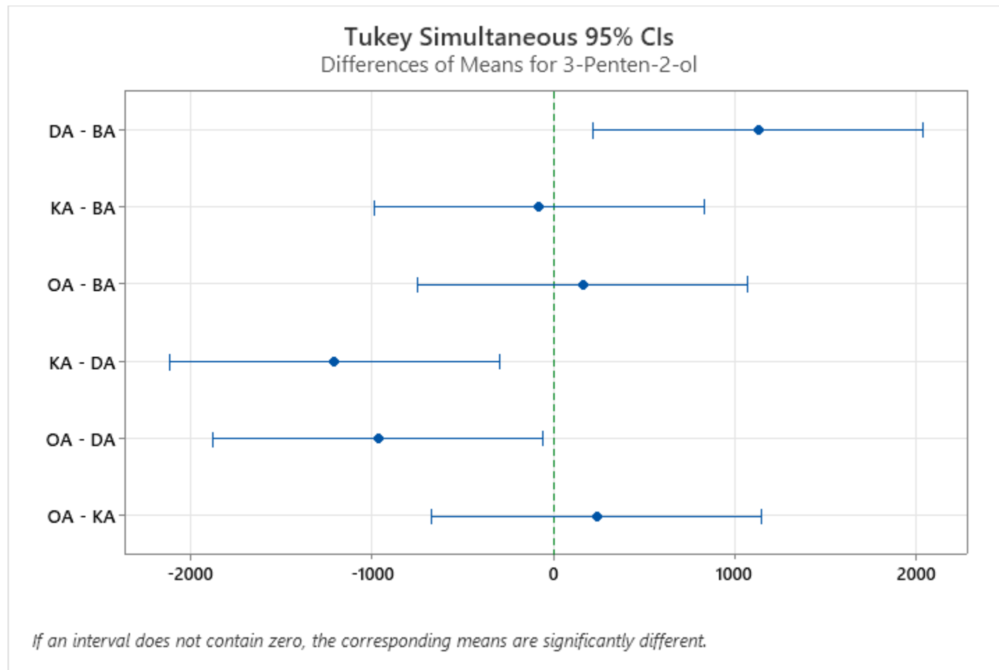
Pooled StDev = 347.115

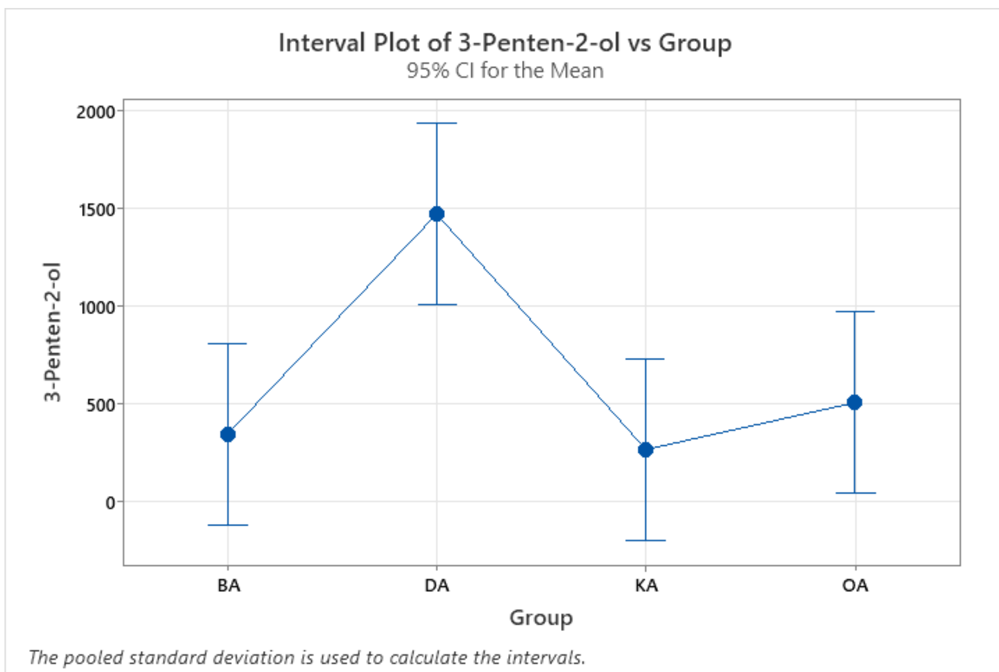
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	1471	A	
OA	3	506.9		B
BA	3	345.20		B
KA	3	266.98		B

Means that do not share a letter are significantly different.





One-way ANOVA: 2-Hexanol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	102119	34040	21.82	0.000
Error	8	12482	1560		
Total	11	114600			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
39.4994	89.11%	85.02%	75.49%

Means

Group	N	Mean	StDev	95% CI
BA	3	269.6	18.5	(217.0, 322.2)
DA	3	207.3	24.4	(154.7, 259.9)
KA	3	33.78	2.94	(-18.81, 86.36)
OA	3	244.8	72.8	(192.2, 297.4)

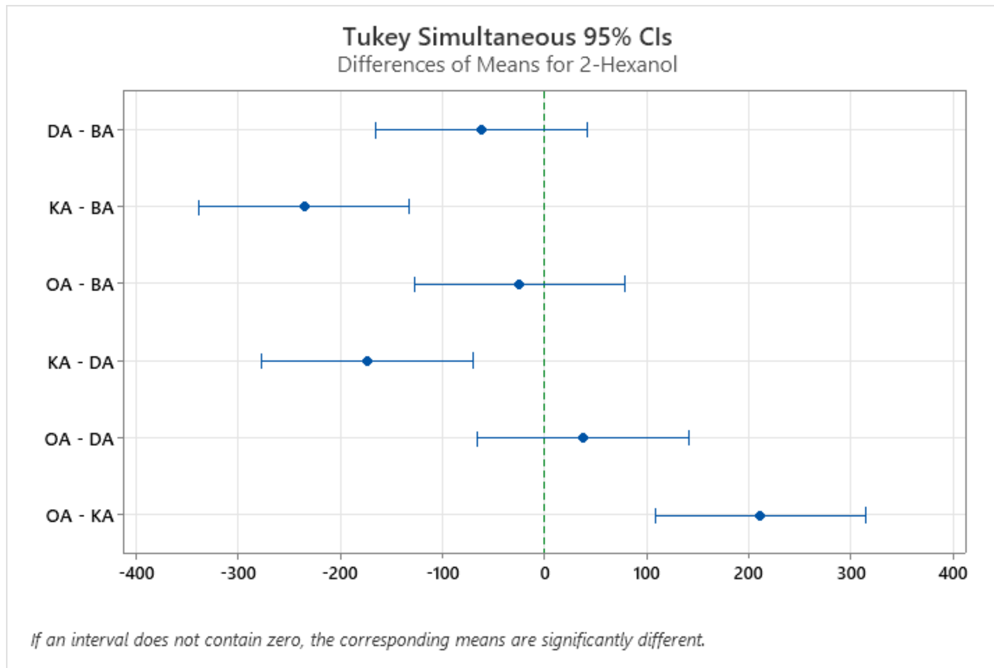
Pooled StDev = 39.4994

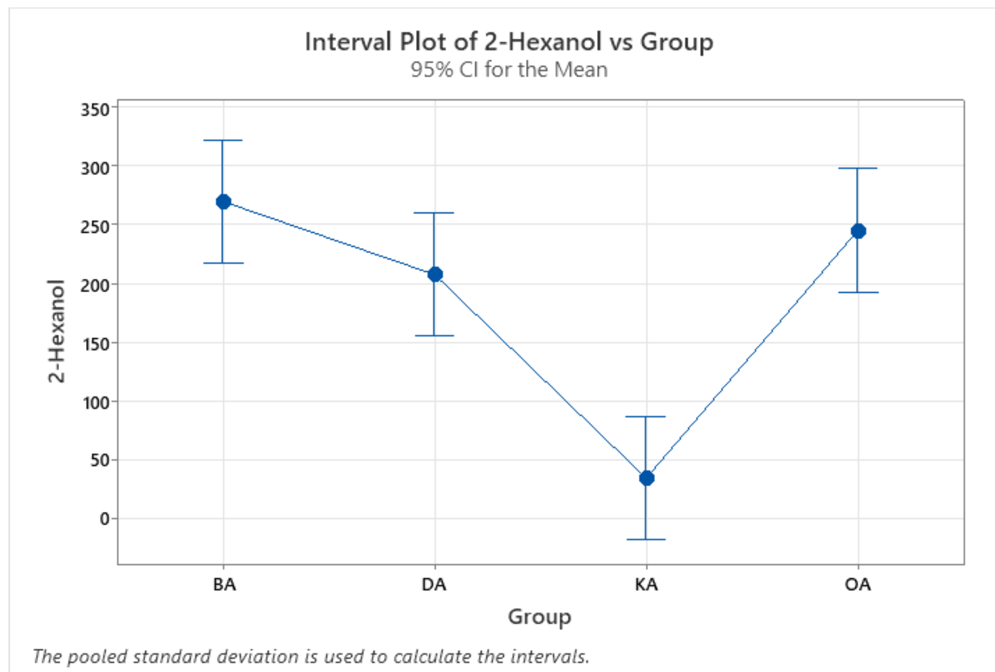
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
BA	3	269.6	A	
OA	3	244.8	A	
DA	3	207.3	A	
KA	3	33.78		B

Means that do not share a letter are significantly different.





One-way ANOVA: 1-Hexanol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	1599237	799619	89.76	0.000
Error	6	53449	8908		
Total	8	1652686			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
94.3830	96.77%	95.69%	92.72%

Means

Group	N	Mean	StDev	95% CI
BA	3	456.5	38.6	(323.2, 589.9)
DA	3	593.7	61.3	(460.4, 727.0)
OA	3	1411.4	146.5	(1278.1, 1544.8)

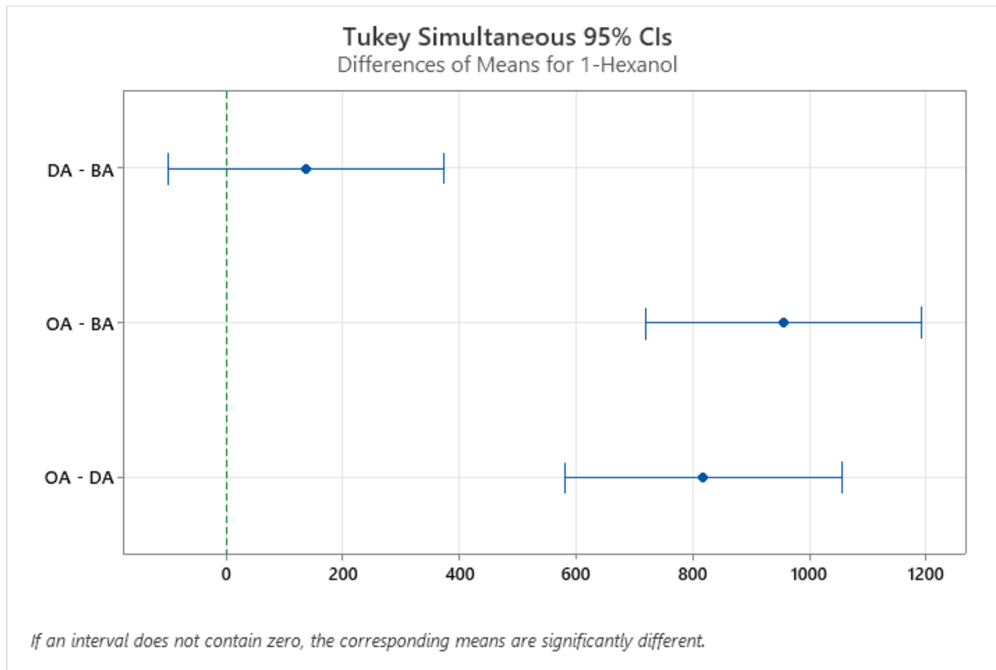
Pooled StDev = 94.3830

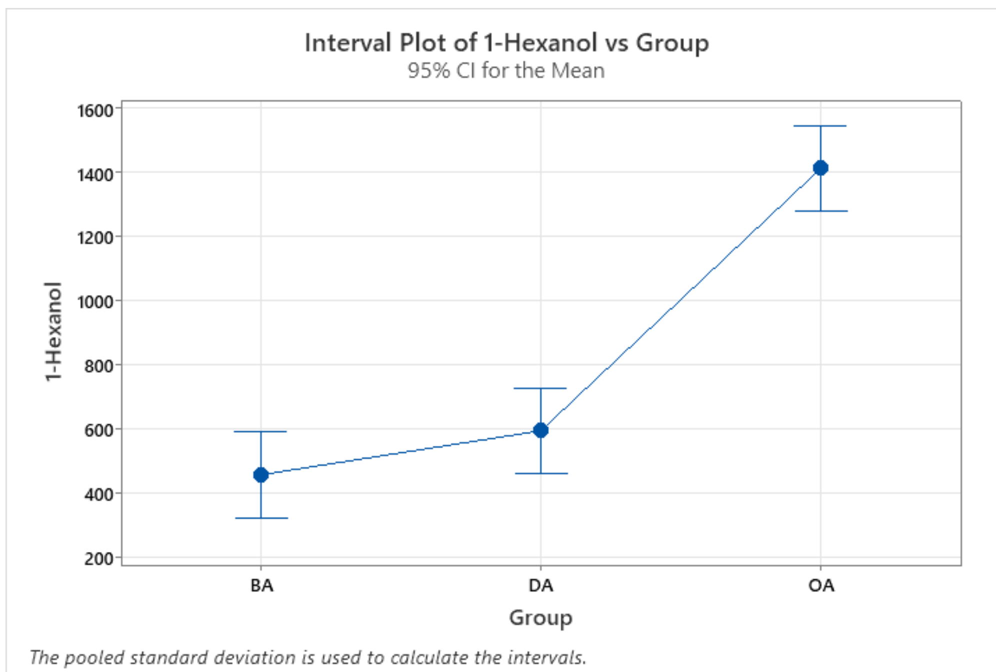
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	1411.4	A	
DA	3	593.7		B
BA	3	456.5		B

Means that do not share a letter are significantly different.





One-way ANOVA: 2-Octanol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	66772	33386	0.01	0.986
Error	6	14365365	2394227		
Total	8	14432137			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1547.33	0.46%	0.00%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	2345	1316	(159, 4531)
DA	3	2340	2063	(154, 4526)
OA	3	2160	1093	(-26, 4346)

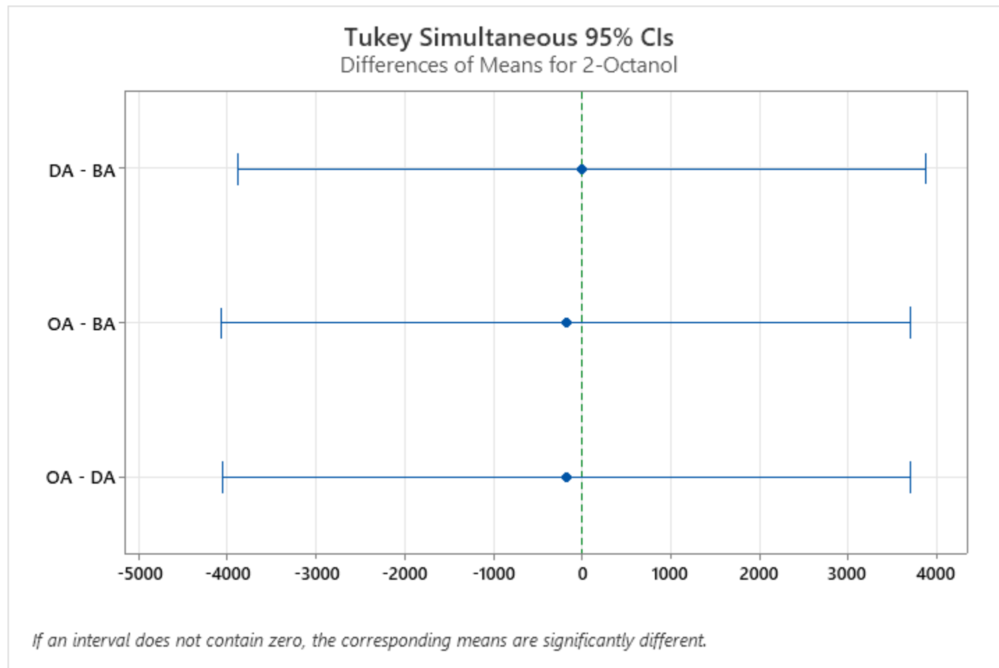
Pooled StDev = 1547.33

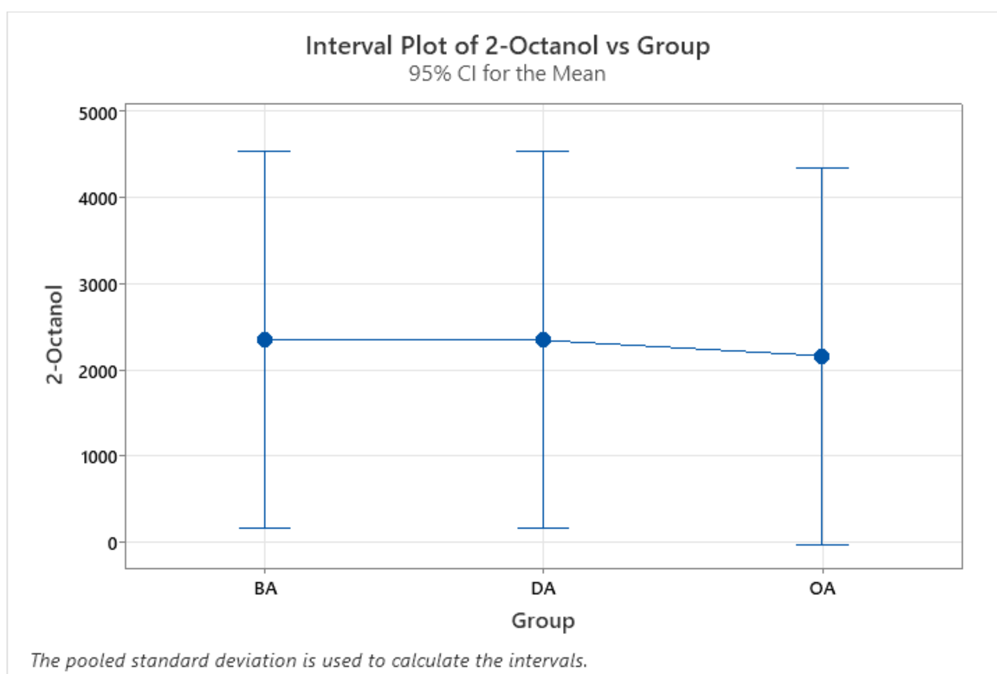
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
BA	3	2345	A
DA	3	2340	A
OA	3	2160	A

Means that do not share a letter are significantly different.





One-way ANOVA: Isoamyl acetate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	4103221	1367740	9.12	0.006
Error	8	1199985	149998		
Total	11	5303207			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
387.296	77.37%	68.89%	49.09%

Means

Group	N	Mean	StDev	95% CI
BA	3	1627.6	58.4	(1111.9, 2143.2)
DA	3	1819	635	(1304, 2335)
KA	3	807.9	59.8	(292.2, 1323.5)
OA	3	2445	436	(1929, 2961)

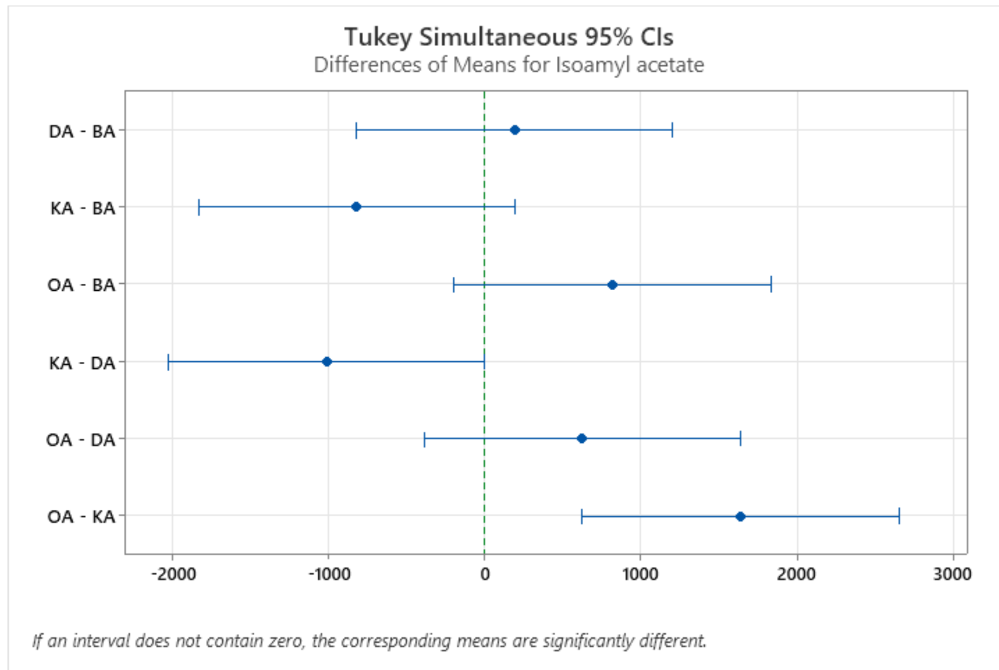
Pooled StDev = 387.296

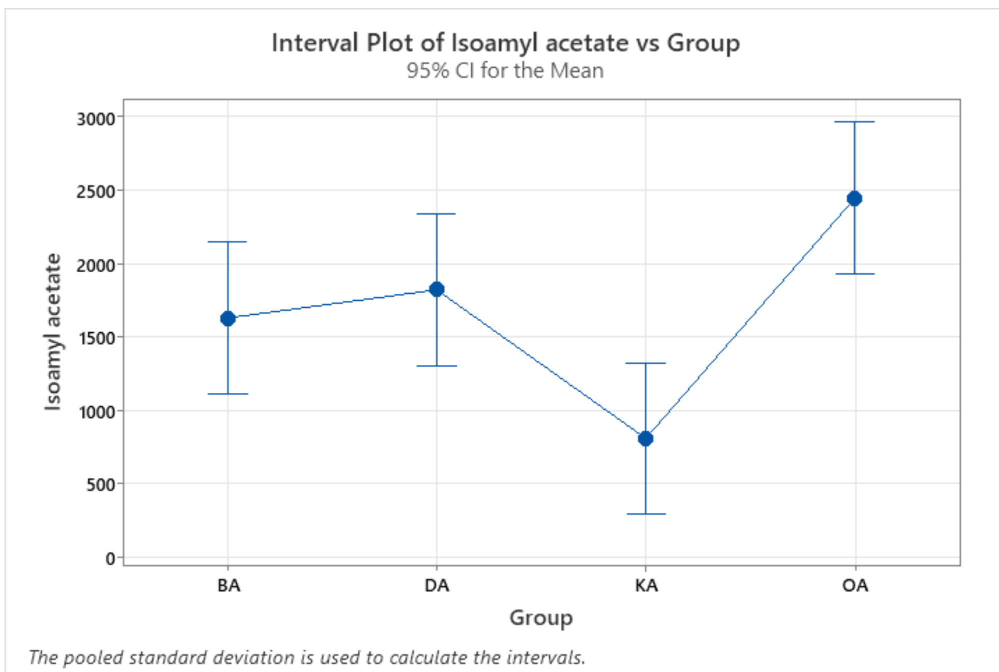
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	2445	A	
DA	3	1819	A	B
BA	3	1627.6	A	B
KA	3	807.9		B

Means that do not share a letter are significantly different.





One-way ANOVA: 2-Phenylethyl acetate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	319424	106475	17.18	0.001
Error	8	49591	6199		
Total	11	369015			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
78.7327	86.56%	81.52%	69.76%

Means

Group	N	Mean	StDev	95% CI
BA	3	222.56	15.32	(117.73, 327.38)
DA	3	286.6	106.5	(181.7, 391.4)
KA	3	226.46	14.21	(121.64, 331.28)
OA	3	617.4	114.1	(512.6, 722.2)

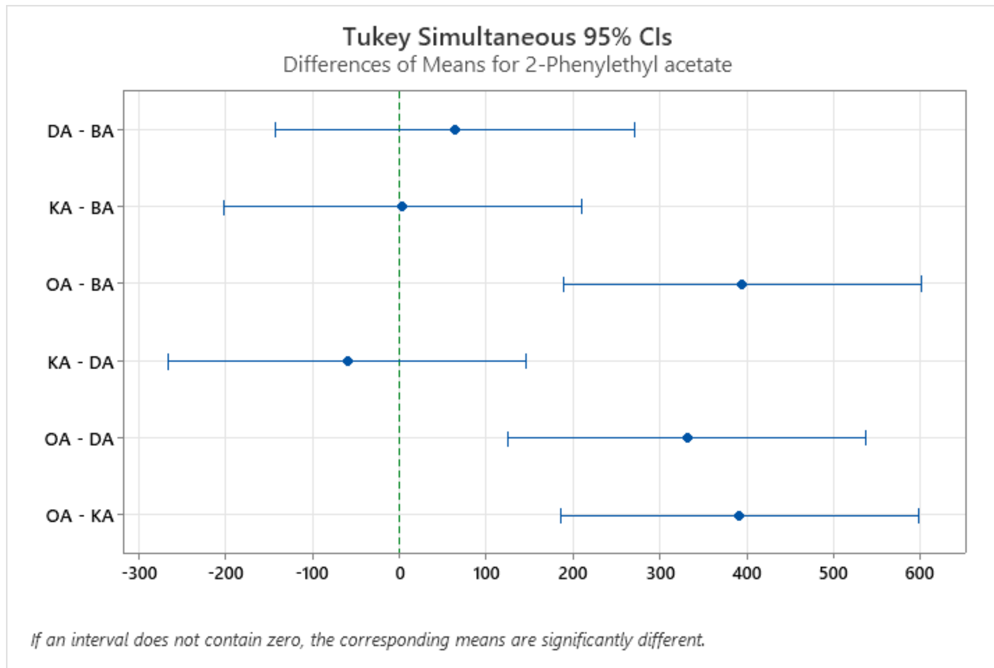
Pooled StDev = 78.7327

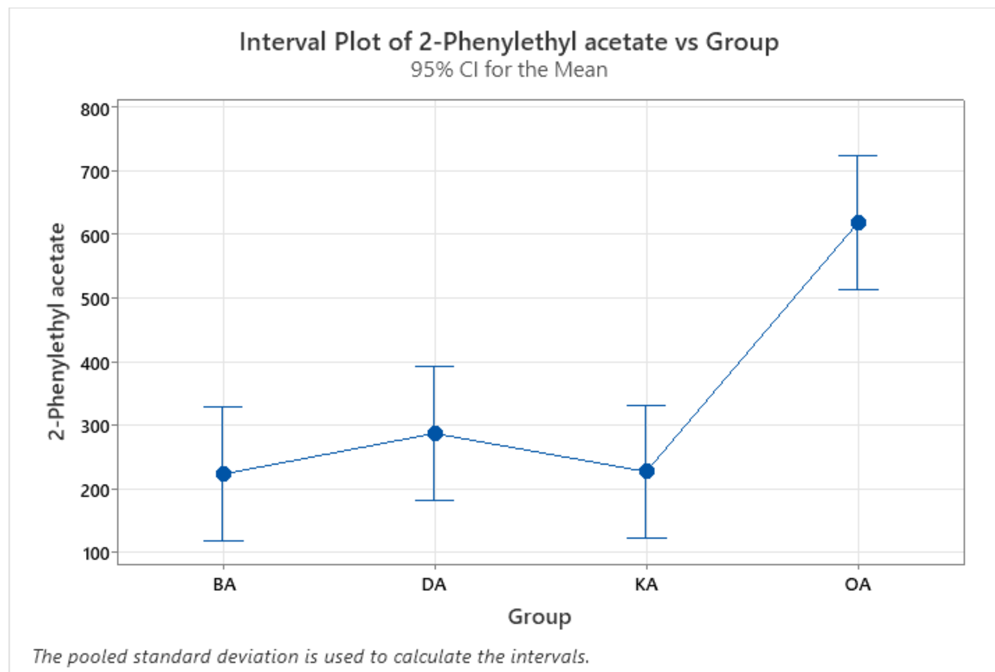
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	617.4	A	
DA	3	286.6		B
KA	3	226.46		B
BA	3	222.56		B

Means that do not share a letter are significantly different.





