YEASTS AUTOLYSIS ON THE MANUFACTURE OF SPARKLING WINES; INFLUENCE OF AGING TIME ON THE RELEASE OF POLYSACCHARIDES AND PROTEINS AND THE CONSUMPTION OF OXYGEN BY THE LEES

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PERE PONS MERCADÉ

DOCTORAL THESIS
2021

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Doctoral Thesis

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(TECNENOL)

UNIVERSITAT ROVIRA i VIRGILI

Tarragona

2021

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Fernando Zamora Marín, Joan Miquel Canals Bosch i Nicolas Rozès, professors titulars del Departament de Bioquímica i Biotecnologia de la Facultat d'Enologia de la Universitat Rovira i Virgili,

FAN CONSTAR que aquest treball, titulat "**Yeasts autolysis on the manufacture of sparkling wines; Influence of aging time on the release of proteins and polysaccharides and the consumption of oxygen by the lees**", que presenta **Pere Pons Mercadé** per l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta Universitat

Tarragona 03 de Juny del 2021

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Funding: This research was funded by the Spanish Ministry of Science and Innovation, Centre for the Development of Industrial Technology (CDTI) (Program CIEN, Project GLOBALVITI: Global solution to improve wine production against climate change based on robotics, IT technology and biotechnological strategies and vineyard management; Program CIEN, Project CAVAWINNER: Study and technological improvement of the traditional processes of Cava elaboration) in collaboration with Juvé & Camps SA.

Grant: This PhD Thesis was performed with a grant provided by the *Departament d'Empresa i Coneixement* of the *Generalitat de Catalunya* under the program *Ajuts per a la contractació de personal investigador FI*. Expedient 2018FI_B1_00163

AGRAÏMENTS

L'acció d'agrair, esta sempre vinculada a el fet de mirar enrere, recordar, reflexionar i sobretot valorar l'esforç i la dedicació de les persones que t'han envoltat, en el cas que em pertoca, en el recorregut de la consecució de la Tesi Doctoral. Entenc també, que l'acció d'agrair és una acció generosa, i per això, vull compartir aquest document amb totes les persones, que juntament amb mi, són igualment partícips de tots i cadascun dels mots impresos en les pàgines que venen a continuació. Agrair vol dir regalar. Regalem un bocí de la feina feta a tothom qui ha tingut part de protagonisme en la consecució de la fita. Soc conscient de que no hauria sigut possible sense la participació — i es pot participar de mil maneres diferents — d'un conjunt de persones a les que haig d'agrair l'acompanyament, el coneixement, el recolzament, l'oxigen i tantes altres coses.

El Dr. Fernando Zamora Marín i el Dr. Joan Miquel Canals Bosch per confiar en mi i donar-me l'oportunitat de ser un més del seu equip i dedicar-me tot el temps i recursos necessaris per desenvolupar la tesi redactada aquí. *Merci* també al Dr. Nicolas Rozès, per sumar-te al camí i estar sempre predisposat a col·laborar i ajudar en el que fes falta. Als tres, gràcies, a més, per ser excel·lents docents i no escatimar mai en compartir el vostre immens coneixement sobre qualsevol tema relacionat amb el món del vi. El simple fet de compartir hores i hores amb vosaltres a la bodega, als laboratoris, a la sala de tast i als despatxos és el millor que me'n duc d'aquesta experiència. Aprendre al vostre costat a fer vi, entendre'l, apreciar-lo i gaudir-lo, és una experiència única que m'acompanyarà per sempre.

Gràcies també a tot l'equip humà que forma el Departament de Bioquímica i Biotecnologia pel recolzament en tot moment, en especial a la Rosa i al Braulio, imprescindibles perquè tot funcioni. Gràcies per tindre el tren sempre alineat sobre les vies, les pàgines de materials i mètodes són vostres, us les mereixeu. Gràcies també als professos que conformen al Departament, per la seva generositat en compartir temps i coneixements dia a dia.

Gràcies també a Glòria Vilomara, Marta Conde i Antoni Cantos, i a la resta de l'equip de Juvé & Camps per compartir part de la vostra història en forma d'ampolles de Cava de nou anyades consecutives. Vàreu posar les primeres ampolles de Cava de la rima que hem

anat construint aquí. Gràcies també al Pepe Heras, Nathalie Sieczkowski i a Lallemand per estar oberts a noves idees i per la confiança en la innovació.

A tots els que formen part del grup Tecnenol, als que han passat i als que hi són, per ser uns immillorables companys de vida i de feina, vostres són les pagines dels resultats, resultats on tots i cadascun de vosaltres hi heu aportat granets de sorra. Diuen que és complicat gaudir mentre treballes, però gràcies a vosaltres ha sigut complicat no gaudir en cada moment. Aprenem molta ciència entre matrassos, espectrofotòmetres i HPLC, però segurament el que més aprenem al laboratori 120 és a treballar en equip, a ser solidaris, a arribar on no arriba l'altre, a recolzar-nos i a compartir qualsevol idea que suposi millorar el treball de l'altre o a la millora del bé comú. Els que em precedeixen saben de que parlo, els que hi sou sigueu-ne un reflex i els que vindreu no ho deixeu perdre mai.

