APPLICATION OF INFRARED SPECTROSCOPY IN MID-INFRARED RANGE COMBINED WITH MULTIVARIATE ANALYSIS TO STUDY YEASTS INVOLVED IN WINE PRODUCTION

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Application of Infrared Spectroscopy in Mid-Infrared Range Combined with Multivariate Analysis to Study Yeasts Involved in Wine Production

DOCTORAL THESIS

Supervised by

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Departament d'Enginyeria Química

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FAIG CONSTAR que el present treball, titulat "Application of Infrared Spectroscopy in Mid-Infrared Range Combined with Multivariate Analysis to Study Yeasts Involved in Wine Production", que presenta Miquel Puxeu Vaqué per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament d'Enginyeria Química d'aquesta universitat.

Tarragona, 13 d'Octubre 2015

La directora de la tesi doctoral

Dra. Sílvia de Lamo Castellví

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ABBREVIATIONS

AF	Alcoholic fermentation
ASB	Alkali-sensitive bonds
ATR	Attenuated Total Reflectance
ATR-FTIR	Attenuated Total Reflectance Infrared Spectroscopy
CWP	Cell wall proteins
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	Nucleoside Triphosphate
ES	Spain
FR	France
FTIR	Fourier Transform Infrared Spectroscopy
GlcNac	N-acetylglucosamine
GPI	Glycosyl-phosphatidylinositol
ICD	Interclass Distance
IR	Infrared
LAB	Lactic acetic bacteria
MCFA	Medium Chain Fatty Acids
MIR	Mid infrared spectroscopy
MLF	Malolactic fermentation
mt-RFLP	Mitochondrial DNA Restriction Fragment Length Polymorphism
NIR	Near infrared spectroscopy
NTU	Nephelometric Turbidity Units
OIV	Organisation Internationale de la Vigne et du Vin
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

PIR	Proteins
QPCR	Quantitative Polymerase Chain Reaction
RAPD-PCR	Random Amplified Polymorphic Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic acid
Rem	Remnant GPI anchor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SIMCA	Soft Independent Modeling Class of Analogy
SS	Saline Solution
SYBR Green	Chemical compound with chemical formula$C_{32}H_{37}N_4S$
TGGE	Temperature Gradient Gel Electrophoresis
UK	United Kindom
USA	United States of America
UV	Ultraviolet
YAN	Yeast Assimilable Nitrogen

SYMBOLS

C=O	Carbon-Oxygen double bond
C-N	Carbon-Nitrogen double bond
C-O	Single bond Carbon-Oxygen
C-O-C	Single bond Carbon-Oxygen-Carbon
C-OH	Single bond Carbon-Hydroxyl
H_2S	Hydrogen sulphide
$MgCl_2$	Magnesium chloride
N-H	Nitrogen-Hydrogen double bond
P=O	Double bond Phosphorous-Oxygen
ZnSe	Zinc Selenide
β	Indicates oxygen linkage to carbon atom
λ	Wavenumber

SUMMARY

In oenology, microorganisms have been traditionally analysed using conventional techniques based on morphological tests supplemented with physiological tests or molecular techniques. These techniques allow obtaining accurate, repetitive and reproducible results, but requiring trained personnel and laborious and time consuming sample preparation. In the case of physicochemical parameters of grape must and wine, the conventional methodologies such as distillations and alkalimetric or iodimetric titrations are expensive, time consuming and poor environmentally friendly and have been gradually replaced by faster analytical technique such as spectrophotometric or enzymatic analysis specially in mid and large size wineries. Moreover, near infrared (**NIR**) and mid infrared (**MIR**) spectroscopies had been a revolution for wineries increasing extensively, the number of parameters of grape musts and wines analysed per hour. Infrared spectroscopy combined with multivariate analysis has been successfully used to study, discriminate and classify microorganisms. Attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) provides bands from all the cellular components of microorganisms, mainly from cell membrane and cell wall that permit the classification of microorganisms. With the present work, we propose to extend the current applications of infrared technology to build up spectral models to study, discriminate and classify "unknown" wine yeasts species. The results from this project can provide the wine industry and research communities with simple, fast, non-destructive, accurate and sensitive technique for discriminating yeast strains commonly used for wine fermentation that are difficult to isolate and identify. The technique is simple, cost-effective, and requires low sample volume. Furthermore, once the instrument is purchased, there is minimal operational cost involved on performing this technique. Due to the use of **MIR** spectroscopy in wine quality control, the wine industry is already familiar with this technology.

The first experimental study was based on performing the spectral analysis of different *Saccharomyces cerevisiae* strains fermenting grape must to obtain their unique signature profiles. Three commercial *S. cerevisiae* strains (ES454, E491, and ES181) and two grape musts (Grenache Blanc from Gandesa, Terra Alta, and Chardonnay musts from Lleida, Costers del Segre, respectively) were used. **DNA** extraction and **PCR** amplification were applied as reference techniques to prove strain differentiation. Microvinifications were performed with 150 mL of cleaned must at 17°C. Fermented

juices at exponential (48 and 120 h for Chardonnay and 198 h for Grenache blanc) and stationary phase (172 h for Chardonnay and 344 h for Grenache blanc) were centrifuged, and the pellets obtained were placed onto **ZnSe** crystal. Spectra were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000–700 cm^{-1}) and analysed by a multivariate analysis technique, soft independent modeling of class analogy (**SIMCA**). Results showed that **ATR-FTIR** coupled to **SIMCA** analysis was a powerful tool to discriminate *S. cerevisiae* cells at strain level and detect biochemical changes experimented by yeast strains depending on their growth phase. The biochemical differences detected between *S. cerevisiae* strains at exponential and stationary phases were mainly related to differences in their cell wall composition. At exponential phase the main groups were related with glucans **β**(1→4) or **β**(1→6) and mannoproteins, and at stationary phase the main groups were linked to glucans, manoproteins and lipids.

One of the common oenological practices is the addition of nitrogen to grape must to ensure optimal nutritional conditions of yeasts and prevent fermentation problems such as slow and sluggish fermentations. In the second study, we mainly focused on studying the potential of using **ATR-FTIR** in the mid-infrared range (**MIR**) combined with **SIMCA** analysis to study biochemical changes of *S. cerevisiae* cells supplemented with nitrogen at the beginning of alcoholic fermentation process. Microvinifications were performed with 150 mL of Grenache blanc cleaned must inoculated with a commercial strain of *S. cerevisiae* E491 with and without the addition of commercial inorganic and organic nitrogen preparations: 10 g/hL ammonium salts with thiamin nutrient as a inorganic source and 30 g/hL for organic nitrogen source rich in amino acids, vitamins and minerals. Fermentations were performed at 21°C and samples by **IR** analysis were taken at 0 h (initial point), 18 h (early exponential phase), 42 h (exponential phase) and 90 h (stationary phase). Samples (1.5 mL) were taken from fermentation batches using sterile material and centrifuged (15900 g for 5 min at room temperature). After centrifugation, the supernatant was carefully removed, and the pellets were washed three times under the same conditions described above using 1 mL of saline solution. After the cleaning process, 1.5 µL of each pellet was placed onto Zinc Selenide (**ZnSe**) crystal in order to acquire the spectral data. Six spectra per each sample and time (0 h, 18 h, 42 h and 90 h) were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000-800 cm^{-1}).

The **SIMCA** analysis confirmed that *S. cerevisiae* cells grown with the addition of inorganic and organic nitrogen were biochemically different from those cells grown without extra nitrogen added. Depending on the source of nitrogen used and the physiological phase studied, the yeast cell wall components that were revealed to differentiate no supplemented and supplemented *S. cerevisiae* cells were different. In the case of inorganic source of nitrogen, at exponential phase the components were protein structures and at stationary phase there was a contribution of lipid esters. Nevertheless, when a source of organic nitrogen was used, the main changes at exponential and stationary phase were produced by changes on nucleic acids and lipid esters.

Wineries performing spontaneous fermentations, consider Non-*Saccharomyces* yeast species a key factor for their role on providing desired and distinct regional characteristics to their wines and for improving wine characteristics such as mouth-feel, complexity and integration of flavours. Traditionally, methods to discriminate yeasts are based on morphological tests supplemented with physiological tests. Molecular techniques such as restriction fragment length polymorphism analysis of **PCR**-Amplified Fragments (**PCR-RFLP**) could be used for the identification of different wine yeast species. Nonetheless, molecular techniques required trained personnel and sample preparation is laborious and time consuming. Therefore, there is a need for simple, high-throughput, and reliable technique for rapid discrimination of *Saccharomyces cerevisie* and Non-*Saccharomyces* species in the wine sector.

In the third study, the potential of using attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) combined with multivariate analysis to discriminate *S. cerevisie* and Non-S*accharomyces* wine yeast species was investigated. Thirty eight strains (twenty nine *S. cerevisie* and nine Non-*Saccharomyces*) isolated from Spanish wines and identified by molecular techniques, were inoculated to Tempranillo thermovinificated red must and fermented juices were taken after 48 h at 28ºC. Pellets obtained after a centrifugation process, were placed onto diamond crystal. Spectra were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000–800 cm^{-1}) and were analysed by a multivariate analysis technique (**SIMCA**). To discriminate between yeast strains, 2-classes **SIMCA** model of Non-*Saccharomyces* and *S. cerevisiae* strains was built up showing tight clustering but close grouping (interclass distance of 1.7). Then, two **SIMCA** models were created separately with **IR** data from Non-*Saccharomyces* and *S. cerevisiae* strains and were validated obtaining scores above 89%. Physiological growth

phase of each strain was taken into consideration to build up **SIMCA** models improving substantially the close grouping detected between yeasts strains or species. Discrimination between Non-*Saccharomyces* and *S. cerevisiae* strains was linked to cell wall components.

To summarize, there is a need for simple, high-throughput, and reliable technique for rapid analysis of microorganism in the wine sector. The technology proposed after complete development, is desired to be able for rapid characterization of yeast used in winemaking sector. Some companies that are selling **IR** equipment's have already developed applications with Fourier transform mid or near infrared spectroscopy to analyse quality routine and quality control parameters in wine or grape must but they have not develop any application to study yeast or bacteria. With the present work we offer the possibility of providing new applications of the traditional uses of infrared spectrometer for the winemaking sector.

RESUM

En l'enologia, els microorganismes han estat analitzats tradicionalment mitjançant l'ús de tècniques convencionals o bé mitjançant tècniques moleculars. Aquestes tècniques permeten obtenir resultats precisos, repetitius i reproduïbles, però requereix personal format i una preparació complexa de la mostra. En el cas de paràmetres fisicoquímics de most de raïm i vi, les metodologies convencionals com ara destil·lacions i valoracions alcalimètriques o iodimètriques que són cares, laborioses i poc respectuoses amb el medi ambient. Aquestes, han estat reemplaçades gradualment per tècniques analítiques més ràpides com ara anàlisis espectrofotomètrics o enzimàtics, especialment en cellers mitjans i de grans dimensions. D'altra banda, l'espectroscòpia d'infraroig ja sigui proper (Near Infrared, **NIR**) o mitja (Mid Infrared, **MIR**), ha estat una revolució per als cellers augmentant àmpliament el nombre de paràmetres de mosts i vi analitzat per hora. L'espectroscòpia d'infraroig combinada amb l'anàlisi multivariant s'ha utilitzat amb èxit per estudiar, discriminar i classificar microorganismes. L'espectroscòpia infraroja de reflectància total atenuada (Attenuated Total Reflectance – Fourier Transform Infrered, **ATR-FTIR**) proporciona bandes de tots els components cel·lulars dels microorganismes, principalment de la membrana i la paret cel·lular que en permeten la seva classificació. Amb el present treball, es proposa estendre les aplicacions actuals de la tecnologia d'infraroig per construir models espectrals per estudiar, discriminar i classificar espècies "desconegudes" de llevats en el vi. Els resultats d'aquest projecte poden proporcionar a la indústria vitivinícola i a les comunitats científiques un tècnica simple, ràpida, no destructiva, precisa i sensible per discriminar diferents soques de llevat utilitzades habitualment per a la fermentació del vi. La tècnica és senzilla i rentable, i requereix un baix volum de mostra. A més, una vegada adquirit l'instrument, hi ha un cost operacional mínim implicat en la utilització d'aquesta tècnica. Atès a l'actual ús de l'espectroscòpia **MIR** en el control de qualitat del vi, la indústria vitivinícola ja hi està familiaritzada.

El primer estudi experimental es basà en l'anàlisi espectral de diferents soques de *Saccharomyces cerevisiae* durant la fermentació alcohòlica de most de raïm per tal d'obtenir els seus perfils. Es van utilitzar tres soques comercials de *S. cerevisiae* (ES454, E491 i ES181) i dos mostos de raïm (Garnatxa Blanc de Gandesa, Terra Alta i Chardonnay de Lleida, Costers del Segre, respectivament). L'extracció d'àcid desoxiribonucleic (**ADN**) i l'amplificació per Polymerase Chain Reaction (**PCR**) es van utilitzar com a tècniques de referència per demostrar que les soques utilitzades eren

diferents. Les microvinificacions es van realitzar amb 150 mL de most net a 17 °C. Les mostres de most fermentant es van extreure en fase exponencial (48 i 120 h per Chardonnay i 198 h per Garnatxa blanca) i en fase estacionària (172 h per Chardonnay i 344 h per Garnatxa blanca). Posteriorment es van centrifugar, i els pellets obtinguts es van col·locar sobre el cristall de selenur de zinc (**ZnSe**) posteriorment a la neteja per al seu anàlisi. Els espectres es van recollir mitjançant un accessori de reflectància total atenuada (**ATR**) en la regió de infraroig mitjà (4000-700 cm^{-1}) i posteriorment van ser analitzats mitjançant una tècnica d'anàlisi multivariant, Soft Independent Modeling Class Analogies (**SIMCA**). Els resultats van mostrar que la tècnica **ATR-FTIR** acoblada a l'anàlisi **SIMCA** era una poderosa eina d'anàlisi per discriminar cèl·lules de *S. cerevisiae* a nivell de soca i detectar els canvis bioquímics que experimenten les diferents soques de llevat en funció de la seva fase de creixement. Les diferències bioquímiques detectades entre les soques de *S. cerevisiae* en les fases exponencial i estacionària, estaven relacionades amb diferents composicions a nivell de paret cel·lular. En la fase exponencial els grups principals es relacionaven amb glucans **β**(1→4) o **β**(1→6) i manoproteïnes, i en fase estacionària els grups principals estaven relacionats amb glucans, manoproteïnes i lípids.

Una de les pràctiques enològiques comunes és l'addició de nitrogen al most per assegurar unes condicions nutricionals òptimes per als llevats i prevenir parades i/o fermentacions lentes. El segon estudi es va centrar en la possibilitat d'utilitzar la tècnica de **ATR-FTIR** en la regió de l'infraroig mitjà (**MIR**) en combinació amb l'anàlisi **SIMCA** per avaluar els canvis bioquímics de les cèl·lules de *S. cerevisiae* quan diferents tipologies de nutrients eren afegits a principis del procés de fermentació alcohòlica. Les microvinificacions es van dur a terme amb 150 mL de Garnatxa blanca, inoculant amb una soca comercial de *S. cerevisiae* E491 amb i sense l'addició de preparats de nitrogen inorgànics i orgànics comercials: 10 g/hL amb sals d'amoni de nutrients tiamina com una font inorgànica i 30 g/hL per a la font de nitrogen orgànic ric en aminoàcids, vitamines i minerals. Les fermentacions es van realitzar a 21°C i els mostrejos es van realitzar a les 0 h (punt inicial), 18 h (a principis de la fase exponencial), 42 h (fase exponencial) i 90 h (fase estacionària). Les mostres (1,5 mL) es van recollir de les diferents microvinificacions utilitzant material estèril i posteriorment es van centrifugar (15.900 g durant 5 min a temperatura ambient). Després de la centrifugació, el sobrenedant es va retirar amb cura, i el pellet resultant es van rentar tres vegades en les mateixes condicions descrites anteriorment usant 1 mL de solució salina. Després del procés de neteja, 1,5 µL de cada

pellet es va col·locar sobre un cristall **ZnSe** per tal d'adquirir els espectres. Sis espectres per cada mostra i temps de mostreig (0 h, 18 h, 42 h i 90 h) es van recollir utilitzant l'accessori de reflectància total atenuada (**ATR**) en la regió de l'infraroig mitjà (4000-800 cm^{-1}).

L'anàlisi mitjançant **SIMCA** dels espectres va confirmar que les cèl·lules de *S. cerevisiae* inoculades en un most amb addició de nitrogen inorgànic i orgànic eren bioquímicament diferents d'aquelles cèl·lules inoculades en un most sense addició de nitrogen extra. Depenent de la font de nitrogen utilitzada i la fase fisiològica estudiada, els components de la paret cel·lular del llevat que van ser detectats per diferenciar cèl·lules de *S. cerevisiae* en mostos amb i sense addició eren diferents. En el cas de la font inorgànica de nitrogen, en la fase exponencial els components responsables de la diferenciació eren estructures de proteïnes, i en fase estacionària a diferència de la fase exponencial hi havia una contribució d'èsters lipídics. No obstant això, quan es va utilitzar una font de nitrogen orgànic, els principals canvis en la fase exponencial i estacionària van ser produïts per canvis en els àcids nucleics i èsters de lípids.

Els cellers que realitzen fermentacions espontànies, consideren l'ús d'espècies de llevat no-*Saccharomyces* un factor clau per a dotar els seus vins amb qualitats diferents a la resta, proporcionant-l'hi característiques regionals i millorant les característiques del vi, com ara sensació en boca, la complexitat i la integració de sabors. Tradicionalment, els mètodes per discriminar els llevats es basen o bé en proves morfològiques completades amb proves fisiològiques o bé amb tècniques moleculars com ara l'anàlisi de polimorfismes de longitud dels fragments de restricció amplificats per **PCR** (Polymerase Chain Reaction - Restriction fragment length polymorphism, **PCR-RFLP**) que poden ser utilitzats per a la identificació de diferents espècies de llevats. No obstant això, les tècniques moleculars requereixen disposar de personal capacitat i d'una preparació de mostra laboriosa i complexa. Per tant, en el sector vitivinícola existeix la necessitat de tècniques simples i fiables per a la discriminació ràpida de *Saccharomyces cerevisiae* i espècies no-*Saccharomyces*.

En el tercer estudi, s'ha avaluat el potencial de l'ús de reflectància total atenuada i l'espectroscòpia infraroja (**ATR-FTIR**) combinada amb l'anàlisi multivariant per a la discriminació d'espècies de *S. cerevisiae* i no-*Saccharomyces*. Un total de trenta-vuit soques de llevat (vint-i-nou de *S. cerevisiae* i nou no-*Saccharomyces*) aïllades de raïm de

varietats autòctones espanyoles i identificades mitjançant tècniques moleculars van ser inoculades en most de la varietat Ull de Llebre que va fermentar a 28ºC durant 48h. Els pellets obtinguts després d'un procés de centrifugació i neteja, es van col·locar en un cristall de diamant. Els espectres es van recollir mitjançant la reflectància total atenuada (**ATR**) a la regió mitjana de l'infraroig (4000-800 cm^{-1}) i es van analitzar mitjançant una tècnica d'anàlisi multivariant (**SIMCA**). Per discriminar entre les soques de llevat, es van construir models **SIMCA** de 2 classes de les soques no-*Saccharomyces* i *S. cerevisiae* mostrant una bona agrupació amb una distancia no suficientment gran (distància entre classes d'1,7). Llavors, es van crear dos models **SIMCA** per separat amb les dades d'**IR** de les soques no-*Saccharomyces* i *S. cerevisiae*. Aquests van ser validats interna i externament obtenint un percentatge d'encert superior al 89%. Per millorar substancialment l'agrupació entre soques es va tenir en compte la fase de creixement de cada soca. Els models **SIMCA** van millorar substancialment fent més estreta l'agrupació entre les soques d'una mateixa espècie i major la distància entre soques de diferents espècies. La discriminació entre soques no-*Saccharomyces* i soques de *S. cerevisiae* estat en tots els casos vinculada a diferents components de la paret cel·lular.

En resum, en el sector vitivinícola existeix una necessitat de metodologies simples, fiables i rentables per a l'anàlisi de microorganismes. La tecnologia proposada en el present estudi després del seu total desenvolupament, és possible que sigui capaç d'oferir una caracterització ràpida de llevats complint amb les característiques anteriors. Diverses empreses comercialitzadores d'equips d'**IR** han desenvolupat aplicacions per l'anàlisi de múltiples paràmetres rutinaris de control de qualitat en most i vi, però no han desenvolupat encara cap aplicació per a l'estudi de llevats o altres microorganismes en el sector enològic. Amb el present treball s'ofereix la possibilitat d'incrementar les aplicacions dels tradicionals espectròmetres d'infraroig en el sector vitivinícola.

UNIVERSITAT ROVIRA I VIRGILI
APPLICATION OF INFRARED SPECTROSCOPY IN MID-INFRARED RANGE COMBINED WITH MULTIVARIATE ANALYSIS TO STUDY YEASTS INVOLVED IN WINE PRODUCTION
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CHAPTER 1

Introduction & Objectives

UNIVERSITAT ROVIRA I VIRGILI
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1.1. Fundamentals of Infrared Spectroscopy

Infrared spectroscopy is an analytical technique based on the interaction of electromagnetic radiation at infrared range with molecules (Sandt et al., 2003). When infrared radiation interacts with the sample, some of the radiation is absorbed by the chemical bounds of its molecules and can be easily measured. Each functional group absorbs at specific wavenumber producing a bound vibration (Colthup et al., 1975; Griffith and de Heaseth, 2007). The spectrum of each sample is unique being a "fingerprint" that allows its identification with certainty (Stuart, 2006; Griffiths and de Haseth, 2007; Smith, 2011). Mainly types of vibrations are observed in infrared spectroscopy, vibrations along chemical bonds called stretching vibrations (**ν**) that produce bond-length changes that can be symmetrical (stretching in phase) or asymmetrical (stretching out of phase), vibrations linked to changes in bond angles called bending vibrations that can be produced in plane (**δ,** called scissoring) or out-of-plane (π, called twisting) or rocking and wagging were bond length and bond angle is maintained stationary (Stuart, 2005). These main types of vibrations are summarized in **Figure 1.1.**

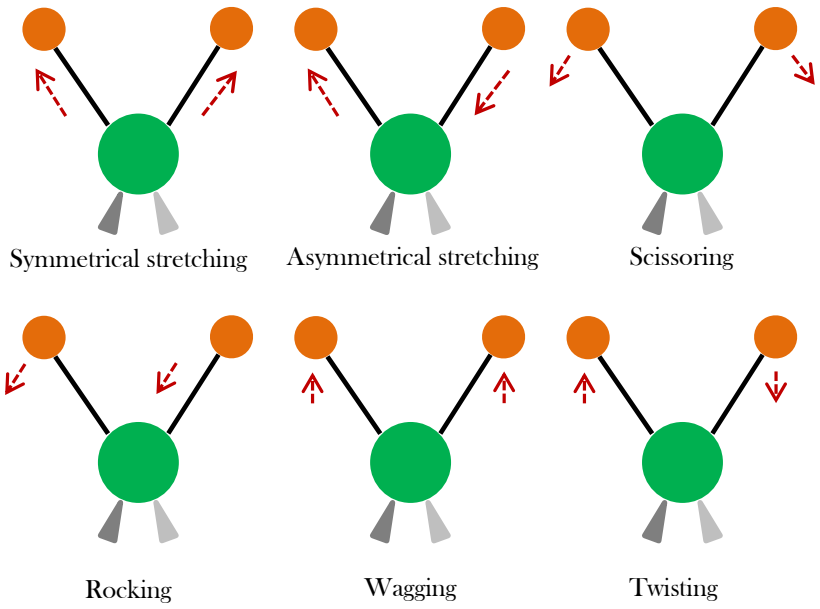

Figure 1.1. Main types of molecular vibrations in infrared spectroscopy.

For instance, for a nonlinear molecule with **Y** number of atoms, there are 3**Y**-6 fundamental motions of the molecule atoms, or 3**Y**-6 fundamental vibrations or normal modes. A normal mode of vibration is infrared active if there is a change in the dipole moment of the molecule during the course of the vibration (Berthomieu et al., 2009). When a positive (**+z**) and negative charge (**–z**) is separated by a distance (**d**), the dipole moment (**μ**) is equal to the magnitude of the charge multiplied by this distance (**Equation 1.1**).

$$\mu = z \cdot d \qquad (1.1)$$

In order to have an infrared spectrum, the molecule basically needs to have a dipole moment due to the fact that symmetric vibrations are usually not detected at the infrared range especially when molecules have centres of symmetry (Berthomieu et al., 2009). Nitrogen and chloride in gas phase are examples of infrared inactive molecules. The infrared region of the electromagnetic spectrum ranges from 12.500 to 10 cm^{-1} and is divided into three different regions according to their relation with the visible spectrum. These regions are called Near-infrared (**NIR**, from 12.500 to 4.000 cm^{-1}), Mid-infrared (**MIR**, from 4.000 to 400 cm^{-1}) or Far-infrared (**FIR**, from 400 to 100 cm^{-1}) (Kümmerle et al., 1998). **Figure 1.2** shows the location of **NIR** and **MIR** on the set of spectral regions is shown:

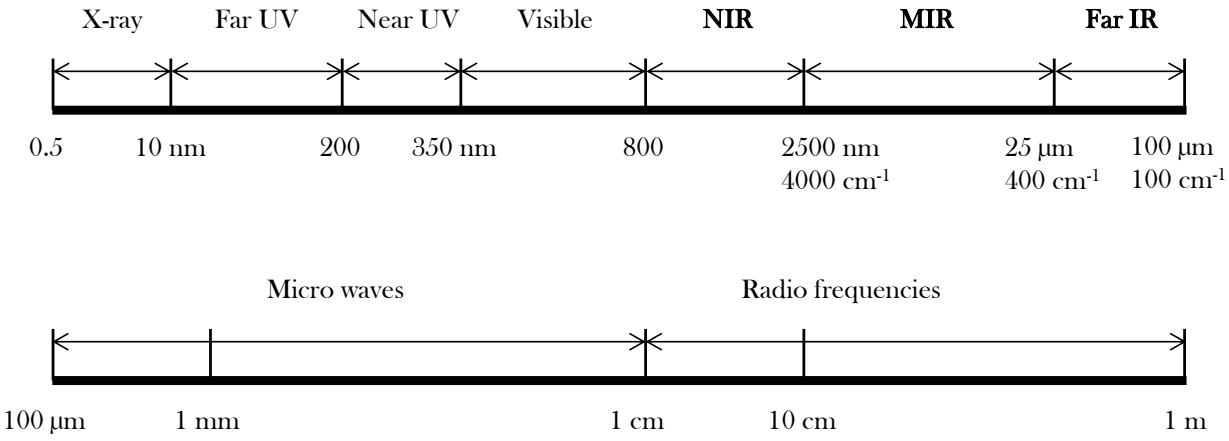

Figure 1.2. Spectral regions of interest for analytical purposes (adapted from Dufour, 2009 in Infrared Spectroscopy for Food Quality Analysis and Control, Chapter 1).

Usually, a **NIR** spectrum is quite complex with road overlapping bands and large baselines variations leading to difficult interpretations. However, mathematical processing techniques such as derivatization and deconvolution can be used as powerful tools to improve spectral characteristics removing baselines shifts, improving bands resolution and reducing the variability between replicates, and also reducing bands width and resolving overlapped bands, respectively (Tooke, 1988; Subramanian and Rodríguez-Saona, 2009). In **NIR** spectrum, the main absorption bands detected are produced by stretching vibrations of hydrogen atoms covalently bonded to carbon, oxygen or nitrogen. Therefore, a large number of organic materials and food and agricultural products are suitable to be analyzed by **NIR** spectroscopy (Osborne et al., 1993; Batten 1998; Gishen et al., 2005). For instance, **NIR** spectroscopy has been extensively used to determine physico-chemical parameters in a large variety of food products such as meat, fruit and vegetables, dairy products, cereals, beverages and tea (Woodcock et al., 2008; Lin et al., 2014; Jamshidi et al., 2015; Lorente et al., 2015; Schmutzler et al., 2015). More recent **NIR** spectroscopy applications are focused on determination of total phenols in cocoa beans (Huang et al., 2014), sorting of pistachio nuts in-shell and without at real time (Haff et al., 2008), discrimination of honey taking into account their flower origin (Tewari and Irudayarj, 2005) and identification of transgenic foods in order to be compared with traditional methodologies (Alishahi et al., 2010). Moreover, **NIR** spectroscopy combined with chemiometric analysis has been also used as a rapid method to discriminate microorganisms such as *Bacillus* species and pathogenic and non-pathogenic *E. coli* strains (Sivakesava et al., 2004; Yue et al., 2010; Ghosh et al., 2015).

MIR is a very robust and reproducible region allowing structural elucidation and compound identification even with samples with subtle chemical differences (Naumann, 2006; Stuart, 2006). **MIR** spectrum is mainly divided into three regions: the region above 3000 cm^{-1} that include bands of hydroxyl or amino groups, the region between 3000 and 1500 cm^{-1} that include stretching vibrations of acyl chain, carbonyl and alkenes groups and the region below 1500 cm^{-1} characterized mainly by bending vibrations and some stretching vibrations of acyl chain and functionalized groups (Pico, 2012). In the last two decades, **MIR** spectroscopy has grown significantly, especially for food applications (Pico, 2012). There are commercial **MIR** equipment's and spectral data bases created for several food applications such as the detection of honey adulteration with sugars (Sivakesava and Irudayaraj, 2002), extra virgin olive oil adulteration with cheaper oils

(Yang and Irudayaraj, 2001; Yang et al., 2005), the quantification of astringency in wine (Costa et al., 2015), for wine quality or authenticity (Banc et al., 2014) or phenolic compounds quantification during red winemaking process (Fragoso et al., 2011ab). **MIR** spectroscopy combined with chemiometric analysis has been also used as a rapid and reproducible method to detect, identify and characterize bacteria and other microorganisms (Sivakesava et al., 2004; Dziuba et al., 2007; Dziuba et al., 2012; Wenning et al., 2013).

FIR region is considered an uncomfortable region where conventional infrared methods cease to be efficient while microwave techniques cannot yet be applied in a straightforward way (Rothschild et al., 1970). A large part of the far-infrared frequency range is also accessible by Raman spectroscopy, although the energy levels concerned are the same in both cases, the selection rules governing transitions between these levels are different (Stuart et al., 2004). Raman spectroscopy has been introduced as a simple, fast and reliable technique for food analysis in the same way that **NIR** and **MIR** spectroscopy since it provides useful information about the molecular structure. Raman spectra have fingerprint properties making them very useful for analytical purposes (El-Abassy et al., 2015). Despite the fact that the number of Raman spectroscopy applications has been steadily increasing in various scientific fields over the last decades, this technique has still to be considered as just emerging technique in food industry (El-Abassy et al., 2015). Some applications had been developed for rapid determination of free fatty acids in extra virgin olive oil (El-Abassy et al., 2009), analytical discrimination between caffeine and demethylated analogues of pharmaceutical relevance (Edwards et al., 2005), determination of fat milk content (El-Abassy et al., 2011a) and discrimination between Arabica and Robusta green coffe (El-Abassy et al., 2011b).

1.1.1. The Interferometer (Michelson Interferometer)

An interferometer is an optical device that allows the generation of interference patterns or interferograms in a controlled form (Stuart et al., 2004; Subramanian and Rodríguez-Saona, 2009). The main parts are the source (that emits light in the **IR** region), the beam splitter (that divides the **IR** light in two and recombines the light from two mirrors), the movable mirror (it moves along the axis from and towards the beam splitter) and the stationary mirror (located back to the beam splitter) (**Figure 1.3**). The position of the two mirrors modifies the distance travelled by the two light beams and is called optical path difference (**OPD**) (Ikemoto et al., 2015).

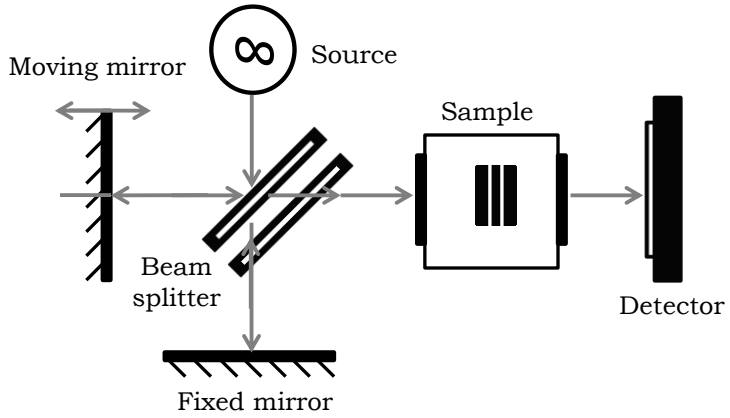

Figure 1.3. Diagram of interferometer designs by Michelson (adapted from Subramanian and Rodríguez-Saona, 2009 in Infrared Spectroscopy for Food Quality Analysis and Control, Chapter 7).

When the two mirrors are at the same distance (zero path difference, **ZPD**) the reflected light beams are in phase and interfere constructively obtaining the highest intensity during constructive interference. This case only occurs when the **OPD** between the mirrors is an integer (**n**) multiple of the wavelength (**λ**). Otherwise, if the mirrors are not in phase, the interference is destructive, leading to a beam of low intensity. Moreover, a completely destructive interference could be created when the path difference is (**n** + ½) multiple of the wavelength. In other cases, the path differences constructive and destructive take place obtaining light intensity variations. (Subramanian and Rodríguez-Saona, 2009).

The plot resulting of the intensity of light (in volts) over the **OPD** is known as interferogram (**Figure 1.4**). The main parts are known as Centerburst and Wings. The Centerburst represents the total intensity of the source without the sample signal

interference and provides the total amount of energy from the source. The Wings are the parts of the interferogram at either side of the Centerburst and are where constructive and destructive interferences take place at varying level (Smith, 2011).

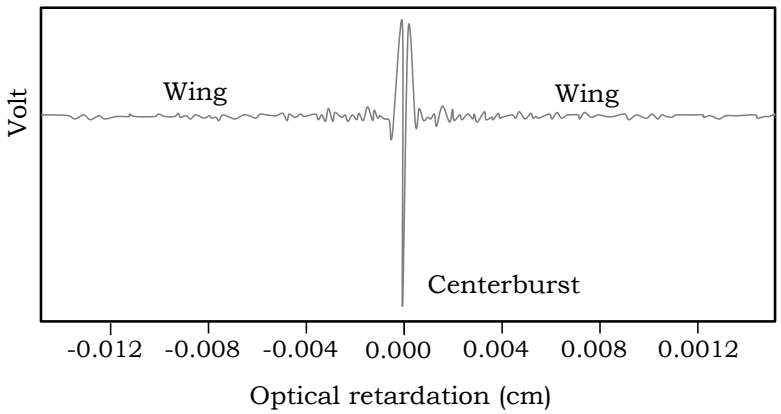

Figure 1.4. Typical interferogram acquired by a modern **FTIR** (adapted from Smith, 2011).

1.1.2. Fourier Transformation

Fourier transform (**FT**) is the mathematical procedure applied to interferograms to obtain the desired spectrum. In **Figure 1.4** could be seen two different interferograms with and without the presence of sample, the **FT** of the both interferograms and the resulting spectrum obtained after rationing truncation (Subramanian and Rodríguez-Saona, 2009; Smith, 2011).

The invention of fast Fourier transform (**FFT**), was an improvement of the first **FT**, by Cooley and Tukey (1965) improving the performances of these early **FTIR** spectrometers being the first commercial **FTIR** spectrometers in late 1960s (Cooley and Tukey et al., 1965). Nowadays, due the rapid commercial development and extensive research, **FTIR** is considered one of the most powerful techniques for chemical analysis because its simplicity, sensitivity, versatility, speeds of analysis and wide range of applications (Subramanian and Rodríguez-Saona, 2009).

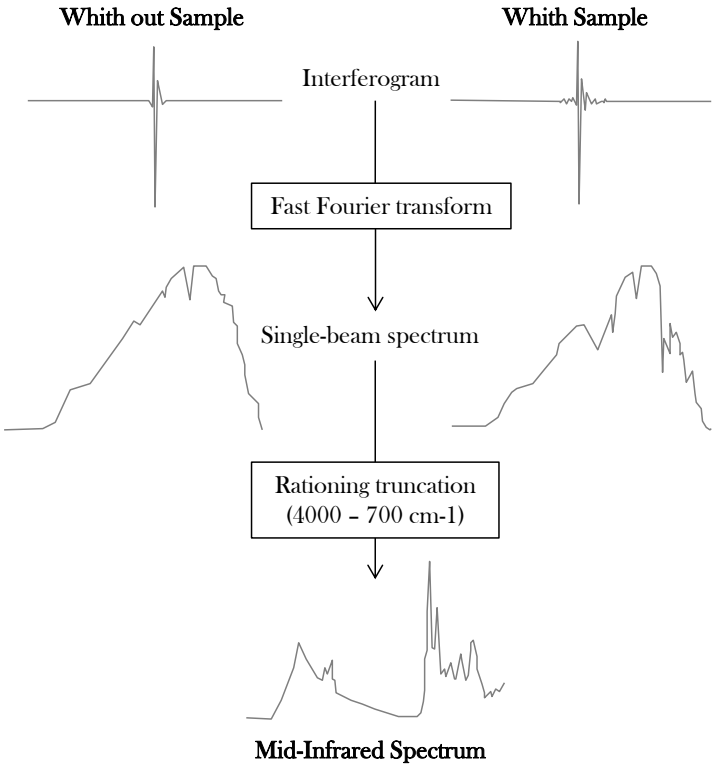

Figure 1.5. Illustration of how a mid-infrared spectrum is obtained from the interferogram (adapted from Subramanian and Rodríguez-Saona, 2009 in Infrared Spectroscopy for Food Quality Analysis and Control, Chapter 7).

1.1.3. Infrared Spectroscopy Work Modes

Depending on the way that infrared radiation interacts with the sample, two modes of work with infrared spectroscopy can be defined (Stuart, 2005; Naumann, 2006; Alvarez-Ordoñez et al., 2011) Transmission and Reflectance methods (Attenuated Total Reflectance spectroscopy, Specular Reflectance spectroscopy, Diffuse Reflectance spectroscopy) (Stuart, 2005; Subramanian and Rodríguez-Saona, 2009). A third way of working is Infrared Spectroscopy is using a microscope coupled to a **FTIR**, called **FTIR** microspectroscopy.

1.1.3.1.Transmission Methods

Transmission spectroscopy is the oldest and most used straightforward infrared method (Stuart, 2004). This technique is based on de the absorption of infrared radiation at a specific wavenumber as it passes through a sample (Stuart, 2005; Naumann, 2006). Using this approach, it is possible to analyse samples in liquid, solid or gas state using specific transmission cells per each application (Subramanian and Rodríguez-Saona, 2009). For instance, when samples are liquid, fixed and semi-permanent cells filled for instance with syringe port or peristaltic pumps can be used. Some authors have used these systems to perform multi-component analysis such as alcoholic strength, total acidity, volatile acidity, pH, malic and lactic acid, glucose and fructose content for wine and must (Patz et al., 1999; Nieuwoudt et al., 2004; Patz et al., 2004; Lachenmeier, 2005; Triebel et al., 2007). Transmission spectroscopy has been also used to analyse microorganisms (Foster et al., 2004). For instance, Baldauf et al., (2007) used transmission **MIR** spectroscopy to differentiate *Salmonella enterica* serovars and Dziuba et al., (2006) to identify lactic acid bacteria at genus level.