One-way ANOVA: Ethyl lactate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	3392427	1130809	11.05	0.003
Error	8	818924	102366		
Total	11	4211351			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
319.946	80.55%	73.26%	56.25%

Means

Group	N	Mean	StDev	95% CI
BA	3	609.5	24.1	(183.5, 1035.5)
DA	3	1402	603	(976, 1828)
KA	3	2059.5	125.8	(1633.6, 2485.5)
OA	3	1037.4	172.6	(611.5, 1463.4)

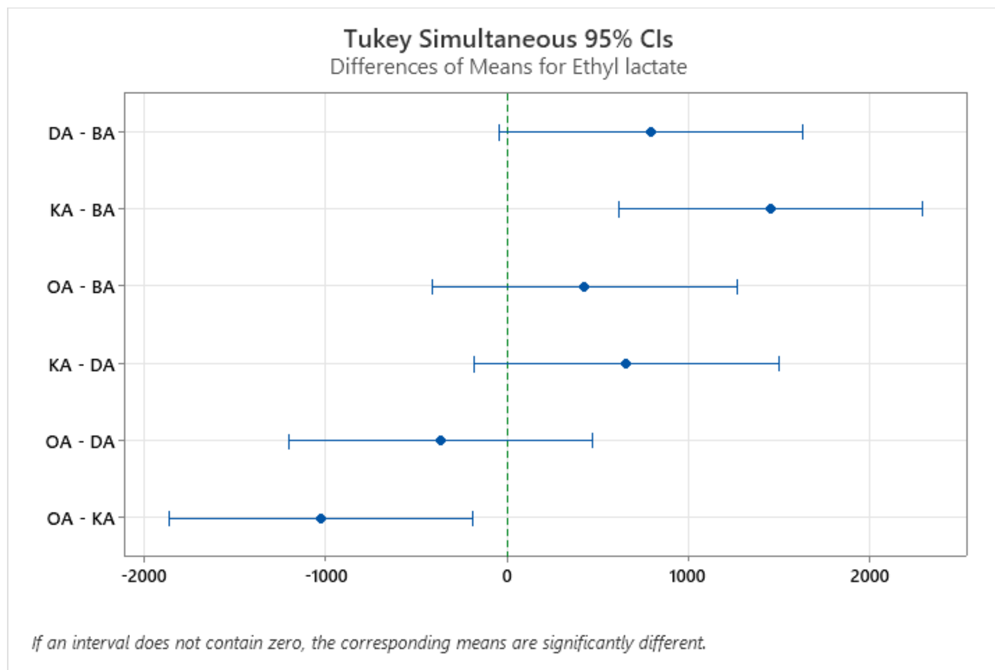
Pooled StDev = 319.946

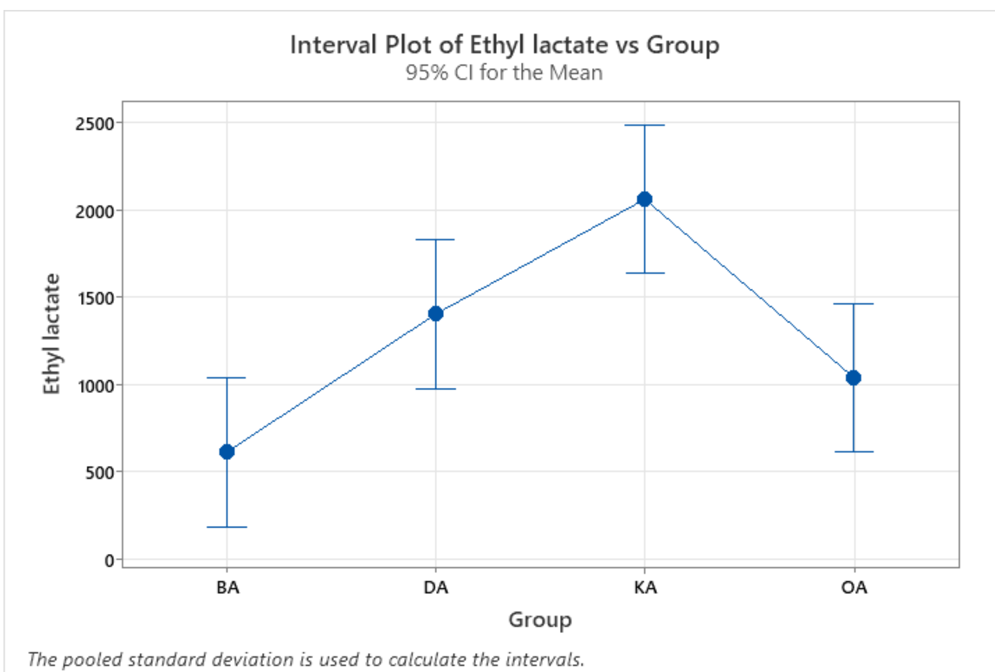
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
KA	3	2059.5	A	
DA	3	1402	A	B
OA	3	1037.4		B
BA	3	609.5		B

Means that do not share a letter are significantly different.





One-way ANOVA: Ethyl octanoate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	901.4	300.5	0.51	0.689
Error	8	4759.1	594.9		
Total	11	5660.5			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
24.3903	15.92%	0.00%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	161.21	4.47	(128.74, 193.68)
DA	3	155.52	11.42	(123.05, 187.99)
KA	3	150.67	6.74	(118.20, 183.14)
OA	3	173.9	46.7	(141.4, 206.3)

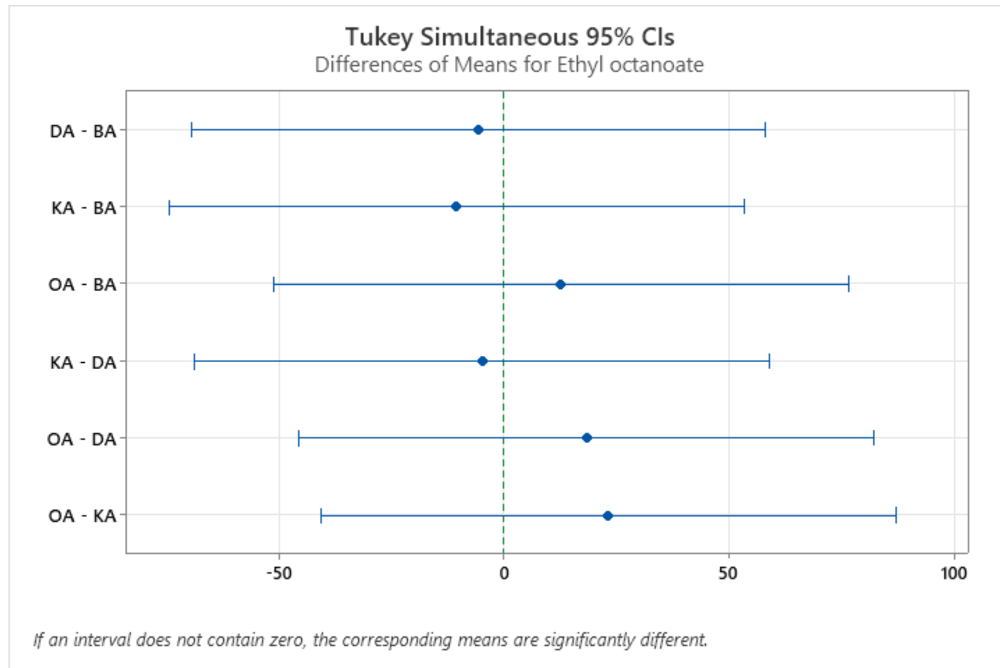
Pooled StDev = 24.3903

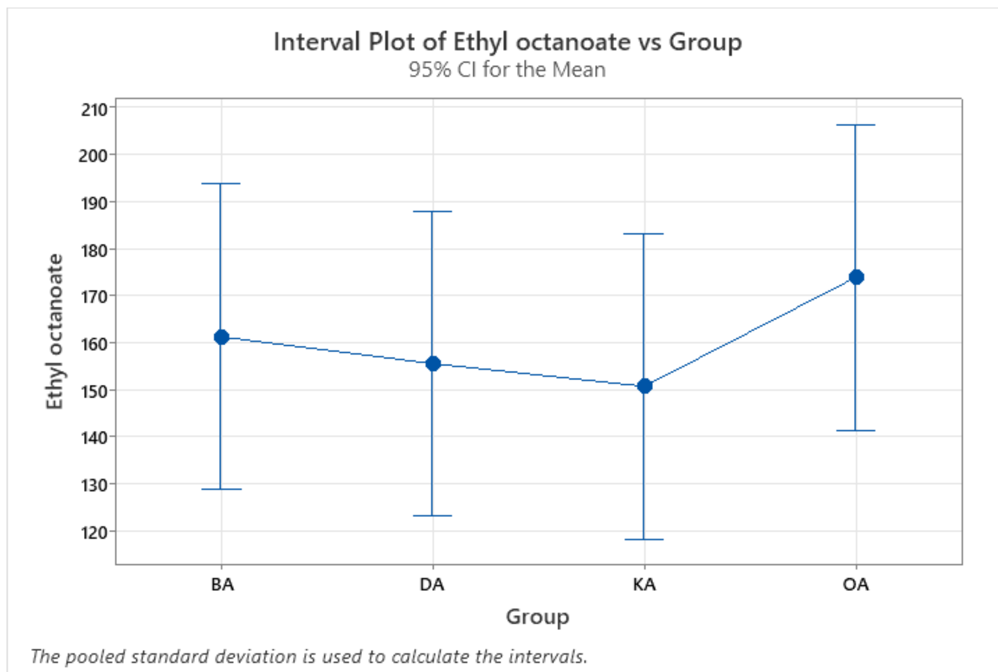
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
OA	3	173.9	A
BA	3	161.21	A
DA	3	155.52	A
KA	3	150.67	A

Means that do not share a letter are significantly different.





One-way ANOVA: Ethyl 3-hydroxybutyrate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	96398	32133	14.56	0.001
Error	8	17652	2206		
Total	11	114050			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
46.9734	84.52%	78.72%	65.18%

Means

Group	N	Mean	StDev	95% CI
BA	3	175.46	10.93	(112.92, 238.00)
DA	3	396.2	61.5	(333.7, 458.8)
KA	3	245.6	24.5	(183.1, 308.2)
OA	3	366.9	65.8	(304.3, 429.4)

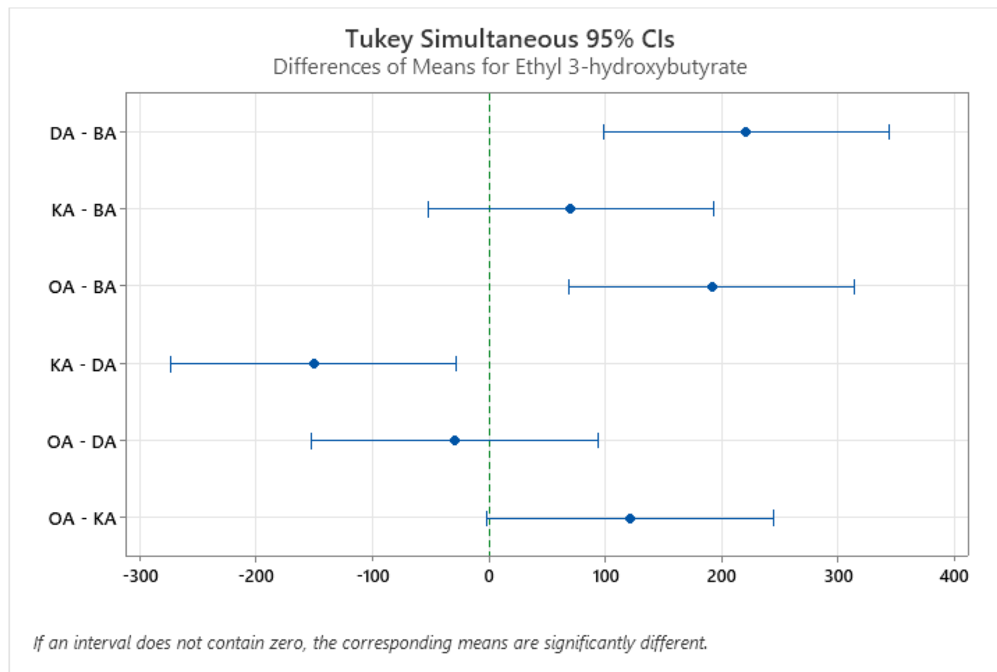
Pooled StDev = 46.9734

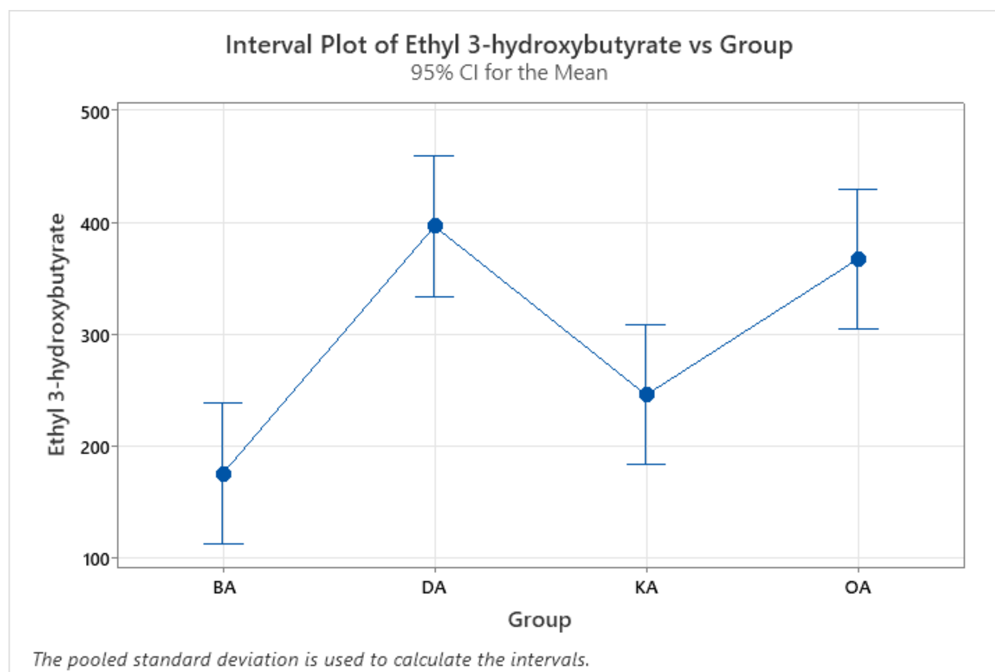
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
DA	3	396.2	A		
OA	3	366.9	A	B	
KA	3	245.6		B	C
BA	3	175.46			C

Means that do not share a letter are significantly different.





One-way ANOVA: Diethyl succinate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	35897	11966	3.38	0.075
Error	8	28360	3545		
Total	11	64258			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
59.5401	55.86%	39.31%	0.70%

Means

Group	N	Mean	StDev	95% CI
BA	3	265.7	18.4	(186.5, 345.0)
DA	3	223.2	46.5	(143.9, 302.5)
KA	3	259.4	19.8	(180.1, 338.7)
OA	3	370.1	106.2	(290.8, 449.3)

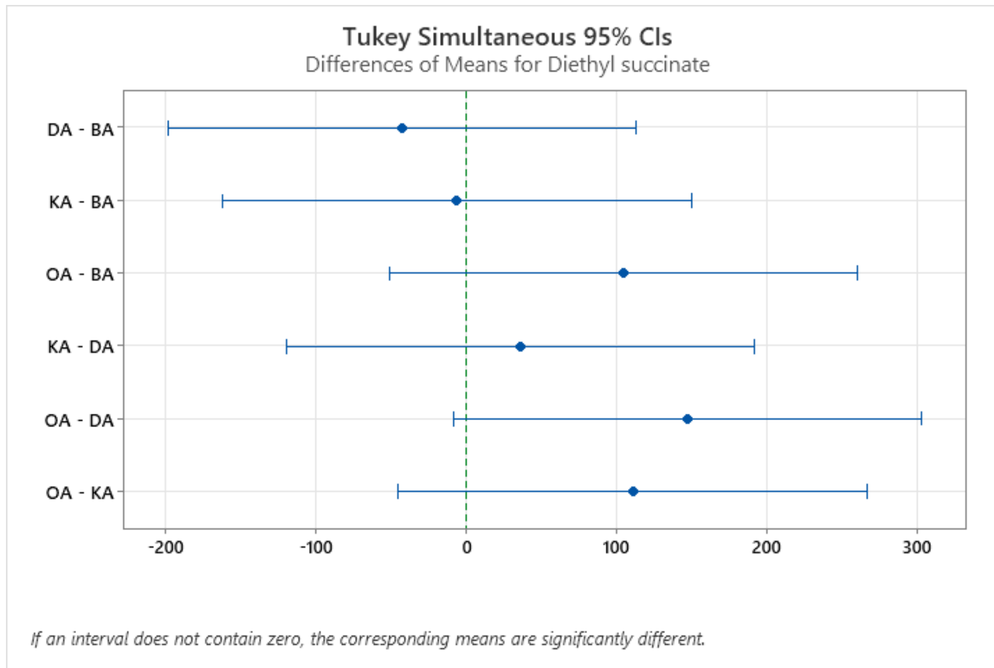
Pooled StDev = 59.5401

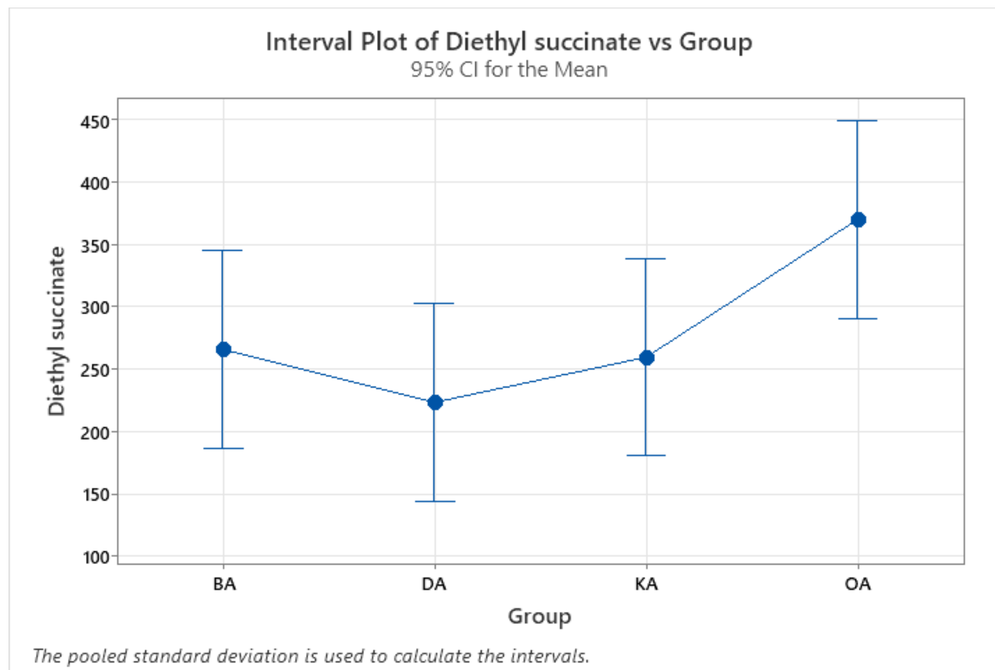
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
OA	3	370.1	A
BA	3	265.7	A
KA	3	259.4	A
DA	3	223.2	A

Means that do not share a letter are significantly different.





One-way ANOVA: Ethyl 4-hydroxybutanoate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	50505355	16835118	41.91	0.000
Error	8	3213703	401713		
Total	11	53719058			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
633.808	94.02%	91.77%	86.54%

Means

Group	N	Mean	StDev	95% CI
BA	3	2279.3	138.2	(1435.4, 3123.1)
DA	3	4363	572	(3519, 5206)
KA	3	3684.7	129.9	(2840.9, 4528.5)
OA	3	7851	1115	(7007, 8695)

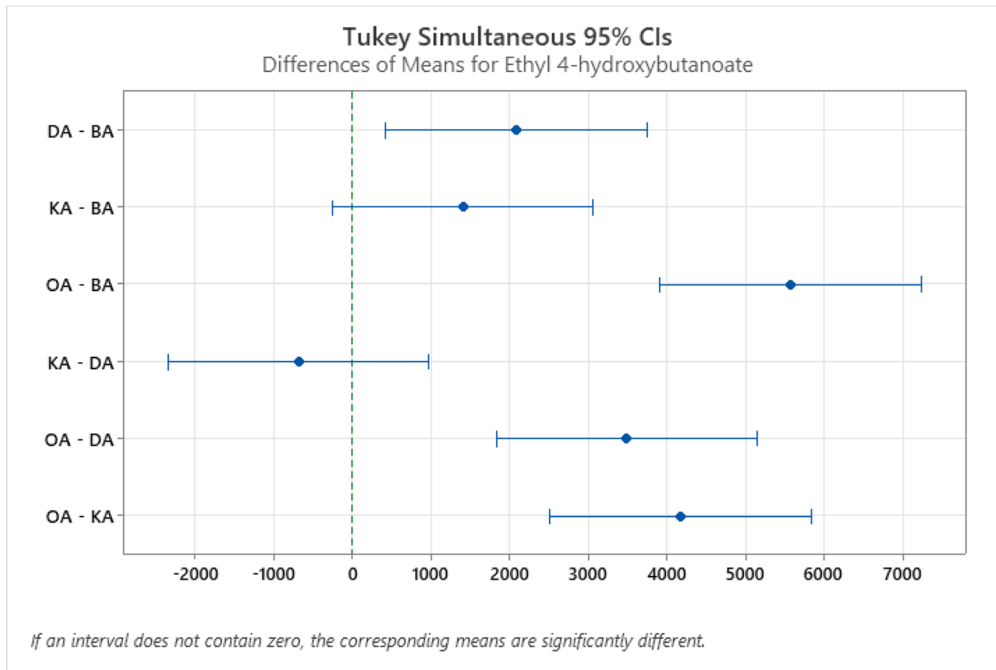
Pooled StDev = 633.808

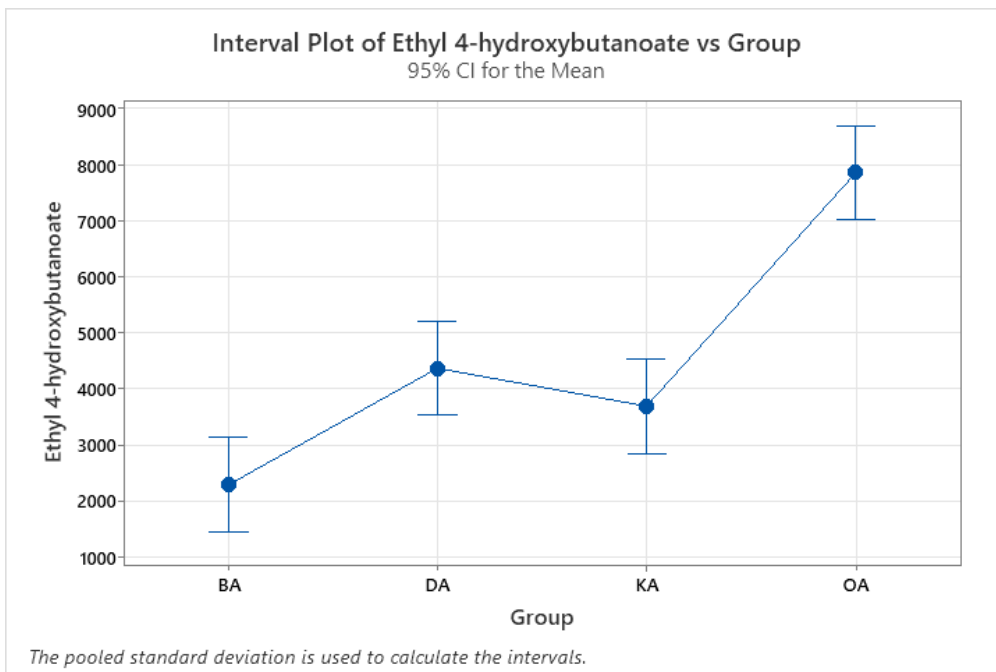
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
OA	3	7851	A		
DA	3	4363		B	
KA	3	3684.7		B	C
BA	3	2279.3			C

Means that do not share a letter are significantly different.