Als tots i totes els que englobeu el terme *musicorquestrals*, per vosaltres van totes les pàgines en blanc, que si fullegeu la tesi, en són moltes. Les pàgines en blanc serveixen per respirar, per calmar-se, per acompanyar i per valorar tota la resta. Les pàgines en blanc també són un univers per escriure o un espai on crear com tot el que ens queda per viure i per descobrir plegats.

Als *Biobotiflers*, sentiu-vos vostres les introduccions, inici de tota història. Com la que s'explica aquí, una història que neix entre classes i laboratoris de Biotecnologia a Barcelona on enllacem camins que, tot i que avui en dia la majoria del temps transcorrin per separat, sempre trobin la drecera fins al Rovellar.

A la família, sempre hi he trobat referents d'esforç, treball i dedicació. Per això, a la família us he guardat les pàgines de referències, potser no són les més glamuroses ni divertides, però són les que ho sustenten i ho recolzen tot. Gràcies per ser sempre un refugi d'amor i tranquil·litat.

Als pares us guardo dues pàgines, la primera, per la immensa estima que us tinc i el reconeixement que us mereixeu, tot això és gràcies a vosaltres. I us guardo també la última, que curiosament està en blanc, llesta per escriure la següent tesi plegats: al taller i a la cava.

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1. RESUME

Cava is a sparkling wine produced by the traditional method which involves two fermentations. In the first fermentation the grape juice is transformed into a base wine according to a standard winemaking process. Then sugar, processing aids and a yeast starter culture, previously adapted, is added to ensure that the second fermentation reaches a successful end. When the second fermentation is complete, the sparkling wines are aged in the bottle in contact with yeast (also called lees) for a certain length of time, while the process of autolysis takes place. During this ageing time, lees release macromolecules to sparkling wine matrix modulating the final composition and the quality of Sparkling wines. In addition, it was also thought by the Sparkling wine industry, that lees enable to age longer due to their protective effect against oxidation. For this reason, winemakers say that this long contact with the lees are the main difference between sparkling wines produced by the traditional method (or *méthode champenoise*) and those produced by other procedures.

In basis of these hypothesis, two objectives were set: first, characterization of yeast autolysis in sparkling wine produced by traditional method (Cava) over long aging time inside the bottle by: quantification of the polysaccharides and protein released by lees over long aging time, following evolution of the sensory characteristics, color and foaming properties, monitoring visual degradation of yeast during autolysis over aging time using Scanning Electron Microscopy (SEM) and quantify the impact of the lees released polysaccharide and proteins in the total sparkling wine macromolecules composition. Second, evaluate the oxygen consumption capacity of lees of sparkling wine elaborated by traditional method and its variation over long aging time.

Furthermore, after confirming that lees really consume oxygen, a third objective were set in order to confirm and understand better the mechanisms of the protective effect against oxidation by inactivated dry yeast (IDY). Three IDY were evaluated by quantifying oxygen consumption rate was measured. These oxygen consumption rates were compared with the most common antioxidants used in winemaking: sulfur dioxide, ascorbic acid and glutathione to evaluate their effectiveness.

2. INTRODUCTION

2.1. Sparkling wines

2.1.1. Definition and types of sparkling wines

According to the European Regulation CE 1493/99, sparkling wines differ from still wines in the level of internal pressure of carbon dioxide which must be higher than 3 bars. Sparkling wines are classed in function of the CO2 origin in gasified wines. Hence, there are two main categories: **Gasified Wines** when the carbon dioxide is from an exogenous source and **Natural Sparkling Wines** when it comes from endogenous fermentation.

Gasified wines are produced simply by injecting carbon dioxide until reaching the desired internal pressure. Normally, these gasified wines are usually low-priced, have no geographical reference and they also have much lower sensory quality than natural sparkling wines. Given their small interest under their sensory point of view they will not be considered in this introduction. In contrast, natural sparkling wines are obtained by means of a natural fermentation keeping all or a proportion of the carbon dioxide inside the vessel in which they have been fermented. There are different elaboration methods of sparkling wines depending on the type of vessel (bottle or tank), time of lees contact, the procedure of eliminating the lees, or the number of alcoholic fermentations they have been through, which may be one or two. Moreover, some of these natural sparkling wines are protected by *Appellation d'origine contrôlées* (AOC) such as Champagne, Cava, Prosecco, Francia Corta, Asti, Crémant de Bourgogne, etc… In this case, any AOC determines the elaboration method, authorized varieties and aging time. The different elaboration methods of natural sparkling wines are described below.