1.1.3.2.Reflectance Methods

Reflectance methods are mainly divided into two groups depending if the reflexion of the **IR** light is internal or external. Attenuated total reflectance (**ATR**) is a type of internal reflectance technique based on the total internal reflection phenomenon. 2011). Studied sample is placed onto crystal with a high refractive index (higher than studied sample) and when the IR radiation interacts with the crystal, an evanescent wave is

produced which penetrates into the sample and into the detector (Naumann, 2006; Stuart, 2006; Smith, 2011). **Figure 1.6** shows a diagram of **ATR** work mode:

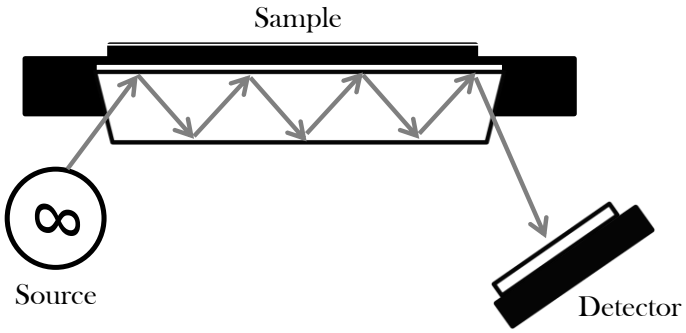

Figure 1.6. Schematic diagram of **ATR** transmission **FTIR** sampling techniques (adapted Stuart, 2004).

Reflectance techniques may be used for samples that are difficult to analyse by the conventional transmittance methodology. The intensity of the reflected light depends on the sample preparation size, the shape of its particles and its molecular organization (Smith, 2011). Unlike transmittance, reflectance involves easier and faster sample preparation, the depth of radiation penetration into the sample is not exactly known and the surface of the sample influences the spectra more than the interior (Subramanian et al., 2006; Subramanian et al., 2007).

Attenuated total reflectance (**ATR**) is a type of reflectance technique based on total internal reflection (Stuart, 2005; Naumann, 2006; Smith, 2011; Alvarez-Ordóñez et al., 2011). The **IR** radiation travels from a medium with higher refractive index (**ATR** crystal) to a medium with lower refractive index (sample), and some amount of light is reflected back into the low refractive index medium (Stuart, 2005; Smith, 2011). The depth of penetration of **IR** radiation in the **ATR** crystal is a function of wavelength (λ), the refractive index of the sample (n_1) the refractive index of the crystal, (n_2) and the angle of incident radiation, (θ). The depth of penetration (d_p) for a non-absorbing medium is given by the following **Equation 1.2** (Stuart, 2005):

$$d_p = (\lambda/n_1)/(2\pi[sin\theta - (n_1/n_2)^2]^{\frac{1}{2}}) \qquad (1.2)$$

The **ATR** crystals are selected by their low solubility in water and their high refractive index. The most used crystals are zinc selenide (**ZnSe**), germanium (**Ge**), diamond or thallium/iodide (**KRS-5**) (Stuart, 2005). Their properties are shown in **Table 1.1.**

Table 1.1. Main properties of common **ATR** crystals (adapted from Smith, 2011).

ATR Crystal	Refractive index	Wavenumber Range	pH Range
ZnSe	2.42	15 000 – 600	5 – 9
Ge	4.00	5 500 – 600	1 – 14
Diamond	2.42	30 000 – 2 200 2000 - 400	1 - 14
KRS-5	2.37	20 000 - 250	5 - 8

Numerous researchers have used **ATR** spectroscopy combined with chemometrics to analyse microorganisms being the **ZnSe** crystal one the most suitable for this application (Borel et al., 1993; Sivakesava et al., 2004; Branan et al., 2007). For example, Suci et al., (1998) studied the interactions between antimicrobial agents and bacterial biofilm formation. Kümmerle et al., (1998) obtained fast and reliable identification of food-borne yeasts developing a standardized sample preparation procedure and to select the most significant spectral windows for efficient identification. Gupta et al., (2007) analysed food-borne pathogens such as *Escherichia coli* O26, *S. typhimurium, Yersinia enterocolitis* and *Shigella boydii*. It is worty to mention that **ATR** spectroscopy is generally used to analyse samples with high water content (Friedel et al., 2013).

Diffuse reflectance is based on external reflectance measurements when the **IR** beam is reflected back from the sample surface in random direction, involving both absorption and scattering (Stuart, 2005; Naumann, 2006; Subramanian and Rodríguez-Saona, 2009). The scattering is due to the rough sample surface and this technique is well suited for highly scattering samples such as freeze-dried biological samples or powder (Naumann, 2006). Diffuse reflectance spectra could be nosier than **ATR** spectra due to light losses when **IR** radiation is reflected from rough surface (Smith, 2011). The packaging density and particle size influence the intensity of the output beam and hence the spectral intensity (Subramanian and Rodríguez-Saona, 2009). A diffuse reflectance

accessory employs two flat mirrors to direct the light and one concave focusing mirror exactly above the sample to concentrate the **IR** beam on to the sample (Subramanian and Rogríguez-Saona, 2009). A diagram of Reflectance sampling technique is shown in **Figure 1.7**.

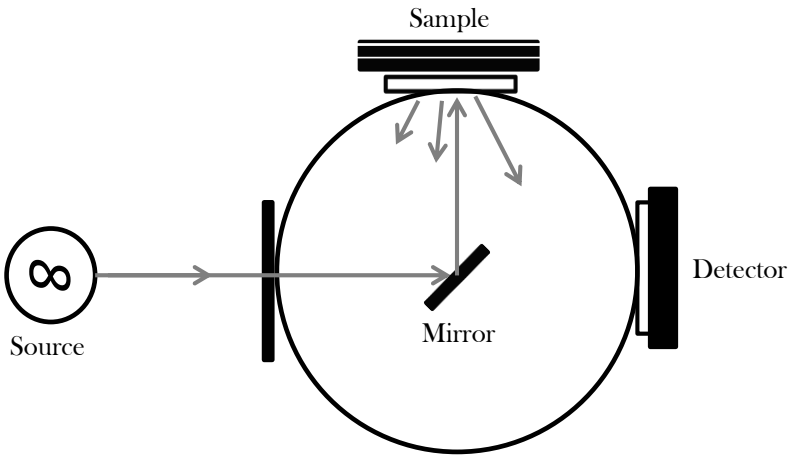

Figure 1.7. Schematic diagram of Diffuse Reflectance transmission **FTIR** sampling techniques (adapted from Subramanian and Rodríguez-Saona, 2009 in Infrared Spectroscopy for Food Quality Analysis and Control, Chapter 7).

Diffuse reflectance technique has been also tested to study microorganisms in different food matrices (Goodacre et al., 2000; Rodríguez-Saona et al., 2004). When this technique is used to differentiate bacteria, the spectra obtained shows the entire cell biochemistry (Winder et al., 2004a). Nonetheless, some studies have stated that bacteria differentiation at strain level is more suitable when **IR** spectra are collected using **ATR-FTIR** (cell surface chemistry) rather than diffuse reflectance spectroscopy (Winder et al., 2004ab).

1.1.3.3.**FTIR** Microspectroscopy

This technique combines an infrared spectrometer with a microscope in order to study very small sample quantities (Stuart et al., 2005). **FTIR** microspectroscopy offers high quality spectra recorded from much less sample material. For microbiological analysis, this technique can reduce the incubation time from approximately 24 h to 6-10 h (Choo-Smith et al., 2001; Maquelin et al., 2003). For example, Yu et al., (2005) used **FTIR** microspectroscopy to discriminate and detect pathogenic strains of S. *enteritidis*, S. *typhimurium*), *E. coli* (serotypes O26, O27 and O157:H7), *Y. enterocolitica* and *Shigella*

boydii. Wenning et al., (2002) used **FTIR** microspectroscopy to identify a total number of 63 yeast strains.

1.2. Multivariate Analysis

Multivariate analysis is a statistical method used to analyse multiple variables of data such as **IR** spectra data applying mathematical models (Miller and Miller, 2005; Naumann, 2006; Oliveri et al., 2011). Multivariate analysis is divided into two different categories, unsupervised and supervised (Beebe et al., 1998; Brereton, 2003; Avarez-Ordóñez and Prieto, 2012) depending on the previous knowledge of the data analysed.

Unsupervised techniques do not require dependent variable for modeling. Instead, these techniques search for patterns among the independent variables, and different group of samples are formed based on the structure of variables (Anzanello et al., 2014ab). Some examples of unsupervised methods are Principal Component Analysis (**PCA**) and Hierarchial Cluster Analysis (**HCA**), among others (Dunn and Wold, 1995; Kansiz et al., 1999; Brereton, 2003; Naumann, 2006; Alvarez-Ordoñez et al., 2011; Anzanello et al., 2014). Supervised multivariate methods are applied on two groups of variables (independent and dependent) establishing a relationship between independent and dependent variable, yielding a model to classify new samples into categories (Huang et al., 2015). Some examples of supervised methods are K-Nearest Neighbor (**KNN**), Linear Discriminant Analysis (**LDA**) and Soft Independent Modeling Class of Analogy (**SIMCA**), among others (Dunn and Wold, 1995; Kansiz et al., 1999; Brereton, 2003; Naumann, 2006; Naumann, 1991; Anzanello et al., 2014ab). Moreover, for quantitative prediction, multivariate regression methods such as Partial Least Squares Regression (**PLSR**) and Principal Component Regression (**PCR**) may be used (Haaland and Thomas, 1988; Brereton, 2003; Miller and Miller, 2005).

1.2.1. Unsupervised Techniques

1.2.1.1.Principal Component Analysis

Principal Component Analysis (**PCA**) is based on the assumption that high variance value or high variability is synonymous of high amount of information (Dunn

and Wold, 1995; Brereton, 2003). Therefore, **PCA** algorithms search for the maximum variance direction in the multidimensional space originated by all the data analysed. The direction in which exists the high variance represents the first principal component (**PC**), the direction that contains the maximum information after the first **PC**, and at the same time is orthogonal to the first is called the second component. The process continues until desired variance is explained or until all variance is explained (Jolliffe, 1982). All the studied samples could be projected in the space created by the new **PC**, and the coordinate values are called scores. At the same time, the **PC**s are expressible as a lineal combination of the original variables and the coefficients which multiply each variable are called loadings (Dunn and Wold, 1995). **PCA** has been used in **NIR** and **MIR** spectroscopy by numerous authors in the last decade (Kansiz et al., 1999; Rodríguez-Saona et al., 2001; Lin et al., 2004; Al-Qadiri et al., 2008; Sundaram et al., 2012).

1.2.1.2.Hierarchial Cluster Analysis

In Hierarchial Cluster Analysis (**HCA**) the clustering of samples is based in inter-object distances in high dimensional space (Miller and Miller, 2005). Different distances measurements such as Euclidean and Mahalanobis could be used to determine the similarity between samples (Naumann, 2006). Sample classification by **HCA** involves calculating the distance between pairs of samples and grouping the samples with smallest distance into the same cluster. Then, the distance of all the remaining samples and this first clusters is calculed, and samples with the closest intercluster distance are grouped in a new single cluster. This procedure is repeated different times until all samples are grouped in a cluster (Naumann, 2006). Results of **HCA** are shown in dendrograms helping the identification of groups of similar individuals (Sandt et al., 2003).

In infrared spectroscopy, **HCA** has been also used to discriminate microorganisms (Kümmerle et al., 1998; Gomez et al., 1999; Ngo Thi and Naumann, 2007; Alvarez-Ordoñez and Prieto, 2010). Nonetheless, some studies have shown that **HCA** was not useful to differentiate bacteria such as *Lactobacillus* species analyzed by**FTIR** spectroscopy (Curk et al., 1994; Oust et al., 2004).

1.2.2. Supervised Techniques

1.2.2.1.Soft Independent Modeling Class Analogy

Soft Independent Modeling Class Analogy (**SIMCA**) is a classification methodology based on individual Principal Component Analysis modelling of each class that can classify samples according to the training sample data (Brereton, 2003; Alvarez-Ordóñez et al., 2011; Al-Qadiri et al., 2008). **SIMCA** technique is used to predict unknown samples and three different situations could be presented: a new sample may be classified as no belonging to any of the set of known classes, a new sample fits to one of the pre-defined classes, or finally fit to more than one pre-defined classes (Ergon et al., 2002; Naes, et al., 2001). Moreover, **SIMCA** also allows knowing the important variables in the training sample (Brereton, 2003). The outputs are mainly class pojections, interclass distances, misclassifications and discriminating power (Brereton, 2003). Class projections are three dimensional representations of the samples clusters where 95% probably clouds are built around the clusters based on **PCA** scores (Subramanian et al., 2007; Grasso et al., 2009). Misclassifications algorithm indicates that the training set is homogeneous and all samples are correctly classified into their corresponding category. Interclass distances (**ICD**) are Euclidian distances between centers of clusters, and above 3.0 are considered significant to identify two clusters as different classe (Dunn and Wold, 1995; Brereton, 2003). Discriminating power is a measure of variable importance in infrared frequencies and contributes to the development of the classification models (Brereton, 2003).

SIMCA have been applied to discriminate, classify and predict vegetative cells and spores (Whittaker et al., 2003; Baldauf et al., 2006; Manning et al., 2008; Kansiz et al., 2009; De Lamo-Castellví and Rodríguez-Saona et al., 2011; Grewal et al., 2015).

1.2.3. Signal Pre-Processing

The signals generally required to be suitably pre-treated since the analytical information is not the exclusive component due a number of different variations generally affect signals. Due to these unwanted variations, signal pre-processing aimed improves the quality of signals and the conversion of data to valuable information (Pico, 2012). In particular the main three objectives of data pre-processing are reduction of random noise,

reduction of systematic unwanted variations and reduction of data size. The techniques for reduction random noise include the moving average filters, the Savitzky-Golay smoothing (Savitzky and Golay, 1964) and Fourier Transform based filters (Reis et al., 2009).

In order to minimize or remove unwanted systematic effects a number of mathematical methods should be employed, such as the standard normal variate (**SNV**) transform and derivatives. **SNV** or row auto-scaling is particularly applied in spectroscopy since it is useful to correct baseline shifts and global intensity variations (Barnes et al., 1989). Each signal is row-centred by subtracting its mean from each single value, and then scaled by dividing the signal standard deviation. After the transformation, each single signal presents mean equal to zero and standard deviation equal to one. **SNV** has the peculiarity of possibly shifting informative regions along the signal range, so should be performed with caution (Fearn et al., 2009). Derivatives are the main pre-processing techniques used in order to reduce random noise and unwanted systematic effects in spectroscopy. Derivatives profiles usually exhibit an increased apparent resolution of overlapping bands and may accentuate small structural differences between nearly identical signals (Taavitsainen et al., 2009). First derivative of a signal, in our case a spectrum signal ($y=f(x)$), consist in the change of y with x ($y'=\mathrm{d}y/\mathrm{d}x$). The way to interpret the first derivative is as the slope of the tangent line to the signal, providing a correction for the baseline shift (Pico, 2012). Otherwise, the second derivative could be understood as a further derivative of the first derivative ($y''=\mathrm{d}^2y/\mathrm{d}x^2$) representing a measure of the curvature of the original signal. The transformation allows the correction of both baseline and shifts. But second derivative signal pre-processing also have a disadvantage, which is the enhancement of the random noise of the signal.

The analysis of microorganism by **NIR** or **MIR** spectroscopic methods has yielded to high dimension vectors containing a multiple dependent variables (Shaw et al., 1999). Therefore, advanced data pre-processing and statistical analysis are required to distinguish minor differences in the spectral features (Lu et al., 2011). Different options of data processing have been selected by researchers working with **FTIR** spectroscopy. For instance, Yu et al., (2004) used first derivative instead of the original spectra to enhance enhancing the important **IR** bands of different microorganisms and to amplify minor differences in the spectra. Wenning et al., (2002) used second derivative for a better identification of yeasts analysed by identification by microspectroscopy. These authors

also applied smoothing to diminish the effects of baseline shifts and to enhance the resolution of complex bands.

1.3. *Saccharomyces cerevisiae* Cell Wall Structure and Composition

Saccahromyces cerevisiae cells are well known to have an important role on the production of a variety of fermented foods and beverages such as bread, beer and wine (Querol and Fleet, 2006; Braconi et al., 2011). *S. cerevisiae* cells are eukaryotics and have mainly two envelopes: the cell wall and the cellular membrane separated by the periplasmic space (Braconi et al., 2011). Their cell wall is composed of a 10 nm thick layer of polysaccharides, predominantly β-glucans and mannoproteins. It serves as the interface between the cell and the neighbouring environment providing osmotic and physical protection and determining the shape of the cell (Klis et al., 2002). The major load-bearing polysaccharide is a moderate branched **β**(1→3) glucan (Fleet, 1991; Kitagaki et al., 1997). Due to the presence of side-chains, **β**(1→3) glucan molecules can only locally associate through hydrogen bonds, resulting the formation of a continuous, three dimensional network. In **Figure 1.8** schematic representation of cell wall components is shown:

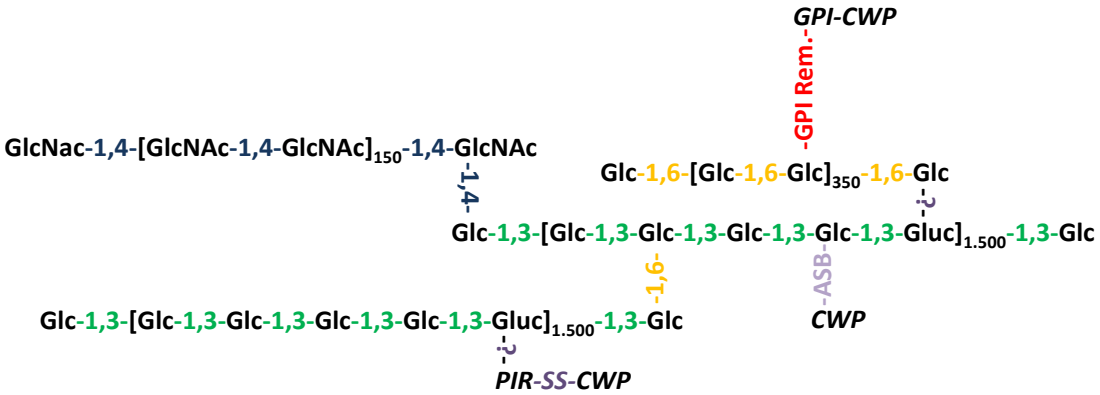

Figure 1.8. Schematic representation of cell wall components and their linkages. The **β**(1→3), **β**(1→4) and **β(1→6)** glucosidic bonds are represented as green, blue, and orange respectively. GlcNac: N-acetylglucosamine; **CWP**: Cell wall proteins; **PIR**: proteins; **ASB**: Alkali-sensitive bonds; **GPI**: Glycosyl-phosphatidylinositol; GPI Rem: remnant GPI anchor. (adapted from Lesage et al., 2006).

Numerous studies have shown that the cell wall external protein layer has at least 20 different glycoproteins and its composition, structure and thickness may vary depending on growing and environmental conditions (Shimoi et al., 1998; Aguilar-

Uscanga et al., 2003; De Groot et al., 2004). For instance, all these cell wall components contribute substantially to the chemical and sensory profile of wines (Braconi et al., 2011).In **Table 1.2** is shown *S. cerevisiae* cell wall components:

Table 1.2. Macromolecules of the *S. cerevisiae* cell wall from the outside to the inside (adapted from Klis et al., 2006).

Macromolecule	% of wall mass	Mean Mr (DP)(kDa)
Mannoproteins	30-50	Highly variable
β(1→6) Glucan	5-10	24 (150)
β(1→3) Glucan	30-45	240 (1500)
Chitin	1.5-6	25 (120)

Mannoproteins can be linked to **β**(1→6) glucose chains through a processed glycosylphosphatidylinositol (**GPI**) anchor or to **β**(1→3) glucans through an alkali-labile bond (Kollar et al., 1995; Kollar et al., 1997; Kapteyn et al., 1999; Dijkgraaf et al., 2002; Klis et al., 2002).

Chitin is a linear polymer of **β**(1→4) linked to N-acetylglucosamine (**GlcNAc**) that forms microfibrils stabilized by hydrogen bonds. The lengths of chitin chains in the cell wall and in bud scars are estimated to be approximately 100 and 190 **GlcNAc** residues, respectively (Kang et al., 1984; Klis et al,. 2002). In the cell wall, about 40 to 50% of the chitin chains are linked to the non-reducing end of **β**(1→3) glucan via a **β**(1→4) bond engaging the reducing end of the chitin polymer (Kollar et al., 1995). Its crystalline structure confers stretching resistance to the cell wall.

1.3.1. Wine Definition and Fermentation Processes

Wine is a complex mixture of chemical compounds in a hydro-alcoholic solution (Ribéreau-Gayon et al., 2006). These chemical compounds contribute to the wine characteristics such as colour, aromatic properties, phenol composition, and mouth feeling among others. Moreover, the wine characteristics also depend on other factors such as viticulture and oenological practices, grape variety, grape maturation, storage conditions, oxygen management and yeast strain used during the alcoholic fermentation

(Esteruelas et al., 2015; Hernandez-Orte et al., 2015; Kizildeniz et al., 2015). Grapes are the raw material used to elaborate wine and are pressed or macerated to avoid or increase the contact area between liquid phase and the grape skins to produce different types of wines, white and red. (**Figure 1.9**).

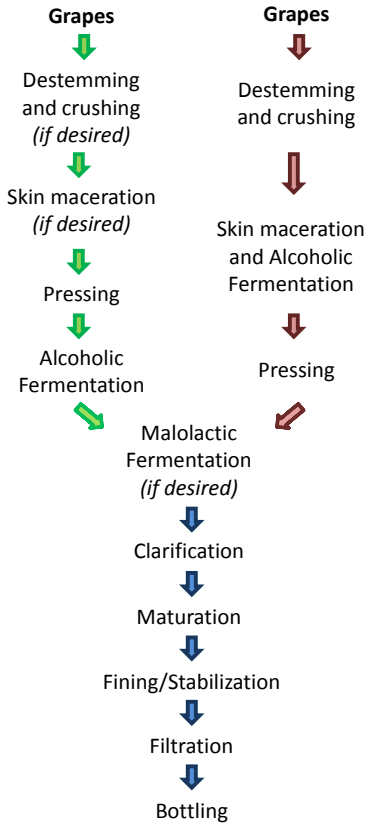

Figure 1.9. Main steps outline of white and red wine production process from white and red grapes respectively (adapted from Pretorius et al., 2000). Green: white wine; Red: red wine and Blue: common steps in white and red.

Grape must is mainly composed by sugars (glucose and fructose) and organic acids (tartaric, malic and lactic acids and in less amount succinic and ceto acids). The main process in wine production involves the transformation of grape must into wine and happens through an alcoholic fermentation (**AF**) perform by one or more strains of yeast, typically *S. cerevisiae* (Sumby et al., 2014). The main role of yeasts during the fermentation process is the biotransformation of sugars into ethanol and carbon dioxide. In 1890, Müller-Thurgau introduced the concept of inoculating pure yeast strains for wine fermentation. This revolutionary strategy was vastly applied by wine producers around the world (Pretorius et al., 2000) challenging the tension existing between tradition and

innovation (Pretorius et al., 1999). Nowadays, the focal point in winemaking for innovation is using genetically modified grape cultivars and wine yeast (Pretorius et al., 2000). **AF** could be performed by other species of yeast called Non-*Saccharomyces* wine species such as *Hansensiaspora, Lachancea, Phichia* among others. Recent studies have shown that these species mainly present in grape skins are able to carry on the **AF** and provide the wines with other desirable properties or attributes such as release of mannoproteins (Domizio et al., 2014), lowering alcohol levels by sugar respiration (Quiros et al., 2014) and release of cell wall polysaccharides (Giovani et al., 2012).

After the **AF**, a secondary fermentation also known as malolactic fermentation (**MLF**) could be performed. It is carried out by lactic acid bacteria (**LAB**), typically *Oenococcus oeni* (Carr et al., 2002). It is more common in red wines than in white wines, but is often undertaken. The **MLF** involves the bioconversion of malic acid to lactic acid and carbon dioxide, producing an important impact on wine acidity. **LAB** of grape must and wine belong to the genera Lactobacillus, *Leuconostoc, Oenococcus* and *Pediococcus* (Rebéreau-Gayon et al., 2006).

In white wines, a second alcoholic fermentation could be performed in order to obtain sparkling wines. Sparkling wines produced by the traditional Méthode Champenoise require a second in-bottle alcoholic fermentation of a base wine (Kemp et al., 2014). A mixture of sugars and yeast is added to white wines in order to produce small quantities of ethanol and carbon dioxide. Comparing the first and the second alcoholic fermentations, the second one is performed in a medium with elevated ethanol content and increasing the carbon dioxide pressure summiting the yeast cells to an altered environmental (Ganss et al., 2011). This second fermentation is followed by prolonged ageing in contact with yeast cells, also know lees. During the ageing time, the autolysis of yeast occurs, releasing different compounds as mannoproteins and polysaccharides that modify the organoleptic properties of the wine increasing the roundness and flavour characteristics (Kemp et al., 2014).

Another microorganism could play an important role in wine quality, acetic acid bacteria (**AAB**). They are very prevalent and can grow and adapted in sugar and alcohol rich media. Their principal propriety is the oxidation of ethanol into acetic acid in two different steps: firstly to acetaldehyde and secondly to acetic acid. **AAB** belong to *Acetobacteraceae* family, which could be separated into the genera: *Acetobacter,*

*Gluconobacter, Gluconacetobacter*among others. (Drysdale and Fleet, 1989; Henick-kling, 1993; Bartowsky et al., 2002; Matthews et al., 2004)

1.3.1.1.Nutrient Supplementation

One of the main environmental variations that yeast could experiment during the alcoholic fermentation is the addition of several additives to the must by winemakers, being nutrients one of the most common products (Gobbi et al., 2013). The availability of diverse nutrients in grape musts as vitamins, sterols, unsaturated fatty acids, pantothenic acid, or nitrogen is often limited and one of the most restricting yeast cell growth factor (Bely et al., 1990ab). In the case of grape must nitrogen, its concentration could vary from 60 to 500 mg/L of yeast assimilable nitrogen **(YAN)** depending on many factors such as climate conditions, viticulture practices, grape variety, grape processing (Bely et al., 1990ab). In fact, nitrogen between 120 and 180 mg/L of **YAN** is considered sufficient concentration for yeast to carry on a proper alcoholic fermentation (Bely et al., 1990a; Sablayrolles et al., 1996).

The most common methodologies to analyse total nitrogen content in musts are distillation and back-titration according to the Kjeldahl mineralization method (Scheiner, 1976) and ammonia concentration measured using an enzymatic method (Francis, 2006) and the concentration of free amino acid nitrogen determination using a spectrophotometric assay (Munik et al., 2002). The addition of ammonium and amino acids in terms of nitrogen represents the yeast assimilable nitrogen (**YAN**) (Dukes et al., 1998).

Numerous studies were carried out to study the effects of nutrient supplementation in different parameters such as alcoholic fermentation kinetics; H_2S evolution and aroma profile production (Ugliano et al., 2010; Bohlscheid et al., 2011; Ugliano et al., 2011; Barbosa et al., 2012; Gobbi et al., 2013) or also in order to study de contribution of yeast fermentation metabolites to the aromatic profile of wine in function of the nutrient availability (Carrau et al., 2008; Barbosa et al., 2012; Mouret et al., 2014; Rollero et al., 2015). These studies have concluded that nitrogen supplementation have an important effect on these parameters an also have pointed out that it is important to consider the source of nitrogen used (organic such as glutathione or inorganic such as ammonium phosphate) (Gobbi et al., 2013) as well as the alcoholic fermentation step and

yeast physiological growth phase (Schulze et al., 1996; Beltran et al., 2005; Brice et al., 2014; Lage et al., 2014).

1.3.1.2. Non-*Saccharomyces* Wine Yeast Species

As has been aforementioned above, *S. cerevisiae* has been traditionally used for their oenological properties to successfully produce alcoholic fermentations transforming grape sugar into alcohol and carbon dioxide (Jolly et al., 2006). But other indigenous Non-*Saccharomyces* yeast species can be also present in the grape must and frequently in greater numbers that *S. cerevisiae*. These yeasts are commonly well adapted to this specific environment and present in active growing phase (Cray et al., 2013). Originally, these Non-*Saccharomyces* yeast species have been considered responsible of microbial problems in wine production, mainly due to their isolation from spoiled wines (Le Roux et al., 1973; De Benedictis et al., 2011; Dashko, et al., 2015). Wineries performing spontaneous fermentations, consider autochthonous yeast a key factor of their winemaking process for their role on providing desired and distinct regional characteristics to their wines (Romano et al., 1996; Romano et al., 1998) and for improving wine characteristics such as mouth-feel, complexity and integration of flavours (Gil et al., 1996; Soden et al., 2000; Varela et al,. 2009; Dashko et al., 2015).

The origin of *Non-Saccharomyces* yeast species is diverse, within the winemaking environment grape berry surfaces, cellar equipment surface and grape could be considered specific niches where wine-related yeasts form communities (Polsinelli et al., 1996; Gayevskiy and Goddard, 2012). Despite the variables in grape harvest and wine production, yeast species are generally found on grapes and in wines similar through the world (Amerine et al., 1967; Longo et al., 1991; Constantí et al., 1997; Jolly et al., 2006).

Otherwise, there are other Non-*Saccharomyces* with a recognized spoilage action in winemaking process as *Dekkera/Brettanomyces*. This specie is normally left out of the description of Non-*Saccharomyces* or autochthonous yeasts description. *Dekkera/Brettanomyces bruxellensis* is considered a one of the main microorganisms with a spoilage action in wine, and responsible of 4-ethylphenol and 4-ethylguayacol off-flavours related to medicinal, barnyard or sewage descriptors becoming a global problem for the global wine industry (Curtin et al., 2013; Sturm et al., 2015). When favourable conditions are conducive for growth of *Dekkera Brettanomyces* in wine, they efficiently

convert non-volatile hydroxycinnamic acids into aroma-active ethylphenols, and thereby reducing the quality of the wine.

1.4. Conventional Techniques to Study Yeast

Wine microbial ecology has been well studied due to its impact and importance in the wine final quality (Pretorius, 2000; Fleet, 2003; Barata et al., 2012; Pinto et al., 2015). Traditional methods for detecting and quantifying different wine microorganisms are based on morphological and physiological tests that help to determine enzyme production profiles and growth characteristics, respectively (Barnett et al., 2000).

Yeast can be identified and classified following the schemes described by Barnett et al., (2000). Several tests are needed to be applied to identify most yeast at specie level, requiring time-consume techniques and considerable expertise for an accurate interpretation of the results. The first microbiological test, is the examination of the microorganism morphology shape (cocci, rods, pointed ends, bowling pin, egg, ogival, elongated, groups or chains), size and arrangements (single, pairsm tetrads, proups or chains) of the cells using a phase contrast microscope. This test can lead to misinterpretations due to the fact that cell morphology or cell appearance can change during cell aging and depending on the culture conditions used to grown these cells (Andorrà, 2010). Moreover, in order to correctly analyse the cell morphology it is important to consider the characteristics of the colonies created in a specific medium like shape (circular, irregular or rhizoid), size (dimensions as well), topography (flat, raised, convex, concave or umbonate), presence of pigments, opacity (transparent, translucent or opaque), surface (smooth, rough, dull or glistening), edge (entire, undulated, lobate, denate or rhizoid) or any changes to agar medium (Fugelsang et al., 1997; Fugelsang, 2007). Once isolated, unknown microorganisms can be characterized using physiological traits such as the assimilation of carbon and nitrogen sources, the carbohydrate fermentation and the formation of carbon dioxide from sugars among other numerous tests (Fugelsang, 1997; Fugelsang et al., 2007).

In the wineries, the traditional methods used to analyse qualitatively yeast or bacteria are counts under the microscope and counts direct plating. Both techniques had advantages and disadvantages.

Microscope technique is faster than plating but require a minimum of 10^4 cell x ml^{-1}, lower populations needs to be concentrated. Otherwise, lower population densities, direct plating is normally used. Counting by microscope consist of quantification using a microscope counting chamber, such as Neubauer chambrer (Rodríguez et al., 1993). Themain drawbacks are that by this technique the cells are quantified without distinction and that the final counts include viable, non-viable and dead cells. Otherwise, plate enumeration consists of growing different microorganisms in selected media, and after that, counting the colonies formed (Andorrà, 2010). To overcome these limitations, plating media need to be used with selective media to suppress the growth of undesirable species. Lysine agar is an example of a selective medium which prevents *S. cerevisiae* from growing because this yeast is unable to grow if Lysine is the sole nitrogen source (Angelo and Siebert, 1987). Otherwise, this medium is effective at isolating and enumerating Non-*Saccharomyces* yeast. Plating techniques takes a long time to growth of different microorganisms, yeast usually need 48 h and **LAB** or **AAB** might need 5 to 10 days (Andorrà, 2010).

Unlike the classic techniques, molecular methods can genotype, identify and quantify the various wine microorganisms as a function of their variability in the genome. Compared with the previous traditional methods, these are generally, faster, more specific, more sensitive, more accurate and more expensive (Andorrà, 2010). Modern molecular techniques methods such as Random Amplified Polymorphic **DNA (RAPD-PCR)** will correctly differentiate at strain level (Sohier et al., 1999). In indirect analysis the sample is plated, the microorganisms are allowed to grow, and then the **DNA** is isolated from the sample and used most often for identification. The analysis of the microorganism's population in these cases is not conducted on the original population; these methods are considered to be indirect methods (Mills et al., 2008). The second way of using molecular methods is to analyse the microorganism's population directly from the sample. A classic example of direct analysis, which has been used from the vineyard through bottling, would be denaturing gradient gel electrophoresis (**DGGE**) where **DNA** is isolated directly from the fermentation or the grape, amplified by **PCR** and analysed by gradient gel electrophoresis without ever having to culture the microorganisms presents in the original sample (Ivey and Phister, 2011). Indirect analyses are typically more sensitive, being able to identify organisms to the strain level. While direct methods are often faster, they are typically less specific, being able to provide the genus and possibly species-level

information for identification. They are however, useful for rapid identification and profiling of communities often being able to detect non culturable organisms (Millet and Lonvaud-Funel, 2000). The major disadvantage of direct methods when compared to the traditional indirect methods is the inability of many direct methods to differentiate viable from dead cells, as both many contain **DNA** or **RNA**. **DNA** is very stable and will often persist long after the cells have died; **RNA** may have a short half-life and, in some cases may be a useful marker for cell viability. The molecular methods used in detection and identification of wine-related microorganisms can be separated in four groups: Hybridization methods, sequencing methods, Fingerprinting methods, **PCR** detection and **QPCR**.

DNA or **RNA** hybridization is most often used as an indirect method to identify various microorganisms and may include simple probes or microarrays to examine similarities in whole genomes. Hybridization is performed by adding a labelled probe of single-stranded **DNA**, specific to a gene or species in the case of total genomic **DNA** probes, to a sample of **DNA** isolated from an unknown organism. The mixture is then heated and the double helix of the **DNA** unravels or denatures to form single strands. After cooling, the helices will begin to reform and if there is similarity, the labelled probe will bind to the unknown **DNA**. Most common methods in this group are Fluorescence in situ hybridization (Indirect/Direct method, allows discrimination from groups to genus and species) (Bilhere et al., 2009; Borneman et al., 2010), Flow cytometry (Direct method, allows discrimination at genus and species level) (Malacrino et al., 2001; Graca da Silveira et al., 2002) and complete genome hybridization (Indirect method, allows from groups to specific strains) (Ivey and Phister, 2011).

In the case of sequencing methods the most commonly used method to identify wine-related microbes is **rDNA** sequencing. It is a powerful tool for rapid and accurate microbial identification and is even used in conjunction with many direct analysis techniques such as **DGGE**. However, it is still common to isolate **DNA** from a colony on a plate and sequence a gene from that **DNA**. The resulting gene sequence is then compared to other genes in a database to identify that colony (Ivey and Phister, 2011).

Fingerprinting methods, in general, examines the whole genome of an organism, often creating a banding pattern by digesting or amplifying regions of the genome, which can be compared between organisms. The fingerprinting methods, such as amplified

ribosomal DNA restriction analysis, can be used to differentiate species. However, most fingerprinting methods are only able to differentiate between strains of a particular organism (Ivey and Phister, 2011). Different Finger printing methods are **RAPD-PCR** (Indirect method, allows strain level discrimination) (Zavaleta et al., 1997; Urso et al., 2008), δ-sequence amplification (Indirect method, allows strain-yeast level discrimination) (Legras et al., 2003; Schuller et al., 2004; Charpentier et al,. 2009), **RFLP mt-DNA** (Indirect method, allows strain-yeast discrimination level) (Schuller et al., 2004), microsatellite (Indirect method, allows strain level discrimination) (Schuller et al., 2004; Gallego et al., 2005) or **DGGE/TGGE** (Direct method, allows species discrimination, but many identity strains depending on targets for **PCR**).

Traditional **PCR** has been used to detect target populations. The targeted gene for the assay can differ greatly among species, but is generally chosen because it is unique to that specie (Zapparoli et al., 1998). A target gene can help ensure specificity, but **PCR** can also be used with just a small random **DNA** sequence that is unique to the species (Ibeas et al., 1996; Ivey and Phister, 2011). **QPCR**, real time polymerase chain reaction (**QPCR**) is similar to traditional **PCR** but incorporates a fluorescent dye, and after each **PCR** cycle, the fluorescence increases. **DNA** amplification is linked to fluorescence in one of two ways, either through the addition of a **DNA** binding dye such as **SYBR** Green or by the addition of a probe labelled with a fluorophore (Ivey and Phister, 2011).

All these techniques can help to know and carry on the wine alcoholic fermentation and winemaking process as a complex microbiological process in which yeast predominate. However, the application of methodologies described above in routine analysis of yeast in the food or winemaking industry is limited by their high cost and the requirement for highly skilled personnel (Wenning et al., 2002).

1.5. Infrared Spectroscpy

1.5.1. Applications of Infrared Spectroscopy for the Wineries

Infrared spectroscopy has been extensively used for routine wine analysis (Dambergs et al., 2015). Numerous physicochemical parameters could be measured using infrared spectroscopy such as ethanol and sugars content, pH value, titratable acidity, volatile acidity, glycerol content and tartaric, malic and lactic acids concentration in musts and wine (Kupina and Shrikhande, 2003). Commonly, **MIR** spectroscopy in transmission mode (Gallignani et al., 1994; Nieuwoudt et al., 2006; Lachenmeier et al., 2007) combined with multivariate analysis has been used for the wine sector as a fast and reliable method for applying simultaneous estimation of multiple physicochemical parameters in wine and must (Wold et al., 2001; Balabin and Smirnov, 2011). **FTIR** equipments developed by the wine sector, most commonly use transmission cuvettes with fixed and variable pathlengths.

When the concentration of the chemical parameter analysed is low (eg. organic acids), using transmission work modes with large pathlengths allow to obtain more reliable results. Nonetheless, for chemical parameters present in higher concentrations such as ethanol and sugars, the results obtained with an **ATR** work mode are comparable or better than those obtained with transmission instruments with long pathlengths (Friedel et al., 2013). Therefore, equipments working with **ATR** work mode have been developed for the wine sector (Shah et al., 2010; Cozzolino et al., 2011; Silva et al., 2014). Moreover, **MIR** spectroscopy has been also used to analyze other components such as phenols and anthocyanins (Laghi et al., 2011; Silva et al., 2014). It is well known that these components are present in grapes and are released from grape skins into the wine during the maceration process (Argyri et al., 2005). While some colour compounds contribute to the taste and sensorial profile of wine (Gawel, 1998; Landon et al., 2008), the colour itself is considered an important marker of the degree of extraction of skin components (Kennedy, 2010). Some methodologies such as **UV-Vis** spectroscopy have been developed over the years to predict wine colour (Harbertson et al., 2003). In addition, there are several assays based on spectrophotometric techniques that allow wineries to obtain the total anthocyanin content, the copigmentation index, the polymeric pigments and the total phenols content in wine in a very easy way (Somers and Evans, 1977; Harbertson et al., 2003).