One-way ANOVA: Monoethyl succinate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	18701062	6233687	68.12	0.000
Error	8	732066	91508		
Total	11	19433128			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
302.503	96.23%	94.82%	91.52%

Means

Group	N	Mean	StDev	95% CI
BA	3	54.13	3.23	(-348.62, 456.87)
DA	3	1444.2	131.5	(1041.4, 1846.9)
KA	3	2107	178	(1704, 2510)
OA	3	3522	563	(3119, 3925)

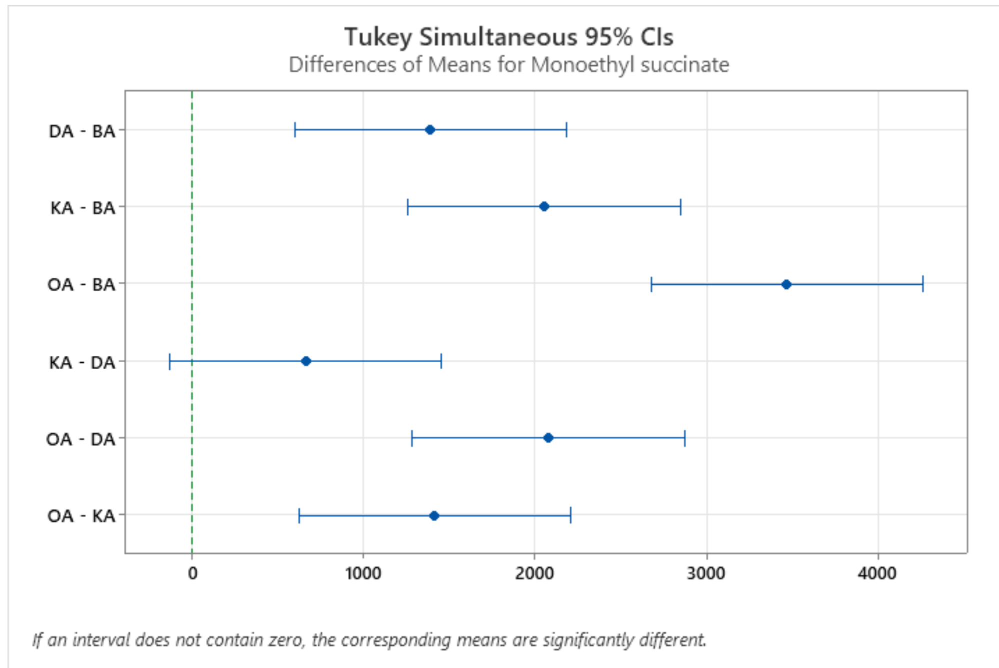
Pooled StDev = 302.503

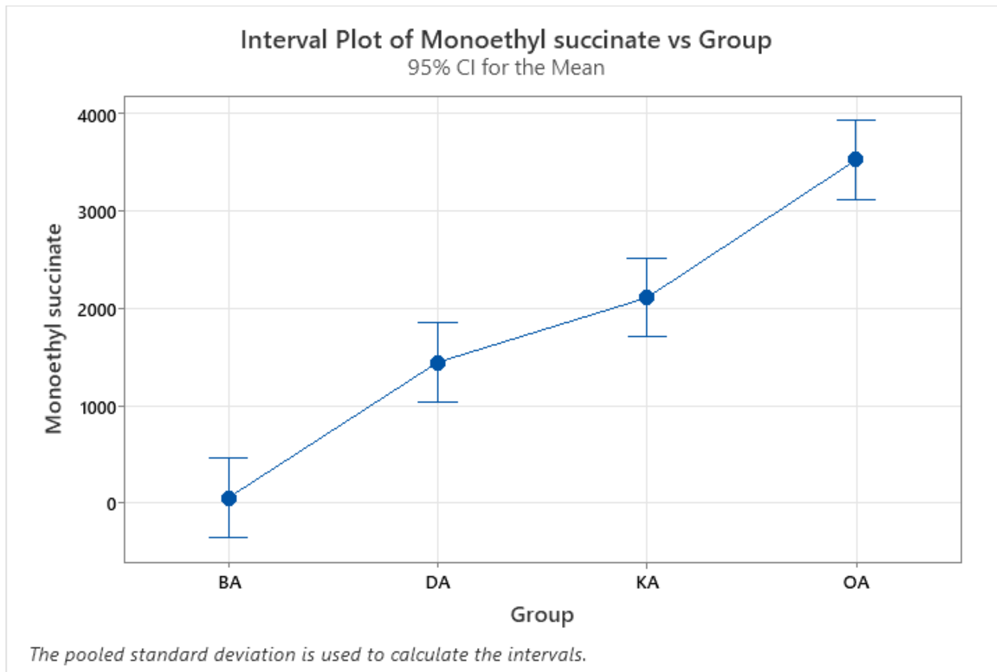
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
OA	3	3522	A		
KA	3	2107		B	
DA	3	1444.2		B	
BA	3	54.13			C

Means that do not share a letter are significantly different.





One-way ANOVA: Ethyl 2-hydroxy-3-phenylpropano versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	5816	2908	2.23	0.189
Error	6	7833	1306		
Total	8	13649			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
36.1322	42.61%	23.48%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	113.30	5.95	(62.26, 164.35)
DA	3	174.8	61.3	(123.8, 225.9)
KA	3	135.76	11.14	(84.71, 186.80)

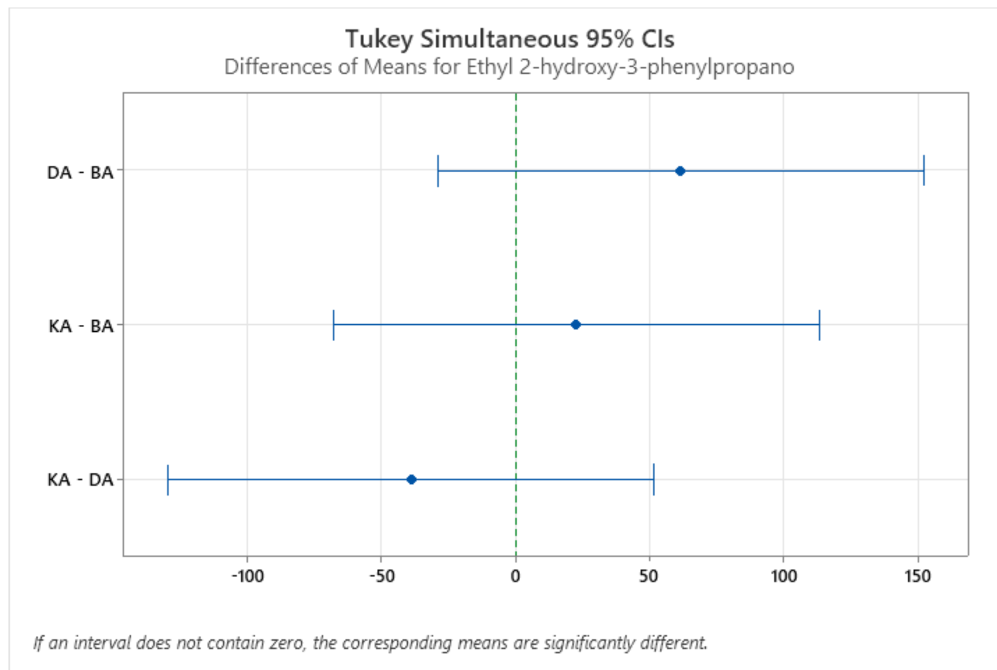
Pooled StDev = 36.1322

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
DA	3	174.8	A
KA	3	135.76	A
BA	3	113.30	A

Means that do not share a letter are significantly different.



One-way ANOVA: Ethyl hexanoate versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	110953	36984	36.28	0.000
Error	8	8156	1020		
Total	11	119109			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
31.9301	93.15%	90.58%	84.59%

Means

Group	N	Mean	StDev	95% CI
BA	3	498.3	36.6	(455.8, 540.8)
DA	3	367.3	36.4	(324.8, 409.8)
KA	3	249.9	23.0	(207.4, 292.5)
OA	3	462.1	29.7	(419.6, 504.6)

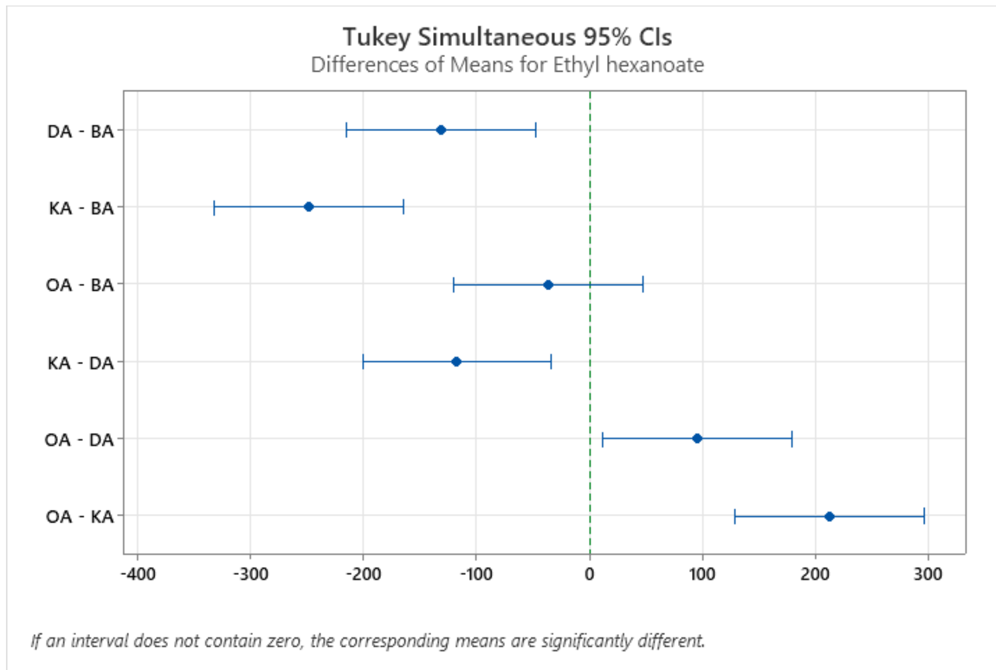
Pooled StDev = 31.9301

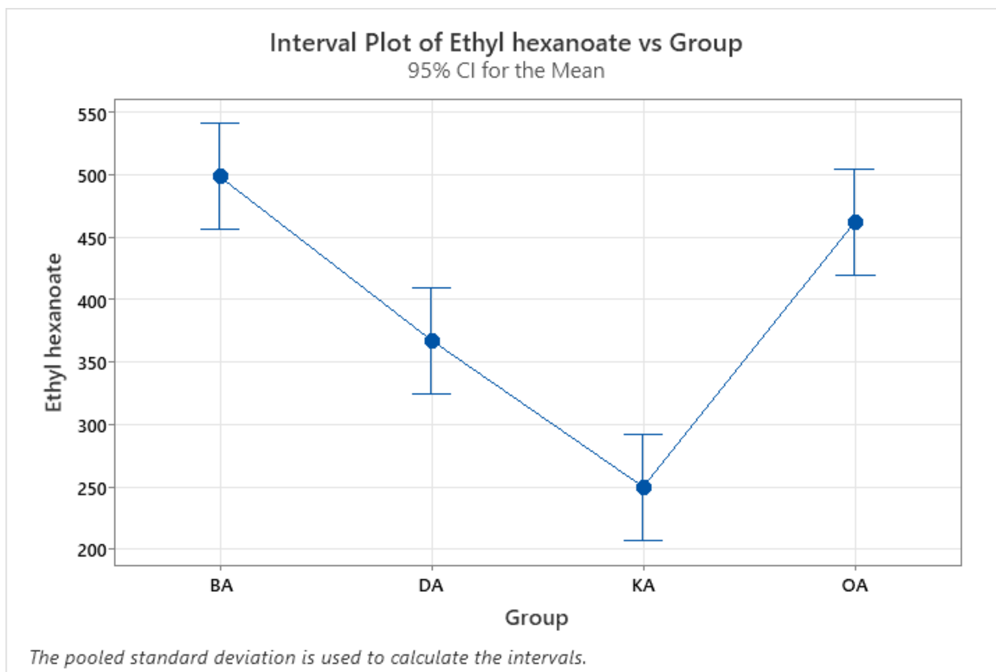
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
BA	3	498.3	A		
OA	3	462.1	A		
DA	3	367.3		B	
KA	3	249.9			C

Means that do not share a letter are significantly different.





One-way ANOVA: Acetoin versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	1557421	519140	112.09	0.000
Error	8	37051	4631		
Total	11	1594472			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
68.0539	97.68%	96.80%	94.77%

Means

Group	N	Mean	StDev	95% CI
BA	3	964.0	29.4	(873.4, 1054.7)
DA	3	357.7	105.1	(267.0, 448.3)
KA	3	1292.3	26.8	(1201.7, 1382.9)
OA	3	567.0	76.9	(476.4, 657.6)

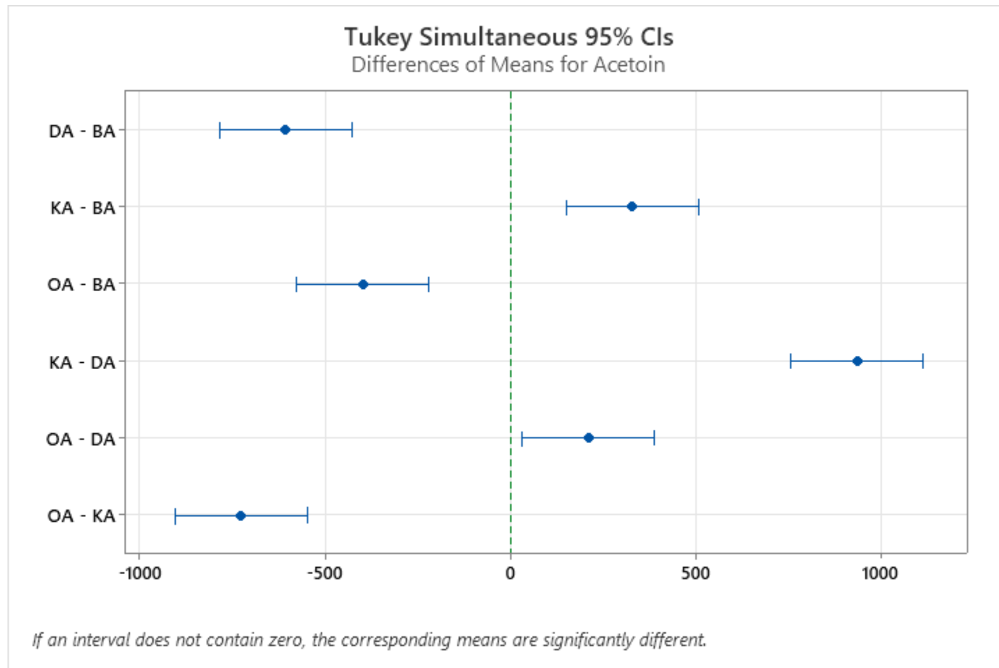
Pooled StDev = 68.0539

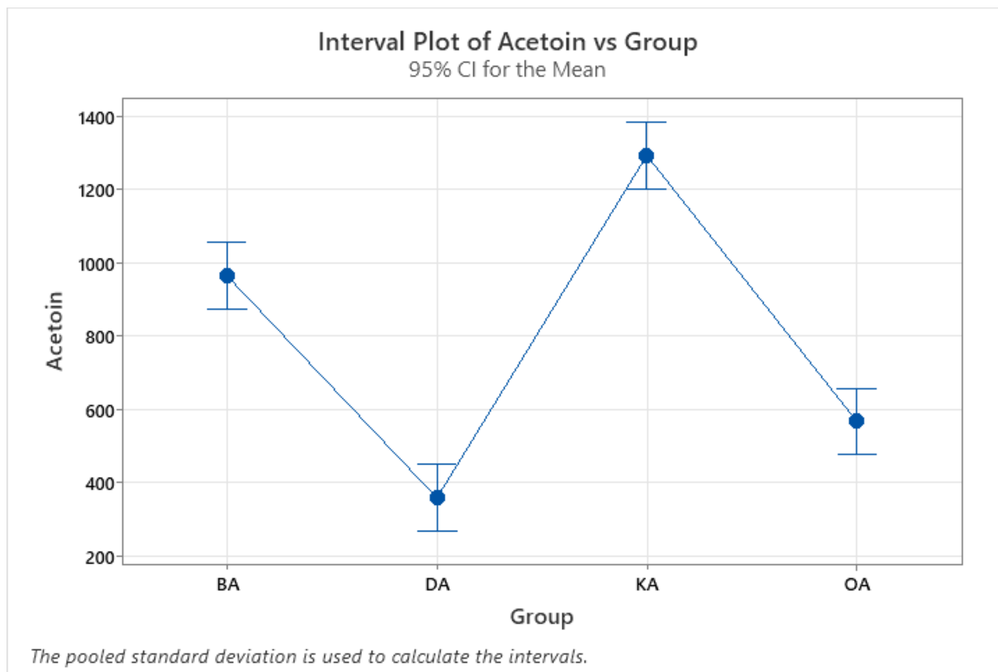
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping			
KA	3	1292.3	A			
BA	3	964.0		B		
OA	3	567.0			C	
DA	3	357.7				D

Means that do not share a letter are significantly different.





One-way ANOVA: Butanoic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	209752	69917	11.04	0.003
Error	8	50650	6331		
Total	11	260402			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
79.5694	80.55%	73.26%	56.24%

Means

Group	N	Mean	StDev	95% CI
BA	3	141.25	10.45	(35.32, 247.19)
DA	3	172.2	54.0	(66.3, 278.2)
KA	3	161.70	12.85	(55.77, 267.64)
OA	3	462.6	148.8	(356.7, 568.6)

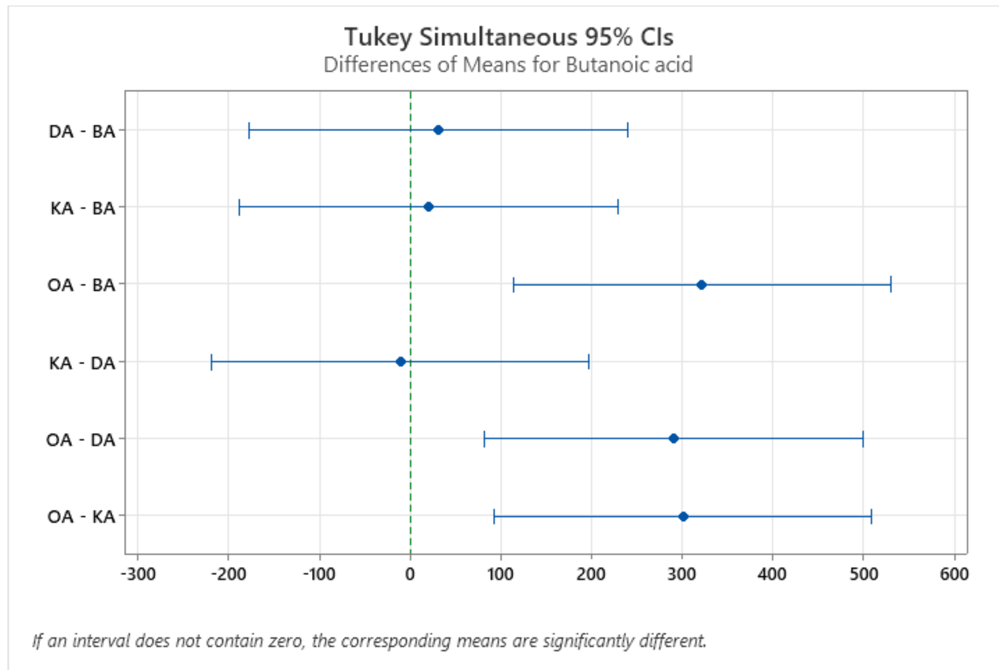
Pooled StDev = 79.5694

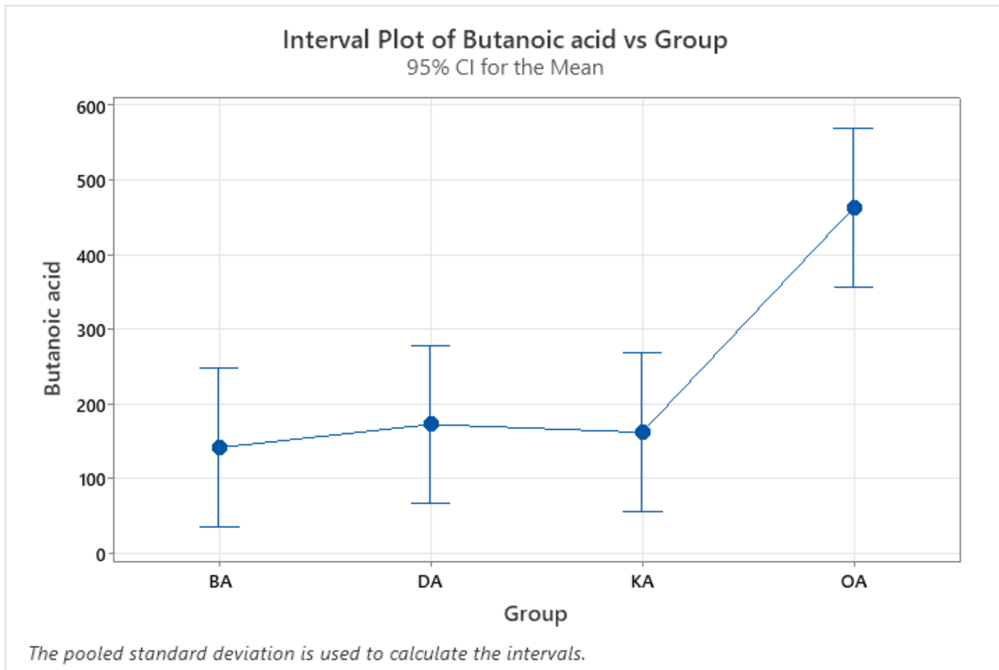
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	462.6	A	
DA	3	172.2		B
KA	3	161.70		B
BA	3	141.25		B

Means that do not share a letter are significantly different.





One-way ANOVA: Hexanoic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	1266778	422259	11.96	0.003
Error	8	282408	35301		
Total	11	1549186			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
187.886	81.77%	74.93%	58.98%

Means

Group	N	Mean	StDev	95% CI
BA	3	313.5	28.6	(63.3, 563.6)
DA	3	594	212	(344, 844)
KA	3	556.2	50.7	(306.1, 806.4)
OA	3	1196	305	(946, 1446)

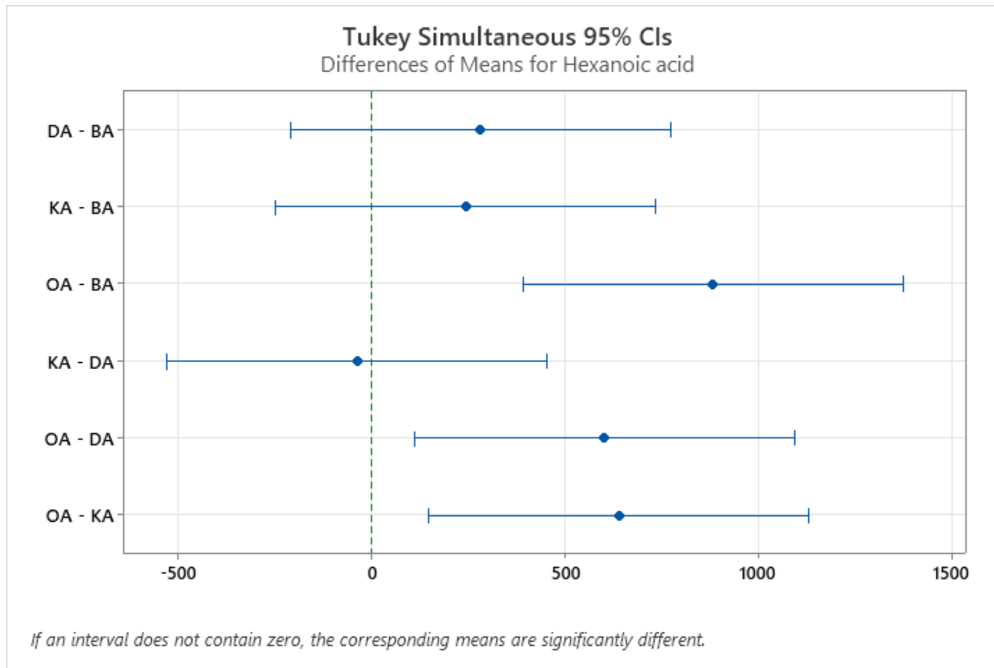
Pooled StDev = 187.886

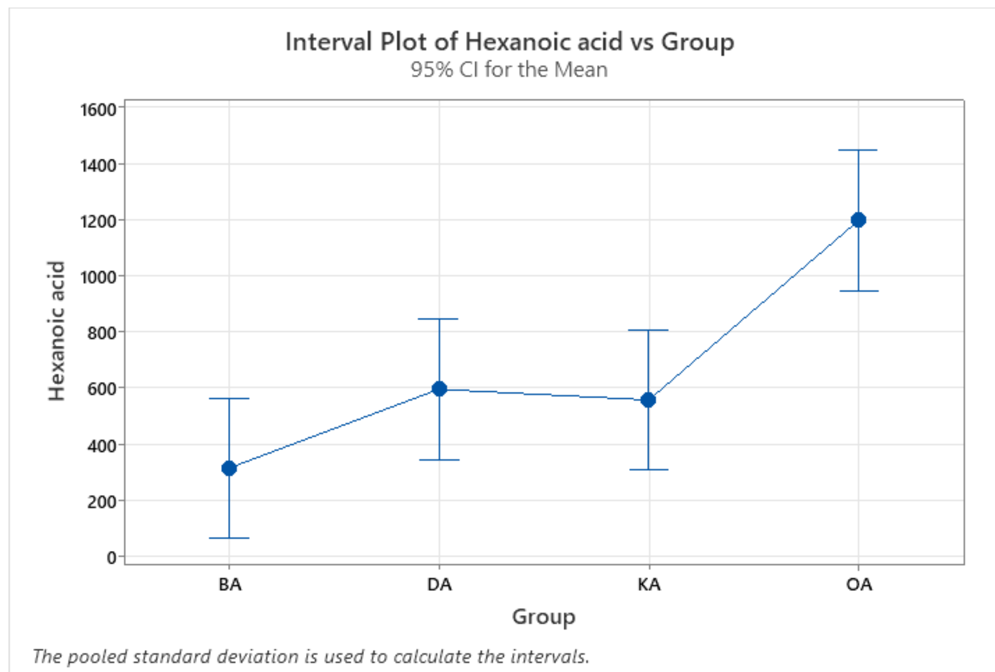
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
OA	3	1196	A
DA	3	594	B
KA	3	556.2	B
BA	3	313.5	B

Means that do not share a letter are significantly different.