2.1.2. Traditional method

Sparkling wines considered as top quality are mainly produced by the traditional method. Cava and Champagne are both produced following the traditional method, also called for Champagne AOC *méthode champenoise*. The main characteristic of the traditional method is that after a first fermentation to obtain the base wine, a second fermentation, also called *prise de mousse,* is performed inside a closed bottle (BOE, 2007; Journal Officiel de la Republique Française, 2010). This second fermentation inside the bottle,

and especially the aging time in contact with the lees, allows a better integration of the CO_2 dissolved giving the ability to constant bubbling and persistent foaming when it is served in a glass.

2.1.2.1. First fermentation

The first fermentation to produce base wine follows generally the normal path for white and rose winemaking: Pressing, settling and fermentation. This first fermentation, which differs in some aspects from the standard fermentation of still wines, is a key point to achieve quality sparkling wines for several reasons. Normally the grapes used for these wines are less ripe, having a lower potential ethanol content (between 8.5% and 11.0%), higher titratable acidity and lower pH. These characteristics are needed for a better final balance in the sparkling wine and also to prevent stuck and sluggish fermentation in the bottle.

Some wineries prefer to press directly the grapes without destemming and crushing to minimize the contact between juice and grape skin. This aspect is absolutely needed in the case of using red grapes to obtain white wines (*Blanc de Noirs*) and for that reason the Champagne AOC establishes that the grapes cannot be destemmed and/or crushed. In the Case of Cava AOC, the pressing procedure is left to the free choice of the winery. It is generally recognized that pressing directly the grapes gives cleaner musts with lower astringency, bitterness and herbaceous character, being even able sometimes to be fermented without settling. In any case, all the sparkling wine AOCs establish a limit in the proportion of grape juice that can be extracted by pressing.

Normally, the grape juices from different varieties are usually vinified separately in order to keep their qualities for the further blending (Ribéreau-Gayon, Dubourdieu and Donèche, 2000).

The alcoholic fermentation is performed by *Saccharomyces cerevisiae* normally inoculated at 10^6 cell/mL to ensure that the process is completed uninterruptedly until the amount of reducing sugars is lower than 2 g/L. The fermentation usually last two or three weeks at 15 to 20 ^oC. At the end of the fermentation, the wine is racked in order to remove the lees. At that point *coupage* is done by blending different base wines made from

different varieties and/or origins. Then, the base wine must be stabilised to prevent protein haze or tartrate crystallization. The use of bentonite for avoiding protein haze should be more carefully controlled than in still wines since an excess of this technological aid can seriously affect the foaming properties of the sparkling wines. Subsequently, tartrate stabilization must be performed. Probably, the most used procedure is cold stabilization, However, nowadays the employment of other physical procedures such as cationic exchange or electrodialysis, or chemical procedures such as carboxymethyl cellulose (CMC) or potassium polyaspartate (KPA) are more and more common. After filtering, the base wine is ready for the second fermentation. Some wineries undergo the base wine to a malolactic fermentation before the second fermentations to reduce acidity, to obtain more flavour complexity and more stability against future bacterial contamination.

2.1.2.2. *Tirage*, second Fermentation and ageing

The process of wine bottling with the addition of sugar and adapted yeast is called *tirage*. The second fermentation or *prise de mousse* is performed after adding sucrose to the base wine since their reducing sugar concentration are normally below 2 g/L. The sugar consumption by yeast during the alcoholic fermentation (Figure 1) occurring in a closed bottle produces an increase of inner CO_2 pressure.

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

Figure 1: Global alcoholic fermentation balance

This internal pressure should be between 5 and 6 bars at 10 ºC to guarantee the correct balance of the sparkling wine. So, to reach a pressure of 5 to 6 bars in the bottle, the amount of sugar added in *tirage* must be between 20 to 24 g/L (Kemp *et al.*, 2015). Normally the added sugar concentration varies regardless of the aging time. The greater the aging time the higher the added sugar concentration in order to compensate for the slight losses of CO_2 internal pressure that permeates across the stoppers.

Those bottles used for sparkling production by the traditional method must be thicker and heavier than current bottles so as to be able to support overpressure.

The base wine is a very aggressive medium for yeast since it contains ethanol (around 10.5%), has a low pH (around 3.0) and has very low yeast assimilable nitrogen (YAN).

Moreover, some toxic fermentation sub-products such as short chain fatty acids can be present in the base wine hindering the yeast growth. These conditions create a difficult environment for sugar fermenting (Alexandre, 2019). Thus, a process of yeast reactivation and acclimation to achieve a suitable physiological condition is necessary. This starter, called *pied de cuve*, is not only important for adapting yeast to the wine conditions but also for multiplying yeast population in order to seed 1.5 million cell/ml in each bottle.

Finally, some adjuvants such as bentonite or alginate, are added to facilitate the riddling and disgorgement processes (Ribéreau-Gayon, Dubourdieu and Donèche, 2000; Vanrell *et al.*, 2007). Bottles are mainly closed with bidule and crown cap (Figure 2) but some winemakers also use cork stoppers even though in that case disgorgement automation is not possible.

Figure 2: Image of shutter (left) crown cap (right).