More recently, spectroscopic techniques combined with multivariate analysis have been used to measure and predict red wine colour (Laghi et al., 2011). In the 1680-900 cm^{-1} region can be found numerous bands originating from wine phenols, among others (Coates et al., 2000; Picque et al., 2001). Wine authentication by **IR** spectroscopy is another important application for the wine sector to prevent adulteration and certify origin (Dixit et al., 2005; Liu et al., 2006; Martelo-Vidal et al., 2013). For instance, Cozzolino et al., (2006) used **MIR** and **NIR** spectroscopy combined with chemometrics to classify wines of different varieties. Martelo-Vidal and Vazquez et al., (2014ab) combined ultraviolet (**UV**), **NIR** and **ANN** analysis as a rapid method to classify wine from different Designation of Origen from Galicia showing that the worst classification was 96.7%.

1.5.2. Hand-held and Portable Infrared Spectrometers

Miniaturization of vibrational spectroscopy components has allowed the development of portable or hand-held systems that are simple to use, require minimal or no sample preparation and performance similar than a laboratory bench top instrument (Mossoba et al., 2012; Mossoba et al., 2014). Hand-held and portable infrared spectrometers are ideal instruments to be used in food quality control, providing sensitivity and portability for in situ analysis. Several applications such as determination quality factors of fruits and vegetables have been studied (Teixeira dos Santos et al. 2014). For instance, Ayvaz and Rodriguez-Saona (2014) have used hand-held and portable infrared spectrometers to screen the acrylamide content in commercial potato chips. Moreover, hand-held infrared spectrometers have been also used to detect food adulteration (Rodríguez-Saona et al., 2011) and food authenticity (Reida et al., 2006). In the wine sector, some portable infrared spectrometers (**UV/NIR)** have been developed mainly to determine grape quality parameters (Carrara et al., 2008). Nevertheless, the potential of using hand-held infrared spectrometers to discriminate between different strains of bacteria or yeast has still not been examined.

1.5.3. Infrared Spectroscopy and Yeasts

Infrared spectroscopy combined with chemometrics has been used by several researchers to identify microbial genera and species with a high degree of confidence (Kümmerle et al., 1998; Kirschner et al., 2001; Sockalingum et al., 2002). In addition, **FTIR** spectroscopy has been proved to be a very sensitive technology to detect subtle biochemical changes in cell composition (Orsini et al., 2000; Galichet et al., 2001; Burattini et al., 2008) leading to the hypothesis that the identification of yeasts at strain level might be possible under well-controlled conditions (Schmalreck et al., 1998; Sockalingum et al., 2002; Sandt et al., 2003). It is important to point out the key role of yeasts on the production of food and beverages (Shapaval et al., 2013ab) and the existing need of developing a rapid method to monitor directly yeast cells during an undergoing fermentation process (Correa-García et al., 2014; Puxeu et al., 2014). Kümmerle et al. (1998) used **MIR** spectroscopy with an **ATR** work mode combined with **HCA** to identify 722 unknown yeast isolates based in 332 food-borne yeast strains spectra library. These authors analysed by **IR** scraped yeast cultures diluted in water and an aliquot was dried out on the surface of the **ZnSe** crystal at 42ºC for 1 h. Timmins et al., (1998) studied the identification of brewing yeast strains and *Candida* species by pyrolysis gas chromatography and infrared spectroscopy obtaining excellent discrimination between yeast species at species and subspecies level. Wenning et al., (2002) compared microspectroscopy and macrospectroscopy technologies to discriminate yeast cells. In this study, the authors used different sample preparation. For the macro spectroscopy analysis used scraped yeast cultures diluted in water. For the microspectroscopy, colonies, grown directly from the agar plate, were transfer the **IR** transparent **ZnSe** carrier to be analysed by **IR**. Under these conditions, microspectroscopy was the technology that offered a higher discrimination between the yeast strains tested. In wine, Burattini et al., (2008) used **FTIR** microspectroscopy in the **MIR** range in transmission and in attenuated total reflectance (**ATR**) mode combined with **PCA** and **HCA** analysis to study the major biochemical changes associated with autolysis of *S. cerevisiae* cells in model wine medium and in a Chardonnay base wine. The comparison performed between **ATR** and transmission modes allowed to conclude that **ATR** measurements were more sensitive to the biochemical changes produced by the autolytic process. Moreover, these authors proposed a list of **IR** bands characteristics of *S. cerevisiae* cells that are shown in **Table 1.3**:

Table 1.3. Relation between absorption bands (cm^{-1}) in the **FTIR** of *S. cerevisiae* and chemical assignments (adapted from Burattini et al., 2008).

Absorption bands (cm^{-1})	Main assignments
~ 2960	$\nu_{asym}CH_3$ lipids
~ 2925	ν_{asym} CH_2 lipids
~ 2890	CH deformation of CH_3 lipid, proteins, and peptides
~ 2875	ν_{sym} CH_3 lipids
~ 2855	ν_{sym} CH_2lipids
~ 1740	C=O stretching in lipid esters
~ 1670	Amide I: C=O vibrations of different protein structures
~ 1550	Amide II: N-H and C-N vibrations of the peptide bond in different protein conformations
~ 1470	CH_2 scissoring in lipids
~ 1455	Various CH_2/CH_3 bending vibrations in lipids and proteins
~ 1440	CH_2 deformation mainly in proteins and peptides
~ 1415	C-O-H in plane bending in proteins
~ 1405	$C(CH_3)_2$ stretching mainly in proteins
~ 1390	C=O of COO^- symmetric stretching in proteins
~ 1370	CH_2 wagging vibrations in lipids and $\beta(1\rightarrow3)$ glucans
~ 1350	CH_2 wagging vibrations in lipids
~ 1340	CH_2 wagging vibrations in lipids
~ 1300	Amide III: C-N and C-O stretching, N-H and O=C-N bending
~ 1240	$\nu_{sym}PO_2^-$ in **DNA**, **RNA** and phospholipids

~ 1215	C-O stretching free nucleotides
~ 1200	C-O-C carbohydrates
~ 1156	C-O, C-OH carbohydrates, various contributions
~ 1135	mannans and **β**(1→3) glucans
~ 1080	$\nu_{sym}PO_2^-$ mainly from **RNA**
~ 1050	mannans
~ 972	mannans
~ 1108	**β**(1→3) glucans
~ 1025	**β**(1→4) glucans
~ 998	**β**(1→6) glucans
~ 915	pyranose ring asymmetric vibrations
~ 905	mannans
~ 880	**β**-glycosidic linkage vibrations
~ 860	**α**-glycosidic linkage vibrations
~ 822	mannans
~ 808	mannans
~ 780	Pyranose ring symmetric vibrations, **GMP** ring stretching

Cavagna et al., (2010) continued using **FTIR** microspectroscopy combined with **PCA** to monitor autolysis of *S. cerevisiae* cells in a base wine. These authors identified seven regions of the *S. cerevisiae* cells spectra most affected by the autolytic process, 3700-2990 cm^{-1} linked to increase of lipids due membrane disorganization, 2875-1670 cm^{-1} associated to degradation of proteins and mannoproteins, 1450-1400 cm^{-1} related to membrane disorganization and degradation of proteins, 1350-1200 cm^{-1} linked to degradation of proteins, increase of free nucleotides and wall polysaccharides, 1050-970 cm^{-1} related to increase mannans and decrease glucans and finally, 970-780 cm^{-1} associated to increase and hydrolysis of mannans and loss of glucose residues.

On the other hand, Adt et al., (2010) studied the potential of using Fourier-transform infrared absorption spectroscopy combined with **PCA** to discriminate *S.*

cerevisiae and *S. bayanus* strains. The main spectral regions taken into account to discriminate between *Saccharomyces* and Non-*Saccharomyces* strains were 700-900 cm^{-1}, 900-1200 cm^{-1}, 1200-1500 cm^{-1}, 1500-1750 cm^{-1} and 2800-3000 cm^{-1} linked to the "fingerprint", polysaccharides, mixed (proteins, nucleic acids, polysaccharides and fatty acids), protein, lipids, and fatty acid absorbing regions, respectively. This study showed the potential of **FTIR** spectroscopy to identify *S. cerevisiae* and *S. bayanus* at the strain level, but stated that the identification at specie level was more difficult to achieve. Finally, Correa-García et al., (2014) used **FTIR** spectroscopy combined with **PCA** to discriminate *S. cerevisiae* yeast cells in two different preparations, fresh and lyophilized. These authors concluded that **FTIR** spectroscopy could be used to discriminate and classify yeast samples based on IR spectral features related to metabolomics changes induced by the type of minimal growth media used regardless the nature of the sample, lyophilized or fresh pellet.

UNIVERSITAT ROVIRA I VIRGILI
APPLICATION OF INFRARED SPECTROSCOPY IN MID-INFRARED RANGE COMBINED WITH MULTIVARIATE ANALYSIS TO STUDY YEASTS INVOLVED IN WINE PRODUCTION
Miquel Puxeu Vaqué

1.6. Aim of the Thesis

The main objective of this work was to evaluate the potential of using attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) in the mid-infrared region combined with multivariate analysis to discriminate, classify and analyse yeasts involved in wine production. This main objective was achieved by fulfilling secondary goals:

- To study the ability of **ATR-FTIR** combined with soft independent modeling of class analogy (**SIMCA**) to discriminate and classify *Saccharomyces cerevisiae* strains.
- To study the biochemical changes of *S. cerevisiae* strains during a fermentation process depending on their physiological phase (lag, exponential and stationary)
- Evaluate the biochemical changes experimented by *S. cerevisiae* during an alcoholic fermentation process when two different sources of nitrogen (organic and inorganic) are added to the grape must
- To obtain mid-infrared spectroscopy profiles of *S. cerevisiae* and Non-*Saccharomyces* wine species.
- To develop multivariate classification and prediction models for discrimination of *S. cerevisiae* and Non-*Saccharomyces* wine species.

1.7. References

Adt, I., Kohler, A., Gognies, S., et al., (2010). FTIR spectroscopic discrimination of *Saccharomyces cerevisiae* and *Saccharomyces* bayanus strains.*Canadian Journal of Microbiology*, 56:793-801.

Aguilar-Uscanga, B., and Francois, J.M. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation.*Letters in Applied Microbiology*, 37:268-274.

Alishahi, A., Farahmand, H., Prieto, N. et al., (2010). Identification of transgenic foods using NIR spectroscopy: A review. *Sprectochimica Acta Part A-Molecular and Biomolecular Spectroscopy*, 75:1-7.

Al-Qadiri, H.M., Lin, M., Al-Holy, M.A., et al., (2008). Detection of sublethal thermal injury in *Salmonella enterica* serotype typhimurium and *Listeria monocytogenes* using Fourier transform infrared (FT-IR) spectroscopy (4000 to 600 cm-1). *Journal of Food Science*, 73:54-61.

Alvarez-Ordoñez, A., and Prieto, M. (2010). Changes in Ultrastructure and Fourier transform infrared spectrum of *Salmonella enterica* serovar typhimurium cells after exposure to stress conditions. *Applied and Environmental Microbiology* , 76:7598-7607.

Alvarez-Ordoñez, A., and Prieto, M. Technical and methodological aspects of Fourier transform infrared spectroscopy in food microbiology Research. In Fourier Transform Infrared Spectroscopy in Food Microbiology, Springer US: 2012; pp 1-18.

Alvarez-Ordoñez, A., Mouwen, D. J. M., Lopez, M., et al., (2011). Fourier transform infrared spectroscopy as a tool to characterize molecular composition and stress response in foodborne pathogenic bacteria. *Journal of Microbiological Methods*, 84:369-378.

Amerine, M. A., Berf, H. W. and Cruess, W. V. Technology of wine Making. 2nfd ed. Westport: Avi Publishing Company Inc. (1967).

Andorra, I. 2010. Adaptation and development of culture-independent techniques for the identification and enumeration of microorganism in wine fermentations. Ph. D. thesis, Universitat Rovira i Virgili, Tarragona, Spain.

Andorra, I., Esteve-Zarzoso, B., Guillamon, J. M. et al., (2010). Determination of viable wine yeast using DNA binding dyes and quantitative PCR.*International Journal of Food Microbiology*, 144:257-262.

Angelo, J., Siebert, K.J. (1987). A new medium for the detection of wild strains in brewing culture yeast,*Journal of the American Society of Brewing Chemist*, 45:135-140.

Anzanello, M. J., and Fogliatto, F. S. (2014a). A review of recent variable selection methods in industrial and chemometrics applications.*European Journal of Industrial Engineering*, 8:619-645.

Anzanello, M.J., Ortix, R.S., Limberger, R., et al., (2014b). Performance of some supervised and unsupervised multivariate techniques for grouping authentic and unauthentic Viagra and Cialis. Egyptian Journal of Forensic Sciences, 4:83-89.

Argyri, K., Proestos, C., Komaitis, M., et al. (2005). Phenolic compounds in red wine digested in vitro in the presence of iron and other dietary factors. *International Journal of Food Sciences and Nutrition*, 56:213-222.

Ayvaz, H., and Rodriguez-Saona, L.E. (2014). Application of handheld and portable spectrometers for screening acrylamide content in commercial potato chips.*Food Chemistry*, 174:154-162.

Ayvaz, H., Plans, M., Riedl, K.M., et al. (2013). Application of infrared microspectroscopy and chemometric analysis for screening the acrylamide content in potato chips. Analytical methods, 5:2020-2027.

Balabin, R. M., and Smirnov, S. V. (2011). Variable selection in near-infrared spectroscopy: Benchmarking of feature selection methods on biodiesel data. *Analytica Chimica Acta*, 692(1–2), 63–72.

Baldauf, N. A., Rodriguez-Romo, L. A., Männig, A., et al. (2007). Effect of selective growth media on the differentiation of *Salmonella enterica* serovars by Fourier transform mid-infrared spectroscopy,*Journal of Microbiological Methods*, 68, 106–114.

Banc, R., Loghin, F., Miere, D., et al., (2014).Romanian Wines Quality and Authenticity Using FT-MIR Spectroscopy Coupled with Multivariate Data Analysis. *Notulae Botanica e Horti Agrobotanici Cluj-Napoca*, 42:556-564.

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *International Jorunal of Food Microbiology*, 153:243-259.

Barbosa, C., Mendes-Faia, A., Mendes-Ferreira, A. (2012). The nitrogen source impacts major volatile compounds released by *Saccharomyces cerevisiae* during alcoholic fermentation. *International Journal of Food Microbiology*, 160:87-93.

Barnes, R.J., Dhanoa, M.S., Lister, S.J. (1989). Standard Normal Variate Transformation and de-trending od Near-Infrared Diffuse Reflectance Spectra. *Applied Spectroscopy*, 43:772-777.

Barnett, J. A., Payne, R.W. and Yarrow, D. (2000). Yeasts: Characteristics and Identification. 3rd edn Cambridge University Press, Cambridge, UK.

Bartowsky, E.J., Francis, I.L., Bellon, J.R. (2002). Is buttery aroma perception in wines predictable from the diacetyl concentration?.*Australian Journal of Grape and wine Research*, 8:180-185.

Batten, G.D. (1998). Plan analysis using near infrared reflectance spectroscopy: the potential and the limitations. *Australian Journal of experimental Agriculture*, 38:697-706.

Beebe, K.R., Pell, R.J., Seasholtz, M.B. Chemometrics: A pactical guide, Johan Wiley & Sons: New York, 1998.

Beltran, G., Esteve-Zarzoso, B., Rozes, N., et al., (2005). Influence of the timing of nitrogen additions during symthetuc grape must fermentations on fermentations kinetics and nitrogen consumption. *Journal of Agricultural and Food Chemistry*, 53:996-1002.

Bely, M., Sablayrolles, J.M. and Barre, P. (1990a). Automatic detection of assimilable nitrogen deficiencies during fermentation in enological conditions,*Journal of Fermentation and Bioengineering*, 70, 246-252.

Bely, M., Sablayrolles, J.M. and Barre, P. (1990b). Description of alcoholic fermentation kinetics, its variability and significance.*American Journal of Enology and Viticulture,* 41, 319-324.

Berthomieu, C., and Hienerwadel, R. (2009). Fourier transform infrared (FTIR) spectroscopy. *Photosynthesis Research*, 101:157-170.

Bilhere, E., Lucas, P.M., Olivier, C., et al., (2009). Multilocus Sequence Tyoing of Oenoccocus oeni: Detection of Two Subpopulations Shaped by Intergenic Recombination. *Applied and Environmental Microbiology*, 75:1291-1300.

Bohlscheid, J.C., Osborne, J.P., Ross, C.F. et al. (2011). Interactive effects of selected nutrients and fermentation temperature on H_2S production by winestrains of *Saccharomyces*.*Journal of Food Quality*, 34:51-55.

Borel, M., and Linch, B.M. (1993). A study of differentiation of living bacteria by ATR FTIR spectroscopy.*Canadian Journal of Applied Spectroscopy*, 38:18-21

Borneman, A.R., Bartowsky, E., McCarthy, J., et al., (2010). Genotypic diversity in *Oenococcus oeni* by hight-density microarray comparative genome hybridization and whole genome sequencing.*Applied microbiology and Biotechnology*, 86:681-691.

Braconi, D., Amato, L., Bernardini, G., et al., (2011). Surfome analysis of a wild-type wine *Saccharomyces cerevisiae* strain.*Food Microbiology*, 28:1220-1230.

Branan, N., and Wells, T.A. (2007). Microorganism characterization using ATR-FTIR on an ultrathin polystyrene layer. *Vibrational Spectroscopy*, 44:192-196.

Brereton, R.G. *Chemometrics: Data analysis for the laboratory and chemical plant.* John Wiley & Sons, Ltd.: Chichester, England, 2003.

Brice, C., Sanchez, I., Tesniere, C., et al., (2014). Assessing the Mechanisms Responsible for Differences between Nitrogen Requirements of *Saccharomyces* cerevisiae Wine Yeast in Alcoholic Fermentation.*Applied and Environmental Microbiology*, 80:1330-1339.

Burattini, E., Cavagna, M., Dell'Anna, R., et al., (2008). A FTIR microspectroscopy study of autolysis in cells of the wine yeast. *Vibrational Spectroscopy*, 47:139-147.

Carr, F.J., Chill, D., and Maida, N. (2002). The lactic acid bacteria: A literature survey. Critical Reviews in Microbiology, 28:281-370.

Carrara, M., Catania, P., Vallone, M., et al,. (2008). Mechanical harvest of grapes: Assessment of the physical-mechanical characteristics of the berry in order to improve the quality of wines. In Proc. Intl. Conf. on Agricultural Engineering: Agricultural and Biosystems Engineering for a Sustainable World (AgEng 2008). Hersonissos, Crete - Greece.

Carrau, F.M., Medina, K., Farina, L., et al., (2008). Production of fermentation aroma compounds by *Saccharomyces* cerevisiae wine yeasts: effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research*, 8:1196-1207.

Cavagna, M., Dell'Anna, R., Monti, F., et al., (2010). Use of ATR-FTIR microspectroscopy to monitor autolysis of *Saccharomyces cerevisiae* cells in a base wine.*Journal of Agricultural and Food Chemistry*, 58, 39–45.

Charpentier, C., Colin, A., Alais, A., et al., (2009). French Jura flor yest: genotype and technological diversity. *Antonie Van Leeuwenhoek Internationl Journal of General and Molecular Microbiology*, 95:263-273.

Choo-Smith, L.P., Maquelin, K., van Vreeswijk, T., et al., (2001). Investigating microbial (micro)colony heterogeneity by vibrational spectroscopy. *Applied and Environmental Microbiology*, 67:1461-1469.

Coates, J. (2000). Interpretation of infrared spectra, a practical approach. In: Meyers, R.A. (Ed.), Encyclopaedia of Analytical Chemistry. John Wiley & Sons Ltd., Chichester, pp. 10815-10837.

Colthup, NB, Daly, L.H., and Wiberly SE. (1975). Introduction to infrared and Raman spectroscopy. Academic Press, New York.

Constantí, M., Poblet, M., Arola, L., et al., (1997). Analysis of yeast populations during alcoholic fermentation in a newly established winery.*American Journal of Enology and Viticulture*, 48:339-344.

Cooley, J.M., and Tukey, J.W. (1965). An Algorithm for the Machine Calculation of Complex Fourier Series. *Mathematics of Computation*, 19:297-301.

Correa-Garcia, S., Bermudez-Moretti, M., Travo, Adrian., et al., (2014). FTIR spectroscopic metabolome analysis of lyophilized and fresh *Saccharomyces cerevisiae* yeast cells.*Analytical Methods*, 6:1855-1861.

Costa, A.M., Costa, M.M., Delgadillo, I., et al., (2015). Astringency quantification in wine: comparison of the electronic tongue and FT-MIR spectroscopy. *Sensors and actuators B-Chemical*, 207:1095-1103.

Cozzolino, D., Cynkar, W., Shah, N., et al., (2011). Feasibility study on the use of attenuated total reflectance mid-infrared for analysis of compositional parameters in wine. Food Research International, 44:181-186.

Cozzolino, D., Parker, M., Dambergs. R.G., et al., (2006). Chemiometrics and visible-near sprectoscopic monitoring f red wine fermentation in a pilot scale. *Biotechnology and Bioengineering*, 95:1101-1107.

Cray, J.A., Bell, A.N.W., Bhaganna, P., et al., (2013). The biology of habitat dominance; can microbes behave as weeds?.*Microbial Biotechnology*, 6:453-492.

Curk, M.C., Peladan, F., and Hubert, J.C. (1994). Fourier-Transform Infrared (FTIR) Spectroscopy for Identifying *Lactobacillus* Species.*FEMS Microbiology Letters*, 123:241-248.

Curtin, C.D., Langhans, G., Henschke, P.A., et al., (2013). Impact of Australian Dekkera bruxellensis strains grown under oxygen-limited conditions on model wine composition and aroma.*Food Microbiology*, 36:241-247.

Dambergs, R., Gishen, M., and Cozzolino, D. (2015). A review of the state of the art, limitations, and prespectives of infrared spectroscopy for the analysis of wine grapes, must, and grape tissue.*Applied Spectroscopy Reviews*, 50:261:278.

Dambergs, R.G., Cozzolino, D., Cynkar, W., et al., (2003). Strategies to minimise matrix-related error with NIR analysis of wine grape quality parameters.In Proc. 11th Intl. Conf. on Near Infrared Spectroscopy, 183-186. A. M. C. Davies and A. Garido-Varo, eds. Chichester, U.K.: NIR Publications.

Dambergs, R.G., Cozzolino, D., Cynkar, W.U., et al., (2006). The determination of red grape quality parameters using LOCAL algorithm,*Journal of Near Infrared Spectroscopy*, 14:71-79.

Dashko, S., Zhou, N., Tinta, T., et al., (2015). Use of non-conventional yeast improves the wine aroma profil·le of Ribolla Gialla. *Journal of Industrial Microbiology & Biotechnology*, 42:997-1010.

De Benedictis, M., Bleve, G., Grieco, F., et al., (2011). An optimized procedure for the enological selection of Non-*Saccharomyces* starter cultures.Antonie Van Leeuwenhoek *International Journal of Generl and Molecular Microbiology*, 99:189-200.

De Groot, P.W.J., de Boer, A.D., Cunningham, J., et al., (2004). Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryotic Cell*, 3, 955–965.

De Lamo-Castellví, S., and Rodríguez-Saona, L.E. (2011). Use of Attenuated Total Reflectance infrared Microspectroscopy to Discriminate Bacillus Spores. Journal of Food Safety, 31:401-407.

Deak, T., and L. R. Beuchat.(1995). Evaluation of the MicroScan enzyme-based system for identification of foodborne yeast.*Journal of Applied Bacteriology*, 79:439-446.

Denise L.D., Janice, A.P. (1999). In Situ Monitoring of an Escherichia coli Fermentation Using a diamond Composition ATR prove and Mid-infrared Spectroscopy. Biotechnology Progress, 15:529-539.

Dijkgraaf, G.J.P., Abe, M., Ohya, Y., et al., (2002). Mutation in Fks1p affects the cell Wall content of beta-1,3- and beta-1,6-glucan in *Saccharomyces cerevisiae*. *Yeast*, 19:671-690.

Dixit, V., Tewari, J.C., Cho, B.K., et al., (2005). Identification and quantification of industrial grade glycerol adulteration in red wine with Fourier transform infrared spectroscopy using chemometrics and artificial neural networks. *Applied Spectroscopy*, 59:1553-1561.

Domizio, P., Liu, Y., Bisson, L.F., et al., (2014). Use of Non-Saccharomyces wine yeasts as novel sources of mannoproteins in wine. *Food Microbiology*, 43:5-15.

Drysdale, G.S., and Fleet, G.H. (1989). The effect of acetic acid bacteria upon the growth and metabolism of yeasts during the fermentation of grape juice.*Journal of Applied Bacteriology*, 67:471-481.

Dufour, E. 2009. . Infrared spectroscopy Spectroscopy for Food Quality Analysis and Control: Principes of Infrared Spectroscopy, Ch. 1. Ed. By Da-Wenn Sun. ISBN 978-0-12-3741363.

Dukes, B. C., and Butzke, C. E. (1998). Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay.*American Journal of Enology and Viticulture*, 49:125-134.

Dunn W.J.; Wold, S. SIMCA pattern recognition and classification.*In:* van de Waterbeemd H. (Ed) Chemometric methods in molecular design. VCH Publishers, NY 1995.

Dziuba, B., and Nalepa, B. (2012).Identification of Lactic Acid Bacteria and Propionic Acid Bacteria using FTIR Spectroscopy and Artificial Neural Networks.*Food Technology and Biotechnology*, 50:399-405.

Dziuba, B., Babuchowski, A., and Nalecz, D. (2007). Identification of lactic acid bacteria using FTIR spectroscopy and cluster analysis.*International Dairy Journal*, 17:183-189.

Dziuba, B., Babuchowski, A., Niklewicz, M., et al., (2006). FTIR spectral characteristics of lactic acid bacteria - a spectral library.*Milchwissensachaft-Milk Science International*, 61:146-149.

Edwards, H. G. M., Munshi, T., and Anstis, M. (2005). Raman spectroscopic characterisations and analytical discrimination between caffeine and demethylated analogues of pharmaceutical relevance.*Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 61:1453-1459.

El-Abassy, R. M., Donfack, P., and Materny, A. (2009). Rapid determination of free fatty acid in extra virgin olive oil by Raman spectroscopy and multivariate analysis,*Journal of the American Oil Chemists' Society*, 86:507-511.

El-Abassy, R. M., Donfack, P., and Materny, A. (2011b). Discrimination between Arabica and Robusta green coffee using visible micro Raman spectroscopy and chemometric analysis. *Food Chemistry*, 126:1443e1448.

El-Abassy, R. M., Eravuchira, P., Donfack, P., et al., (2011a). Fast determination of milk fat content using Raman spectroscopy. *Vibrational Spectroscopy*, 56:3-8.

El-Abassy, R.M., von der Kammer and Materny, A. (2015). UV Raman spectroscopy for the characterization of strongly fluorescing beverages.*Food Science and Technology*, 64:56-60.

Ergon, R., and Esbensen, K.H. (2002). PCR/PLSR optimization based on noise covariance estimation and Kalman filtering theory,*Journal of Chemometrics*, 16:8-10.

Esteruelas, M., Gonzalez-Royo, E., Kontoudakis, N., et al., (2015). Influence of grape maturity on the foaming properties of base wines and sparkling wines (Cava). *Journal of the Science of Food and Agriculture*, 95:2071-2080.

Fearn, T., Riccioli, C., Garrido-Varo, A., et al., (2009). On the geometry of SNV and MSC.*Chemiometrics and Intelligent Laboratory Systems*, 96:22-26.

Fleet, G.H. (1991).Cell walls.In The yeasts.Volume 4.Yeasts organelles; Rose, A. H., Harrison, J. S., Eds; Academic Press: London.

Fleet, G.H., (2003). Yeast interactions and wine flavour. International Journal of Food Microbiology, 86:11-22.

Foster, N.S., Thompson, SE., Valentine, NB., et al., (2004). Identification of sporulated and vegetative bacteria using statistical analysis of Fourier transform mid-infrared transmission data. *Applied Spectroscopy*, 58:203-211.

Fragoso, S., Acena, L., Guasch, J., et al., (2011a). Application of FT-MIR Spectroscopy for Fast Control of Red Grape Phenolic Ripening,*Journal of agricultural and Food Chemistry*, 59:2175-2183.

Fragoso, S., Acena, L., Guasch, J., et al., (2011b). Quantification of Phenolic Compounds during Red Winemaking Using FT-MIR Spectroscopy and PLS-Regression,*Journal of Agricultural and Food Chemistry*, 59:10795-10802.

Francis, P.S. (2006).The determination of urea in wine – a review.*Australian Journal of Grape and Wine Research*, 12:97-106.

Friedel, M., Patz, C., and Dietrich, H. (2013). Comparison of different measurement techniques and variable selection methods for FT-MIR in wine analysis.*Food Chemistry*, 141:4200-4207.

Fugelsang, K.C. (1997). Wine Microbiology. Chapman & Hall, New York, USA.

Fugelsang, K.C., and Edwards, C.G. (2007). Wine microbiology: Practical Applications and Procedures. Springer US, London.

Galichet, A., Sockalingum, G.D., Belarbi, A., et al., (2001). FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype. *FEMS Microbiology Letters*, 197:179–186.

Gallego, F.J., Pérez, M.A., Núñez, Y., et al., (2005). Comparison of RAPDs, AFLPs and SSR markers for the genetic analysis of yeast strains of Saccharomyces cerevisiae. *Food Microbiology*, 22,:561-566.

Ganss, S., Kirsch, F., Winterhalter, P., et al., (2011). Aroma changes due to second fermentation and glycosylated precursors in Chardonnay and Riesling sparkling wines.*Journal of Agricultural and Food Chemistry*, 59:2524–33.

Gawel, R. (1998). Red wine astringency: a review. *Australian Journal of Grape and Wine Research*, 4:74-95.

Gayevskiy, V., and Goddard, M. R. (2012). Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. *ISME Journal*, 6:1281-1290.

Ghosh, S.B., Bhattacharya, K., Nayak, S., et al., (2015). Identification of different species of *Bacillus* isolated from Nisargruna Biogas Plant by FTIR, UV-Vis and NIR spectroscopy. *Spectrochima Acta Part A.: Molecular and Biomolecular Spectroscopy*, 148:420-426.

Gil, J.V., Mateo, J.J., Jimenez, M., et al., (1996). Aroma compounds in wine as influenced by apiculate yeasts. *Journal of Food Science*, 61:1247-1256.

Giovani, G., Rosi, I., and Bertuccioli, M. (2012). Quantification and characterization of cell wall polysaccharides released by Non-Saccharomyces yeast strains during alcoholic fermentation. International Journal of Food Microbiology, 160:113-118.

Gishen, M., and Dambergs, B. (1998). Some preliminary trials in the application of scanning near infrared spectroscopy (NIRS) for determining the compositional quality of grapes, wine, and spirits.*Australian Grapegrower and Winemaker,* 414a:43-45.

Gishen, M., Dambergs, RG., and Cozzolino, D. (2005). Grape and wine analysis - enhancing the power of spectroscopy with chemometrics.A review of some applications in the Australian wine industry.*Australian Journal of Grape and Wine Research*, 11:296-305.

Gobbi, M., Comitini, F., D'Ignazi, G., et al., (2013). Effects of nutrient supplementation on fermentation kinetics, H2S evolution, and aroma profile in Verdicchio DOC wine production. *European Food Research and Technology*, 236:145-154.

Goodacre, R., Shann, B., Gilbert, R. J., et al., (2000). Detection of dipicolinic acid biomarker in *Bacillus* spores using curie-point pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. *Analytical Chemistry*, 72:119-127.

Graca da Silveira, M., Victoria San Romano, M., Loureiro-Dias, M.C. et al., (2002). Flow cytometric assessment of membrane integrity of etanol-stressed *Oenoccoccus oeni* cells. *Applied and Environmental Microbiology*, 68:6087-6093.

Grasso, E. M., Yousef, A.E., de Lamo Castellvi, S., et al., (2009). Rapid Detection and Differentiation of Alicyclobacillus Species in Fruit Juice Using Hydrophobic Grid Membranes and Attenuated Total Reflectance Infrared Microspectroscopy. *Journal of Agricultural and Food Chemistry*, 57:10670-10674.

Grewal, M.K., Jaiswal, P., and Jha, S.N. (2015). Detection of poultry meat specific bacteria using FTIR spectroscopy and chemometrics. Journal of Food Science and Technology-Mysore, 52:3859-3869.

Griffiths, P., and de Haseth, J. A. Fourier transform infrared spectrometry. 2nd Edition ; John Wiley & Sons, Inc.: New York, 2007.

Gupta, M. J., Irudayaraj, J. M., Schmilovitch, Z., et al., (2007). Identification and quantification of foodborne pathogens in different food matrices using FTIR spectroscopy and artificial neural networks. *Transactions of the Asabe*, 49:1249–1255.

Haaland, D.M., and Thomas, E.V. (1988). Partial Least-Squares Methods for Spectral Analyses. 2. Application to Simulated and Glass Spectral Data. *Analytical Chemistry*, 60:1202-1208.

Haff, R.P., Jackson, E. (2008). Low cost real-time sorting of in-shell pistachio nuts from kernels.*Applied Engineering in Agriculture*, 24:487-490.

Harbertson, J.F., Piccotto, E.A. and Adams, D.O. (2003). Measurement of polymeric pigments in grape berry extracts and wines using protein precipitation assay combined with bisulfide bleaching. *American Journal of Enology and Viticulture*, 54:301-306.

Helm, D., Labischinski, H., Schallehn, G., et al., (1991). Classification and Identification of Bacteria by Fourier-Transform Infrared Spectroscopy,*Journal of General Microbiology*, 137:69-79.

Henick-Kling, T. (1993).Malolactic fermentation. In: Wine Microbiology and Biotechnology. Fleet, GH (ed). Pp, 289-326. Harwood Academic Publisher, Chur, Switzerland.

Hernandez-Orte, P., Concejero, B., Astrain, Jesus.,et al., (2015). Influence of viticulture practices on grape aroma precursors and their relation with wine aroma. *Journal of the Science of Food and Agriculture*, 95:688-701.

Huang, S., Yang, D., Ge, Y.G., et al., (2015). Combined supervised information with PCA via discriminative component selection. Information Processing Letters, 115:812-816.

Huang, X., Teye, E., Sam-Amoah, L., et al., (2014). Rapid measurement of total polyphenols content in cocoa beans by data fusion of NIR spectroscopy and electronic tongue.*Analytical Methods*, 6:5008-5015.

Ibeas, J., Lozano, J., Perdigones, L., et al., (1996). Detection of *Dekkera/Brettanomyces* strains in sherry by a nested PCR method. *Applied Environmental Microbiology*, 62: 998-1003.

Ikemoto, Y., Okamura, H., Moriwaki, T., et al., (2015). Improvement of infrared near-field spectrum by asymmetric interferometer configuration,*Japanese Journal of Applied Physics*, 54:DOI: 10.7567/JJAP.5..082402.

Ivey, M.L., and Phister, T.G. (2011). Detection and identification of microorganisms in wine: a review of molecular technique. *Journal of Industrial Microbiology & Biotechnology*, 38:1619-1634.

Jamshidi, B., Mohajerani, E., Jamshidi, J., et al., (2015). Non-destructive detection of pesticide residues in cucumber using visible/near-infrared spectroscopy.*Food Additives and Contaminants Part A – Chemistry Analysis Control Exposure & Risk Assessment*, 32:857-863.

Jolliffe, I.T. (1982). A note on the Use of Principal Components in Regression.*AppliedStatistics Journal of the Royal Statistical Society Series*, 31:300-303.

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2006). The role and use of Non-*Saccharomyces* yeasts in wine production. *South African Society for Enology & Viticulture*, 27:15-38.

Kang, M.S., Elango, N., ;Mattia, E., et al., (1984). Isolation of chitin synthetase from Saccharomyces cervisiae – Purification of an enzyme by entrapment in the reaction product. *Journal of Biological Chemistry*, 259:4966-4972.

Kansiz, M., Heraud, P., Wood, B., et al., (1999). Fourier Transform Infrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains.*Phytochemistry*, 52:407-417.

Kapteyn, J.C., Van Den Ende, H., and Klis, F.M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall.*Biochimica et Biophysica Acta*, 1426: 373–383.

Kemp, B., Hervé, A., Robillard, B., et al., (2015). Effect of Production Phase on Bottle-Fermented Sparkling Wine Quality. *Journal of Agricultural and Food Chemistry*, 63:19-38.

Kennedy, J.A. (2010). Wine colour. Managing Wine Quality, Vol. 1: *Viticulture and wine Qualiy*, 187:73-104.

Kirschner, C.K., Maquelin, P. Pina, N.A., et al., (2001).Classification and Identification of *enterococci*: a comparative phenotypic, genothypic, and vibrational spectroscopy study. *Journal of Clinical Microbiology*, 39:1763-1770.

Kitagaki, H., Shimoi, H., and Itoh, K. (1997). Identification and analysis of a static culture-specific cell wall protein, Tir1p/Srp1p in *Saccharomyces cerevisiae.European Journal of Biochemistry*, 249:343-349.

Kizildeniz, T., Mekni, I., Santesteban, H., et al., (2015). Effects of climate change including elevated CO_2 concentration, temperature and water deficit on growth, water status, and yield quality of grapevine (Vitis vinifera L.) cultivars. *Agricultural Water Management*, 159:155-164.

Klis, F. M., Boorsma, A., and De Groot, P. W. J. (2006). Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*, 23, 185–202.

Klis, F. M., Mol, P., Hellingwerf, K., and Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*, 26(3), 239–256.

Kollar, R., Petrakova, E., Ashwell, G., et al., (1995). Architecture of the yeast cell wall – the linkage between chitin and beta (1-3) glucan. Journal of Biological Chemistry, 270:1170-1178.

Kollar, R., Reinhold, B.B., Petrakova, E., et al., (1997). Architecture of the yeast cell wall beta (1-6) glucan interconnect mannoprotein, beta (1-3) glucan, and chitin. Journal of Bilogical Chemistry, 272:17762-17775.

Kuligowski, J., Quintas, G., Herwing, C. et al., (2012). A rapid method for the differentiation of yeast cell grow under carbon and nitrogen-limited conditions by means of partial least squares discriminant analysis employing infrared micro-spectroscopic data of entire yeast cells. *Talata*, 99:566-573.

Kümmerle, M., Scherer, S., and Seiler, H. (1998). Rapid and reliable identification of food-borne yeast by Fourier-transform infrared spectroscopy.*Applied and Environmental Microbiology*, 64:2207-2214.

Kupina, N.C., Shrikhande, A.J. (2003). Evaluation of Fourier transform infrared instrument for rapid quality-control wine analyses. *American Journal of Enology and Viticulture*, 54:131-134.

Lachenmeier, D.W. (2007). Rapid quality control of spirit drinks and beer using multivariate data analysis of Fourier transform infrared spectra. *Food Chemistry*, 101:825-832.

Lage, P., Barbosa, C., Mateus, B., et al., (2014). H. *Guilliermondii* impacts growth kinetics and metabòlic activity of S. cerevisiae: The role of initial nitrogen concentration. *International Journal of Food Microbiology*, 172:62-69.

Laghi, L., Versari, A., Parpinello, G.P., et al., (2011). FTIR spectroscopy and direct orthogonal signal correction preprocessing applied to selected phenolic compounds in red wines. *Food Analytical Methods*, 4: 619–625.

Le Roux, G., Eschenbruch, R., and de Bruin, S.I. (1973). The microbiology of South African wine-making. Part VIII. The microflora of healthy and *Botrytis cinerea* infected grapes. *Phytophylactica*, 5, 51-54.

Legras, J.L., and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomycescerevisiae* strain characterisation. FEMS *Microbiology Letters*, 221:249–255.