One-way ANOVA: Octanoic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	1591247	530416	15.38	0.001
Error	8	275957	34495		
Total	11	1867205			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
185.727	85.22%	79.68%	66.75%

Means

Group	N	Mean	StDev	95% CI
BA	3	282.4	29.0	(35.1, 529.6)
DA	3	737	226	(490, 985)
KA	3	433.9	35.2	(186.6, 681.2)
OA	3	1236	291	(988, 1483)

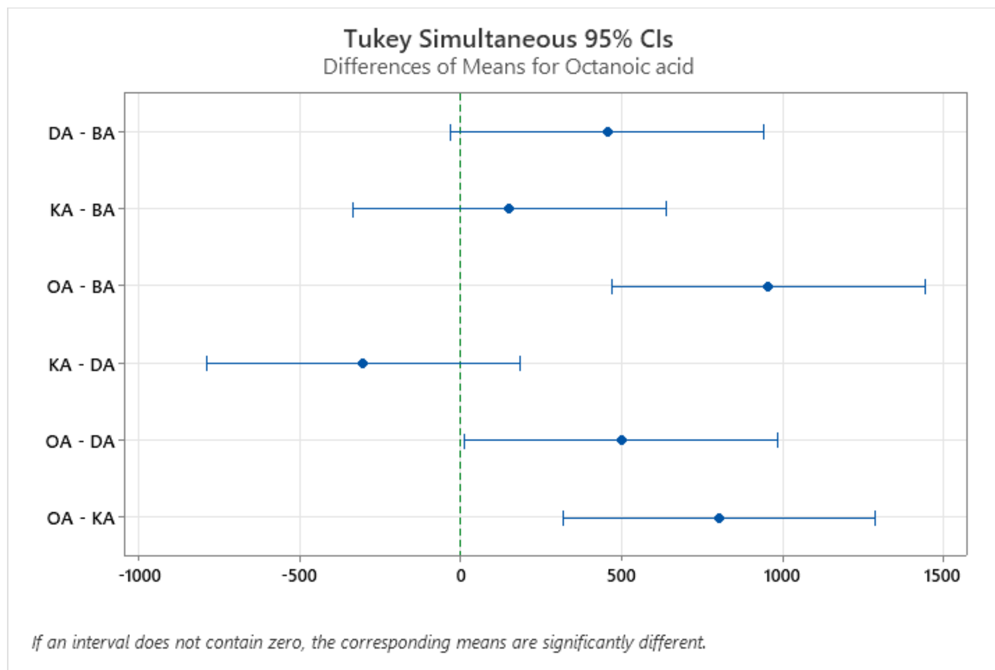
Pooled StDev = 185.727

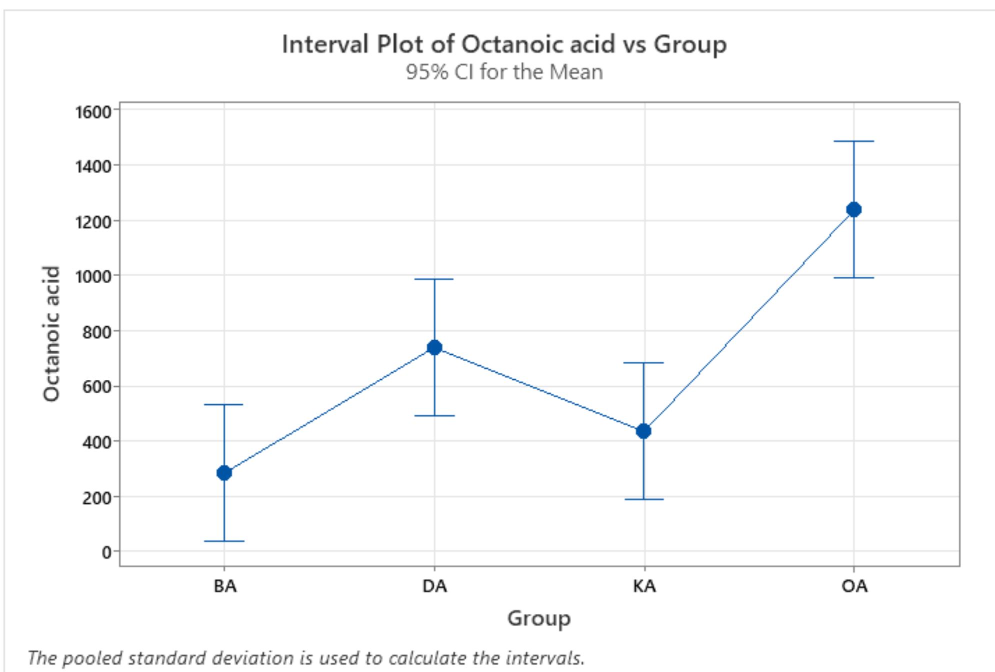
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
OA	3	1236	A
DA	3	737	B
KA	3	433.9	B
BA	3	282.4	B

Means that do not share a letter are significantly different.





One-way ANOVA: Propanoic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	19121	6373.5	8.47	0.007
Error	8	6023	752.9		
Total	11	25144			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
27.4386	76.05%	67.06%	46.10%

Means

Group	N	Mean	StDev	95% CI
BA	3	60.42	5.22	(23.89, 96.95)
DA	3	157.0	40.5	(120.5, 193.6)
KA	3	124.90	5.71	(88.37, 161.43)
OA	3	159.3	36.2	(122.8, 195.8)

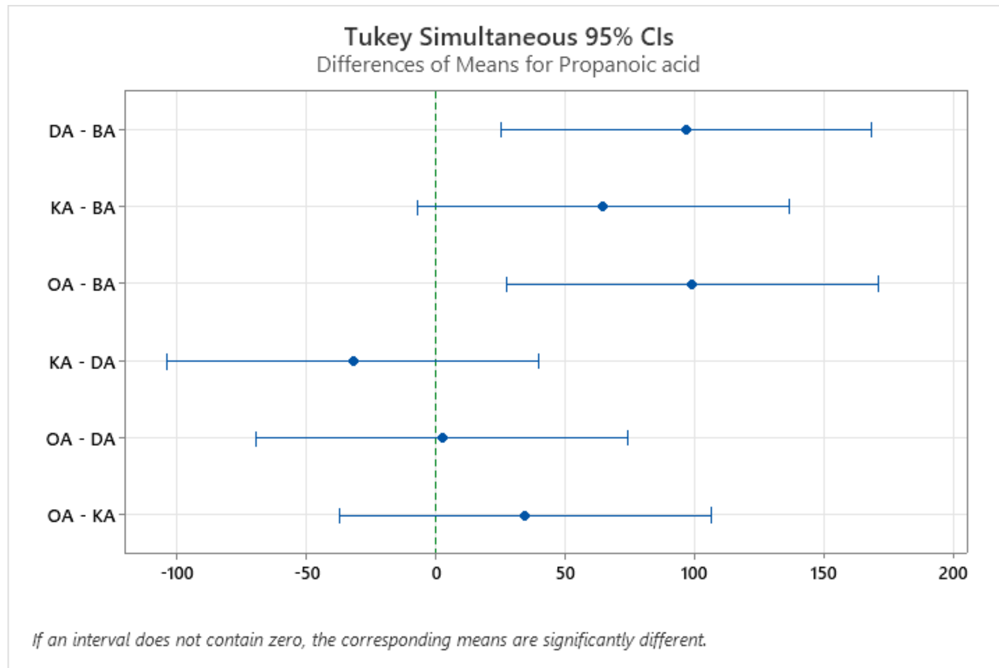
Pooled StDev = 27.4386

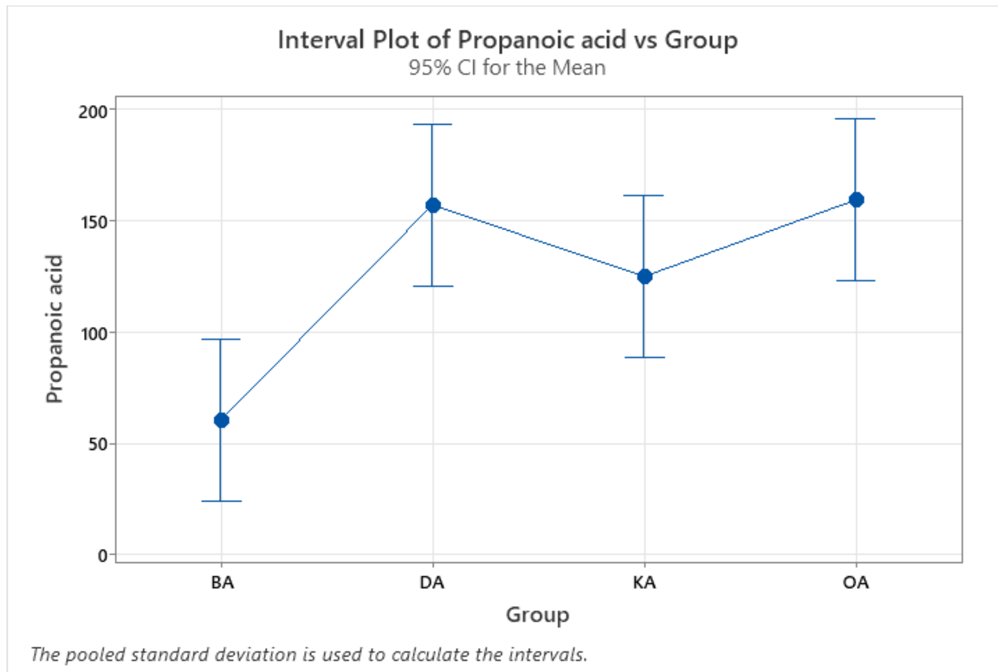
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	159.3	A	
DA	3	157.0	A	
KA	3	124.90	A	B
BA	3	60.42		B

Means that do not share a letter are significantly different.





One-way ANOVA: Nonanoic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	33584	11194.7	14.24	0.001
Error	8	6287	785.9		
Total	11	39871			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
28.0334	84.23%	78.32%	64.52%

Means

Group	N	Mean	StDev	95% CI
BA	3	106.30	6.09	(68.98, 143.62)
DA	3	63.2	17.6	(25.9, 100.6)
KA	3	22.812	0.733	(-14.511, 60.135)
OA	3	165.5	52.9	(128.2, 202.8)

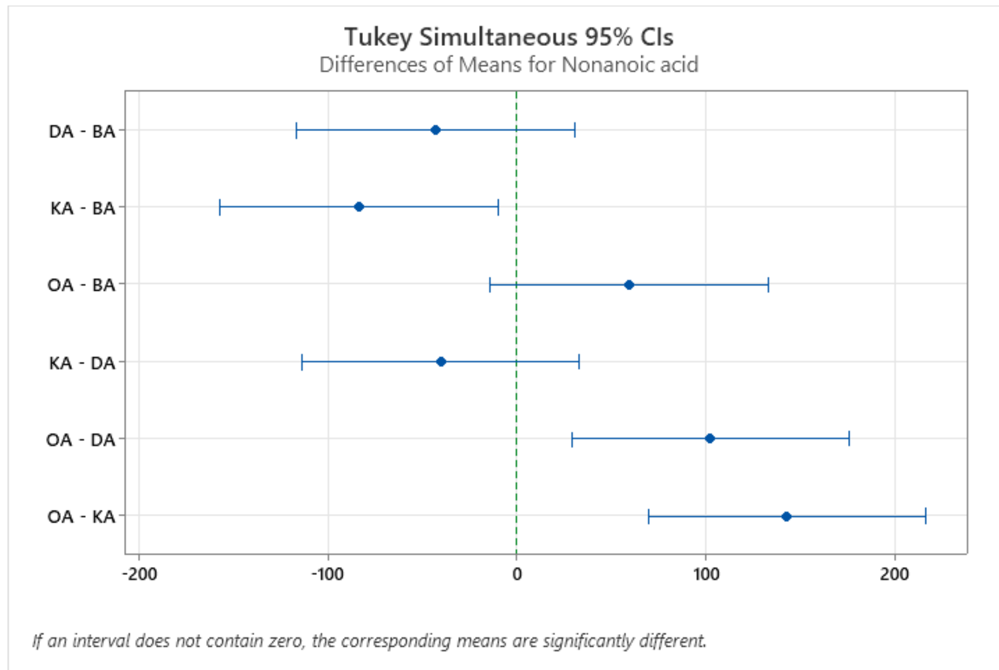
Pooled StDev = 28.0334

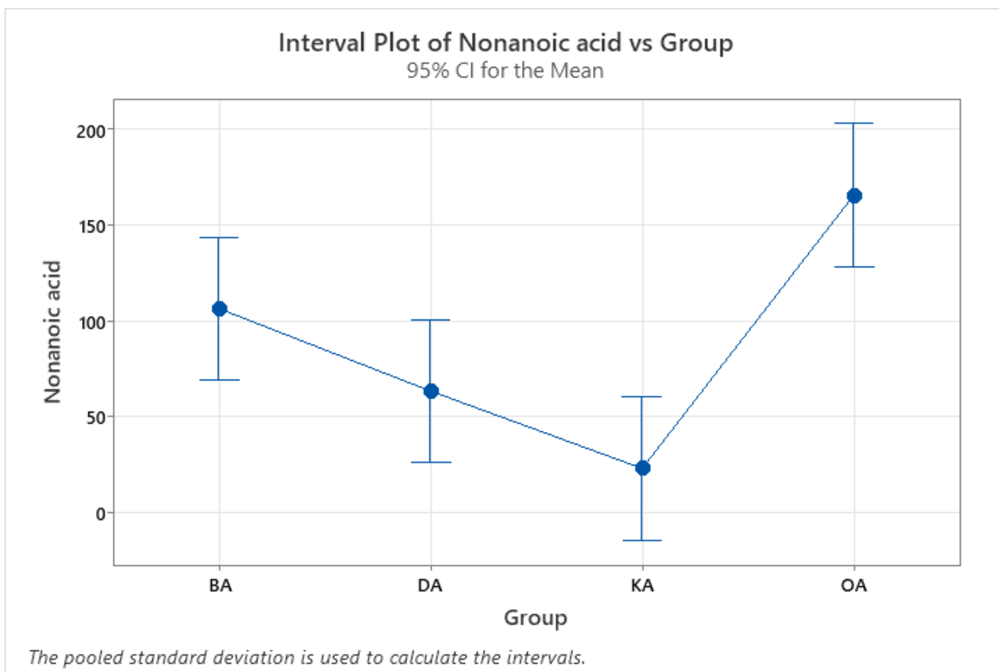
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
OA	3	165.5	A		
BA	3	106.30	A	B	
DA	3	63.2		B	C
KA	3	22.812			C

Means that do not share a letter are significantly different.





One-way ANOVA: Isobutyric acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	331532	110511	8.73	0.007
Error	8	101297	12662		
Total	11	432829			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
112.526	76.60%	67.82%	47.34%

Means

Group	N	Mean	StDev	95% CI
BA	3	354	206	(205, 504)
DA	3	688.80	8.81	(538.98, 838.61)
KA	3	389.6	29.9	(239.8, 539.4)
OA	3	717.0	84.9	(567.2, 866.8)

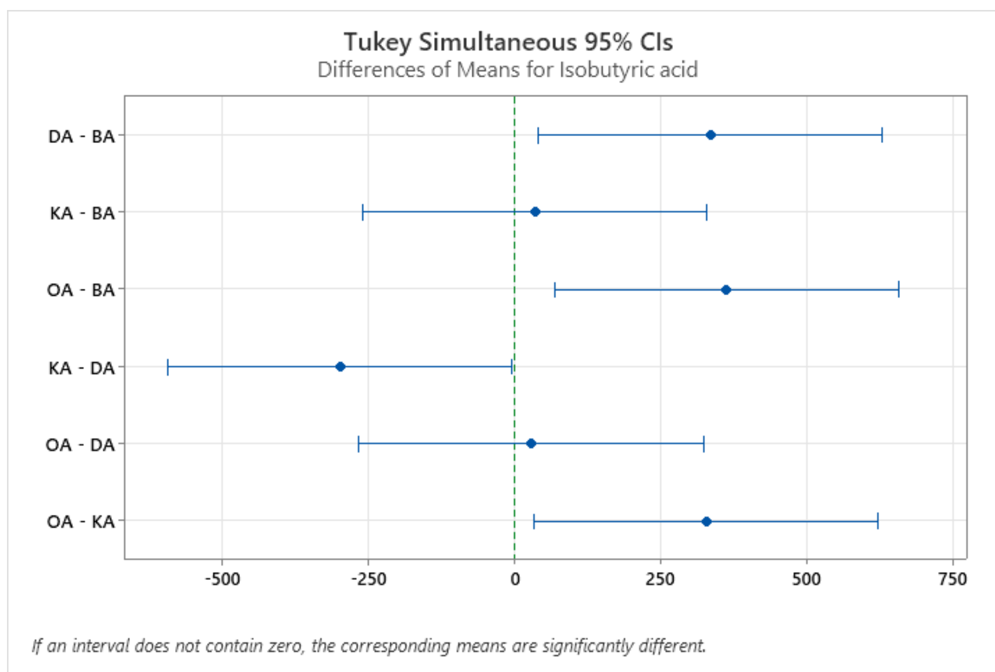
Pooled StDev = 112.526

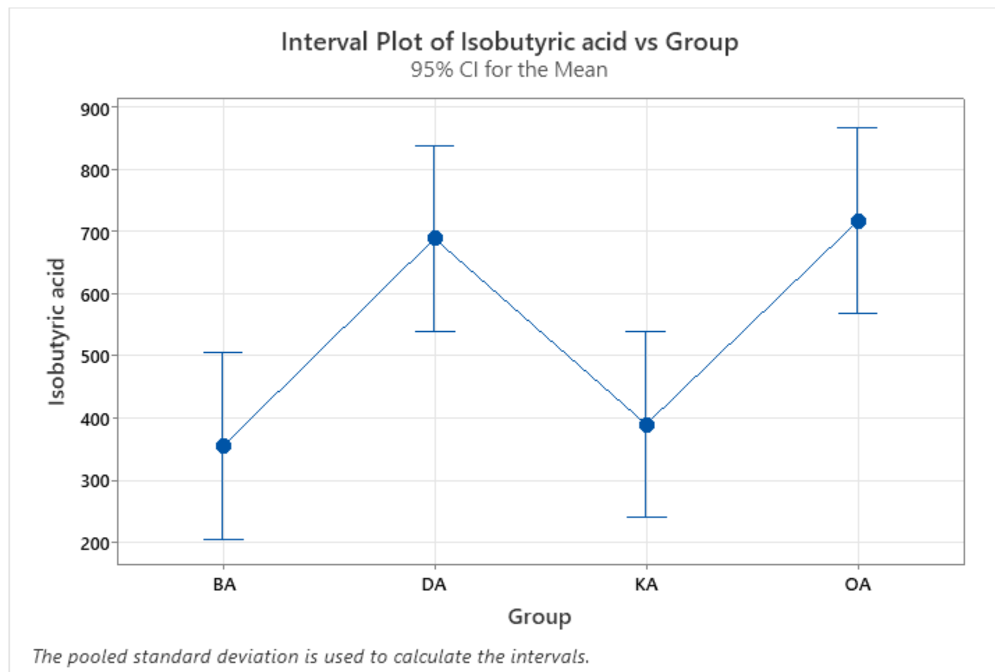
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	717.0	A	
DA	3	688.80	A	
KA	3	389.6		B
BA	3	354		B

Means that do not share a letter are significantly different.





One-way ANOVA: Acetic acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	15633112	5211037	130.02	0.000
Error	8	320642	40080		
Total	11	15953754			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
200.201	97.99%	97.24%	95.48%

Means

Group	N	Mean	StDev	95% CI
BA	3	212.67	8.21	(-53.87, 479.21)
DA	3	1533	333	(1267, 1800)
KA	3	3414.6	56.5	(3148.1, 3681.2)
OA	3	1928	215	(1661, 2194)

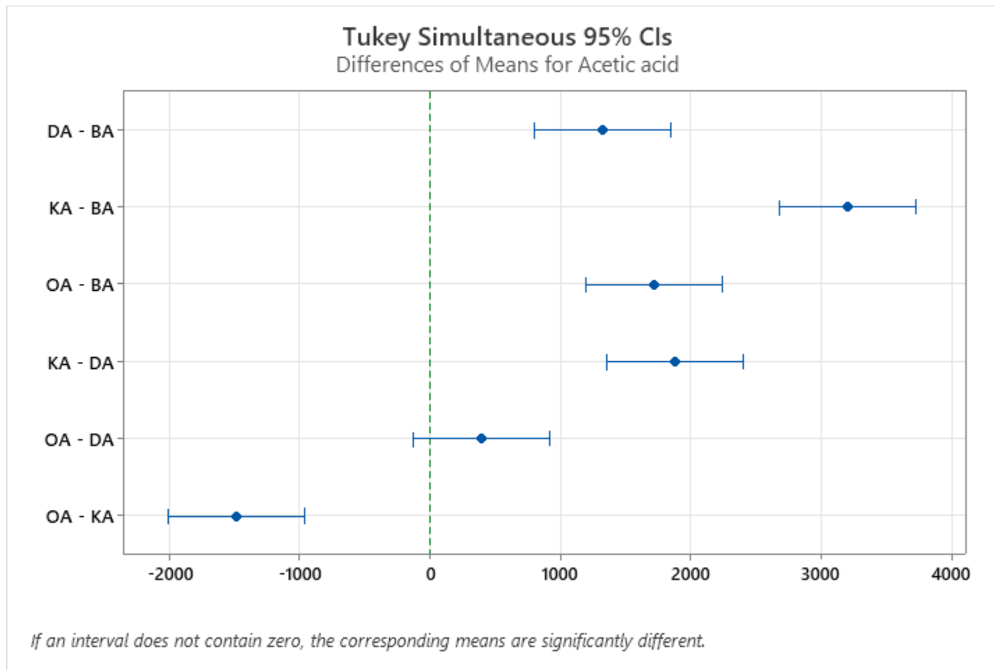
Pooled StDev = 200.201

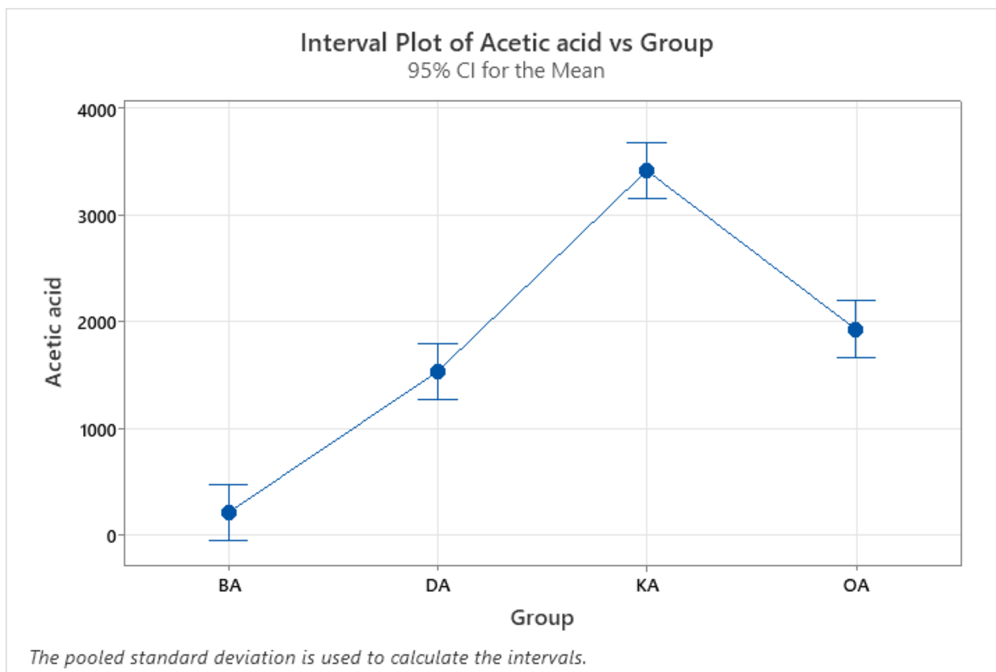
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
KA	3	3414.6	A		
OA	3	1928		B	
DA	3	1533		B	
BA	3	212.67			C

Means that do not share a letter are significantly different.