After *tirage*, all bottles lay in horizontal position for the second alcoholic fermentation and ageing. The consumption of sugar and CO_2 production by yeast occurs in approximately in 1 or 2 months. The yeast viability decreases from 1 to 3 months, and after 6 months no viable cells are found (Nunez *et al.*, 2005). Then, these bottles remain horizontally stocked in the cellar at 10 to 15 ºC for the ageing period. This period can last different periods of time depending on the minimum established by the AOCs (Table 1) for the different categories or the winemaker's criteria. During the ageing, the horizontal position enhances the surface of contact between wine sediments (lees) and wine. The sediments composition is based mainly on dead yeasts (lees), bentonite and other substances dragged by it as proteins, tartrate crystals and other solids (Alexandre and Guilloux-Benatier, 2006). During ageing, yeasts release substances into the wine, first by exsorption and later on, once yeasts start to be degraded, by the autolysis process. This interaction between lees and wine during ageing improves the sparkling wine quality (Ribéreau-Gayon, Dubourdieu and Donèche, 2000). These lees also act as a redox buffer being able to protect wine against oxidation (Pons-Mercadé *et al.*, 2021).

AOC	Classification		Ageing time
CAVA	Cava de Guarda		9 months
	Cava de Guarda Superior	Reserva	18 months
		Gran Reserva	30 months
		Paratge Qualificat	36 months
CHAMPAGNE	Non-Vintage		12 months
	Vintage		36 months
FRANCIACORTA	Non-vintage		18 months
	Vintage		30 months
CREMANT BOURGOGNE	Eminent		24 months
	Grand Eminent		36 months

Table 1: Comparison of different AOCs and type products by the time of ageing in contact with lees.

2.1.2.3. Riddling, disgorging and expedition

When the ageing period is finished the next step is to remove the wine sediments. For this proposal, the sediments must descend to the neck of the bottle. This process is performed by gradually moving the bottles from a horizontal to a vertical position, while making rotatory movements on the bottle's central angle to facilitate the glide of sediments (Figure 3). The rotatory movement should be done clockwise in the northern hemisphere and counter clockwise in the southern hemisphere to be favoured by the Coriolis acceleration. Traditionally this process had been done manually, taking one month of time which increase costs. Nowadays, riddling is mainly done by a mechanized and programmed cycles system called *gyropallet* that reproduces the manual procedure and reduces the time to one week at the most. In fact, without the use of bentonite and other riddling agents added in *tirage* this process should be very slow and in some sparkling wines nearly impossible.

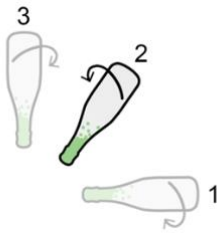

Figure 3: Riddling process from horizontal (1) to vertical (2) position.

The disgorging is carried out taking advantage of the inner bottle pressure. When sediments are settled on the cap, the bottle is opened removing the crown cap and the bidule, which contains the sediments, is expelled. This process implies losing a few millilitres of wine that must be compensated by adding the expedition liqueur. Then, the bottle is closed with the final cork that is immediately fixed with the muzzle, cleaned and labelled. Nowadays, in order to industrialize and facilitate the process, the neck is frozen before disgorging.

	Blend at the time of bottling	After second fermentation and addition of dosage for *brut* quality
Mass density at 20°C (g/dm^3)	990.5	993.9
Alcohol at 20°C (% vol.)	11.0	12.2
Sugars (g/l)	1.3	12.7
pH	3.02	3.05
Total acidity (g/l H_2SO_4)	4.7	4.7
Volatile acidity (g/l H_2SO_4)	0.27	0.3
Free SO_2 (mg/l)	8	8
Total SO_2 (mg/l)	38	58
Tartaric acid (g/l)	3.5	3.2
Malic acid (g/l)	0.2	0.2
Potassium (mg/l)	330	325
Calcium (mg/l)	85	70
Copper (mg/l)	0.17	0.13
Iron (mg/l)	2.1	2.8
Sodium (mg/l)	8	12
Magnesium (mg/l)	60	70
Total nitrogen (mgN/l)	303	410
Ammoniacal nitrogen (mg/l)	13	20
OD 520 nm	0.038	0.028
OD 420 nm	0.087	0.106
Color intensity	0.125	0.134
Shade	2.59	3.89
Conductivity (mS/cm) at 20°C	1.32	1.32
Saturation temperature at 20°C	10°C	12.1°C

Table 2: Base wine composition and Sparkling wine composition after dosage liquor addition. Table extracted from (Ribéreau-Gayon, Dubourdieu and Donèche, 2000).

At that point, prior to the close of the bottle, an expedition liquor made of wine and sugar can be added to regulate the final sugar concentration. This operation is called *Dosage*. In Cava appellation, depending on the final wine sugar concertation, the product can be labelled as *Brut Nature* (less than 3 g/L), *Brut* (less than 12 g/L), *Semi-Sec* (from 32 to 50 g/L), or sweet (more than 50g/L). Expedition Liquor is also used by winemakers to finally

adjust acidity and SO_2 levels. Table 2 shows a comparison of the most relevant physicochemical properties between a base wine and an ended *Brut* Sparkling wine.