Lin, C., Ayvaz, H., and Rodriguez-Saona, L. E. (2014). Application of Portable and Handheld Infrared Spectrometers for Determination of Sucrose Levels in Infant Cereals.*Food Analytical Methods*, 7:1407-1414.

Lin, M.S., Al-Holy, M., and Al-Qadiri, H., et al., (2004). Discrimination of intact and injured *Listeria monocytogenes* by fourier transform infrared spectroscopy and principal component analysis. *Journal of Agricultural and Food Chemistry*, 52:5769-5772.

Liu, L., Cozzolino, D., Cynkar, W., et al., (2006). Georaphic classification of Spanish and Australian tempranillo red wines by visible and near-infrared spectrsocopy combined with multivariate analysis. *Journal of Agricultural and Food Chemistry*, 54:674-6759.

London, J.L., Weller, K., Harberstson, J.F., et al., (2008). Chemical and sensory evaluation of estringency in Washington State red wines.*American Journal of Enology and Viticulture*, 59:153-158.

Longo, E., Cansado, J., Agrelo, D., et al., (1991). Effect of Climatic Conditions on Yeast Diversity in GrapeMusts from North West Spain.*American Journal of Enology and Viticulture*, 42:141-144.

Lorente, D., Escandell-Montero, P., Cubero, S., et al., (2015). Visible-NIR reflectance spectroscopy and manifold learning methods applied to the detection of fungal infections on citrus fruit. *Journal of Food Engineering*, 163:17-24.

Lu, X., Al-Qadiri, H., Lin, M., et al., (2011). Application of Mid-infrared and Raman Spectroscopy to the Study of Bacteria.*Food and Bioprocess Technology*, 4:919-935.

Mac Naughton, D., Romeo, M., Kansiz, M., et al., Otto (Eds.), Spectroscopy of Biological Molecules: New Directions, Kluwer Academic Publistars, Dordrecht, The Netherlands, 1999.

Malacrino, P., Zapparoli, G., Torriani, S., et al., (2001). Rapid detection of viable yeasts and bacteria in wine by flow cytometry,*Journal of Microbiological Method*s, 45:127–134.

Maquelin, K., Kirschner, C., Choo-Smith, L., et al., (2003). Prospective study of the performance of vibrational spectroscopies for rapid identification of bacterial and fungal pathogens recovered from blood cultures. *Journal of Clinical Microbiology*. 41:324:329.

Martelo-Vidal, M.J,.and Vazquez, M. (2014a). Rapid authentication of White Wines. Part 1: Classsification and designation of origin. Agro Food Industry Hi-Tech, 25:44-48.

Martelo-Vidal, M.J,.and Vazquez, M. (2014b). Rapid authentication of White Wines. Part 2: Classsification by grape variety. Agro Food Industry Hi-Tech, 25:44-48.

Martelo-Vidal, M.J., Dominguez-Agis, F., and Vazquez, M. (2013). Ultraviolet/visible/near-infrared spectral analysis and chemometric tools for the discrimination of wines between

subzones inside a controlled designation of origin: a case study of Rias Baixas. *Australian Journal of Grape and Wine Research*, 19:62-67.

Matthews, A., Grimaldi, A., Walker, M., et al., (2004). Lactic acid bacteria as a potential source of enzymes for use in vinification.*Applied Environmental Microbiology*, 70:5715–5731.

Miller J.N.; Miller, J.C. Statistics and Chemometrics for Analytical Chemistry. 5th ed.; Pearson Education Limited: Essex, 2005.

Millet, V., and Lonvaud-Funel, A. (2000). The viable but non-culturable state of wine microorganisms during storage.*Letters in Applied Microbiology*, 30:136–141.

Mills, D.A., Phister, T., Neeley, E. and Johannsen, E. (2008). Wine fermentation. In: Molecular techniques in the microbial ecology of fermented food. Cocolin L & Ercolini D (eds). Pp, 162-192. Springer, New York, USA.

Mossoba, M.M., Kramer, J.K.G., Azizian, H., et al., (2012). Application of a Novel. Heated, nine-reflection ATR crystal and a portable FTIR spectrometer to the rapid determination of total trans fat. *Journal of the American Oil Chemists' Society*, 89:419–429.

Mossoba, M.M., Tyburzy, C., Farris, S., et al., (2014). Evaluation of the Performance of a Portable Mid-Infrared Analyzer for the Rapid Determination of Total Trans Fat in Fast Food *Journal of the American Oil Chemists' Society,* 91:1651-1663.

Mouret, J.R., Camarasa, C., Angenieux, M., et al., (2014). Kinetic analysis and gas-liquid balances of the production of fermentative aromes during winemaking fermentations: Effect of assimilable nitrogen and temperature. *Food Research International*, 62:1-10.

Munik, B., Edelman, A., Lendl, B., et al., (2002). Determination of yeast assimilable nitrogen content in wine fermentations by sequential injection analysis with spectrophotometric detection.*Analytical and Bioanalytical Chemistry*, 374:167-172.

Naes, T., and Mevik, B.H. (2001). Understanding the collinearity problem in regression and discriminant analysis,*Journal of Chemiometrics*, 15:413-426.

Naumann, D. Infrared spectroscopy in microbiology. In Encyclopedia of Analytical Chemistry, John Wiley & Sons, Ltd: 2006.

Naumann, D., Helm, D., and Labischinski, H. (1991). Microbiological Characterization by FT-IR Spectroscopy.*Nature*, 351:81-82.

Ngo Thi, N.A. and Nauman, D. (2007). Investigating the heterogeneity of cell growth in microbial colonies by FTIR microspectroscopy.Analytical and Bioanalytical Chemstry, 387:1769-1777.

Nieuwoudt, H. H., Prior, B.A., Pretorius, I. S. et al., (2004). Principal component analysis applied to Fourier transform infrared spectroscopy for the design of calibration sets for glycerol prediction models in wine and for the detection and classification of outlier samples. *Journal of Agricultural and Food Chemistry*, 52:3726-3735.

Nieuwoudt, H., Pretorius, I.S., Bauer, F.F., et al., (2006). Rapid screening of the fermentation profiles of wine yeasts by Fourier transform infrared spectroscopy. Journal of Microbiological Methods, 67:248-256.

Oliveri, P., Di Egidio, V., Woodcock, T., et al., (2011). Application of class-modelling techniques to near infrared data for food authentication purposes.*Food Chemistry*, 125:1450-1456.

Orsini, F., Ami, A. M., Villa, G., et al., (2000). FT-IR microspectroscopy for microbiological studies.*Journal of Microbiological Methods,* 42:17–27.

Osborne, B. G., T. Fearn and P. H. Hindle. (1993). Practical NIR spectroscopy with applications in food and beverages analysis. Longman Scientific and Technical, Harlow.

Oust, A., Moretro, T., Kirschner, C., et al., (2004). Evaluation of the robustness of FT-IR spectra of *lactobacilli* towards changes in the bacterial growth conditions.*FEMS Microbiology Letters*, 239:111-116.

Patz, C. D., Blieke, A., Ristow, et al., (2004). Application of FT-MIR spectrometry in wine analysis.*Analytica Chimica Acta*, 513:81-89.

Patz, C. D., David, A., Thente, K., et al., (1999). Wine analysis with FTIR spectrometry.*Die Win-Wissenschaft*, 54:80-87.

Pico, Y., Chemical Analysis of Food: Techniques and Applications. 1st ed., Elsevier, 2012.

Picque, D., Cattenoz, T., and Corrieu, G. (2001). Classification of red wines analysed by middle infrared spectroscopy of dry extract according to their geographical origin.*Journal International des Sciences de la Vigne et du Vin*, 35:165-170.

Pinto, C., Pinho, D., Cardoso, R., et al., (2015). Wine femrention microbiome: a landscape from different Portuguese wine applellations. *Frontiers in Microbiology*, 6.

Pollard, D.J., Buccino, R., Connors, N.C., et al., (2001). Real-time analyte monitoring of a fungal fermentation, at pilot scale, using in situ mid-infrared spectroscopy.*Bioprocess and Biosystems Engineering*, 24:13-24.

Polsinelli, M., Romano, P., Suzzi, G., et al., (1996). Multiple strains of *Saccharomyces cerevisiae* on a single grape vine. *International in Applied Micobiology*, 23:110-114.

Pretorius, I.S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast, 16:675-729.

Pretorius, I.S., van der Westhuizen, T.J., and Augustyn, O.P.H. (1999). Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *South African Society for Enology & Viticulture*, 20, 61-70.

Puxeu, M., Andorrà, I., andDe Lamo-Castellví, S. (2014). Monitoring Saccharomyces cerevisiae Grape Must fermentation Process by Attenuated Total Reflectance Spectroscopy, Food Bioprocess and Technology, 8: 637-646.

Querol, A., and Fleet, G.H. Yeasts in Food and Beverages.Springer Science & Business Media, 2006.

Quiros, M., Rojas, V., Gonzalez, R., et al., (2014). Selection of Non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugarrespiration. International Journal of Food Microbiology, 181:85-91.

Reida, L.M., Colm, P., O'Donnellb, Gerard, D. (2006). Recent technological advances for the determination of food authenticity. *Trends in Food Science and Technology*, 17:344-353.

Reis, M. S., Saraiva, P. M., and Bakshi, B. R. Comprehensive Chemometrics: Chemical and Biochemical Data Analysis. Edited by: Brown, SD; Tauler, R; Walczak, B., Vol. 1-4, 2009.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., et al., (2006). Handbook of Enology.The Microbiology of Wine and Vinifications.Vol 1.2nd ed. John Wiley & Sons Ltd. West Sussex, England.

Rodríguez, A., Vaz, C.P., da Fontesca, A.F., et al., (1993). Evaluating the concentration of a Candid albicans suspensiopn.*Infectious diseases in obstetrics and gynecology*, 1:134-136.

Rodríguez-Saona, L.E., and Allendorf, M.E. (2011). Use of FTIR for Rapid Authentication and Detection od Adulteration of Food. Ed. By Doyle M.P and Klaenhammer, TR. *Annual Review of Food Science and Technology*, 2:467-483.

Rodríguez-Saona, L.E., Khambaty, F.M., Fry, F.S., et al., (2001). Rapid detection and identification of bacterial strains by Fourier transform near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, 49:574-579.

Rodríguez-Saona, L.E., Khambaty, F.M., Fry, F.S., et al., (2004). Detection and identification of bacteria in a juice matrix with Fourier transform-near infrared spectroscopy and multivariate analysis. *Jouranl of Food Protection*, 67:2555-2559.

Rollero, S., Bloem, A., Camarasa, C., et al., (2015). Combined effects of nutrients and temperature on the production of fermentative aromes by *Saccharomyces* cerevisiae during wine fermentation.*Applied Microbiology and Biotechnology*, 99:2291-2304.

Romano, A., Casaregola, S., Torre, P., et al., (1996). Use of RAPD and mitochondrial DNA RFLP for typing of *Candida zeylanoides* and *Debaryomyces hansenii* yeast strains isolated from cheese. *Systematic and Applied Microbiology*, 19:255-264.

Romano, P., Monteleone, E., Paraggio, M., et al., (1998). A methodological approach to the selection of *Saccharomyces* cerevisiae wine strains. *Food Technology and Biotechnology*, 36:69-74.

Rothschild, W.G. and Moller, K.D. (1970). Far-Infrared Spectroscopy.*Physics Today*, 23:44-&.

Sablayrolles J.Mm, Dubois C., Manginot C., et al., (1996). Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations,*Journal of Fermentation and Bioengineering*, 82, 377–381.

Sandt, C., Sockalingum, GD; Aubert, D., et al., (2003). Use of Fourier-transform infrared spectroscopy for typing of *Candida albicans* strains isolated in intensive care units. *Journal of Clinical Microbiology*, 3:954-959.

Savitzky, A., and Golay, M.J.E. (1964). Smoothing + Differentiation of Data by Simplified Least Squares Procedures. Analytical Chemistry, 36:1627-1634.

Scheiner, D. (1976). Determination of Ammonia and Kjeldahl Nitrogen by Indophenol method. *Water Research*, 10:31-36.

Schmalreck, A.F., Trankle, P., Vanca, E., et al., (1998). Differenciation and characterization of yeast pathogenic for humans (*Candida albicans, Exophiala dermatitidis*) and algae pathogenic for animals (*Prototheca spp.*) using Fourier transform infrared spectroscopy (FTIR) in comparison with conventional methods. *Mycoses*, 4:71–77.

Schmutzler, M., Beganovic, A., Boehler, G., et al., (2015). Methods for detection of pork adulteration in veal product based on FT-NIR spectroscopy for laboratory, industrial and on-site analysis.*Food Control*, 57:258-267.

Schuller, D., Valero, E., Dequin, S., et al., (2004). Survey of molecular methods for the typing of wine yeast strains.*FEMS Microbiology Letters*, 231:19-26.

Schulze, U., Liden, G., Nielsen, J., et al., (1996). Physiological effects of nitrogen starvation in an anaerobic batch culture of *Saccharomcyes cerevisiae*. *Microbiology-UK*, 142:2299-2310.

Shah, N., Cynkar, W., Smith, P., et al., (2010). Use of Attenuated Total Reflectance Mid infrared for Rapid and Real-Time Analysis of Compositional Parameters in Commercial White Grape Juice. *Journal of Agricultural and Food Chemistry*, 58:3279-3283.

Shapaval, V., Schmitt, J., Moretro, T., et al., (2013a). Characterization of food spoilage fungi by FTIR spectroscopy,*Journal of Applied Microbiology*, 114:788-796.

Shapaval, V., Walczak, B., Gognies, S., et al., (2013b). FTIR spectroscopic characterization of differently cultivated food related yeasts. *Analyst*, 138:4129-4138.

Shaw, A. D., Winson, M. K., Woodward, A. M., et al., (1999). Rapid Analysis of High-Dimensional Bioprocesses using Multivariate Spectroscopies and Advanced Chemiometrics. *Advances in Biomedical Engineering and Biotechnology*, 66:83-113.

Shimoi, H., Kitagaki, H., Ohmori, H., et al., (1998). Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. *Journal of Bacteriology*, 180:3381-3387.

Silva, S., Feliciano, R.P, Boas, L.V., et al., (2014). Application of FTIR-ATR to Moscatel dessert wines for prediction of total phenolic and flavonoid contents and antioxidant capacity. *Food Chemistry*, 150:489-493.

Sivakesava, S., and Irudayaraj, J. (2002). Classification of simple and complex sugar adulterants in honey by mid-infrared spectroscopy.*International Journal of food Science and technology*, 37:351-360.

Sivakesava, S., Irudayaraj, J., Ayaraj, J., et al., (2004). Differentiation of microorganisms by FTIR-ATR and NIR spectroscopy. *Transactions of the ASAE*, 47:951-957.

Smith, B.C. Fundamentals of Fourier transform infrared spectroscopy. 2nd Edition; Taylor & Francis Group, LLC: New York, 2011.

Sockalingum, G. D., Sandt, D., Toubas, J., et al., (2002). FTIR characterization of *Candida species*: a study on some reference strains and pathogenic *C. albicans* isolates from HIV patients. *Vibrational Spectroscopy*, 28:137–146.

Soden, A,, Francis, I.L., Oakey, H., et al., (2000). Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Australian Journal of Grape Wine Research*, 6, 21-30.

Sohier, D., Coulon, J., and Lonvaud-Funel, A. (1999). Molecular identification of Lactobacillus hilgardii and genetic relatedness with Lactobacillus brevis.*International Journal of Systematic Bacteriology*, 49:1075-1081.

Somers, T.C., and Evans, M.E. (1977). Spectral Evaluation of young red wines – anthocyanin equilibria, total phenolics, free and molecular SO2, chemical age. Journal of the Science of Food and Agriculture, 28:279-287.

Stuart, B.H. Infrared Spectroscopy of biological applications. In Encyclopedia of Analytical Chemistry, John Wiley & Sons, Ltd: 2005.

Stuart, B.H. Infrared Spectroscopy of biological applications. In Encyclopedia of Analytical Chemistry, John Wiley & Sons, Ltd: 2006.

Stuart, B.H. Infrared Spectroscopy: Fundamentals and Applications. John Wiley & Sons. 2004.

Sturm, M.E., Assof, M., Fanzone, M., et al., (2015). Relation between coumarate decarboxylase and vinylphenol reductase activity with regard to the production of volatile phenols by

native *Dekkera bruxellensis* strains under 'wine-like' conditions. *International Journal of Food Microbiology*, 206:51-55.

Subramanian, A., , Ahn, J., Balasubramaniam, V.M., et al., (2006). Determination of spore inactivation during thermal and pressure-assisted thermal processing using FT-IR spectroscopy.*Journal of Agricultural and Food Chemistry*, 54:10300-10306.

Subramanian, A., Ahn, J., Balasubramaniam, V.M., and Rodriguez-Saona, L. (2007). Monitoring biochemical changes in bacterial spore during thermal and pressure-assisted thermal processing using FT-IR Spectroscopy.*Journal of Agricultural and Food Chemistry*, 55:9311-9317.

Subramanian, A., and Rodriguez-Saona, L. (2009). Infrared spectroscopy Spectroscopy for Food Quality Analysis and Control: Fourier Transform Infrared (FTIR) Spectroscopy, Ch. 7. Ed. By Da-Wenn Sun. ISBN 978-0-12-3741363.

Suci, P. A., Vrany, J. D., and Mittelman, M. W. (1998). Investigation of interactions between antimicrobial agents and bacterial biofilms using attenuated total reflection Fourier transform infrared spectroscopy. *Biomaterials*, 19:327-339.

Sumby, K.M., Grbin, P.R., and Jiranekk, V. (2014). Implications of new research and technologies for malolactic fermentation in wine.*Applied Microbiology and Biotechnology*, 98:8111-8132.

Sundaram, J., Park, B., and Hinton, A,Jr., et al., (2012). Classification and Structural Analysis of Live and Dead Salmonella Cells Using Fourier Transform Infrared Spectroscopy and Principal Component Analysis. *Journal of Agricultural and Food Chemistry*, 60:991-1004.

Taavitsainen, V.M. Denoising and Signal-to-Noise Ratio Enhancement: Derivatives, Vol. 1-4. Brown, SD; Tauler, R; Walczak, B. Comprensive Chemiometrics.Chemical and Biochemical Data Analysis.Vol. 1-4, 2009.

Teixeira dos Santos, C.A., Lopo, M., Pascoa, R.N.M.J., et al. (2013). A Review on the Applications of Portable Near-Infrared Spectrometers in the Agro-Food Industry.*Applied Spectroscopy*, 67:1214-1233.

Tewari, J.C., and Irudayaraj, J.M.K. (2005). Floral classification of honey using mid-infrared spectroscopy and surface acoustic wave based z-Nose sensor. *Agricultural and Food Chemistry*, 53:6955-6966.

Timmins, E. M., Quains, D.E. and Goodacre, R. (1998). Differentiation of brewing yeast strains by pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. *Yeast*, 14:885–893.

Tooke, P. B. (1988). Fourier self-deconvolution in IR spectroscopy.*TrAC Trends in Analytical Chemistry*, 7:130-136.

Travo, A., Despalt, V., Barron, E., et al., (2012). Basis of a FTIR spectroscopy methodology for automated evaluation of Akt kinase inhibitor on leukemic cell lines used as model. *Analytical and Bioanalytical Chemistry*, 404:1733-1743.

Triebel, S., Sproll, C., Reusch, H., et al., (2007). Rapid analysis of taurine in energy drinks using amino acid analyser and Fourier transform infrared (FTIR) spectroscopy as basis for toxicological evaluation. *Amino Acids*, 33:451-457.

Ugliano, M., Kolouchova, R., and Henschke, P.A. (2011). Ocurrence og hidrogen sulfide in wine and in fermentation: influence of yeast strain and supplementation of yeast available nitrogen. *Journal of Industrial Microbiology & Biotechnology*, 38:423-429.

Ugliano, M., Travis, B., Francis, I.L., et al., (2010). Volatile compositionand Sensory Properties of Shiraz Wines As Affected by Nitrogen Supplementation and Yeast Species: Rationalizing Nitrogen Modulation of Wine Aroma. *Journal of Agricultural and Food Chemstry*, 58:12417-12425.

Urso, R., Rantsiou, K., Dolci, P., et al., (2008). Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. *FEMS Yeast Research*, 8:1053-1062.

Varela, C., Siebert, T., Cozzolino, D., et al., (2009). Discovering a chemical basis for differentiating wines made by fermentation with 'wild' indigenous and inoculated yeasts: role of yeast volatile compounds.*Australian Journal of Grape and Wine Research*, 15:238-248.

Veale, E.L., Irudayaraj, J., and Demirci, A. (2007). An on-line approach to monitor ethanol fermentation using FTIR spectroscopy.*Biotechnology Progress*, 23:494-500.

Wenning, M., and Scherer, S. (2013). Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. *Applied Microbiology and Technology*, 97:7111-7120.

Wenning, M., Seiler, H., Scherer, S. (2002). Fourier-transform infrared microspectroscopy, a novel and rapid tool for identification of yeasts.*Applied and Environmental Microbiology*, 68, 4717-4721.

Whittaker, P., Mossoba, M.M., Al-Khaldi, S., et al. (2003). Identification of foodborne bacteria by infrared spectroscopy using cellular fatty acid methyl esters. Journal of Microbiological Methods, 55:709-716.

Winder, C. L., and Goodacre, R. (2004a). Comparison of diffuse-reflectance absorbance and attenuated total reflectance FT-IR for the discrimination of bacteria.*Analyst*, 129:1118-1122.

Winder, C. L., Carr, E., Goodacre, R., et al., (2004b). The rapid identification of Acinetobacter species using Fourier transform infrared spectroscopy. *Journal of Applied Microbiology*. 62:329-339.

Wold, S., Sjostrom, M., and Eriksson, L. (2001). PLS-regression: A basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems*, 58(2), 109–130.

Woodcock, T., Downey, G. and O'Donnell, C.P. (2008). Better quality food and beverages: the role of near infrared spectroscopy. *Journal of Near Infrared Spectroscopy*, 16:1-29.

Yang, H., and Irudayaraj, J. (2001). Comparison of near-infrared, Fourier transform-infrared, and Fourier transform-Raman methods for determining olive pomace oil adulteration in extra virgin olive oil. *Journal of the American Oil chemist Society*, 78:889-895.

Yang, H., Irudayaraj, J., and Paradkar, M.M. (2005). Discriminant analysis of edible oils and fats by FTIR, FT-NIR and FT-Raman spectroscopy.*Food Chemistry*, 93:25-32.

Yu, C. X., Irudayaraj, J., Debroy, C., et al., (2004). Spectroscopic differentiation and quantification of microorganisms in apple juice.*Journal of Food Science*, 69, 268–272.

Yu, C., and Irudayaraj, J. (2005). Spectroscopics Characterization of Microorganisms by Fourier Transform Infrared Microspectroscopy. *Byopolymers*, 77:368-377.

Yue, T.L., Wang, J., Yuan, Y.H., et al., (2010). Rapid Identification of Microorganisms Based on Fourier Transform Near Infrared Spectroscopy. *Spectroscopy and Spectral Analysis*, 30:2945-2949.

Zapparolli, G., Torriani, S., Pesente, P., et al., (1998). Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Letters in Applied Microbiology*, 27:243-246.

Zavaleta, Al., Martínez-Murcia, A.J.,and Rodríguez-Varela, F., (1997). Intraspecific genetic diversity of Oenococcus oeni as derived from DNA fingerprint and sequence analyses. *Applied and Environmental Microbiology*, 63:1261-1267.

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APPLICATION OF INFRARED SPECTROSCOPY IN MID-INFRARED RANGE COMBINED WITH MULTIVARIATE ANALYSIS TO STUDY YEASTS INVOLVED IN WINE PRODUCTION
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CHAPTER 2

Monitoring *Saccharomyces cerevisiae* Grape Must Fermentation Process by Attenuated Total Reflectance Spectroscopy

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Abstract

Attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) combined with soft independent modeling of class analogy (**SIMCA**) was used to study the physiological state (exponential and stationary) of three commercial strains of *Saccharomyces cerevisiae* (ES454, E491, and ES181) fermented in Grenache blanc and Chardonnay musts. Microvinifications were performed with 150 mL of cleaned must at 17°C. Fermented juices at exponential and stationary phase were centrifuged, and the pellets obtained were placed onto **ZnSe** crystal. Spectra were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000–700 cm^{-1}) and were analysed by a multivariate analysis technique, **SIMCA**. The chemical differences detected between *S. cerevisiae* strains at exponential and stationary phases were mainly related to differences in their cell wall composition. **ATR-FTIR** combined with multivariate technique was a rapid and simple method to study psychological states of *S. cerevisiae* during wine fermentation.

2.1. Introduction

Fourier transform infrared spectroscopy (**FTIR**) technique has been widely used in winemaking sector as a rapid method to determine control parameters such as alcoholic strength, acetic acid, pH, and total acidity (Ferreira et al., 2009; Lachenmeier et al., 2010). Moreover, in wine research, this technique has been found useful to perform a variety of analyses such as color prediction (Versari et al., 2012), classify wines in function of their origin, and determine phenol compounds (Laghi et al., 2011).

Attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) provides bands from all the cellular components of microorganisms, mainly from cell membrane and cell wall that permit the classification of microorganisms (Baldauf et al., 2007). Microorganisms can be classified at the strain and species/variety level using supervised multivariate classification models. **ATR -FTIR** has been used to detect *Alicyclobacillus* in fruit juice (Grasso et al., 2009), *Lactobacilli* in meat and cheese (Oust et al., 2004), and *Salmonella* in apple juice (Yu et al., 2004). Burattini et al., (2008) and Cavagna et al., (2010) studied the autolysis of *Saccharomyces* cerevisiae in sparkling wines. These authors showed that **FTIR** spectroscopy in the mid-infrared range is a rapid and accurate technique to determine simultaneously the main biochemical modifications induced by autolysis when the cells enter to autolysis stage. They also demonstrated that mid infrared microspectroscopy in **ATR** mode combined with principal component analysis (**PCA**) allowed to monitor the three main biochemical processes involving the degradation of lipids, proteins, and polysaccharides.

The application of **ATR-FTIR** to study microorganisms during the fermentation, stock, or aging processes would be of great interest in the winemaking sector. **ATR-FTIR** technique has several advantages such as short time of analysis, little sample preparation, and no need for qualified employees among others. Moreover, analysing the **IR** raw spectra with multivariate analysis, the physiological state of *S. cerevisiae* could be determinate, in order to prevent stuck and sluggish fermentations and problems with the end of fermentations. All previous uses would increase the applications of **ATR-FTIR** equipments present in many wineries.

The main objective of this research is to evaluate the potential of using **ATR-FTIR** combined with soft independent modeling of class analogy (**SIMCA**) to discriminate and classify *S. cerevisiae* strains and analyze their chemical changes produced by their

different physiological states during a fermentation process. Developing a simple, rapid, reproducible, and sensitive infrared spectroscopy method based on analysing yeast pellets to study wine fermentation will be a breakthrough for wine industry.

2.2. Material and Methods

2.2.1. Must Preparation

Chardonnay and Grenache blanc grapes were acquired from Lleida (Costers del Segre, **ES**) and Gandesa (Terra Alta, **ES**), respectively. Must (2.3 L) was obtained by pressing the grapes with a manual press with total capacity of 5 kg (Magusa, Vilafranca del Penedès, **ES**) until obtaining 47 and 49% of must yield for Chardonnay and Grenache blanc, respectively. After adding 40 mg/L of sulphur dioxide and 1 g/hL of depectil clarification enzyme (Martin Vialatte, Sant Miquel d'Olèrdola, **ES**), the grape must was settled for 24 h to remove the solid fraction. The clarification performance was measured by calculating the ratio between must weight obtained from the grape weight (72% for Chardonnay and 78% for Grenache blanc).

2.2.2. Must Characterization

Brix degree and alcoholic potential strength by refractometry, pH, total acidity, and final alcoholic strength by official European method, turbidity in nephelometric turbidity units (**NTU**), and assimilable nitrogen were analysed following the procedure of the Office International de la Vine et du Vin (**OIV**, 2008). All these parameters were used for characterization of the musts and identifying the main differences between two types of grapes used in this study. The analyses were performed in triplicate for each sample tested.

2.2.3. Microvinification Process

A volume of 150 mL of clarified must was fermented in sterile Erlenmeyer using three commercial strains of *S. cerevisiae*, E491 (Station Oenotechnique de Champagne, Cormontreuil, **FR**), ES454, and ES181 (SEPSA Enartis, Vilafranca del Penedès, **ES**).

Each strain is recommended by the commercial supplier for white grapes such as Chardonnay and Grenache blanc. In each case, inoculum was prepared according to the instructions provided by the supplier to reach an initial concentration of 10^6 cfu/mL *S. cerevisiae* in the grape must. Fermentation was performed at 17°C using a thermostatic water bath (Grupo-Selecta, Abrera, ES) and was daily monitored by controlling density with an electronic densimeter (Metler Toledo, Hospitalet de Llobregat, ES) until the total glucose and fructose concentration was lower than 5 g/L.

Fermentation was complete after 240 and 384 h approximately for Chardonnay and Grenache blanc, respectively. All fermentations were performed in triplicate, after the fermentation residual sugars (sum of the concentrations of glucose and fructose) and acetic acid contents were analysed by an enzymatic method following the procedures of OIV (OIV 2008) using an automatic enzymatic device (Biosystems, Barcelona, ES). For the IR analysis, samples were taken when yeast cells where at exponential phase (48 and 120 h for Chardonnay and 198 h for Grenache blanc) and stationary phase (172 h for Chardonnay and 344 h for Grenache blanc).

2.2.4. Genetic Test

DNA extraction was performed as described by Querol et al., (1992). The characterization of *S. cerevisiae* strains was performed using the primer pair delta 12 and delta 21 described by Legras and Karst (2003). PCR amplifications were carried out in 25 µL reaction containing 5–20 ng yeast DNA, 0.2 mg/mL of BSA, 0.2 mM of dNTPs, 1 µM of each primer, 2.5 mM of $MgCl_2$, 1× of reaction buffer (Biotaq™, Bioline Reagents, UK), and 0.5 U de TaqDNA polymerase (Bioline). Amplification reactions were performed with a Bio-Rad T11 thermal cycler (Bio-Rad, Alcobendas, ES) following the program described by Legras and Karst (2003). Amplification reactions were separated by electrophoresis on 0.8% agarose (Ecogen, Barcelona, ES) gels supplemented with 0.6× of Red safe TM nucleic acid staining solution (iNtRon Biotechnology, Sevilla, ES) submitted to 100 V for 1 h in 1× TBE buffer. Gels were then scanned with a Gel Doc™ XR+ apparatus (Bio-Rad). Yeast profiles were normalized and compared with the Image Lab™ software (Bio-Rad). Restriction analyses of mitochondrial DNA were also performed in order to genotype *S. cerevisiae* strains belonging to *S. cerevisiae* species and were differentiated at strain level according to their mitochondrial (mt) DNA patterns. Total

DNA extraction and mtDNA restriction analysis were performed as described by Querol et al. (1992) with the restriction endonuclease HinfI (Roche Molecular Biochemicals, Barcelona, ES).

2.2.5. Sample Preparation

Fermented juices (1.5 mL) were centrifuged (15,900g for 5 min at room temperature) and washed three times under the same conditions using 1 mL of saline solution (SS, 0.88% NaCl) to obtain clean yeast cells and remove phenols, anthocyanins, organic acids, and other potential remaining components to ensure that IR spectra were not influenced by these compounds. After the cleaning process, 1.5 µL of pellet was placed onto ZnSe crystal. Six spectra per each sample and day of experiment were collected in the attenuated total reflectance (ATR) mode in the mid-infrared region (4000–800 cm^{-1}).

2.2.6. FTIR Used in ATR Mode

Spectral data of *S. cerevisiae* pellets were obtained using an FTIR spectrometer Nicolet 380 (Thermo Scientific, Madrid) adapted with an ultra-high performance ATR plate of Zinc Selenide (ZnSe) crystal (Smart iTR, Thermo Scientific, Madrid, ES). Spectra were collected from 4000 to 800 cm^{-1} with a resolution of 2 cm^{-1}. The spectrum of each sample was obtained by taking the average of 32 scans to improve the signal-noise ratio. Spectra were displayed in terms of absorbance obtained by rationing the single beam spectrum against that of the air background.

2.2.7. Multivariate Analysis

Spectra were exported to the Pirouette® multivariate analysis software (version 4.0, InfoMetrix, Inc., Woodville, USA). The FTIR spectral data were mean-centered, transformed to their second derivative using a 25-point Savitzky-Golay polynomial filter, and vector-length normalized; sample residuals and Mahalanobis distance were used to determine outliers (Kansiz et al., 1999; Hruschka, 2001). Soft independent modeling of

class analogy (**SIMCA**) was used to build a predictive model based on the construction of separate **PCA** models for each class to describe and model the variation (Kansiz et al., 1999). **SIMCA** class models were interpreted based on class projections, misclassifications, discriminating power, and interclass distances. Class projections were visible through a three dimensional graphic of clustered samples and are built around the clusters based on **PCA** scores, allowing **SIMCA** to be used as a predictive modeling system. Variable importance, also known as discriminating power, was used to define the variables (wavenumbers) that have a predominant effect on sample classification, minimizing the difference between samples within a cluster and maximizing differences between samples from different clusters (Dunn and Wold, 1995). Total misclassifications were analysed and interpreted for the input data.

2.3. Results and Discussion

2.3.1. Must Characterization

Physical-chemical characteristics of the Chardonnay and Grenache blanc musts used for our fermentations are shown in **Table 2.1**.

Table 2.1. Physical-chemical characteristics of the Chardonnay and Grenache blanc musts and wines.

Must	Chardonnay	Grenache blanc
Brix degree	17.2 ± 0.1	19.3 ± 0.2
Alcoholic potential strength (%vol.)	9.7 ± 0.1	11.4 ± 0.2
Assimilable Nitrogen (mg/lL)	196 ± 4	252 ± 6
pH	2.8 ± 0.1	3.0 ± 0.1
Total acidity (g/L)	13.4 ± 0.1	5.9 ± 0.1
Turbidity (**NTU**[a])	13.0 ± 2.0	11.0 ± 2.0
Press yield (%)	46.9 ± 0.7	49.4 ± 0.7
Clarification yield (%)	71.9 ± 0.7	78 ± 0.7

Results are shown as mean ± standard deviation ; [a]Nephelometric turbity units

No significant differences between Chardonnay and Grenache blanc alcoholic potential strengths were detected being these values low enough to not produce problems during the alcoholic fermentation. No important differences were detected between press and clarification yields resulting in similar values of final turbidity of the musts. Assimilable nitrogen content was appropriate in Chardonnay and Grenache blanc musts for yeast to grow during early exponential phase and carry on the fermentation during the exponential phase until the end without any nutritional problem. Total acidity showed the highest differences between values in both varieties, mainly due to the different stage of grape ripening. To support this statement, the initial concentration of sugars was calculated to detect differences between maturity of the two varieties (Walker, 1998) being 171 and 213 g/L for Chardonnay and Grenache blanc, respectively.

2.3.2. Fermentation Behaviour

Fermentation performance was assessed through the daily measurement of density (**Figure 2.1**) and the residual sugar at the end of fermentation (lower than 5 g/L, **Table 2.2**). In all fermentation processes tested, the kinetics curves obtained showed typical shape without detecting stuck fermentations or any other alterations such as long early exponential phase produced by yeast acclimatization and slow fermentation speed in the stationary phase due to the concentration of ethanol.

Fermentations performed with Chardonnay must inoculated with *S. cerevisiae* ES454 and E491 strains (**Figure 2.1 a, c**), lag phase lasted less than 24 h, starting the exponential phase after 24 h being the density and sugar values 1063 and 163 g/L respectively for both yeast. Whereas for the *S. cerevisiae* ES181 strain (**Figure 2.1b**), the lag phase lasted for 48 h, being the density and sugar values 1081 and 163 g/L, respectively. In the case of fermentations produced using Grenache blanc inoculated with *S. cerevisiae* ES454 and E491 strains (**Figure 2.1 d, f**), yeast cells started the exponential phase after 72 h of lag phase.

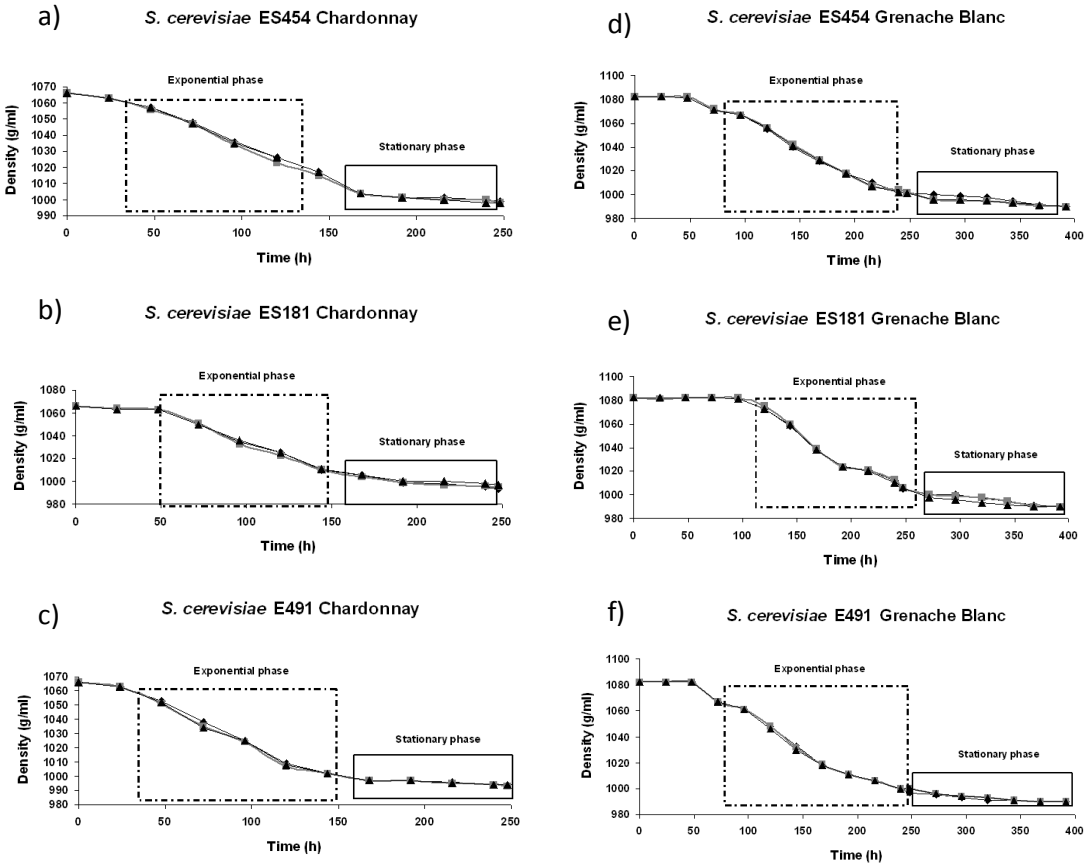

Figure 2.1. Density plots for fermentation kinetics: **a)** strain ES454 for Chardonnay must, **b)** strain ES181 for Chardonnay must, **c)** strain E491 for Chardonnay must, **d)** strain ES454 for Grenache Blanc must, **e)** strain ES181 for Grenache must and **f)** strain E491 for Grenache must.

Density and sugar values were 1071 and 186 g/L for strain ES454 and 1067 and 174 g/L for the strain E491. For strain ES181 (**Figure 3.1e**), the lag phase lasted almost 48 h more to starting the exponential phase after 120 h of being inoculated, being the density and sugar values 1073 and 191 g/L, respectively.

Table 2.2. Physical-chemical characteristic of the Chardonnay and Grenache blanc wines obtained.