One-way ANOVA: Isovaleric acid versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	2	877118	438559	43.92	0.000
Error	6	59913	9986		
Total	8	937031			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
99.9279	93.61%	91.47%	85.61%

Means

Group	N	Mean	StDev	95% CI
BA	3	270.7	24.8	(129.5, 411.9)
DA	3	1011.9	99.0	(870.7, 1153.0)
OA	3	804.2	139.8	(663.1, 945.4)

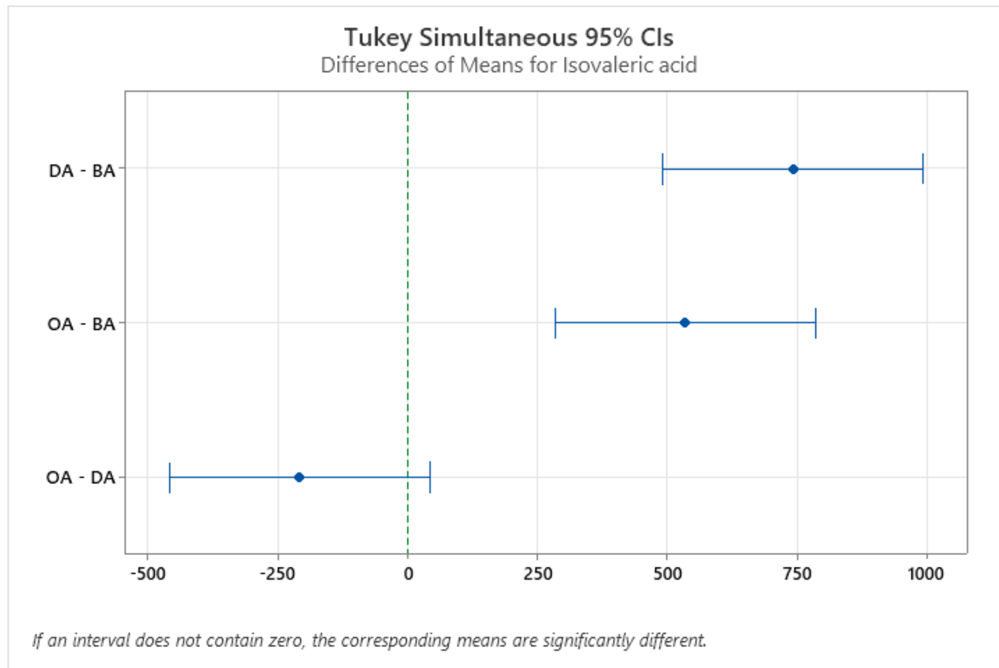
Pooled StDev = 99.9279

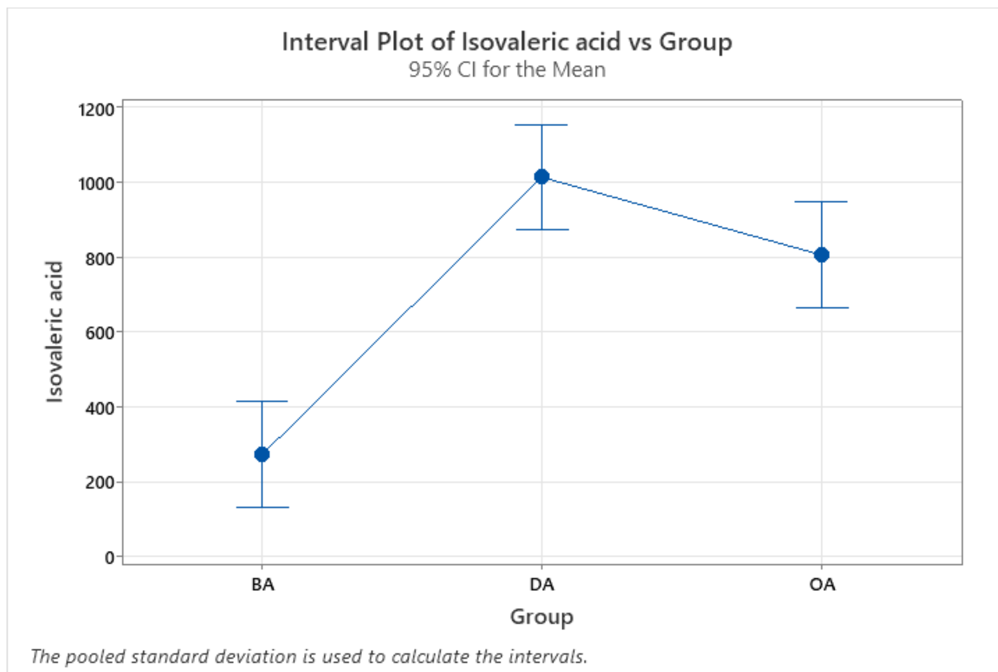
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	1011.9	A	
OA	3	804.2	A	
BA	3	270.7		B

Means that do not share a letter are significantly different.





One-way ANOVA: γ-Butyrolactone versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	779519	259840	1.33	0.330
Error	8	1557717	194715		
Total	11	2337237			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
441.265	33.35%	8.36%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	1356.0	109.8	(768.5, 1943.5)
DA	3	1537	738	(950, 2125)
KA	3	1176.0	30.3	(588.5, 1763.5)
OA	3	1866	470	(1278, 2453)

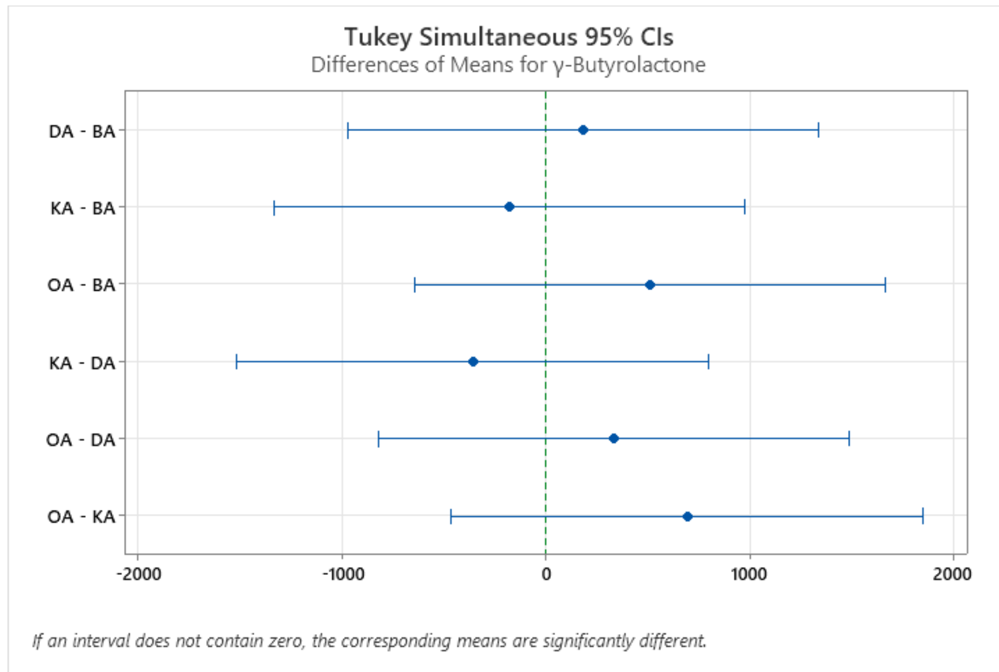
Pooled StDev = 441.265

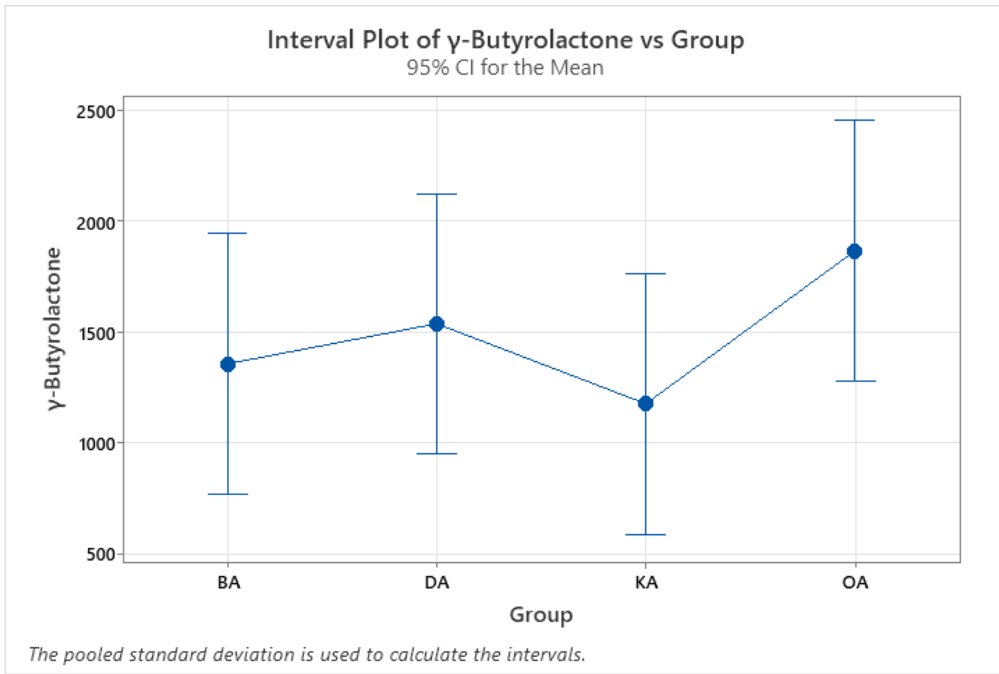
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
OA	3	1866	A
DA	3	1537	A
BA	3	1356.0	A
KA	3	1176.0	A

Means that do not share a letter are significantly different.





One-way ANOVA: Pantolactone versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	26921	8974	5.85	0.020
Error	8	12278	1535		
Total	11	39199			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
39.1754	68.68%	56.93%	29.53%

Means

Group	N	Mean	StDev	95% CI
BA	3	106.10	15.08	(53.95, 158.26)
DA	3	105.87	7.40	(53.71, 158.03)
KA	3	77.35	3.88	(25.19, 129.50)
OA	3	202.4	76.4	(150.3, 254.6)

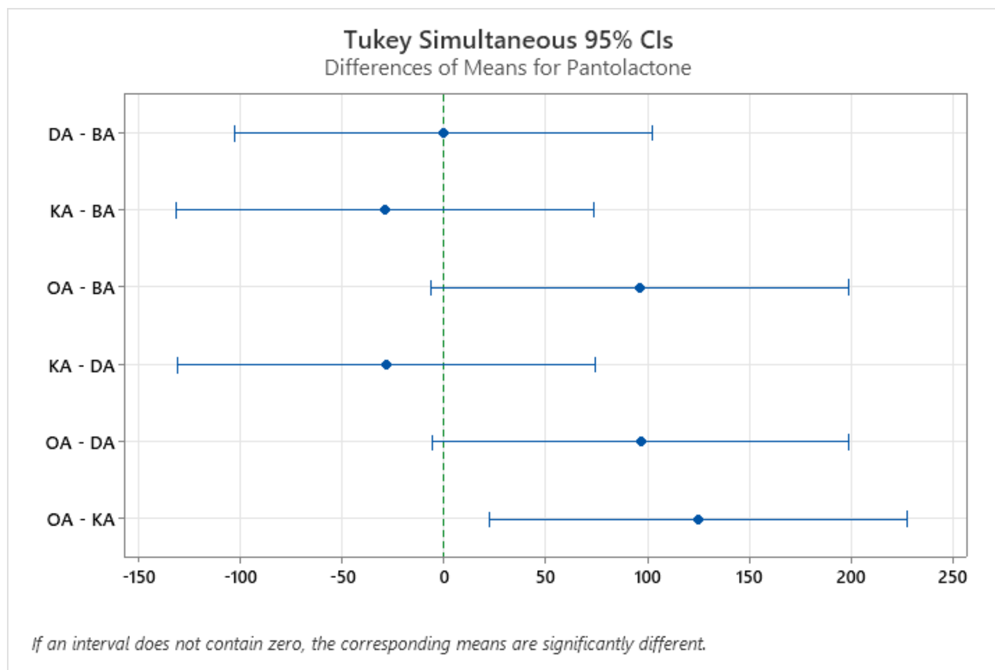
Pooled StDev = 39.1754

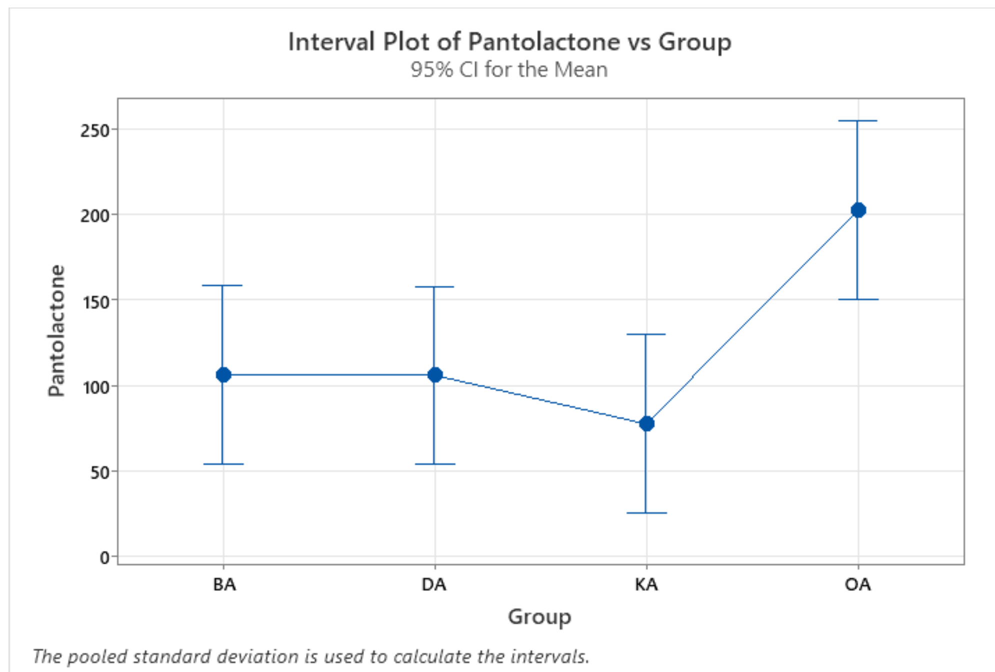
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
OA	3	202.4	A	
BA	3	106.10	A	B
DA	3	105.87	A	B
KA	3	77.35		B

Means that do not share a letter are significantly different.





One-way ANOVA: 2-Methoxy-4-vinylphenol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	13128	4376	1.87	0.213
Error	8	18714	2339		
Total	11	31842			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
48.3654	41.23%	19.19%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	101.68	8.50	(37.28, 166.07)
DA	3	191.5	94.9	(127.1, 255.9)
KA	3	153.11	7.84	(88.72, 217.50)
OA	3	128.42	14.54	(64.03, 192.81)

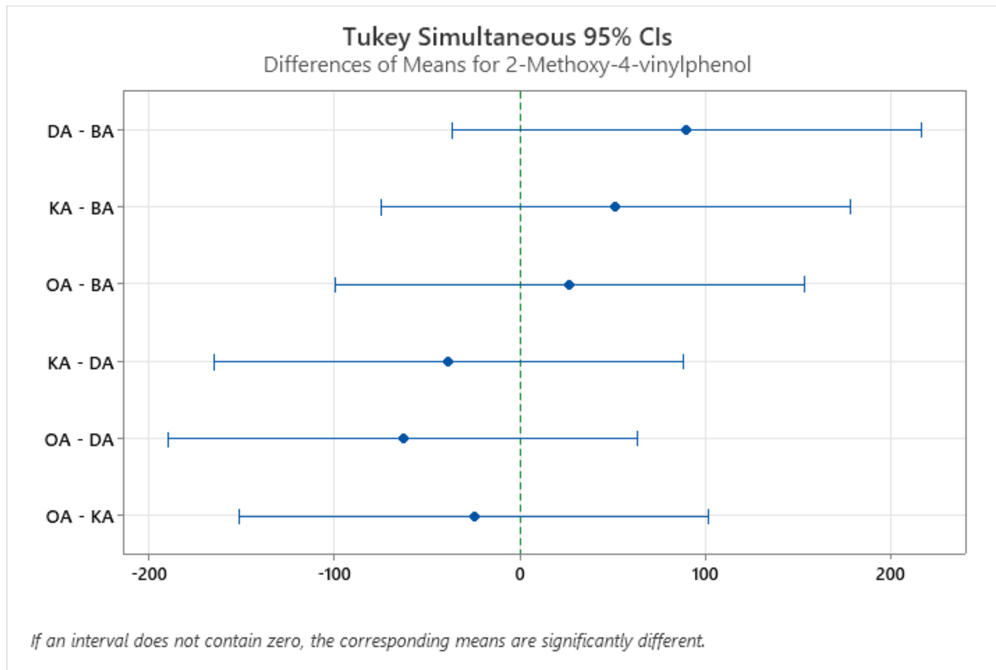
Pooled StDev = 48.3654

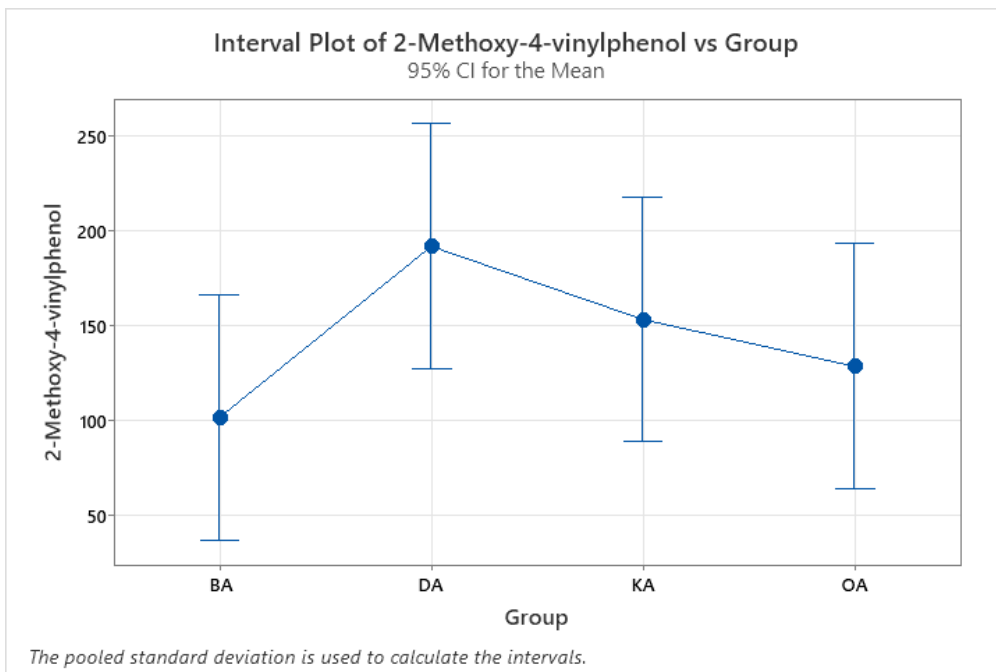
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
DA	3	191.5	A
KA	3	153.11	A
OA	3	128.42	A
BA	3	101.68	A

Means that do not share a letter are significantly different.





One-way ANOVA: Soleron versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	41857	13952	4.57	0.038
Error	8	24400	3050		
Total	11	66256			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
55.2264	63.17%	49.36%	17.14%

Means

Group	N	Mean	StDev	95% CI
BA	3	135.36	12.69	(61.83, 208.88)
DA	3	249.1	105.5	(175.5, 322.6)
KA	3	97.06	8.48	(23.53, 170.59)
OA	3	204.5	29.0	(130.9, 278.0)

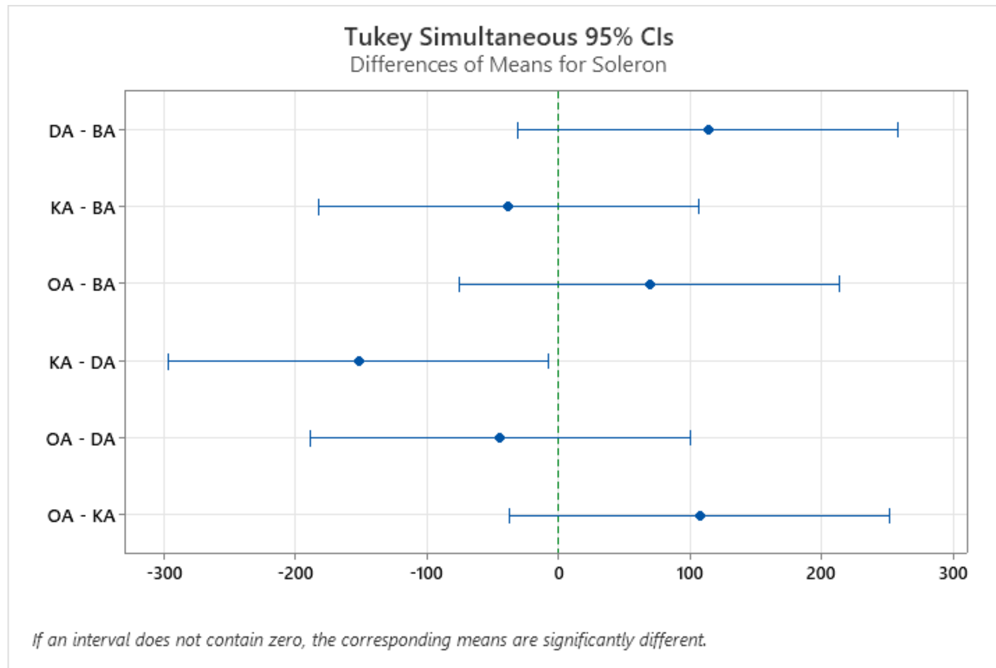
Pooled StDev = 55.2264

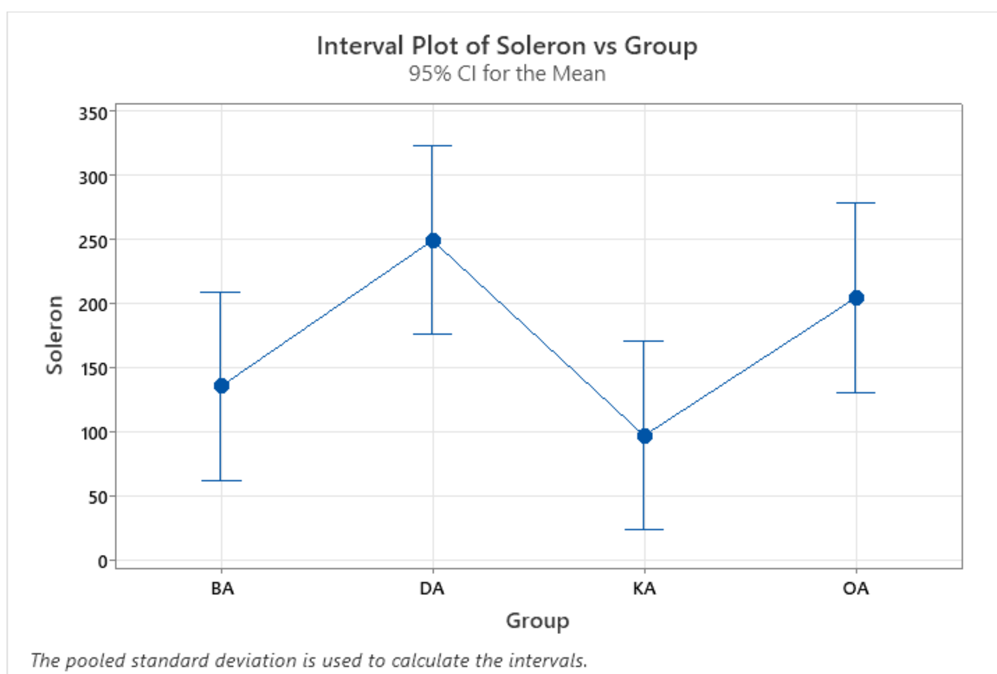
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping	
DA	3	249.1	A	
OA	3	204.5	A	B
BA	3	135.36	A	B
KA	3	97.06		B

Means that do not share a letter are significantly different.