2.1.2.4. AOC Cava

AOC Cava is the appellation that includes the majority of sparkling wines made by the traditional method in Spain with an epicenter in the Catalan region of *Penedès* (south-west from Barcelona). Historically *Cava* name appears for the first time in an official legislation in 1959 referencing the sparkling wine products from the *Penedès* region (BOE, 1959). Nevertheless, the first references of Catalan sparkling wine were dated in the middle XIX century as an emulation of champagne and even these first sparkling wines were named as *Xampany* o *Champaña*. But in 1958 and because of the Lisbon Agreement for the Protection of Appellations of Origin and their International Registration, Catalan producers were no longer able to longer use the name *Xampany* o *Champaña* in their products. After that event, a process of legislation around Cava production, quality and region started.

In 1966, in the *Reglamentación de Vinos Espumosos y gasificados* (BOE, 1966) Cava products were established to be only produced by the traditional method of second fermentation and ageing inside the bottle. In 1970, after the enactment *Estatuto de la Viña, del vino y de los alcoholes* Cava producing regions were specifically determined (BOE, 1970). Later, when Spain entered the European Economic Community in 1986, Cava was recognized as a quality sparkling wine produced in a determined region. Consequently, in 1991 a regulation of Cava production was published establishing three different products based on minimal ageing times: *Cava* (9 months), *Cava Reserva* (15 months) and *Cava Gran Reserva* (30 months).

Varieties accepted for Cava production are Macabeu, Xarel·lo, Parellada, Chardonnay, Garnatxa Negre, Trepat, Pinot Noir, Subirat Parent and Monastrell in descending order of planted varieties. Nowadays there are 38.151,74 hectares of planted vineyards divided in four regions: Comtats de Barcelona, Valle del Ebro, Zona de Levante and Viñedos de Almendralejo (Figure 4). In 2020, 209 producers were registered in the AOC and approximately 215 million bottles were produced. Since 2020, the denomination regarding the ageing period of cavas has been changed. Hence, those cavas with minimum

legal ageing time of 9 months receive the denomination of *Cava de Guarda*, those undergoing a process of at least 18 months are labelled as *Cava de Guarda Superior Reserva*, those with more than 30 months are known as *Cava de Guarda Superior Gran Reserva*, and finally the ones produced by a recognized single vineyard with more than 36-month ageing process are known as *Cava de Guarda Superior Reserva de Paratge Qualificat*.

Figure 4: AOC Cava producing regions (adapted from www.cava.wine)

2.1.3. Ancestral method

The Ancestral method consists in producing a sparkling wine using only a single fermentation. This method was created by the Benedictine monks of *Saint-Hilaire* in the XVI century. At the present time, the most representative AOC using this method is *Blanquette de Limoux* in the southwest of France. The fermentation is performed following the normal steps of base wine winemaking, until this fermentation is around 20 g of sugar/L. At that moment, the wine is bottled and corked, usually using a bidule and crown cap. This way, the wine completes the alcoholic fermentation in the bottle generating CO_2 which is retained inside. Specifically, the sugar used for the CO_2 production comes from the grape not needing to add external sugar. This procedure is simpler than the traditional method since it allows us to obtain sparkling wines in the bottle by means of a single fermentation. In the past, neither disgorging nor dosage were applied, but nowadays these processes are used in order to commercialize limpid sparkling wines. Even so, some producers disgorge just after the end of the fermentation to release part of the lees and to avoid further reductions of taint.

Usually, sparkling wines elaborated with the ancestral method have low alcohol content and higher sweetness due to the difficulties of the yeast to end the fermentation.

2.1.4. Charmat method

The Charmat method is characterized by the fact that a second fermentations is undergone in a large-scale pressurized tank. *Prosecco*, some *crémants* and most of the New World sparkling wines are produced using this method. In this method, the blended base wine, yeast starter and sugar are added in a fermenting tank in order to produce up to 5 bars of CO_2 pressure. Then, the wine is refrigerated, isobarically filtered and bottled. A pressurized system is needed for all the production process and also wineries need to work at low temperatures to make CO_2 more soluble in wine and reduce leak.

In this method, the contact with lees is negligible because of the great size of tank and the short period that they are in contact one each other. So, all quality aspects achieved by bottle ageing in the traditional method are not found in this method. However, there are some techniques to enhance wine and yeast interaction based on generating a constant suspension of yeasts or adding enzymes that favour the wall yeast cell degradation (Howe, 2003).

2.1.5. Transfer method

The Transfer method follows the Traditional method until the end of ageing, afterwards, the riddling and disgorging is avoided. When the ageing is completed, sparkling wine is isobarically filtered to remove all yeast and solids from wine. Immediately after, the expedition liqueur is added and the wine is bottled and corked. Therefore, one bottle has been used for fermentation and ageing, and another one for the final commercialization of the product. The Transfer method takes advantage of all the benefits of ageing wine in contact with lees, and it simplifies and reduces the time devoted to all the riddling and disgorgement process.