Parameter	Methodology	Strain	Chardonnay	Grenache blanc
Glucose + Fructose (g/L)	Enzymatic	ES454	3.1 ± 0.2	2.3 ± 0.2
		ES181	2.9 ± 0.5	3.9 ± 0.3
		E491	0.8 ± 0.2	2.9 ± 0.3
Acetic acid (g/L)	Enzymatic	ES454	0.30 ± 0.03	0.48 ± 0.05
		ES181	0.34 ± 0.03	0.25 ± 0.02
		E491	0.51 ± 0.04	0.37 ± 0.05
Final alcoholic strength (%vol.)	Distillation and hydrostatic balance	ES454	10.3 ± 0.1	12.9 ± 0.1
		ES181	10.4 ± 0.1	12.7 ± 0.1
		E491	10.6 ± 0.1	13.0 ± 0.1

Results are shown as mean ± standard deviation

In conclusion, *S. cerevisiae* ES454 and E491 strains needed less time to adapt in both grape must varieties than strain ES181. Their adaptation was better in Chardonnay than in Grenache Blanc due their lower sugar concentration (171 g/L for Chardonnay and 213 g/L for Grenache blanc) and their final alcoholic strength.

2.3.3 Genetic Results

The **PCR** amplification of delta sequences interspersed regions showed different patterns for three yeast strains studied (**Figure 3.2 a**). The **RFLP** of mitochondrial **DNA** also showed different patterns for the different yeast studied (**Figure 3.2 b**). The analysis of the genetic variability of three commercial *S. cerevisiae* wine strains by both techniques indicated that the three species are different at strain level.

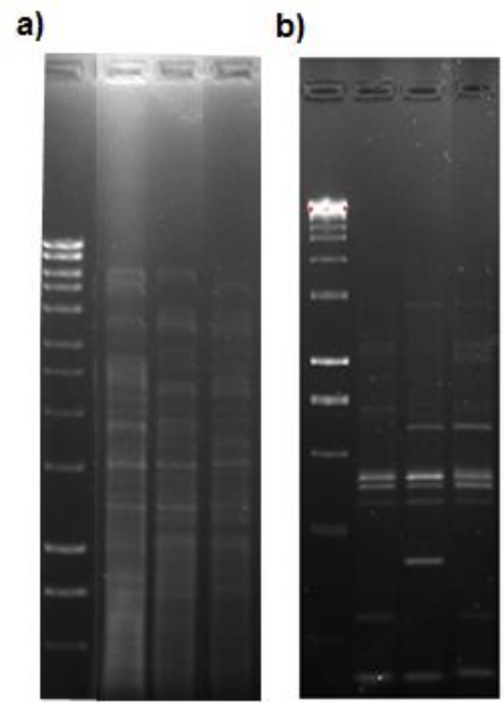

Figure 2.2. Characterization of *S. cerevisiae* strains by: **a)** restriction analyses of mitochondrial **DNA** (order: marker, *S. cerevisiae* E454, *S. cerevisiae* E491 and *S. cerevisiae* ES181) and **b)** primer pair delta 12 and delta 21 (order: marker: *S. cerevisiae* ES181, *S. cerevisiae* E454 and *S. cerevisiae* E491).

2.3.4. Discrimination of Saccharomyces cerevisiae *Strains at Exponential Phase by* ***ATR-FTIR*** *Combined with* ***SIMCA***

Multivariate analysis technique **SIMCA** was used to discriminate between three *S. cerevisiae* strains fermenting in Chardonnay and Grenache blanc during exponential phase (**Figure 2.3** and **Figure 2.4**). In each principal component direction, a 95% confidence interval probability cloud is assigned around each class (Subramanian et al., 2007; Grasso et al., 2009). Clusters permitted tight clustering and clear differentiation between *S. cerevisiae* strains analysed at the early (48 h, Chardonnay) and late (120 h Chardonnay and 172 h Grenache blanc) exponential phase.

Interclass distances (**ICD**) are Euclidian distances between centers of clusters, and above 3.0 are considered significant to identify two clusters as different classes (Dunn and Wold, 1995). **ICD** ranged from 2.4 to 15.1 in the case of Chardonnay (**Table 2.2**) and from 2.9 to 5.4 in the case of Grenache blanc (**Table 2.3**), showing differences between their biochemical composition, excepting between *S. cerevisiae* ES181 and E491 strains

fermented for 120 and 198 h in Chardonnay must and between *S. cerevisiae* E491 and ES181 at 344 h in Grenache Blanc must.

SIMCA's misclassification algorithm indicated that the training set was homogeneous, and all *S. cerevisiae* strains were correctly classified into their corresponding categories. **Figures 2.3 d,e** and **Figure 2.4 c** shows the wavenumbers that had a predominant effect on discrimination of *S. cerevisiae* strains during their exponential phase of fermentation in Chardonnay and Grenache blanc musts, respectively.

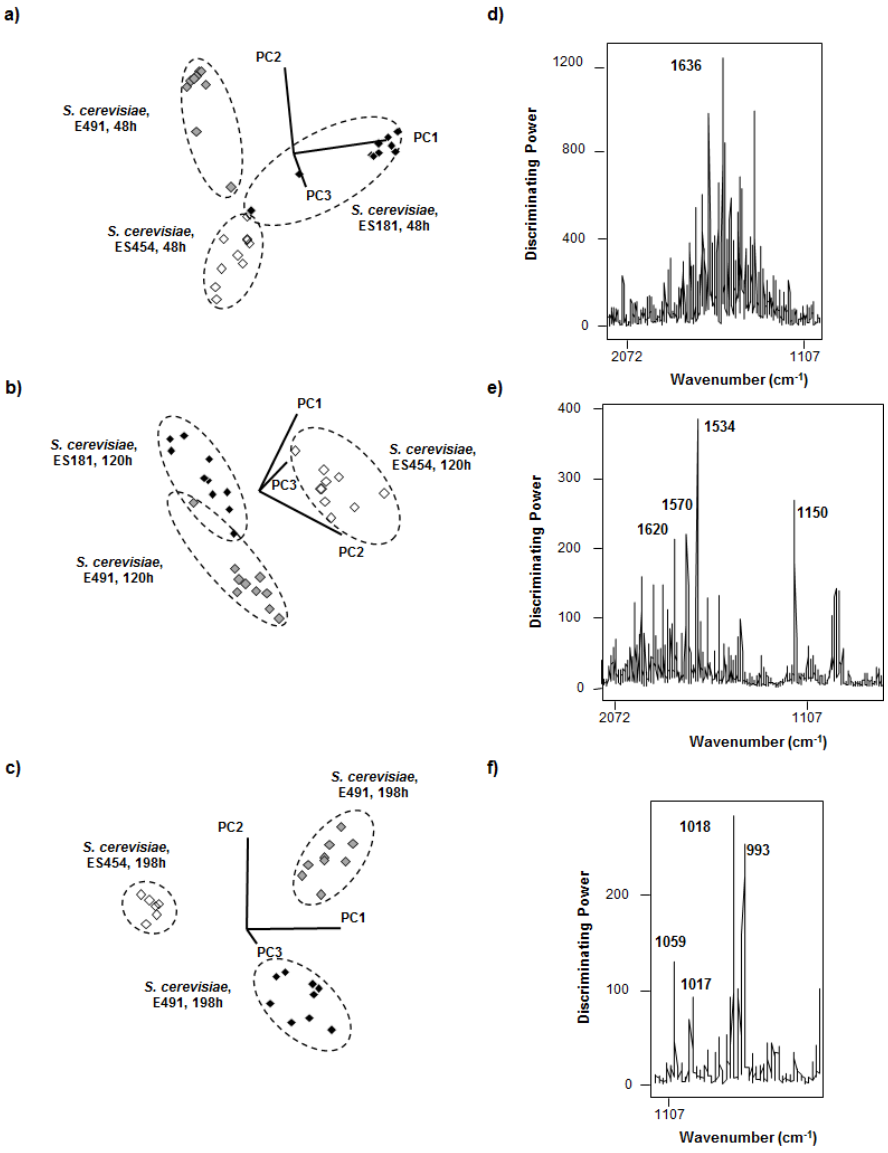

Figure 2.3. Soft independent modeling of class analogy (**SIMCA**) class projections (**a–c**) and discriminating power (**d–f**) f transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}). *S. cerevisiae* strains fermented in Chardonnay must in early exponential phase at 48 h (**a, d**), late exponential phase at 120 h (**b, e**), and stationary phase at 198 h (**c, f**). Experiments were performed in triplicate.

Discriminating power of *S. cerevisiae* strains fermented for 48 h in Chardonnay must showed mainly three spectral bands at 1752, 1636, and 1489 cm^{-1}, linked to C=O stretching in lipid esters, amide I absorption of proteins, and CH_2 scissoring in lipids, respectively (Cavagna et al., 2010). In the case of *S. cerevisiae* strains fermented for 120 h in Chardonnay, the major discriminating bands were 1534 and 1150 cm^{-1} associated to protein amide II band (N–H and C–N vibrations) of the peptide bond in different protein conformations (Cavagna et al., 2010) and C–O–C stretching (glycosidic linkages) of **β**(1→3) glucans (Naruemon et al., 2013) or C–O, C–OH stretching of carbohydrates (Cavagna et al., 2010), respectively. Analysing the most predominant bands detected in the case of *S. cerevisiae* strains fermented for 172 h in Grenache blanc, two spectral bands 1612 and 1374 cm^{-1} related to protein amide II band and CH_2 wagging vibrations in lipids, and **β**(1→3) glucans (Cavagna et al., 2010), respectively, were observed. The polysaccharide absorbing region including component structures of mannans and **β**-glucans of *S. cerevisiae* strains has been assigned to the spectral range of 925–1190 cm^{-1} (Galichet et al., 2001).

Since cell walls of *S. cerevisiae* are mainly composed of mannoproteins that form radially extending fibrillate at the outside of the cell wall (Kapteyn et al., 1999) and **β**(1→6) and **β**(1→3) glucans (85–90% of the cell wall dry mass) (Kapteyn et al., 1999; Lesage and Bussey, 2006; Huang et al., 2008), these seem to be the main components responsible of the discrimination between different *S. cerevisiae* strains during their exponential phase of growth.

Moreover, lipids may also play a role to explain the biochemical differences between *S. cerevisiae* ES181, E491, and ES181 strains, especially at early exponential phase in Chardonnay must. Glycoprotein anchors that appear in the cell wall or at the plasma membrane of *S. cerevisiae* are mainly composed by glycosylphosphatidylinositol (Komano and Fuller 1995). Another hypothesis to explain this result is that lipid fraction of yeast cells mainly changes during wine fermentation due to several factors: adaptation to environmental agents such as temperature, oxygen, nutrient limitation (Ratledge and Evans, 1989; Beltran et al., 2008), and environment's lipid composition (Rosi and Bertuccioli, 1992).

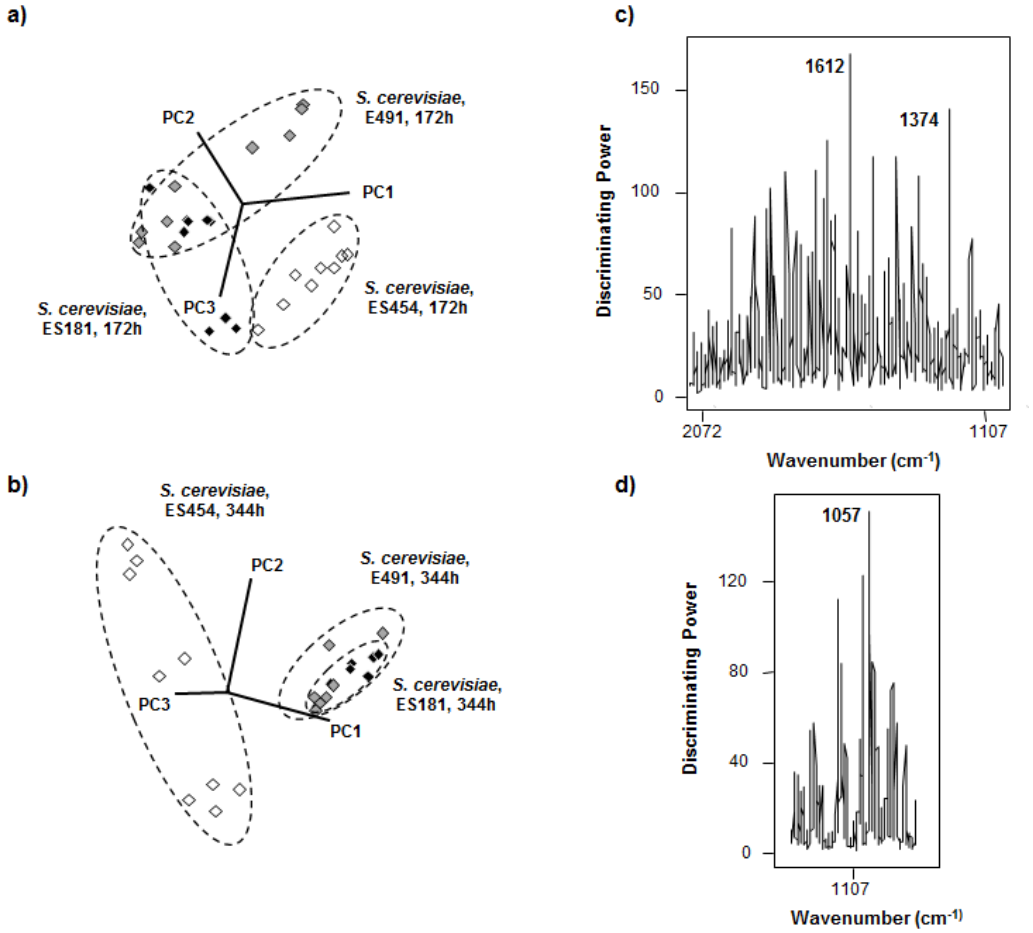

Figure 2.4. Soft independent modeling of class analogy (**SIMCA**) class projections (**a, b**) and discriminating power (**c, d**) f transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}). *S. cerevisiae* strains fermented in Grenache blanc must in exponential phase at 172 h (**a, c**) and stationary phase at 344 h (**b, d**). Experiments were performed in triplicate.

2.3.5. Discrimination of Saccharomyces cerevisiae *Strains at Stationary Phase by* ***ATR-FTIR*** *Combined with* ***SIMCA***

Infrared spectra analyses (2100–900 cm^{-1}) using **SIMCA** classification models of three *S. cerevisiae* strains fermented in Chardonnay and Grenache blanc musts during stationary phase are shown in **Figures 2.3c, 2.3f** and **2.4b** and **2.4d** respectively. The **SIMCA** class projection plots showed well-separated grouping of the samples analysed in a three-dimensional pattern. **ICD** values varied from 1.5 to 4.0 in the case of Chardonnay and from 2.8 to 4.1 in the case of Grenache blanc showing significant differences between the samples compared excepting *S. cerevisiae* ES181 and E491 strains fermented in Chardonnay must for 198 h (**Tables 2.3** and **2.4**).

Table 2.3. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* strains fermented in Chardonnay must at early exponential phase (48 h) and late exponential phase (120 h) and stationary phase (198 h) of transformed (second derivative, 25 points window) Attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectrum.

48 h	*S. cerevisiae* ES454	*S. cerevisiae* ES181	*S. cerevisiae* E491
S. cerevisiae ES454	0.0		
*S. cerevisiae*ES181	8.6	0.0	
S. cerevisiae E491	10.0	15.1	0.0
120 h	***S. cerevisiae* ES454**	***S. cerevisiae* ES181**	***S. cerevisiae* E491**
S. cerevisiae ES454	0.0		
*S. cerevisiae*ES181	4.1	0.0	
S. cerevisiae E491	4.3	2.4	0.0
198 h	***S. cerevisiae* ES454**	***S. cerevisiae* ES181**	***S. cerevisiae* E491**
S. cerevisiae ES454	0.0		
*S. cerevisiae*ES181	4.0	0.0	
S. cerevisiae E491	5.2	1.5	0.0

Discriminating power of **SIMCA** model build up with *S. cerevisiae* strains fermented in Chardonnay until reaching the stationary phase (198 h, **Figure 2.2 f**) showed three spectral bands at 1018, 993, and 1059 cm^{-1} linked to **β**(1→4) glucans, **β**(1→6) glucans, and mannans, respectively (Galichet et al., 2001).

In the case of *S. cerevisiae* strains fermented in Grenache blanc at 344 h (**Figure 2.3 d**), the discriminating power showed mainly one band at 1057 cm^{-1} responsible of their biochemical differences and was related to mannans (Cavagna et al., 2010). Stationary-phase cells have thick, less porous cell walls, and their resistance to degradative enzymes has been related to changes in their mannoprotein structure (Werner-Washburne et al., 1993).

Table 2.4. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* strains fermented in Grenache blanc must at exponential phase (172 h) and stationary phase (344 h) of transformed (second derivative, 25 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

172 h	*S. cerevisiae* ES454	*S. cerevisiae* ES181	*S. cerevisiae* E491
S. cerevisiae ES454	0.0		
*S. cerevisiae*ES181	5.4	0.0	
S. cerevisiae E491	4.3	2.9	0.0
344 h	***S. cerevisiae* ES454**	***S. cerevisiae* ES181**	***S. cerevisiae* E491**
S. cerevisiae ES454	0.0		
*S. cerevisiae*ES181	4.1	0.0	
S. cerevisiae E491	3.7	2.8	0.0

2.3.6. Discrimination of Saccharomyces cerevisiae *strains during the Fermentation Process in Chardonnay and Grenache Blanc Musts by* ***ATR-FTIR*** *Combined with* ***SIMCA***

It was also important to study which functional groups were mainly responsible of the biochemical differences between each *S. cerevisiae* strain at exponential and stationary phase. The **SIMCA** class projection plot showed well-separated and non-overlapping clusters between *S. cerevisiae* ES454, ES181, and E491 (**Figures 2.5** and **2.6**) strains. Moreover, **ICD** were ranging from 3.7 to 17.7 (**Tables 2.5** and **2.6**) showing differences in their biochemical patterns.

In the case of *S. cerevisiae* ES454 fermented in Chardonnay and Grenache blanc, discriminating power of **SIMCA** showed two and three strong spectral bands per each type of must tested at 1570 and 1009 cm^{-1} (**Figure 2.5 d**) and at 1060, 1380, and 1270 cm^{-1} (**Figure 2.6 d**), respectively.

The absorption band at 1570 cm^{-1} was linked to N–H and C–N vibrations of the peptide bond in different protein conformations (Cavagna et al., 2010); the IR band at 1009 cm^{-1} was associated to **β**(1→4) or **β**(1→6) glucans (Galichet et al., 2001). The **IR** band at 1060 cm-1 was related to mannans, at 1237 cm^{-1} to asymmetric stretching of PO_2^-

in **RNA** and phospholipids, and at 1380 cm^{-1} to CH_2 wagging vibrations in lipids and **β**(1→3) glucans. Two bands had a predominant effect on the classification *S. cerevisiae* ES181 strain fermented in Chardonnay, 1695 cm^{-1} (**Figure 2.5 e**) linked to amide I group vibrations of peptides and **α**-helix secondary protein structure and 1079 cm^{-1} associated to symmetric stretching of P=O (PO_2^-) in phosphodiesters.

Table 2.5. Soft independent modelling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* ES181 strain fermented in Chardonnay must at early exponential phase (48 h) and late exponential phase (120 h) and stationary phase (198 h) of transformed (second derivative, 25 point window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	***S. cerevisiae* ES454 48 h**	***S. cerevisiae* ES454 120 h**	***S. cerevisiae* ES454 198 h**
S. cerevisiae ES454 48 h	0.0		
S. cerevisiae ES454 120 h	4.3	0.0	
S. cerevisiae ES454 198 h	4.8	5.2	0.0
	***S. cerevisiae* ES181 48 h**	***S. cerevisiae* ES181 120 h**	***S. cerevisiae* ES181 198 h**
S. cerevisiae ES181 48 h	0.0		
S. cerevisiae ES181 120 h	7.6	0.0	
S. cerevisiae ES181 198 h	7.6	3.7	0.0
	***S. cerevisiae* E491 48 h**	***S. cerevisiae* E491 120 h**	***S. cerevisiae* E491 198 h**
S. cerevisiae E491 48 h	0.0		
S. cerevisiae E491 120 h	9.3	0.0	
S. cerevisiae E491 198 h	17.7	4.2	0.0

In the case of *S. cerevisiae* ES181 strain fermented in Grenache blanc (**Figure 2.6 e**), only one band at 1511 cm^{-1} related to amide II group vibrations of N–H and C–N vibrations of peptide bonds in different protein conformations (Cavagna et al., 2010) was found mainly responsible for the biochemical differences of this strain during the fermentation process.

On the other hand, three bands had a predominant effect on the classification of *S. cerevisiae* E491 strain fermented in Chardonnay (**Figure 2.5 f**): 1051, 1722, and 990 cm^{-1}.

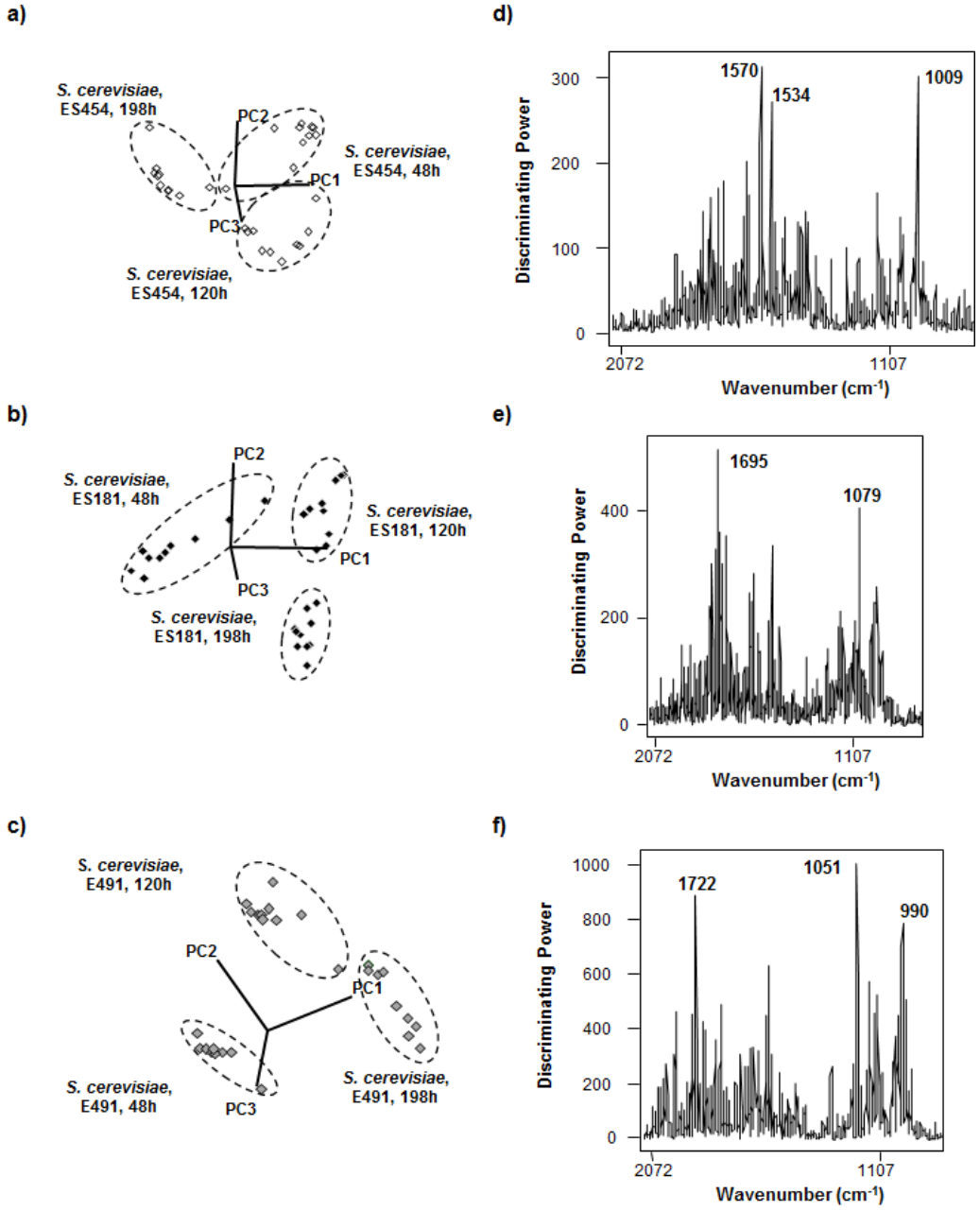

Figure 2.5. Soft independent modeling of class analogy (**SIMCA**) class projections (**a–c**) and discriminating power (**d–f**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}). *S. cerevisiae* ES454 (**a, d**), ES181 (**b, e**), and ES491 (**c, f**) strains fermented in Chardonnay must at 48, 120, and 198 h. Experiments were performed in triplicate.

These bands were associated to vibration of mannans, C–O stretching in lipid esters, and **β**(1→6) glucans bounds present in their cell wall (Galichet et al., 2001; Maquelin et al., 2002; Huang et al., 2008). In the case of this yeast strain fermented in Grenache blanc (**Figure 3.5 f**), the absorption band at 1080 cm^{-1} was mainly responsible of the biochemical differences and was linked to symmetric stretching of PO_2^- mainly from **RNA** (Cavagna et al., 2010).

Table 2.6. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* ES181 strain fermented in Grenache blanc must at exponential phase (172 h) and late exponential phase (344 h) and stationary phase (198 h) of transformed (second derivative, 25 point window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	***S. cerevisiae* ES454 172 h**	***S. cerevisiae* ES454 344 h**
S. cerevisiae ES454 172 h	0.0	
S. cerevisiae ES454 344 h	3.7	0.0
	***S. cerevisiae* ES181 172 h**	***S. cerevisiae* ES181 344 h**
S. cerevisiae ES181 172 h	0.0	
S. cerevisiae ES181 344 h	4.8	0.0
	***S. cerevisiae* E491 172 h**	***S. cerevisiae* E491 344 h**
S. cerevisiae E491 172 h	0.0	
S. cerevisiae E491 344 h	5.3	0.0

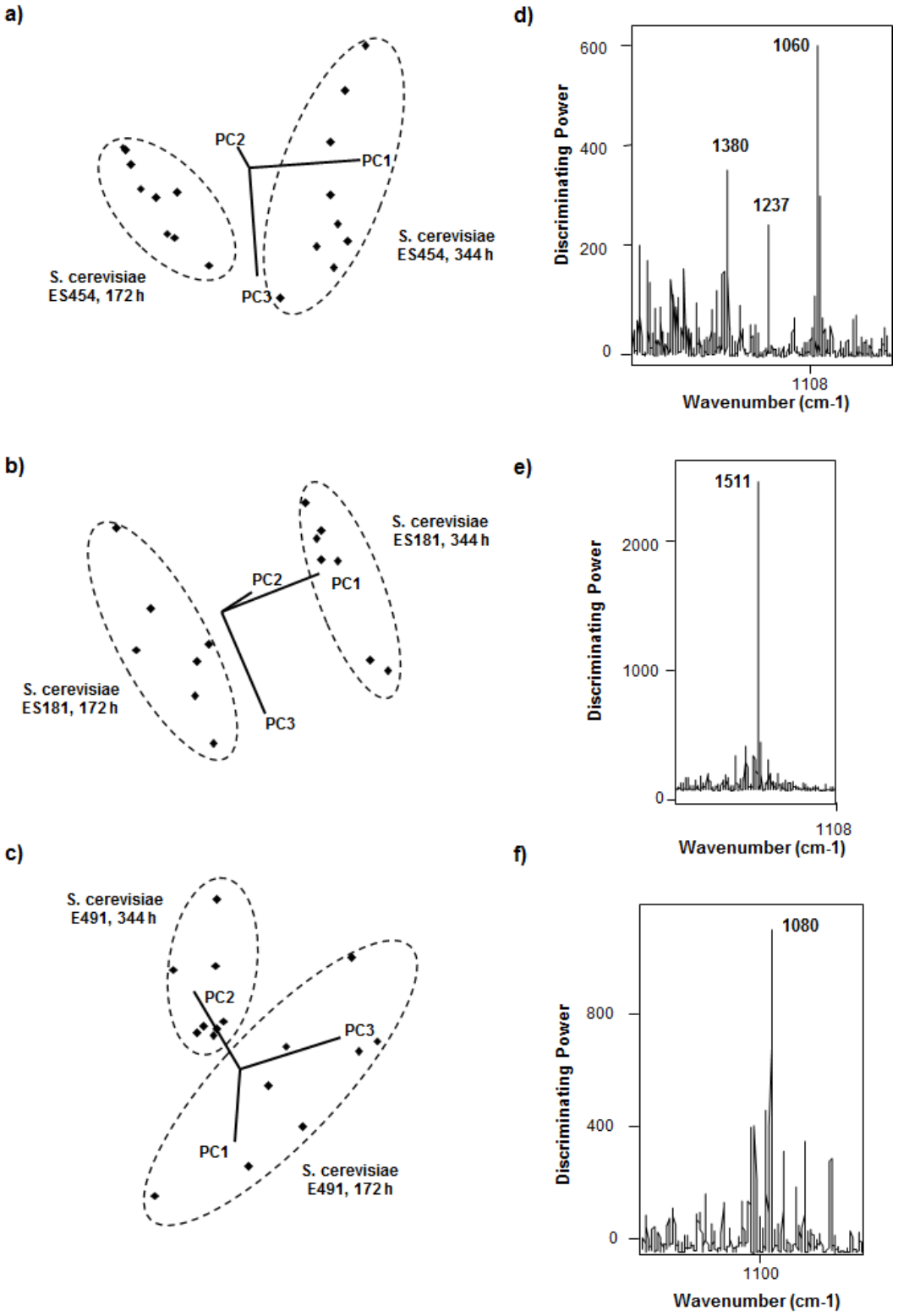

Figure 2.6. Soft independent modeling of class analogy (**SIMCA**) class projections (**a–c**) and discriminating power (**d–f**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}). *S. cerevisiae* ES454 (**a, d**), ES181 (**b, e**), and ES491 (**c, f**) strains fermented in Grenache blanc must at 172 and 344 h. Experiments were performed in triplicate.

2.4. Conclusions

The combination of **ATR-FTIR** with **SIMCA** to monitor *S. cerevisiae* grape must fermentation showed promising results to discriminate between different yeast strains and study biochemical changes produced during the fermentation process. Their differentiation was mainly associated with **IR** frequencies of *S. cerevisiae* cell wall. Depending on the physiological state, the components that played an important role to discriminate *S. cerevisiae* strains changed. At exponential phase, the cell wall components were mainly glucans, mannoproteins, and lipids and, at stationary phase, were mainly glucans and mannans.

2.5. References

Baldauf, N.A., Rodriguez-Romo, L.A., Männig, A., et al.,(2007). Effect of selective growth media on the differentiation of *Salmonella enterica* serovars by Fouriertransform mid-infrared spectroscopy. *Journal of Microbiological Methods*, 68: 106–114.

Beltran, G., Novo, M., Guillamón, et al., (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *International Journal of Food Microbiology*, 121: 169–177.

Burattini, E., Cavagna, M., Dell'Anna, R., et al., (2008). A FTIR microspectroscopy study of autolysis in cells of the wine yeast *Saccharomyces cerevisiae*. *Vibrational Spectroscopy*, 47: 139–147.

Cavagna, M., Dell'Anna, R., Monti, F., et al., (2010). Use of ATR-FTIR microspectroscopy to monitor autolysis of *Saccharomycescerevisiae* cells in a base wine.*Journal of Agricultural and Food Chemistry*, 58: 39–45.

Dunn, W.J., and Wold,S. (1995). SIMCA pattern recognition and classification.In H. van de Waterbeemd (Ed.), Chemometric methods in molecular design (pp. 179–193).EUA: VCH Publishers.

Ferreira, M.L., Costa, A.M., Ribeiro, N., et al., (2009). Quality control in FTIR wine analysis acceptance of analytical results.*Ciencia e Tecnica Vitivinicola*, 24: 47–53.

Galichet, G. D., Sockalingum, A., Belarbi, et al., (2001). FTIR spectroscopic analysis of *Saccharomycescerevisiae* cell walls: study of an anomalous strain exhibiting a pinkcolored cell phenotype. *FEMS Microbiology Letters*, 197: 179–186.

Grasso, E.M., Yousef, A.E., De Lamo-Castellvi, *S.*, et al., (2009). Rapid detection and differentiation of *Alicyclobacillus* species in fruit juice using hydrophobic grid membranes and attenuated total reflectance infrared microspectroscopy. *Journal of Agricultural and Food Chemistry*, 57: 10670–10674.

Hruschka, W.R. (2001). In P. Williams & K. Norris (Eds.), Near-Infrared technology in the agricultural and food industries (pp. 39–58). EUA: AACC, Inc.

Huang, K.C., Mukhopadhyay, R., Wen, B., et al., (2008). Cell shape and cell-wall organization in Gram-negative bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 105: 19282–19287.

Kansiz, M., Heraud, P., Wood, B., et al., (1999). Fourier transform infrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains. *Phytochemistry*, 52: 407–417.

Kapteyn, J.C., Van Den Ende, H., and Klis, F.M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall.*Biochimica et Biophysica Acta*, 1426: 373–383.

Komano, H., and Fuller, R.S. (1995). Shared functions in vivo of a glycosyl phosphatidylinositol-linked aspartyl protease, Mkc7, and the protein processing protease Kex2 in yeast.*Proceedings of the National Academy of Sciences of the United States of America*, 92: 10752–10756

Lachenmeier, D.W., Godelmann, R., Steiner, M., et al., (2010). Rapid and mobile determination of alcoholic strength in wine, beer and spirits using a flow-through infrared sensor. *Chemistry Central Journal*, 4: 5–6.

Laghi, L., Versari, A., Parpinello, G.P., et al., (2011). FTIR spectroscopy and direct orthogonal signal correction preprocessing applied to selected phenolic compounds in red wines. *Food Analytical Methods*, 4: 619–625.

Legras, J.L., and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiology Letters, 221, 249–255.

Lesage, G., and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, 70: 317– 343.

Maquelin, K., Kirschner, C., Choo-Smith, L.P., et al., (2002). Identification of medically relevant microorganisms by vibrational spectroscopy. *Journal of Microbiological Methods*, 51: 255–271.

Naruemon, M., Romanee,S., Cheunjit, P., et al., (2013). Influence of additives on *Saccharomy ces cerevisiae* β -glucan production. *International Food Research Journal*, 20: 1953–1959.

OIV, Organisation Internationale de la Vigne et du Vin (2008). Compendium of international méthods of wine and must analysis 2008 edition. OIV, Paris.

Oust, A., Mθretrθ, T., Kirschner, C., et al., (2004). FT-IR spectroscopy for identification of closely related lactobacilli,*Journal of Microbiological Methods*, 59: 149–162.

Querol, A., Barrio, E., and Ramon, D. (1992). A comparative study of different methods of yeast strain characterization. *Systematic and Applied Microbiology*, 15, 439–446.

Ratledge, C., and Evans, C.T. (1989). Lipids and their metabolism. In A. H. Rose & J. *S.* Harrison (Eds.), The yeasts (pp. 367–455). London: Academic Press.

Rosi, I., and Bertuccioli, M. (1992). Influences of lipid addition on fatty acid composition of *Saccharomyces cerevisiae* and aroma characteristics of experimental wines. *Journal of the Institute of Brewing*, 98: 305–314.

Subramanian, A., Ahn, J., Balasubramaniam, V.M., et al., (2007). Monitoring biochemical changes in bacterial spore during thermal and pressure-assisted thermal processing using FT-IR spectroscopy. *Journal of Agricultural and Food Chemistry*, 55: 9311–9317.

Versari, A., Parpinello, G.P., and Laghi, L. (2012). Application of infrared spectroscopy for the prediction of color components of red wines. *Spectroscopy*, 27: 36–43.

Walker, G.M. (1998). Yeast physiology and biotechnology (pp. 131– 135). UK: Wiley.

Werner-Washburne, M., Braun, E., Johnston, G. C., et al., (1993). Stationary phase in the yeast *Saccharomycescerevisiae*. *Microbiology Reviews*, 57: 383–401.

Yu, C.X., Irudayaraj, J., Debroy, C., et al., (2004). Spectroscopic differentiation and quantification of microorganisms in apple juice. *Journal of Food Science*, 69: 268–272.

UNIVERSITAT ROVIRA I VIRGILI
APPLICATION OF INFRARED SPECTROSCOPY IN MID-INFRARED RANGE COMBINED WITH MULTIVARIATE ANALYSIS TO STUDY YEASTS INVOLVED IN WINE PRODUCTION
Miquel Puxeu Vaqué

CHAPTER 3

Fourier-Transform Infrared Spectroscopy to Study *Saccharomyces cerevisiae* Nitrogen Supplementation

This chapter has been submitted as: Puxeu, M., Andorrà, I., Brull, A., and De Lamo-Castellví, S. Fourier-Transform Infrared Spectroscopy to Study *Saccharomyces cerevisiae* Nitrogen Supplementation. *Canadian Journal of Microbiology*.

Abstract

The main objective of this research was to use attenuated total reflectance (**ATR**) in the mid-infrared range (**MIR**) combined with multivariate analysis to study biochemical changes of *Saccharomyces cerevisiae* cells supplemented with nitrogen during an alcoholic fermentation process. Microvinifications were performed with 150 mL of Grenache blanc cleaned must inoculated with a commercial strain of *S. cerevisiae* E491 with and without the addition of commercial inorganic and organic nitrogen preparations: 10 g/hL ammonium salts with thiamin nutrient as a inorganic source and 30 g/hL for organic nitrogen source rich in amino acids, vitamins and minerals. Spectra were collected in the attenuated total reflectance mode in the mid-infrared region (4000-800 cm^{-1}). The **SIMCA** analysis confirmed that *S. cerevisiae* cells grown with the addition of inorganic and organic nitrogen were biochemically different from those cells grown without extra nitrogen added. The highest discrimination was detected at exponential and stationary phase and was related to proteins and lipid esters structures from yeast cell wall.

3.1. Introduction

It is well known that adequate nutritional conditions are essential to the correctly growth of *Saccharomyces cerevisiae* for a proper alcoholic fermentation (Bell et al., 2005). Grape juice is the main source of nutrients and quantitative and qualitative composition influences strongly the kinetics of fermentation and its duration. Nitrogen is a particularly significant nutrient being essential to the growth and metabolism of *Saccharomyces cerevisiae* (Bezenger et al., 1988; Bely et al., 1990; Manginot et al., 1998; Bell et al., 2005). Qualitative and quantitative composition of nitrogen fraction of the grape juice influences the kinetics fermentation, the final aroma, and the health-related metabolic by-products (Amerine et al., 1980; Torrea et al., 2011). Yeast available nitrogen (**YAN**) is essential for a successful fermentation. A rich supply of nitrogen allows high growth rated and biomass yield and stimulates fermentation activity and the formation of the end-products. Otherwise, a limited supply of nitrogen directly restricts the metabolic activity and growth of yeast ending in a sluggish or even non-existent alcoholic fermentation (Jiranek et al., 1995). Sluggish or stuck fermentation, together with the production of undesirable by-products such as H_2S formation are the main problems produced by a deficient nutrition environment (Bisson et al., 1999; Spiropoulos et al., 2000).

Various studies had shown that *S. cerevisiae* cells required minimum level of **YAN**/L 120 to 180 mg to obtain optimum fermentation kinetics (Bely et al., 1990; Sablayrolles et al., 1996). Nitrogen supplementation is recommended to correct the must deficiencies and ensure fine fermentation (Bely et al., 1990). The timing of nutrients addition is also important in winemaking process. Nitrogen is most effective when is added halfway through the fermentation process, during the acclimation phase at the beginning of the alcoholic fermentation (Bely et al., 1990; Sablayrolles et al., 1996). At this stage, it is assimilated and has immediate effects on the kinetics of fermentation, through the protein synthesis reactivation and particularly on sugar transporters (Bely et al., 1994). On the other hand, the late addition of **YAN** avoids its transportation and assimilation by the yeast cells (Bisson et al., 1991). Moreover, it has been reported that the supplementation of grape musts with inorganic sources of nitrogen mainly ammonium chloride and diammonium phosphate salts can decrease risks of slow and sluggish fermentations and

reduce the formation of undesirable volatile sulphur compounds (Bell et al., 2005; Torrea et al., 2011).