One-way ANOVA: Guaiacol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	10097	3366	2.02	0.190
Error	8	13351	1669		
Total	11	23448			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
40.8521	43.06%	21.71%	0.00%

Means

Group	N	Mean	StDev	95% CI
BA	3	164.87	10.69	(110.48, 219.26)
DA	3	129.7	57.7	(75.4, 184.1)
KA	3	94.70	4.78	(40.31, 149.08)
OA	3	164.5	56.7	(110.1, 218.9)

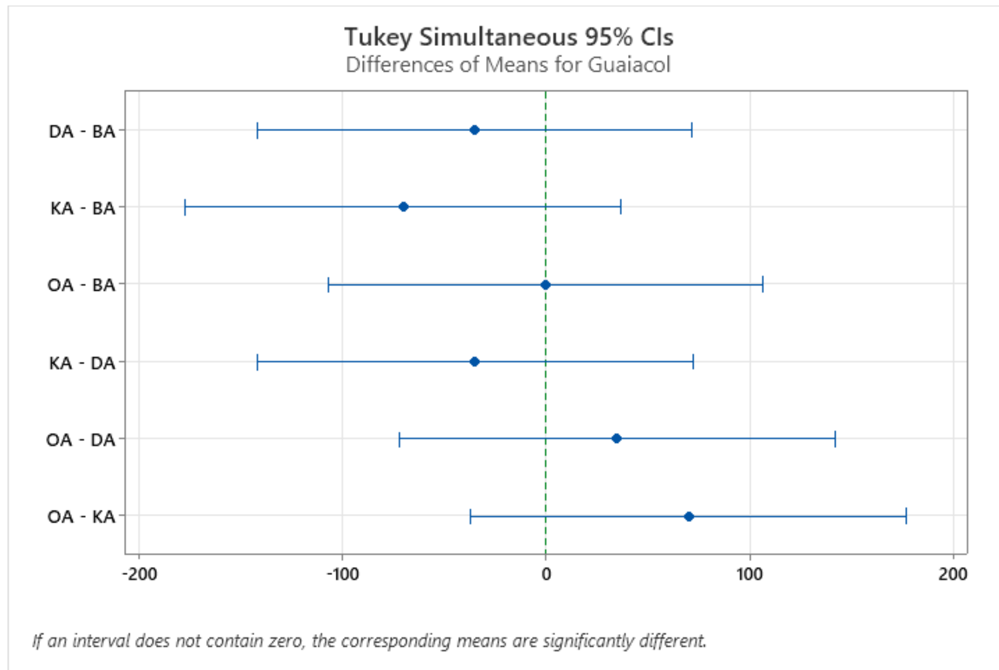
Pooled StDev = 40.8521

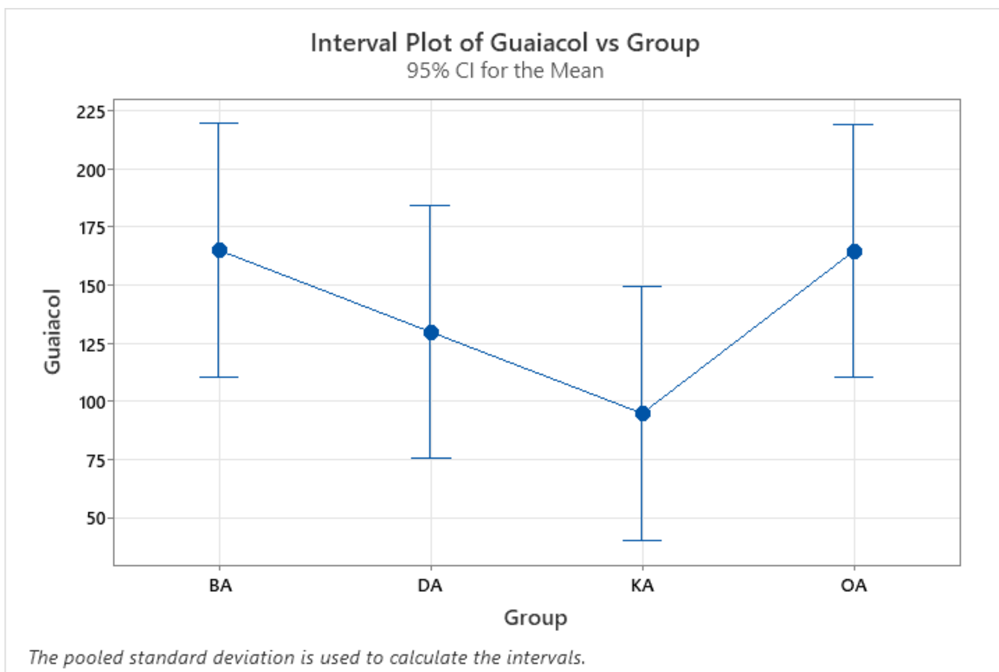
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping
BA	3	164.87	A
OA	3	164.5	A
DA	3	129.7	A
KA	3	94.70	A

Means that do not share a letter are significantly different.





One-way ANOVA: Syringol versus Group

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Group	3	51636	17212	12.64	0.002
Error	8	10891	1361		
Total	11	62526			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
36.8962	82.58%	76.05%	60.81%

Means

Group	N	Mean	StDev	95% CI
BA	3	143.90	10.93	(94.78, 193.02)
DA	3	251.1	26.3	(202.0, 300.2)
KA	3	190.719	0.512	(141.597, 239.842)
OA	3	318.7	68.1	(269.6, 367.8)

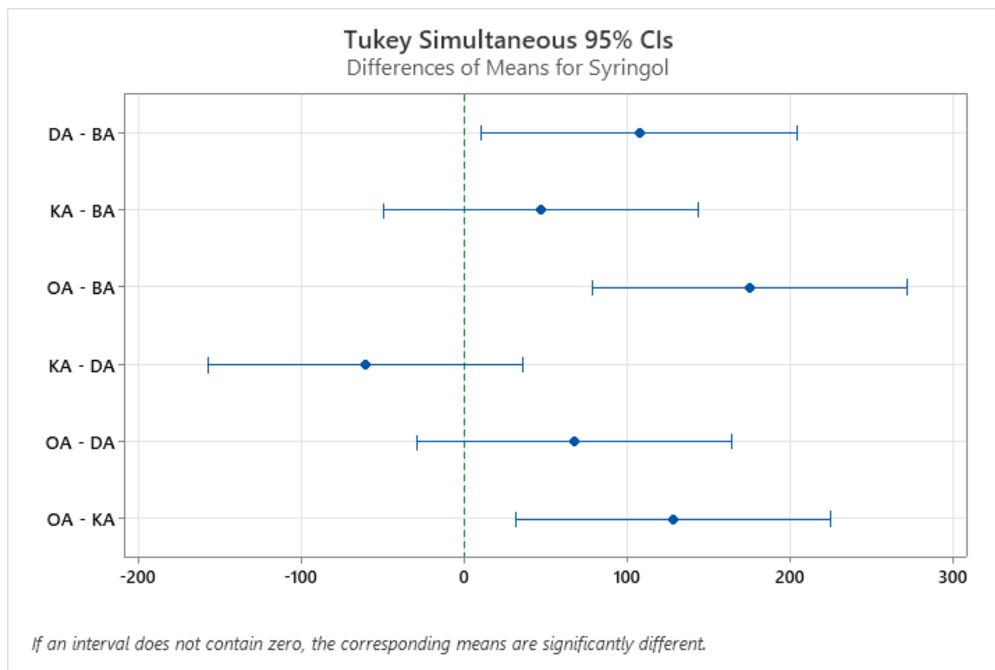
Pooled StDev = 36.8962

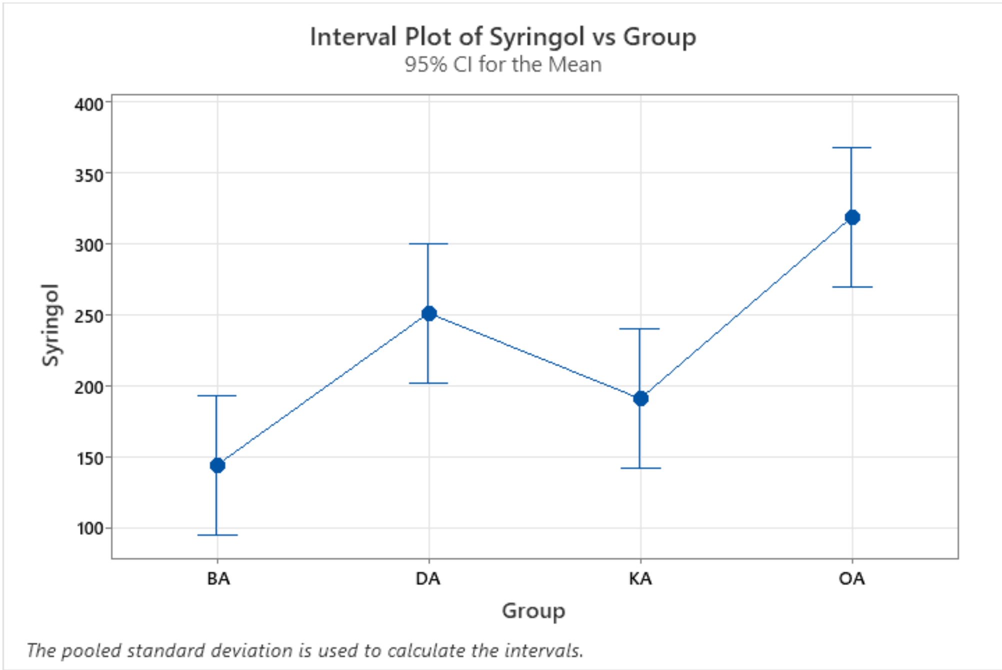
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Group	N	Mean	Grouping		
OA	3	318.7	A		
DA	3	251.1	A	B	
KA	3	190.719		B	C
BA	3	143.90			C

Means that do not share a letter are significantly different.





Interval Plot of Syringol vs Group
95% CI for the Mean
Syringol
400
350
300
250
200
150
100
BA
DA
KA
OA
Group
The pooled standard deviation is used to calculate the intervals.

B. Amplification Plots of Kalecik Karası Grape Must or Wine Samples Obtained by Using Specific Primers

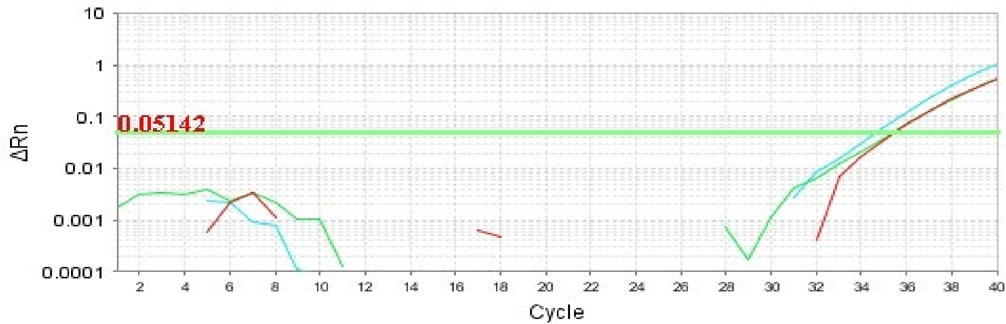


Figure B-1 Amplification plot of must/wine samples of Kalecik Karası with *Candida glabrata* primer. Red colored, 2 (M); green colored,4 (M); light blue colored, 14 (F) samples.

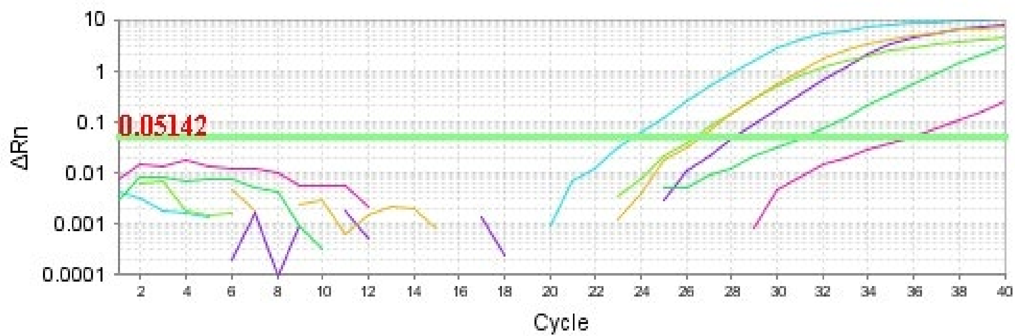


Figure B-2 Amplification plot of must/wine samples of Kalecik Karası with *Candida zemplinina* primer. Pink colored, 4 (M); dark green colored, 4(CM); purple colored, 14 (F); light green colored, 0 (CM); yellow colored, 2 (CM); light blue colored, 2(M) samples.

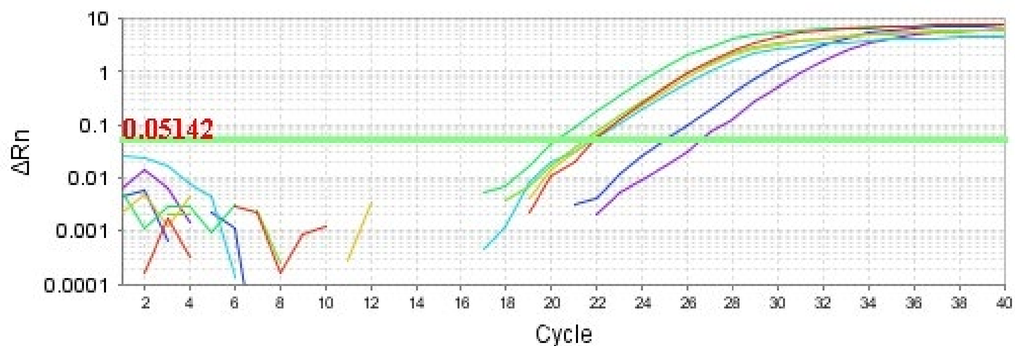


Figure B-3 Amplification plot of must/wine samples of Kalecik Karası with *Hanseniaspora* ssp. primer. Purple colored, 28 (F); dark blue colored,14 (F); light green colored, 0 (CM); light blue colored, 4 (M); yellow colored, 2 (CM); red colored, 4 (CM); dark green colored, 2 (M) samples.

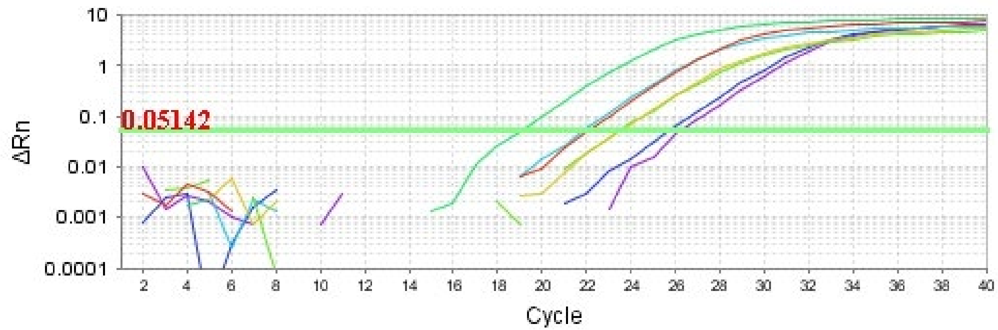


Figure B-4 Amplification plot of must/wine samples of Kalecik Karası with *Hanseniaspora uvarum* primer. Purple colored, 28 (F); dark blue colored,14 (F); light green colored, 2 (CM); yellow colored 4 (CM); light blue colored, 0 (CM); red colored, 4 (M); dark green colored, 2 (M) samples.

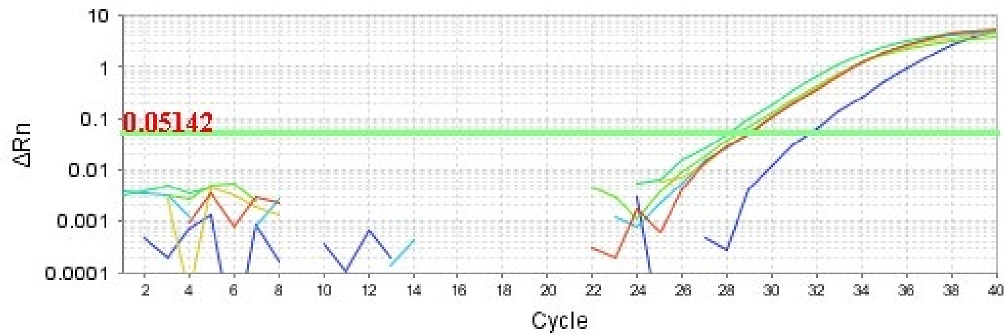


Figure B-5 Amplification plot of must/wine samples of Kalecik Karası with *Issatchenkia orientalis* primer. Dark blue colored, 14 (F); light green colored, 4 (M); light blue colored, 0 (CM); yellow colored, 2 (CM); red colored, 4 (CM); dark green colored, 2 (M) samples.

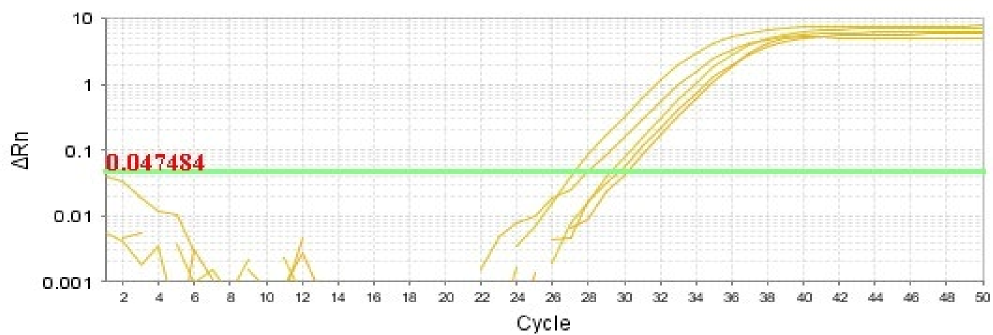


Figure B-6 Amplification plot of must/wine samples of Kalecik Karası with *Lachancea thermotolerans* primer. From minimum to maximum C_T: 0 (CM), 2 (CM), 4 (M), 4 (CM), 2 (M), 4 (M) samples.

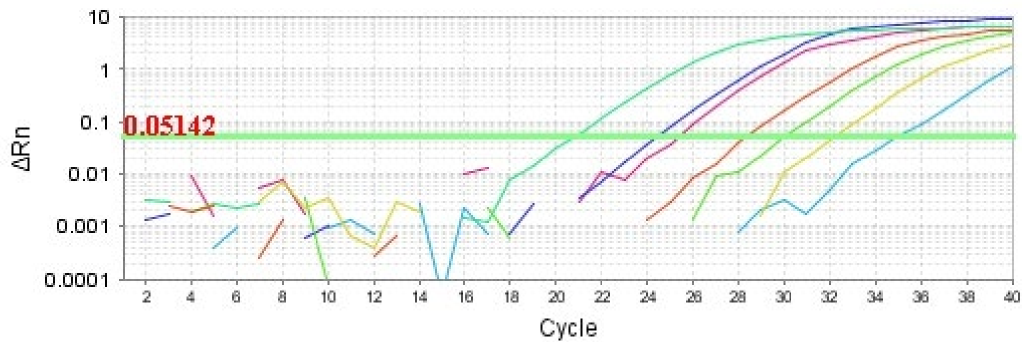


Figure B-7 Amplification plot of must/wine samples of Kalecik Karası with *Pichia anomala* primer. Light blue colored, 4 (M); yellow colored, 2 (CM); light green colored, 4 (CM); red colored 0 (CM); pink colored, 28 (F); dark blue colored, 14 (F); dark green colored, 2 (M) samples.

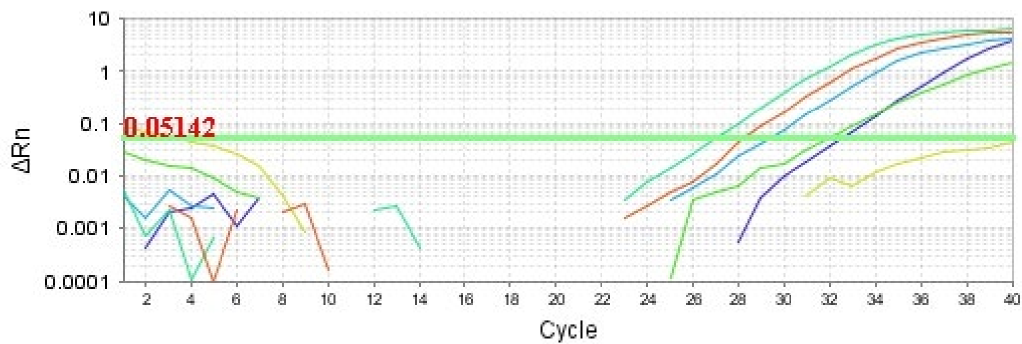


Figure B-8 Amplification plot of must/wine samples of Kalecik Karası with *Pichia kluyveri* primer. Dark blue colored,4 (M); dark green colored, 2 (M); light blue colored, 0 (CM); red colored, 2 (CM); light green colored, 4 (CM) samples.

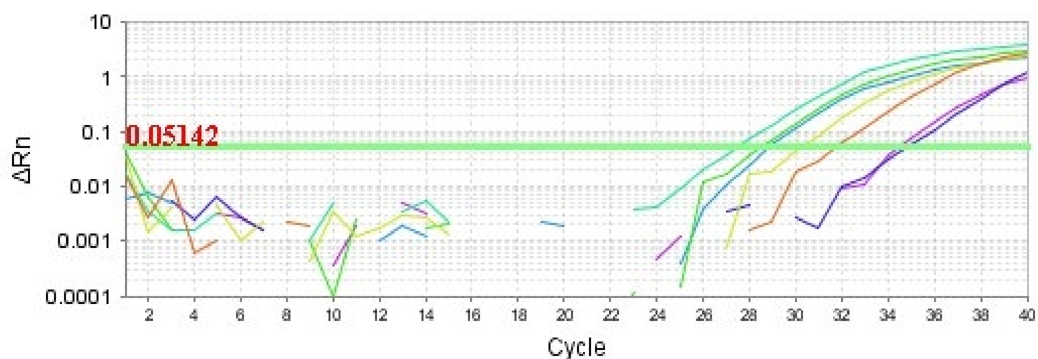


Figure B-9 Amplification plot of must/wine samples of Kalecik Karası with *Saccharomyces* spp. primer. Dark blue colored, 2 (CM); purple colored, 4 (CM); red colored, 0 (CM); yellow colored, 28 (F); dark green colored, 14 (F); light green colored, 4 (M); light blue colored, 2 (M) samples.

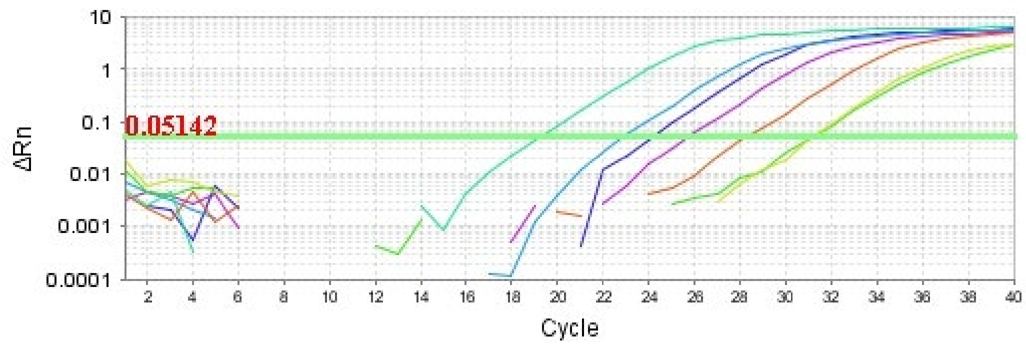


Figure B-10 Amplification plot of must/wine samples of Kalecik Karası with *Saccharomyces cerevisiae* primer. light green colored, 2 (CM); yellow colored, 4 (CM); red colored, 0 (CM); purple colored, 28 (F); Dark blue colored, 14 (F); light blue colored, 4 (M); dark green colored, 2 (M) samples.

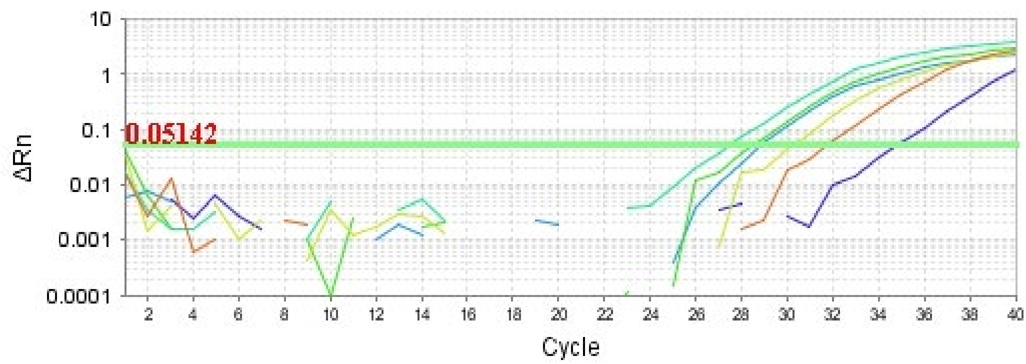


Figure B-11 Amplification plot of must/wine samples of Kalecik Karası with *Torulaspora delbrueckii* primer. Dark blue colored, 14 (F); red colored, 0 (CM); yellow colored, 2 (CM); light blue colored, 4 (M); dark green colored, 4 (CM); light green colored, 2 (M) samples.