On one hand, this method presents some technical issues regarding the filtering and bottling so as not to lose foaming properties. For that reason, wine is cooled down until

minus 5ºC given the fact that at this temperature CO_2 is more soluble. In addition, a pressurized tank and an isobaric filter is needed to assure no CO_2 loss and to prevent degassing.

On the other hand, this method also presents some advantages. First, expedition liquor could be added to an entire batch of product in contrast to the one by one bottle of the traditional method. In this way, it is better and more regularly distributed. Cold stabilization and filtering guarantees a complete yeast elimination and a perfectly limpid wine (Ribéreau-Gayon, Dubourdieu and Donèche, 2000). Nowadays, the transfer method is a good alternative for bottles of lower volume than 0.75cL. In fact, AOC Cava authorize the transfer method only for quarter or half bottles because it reduces the higher oxidation that usually takes place in these small bottles and also because the traditional riddling process is quite complicated in those conditions.

2.1.6. Asti method

The Asti method is mainly used for Muscat sparkling wines production in the AOC Asti (Italy). This method is characterized by carrying out two fermentations in a tank. The first fermentation is temperature controlled to slow the fermentation speed and consequently conserve aromas. When wine reaches 5 to 7% alcohol when it already contains between 80 to 120 g/L of residual sugars. The first fermentation is stopped by freezing. Then, wine is fined and centrifuged until obtaining a stable wine. Subsequently, the different must/wines are blended and the second fermentation is performed similarly than in the Charmat method, but without adding sugar. The second fermentation is also stopped at 0ºC when it reaches around 5 bars of pressure, has an alcohol degree of 6 to 9% and it still contains between 60 to 100 g of sugar/L. At that point, the sparkling wine is stabilized at -4ºC for 10 days and then filtered and bottled. As Charmat and Transfer methods, isobaric filtration and bottling must be pressurized with CO_2 to avoid gas leaks. Some wineries do sterile filtration, and even pasteurization to avoid re-fermentation in bottle due to the high amount of residual sugar present in wine.

One advantage of the Asti method is that it reduces the losses of variety of aromas making it possible to obtain sparkling wines with a higher richness of Muscat variety aromas than other procedures.

2.2. Wine Macromolecules

Proteins and Polysaccharides are macromolecules with an important role in sparkling wine production and quality. These macromolecules come mainly from grapes (Ayestarán, Guadalupe and León, 2004; Esteruelas *et al.*, 2015) and yeast (Ayestarán, Guadalupe and León, 2004; Guadalupe and Ayestarán, 2007). This chemical composition depends on the grape variety and maturity (Esteruelas *et al.*, 2015; Gil *et al.*, 2015), fungal infection (Francioli, Buxaderas and Pellerin, 1999; Cilindre *et al.*, 2008), winemaking process (Vanrell *et al.*, 2007; Cilindre *et al.*, 2010) and autolysis (Martínez-Lapuente *et al.*, 2013). These macromolecules are also related with some physics and organoleptic process associated with wine quality.

2.2.1. Polysaccharides

Polysaccharides play an important role as protective colloids limiting and preventing tannin aggregation (Riou *et al.*, 2002) and tartrate salt crystallization and precipitation (Gerbaud *et al.*, 1997; Gerbaud *et al.*, 1996). It has also been reported that some polysaccharides improve protein stability (Moine-Ledoux and Dubourdieu, 1999) and that others can seriously affect wine filterability (Vernhet *et al.*, 1999). Polysaccharides are also related with some other quality parameters. Specifically, it has been described that polysaccharides improve foaming properties (Martínez-Lapuente *et al.*, 2015), increase wine mouthfeel (Escot *et al.*, 2001; Vidal *et al.*, 2004), reduce astringency and bitterness (González-Royo *et al.*, 2013; Quijada-Morín *et al.*, 2014).

Wine polysaccharides fraction is generally classified by their origin and composition. Polysaccharides rich in arabinose and galactose (named as PRAGs) come from grapes after degrading due to the pectinolytic enzymes during the maturation or winemaking process. The main PRAG types are arabinogalactans (AGs) and arabinogalactan-proteins (AGPs) with neutral character and they represent the 38%-41% of total polysaccharides in wine. AGs can be divided in two types (I and II). AGs-I are the most widespread, with a backbone of β-(1,4)-D-galactose substituted in position 3 by individual or oligomers of arabinose bonded on α-(1,5). The AGs Type II structure is based on a backbone β-(1,3)-

D-galactose backbone with linked β-(1,6)-D-galactose chains highly substituted by arabinose units (Figure 5). AGPs contain less than 10% protein (Ayestarán, Guadalupe and León, 2004; Ribéreau-Gayon *et al.*, 2000; Moreno-Arribas and Polo, 2009; Martínez-Lapuente *et al.*, 2013). PRAGs has a sugar moiety with hydrophilic character and protein moiety with hydrophobic and hydrophilic domains (Martínez-Lapuente *et al.*, 2015).