Fourier transform infrared spectroscopy (**FTIR**) technique has been widely used in winemaking sector as a rapid technique to determine quality control parameters (Ferreira et al., 2009; Lachenmeier et al, 2010). Some researchers have also used **FTIR** to study the main biochemical changes associated with autolysis in yeast cells finding IR bands related to proteins, peptides, mannans and **β**(1→3) glucans that are responsible of those changes (Lesage et al., 2006; Burattini et al., 2008; Cavagna et al., 2010). In a previous research, we used attenuated total reflectance Fourier transform infrared spectroscopy (**ATR-FTIR**) combined with soft independent modeling of class analogy (**SIMCA**) to study *S. cerevisiae* cells at exponential and stationary phase of growth during a fermentation process (Puxeu et al., 2014). The aim of the present research was to use **ATR-FTIR** combined with multivariate analysis to study the biochemical changes experimented by *S. cerevisiae* cells when two different sources of nitrogen (inorganic and organic) were added at the beginning of their alcoholic fermentation process.

3.2. Materials and Methods

3.2.1. Must Preparation and Chemical Analysis

Grenache Blanc grapes were acquired from Gandesa (Terra Alta, **ES**) and must (2.5 L) was obtained by pressing the grapes with a manual press with total capacity of 5 kg (Magusa, Vilafranca del Penedès, **ES**) until obtaining 54% of must yield. After adding 40 mg/L of sulphur dioxide and 1 g/hL of depectil clarification enzyme (Martin Vialatte, Sant Miquel d'Olèrdola, **ES**) the grape must was settled for 24 h to remove the solid fraction. The clarification performance was 81%. Brix degree and alcoholic potential strength by refractometry, pH, total acidity and final alcoholic strength were analysed by official European method, turbidity in Nephelometric Turbidity Units (**NTU**) and assimilable nitrogen were analysed following the procedure of the Office International de la Vigne et du Vin (**OIV**, 2008). All these parameters were used for characterization of the must tested. All the analysis was performed in triplicate.

3.2.2. Nutrients Addition and Microvinification Conditions

Three batches of cleaned must (150 mL) were inoculated with *Saccharomyces cerevisiae* strain E491 (Zymaflore X5®, Laffort, Bordeaux, **FR**). Inoculum was prepared according to the instructions provided by the supplier (20 g/hL) to reach an initial concentration in must of 10^6 cfu/mL. In the first batch, no exogenous nitrogen source was added (Control). In the second and third batch, 10 g/hL of an inorganic nitrogen based on ammonium salts with thiamin (20 mg/L of **YAN**, Thiazote, Laffort, Bordeaux, **FR**) and 30g/hL of an organic nitrogen source rich in amino acids, vitamins and minerals (21 mg/L of **YAN**, NutriStart OrganiQ, Laffort) were added respectively. Nutrients were added during the yeast rehydration following the manufacturer's instructions (Laffort).

Fermentations were performed at 21ºC using a thermostatic water bath and was daily monitored by controlling density with an electronic densimeter (Metler Toledo, Hospitalet de Llobregat, **ES**) and sugars as a sum of glucose and fructose (Biosystems, Barcelona, **ES**). Fermentations were considered ended when the concentration of these sugars was lower than 3 g/L. All fermentations were performed in triplicate. After the fermentation residual sugars (sum of the concentrations of glucose and fructose) and acetic acid contents were analyzed by an enzymatic method following the procedures of OIV (OIV, 2008) using an automatic enzymatic device (Biosystems, Barcelona, **ES**). For **IR** analysis, samples were taken at 0 h (initial point), 18 h (early exponential phase), 42 h (exponential phase) and 90 h (stationary phase). Sampling times were selected to monitor the changes induced by the addition of inorganic and organic sources of nitrogen in the main physiological stages described during alcoholic fermentation (Puxeu et al., 2014).

3.2.3. Sample Preparation

Samples (1.5 mL) were taken from fermentation batches using sterile material and centrifuged (15900 g for 5 min at room temperature). After centrifugation, the supernatant was carefully removed, and the pellets were washed three times under the same conditions described above using 1 mL of saline solution (**SS**, 0.88% **NaCl**) to obtain clean yeast cells, free of phenols, sugars, anthocyanins, organic acids and other potential remaining components to reduce interferences with the **IR** analysis. After the cleaning process, 1.5 µL of each pellet was placed onto Zinc Selenide (**ZnSe**) crystal in

order to acquire the spectral data. Six spectra per each sample and time (0 h, 18 h, 42 h and 90 h) were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000-800 cm^{-1}).

*3.2.4. Fourier Transform Infrared Spectroscopy (**FTIR**) Used in Attenuated Total Reflectance (**ATR**) Mode*

Spectra were obtained using an **FTIR** spectrometer Nicolet 380 (Thermo Scientific, Madrid, **ES**) adapted with an ultra-high-performance attenuated total reflectance (**ATR**) plate of **ZnSe** crystal (Smart **iTR**, Thermo Scientific, Madrid, **ES**). Spectra were collected from 4000 to 800 cm^{-1} with a resolution of 2 cm^{-1}. The spectrometer was controlled using **OMNIC™** control software (Version 7.0, Thermo Scientific, Madrid, Spain). The spectrum of each sample was obtained by taking the average of 32 scans to improve the signal-noise ratio. Spectra were displayed in terms of absorbance obtained by rationing the single beam spectrum against that of the air background.

3.2.4. Statistical and Multivariate Analyses

Analysis of variance was performed using the General Linear Models Procedure of **SAS®** software (**SAS®** System for Windows™, 8.02, 1999; **SAS** Institute, Cary, North Carolina, **USA**). Tukey was used to obtain paired comparisons among sample means. Level of significance was set at $P < 0.05$. Experiments were run three times with duplicate analysis in each replicate.

Spectra were exported to the Pirouette® multivariate analysis software (version 4.0, InfoMetrix, Inc., Woodville, **WA**). The **FTIR** spectral data were mean-centered, transformed to their second derivative using a 15-point Savitzky-Golay polynomial filter, and vector-length normalized; sample residuals and Mahalanobis distance were used to determine outliers (Park et al., 2001; Kansiz et al., 1999). Soft independent modeling of class analogy (**SIMCA**) was used to build models based on the construction of separate **PCA** models for each class to describe and model the variation (Kansiz et al., 1999). **SIMCA** class models were interpreted based on class projections, misclassifications, discriminating power and interclass distances. Total misclassifications were analysed and interpreted for the input data.

3.3. Results and Discussion

3.3.1. Must and Wine Characterization

Physical-chemical characteristics of Grenache blanc must are shown in **Table 3.1.** Grenache blanc must **YAN** content (170 mg/L) was appropriate for *S. cerevisiae* strain E491 to grow during early exponential phase and complete the alcoholic fermentation until all the sugars were consumed without any nutritional problems (Bely et al., 1990; Sablayrolles et al., 1996).

Table 3.1. Physical-chemical characteristics of Grenache blanc must. Results shown as mean ± Standard Deviation.

	Grenache blanc
Brix degree (ºBx)	20.3 ± 0.2
Alcoholic potential strength (% vol.)	11.8 ± 0.2
YAN (mg/L)	170 ± 6
pH	3.1 ± 0.1
Total acidity (g/L)	5.5 ± 0.1
Turbidity (**NTU**[a])	18 ± 2
Press yield (%)	54 ± 0.7
Clarification yield (%)	81 ± 0.8

[A] Nephelometric Turbity Units

A grape must with initial concentration of **YAN** without any extra addition was used as Control to reproduce a common practice applied by the wineries (Torrea et al., 2001). Total acidity (5.5 g/L)and pH (3.1) values were common for Grenache blanc grape at this ripening stage. Other authors had analysed the total acidity and pH values of Grenache blanc at the same ripening state being 5.9 g/L and 3.3, respectively (Ricardo-da Silva et al., 1993).

Table 3.2. Acetic acid and alcoholic strength of Grenache blanc wines obtained. Results are shown as mean ± Standard Deviation.

	Acetic acid (g/L)	Alcoholic strength (%vol.)
Control[1]	$0.42^{a} \pm 0.03$	$12.1^{B} \pm 0.1$
Inorganic nitrogen	$0.38^{a} \pm 0.02$	$12.3^{A} \pm 0.1$
Organic nitrogen	$0.40^{a} \pm 0.02$	$12.3^{A} \pm 0.1$

[a]column means of acetic acid values with different lower case superscripts differ ($P < 0.05$).
[A,B]column means of alcoholic strength values with different upper case superscripts differ ($P < 0.05$).
[1]Without nitrogen supplementation.

When fermentations processes were completed, the content of acetic acid and its alcoholic strength were also analysed (**Table 3.2**) finding significant differences only in the case of alcoholic strength values. Control fermentations had lower alcohol concentration at the end of the fermentation process than those performed adding inorganic and organic sources. These results were consistent with other studies (Bisson et al., 2000; Gobbi et al., 2013) showing that the increase of nitrogen stimulated the fermentation activity and the formation of the end-products.

3.3.2. Fermentation Performance

Fermentation performance was assessed through the daily measurement of sugar (glucose and fructose). Sugar concentration obtained at each time of the fermentation process (18, 42 and 90 h) were statistically compared (**Table 3.3**). At the early exponential phase (18 h), sugar concentration was significantly different between the three batches tested (**Table 3.3**). The batch produced with supplementation of inorganic nitrogen had the highest sugar concentration (164.7 g/L) and the batch fermented adding the organic source of nitrogen showed the lowest one (161.2 g/L). Moreover, significant differences were also detected at exponential and stationary phase (42 and 90 h, **Table 3.3**).

Table 3.3. Mean and Standard deviation of sugars concentration at different fermentation times studied (18, 42 and 90 h).

	Glucose + Fructose (g/L), 18 h	Glucose + Fructose (g/L), 42 h	Glucose + Fructose (g/L), 90 h
Control[1]	$162.1^{ab} \pm 1.5$	$71.6^{c} \pm 1.5$	$1.7^{e} \pm 0.5$
Inorganic nitrogen	$164.7^{b} \pm 1.5$	$56.0^{d} \pm 1.5$	$0.0^{f} \pm 0.0$
Organic nitrogen	$161.2^{a} \pm 0.7$	$87.8^{d} \pm 1.5$	$0.0^{f} \pm 0.0$

[a,b,c,d,e,f] column means of sugars concentration values with different lower case superscripts differ ($P < 0.05$).

[1]Without nitrogen supplementation.

At exponential phase, sugar concentrations were 71.6 g/L for Control, 56.0 g/L for inorganic source of nitrogen and 57.8 g/L organic source of nitrogen. At stationary phase, sugar concentrations were 2 g/L for Control and 0 g/L for the batches supplemented with inorganic and organic nitrogen sources. The nutrient additions at the beginning of alcoholic fermentation is quickly assimilated by yeast and immediately have effects on the kinetics of fermentation (Bely et al., 1994) stimulating fermentation activity and the formation of the end-products (Bisson et al., 2000; Gobbi et al., 2013). This behaviour had been reported by several authors, could explain the significant differences between sugar concentration values detected at exponential and stationary phases.

3.3.3. Raw Spectra and Second Derivative

Typical attenuated total reflectance Fourier transform infrared spectroscopy (**ATR-FTIR**) and their second derivative of fresh pellet of *S. cerevisiae* strain E491 fermented without and with the supplementation of inorganic and organic source of nitrogen are shown in **Figure 3.1**. The **IR** bands of highest proportion in the raw spectra were mainly concentrated in two different regions. The first one was located between 900 and 1200 cm^{-1} associated mainly with the polysaccharide absorbing region of *S. cerevisiae* (Galichet et al., 2001) and the second region was located between 1500 and 1750 cm^{-1} formed mainly by the vibrations of proteins and lipid structures (Cavagna et al., 2010; Puxeu et al., 2014).

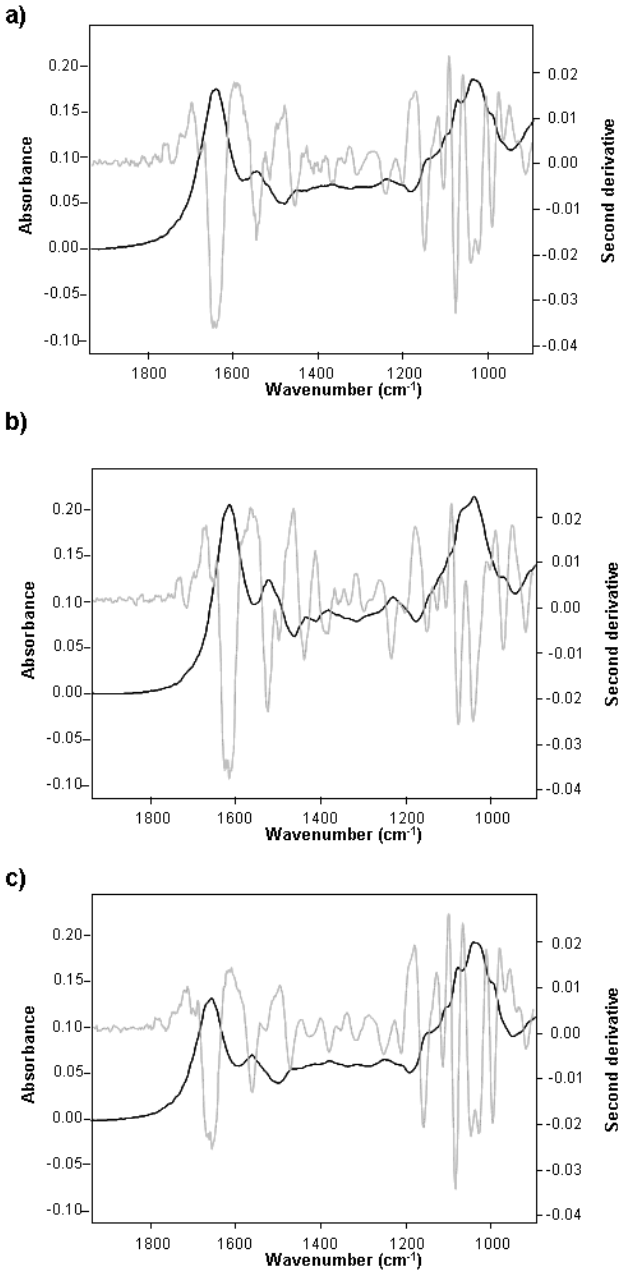

Figure 3.1. Typical attenuated total reflectance infrared spectra (**ATR-FTIR**) and their second derivative of *S. cerevisiae* strain ES491 fermented for 18 h without (**a**) and with a source of inorganic (**b**) and organic (**c**) nitrogen. The spectra were taken with a zinc selenide crystal accessory in reflectance mode.

3.3.4. Biochemical Changes during Alcoholic Fermentation Analysed by ***ATR-FTIR*** *Combined with* ***SIMCA***

Class projections of **SIMCA** classification model of transformed spectra (1900-800 cm^{-1}) of *S. cerevisiae* strain E491 fermented in Grenache blanc must with and without using an extra nitrogen source (**Figure 3.2a, Figure 3.3a** and **Figure 3.4a**) showed clear differentiation between non-fermented (0 h) and fermented *S. cerevisiae* cells (18, 42 and 90 h).

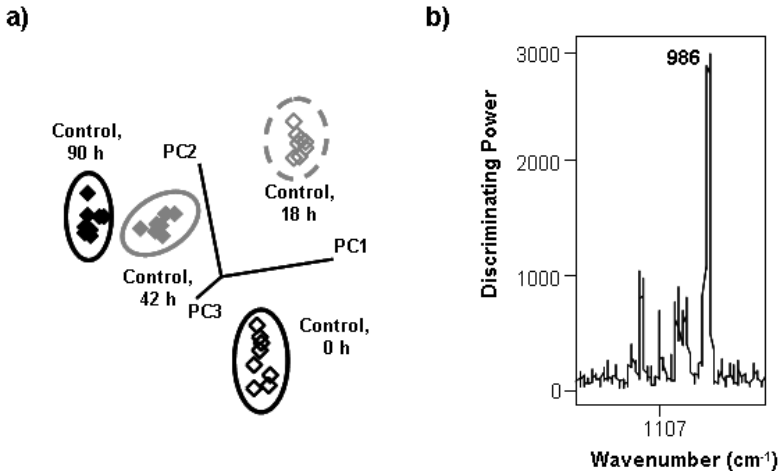

Figure 3.2. Soft independent modeling class analogy (**SIMCA**) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must without nitrogen supplementation (Control) for 0, 18, 42 and 90 h.

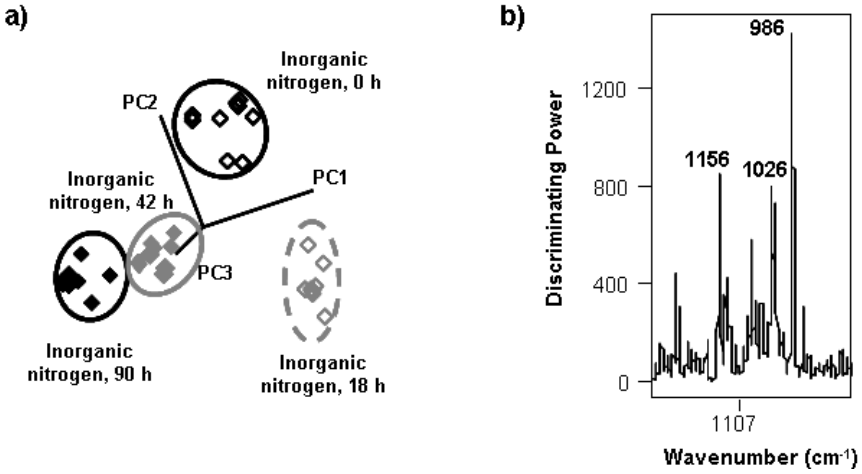

Figure 3.3. Soft independent modeling class analogy (**SIMCA**) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must with supplementation of inorganic source of nitrogen for 0, 18, 42 and 90 h.

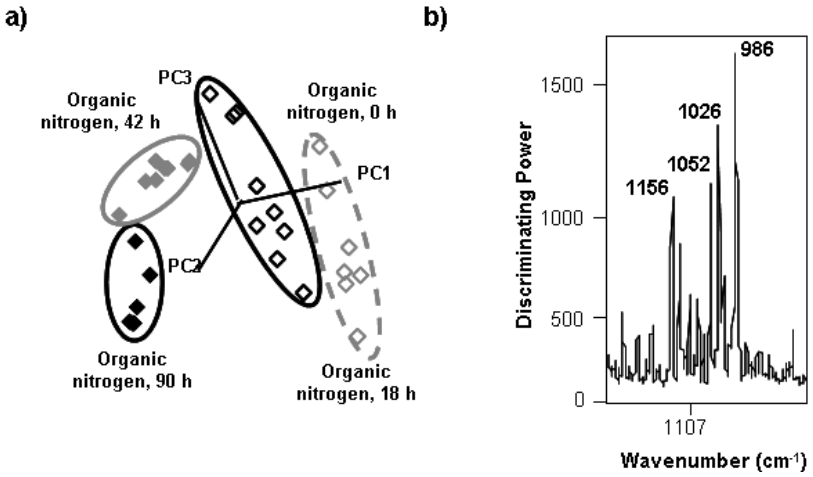

Figure 3.4. Soft independent modeling class analogy (**SIMCA**) of class projections (**a**) and discriminating power (**b**) of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must with supplementation of organic source of nitrogen for 0, 18, 42 and 90 h.

Interclass distances (**ICD**) values further proved these findings. Generally, **ICD** values above 3.0 are considered significant to discriminate two clusters of samples as a different class (Dunn et al., 1995). **ICD** values between non-fermented and fermented *S. cerevisiae* cells without extra nitrogen added, inorganic source and organic source of nitrogen (**Table 3.4**) varied from 4.4 to 22.4 showing different pattern of clustering between *S. cerevisiae* cells at early and exponential phase and at stationary phase. Moreover, *S. cerevisiae* cells cluster fermented for 18 h (early exponential phase) without and with nitrogen supplementation showed the highest values of **ICD** when was compared with the clusters of *S. cerevisiae* cells fermented for 42 h (exponential phase) and 90 h (stationary phase). For instance, in the case of Grenache blanc must supplemented with the organic source of nitrogen (**Table 3.4**) the **ICD** values of *S. cerevisiae* cells fermented for 18 h compared with *S. cerevisiae* cells fermented for 42 h and 90 h were 13.7 and 17.1 respectively. Nonetheless, when *S. cerevisiae* cells fermented for 42 h and 90 h were compared among them, the **ICD** value was 5.8 and 4.4 for inorganic and organic nitrogen sources respectively.

Table 3.4. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* E491 strain fermented in Grenache must without and with inorganic and organic nitrogen supplementation for 0, 18, 42 and 90 h.

Control	**0 h**	**18 h**	**42 h**	**90 h**
0 h	0.0			
18 h	13.3	0.0		
42 h	10.3	15.6	0.0	
90 h	16.0	22.4	6.2	0.0
Inorganic nitrogen	**0 h**	**18 h**	**42 h**	**90 h**
0 h	0.0			
18 h	7.9	0.0		
42 h	7.0	13.9	0.0	
90 h	9.5	16.3	5.8	0.0
Organic nitrogen	**0 h**	**18 h**	**42 h**	**90 h**
0 h	0.0			
18 h	4.7	0.0		
42 h	9.5	13.7	0.0	
90 h	11.7	17.1	4.4	0.0

These results showed that the highest biochemical differences between *S. cerevisiae* cells were found when yeast cells were at early exponential phase in the three matrices tested. Discriminating power of *S. cerevisiae* cells non-fermented and fermented without and with nitrogen supplementation (**Figure 3.2b, Figure 3.3b** and **Figure 3.4b**) showed a common band at 986 cm^{-1} linked to with **β**(1→6) glucans (Cavagna et al., 2010; Kuligowski et al., 2012). In the case of discriminating power of *S. cerevisiae* cells non-fermented and fermented with nitrogen supplementation (**Figure 3.3b** and **Figure 3.4b**), two secondary **IR** bands at 1026 and 1156 cm^{-1} linked to **β**(1→4) glucans and C-O, C-OH carbohydrates present in yeast cell wall (Cavagna et al., 2010; Kuligowski et al., 2012). These results are in agreement with a previous research performed with several strains of

S. cerevisiae fermented in Grenache blanc and Chardonnay musts (Puxeu et al., 2014). In this case, **SIMCA** models were used to detect biochemical changes produced by *S. cerevisiae* cells at different physiological states during a fermentation process. Depending on the strain tested, the compounds mainly responsible of *S. cerevisiae* cells discrimination during the entirely fermentation process in Grenache blanc were mannoproteins, lipids and **RNA**. Moreover, it is important to mention that discrimination power values of 986 cm^{-1} band decreased with the supplementation of inorganic (2990 to 1432 units) (**Figure 3.2b** and **Figure 3.3b**) and organic nitrogen (2990 to 1646 units) (**Figure 3.2b** and **Figure 3.4b**) showing higher discrimination when *S. cerevisiae* cells were fermenting without nitrogen supplementation.

It was also important to study the possibility to discriminate between *S. cerevisiae* cells fermented without and with nitrogen supplementation. For this purpose, **SIMCA** models were built up using **IR** data from non-fermented (0 h) and fermented *S. cerevisiae* cells (18, 42 and 90 h) without and with inorganic and organic nitrogen supplementation (**Table 3.5**). The distance between the clusters of non-fermented without and with nitrogen supplementation and fermented samples increased over the time of fermentation excepting for *S. cerevisiae* cells fermented for 42 h (exponential phase) with the addition of nitrogen and *S. cerevisiae* cells fermented for 90 h (stationary phase) with the addition of organic nitrogen (**Table 3.5**). In this case, **ICD** values of non-fermented and fermented *S. cerevisiae* cells without and with the supplementation of nitrogen varied from 1.6 to 19.2 (**Table 3.5**). In general, *S. cerevisiae* cells fermented without and with the addition of inorganic source of nitrogen were clearly differentiated at early (**ICD** 2.7), exponential (**ICD** 4.2) and at stationary phase (**ICD** 3.6). Nonetheless, *S. cerevisiae* cells fermented without and with the addition of organic nitrogen were only differentiated at exponential phase (**ICD** 2.8). When *S. cerevisiae* cells fermented with the supplementation of inorganic and organic sources of nitrogen were compared, just yeast cells fermented for 42 h (exponential phase) were different (**ICD** 3.1) and the lowest differences were found at stationary phase (**ICD** 1.6). Moreover, *S. cerevisiae* cells cluster fermented for 18 h without and with the supplementation of nitrogen showed the highest values of **ICD** when was compared with the clusters of *S. cerevisiae* cells fermented for 42 h and 90 h (**Table 3.5**). This trend was also detected when **SIMCA** models of fermented *S. cerevisiae* cells without and with nutrient supplementation were built up separately (**Figure 3.2a**, **Figure 3.3a**, **Figure 3.4a** and **Table 3.4**).

Discriminating power (**Figure 3.5**) showed a unique **IR** band at 986 cm^{-1} mainly responsible of the biochemical differences between the samples compared. This band also played an important role on the differentiation of non-fermented and fermented *S. cerevisiae* cells when **SIMCA** models were built up separately.

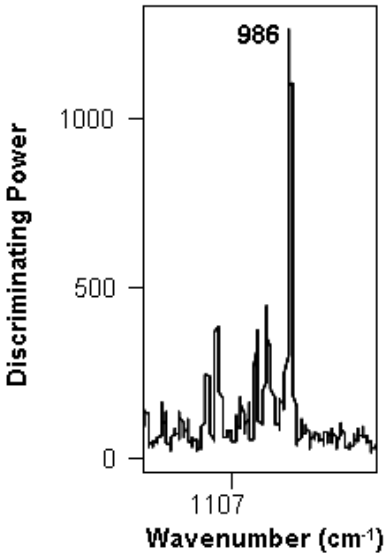

Figure 3.5. Soft independent modeling of class analogy (**SIMCA**) of discriminating power of *S. cerevisiae* E491 strain fermented in Grenache must for 0, 18, 42 and 90 h without and with inorganic and organic nitrogen supplementation.

All the **IR** bands mainly responsible of the biochemical differences between the samples compared were related with compounds presents in the *S. cerevisiae* cell wall (Kapteyn et al., 1999; Klis et al., 2002; Klis et al., 2006). According to these results, supplementing the Grenache blanc must with inorganic and organic sources of nitrogen had an impact on the composition of the yeast cell wall, especially at early exponential phase related to the presence of **β**(1→6) glucans.

Table 3.5. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of non-fermented (0 h) and fermented *S. cerevisiae* E491 cells (18, 42 and 90 h) without and with inorganic and organic nitrogen supplementation.

	Control, 0 h	Inorganic nitrogen, 0 h	Organic nitrogen, 0 h	Control, 18 h	Inorganic nitrogen, 18 h	Organic nitrogen, 18 h	Control, 42 h	Inorganic nitrogen, 42 h	Organic nitrogen, 42 h	Control, 90 h	Inorganic nitrogen, 90 h	Organic nitrogen, 90 h
Control, 0 h	0.0											
Inorganic nitrogen, 0 h	6.0	0.0										
Organic nitrogen, 0 h	3.8	3.8	0.0									
Control, 18 h	9.2	6.5	4.6	0.0								
Inorganic nitrogen, 18 h	9.9	7.2	5.9	2.7	0.0							
Organic nitrogen, 18 h	9.0	6.7	4.6	2.0	2.3	0.0						
Control, 42 h	7.7	5.5	6.6	13.0	12.1	11.7	0.0					
Inorganic nitrogen, 42 h	10.1	7.2	9.6	16.2	13.2	14.0	4.2	0.0				
Organic nitrogen, 42 h	9.5	7.3	9.6	15.2	12.0	13.6	2.8	3.1	0.0			
Control, 90 h	11.0	9.4	12.5	19.2	17.4	17.0	6.2	10.3	7.0	0.0		
Inorganic nitrogen, 90 h	10.4	9.4	13.0	18.9	15.1	16.7	4.2	5.5	4.3	3.6	0.0	
Organic nitrogen, 90 h	8.7	7.8	10.6	17.1	14.9	15.3	4.0	5.5	4.5	2.3	1.6	0.0

*3.3.5. **SIMCA** Biochemical Changes Induced by Inorganic and Organic Nitrogen Supplementation Analysed by **ATR-FTIR** Combined with **SIMCA***

In this research it was also interesting to compare among fermented *S. cerevisiae* cells without and with nitrogen supplementation at early exponential (18 h), exponential phase (42 h) and stationary phase (90 h) to study the biochemical differences present in each physiological phase depending on the source of nitrogen used. At early exponential phase, clusters from fermented *S. cerevisiae* cells without (Control) and with inorganic (**ICD** 4.4) and organic (**ICD** 3.1) nitrogen supplementation were clearly separated (data not shown). The major discriminating bands observed when 2-classes **SIMCA** classification models were developed using transformed spectra from *S. cerevisiae* fermented without (Control) and with inorganic (**Figure 3.6a**) and organic (**Figure 3.6d**) sources of nitrogen were 1538 and 1511 cm^{-1}, respectively. The **IR** bands at 1538 and 1511 cm^{-1} were related to amide II group vibrations of N-H and C-N bounds from peptides (Cavagna et al., 2010; Kuligowski et al., 2012). In addition to these major discriminating bands, there was a small contribution of amide I band resulting from antiparallel pleated sheets and **β**–turns of proteins at 1696 cm^{-1} (Maquelin et al., 2002) in the discrimination of *S. cerevisiae* fermented without (Control) and with organic (**Figure 3.6d**) nitrogen. In the case of *S. cerevisiae* cells at exponential phase (42 h) fermented without (Control) and with inorganic nitrogen (**Figure 3.6b**), the biochemical differences were also linked to **IR** bands (1550 and 1647 cm^{-1}) related to different protein structures. Whereas, when **SIMCA** models were built up using **IR** data from *S. cerevisiae* cells fermented at exponential phase without (Control) and with organic nitrogen (**Figure 3.6e**), the discrimination among clusters was due to two **IR** bands at 1714 and 1744 cm^{-1} related to C-O stretching of carbonic acid or nucleic acids (Maquelin et al., 2002). Finally, at stationary phase (90 h), *S. cerevisiae* cells fermented without (Control) and with inorganic nitrogen were mainly discriminated by **IR** bands related to protein structures (1678 and 1541 cm^{-1}), to asymmetric stretching of PO_2^- in RNA and phospholipids and lipid esters (1285 and 1748 cm^{-1}) and to CH_2 scissoring vibrations of lipids (**Figure 3.6c**). In the case of *S. cerevisiae* cells fermented at stationary phase without (Control) and with organic nitrogen (**Figure 3.6f**) a unique band at 1714 cm^{-1} linked to C-O stretching of carbonic acid or nucleic acids (Maquelin et al., 2002) was mainly responsible of their discrimination.

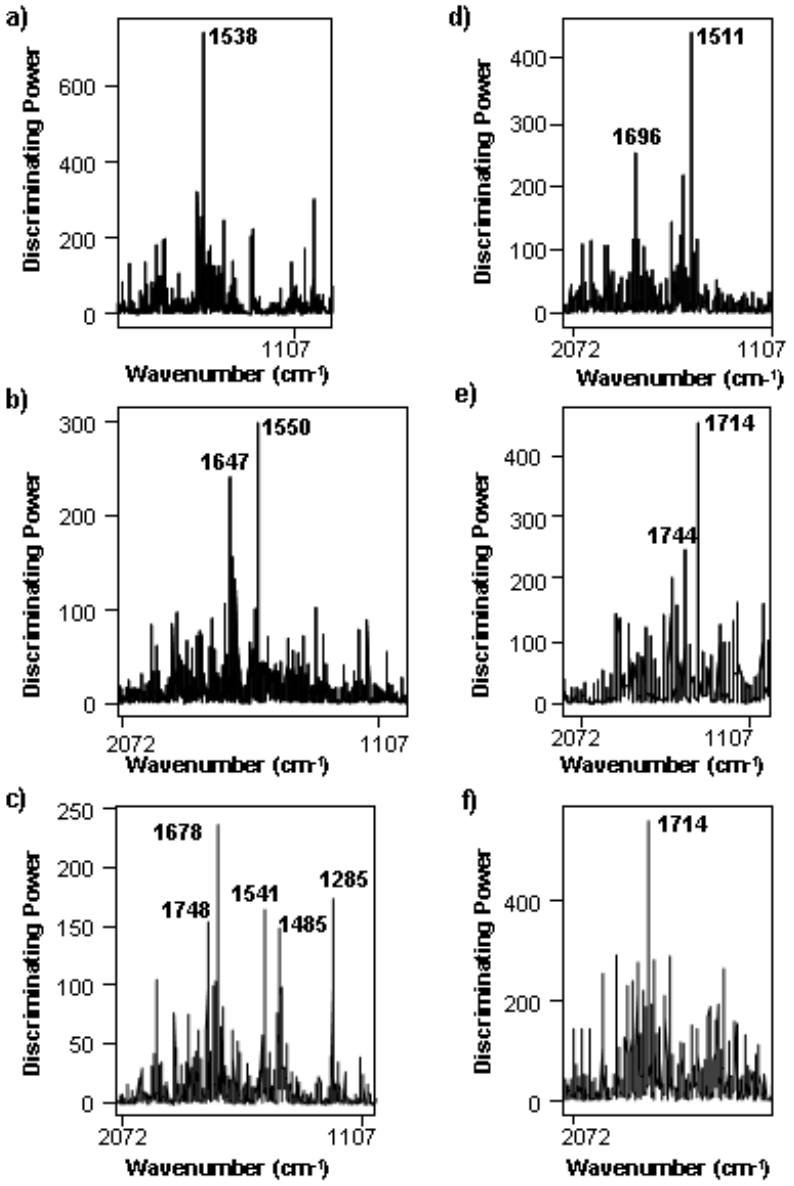

Figure 3.6. Soft independent modeling class analogy (**SIMCA**) of discriminating power of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy spectra of *S. cerevisiae* strain E491 fermented in Grenache must without and with inorganic nitrogen for18 h (**a**), 42 h (**b**) and 90 h (**c**) and without and with organic nitrogen for 18 h (**d**), 42 h (**e**) and 90 h (f).

To summarize, the supplementation of Grenache blanc must with inorganic and organic sources of nitrogen showed biochemical differences especially when *S. cerevisiae* cells were grown at exponential and stationary phase. These results are in agreement with previous studies performed to evaluate the effect of nitrogen supplementation on *S. cerevisiae* cells alcoholic fermentation. Some authors have described the relation between must nitrogen supplementation and the formation of ethyl esters from medium-chain fatty

acids (**MCFA**) (Saerens et al., 2006) derived from the metabolism of sugars and also amino acids by yeast (Lilly et al., 2000; Swiegers et al., 2005; Carrau et al., 2008). The synthesis of **MCFA** ethyl esters is produced by of substrate availability and could be related to the relative increase of fatty acids synthesis due to nitrogen supplementation (Sarens et al., 2006 and 2008). Other studies showed that the addition of organic nitrogen (mixture of amino acid and ammonium nitrogen) resulted in higher concentrations of **MCFA** ethyl esters that those samples supplemented with inorganic sources alone (ammonium salts) (Torrea et al., 2011). In contrast to these results, other authors have suggested that when a high total **YAN** concentration is naturally present in the grape must, the addition of amino acids can reduce ester concentration due to feedback inhibition suppressing amino acid uptake (Miller et al., 2007). From our research, we can conclude that a positive correlation between total nitrogen and esters formation was detected as other authors suggested (Vilanova et al., 2007; Ugliano et al., 2008) and the nature of the source of nitrogen used can influence the ester production and the composition of the yeast cell wall composition.

3.4. Conclusions

The combination of **ATR-FTIR** with **SIMCA** allowed studying the biochemical changes produced during alcoholic fermentation induced by the supplementation of Grenache Blanc with organic and inorganic sources of nitrogen. Depending on the source of nitrogen used and the physiological phase studied, the yeast cell wall components that were revealed to differentiate no supplemented and supplemented *S. cerevisiae* cells were different. In the case of inorganic source of nitrogen, at exponential phase the components were protein structures and at stationary phase there was a contribution of lipid esters. Nevertheless, when a source of organic nitrogen was used, the main changes at exponential and stationary phase were produced by changes on nucleic acids and lipid esters.

3.5. References

Amerine, M.A., Berg, H.W., Kunkee R.E., et al. (1980). The technology of wine making (4th ed.). USA: AVI Publishing Co. (pp.105-123).

Bell, S.J. and Henschke, PA. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research,* 11, 242-295.

Bely, M., Sablayrolles, J.M. and Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during fermentation in enological conditions. *Journal of Fermentation and Bioengineering,* 70, 246-252.

Bely, M., Sablayrolles, J.M. and Barre, P. (1990). Description of alcoholic fermentation kinetics, its variability and significance. *American Journal of Enology and Viticulture* 41, 319-324.

Bely, M., Salmon, J.M. and Barre, P. (1994). Assimilable nitrogen addition and hexose transport system activity during enological fermentation. *Journal of the Institute of Brewing,* 100, 279–282.

Bezenger, M.C. and Navarro, J.M. (1988). Alcoholic fermentation – model accounting for initial nitrogen influence. *Biotechnology and Bioengineering,* 31, 747-749.

Bisson, L.F. (1991). Influence of nitrogen on yeast and fermentation of grapes, in: Proceedings of the international symposium on nitrogen in grapes and wine, USA: *American Society for Enology and Viticulture*, (pp 78–89 and 266–269).

Bisson, L.F. and Butzke, C.E. (2000). Diagnosis and rectification of stuck and sluggish fermentations. *American Journal of Enology and Viticulture,* 51:168-177.

Bisson, L.F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture,* 50, 107-119.

Borst-Pauwels, G.W. (1981). Ion transport in yeast. *Biochimica Et Biophysica Acta,* 650, 88-127.

Burattini, E., Cavagna, M., Dell'Anna, et al. (2008). A FTIR microspectroscopy study of autolysis in cells of the wine yeast *Saccharomyces cerevisiae*. *Vibrational Spectroscopy*, 47, 139-147.

Carrau, F.M., Medina K., Farina L., et al. (2008). Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Research*, 8, 1196-1207.

Cavagna, M., Dell'Anna, R., Monti, F., et al. (2010). Use of ATR-FTIR microspectroscopy to monitor autolysis of *Saccharomyces cerevisiae* cells in a base wine. *Journal of Agricultural and Food Chemistry*, 58, 39–45.

Dunn, W.J., and Wold, S. (1995). SIMCA pattern recognition and classification in Chemiometric Methods in Molecular Design, ed. H. van de Waterbeemd, EUA; VCH Publishers, (pp. 179-193).

Ferreira, M.L.; Costa, A.M.; Ribeiro, N., et al. (2009). Quality control in FTIR wine analysis acceptance of analytical results. *Ciencia e Tecnica Vitivinicola*, 24,47-53.

Galichet, A., Sockalingum, G.D., Belarbi, A. et al. (2001). FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype. *FEMS Microbiology Letters,*197, 179–186.

Gobbi, M., Comitini, F., D'Ignazi, et al. (2013). Effects of nutrient supplementation kinetics, H_2S evolution, and aroma profile in Verdicchio DOC wine production. *European Food Research and Technology*, 236, 145-154.

Jiranek, V. Langridge, P. and Henschke, P.A. (1995). Amino-Acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeast from a chemically-defined medium. *American Journal of enology and Viticulture*, 46, 75-83.

Jiranek, V., Langridge, P. and Henschke, P.A. (1995). Regulation of hirdogen sulfide liberation in wine producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. *Applied and Environmental Microbiology,* 61, 461-467.

Kansiz M., Heraud P., Wood B., et al. (1999). Fourier transform infrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains. *Phytochemistry*, 52, 407-417.