C. Amplification Plots of Boğazkere Grape Must or Wine Samples Obtained by Using Specific Primers

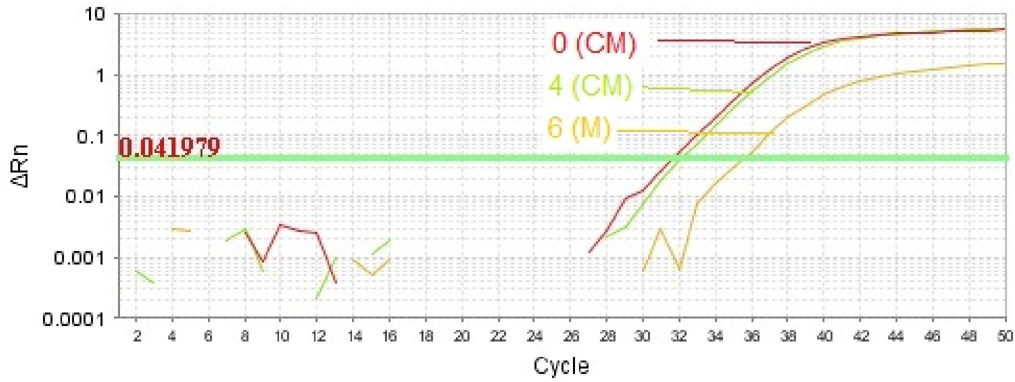


Figure C-1 Amplification plot of must/wine samples of Boğazkere with *Candida glabrata* primer. 0 (CM), 0th day of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

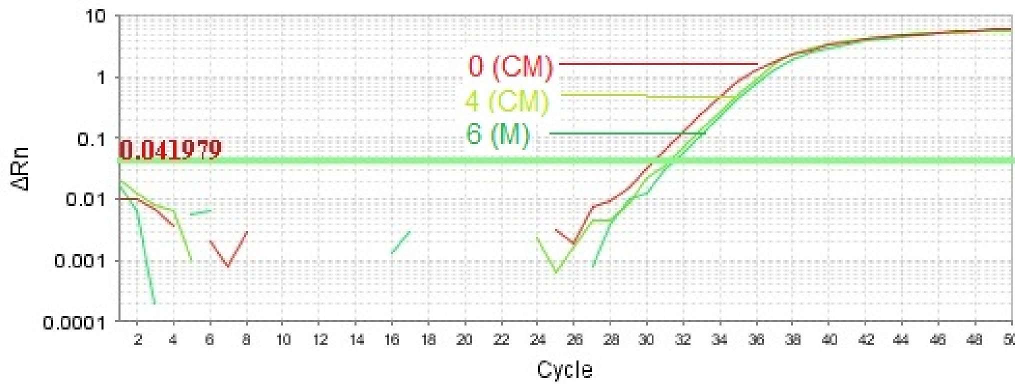


Figure C-2 Amplification plot of must/wine samples of Boğazkere with *Hanseniaspora* spp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

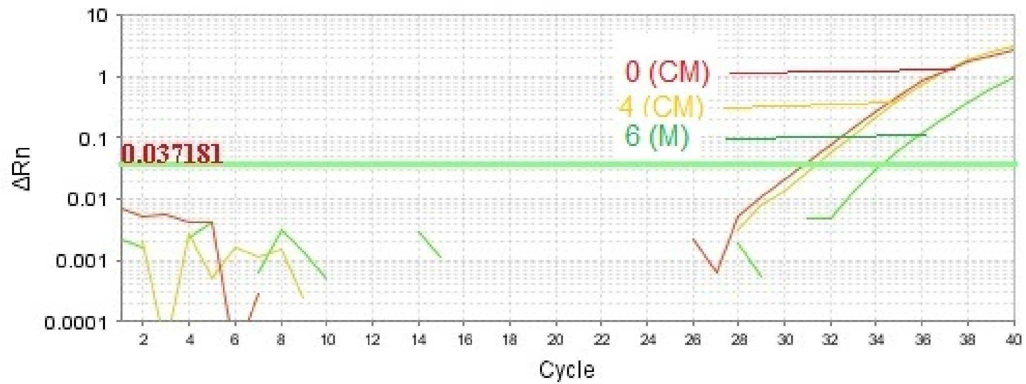


Figure C-3 Amplification plot of must/wine samples of Boğazkere with *Hanseniaspora uvarum* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

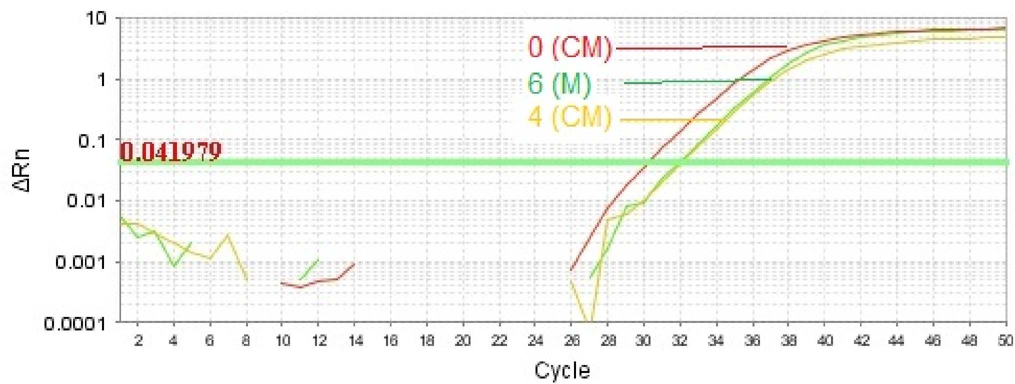


Figure C-4 Amplification plot of must/wine samples of Boğazkere with *Issatchenkia orientalis* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

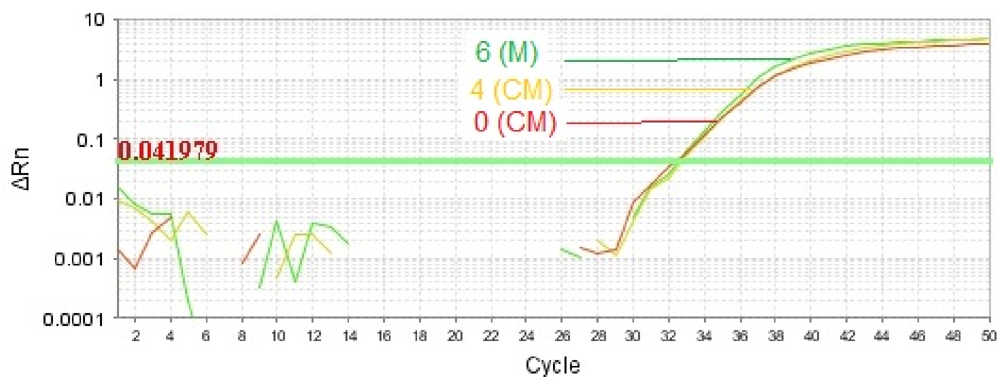


Figure C-5 Amplification plot of must/wine samples of Boğazkere with *Pichia fermentans* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

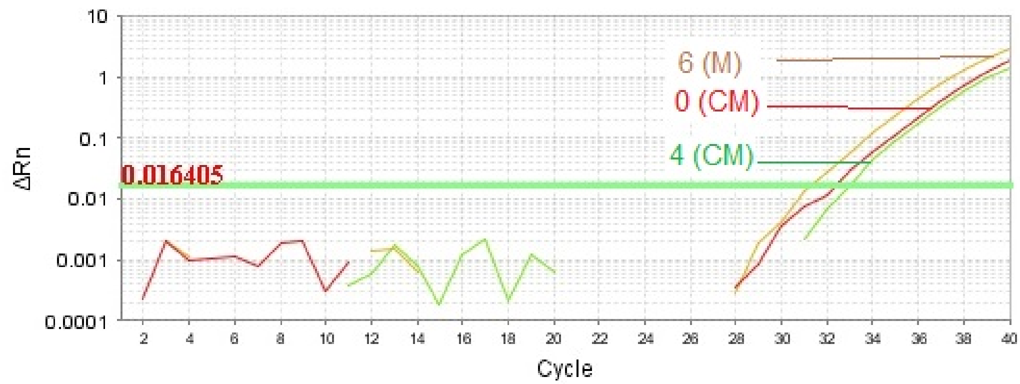


Figure C-6 Amplification plot of must/wine samples of Boğazkere with *Saccharomyces* ssp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

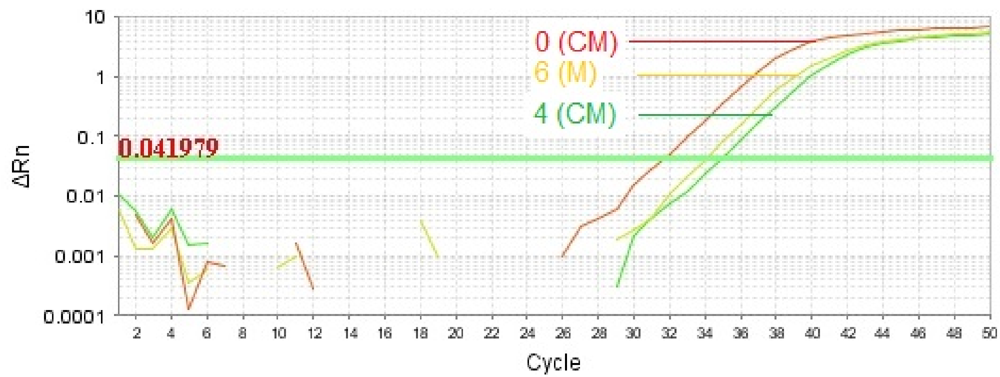


Figure C-7 Amplification plot of must/wine samples of Boğazkere grape with *Saccharomyces cerevisiae* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

D. Amplification Plots of Öküzgözü Grape Must or Wine Samples Obtained by Using Specific Primers

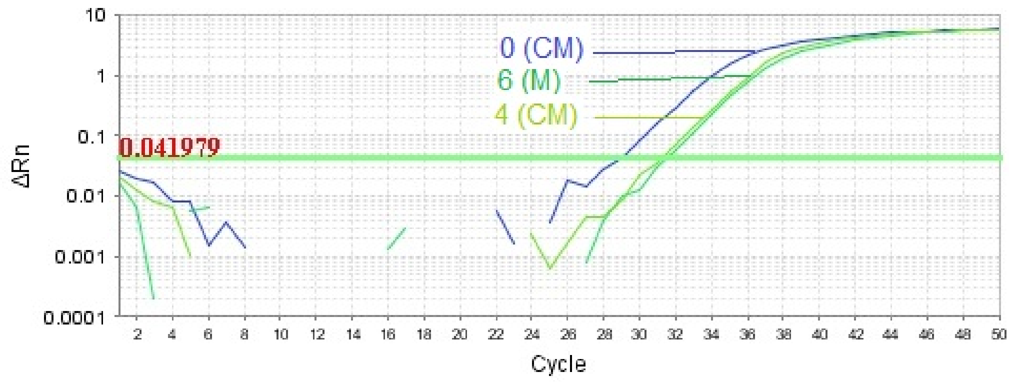


Figure D-1 Amplification plot of must/wine samples of Öküzgözü with *Hanseniaspora* spp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

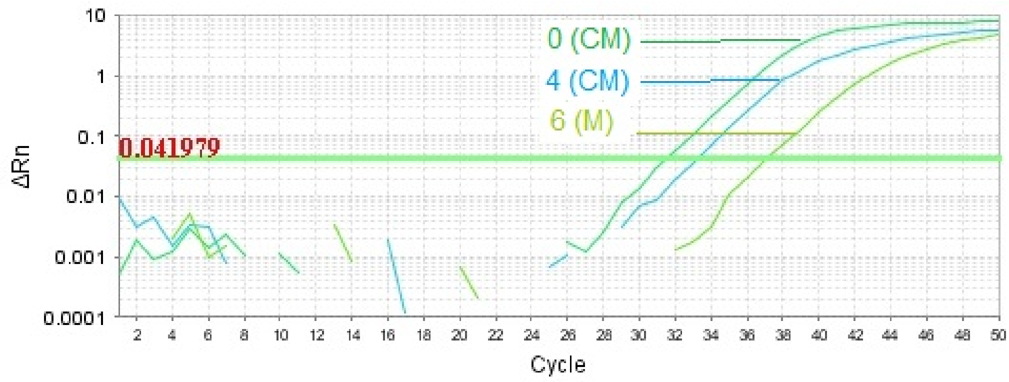


Figure D-2 Amplification plot of must/wine samples of Öküzgözü with *Hanseniaspora uvarum* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

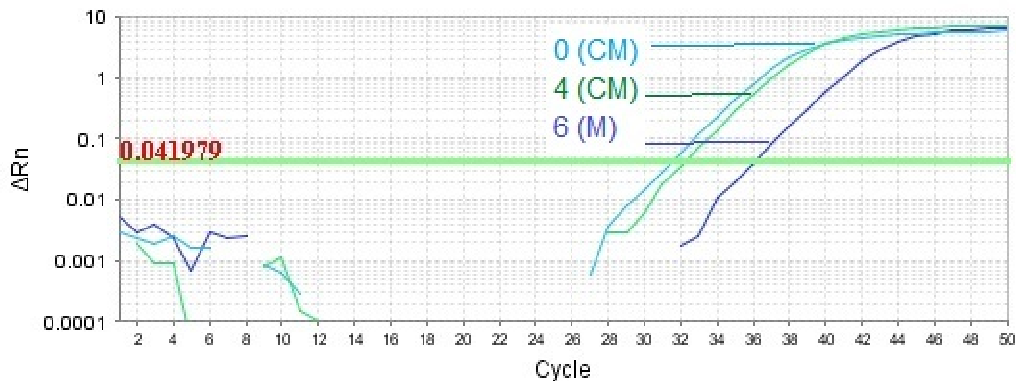


Figure D-3 Amplification plot of must/wine samples of Öküzgözü with *Issatchenkia orientalis* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

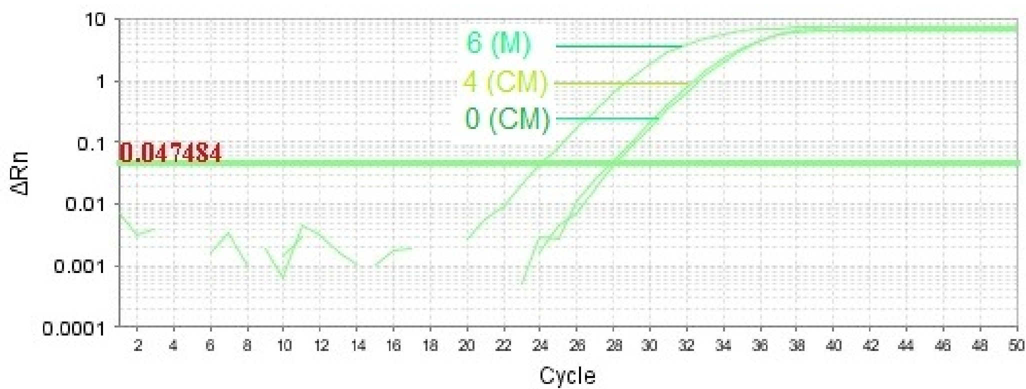


Figure D-4 Amplification plot of must/wine samples of Öküzgözü with *Lachancea thermotolerans* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

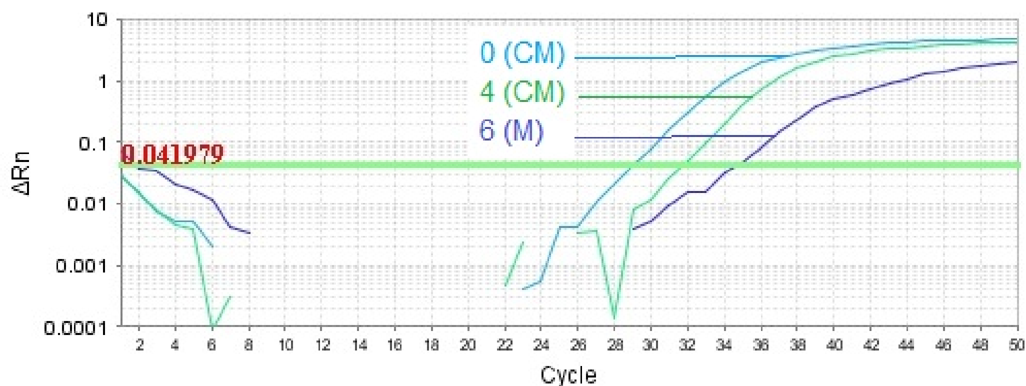


Figure D-5 Amplification plot of must/wine samples of Öküzgözü with *Pichia fermentans* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

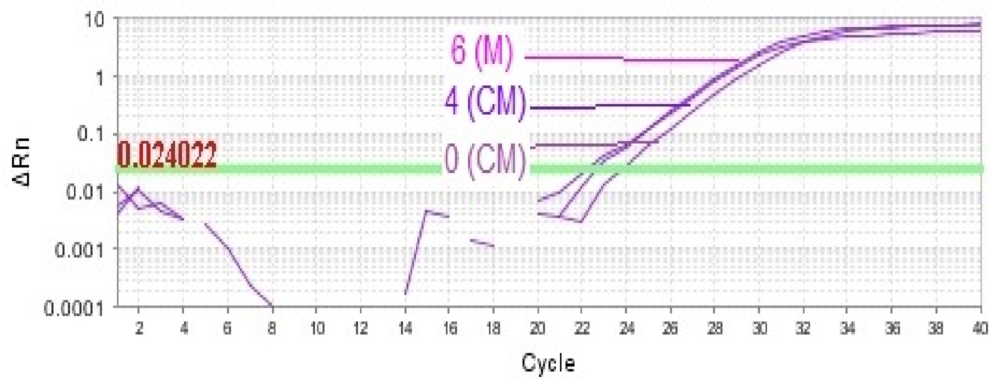


Figure D-6 Amplification plot of must/wine samples of Öküzgözü with *Saccharomyces* spp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

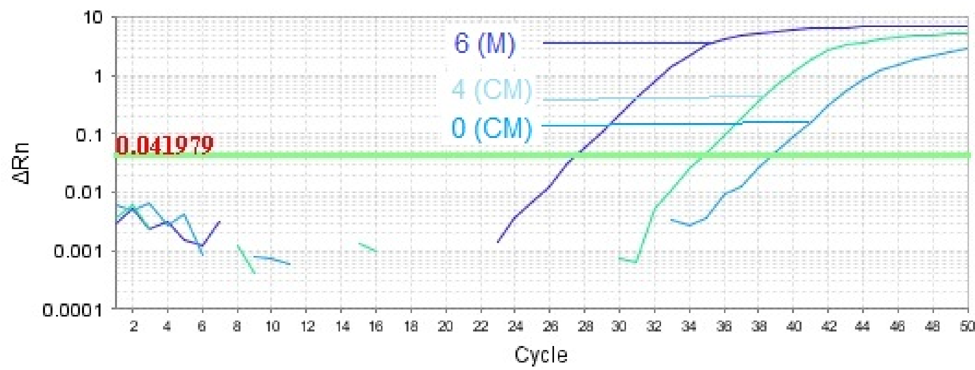


Figure D-7 Amplification plot of must/wine samples of Öküzgözü with *Saccharomyces cerevisiae* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 6 (M), sixth day of maceration.

E. Amplification Plots of Dimrit Grape Must or Wine Samples Obtained by Using Specific Primers

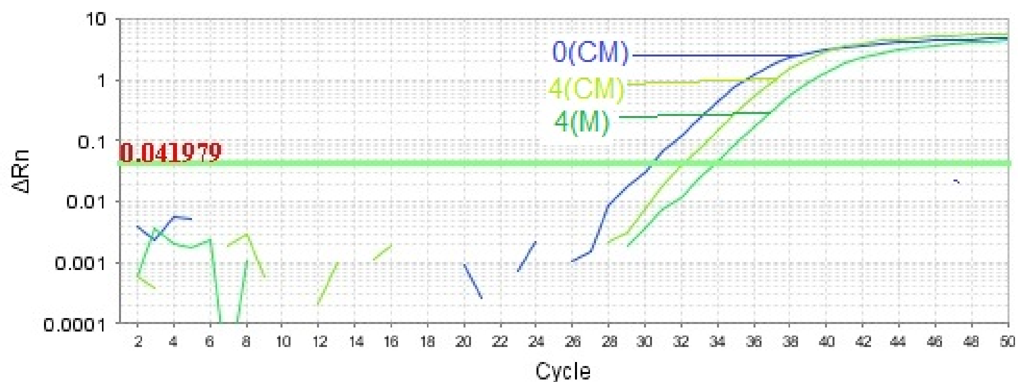


Figure E-1 Amplification plot of must/wine samples of Dimrit with *Candida glabrata* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

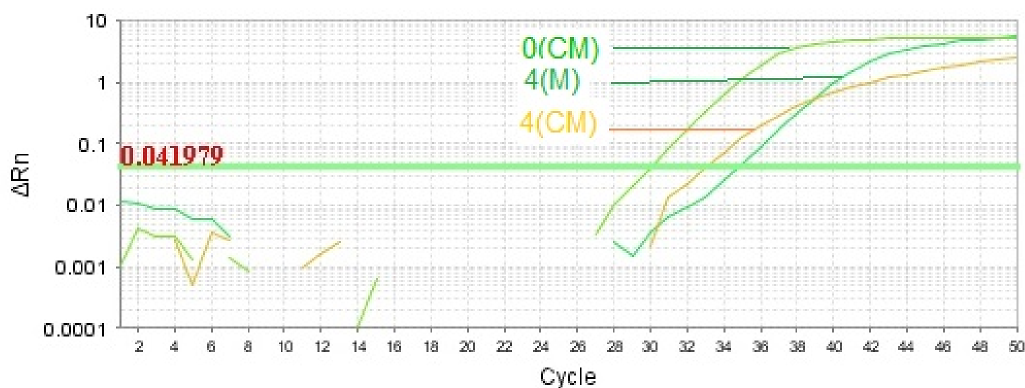


Figure E-2 Amplification plot of must/wine samples of Dimrit with *Candida zemplinina* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

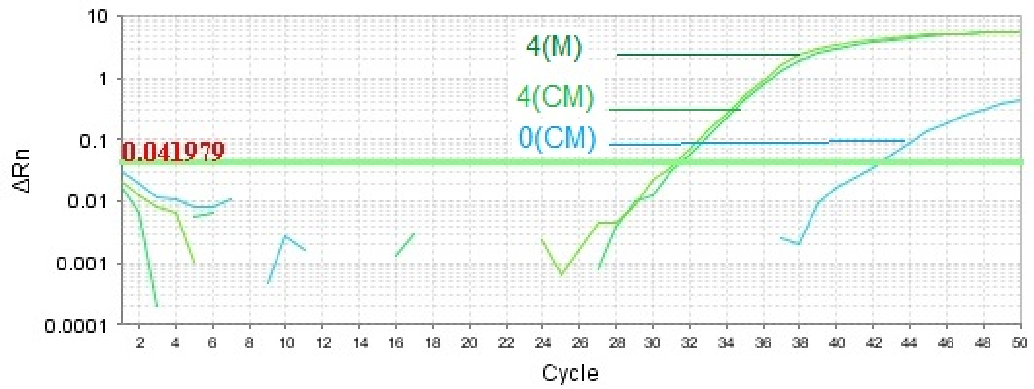


Figure E-3 Amplification plot of must/wine samples of Dimrit with *Hanseniaspora* spp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

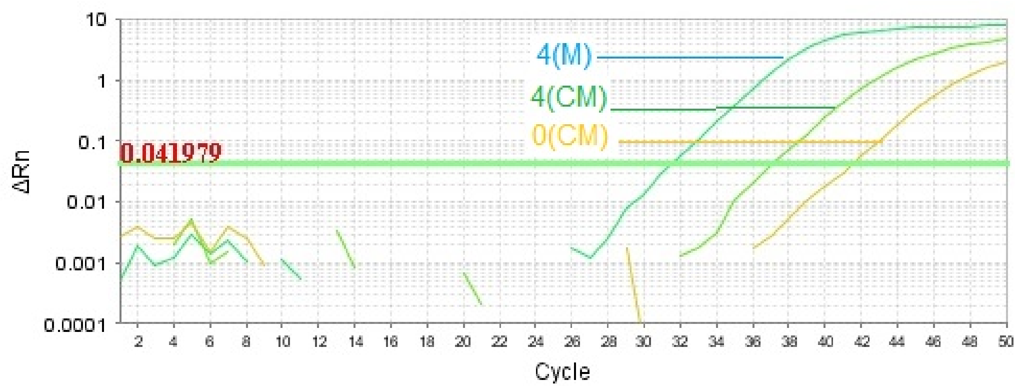


Figure E-4 Amplification plot of must/wine samples of Dimrit with *Hanseniaspora uvarum* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

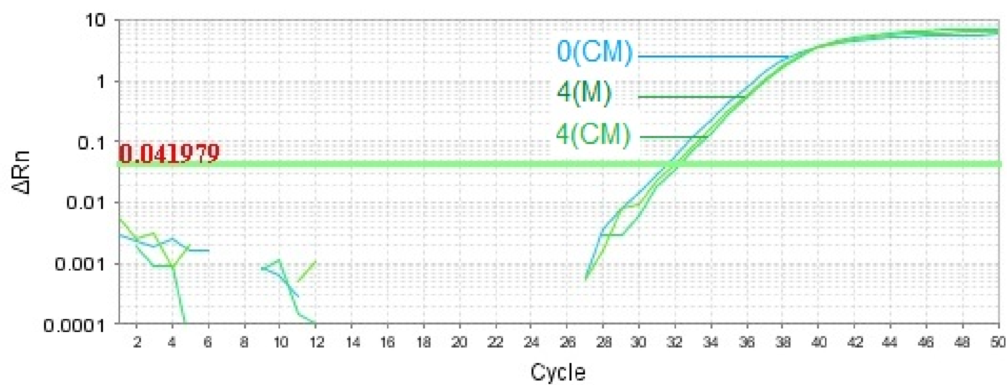


Figure E-5 Amplification plot of must/wine samples of Dimrit with *Issatchenkia orientalis* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

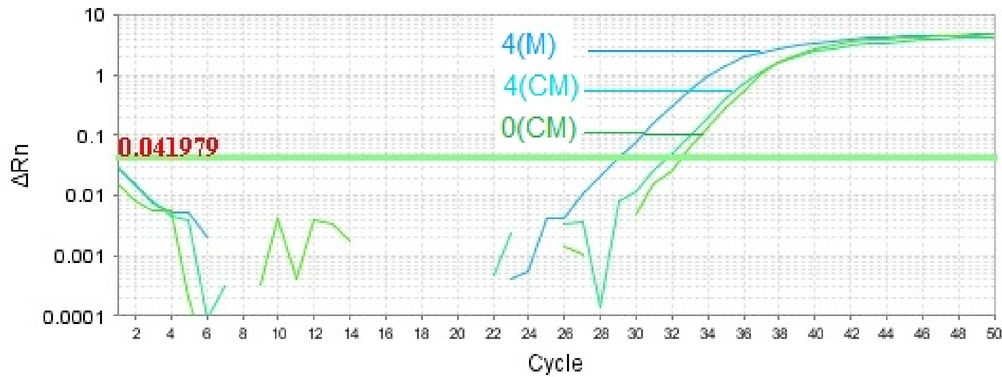


Figure E-6 Amplification plot of must/wine samples of Dimrit with *Pichia fermentans* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

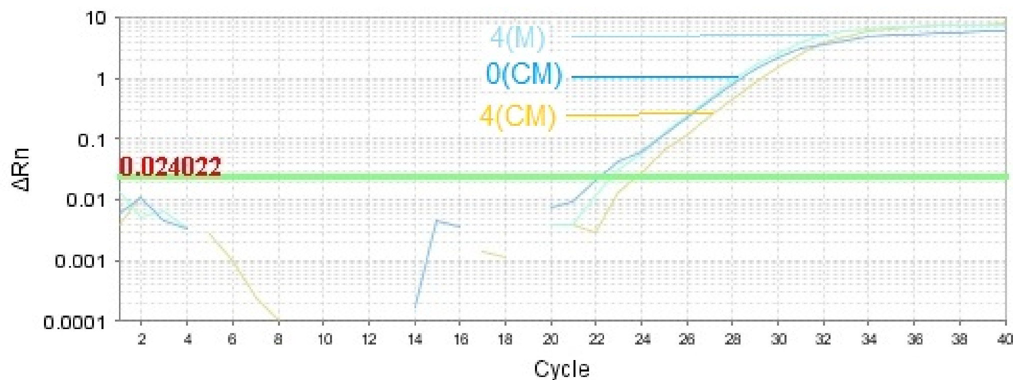


Figure E-7 Amplification plot of must/wine samples of Dimrit with *Saccharomyces* spp. primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

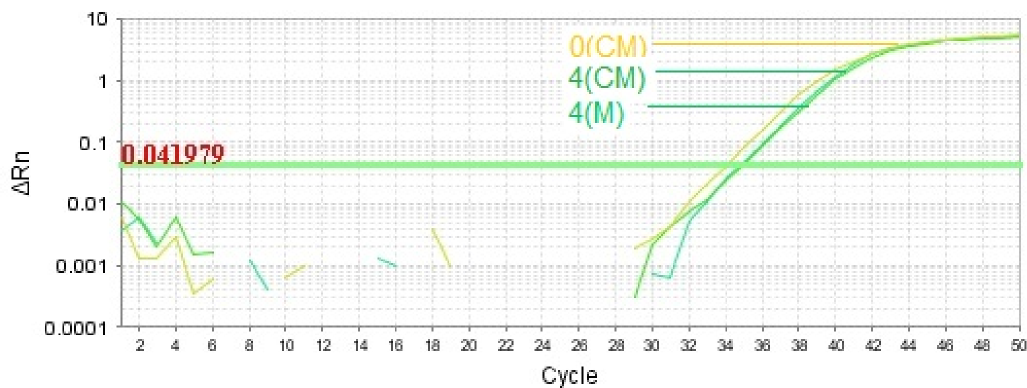


Figure E-8 Amplification plot of must/wine samples of Dimrit with *Saccharomyces cerevisiae* primer. 0 (CM), 0th of cold maceration; 4 (CM), fourth day of cold maceration; 4 (M), fourth day of maceration.