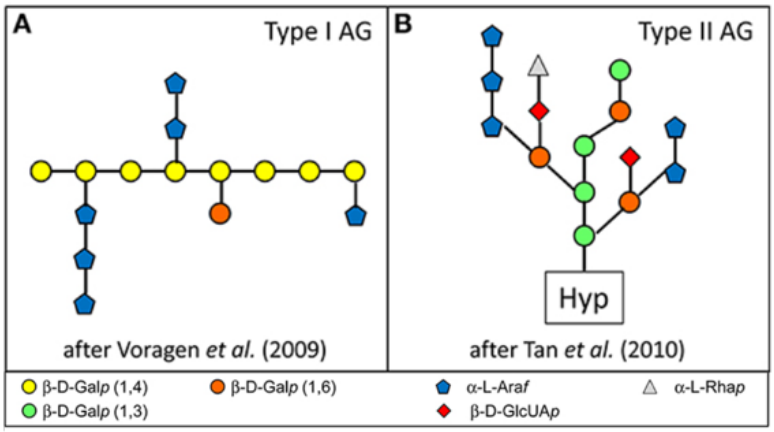

Figure 5: Arabinogalactans molecular scheme. A: Arabinogalactans type A, B: Arabinogalactans type B (adapted from Hijazi *et al.*, 2014)

Rhamnogalacturonans type I (RG-I) and type II (RG-II) and Homogalacturonans (HLs) are acidic substances due to their high proportion of galacturonic acid (GalA) and represent about 20% of soluble wine polysaccharides. The origin of these Polysaccharides is also grape cell walls. RG-II is a really complex polysaccharide based on a backbone composed by units of galacturonic acid repeated several times. This backbone, which is rather short, is branched with four side chains formed by a huge diversity of carbohydrate molecules, such as arabinose (Ara), rhamnose (Rha), fucose (Fuc), galactose (Gal) and galacturonic acid (GlcA). RG-I is a branched polymer with a backbone of disaccharides (α-(1,4)-D-GalA-α-(1,2)-L-Rha) repeated and some other linked carbohydrates. RG-II is present at higher concentration in wine than RG-I (Pellerin *et al.*, 1996; Ayestarán, Guadalupe and León, 2004; Ribéreau-Gayon *et al.*, 2000; Costa and Plazanet, 2016). Homogalacturonans are unbranched homopolymer chains of a α-(1,4)-D-GalA (Moreno-Arribas and Polo, 2009; Costa and Plazanet, 2016). Recently RG-II ability to complex lead (Pb^{2+}) has been described avoiding human ingestion (Pellerin *et al.*, 1997).

Mannoproteins (MPs) are the main polysaccharide fraction released by yeast cell wall (Escot *et al.*, 2001) representing around 35% of total polysaccharides in wine (Vidal *et*

al., 2003). However, this proportion can be higher in wines aged on lees (Martínez-Lapuente *et al.*, 2018). Mannoproteins composition is based on chains of mannose (80-95%) forming covalent complexes with proteins (10-15%) and are anchored in a layer of β-1,3-glucan from the external cell wall (Zlotnik *et al.*, 1984). MPs are highly glycosylated with α-mannose units (α-1,6; α-1,2 and α-1,3) (Klis *et al.*, 2002). This protein and sugar composition give to MPs an amphiphilic character (Martínez-Lapuente *et al.*, 2015). Glucomannoproteins have been also reported in wines but in a lower amount than MPs (Ribéreau-Gayon *et al.*, 2000; Moreno-Arribas and Polo, 2009).

Polysaccharides can also come from bacterial and fungal grape contamination but with less importance than grape and yeast fractions. For example, β-glucans from *Botrytis cinerea* causing fining and filtration problems (Dubourdieu, Ribereau-Gayon and Fournet, 1981).

Other exogenous polysaccharides such as arabic gum and carboxymethyl cellulose (CMC) can be also present in wine because of their technological use (Marchal and Jeandet, 2009; Bosso *et al.*, 2010; Claus *et al.*, 2014) for avoiding wine instability.

2.2.2. Proteins

In wine, proteins come predominantly from grapes (Ferreira *et al.*, 2000; Dambrouck *et al.*, 2003). The protein fraction concentration range in white wine is wide, from 30 to 230 mg/L (Bayly and Berg, 1967) and it is involved in several roles in wine. The most studied one, is the ability to produce the protein haze because their aggregation makes them insoluble. These aggregates lead to protein haze formation and precipitation which affects the wine stability during its storage. Consequently, it is considered as a negative quality parameter (Dawes *et al.*, 1994). It has also been reported that proteins can interact with aromas (Lubbers *et al.*, 1993; Ledoux and Dubourdieu, 1994) and can participate in the foam stabilization of sparkling wines (Girbau-Solà *et al.*, 2002; Liger-Belair, 2005; Vanrell *et al.*, 2007).