Kapteyn, J.C., Van Den Ende, H. and Klis, F.M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochimica et Biophysica Act*a, 1426, 373-383.

Klis, F.M., Boorsma, A. and De Groot, P.W.J. (2006). Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*, 23:185-202.

Klis, F.M., Mol, P., Hellingwerf, K. et al. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews,* 26:239–256.

Kuligowski, J., Quintas, G., Herwig, C., et al. (2012). A rapid method for the differentiation of yeast cells grown under carbon and nitrogen-limited conditions by means of partial least squares discriminant analysis employing infrared micro-spectroscopic data of entire yeast cells. *Talanta*, 99: 566-573.

Lachenmeier, D.W., Godelmann, R., Steiner, M., et al. (2010). Rapid and mobile determination of alcoholic strength in wine, beer and spirits using a flow-through infrared sensor. *Chemistry Central Journal*, 4, 5-6.

Lesage, G. and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, 70, 317–343.

Lilly M., Lambrechts M.G. and Pretorius, I.S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Applied and Environmental Microbiology*, 66, 744-753.

Manginot, C., Roustand, J.L. and Sablayrolles, J.M. (1998). Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enzyme and Microbial Technology*, 23, 511-517.

Maquelin, K., Kirschner, C., Choo-Smith, L.P., et al. (2002). Identification of medically relevant microorganisms by vibrational spectroscopy. *Journal of Microbiological Methods*, 51, 255-271.

Miller, A.C., Wolf, S.R., Bisson, L.F. et al. (2007). Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. *American Journal of Enology and Viticulture*, 58:470-483.

OIV, Organisation Internationale de la Vigne et du Vin (2008). Compendium of international méthods of wine and must analysis 2008 edition. OIV, Paris.

Park, B., Chen, Y.R., Hruschka, W.R., et al. (2001). Principal component regression of near-infrared reflectance spectre for beef tenderness prediction. *Transactions of the American Society of Agriculture Engineers*, 44, 609-615.

Puxeu, M., Andorra, I. and De Lamo-Castellví S. (2014). Monitoring *Saccharomyces cerevisiae* grape must fermentation process by attenuated total reflectance spectroscopy. *Food and Bioprocess Tecnology,* 8, 637-646.

Ricardo-da-Silva, J.M., Cheynier, V., Samson, A., et al. (1993). Effect of Pomace contact, carbonic maceration and hyperoxidation on the procyanidin composition of Grenache blanc wines. *American Journal of Enology and Viticulture*, 44, 168-172.

Sablayrolles J.M, Dubois C., Manginot C., et al. (1996). Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations. *Journal of Fermentation and Bioengineering*, 82, 377–381.

Saerens, S.M.G., Delvaux, F., Verstrepen, K.J., et al. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology,* 74, 454-461.

Saerens, S.M.G., Verstrepen, K.J., Van Laere, S.D.M., et al. (2006). The *Saccharomyces cerevisiae* EHT1 and EBB1 genes encode novel enzymes with medium-chain fatty acid ethyl esters synthesis and hydrolysis capacity. *Journal of Biological Chemistry*, 281, 4446-4456.

Spiropoulos, A., Tanaka, J., Flerianos, I. et al. (2000). Characterization of hydrogen sulfide formation in commercial and natural wine isolates of *Saccharomyces*. *American Journal of Enology and Viticulture*, 51, 233-248.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. et al. (2005). Yeast and bacterial modulation of wine aroma and flavor. *Australian Journal of Grape and Wine Research*, 11, 139-173.

Swingers, J.H. and Pretorius, I.S. (2005). Yeast modulation of wine flavour. *Advances in Applied Microbiology*, 57, 131-175.

Torrea, D., Varela, C., Ugliano, M., et al. (2011). Comparision of inorganic and organic nitrogen supplementation of grape juice. Effect on volatile composition and aroma profile of a Chardonnay wine fermented with *Saccharomyces cerevisiae* yeast. *Food Chemistry*, 127: 1072-1083.

Ugliano, M., Siebert, T., Mercurio, M., et al. (2008). Volatile and color composition of young and model-aged Shiraz wines as affected by diammond phosphate supplementation before alcoholic fermentation. *Journal of Agricultural and Food Chemistry*, 56, 9175-9182.

Vilanova, M., Ugliano, M., Varela, C., et al. (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeast. *Applied Microbiology and Biotechnology*,77: 145-157.

Werner-Washburne, M., Braun, E., Johnston, G.C. et al. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology*, 57: 383-401.

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CHAPTER 4

Discrimination of *Saccharomyces cerevisiae* and Non-*Saccharomyces* Species by Attenuated Total Reflectance Infrared Spectroscopy Combined with Multivariate Analysis

Abstract

The potential of using attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) combined with multivariate analysis to discriminate *Saccharomyces cerevisiae* and Non-*Saccharomyces* wine yeast species was investigated. Thirty eight strains (twenty nine *S. cerevisiae* and nine Non-*Saccharomyces*) isolated from Spanish wines and identified by molecular techniques, were inoculated to Tempranillo thermovinificated red must and fermented juices were taken after 48 h at 28ºC. Pellets obtained after a centrifugation process, were placed onto diamond crystal. Spectra were collected in the attenuated total reflectance (**ATR**) mode in the mid-infrared region (4000–800 cm^{-1}) and were analyzed by a multivariate analysis technique (**SIMCA**). To discriminate between yeast strains, 2-classes **SIMCA** model of Non-*Saccharomyces* and S. *cerevisiae* strains was built up showing tight clustering but close grouping (interclass distance of 1.7). Then, two **SIMCA** models were created separately with **IR** data from Non-*Saccharomyces* and *S. cerevisiae* strains and were validated obtaining scores above 89%. Physiological growth phase of each strain was taken into consideration to build up **SIMCA** models improving substantially the close grouping detected between yeasts strains or species. Discrimination between Non-*Saccharomyces* and S. *cerevisiae* strains was linked to cell wall components.

4.1. Introduction

Fourier-Transform infrared (**FTIR**) spectroscopy in mid infrared range combined with powerful supervised pattern recognition techniques such as Soft Independent Modeling Class Analogy (**SIMCA**) is a non-destructive, simple, fast and highly specific technology that can be used for the differentiation and identification of vegetative cells and spores (Subramanian et al., 2007). The application of chemometrics to **IR** data has allowed the detection of subtle chemical differences between strains of the same species of bacteria and yeast. Some researchers have successfully built up multivariate classification models for discrimination and prediction of food-borne yeasts (Kümmerle et al., 1998) or *S. cerevisiae* and *S. bayanus* strains (Adt et al., 2010). Others authors, have used micro spectroscopy to monitor autolysis of *S. cerevisiae* cells in a base wine (Burattini et al., 2008; Cavagna et al., 2010). There is considerable controversy concerning the effect that different alcoholic fermentation processes exercise on the organoleptic quality of wine. Some researchers have found remarkable differences between fermentations conducted with pure cultures and those with native yeasts (Bisson 1999; Dominzo et al., 2011). The Non-*Saccharomyces* yeasts contain numerous species, dominated numerically by the apiculate yeasts, e.g. *Kloeckera* spp. and *Candida* spp. (Jolly et al., 2006) and each yeast confers specific desired wine properties (Ciani and Maccarelli et al., 1998). For instance, *Metschnikowia pulcherrima* produces highconcentrations of β-glucosidase (Rodríguez et al., 2010), medium chain fatty acids, esters, terpenols and glycerol (Rodríguez et al., 2010; Sadineni et al., 2012). On the other hand, *Debaryomyces hansenii* is capable of maintaining **β**-glycosidase activity in the presence of high ethanol content (up 15% vol) and releases terpens (Yanai and Sato, 1999). Therefore, Non-*Saccharomyces* yeast species can be used to develop unique and singular wine products (Jolly et al., 2003; Ciani et al., 2010). Traditionally, methods to discriminate yeasts are based on morphological tests supplemented with physiological tests (Fugelsang and Edwards 2007). Molecular techniques such as restriction fragment length polymorphism analysis of **PCR**-Amplified Fragments (**PCR-RFLP**) could be used for the identification of different wine yeast species (Esteve-Zarzoso et al., 1999). Nonetheless, molecular techniques required trained personnel and sample preparation is laborious and time consuming. Therefore, there is a need for simple, high-throughput, and reliable technique for rapid discrimination of *S. cerevisiae* and Non-*Saccharomyces* species in the wine sector.

The goal of this work is to study the potential of using infrared spectroscopy combined with multivariate analysis to develop a non-destructive method to discriminate *S. cerevisiae* and Non-*Saccharomyces* yeast species.

4.2. Materials and Methods

4.2.1. Yeast Isolation

Autochthonous yeasts species were isolated from five different Spanish wine regions Penedès, Terra Alta and Ribera Sacra Designation of Origin (**DO**) and Priorat and Rioja Qualified Designation of Origin (**DQO**). From each region typical grape varieties were chosen to isolate their characteristic yeast species: Xarel·lo grapes for **DO** Penedès, Mencia grapes for **DO** Ribera Sacra, Grenache blanc grapes for **DO** Terra Alta, Carignan and Grenache noir grapes for **DOQ** Priorat and Tempranillo grapes for **DOQ** Rioja. In order to simulate industrial conditions, in the case of white musts spontaneous fermentations were carried out using 30 L stainless-steel vats immersed in chiller water bath at 18 ºC and for red grapes 50 L stainless-steel vats placed in a thermostatic chamber at 25ºC were used. In each spontaneous fermentation, carbon dioxide was used to inert the vat headspace in order to prevent must oxidations and acetic acid bacteria appearance. Sugar consumption was daily monitored by measuring the density (g/L) of the fermenting must with an electronic densimeter (Metler Toledo, Hospitalet de Llobregat, **ES**). Fermentations were considered to be finished when the level of reducing sugars was below 2 g/L. All fermentations were performed in triplicate. For yeast isolation, fermented musts were taken at different stages of the spontaneous fermentation, at the beginning (0-2 days), in the middle of alcoholic fermentation (4-6 days), at the end of alcoholic fermentation (8-12 days) and finally from the lees.

Tenfold dilutions of each sample were plated on Yeast Extract-Peptone-Dextrose (**YPD**) (Panreac Química SL, Castellar del Vallés, **ES**). An aliquot of 0.1mL of the appropriated dilution was plated onto Yeast Extract-Peptone-Dextrose Agar (**YPDA**) (Panreac Química SL). In order to differentiate between *Saccharomyces* and Non-*Saccharomyces* yeast, all colonies that grown onto **YPDA** were transfer onto Lysine Medium (LM, Oxoid, Hampshire, United Kindom), this medium only allow the growth of Non-*Saccharomyces* yeast. Twenty five colonies from each fermentation time and

grape must variety were randomly isolated and purified for future identification. DNA from wine samples was extracted according to Querol et al. (1992) and diluted to 1-50 ng/μL. Sample DNA was extracted from 1 mL of must or wine and used for the identification of *S. cerevisiae* strains and other Non-*Saccharomyces* yeast species. The identification of *S. cerevisiae* strains were done by amplification of interdelta regions as described in Legras and Karst (2003). For the identification of Non-*Saccharomyces* yeast the ITS region and 5.8S rDNA gene were amplified as described previously by Esteve-Zarzoso et al. (1999). Consequently, 5 μL of the ITS/5.8S rDNA gene amplified product were digested with the DNA restriction enzimes *Hin*fI according to the supplier's instructions (Roche Diagnostics, Mannhein, GR). *Cfo*I, *Dra*I and/or *Hae*III DNA restriction enzymes were also used for further identification when needed (Roche Diagnostics, Mannhein, Germany). All the amplifications were performed using a T100 Thermal Cycler (Bio-Rad, California, USA). Once yeast species were correctly identified were kept with glycerol at -80 ºC for further analysis. From a total of 1350 strains isolated from the grape varieties studied 29 *S. cerevisiae* different strains and 9 Non-*Saccharomyces* yeast species were selected for the present study (Table 4.1).

Table 4.1. Non-*Saccharomyces* and *Saccharomyces* wine yeast species studied in the present research.

Yeast strain	Grape variety	Wine region
S. cerevisiae RI I	Tempranillo	RI
S. cerevisiae RI II	Tempranillo	RI
S. cerevisiae RI III	Tempranillo	RI
S. cerevisiae RI IV	Tempranillo	RI
S. cerevisiae RI V	Tempranillo	RI
S. cerevisiae RI VI	Tempranillo	RI
S. cerevisiae RI VII	Tempranillo	RI
S. cerevisiae RI VIII	Tempranillo	RI
S. cerevisiae RI X	Tempranillo	RI
S. cerevisiae RI XI	Tempranillo	RI
S. cerevisiae RI XII	Tempranillo	RI
S. cerevisiae TA I	Grenache blanc	TA
S. cerevisiae TA II	Grenache blanc	TA
S. cerevisiae TA III	Grenache blanc	TA

S. cerevisiae TA IV	Grenache blanc	TA
S. cerevisiae RS I	Mencía	RS
S. cerevisiae RS II	Mencía	RS
S. cerevisiae RS III	Mencía	RS
S. cerevisiae RS IV	Mencía	RS
S. cerevisiae PE I	Xarel·lo	PE
S. cerevisiae PE II	Xarel·lo	PE
S. cerevisiae PE III	Xarel·lo	PE
S. cerevisiae PE IV	Xarel·lo	PE
S. cerevisiae PE V	Xarel·lo	PE
S. cerevisiae PR I	Carignan	PR
S. cerevisiae PR II	Carignan	PR
S. cerevisiae PR III	Carignan	PR
S. cerevisiae PR IV	Grenache noir	PR
S. cerevisiae PR V	Grenache noir	PR
S. cerevisiae PE II	Xarel·lo	PE
S. cerevisiae PE III	Xarel·lo	PE
S. cerevisiae PE IV	Xarel·lo	PE
S. cerevisiae PE V	Xarel·lo	PE
S. cerevisiae PR I	Carignan	PR
S. cerevisiae PR II	Carignan	PR
S. cerevisiae PR III	Carignan	PR
S. cerevisiae PR IV	Grenache noir	PR
S. cerevisiae PR V	Grenache noir	PR
Pichia anomala	Grenache blanc	TA
Hanseniaspora uvarum	Grenache blanc	TA
Kluyveromyces thermotolerans PE I	Xarel·lo	PE
Pichia fermentans	Xarel·lo	PE
Metschnikowia pulcherrima PR I	Carignan	PR
Metschinikowia pulcherrima PR II	Grenache noir	PR
Candida zemplinina	Grenache noir	PR
Debaryomyces hansenii	Grenache noir	PR
Kluyveromyces thermotolerans PR I	Grenache noir	PR

RI: **DOQ** Rioja; TA: **DO** Terra Alta; **RS**: **DO** Ribera Sacra; **PE**: **DO** Penedès; **PR**: **DOQ** Priorat

4.2.2. Must Preparation and Characterization

Thermovinificated Tempranillo red must was purchased from Sant Josep Agricultural Cooperative (Bot, **ES**). Sterilized must (2.5 L) was obtained by four cycles of vacuum filtration, decreasing in each cycle the pore size of the cellulose nitrate filter used from 8.0 to 0.2 μm (Sartorius Stedim Biotech GmbH, Göttingen, **GR**). Brix degree and alcoholic potential strength by refractometry, pH, total acidity and final alcoholic strength were analysed by official European method and turbidity in Nephelometric Turbidity Units (**NTU**) were analysed following the procedure of the Office International de la Vigne et du Vin (**OIV,** 2008). All these parameters were used for characterization of the must tested. All the analyses were performed in triplicate.

4.2.3. Growing Conditions

An aliquot of 0.1 mL of the selected strains were revived by platting them onto **YPDA**: 2% glucose, 2% peptone, 1% yeast extract and 2% agar (Panreac Química SL, Castellar del Vallés, **ES**) in the case of *S. cerevisiae* strains and onto Lysine medium (Oxoid) for the Non-*Saccharomyces* yeasts at 28ºC during for 48 h. A colony of each strain was selected and placed into 10 mL of **YPD** (Panreac Química SL) and incubated at 28°C for 18 h. Elapsed this time, cells were counted microscopically by using an improved Neubauer chamber (**OIV**, 2008). Counting was performed by triplicated.

From each pure yeast strain different volumes of the starting culture were taken in order to inoculate 10^6 cell/mL in 7.5 mL of sterile red grape must. Cells were grown at 28ºC for 48 h and kept in constant agitation using a mini orbital shaker (Vidrafoc SA, Barcelona, **ES**). 1,5 mL of sample must were taken for **ATR-FTIR** analysis and 1,5 mL for sugars (**OIV**, 2008) to know the physiological phase of each yeast strain. All the analysis was performed in triplicate.

4.2.4. Sample Preparation

Samples (1.5 mL) were centrifuged (15900 g for 5 min at room temperature) and the pellets obtained were washed three times under the same conditions described above

using 1 mL of saline solution (**SS**, 0.88% **NaCl**). Then, 1.5 µL of each pellet was placed onto diamond crystal in order to acquire the spectral data.

*4.2.5. Fourier Transform Infrared Spectroscopy (**FTIR**) Used in Attenuated Total Reflectance (**ATR**) Mode*

Spectra were obtained using a **FTIR** spectrometer Jasco **FT/IR**-600 Plus (Jasco Comparison Proven, Madrid, Spain) adapted with an ultra-high performance **ATR** plate of Diamond. Spectra were collected from 4000 to 800 cm^{-1} with a resolution of 2 cm^{-1}. The spectrometer was controlled using Spectra Manager software (Jasco Comparison Proven, Madrid, **ES**). The spectrum of each sample was obtained by taking the average of 32 scans to improve the signal-noise ratio. Spectra were displayed in terms of absorbance obtained by rationing the single beam spectrum against that of the air background.

4.2.6. Statistical and multivariate analyses

Analysis of variance was performed using **XLSTAT** 2015 software (Microsoft, Redmond, **USA**). Tukey test was used to obtain paired comparisons among sample means. Level of significance was set at $P < 0.05$. Experiments were run three times with duplicate analysis in each replicate.

Spectra were exported to the Pirouette® multivariate analysis software (version 4.0, InfoMetrix, Inc.,Woodville, **USA**). The **FTIR** spectral data were mean-centered, transformed to their second derivative using a 15-point Savitzky-Golay polynomial filter, and vector-length normalized; sample residuals and Mahalanobis distance were used to determine outliers (Kansiz et al., 1999; Hruschka 2001). Soft independent modeling of class analogy (**SIMCA**) was used to build a predictive model based on the construction of separate **PCA** models for each class to describe and model the variation (Kansiz et al.,1999). **SIMCA** class models were interpreted based on class projections, misclassifications, discriminating power, and interclass distances. Total misclassifications were analysed and interpreted for the input data. Variable importance, also known as discriminating power, was used to define the variables (wavenumbers) that have a predominant effect on sample classification, minimizing the difference between samples

within a cluster and maximizing differences between samples from different clusters (Dunn and Wold 1995). **SIMCA** models can be used for the prediction of unknown samples. The identity of unknown samples can be predicted using the training models with three possible outcomes: the unknown sample is part of one class, the unknown sample is part of more than one class or the unknown sample does not belong to one class. Validation of the model was conducted by internal validation. **SIMCA** was used to build a predictive model based on the **PCA** models constructed separately (Kansiz et al., 1999). The build model was validated using two randomly spectra for strain and evaluating the correct classification of each strain in their correct class in the **SIMCA** build models (Grasso et al., 2009).

4.3. Results and Discussion

4.3.1. Fermentation Conditions

It is well known that culture conditions strongly affect **FTIR** grouping of microorganisms (Mariey et al., 2001; Baldauf et al., 2007; De Lamo-Castellví and Rodriguez-Saona, 2011). Moreover, it has been reported that the concentration of fermented sugars is a key factor for the alcoholic fermentation kinetics (van Dijken et al., 1993). Therefore, for this research, we selected to standardize the growing medium of *S. cerevisiae* and Non-*Saccharomyces* yeast species using commercial Tempranillo thermovinificated red must. Composition parameters for red must are shown in **Table 4.2**. Sugar concentration of 231 g/L was appropriated for Tempranillo grapes in advance stage of ripening. Total acidity (4.6 g/L) and pH (3.8) also indicated the advanced stage of grape maturity. Assimilable nitrogen content of 104 mg/L was sufficient for the growth of yeast species.

Table 4.2. Physical-chemical characterization of thermovinifcated Tempranillo red must. Results are shown as mean ± standard deviation.

	Tempranillo
Sugars (glucose+fructose)	231.1 ± 0.2
Density (mg/mL)	1097 ± 1
Alcoholic potential strength (vol%)	13.2 ± 0.1
pH	3.8 ± 0.1
Total acidity	4.6 ± 0.1
Primary Amino Nitrogen (mg/L)	80.7 ± 5
Ammonium (mg/L)	30 ± 5
Assimilable nitrogen (mg/L)	104 ± 5
Acetic acid (g/L)	0.25 ± 0.03
Malic acid (g/L)	1.8 ± 0.1
Lactic acid (g/L)	0.0 ± 0.1

*4.3.2. Discrimination of Non-*Saccharomyces *Yeast Species by* ATR-FTIR *Combined with* SIMCA

Infrared spectra analyses (800-1800 cm^{-1}) using **SIMCA** classification models of nine Non-*Saccharomyces* wine yeast species are shown in **Figure 4.1**. Class projections illustrate the ability of **SIMCA** to differentiate **IR** data based on the first 3 principal components; however, more principal components were used for actual differentiation (Subramanian et al., 2007; Grasso et al., 2009). This model offered good class separation, tight clustering among yeast species (**Figure 4.1a**) and interclass distances (**ICD**) ranging from 1.2 to 8.3 showing differences between the species compared (**Table 4.3**). The **ICD** are Euclidian distances between centers of clusters and values above 3 are considered good for class discrimination (Dunn and Wold, 1995). Close grouping between samples indicates biochemical similarities. For this model, similarities where found between *C. zemplinina* and *M. pulcherrima* PR I, *K. thermotolerans* PE I and *P. fermentans*, *D. hansenii* and *M. pulcherrima* PR II and *P. fermentans*, *P. anomala* and *H. uvarum* and between *D. hansenni* and *K. thermotolerans* TA I. Discriminating power of **SIMCA** showed two strong spectral bands at 1032 and 1630 cm^{-1} (**Figure 4.1b**) related to **β**(1→4) glucans and

N-H and C-N vibrations of peptide bond in different protein conformations, respectively (Cavagna et al., 2010). Domizio et al. (2011) and Giovani et al. (2012) reported that Non-*Saccharomyces* wine yeasts have a high capacity to release polysaccharides mainly mannoproteins into wine.

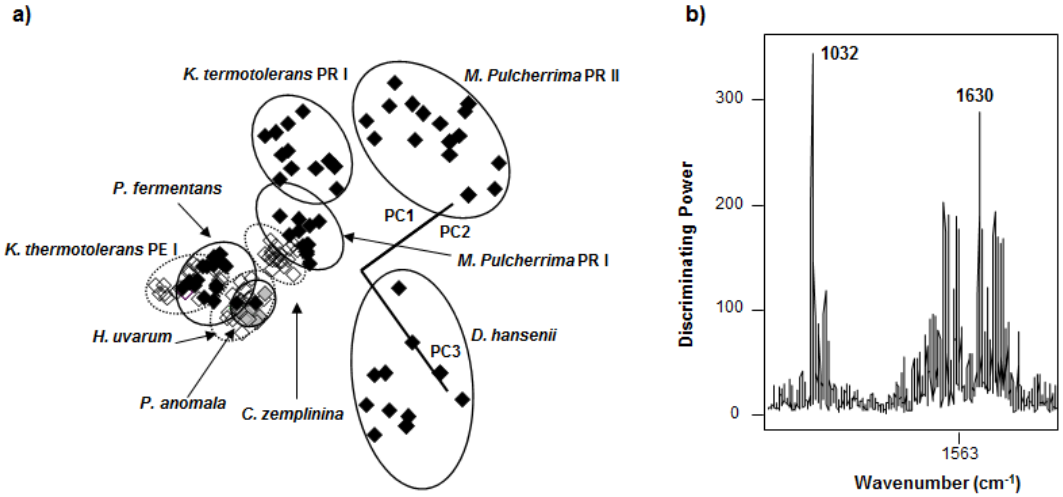

Figure 4.1. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of nine Non-*Saccharomyces cerevisiae* strains fermented in Tempranillo must.

Mannoproteins are polymers with high mannose content that exist as covalent complexes with proteins and are released into the extracellular medium by yeast during yeast growth and autolysis (Llauberes et al., 1987; Boivin et al., 1998; Dupin et al., 2000; Rosi et al., 2000; Alexandre and Guilloux-Benatier, 2006; Palomero et al., 2007). Domizio et al. (2014) also confirmed that the polysaccharides released in the media for Non-*Saccharomyces* wine yeasts species were indeed of cell wall origin and essentially mannoproteins. Moreover, these authors reported that Non-*Saccharomyces* yeasts were growth-independent when they were starting the fermentation process.

Table 4.3. Soft independent modelling of class analogy (**SIMCA**) of interclass distance of nine studies Non-*Saccharomyces* of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	P. anomala	*H. uvarum*	*K. thermotolerans* **PE I**	*P. fermentans*	*M. pulcherrima* **PR I**	*M. pulcherrima* **PR II**	*C. zemplinina*	*D. hansenii*	*K. thermotolerans* **PR I**
P. anomala	0.0								
H.uvarum	1.2	0.0							
K. thermotolerans PE I	3.7	3.0	0.0						
P. fermentans	2.9	2.6	1.6	0.0					
M. pulcherrima PR I	3.4	3.1	4.2	3.5	0.0				
M. pulcherrima PR II	6.4	6.0	7.1	5.7	4.8	0.0			
C. zemplinina	2.9	2.7	3.7	3.3	1.3	6.3	0.0		
D. hansenii	2.7	2.7	2.8	2.3	2.9	2.4	3.5	0.0	
K. thermotolerans PR I	7.1	6.6	7.6	5.8	6.8	3.5	8.3	2.1	0.0

4.3.3. Discrimination of S. cerevisiae Yeast Strains by ***ATR-FTIR*** *Combined with* ***SIMCA***

A **SIMCA** model of *S. cerevisiae* strains was built up. Class projections plot showed well-separated grouping of the strains analysed (**data not shown**). **ICD** ranged from 1.3 to 8.7 (**data not shown**) pointing out that a high number of samples (30 pairs of samples) with close grouping. The wavenumbers that have predominant effect on discrimination of *S. cerevisiae* strains were mainly two 1558 and 1684 cm^{-1} (**Figure 4.2**). These bands were related with N-H and C-N vibration of the peptide bond in different protein conformations (Cavagna et al., 2010) and protein **β**-turns and pleated sheets conformation (Corte et al., 2014), respectively. As we reported in previous research (Puxeu et al., 2014), mannoproteins present in yeast cell wall seem to be the key factor to discriminate *S. cerevisiae* strains.

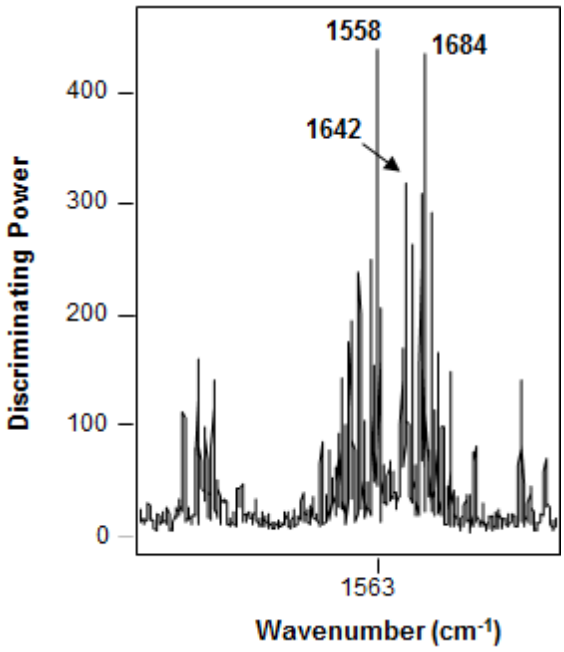

Figure 4.2. Soft independent modeling of class analogy (**SIMCA**) discriminating power of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) for 29 studied *Saccharomyces cerevisiae* strains fermented in Tempranillo must at 48 h at exponential phase.

*4.3.4. Discrimination of Non-*Saccharomyces *and S. cerevisiae Yeast Strains by* ATR-FTIR *Combined with* SIMCA

A 2-classes **SIMCA** model to discriminate Non-*Saccharomyces* wine yeast species and *S. cerevisiae* strains was created (**Figure 4.3a and b**). The two clusters of Non-*Saccharomyces* and *S. cerevisiae* strains were well-separated (**Figure 4.3a**) but the **ICD** was 1.7 showing close grouping. The **IR** bands mainly responsible of their discrimination were 1032 and 1541 (**Figure 4.3b**) linked to **β**(1→6) glucans and N-H and C-N vibrations of the peptide bond in different protein conformations (Cavagna et al., 2010).

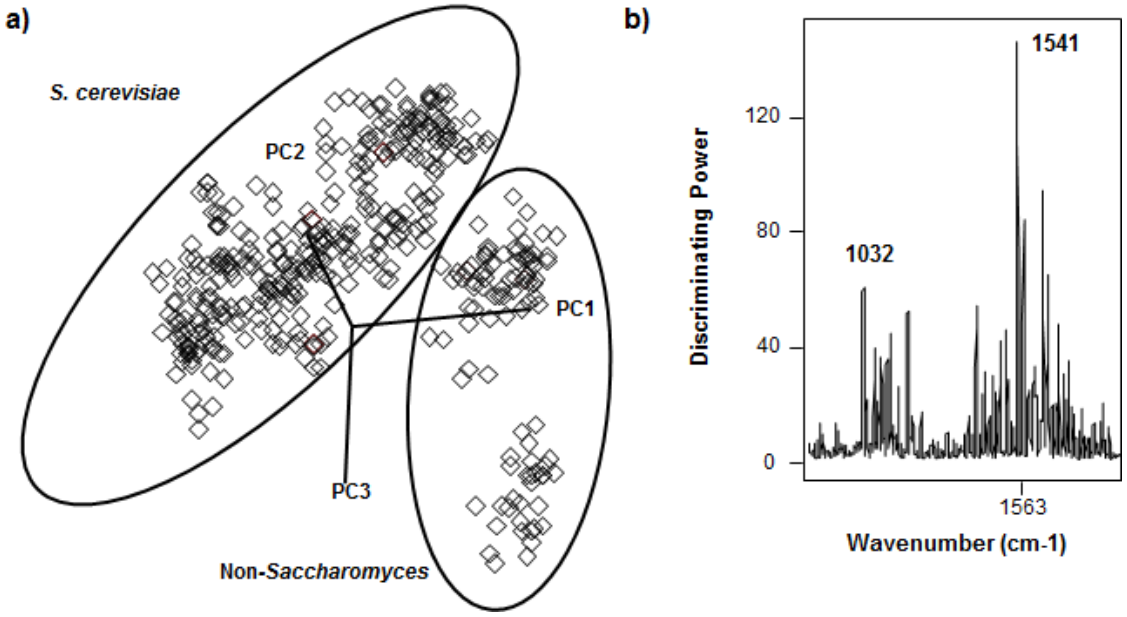

Figure 4.3. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of nine Non-*Saccharomyces cerevisiae* and twenty nine *S. cerevisiae* strains fermented in Tempranillo must.

Under these circumstances, the objective of having a unique model to discriminate between Non-*Saccharomyces* yeast species and *S. cerevisiae* strains was not suitable. Nonetheless, the two **SIMCA** models built up separately with **IR** data of Non-*Saccharomyces* wine yeast species and *S. cerevisiae* strains could be used to predict unknown wine yeast species. To test the performance of these two **SIMCA** models, an internal validation (2 spectra per specie or strain not used to build up the **SIMCA** models) was performed obtaining 100% correct predictions into the appropiate class (**Table 4.4**). Moreover, we decided to apply an external validation using as data set Non-*Saccharomyces* yeast species **IR** data to predict their class into the **SIMCA** model of *S.*

cerevisiae strains and vice versa (**Table 4.4**). In this case, 89% correct predictions were obtained for *S. cerevisiae* strains and 87% were correctly predicted as Non-*Saccharomyces* wine yeast species.

Table 4.4. *Saccharomyces cerevisiae* and Non-*Saccharomyces cerevisiae* models validation by Internal Validation and Cross Validation using 2 spectra per strain in both cases.

SIMCA Models	Internal Validation (2 spectra per strain)	Cross validation (2 spectra per strain)
S. cerevisiae	100% (29 samples)	89% (9 samples)
Non-*Saccharomyces* species	100% (9 samples)	97% (29 samples)

4.3.5. Physiological Growth Phase

In a previous research, we found that depending on the physiological phase, the biochemical components that played an important role to discriminate *S. cerevisiae* strains changed (Puxeu et al., 2014). Moreover, most of the Non-*Saccharomyces* species isolated from wine-related environments have limited fermentation potential, such as low fermentation power and rates, as well as low SO_2 resistance (Jolly et al., 2003). Consequently, the sugar consumption of our selected yeasts was monitored to determine their growing phase after 2 days of fermenting at 28ºC (**Table 4.5** and **Table 4.6**). Even though the growing process started with equal amounts of sugars, grape must fermented by *P. anomala*, *H. uvarum, K. thermotolerans* PE I, *P. fermentans*, *M. pulcherrima* PR I, *M. pulcherrima* PR II and *C. zemplinina*, sugars used up more slowly over the course of two days of fermentation showing low fermentative capacity with sugar values and leading to yeast cells

Table 4.5. Sugar concentration per each Non-*Saccharomyces* wine specie after 48 h of fermentation at 28ºC and its physiological phase of growth. Results are shown as mean ± standard deviation.

Species	Glucose + Fructose (g/L)	Physiological phase
Grape must	$231.1^{a} \pm 0.2$	
P. anomala	$180.7^{bc} \pm 5.5$	Early exponential
H. uvarum	$186.7^{bc} \pm 6.5$	Early exponential
K. thermotolerans PE I	$182.7^{bc} \pm 10.1$	Early exponential
P. fermentans	$200.7^{b} \pm 9.0$	Early exponential
M. pulcherrima PR I	$197.0^{b} \pm 11.1$	Early exponential
M. pulcherrima PR II	$179.0^{bc} \pm 17.1$	Early exponential
C. zemplinina	$185.7^{bc} \pm 11.9$	Early exponential
D. hansenii	$168.7^{c} \pm 3.5$	Exponential.
K. thermotolerans PR I	$116.3^{d} \pm 8.1$	Exponential

mainly at early exponential phase (**Table 4.3**). Nevertheless, *D. hansenii* and *K. thermotolerans* PR I showed the highest fermentative capacity with sugar concentration values of 168.7 and 116.3 g/L, respectively, leading to yeast cells at exponential phase (**Table 4.3**). Several researchers have reported that Non-*Saccharomyces* wine species have different fermentative capacity. For instance, Jolly et al., (2003) has reported that apiculate yeasts such as *Kloeckera apis*, *K. javanica* and *H. uvarum* have low fermentative activity and Santos et al. (2008) found that *Torulaspora delbrueckii* showed the highest fermentation power.

In the case of *S. cerevisiae* strains, *S. cerevisiae* RI IX (83 g/L) showed the highest fermentation capacity and *S. cerevisiae* PE II (202.7 g/L) the lowest (**Table 4.6**). Regarding their physiological phase, six strains were classified at early exponential phase, eighteen at exponential phase and five at late exponential phase (**Table 4.4**).

Table 4.6. Sugar concentration per each *S. cerevisiae* strain after 48 h of fermentation at 28ºC and its physiological phase of growth. Results are shown as mean ± standard deviation.

Strain	Glucose + Fructose (g/L)	Physiological phase	Strain	Glucose + Fructose (g/L)	Physiological phase
Grape	$231^{a} \pm 0.2$	-	*S. cerevisiae* TA IV	$133.7^{ghij} \pm 3.1$	Exponential
S. cerevisiae RI I	$93.7^{lm} \pm 7.8$	Late exponential	*S. cerevisiae* RS I	$128.0^{ghijk} \pm 7.5$	Exponential
S. cerevisiae RI II	$129.7^{ghij} \pm 8.5$	Exponential	*S. cerevisiae* RSII	$145.3^{efgh} \pm 6.4$	Exponential
S. cerevisiae RI III	$125.3^{hijk} \pm 3.5$	Exponential	*S. cerevisiae* RS III	$142.7^{efgh} \pm 7.5$	Exponential
S. cerevisiae RI IV	$99.7^{klm} \pm 4.5$	Late exponential	*S. cerevisiae* RS IV	$182.3^{bc} \pm 9.7$	Early exponential
S. cerevisiae RI V	$175.0^{bcd} \pm 5.0$	Early exponential	*S. cerevisiae* PE I	$166.7^{cde} \pm 17.6$	Exponential
S. cerevisiae RI VI	$179.3^{bc} \pm 5.5$	Early exponential	*S. cerevisiae* PE II	$202.7^{ab} \pm 10.3$	Early exponential
S. cerevisiae RI VII	$106.3^{jklm} \pm 5.5$	Exponential	*S. cerevisiae* PE III	$154.3^{cdefg} \pm 7.1$	Exponential
S. cerevisiae RI VIII	$136.0^{fhgi} \pm 8.7$	Exponential	*S. cerevisiae* PE IV	$164.0^{cdef} \pm 10.8$	Exponential
S. cerevisiae RI X	$86.0^{m} \pm 3.6$	Late exponential	*S. cerevisiae* PEV	$166.0^{cde} \pm 21.2$	Exponential
S. cerevisiae RI XI	$126.3^{ghijk} \pm 6.0$	Exponential	*S. cerevisiae* PR I	$145.3^{efgh} \pm 6.8$	Exponential
S. cerevisiae RI XII	$87.0^{lm} \pm 3.6$	Late exponential	*S. cerevisiae* PR II	$175.0^{bcd} \pm 13.5$	Early exponential
S. cerevisiae TA I	$100.7^{klm} \pm 4.5$	Exponential	*S. cerevisiae* PR III	$165.3^{cde} \pm 11.0$	Exponential
S. cerevisiae TAII	$96.0^{lm} \pm 10.9$	Late exponential	*S. cerevisiae* PR IV	$149.7^{defgh} \pm 14.5$	Exponential
S. cerevisiae TA III	$115.0^{ijkl} \pm 5.6$	Exponential	*S. cerevisiae* PR V	$174.7^{bcd} \pm 7.0$	Early exponential

In order to improve the multivariate classification models developed to differentiate and predict Non-*Saccharomyces* species, the physiological growth phase was taken into account. In this study, we had two species of Non-*Saccharomyces* yeasts at exponential phase and these **IR** data were not used to create a **SIMCA** model due to the lack of sufficient samples and spectra to build up a consistent model. Therefore, **IR** data from Non-*Saccharomyces* species at early exponential phase were used to build up a 7-classes **SIMCA** model (1900–800 cm^{-1}, **Figure 4.4**) obtaining well defined clusters for each Non-*Saccharomyces* wine yeast (**Figure 4.4a**) along with clear differentiation between most of the species tested. These findings were further proved with **ICD** that ranged from 1.2 to 6.7 reducing the numbers of species with close grouping to 3 (*H. uvarum* and *P. fermentans*, *K. Thermotolerans* PE I and *M. pulcherrima* PR I and *M. pulcherrima* PR II and *C. zemplinina*, **Table 4.7**).Discriminating power of this **SIMCA** model (**Figure 4.4b)** showed mainly two spectral bands at 1032 and 1507 cm^{-1}, linked to **β**(1→4)glucans and C=C stretching modes in aromatic rings (Cavagna et al., 2010). Whitener et al., (2015) found that some Non-*Saccharomyces* species such as *M. pucherrima* at early fermentation phase have high production of different aromatic metabolites formed by aromatic rings.