F. Amplification Plots of Emir Grape Must or Wine Samples Obtained by Using Specific Primers

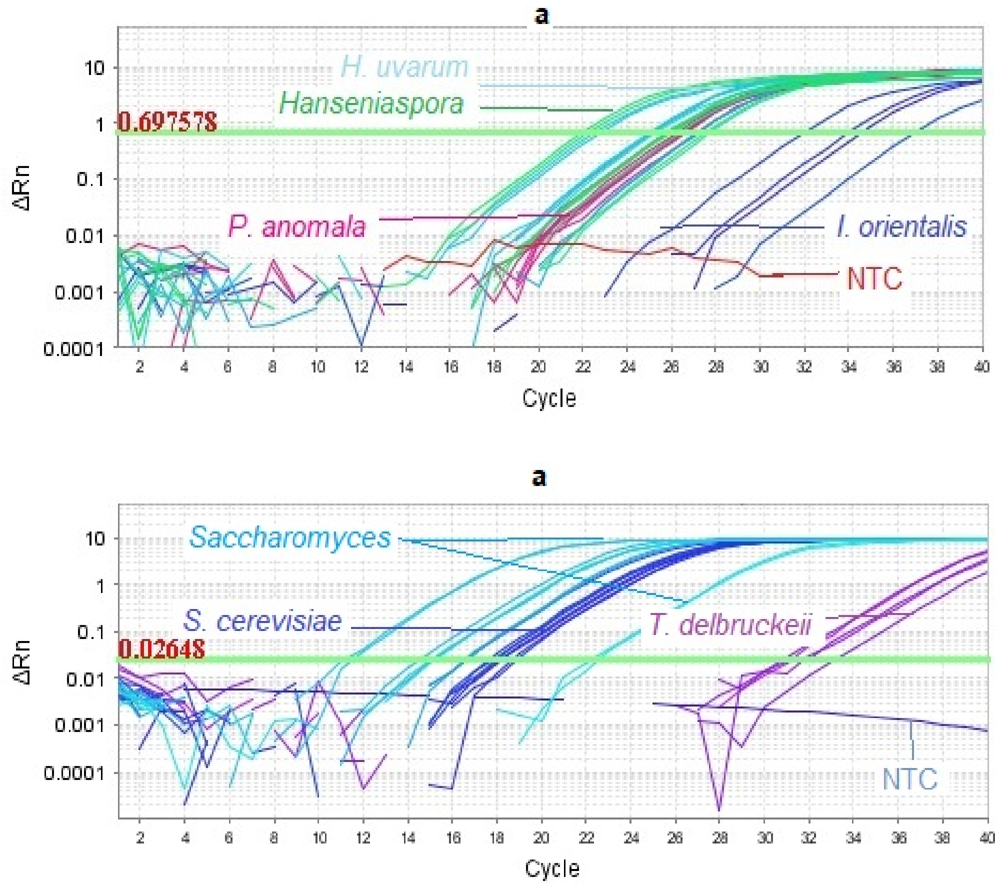


Figure F-1 Amplification plot of must/wine samples of Emir (0, 1, 2, 3, 4th week of alcoholic fermentation) with *Hanseniaspora* spp., *Hanseniaspora uvarum, Issatchenkia orientalis*, *Pichia anomala*, *Saccharomyces* spp., *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii* primers. NTC, no template control.

G. Melting Curve Analysis of Real-Time PCR Products of Kalecik Karası, Boğazkere, Öküzgözü and Dimrit Must/Wine Samples Amplified by Specific Primers

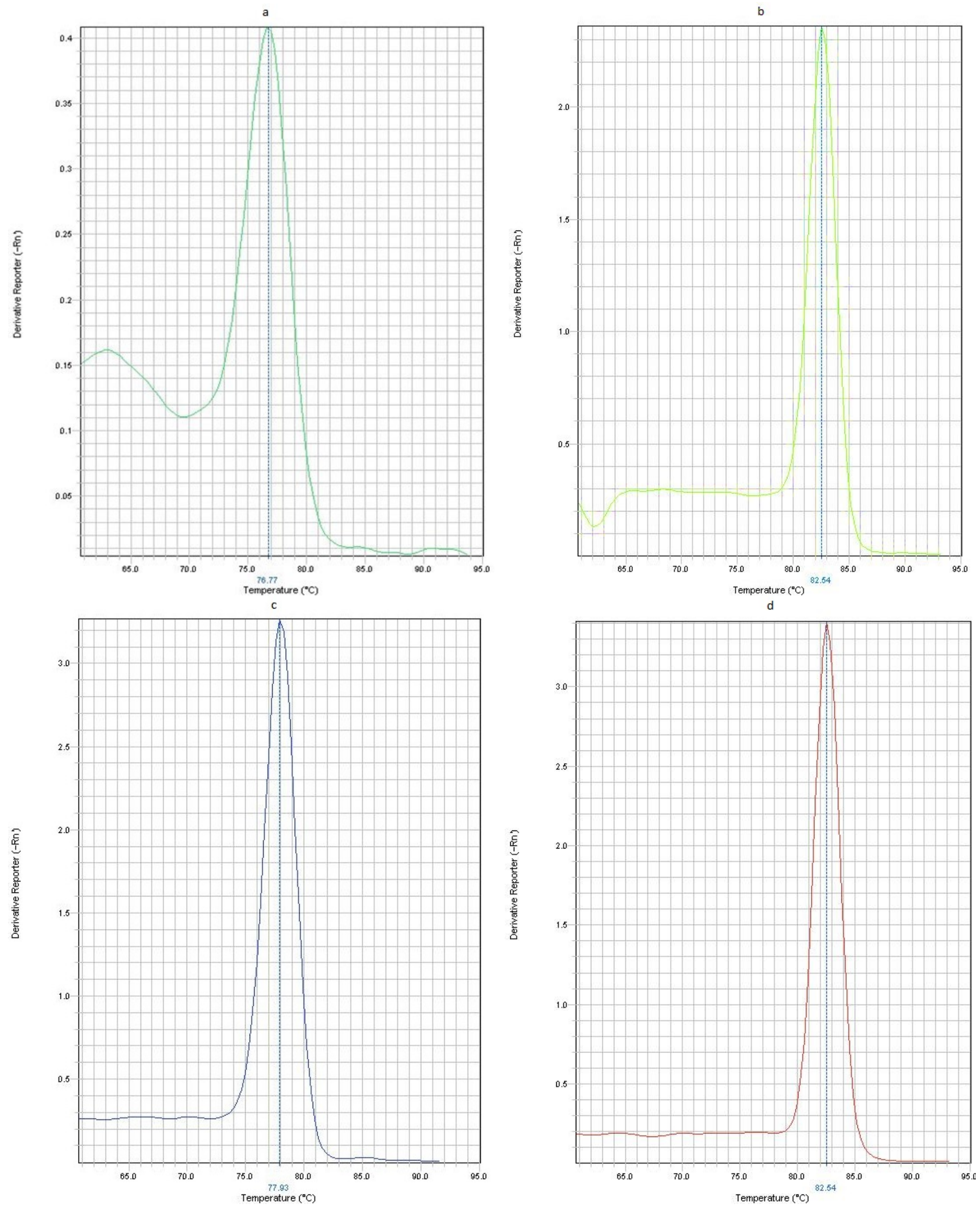


Figure G-1 Melting curve analysis of real-time PCR products of must/wine samples amplified by *Candida glabrata* (a), *Candida zemplinina* (b), *Hanseniaspora* spp. (c), and *Hanseniaspora uvarum* (d) primers indicating the reaction specificity, observed through a single peak in each curve. Y-axis represents the derivative reporter (ΔRn) while x-axis represents the temperature (°C). The figures show a melting temperature (T_m) of real-time PCR products as 76.77, 82.54, 77.93, and 82.54°C, respectively.

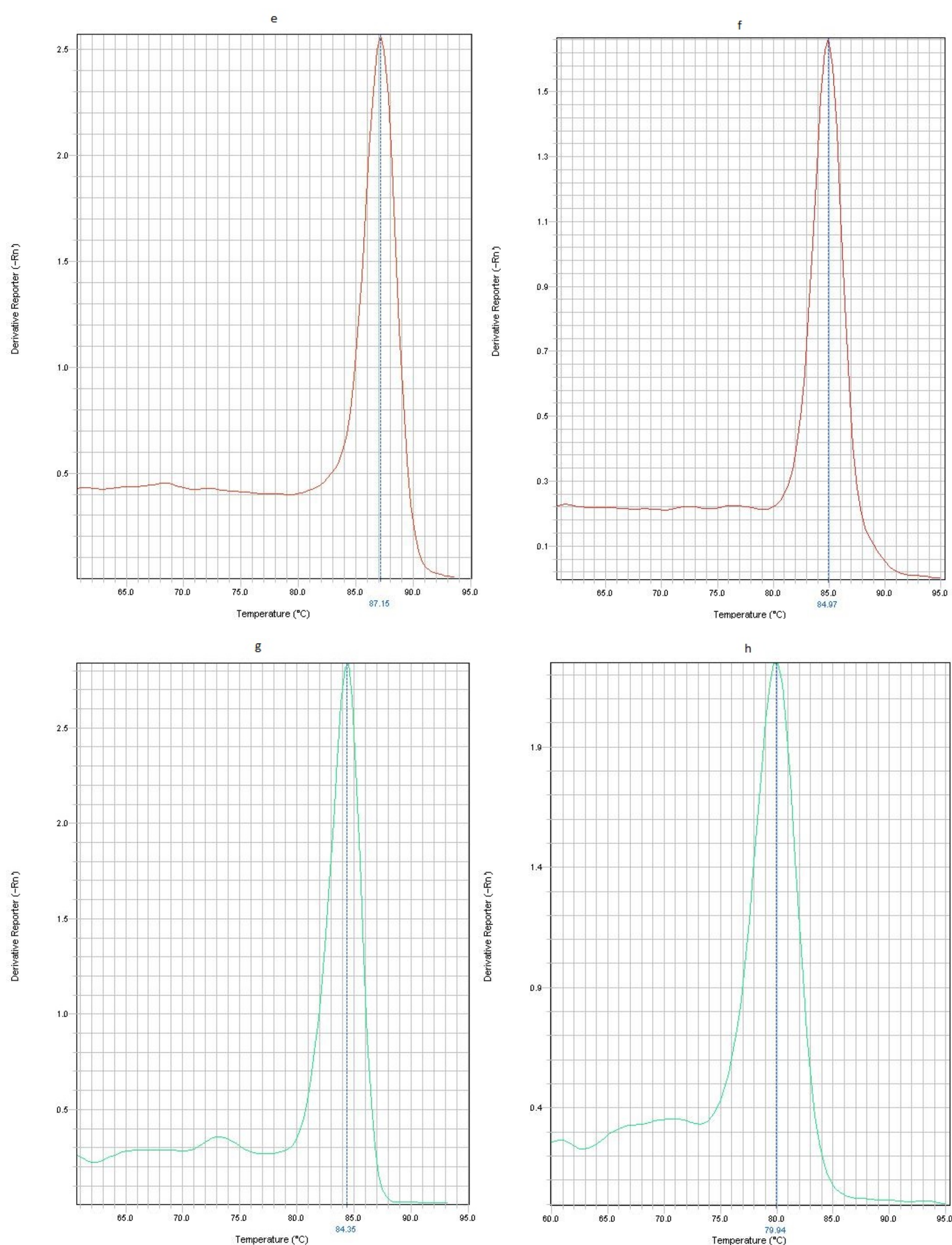


Figure G-2 Melting curve analysis of real-time PCR products of must/wine samples amplified by *Issatchenkia orientalis* (e), *Lachancea thermotolerans* (f), *Pichia anomala* (g), and *Pichia fermentans* (h) primers indicating the reaction specificity, observed through a single peak in each curve. Y-axis represents the derivative reporter (ΔRn) while x-axis represents the temperature (°C). The figures show a melting temperature (T_m) of real-time PCR products as 87.15, 84.97, 84.35, and 79.94°C, respectively.

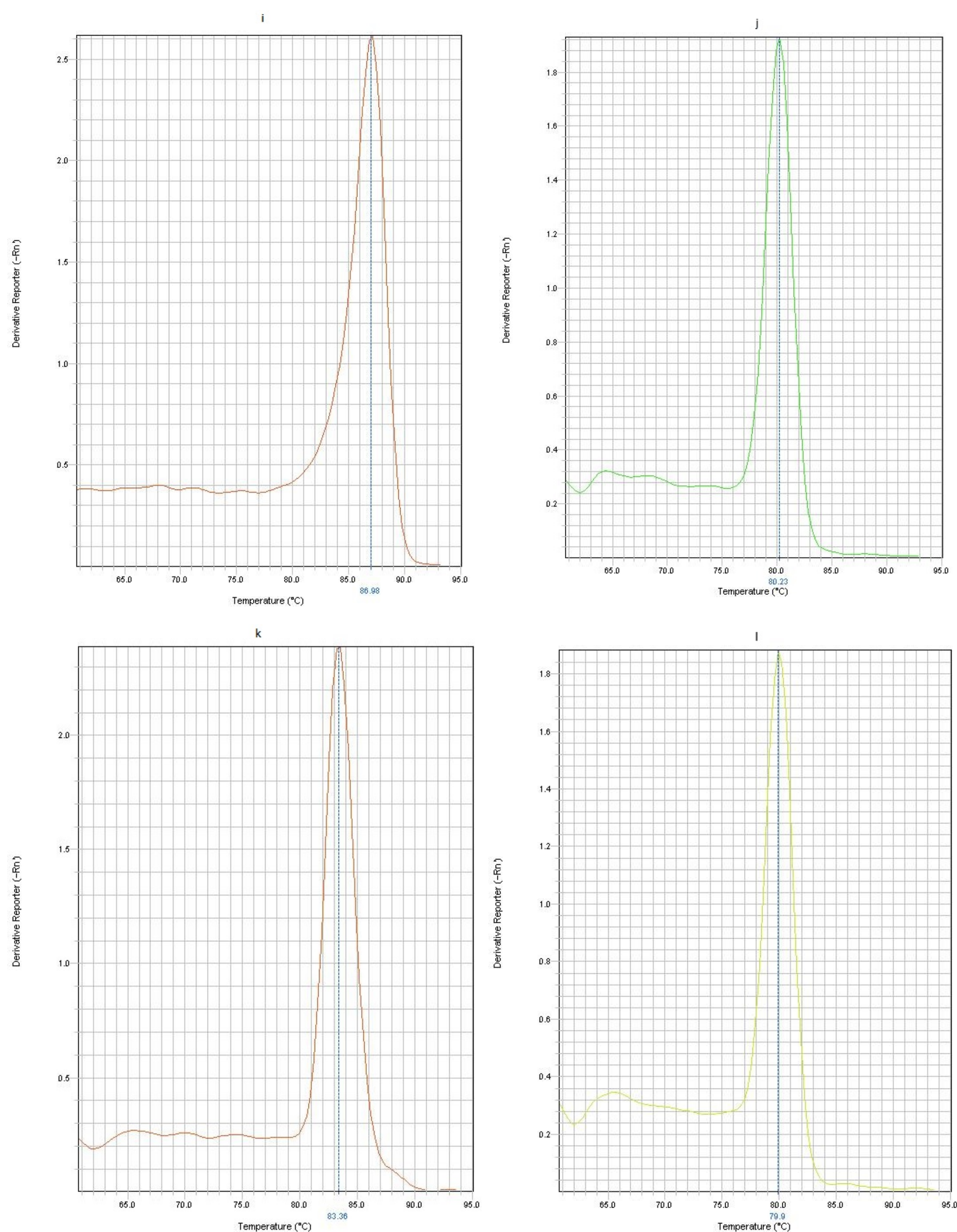


Figure G-3 Melting curve analysis of real-time PCR products of must/wine samples amplified by *Pichia kluyveri* (i), *Saccharomyces* spp. (g), *Saccharomyces cerevisiae* (k), and *Torulaspora delbrueckii* (l) primers indicating the reaction specificity, observed through a single peak in each curve. Y-axis represents the derivative reporter (ΔRn) while x-axis represents the temperature (°C). The figures show a melting temperature (T_m) of real-time PCR products as 86.98, 80.23, 83.36, 79.90°C, respectively.

H. Melting Curve Analysis of Real-Time PCR Products of Emir Must/Wine Samples Amplified by Specific Primers

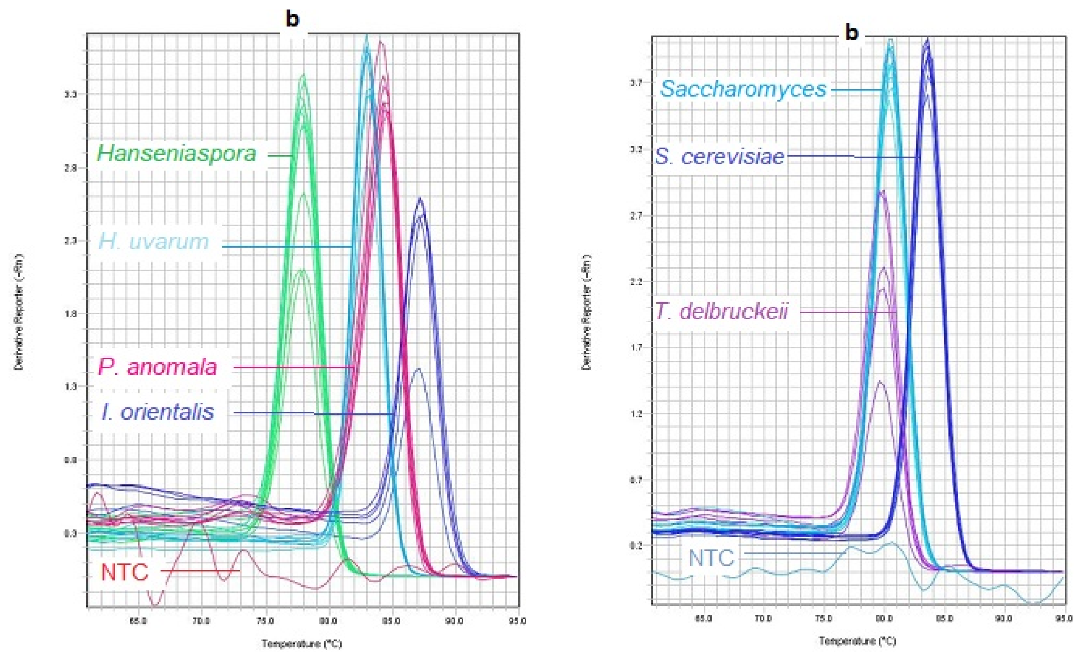


Figure H-1 Melting curves of must/wine samples of Emir (0, 1, 2, 3, 4th week of alcoholic fermentation) with *Hanseniaspora* spp., *Hanseniaspora uvarum, Issatchenkia orientalis*, *Pichia anomala*, *Saccharomyces*, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii* primers indicating the reaction specificity, observed through a single peak in each curve. NTC, no template control.

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EDUCATION

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Thesis Title	Biodiversity of yeasts of local winegrapes in Türkiye and genotyping of *Saccharomyces cerevisiae*

Degree	MSc in Biochemistry
Date	02/2013-12/2014
Institution	Middle East Technical University, Ankara, Türkiye
Field of Specialization	Biochemistry
Supervisor	Prof. Dr. Bülent İçgen
Thesis Title	Monitoring methicillin-resistant bacteria in river water by using *mec*A-specific DNA probe

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Institution	University of Tehran, Iran
Field of Specialization	Agricultural Engineering- Animal Science
Research Title	The effect of the copper level in the growing chicken

-**Master’s thesis was funded** by the scientific and technological research council of Türkiye (TUBITAK) through the support program for scientific and technological research projects (1001) with the project number 113Z198, 2013-12/2014.

- **PhD’s thesis was funded** by the scientific and technological research council of Türkiye (TUBITAK) through the support program for scientific and technological research projects (1001) with the project number 116O521, 05/2017-05/2020.

FOREIGN LANGUAGES

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Department of Basic English, Middle East Technical University, Ankara, Türkiye, 2011-2012.

WORK AND RESEARCH EXPERIENCE

1. Starter Culture Laboratory for biodiversity detection of indigenous non-*saccharomyces* and *saccharomyces* yeasts from grapes, musts and wines of important regions in Türkiye by applying various molecular methods, Department of Food Engineering, Middle East Technical University, Ankara, Türkiye, 05/2017-Current.

2. Microbiology Laboratory of Prof. Dr. Bülent İçgen, for monitoring methicillin-resistant bacteria in river water by using *mec*A-specific DNA probe, researching antibiotic resistant bacteria, and bacterial degradation and transformation and detoxification of xenobiotic compounds, Department of Environmental Engineering, Middle East Technical University, Ankara, Türkiye, 03/2013- 12/2014.

3. Central Laboratory of Abureyhan Campus of University of Tehran for general biochemistry, molecular biology, breeding and animal science researches, University of Tehran, Tehran, Iran, 2006-2007.

4. Pharmacy Manager, Drug store of Ziyaiyan Hospital, Tehran, Iran, 03/2008-09/2010.

PUBLICATIONS

PUBLICATIONS IN REFEREED INTERNATIONAL JOURNALS

1. Gurakan, G. C., Aktuna, I., and **Seyedmonir, E**. (2022). Diversity of wild yeasts during spontaneous fermentation of wines from local grape varieties in Türkiye. American Journal of Enology and Viticulture, ajev.2022.22001.

2. **Seyedmonir, E**., Yilmaz, F., and Icgen, B. (2016). Methicillin-resistant bacteria inhabiting surface waters monitored by *mec*A-targeted oligonucleotide probes Bulletin of Environmental Contamination and Toxicology, 97 (2), 261-271.

3. **Seyedmonir, E**., Yilmaz, F., and Icgen, B. (2015). *mec*A gene dissemination among staphylococcal and non-staphylococcal isolates shed in surface waters Bulletin of Environmental Contamination and Toxicology, 95 (1), 131-138.

CONFERENCE PROCEEDINGS

INTERNATIONAL CONFERENCE PROCEEDINGS

1. Aktuna, I., **Seyedmonir, E**., Çetinkaya, A., and Gurakan, G. C. (2019). Isolation of non-*Saccharomyces* in traditional wines made from two different grape varieties taken from Elazig in Türkiye. 42nd Congress of Vine and Wine, 17th General Assembly of the OIV, 15-19 July, CICG, Geneva, Switzerland.

2. Aktuna, I., **Seyedmonir, E**., Çetinkaya, A., and Gurakan, G. C. (2019). Isolation of non-*Saccharomyces* yeasts in traditional wine made from Kalecik Karası, Ankara in Türkiye. 1st Science and Wine World, 8-10 May, Porto, Portugal.

3. Gurakan, G. C., Aktuna, I., and **Seyedmonir, E**. (2019). Characterization of wild *Saccharomyces* and non-*Saccharomyces* yeasts from must and wine of grapes from Türkiye for the development of new strains for wine production. 1st Science and Wine World, 8-10 May, Porto, Portugal.

4. Gurakan, G. C., Aktuna, I., **Seyedmonir, E**., and Çavdaroğlu, Ç. (2018). Diversity of wild non-*Saccharomyces* yeast population in must and wine during spontaneous wine fermentation. 5th World Congress and Expo on Applied Microbiology, 12-13 November, Edinburgh, Scotland.

NATIONAL CONFERENCE PROCEEDINGS

1. **Seyedmonir, E**., Yilmaz, F., and Icgen, B. (2015). Methicillin-resistant surface water isolates monitored by *mec*A-specific DNA probes. ICOCEE Cappadocia 2015 International Conference on Civil and Environmental Engineering, 20-23 May, Nevsehir, Türkiye.

2. **Seyedmonir, E**., Yilmaz, F., and Icgen, B. (2014). Surface waters as hot spots for *mec*A harbouring staphylococcal and non-staphylococcal isolates. ICCBEE'14 International Conference on Chemistry, Biomedical and Environment Engineering, 7-8 October, Antalya, Türkiye.

3. **Seyedmonir, E**., Yilmaz, F., and Icgen, B. (2014). Prevalence of *mec*A in surface water contaminated with *Staphylococcus*. EURASIA 2014 Waste Management Symposium, 28-30 April, Istanbul, Türkiye.

BOOK CHAPTER

1. Gurakan, G. C., Aktuna, I., and **Seyedmonir, E**. (2022). Starter cultures. In R. E. Anlı and P. Şanlıbaba (Eds.). FOOD: Science, Technology and Engineering (pp. 171-216). Ankara, Türkiye: Nobel Publication.