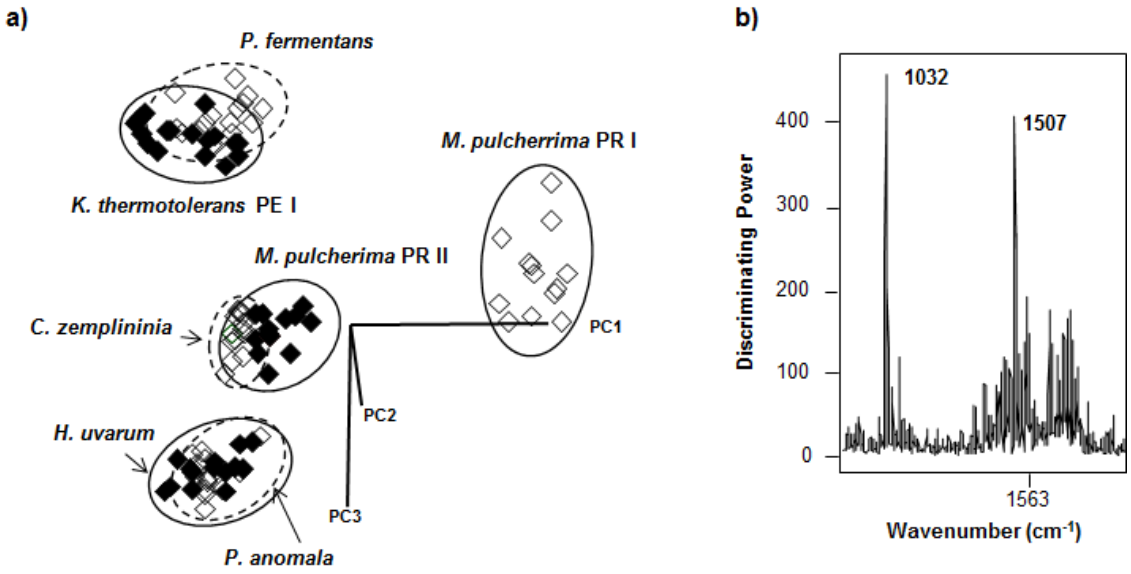

Figure 4.4. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of seven Non-*Saccharomyces cerevisiae* strains fermented in Tempranillo must at early exponential phase.

Table 4.7. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of Non-*Saccharomyces cerevisiae* wine species at early exponential phase of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	P. anomala	*H. uvarum*	*K. thermotolerans* PE I	*P. fermentans*	*M. pulcherrima* PR I	*M. pulcherrima* PR II	*C. zemplinina*
P. anomala	0.0						
H. uvarum	6.7	0.0					
K. thermotolerans PE I	6.2	3.4	0.0				
P. fermentans	5.3	1.6	2.7	0.0			
M. pulcherrima PR I	6.1	3.9	1.4	2.8	0.0		
M. pulcherrima PR II	4.6	3.9	3.1	3.2	3.2	0.0	
C. zemplinina	6.2	3.8	2.9	3.2	3.0	1.2	0.0

Three **SIMCA** models (at early, exponential and stationary phase) were built up for *S. cerevisiae* strains. At early and late exponential phases, clusters from fermented *S. cerevisiae* strains were well separated (**Figure 4.5** and **Figure 4.6**).

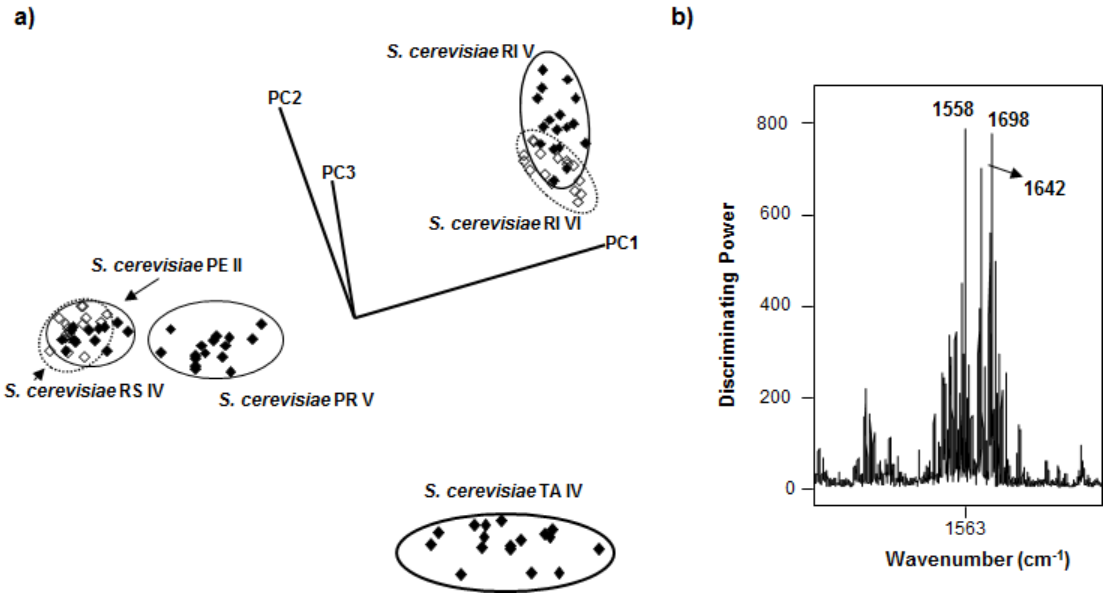

Figure 4.5. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of six *Saccharomyces cerevisiae* strains fermented in Tempranillo must at early exponential phase.

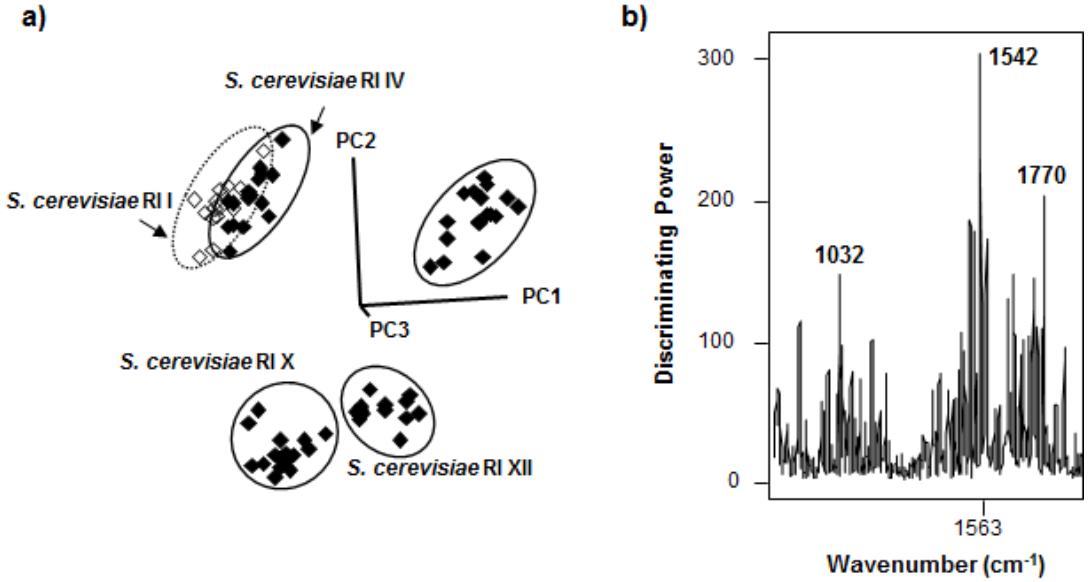

Figure 4.6. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of five *S. cerevisiae* strains fermented in Tempranillo must at late exponential phase.

ICD varied from 1.9 to 8.4 (**Table 4.8** and **Table 4.9**) showing differences between their biochemical composition, excepting between *S. cerevisiae* PR V and *S. cerevisiae* TA IV and *S. cerevisiae* RI IX and *S. cerevisiae* RI XII. At early exponential phase, major discrimination between *S. cerevisiae* strains (**Figure 4.5b**) occurred in all cases, at 1558, 1642 and 1698 cm^{-1}, presumably due to N-H and C-N vibrations of peptide bond in different protein conformations (Cavagna et al., 2010), vibrations of amide I of **β**-sheets (Yu and Irudayaraj, 2005) and protein **β**-turns and pleated sheets conformations (Corte et al., 2014), respectively. At late exponential phase (**Figure 4.6b**), yeast species were also discriminated by the **IR** band at 1032 cm^{-1} linked to **β**(1→4)glucans and the **IR** band at 1771 cm^{-1} associated to C=O stretching in lipid esters (Cavagna et al., 2010).

Table 4.8. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* strains at early exponential phase of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	***S. cerevisiae* RI V**	***S. cerevisiae* RI VI**	***S. cerevisiae* TA IV**	***S. cerevisiae* RS IV**	***S. cerevisiae* PE II**	***S. cerevisiae* PR V**
S. cerevisiae RI V	0.0					
S. cerevisiae RI VI	2.7	0.0				
S. cerevisiae TA IV	8.4	6.9	0.0			
S. cerevisiae RS IV	6.0	4.4	2.9	0.0		
S. cerevisiae PE II	4.4	3.4	4.9	3.3	0.0	
S. cerevisiae PR V	7.6	6.3	2.2	2.6	4.3	0.0

Table 4.9. Soft independent modelling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* strains at late exponential phase of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra.

	S. cerevisiae **RI I**	*S. cerevisiae* **RI IV**	*S. cerevisiae* **RI X**	*S. cerevisiae* **RI XII**	*S. cerevisiae* **TA II**
S. cerevisiae **RI I**	0.0				
S. cerevisiae **RI IV**	1.9	0.0			
S. cerevisiae **RI X**	3.3	3.7	0.0		
S. cerevisiae **RI XII**	4.7	4.8	2.6	0.0	
S. cerevisiae **TA II**	5.6	5.6	5.1	3.3	0.0

Infrared spectra analysis (1900-800 cm^{-1}) using **SIMCA** classification models of *cerevisiae* strains at exponential phase (**data not shown**) permitted tight clustering and zero misclassifications among clusters between strains. **ICD** ranged from 1.7 to 11.4 (**Table 4.10**) showing differences between their biochemical patterns in mostly all classes compared. Major discrimination between these *S. cerevisiae* strains (**Figure 4.7**) also occurred at 1032, 1558, 1642 and 1698 cm^{-1}. These results are in agreement with previous experiments (Puxeu et al., 2014). We have already reported that depending on the physiological phase, the components that played an important role to discriminate *S. cerevisiae* strains changed. At exponential phase, the cell wall components were mainly glucans, mannoprotiens and lipids.

Table 4.10. Soft independent modeling of class analogy (**SIMCA**) of interclass distance of *S. cerevisiae* strains at exponential phase of transformed (second derivative, 15 points window) attenuated total reflectance infrared spectroscopy (**ATR-FTIR**) spectra. *S. c.: Saccharomyces cerevisiae*

	S.c. **RI II**	***S.c.*** **RI III**	***S.c.*** **RI VII**	***S.c.*** **RI VIII**	***S.c.*** **RI XI**	***S.c.*** **TA I**	***S.c.*** **TA III**	***S.c.*** **TA IV**	***S.c.*** **RS I**	***S.c.*** **RS II**	***S.c.*** **RS III**	***S.c.*** **PE I**	***S.c.*** **PE III**	***S.c.*** **PE IV**	***S.c.*** **PE V**	***S.c.*** **PR III**	***S.c.*** **PR IV**
S.c.**RI II**	0.0																
S.c.**RI III**	2.2	0.0															
S.c.**RI VII**	4.0	3.7	0.0														
S.c.**RI VIII**	7.5	6.7	6.0	0.0													
S.c.**RI XI**	4.7	4.0	3.0	3.7	0.0												
S.c.**TA I**	5.3	4.9	4.9	3.7	4.5	0.0											
S.c.**TA III**	3.8	3.7	3.3	5.2	3.7	4.6	0.0										
S.c.**TA IV**	3.6	3.2	4.8	4.9	3.1	4.6	5.0	0.0									
S.c.**RS I**	7.7	7.7	7.9	7.4	5.4	6.2	7.7	4.5	0.0								
S.c.**RS II**	8.5	8.3	8.5	7.7	5.5	6.5	8.4	4.8	1.8	0.0							
S.c.**RS III**	11.2	11.3	11.4	8.7	6.9	7.2	10.4	5.9	2.4	1.7	0.0						
S.c.**PE I**	9.4	9.3	9.7	8.6	5.9	7.0	9.1	4.8	3.4	3.1	3.7	0.0					
S.c.**PE III**	2.9	2.6	3.0	4.0	2.7	3.8	3.4	2.3	3.4	3.5	4.2	3.3	0.0				
S.c.**PE IV**	4.8	4.4	5.0	5.3	3.3	4.8	5.6	2.6	3.1	3.0	3.6	2.2	1.9	0.0			
S.c.**PE V**	7.2	6.9	7.2	7.2	4.7	6.2	7.2	4.0	3.3	3.0	3.7	1.8	2.8	1.8	0.0		
S.c.**PR III**	5.9	5.7	6.1	5.2	3.6	5.2	6.5	3.0	2.9	2.8	5.7	2.9	2.6	2.3	2.9	0.0	
S.c.**PR IV**	5.1	4.8	4.6	4.6	3.5	3.0	5.4	5.5	4.9	4.9	4.8	5.2	3.1	3.4	4.7	3.6	0.0

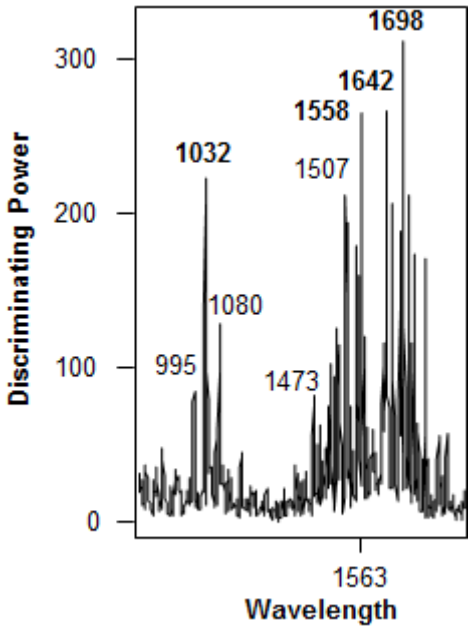

Figure 4.7. Soft independent modeling of class analogy (**SIMCA**) discriminating power of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of eighteen *Saccharomyces cerevisiae* strains fermented in Tempranillo must at exponential phase.

Using the IR data at early exponential phase, a 2-classes **SIMCA** model to discriminate Non-*Saccharomyces* wine yeast species and *S. cerevisiae* strains (**Figure 4.8**) was created. In this case, the two clusters were well-separated (**Figure 4.8a**) and the **ICD** was 3.0 showing good class discrimination and biochemichal differences between the samples compared. The **IR** bands mainly responsible of their discrimination were 1032, 1541 and 1643 cm^{-1} (**Figure 4.8b**). These bands were associated to **β**(1→6) glucans, vibrations of amide I of **β**-sheets (Yu and Irudayaraj, 2005) and N-H and C-N vibrations of the peptide bond in different protein conformations (Cavagna et al., 2010).

Therefore, **SIMCA** models were improved when physiological growth phase was considered. Nonetheless, the lack of Non-*Saccharomyces* species at exponential and late exponential phases did not allow producing 2-classes **SIMCA** models to further proved these findings. Nonetheless, the lack of Non-*Saccharomyces* species at exponential and late exponential phases did not allow producing 2-classes **SIMCA** models to further proved these findings.

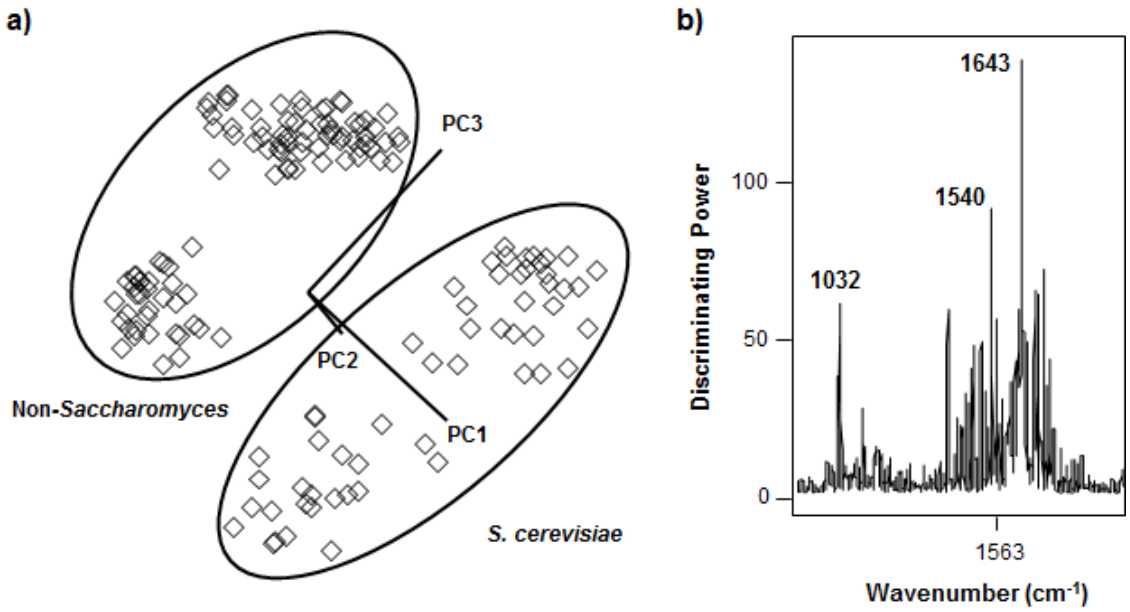

Figure 4.8. Soft independent modeling of class analogy (**SIMCA**) class projections (**a**) and discriminating power (**b**) of transformed attenuated total reflectance (**ATR**) infrared spectroscopy spectra (1900–800 cm^{-1}) of Non-*Saccharomyces cerevisiae* and *S. cerevisiae* strains fermented in Tempranillo must when physiological growth phase.

4.4. Conclusions

This study showed the potential of using **ATR-FTIR** combined with multivariate analysis to differentiate *S. cerevisiae* and Non-*Saccharomyces* wine yeast species. In order to develop appropriate **SIMCA** models to discriminate between *S. cerevisiae* and Non-*Saccharomyces* wine yeast species, their physiological growth phase needed to be taken into account. Non-*Saccharomyces* species were mainly discriminated by **β**-glucans and proteins from the components presents in their cell way. Nevertheless, *S. cerevisiae* were mainly discriminated by proteins compounds.

4.5. References

Adt, I., Kohler, A., and Gognies, S. (2010). FTIR spectroscopic discrimination of *Saccharomycescerevisiae* and *Saccharomyces* bayanus strains. *Canadian Journal of Microbiology*. 65: 793-801.

Alexandre, H., and Guilloux-Benatier, M., (2006). Yeast autolysis in sparkling wine – a review. *Australian Journal of Grape and Wine Research,* 12: 119–127.

Baldauf, N. A., Rodriguez-Romo, L. A., Männig, A., et al., (2007). Effect of selective growth media on the differentiation of Salmonella enterica serovars by Fourier transform mid-infrared spectroscopy. *Journal of Microbiological Methods*, 68: 106–114.

Bisson, L.F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, 50: 107-119.

Boivin, S., Feuillat, M., Alexandre, H., et a. (1998). Effect of must turbidity on cell wall porosity and macromolecule excretion of *Saccharomyces cerevisiae* cultivated on grape juice. *American Journal of Enology and Viticulture*, 49: 325–332.

Burattini, E., Cavagna, M., Dell'Anna, R., et al. (2008). A FTIR microspectroscopy study of autolysis in cells of the wine yeast *Saccharomyces cerevisiae*. *Vibrational Spectroscopy*, 47: 139–147.

Cavagna, M., Dell'Anna, R., Monti, F., et al. (2010). Use of ATR-FTIR microspectroscopy to monitor autolysis of *Saccharomyces cerevisiae* cells in a base wine,*Journal of Agricultural and Food Chemistry*, 58: 39–45.

Ciani, M. and Maccarelli, F. (1998). Oenological properties of Non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology & Biotechnology*, 14: 199-203.

Ciani, M., Comitini, F., Mannazzu, I., et al. (2010). Controlled mixed culture fermentation: a new perspective on the use of Non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, 10: 123–133.

Corte, L., Tiecco, M., Roscini, L., et al. (2014). FTIR analysis of the metabolomic stress response induced by N-alkyltropinium bromide surfactants in the yeasts *Saccharomycescerevisiae* and Candida albicans. *Colloids and Surfaces B-Biointerfaces*, 116: 761-771.

De Lamo-Castellví, S., and Rodríguez-Saona, L.E. (2011). Use of Attenuated Total Reflectance infrared Microspectroscopy to Discriminate Bacillus Spores. *Journal of Food Safety*, 31: 401-407.

Domizio, P., Romani, C., Lencioni, L., et al. (2011). Outlining a future for Non-*Saccharomyces* yeasts: Selection of putative spoilage wine strains to be used in association with *Saccharomycescerevisiae* for grape juice fermentation. *International Journal of Food Microbiology*, 147: 170-180.

Dunn, W. J., and Wold, S. (1995). SIMCA pattern recognition and classification.In H. van de Waterbeemd (Ed.), Chemometric methods in molecular design (pp. 179–193). EUA: VCH Publishers.

Dupin, I.V.S., McKinnon, B.M., Ryan, C., et al. (2000). *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *Journal of Agricultural and Food Chemistry,* 48: 3098–3105.

Esteve-Zarzoso, B., Belloch, C., Urususbu, F., et al. (1999). Identification of yeast by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology*. 49: 329-337.

Fugelsang, K.C., and Edwards, C.G. (2007).Wine microbiology. Practical Applications and Procedures.2nd ed. Springer Science Business Media, LLC, New York, USA.

Giovani, G., Rosi, I., and Bertuccioli, M. (2012). Quantification and characterization of cell wall polysaccharides released by Non-*Saccharomyces* yeast strains during alcoholic fermentation. *International Journal of Food Microbiology,* 160: 113–118.

Grasso, E. M., Yousef, A. E., De Lamo-Castellvi, S., et al. (2009). Rapid detection and differentiation of Alicyclobacillus species in fruit juice using hydrophobic grid membranes and attenuated total reflectance infrared microspectroscopy. *Journal of Agricultural and Food Chemistry*, 57: 10670–10674.

Hruschka,W. R. (2001). In P.Williams & K. Norris (Eds.), Near-Infrared technology in the agricultural and food industries (pp. 39–58).EUA: AACC, Inc.

Huang, K. C., Mukhopadhyay, R.,Wen, B., et al. (2008). Cell shape and cell-wall organization in Gram-negative bacteria. *Proceedings of the NationalAcademy of Sciences of the United States of America*, 105: 19282–19287.

Jolly, N.P., Augustyn, O.P.R., and Pretorius, L.S. (2003). The effect of Non-*Saccharomyces* yeasts on fermentation and wine quality. *South African Journal of Enology and Viticulture,* 24: 8–10.

Kansiz, M., Heraud, P., Wood, B., et al. (1999). Fourier transform infrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains. *Phytochemistry*, 52: 407–417.

Klis, F.M., Boorsma, A., and De Groot PWJ.(2006). Cell wall construction in *Saccharomyces crevisiae*. *Yeast*, 23: 185-202.

Kümmerle, M., Scherer, S., and Seiler, H. (1998). Rapid and reliable identification of food borne yeast by Fourier-transform infrared spectroscopy. *Applied and Environmental Microbiology*. 64: 2207-2214.

Legras, JL. and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterization. *FEMS Microbiology Letters*, 221: 249-255.

Lesage, G., and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, 70: 317–343.

Llauberes, R.M., Dubourdieu, D., and Villetaz, J.C., (1987). Exocellular polysaccharides from *Saccharomyces* in wine. *Journal of the Science of Food and Agriculture*, 41: 277–280.

Mariey, L., Signolle, J.P., Amiel, C., et al. (2001). Discrimination, classification, identification of microorganisms using FTIR spectroscopy and chemometrics. *Vibrational Spectroscopy*, 26: 151-159.

OIV, Organisation Internationale de la Vigne et du Vin (2008). Compendium of international méthods of wine and must analysis 2008 edition. OIV, Paris.

Palomero, F., Morata, A., Benito, S., et al. (2007). Conventional and enzyme-assisted autolysis during ageing over lees in red wines: influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chemistry,* 105: 838–846.

Puxeu, M., Andorrà, I. and De Lamo-Castellví, S. (2014). Monitoring *Saccharomyces cerevisiae* Grape Must Fermentation Process by Attenuated Total Reflectance Spectroscopy.*Food Bioporcess Technology*, 8: 637-646.

Querol, A., Barrio, E., and Ramon, D. (1992). A comparative study of different methods of yeast-strain characterization. *Systematic and Applied Microbiology*. 15: 439-446.

Rodríguez, M.E., Lopes, C.A., Barbagelata, R.J., et al. (2010). Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. *International Journal of Food Microbiology,* 138: 19–25.

Rojas, V., Gil, J., Piñaga, F., et al. (2003). Acetate ester formation in wine by mixed cultures in laboratory fermentations. *International Journal of Food Microbiology,* 86: 181–188.

Rosi, I., Gheri, A., Domizio, P., et al. (2000). Production de macromolecules pariétales de *Saccharomyces cerevisiae* au cours de la fermentation et leur influence sur la fermentation malolactique. *Revue des Oenologues,* 94: 18–20.

Sadineni, V., Kondapalli, N., Obulam, et al. (2012). Effect of co-fermentation with *Saccharomyces cerevisae* and *Torulaspora delbrueckij* of *Metschnikowia pulcherrima* on the aroma and sensory properties of mango wine. *Annals of Microbiology*, 62: 1353-1360.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., et al. (2012). Yeast–yeast interactions revealed by aromatic profile analysis of sauvignon blanc wine fermented by single or co-culture of Non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol,* 32: 243–253.

Santos, J., Sousa, M.J., Carcloso, H., et al. (2008). Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. *Microbiology-SGM*, 154: 422-430.

Subramanian, A., Ahn, J., Balasubramaniam, V.M., et al. (2007). Monitoring biochemical changes in bacterial spore during thermal and pressure-assisted thermal processing using FT-IR Spectroscopy. *Journal of Agricultural and Food Chemistry*, 55: 9311–9317.

van Dijken, J.P., Weusthuis, R.A., Pronk, J.T. (1993). Kinetics of growth and sugar consumption in yeasts.*Growth in the Industrial Environment – Antonie van Leeuwenhoek*, 63: 343-352.

Whitener, M.B.E., Silvia, C., Dan, J., et al. (2015). Early fermentation volatile metabolite profile of Non-*Saccharomyces* yeasts in red and white grape must: A targeted approach. *LWT-Food Science and Technology*, 64: 412-422.

Yanai, T., and Sato, M. (1999). Isolation and properties of beta-glucosidase produced by Debaryomyces hansenni and its application in winemaking. *American Journal of enology and Viticulture*, 50: 231-235.

Yu, C.X., and Irudayaraj, J. (2005). Spectroscopic characterization of microorganisms by Fourier transform infrared microspectroscopy. *Biopolymers*, 77: 368-377.

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CHAPTER 5

General Conclusions

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5.1. Conclusions

Based on the objectives and the background described in the present work, the main conclusions of this thesis are:

- **ATR-FTIR** combined with soft independent modeling of class analogy (**SIMCA**) is a powerful technique to discriminate and classify *Saccharomyces cerevisiae* strains. Detailed spectral analysis of different *S. cerevisiae* strains has provided unique signature profiles allowing their differentiation.

- *S. cerevisiae* strains differentiation was mainly associated with **IR** frequencies of cell wall components.

- The physiological growth phase of yeasts played an important role to *S. cerevisiae* strains discrimination by infrared spectroscopy combined with **SIMCA**. At exponential phase, the cell wall components were mainly glucans, mannoprotiens, and lipids and, at stationary phase, were mainly glucans and mannans.

- The effect of nitrogen supplementation at the beginning of the alcoholic fermentation was successfully evaluated by **ATR-FTIR** combined with **SIMCA.** Depending on the source of nitrogen used and the physiological phase studied, the yeast cell wall components that were revealed to differentiate no supplemented and nitrogen supplemented *S. cerevisiae* cells were different.Two effective algorithm classification **SIMCA** models were developed to differentiate and predict Non-*Saccharomyces* yeast species and *Saccharomyces cerevisiae* strains based upon data obtained from mid-infrared spectroscopy profiles.

- When the physiological growth phase of yeasts was taken into account, better **SIMCA** models to differentiate and predict Non-*Saccharomyces* yeast species and *Saccharomyces cerevisiae* strains were built up.

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CHAPTER 6

Apendixes

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6.1 Apendix 1: About the Author

6.1.1. Published Articles

Puxeu, M., Andorra, I.,& De Lamo-Castellví, S. (2014). Monitoring *Saccharomyces cerevisiae* Grape Must Fermentation Process by Attenuated Total Reflectance Spectroscopy.*Food and Bioprocess Technology*, *8*(3), 637–646.

Diban, N., Arruti, A., Barceló, A., **Puxeu, M.**, Urtiaga, A. & Ortiz, I. Membrane dealcoholization of diferent wine varieties reducing aroma losses. *Innovative Food Science and Engineering Technologies*, 20, 259-268.

6.1.2. Articles under Review

Puxeu, M., Andorra, I., Brull, A. and De Lamo-Castellví, S. (2015) Fourier-Transform Infrared Spectroscopy to Study *Saccharomyces cerevisiae*. Submitted to *Canadian Journal of Microbiology.*

Puxeu, M., Andorra, I., Brull, A. and De Lamo-Castellví, S. (2015). Discrimination of *Saccharomyces cerevisiae* and Non-*Saccharomyces* species by attenuated total reflectance infrared spectroscopy combined with multivariate analysis.

6.1.3. Participation in International Congresses

Puxeu, M., Andorra, I., Brull, A. and De Lamo-Castellví, S. Discrimination of *Saccharomyces* cerevisiae and Non-*Saccharomyces* yeast isolated from Spanish grapes by Attenuated Total Reflectance infrared spectroscopy combined with multivariate analysi. European Symposium of Food Safety (**IAFP**), Cardiff (Walles), **2015**. Conference.

Puxeu, M., Andorra, I., Brull, A., Carmona, J. and De Lamo-Castellví, S. Use of **ATR-FTIR** with multivariate analysis as a rapid method to identify and discriminate *Saccharomyces* an Non-*Saccharomyces* yeast strains. European Federation of Food Science and Technology (**EFFOST**), Uppsala (Sweden), **2014**.Poster presentation.

Puxeu, M. and De Lamo-Castellví, S. Use of **ATR-FTIR** spectroscopy combined with multivariate analysis to study nutrient supplementation during alcoholic fermentation.European Federation of Food Science and Technology. Bologna (Italy), **2013**. Conference.

Tejerina, M., Hernandéz, E., Velado, D., **Puxeu, M.** and Ojeda, H. Influence of water deficit on plant physiology, grape aromatic precursors and wine quality of Vitis vinífera White grenache.Groupe International d'Experts en Systemes vitivinicoles pour la CoOpération (**GiESCO**), Oporto (Portugal), **2013**. Poster presentation.

Puxeu, M. and De Lamo-Castellví, S. Use of **ATR-FTIR** spectroscopy to monitor alcoholic fermentation and control the physiological state of yeast. **ENOFORUM**, Arezzo (Italy), 2013. Conference.

Gelaw, T.K., **Puxeu, M.** and De Lamo-Castellví, S. Discrimination and classification of acetic acid bacteria and *Saccharomyces cerevisiae* strains by attenuated total reflectance microspectroscopy. European Symposium on Food Safety (**IAFP**), Ede (Netherlands), **2011**.

6.1.4. Participation in National Congresses

Puxeu, M., Brull, A., Andorra, I. and De Lamo-Castellví, S. Application of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy combined with multivariate analysis to study yeast involved in winemaking processes. **GIENOL**, Tarragona, **2015**. Poster presentation.

Martin, L., Tomé, J., **Puxeu, M.**, Brull, A., Nart, E. & Andorrà, I. Levaduras Autóctonas para producer vinos libres de dioxide de azufre. **GIENOL**, Tarragona. **2015**. Poster presentation.

Roselló, M., **Puxeu, M.** & Rubio Coque, JJ. Estudio del perfil sensorial de los tapones de corcho naturales para vinos tranquilos. **GIENOL**, Tarragona. **2015**. Poster presentation.

Martin, L., Tomé, J., **Puxeu, M** and Andorrà, I. Selecció, Identificació i caracterització de llevats autòctons per la producción d'un vi sense sulfuròs.II Jornada de Recerca

d'Enologia i Viticultura a Catalunya (**CEICS**), Tarragona, **2014**. Poster Presentation.

Brull, A., **Puxeu, M.**, Nart, E., Rosello, J. and Pellerin, P. Influencia de las enzimas de maceración *Rapidase Extra Color* y *Rapidase Extra Fruit* sobre diferente parámetros de calidad del vino. II Jornada de Recerca d'Enologia i Viticultura a Catalunya (**CEICS**), Tarragona, **2014**. Poster Presentation.

Puxeu, M., Andorra, I., Brull, A. and De Lamo-Castellví, S. Use of **ATR-FTIR** combined with multivariative analysis as a rapid tool to deiscriminate between native *Saccharomyces* and Non-*Saccharomyces* yeast strains isolated from Grenache blanc from Terra Alta. II Jornada de Recerca d'Enologia i Viticultura a Catalunya (**CEICS**), Tarragona, **2014**. Poster Presentation.

Puxeu, M., Andorra, I and De Lamo-Castellví, S. Use of **ATR-FTIR** spectroscopy to monitor alcoholic fermentations and control the physiological state of yeast. I Jornada de Recerca d'Enologia i Viticultura a Catalunya (**CEICS**), Tarragona, **2013**. Poster Presentation.

Puxeu, M., Barceló, A., Brull, A., Andorrà, I. y De Lamo-Castellví, S. Evaluación del cambio químico experimentado por cepas de S*accharomyces cerevisiae* mediante la técnica de **ATR-FTIR**. **GIENOL**, Madrid, **2013**. Poster Presentation.

Roselló, J., **Puxeu, M.**, Andorrà, I., Esteve, B. y Rubio Coque, J. Estudio de la mancha amarilla del corcho como modelo de producción de compuestos organoclorados. **GIENOL**, Madrid, **2013**. Poster presentation.

6.1.5. Books

Palacios, A., San Román, JI., Roselló, J., **Puxeu, M.**, Rubio Coque, JJ., Moutinho, S. (**2015**). Valoración enológica del tapón de corcho: reducción de organoclorados y aportación de compuestos positivos al vino. (1ª ed.), Parc Tecnològic del Vi, Falset. **ISBN**:978-84-606-9209-6.

6.1.6. Main Research Projects

Lucha integral contra el carácter fenolado del vino. Desarrollo de técnicas para el control y eliminación de Brettanomyces en la elaboración de vinos tintos. Ministerio de Ciencia e Innovación. **2014-2016**.

Desarrollo de un itinerario enológico para elaborar vinos de alta calidad libres (**IPT**-2012-0967-060000). Ministerio de Ciencia e Innovación (**MINECO**). **2012-2015**.

Reducción de oxígeno disuelto y desalcoholización en vinos mediante el uso de membranas hidrofóbicas (IPT-060000-2010-016). Ministerio de Ciencia e Innovación (**MINECO**). **2011-2014**.

Elaboración de un vino blanco ecológico de la variedad xarel•lo con mínimo contenido en dióxido de azufre utilizando alternativas de materia prima ecológica. Generalitat de Catalunya – **AGAUR**. **2013-2014**.

Utilización de la espectroscopia de infrarrojo cercano (**FT-NIR**) para estudiar el proceso de fermentación alcohólica a partir de mosto de uva (PTQ-10-03144). Ministerio de Ciencia e Innovación (**MINECO**). **2011-2013**.

ENOR&D. Ministerio de Educación, Política Social y Deporte. 2011-2012.

6.1.7. Main Research Contracts

Project title: Crianza acelerada de tempranillo mediante la adición de microoxigenación y distintas duelas y chips. Role: Investigador. **Start date:** 01/06/2013. **Time:** 6 months.

Project title: determinación del perfil aromático de mostos procedente de variedades autóctonas gallegas. Role: Investigador. **Start date:** 01/06/2013. **Time:** 6 months.

Project title: Determinación del momento óptimo de vendimia para las variedades Garnacha tinta y Cariñena en la DOC Priorato. Role: Investigador. **Start date:** 01/01/2013. Time: 10 months.

Project title: Influencia de la co-inoculación de bacterias lácticas y la microocigenación durante el proceso de eleboración del vino. Role: Investigador. **Start date:** 01/09/2012. **Time:** 9 months.

Project title: Caracterización de Bioactivo en los residuos de la uva y el vino Role: Investigador.**Start date:** 01/07/2012. **Time:** 10 months.

Project title: Ensayo y calibración Equipo IR-Spectron 2012-2013. Role: Investigador. **Start date:** 01/04/2012. **Time:** 6 months.

Project title: Calidad enológica vs edad del viñedo de Cariñena en la DOC Priorato. Role: Investigador. **Start date:** 01/01/2012. **Time:** 9 months.

Project title: Mejora de la eficiencia del riego de soporte en vid para los viticultores de la cooperativa del Masroig. Role: Investigador. **Start date:** 01/01/2012. **Time:** 10 months.

Project title: Estudio de coinoculación de levaduras y bacterias. Impacto en el vino de garnatcha negra de la Denominación de Origen Calificada Priorato. Role: Investigador. **Start date:** 01/01/2011. **Time:** 1 year.

Project title: Estudio y mejora de los vinos elaborados con variedades tradicionales de la província de Tarragona. Role: Coordinador.**Start date:** 01/01/2011. **Time:** 3 years.

Project title: estudio de precios de la uva percibidos por los viticultores de Cataluña. Role: Investigador. **Start date:** 01/01/2011. **Time:** 1 year.

Project title: Bodega Sostenible. Role: Investigador. Start date: 01/06/2010. Time: 6 months.

Project title: Gestión de las aguas residuales en bodegas elaboradoras de vino situades en el interior del casco urbano. Role: Investigador. **Start date:** 01/06/2010. **Time:** 6 months.

Project title: Aptitud de viñas jóvenes y viñas viejas, de la variedad de Garnacha Tinta en las condiciones de la Denominación de Origen Montsant, para la elaboración de vinos tintos de calidad. Role: Investigador. **Start date:** 01/01/2010. **Time:** 1 year.

Project title: Estudio de precios de la uva percibidos por los viticultores de Cataluña. Vendimia 2010. Role: Investigador. **Start date:** 01/01/2010. **Time:** 1 year.

Project title: Modelo de adaptación del viñedo y la maduración para producir vinos de éxito para mercados Internacionales Role: Investigador. **Start date:** 01/01/2010. **Time:** 1 year.

Project title: Optimización del método Biolan de análisis de ácido glucónico comparado con HPLC, infrarrojo y el kit enzimático. Role: Investigador. **Start date:** 01/01/2010. **Time:** 1 year.

Project title: Potenciación de las variedades tradicionales de la DO Terra Alta. Role:Investigador. **Start date:** 01/01/2010. **Time:** 1 year.

Project title: Estudio de la adaptación de un vinyedo situado en Les Garrigues y sometido. a condiciones de gran sequedad y temperatures. Role: Investigador. **Start date:** 01/01/2009. **Time:** 3 years.

Project title: Estudio de la adaptación de un vinyedo situado en Les Garrigues y sometido a condiciones de gran sequedad y temperaturas elevadas para la producción de vinos ecológicos. Role: Investigador. **Start date:** 01/01/2009. **Time:** 2 years.

Project title: Elaboración de microvinificaciones y determinaciones analíticas en mostos y vino . Role: Investigador. **Start date:** 01/01/2008. **Time:** 3 years.

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