Proteins are defined as a chain of amino acids linked by peptide bond whose composition determines their three-dimensional structure. According to pH they may be positively or negatively charged. Generally, in wine proteins are positively charged because the wine

pH is lower than the Isoelectric Point (IP) of the majority of these proteins (4.1-5.8) (Hsu and Heatherbell, 1987; Moreno-Arribas and Polo, 2009). Grapes contain around 41 different proteins with a range of 11.2 kDa to 190 kDa. However, after the winemaking process, juices and wine protein fraction decreases until 25 with a range of 11.2 kDa to 65 kDa (Hsu and Heatherbell, 1987).

Nevertheless, the main proteins found in wine are grape invertase with a molecular weight of 71,5kDa (Dambrouck *et al.*, 2005; Cilindre *et al.*, 2014) and some pathogenesis-related proteins such as thaumatin like proteins and chitinases with molecular weights between 24 and 32 kDa (Waters, Shirley and Williams, 1996). These pathogenesis-related proteins are the main responsible of the natural protein haze (Waters *et al.*, 1998; Esteruelas *et al.*, 2009). Other proteins such as β-1,3-glucanase with molecular weights of 41 kDa have also been reported to participate in the natural protein haze of white wines (Esteruelas *et al.*, 2009).

Recent studies observed that seripauperin, a yeast produced protein associated with alcoholic fermentation, could have a really relevant concentration in wines and especially in sparkling wines (Pegg *et al.*, 2021).

It is thought that the aggregation and further precipitation is a consequence of a high temperature during the storage that leads to a slow denaturation of proteins (Pocock and Waters, 2006). It has been also proposed that phenolic compounds, in particular proanthocyanidins, can favor the appearance of protein haze (Esteruelas *et al.*, 2011). In fact, the possible effect of the tannin release into the wine from the cork has been suggested (Ribéreau-Gayon *et al.*, 2000). Other factors such as polysaccharides, alcohol level and pH might modulate the protein haze formation (Bayly and Berg, 1967; Ribéreau-Gayon *et al.*, 2006).

For those reasons, bentonite fining is generally used to prevent protein haze (Ribéreau-Gayon *et al.*, 2006). Bentonite is a montmorillonite clay, negatively charged, that is used for removing proteins before bottling (Ferreira *et al.*, 2001; Vanrell *et al.*, 2007).

When bentonite, negatively charged, interacts with wine proteins, mainly positively charged, a great proportion of proteins are removed (between 70% to 89% depending on

the wine characteristics and the used dose) (Moreno-Arribas and Polo, 2009). It has been reported that high molecular weight protein fractions are less affected by bentonite stabilization (Esteruelas *et al.*, 2015). However, this treatment negatively affects the foam quality parameters (Martínez-Rodriguez and Polo, 2003; Vanrell *et al.*, 2007; Esteruelas *et al.*, 2015) since proteins have been described as a positive factor for foamability.

2.3. Effervescence and foam

The Foaming properties are one of the most important sensorial characteristics of sparkling wines. A sparkling wine has a good quality foam when its effervescence forms numerous trains of small bubbles that rise from the depth of the liquid up to the surface where it is formed a white and stable crown (Moreno-Arribas and Polo, 2009).

It is obvious that the first aspect that consumers see in a glass of sparkling wine is the spectacle generated by the bubbles and consequently their appreciation of the quality is strongly conditioned by the foam and effervescence. In fact, the foaming properties of sparkling wines are considered as main parameters to determine their quality. Effervescence is the process in which the gas is discharged form liquid by bubbling formation (Gérard Liger-Belair *et al.*, 1999) whereas foam is described as the agglomeration of bubbles that remains as a collar on the wine surface (Moreno-Arribas and Polo, 2009). The foamability, concept that refers to the ability of a wine to produce foam is also divided in foam formation and foam stabilization. The first is related with the amount of bubbles produced by a sparkling wine and the second is the capacity of bubbles to endure in time (Buxaderas and López-Tamames, 2012). The permanence of the crown is conditioned therefore by the balance between the rate of these two processes: bubble formation and destruction (Zamora, 2003).

The responsible for the effervescence and foaming in sparkling wines is the CO_2 produced during the second fermentation. This gas remains dissolved in wine following Henry's Law achieving an over-saturated solution of CO_2. When a sparkling wine bottle is opened, the internal pressure suddenly decreases and the decompression produces the effervescence (Buxaderas and López-Tamames, 2012).

A bubble can be dissected in two main phases (Figure 6), the inner gas phase, full of CO2 and an aqueous liquid phase surrounding the gas phase. Also, between these two phases there is a thin liquid phase known as the lamellar phase (Blasco, Viñas and Villa, 2011). The gas phase is hydrophobic and the liquid phase hydrophilic (Moreno-Arribas *et al.*, 2000).

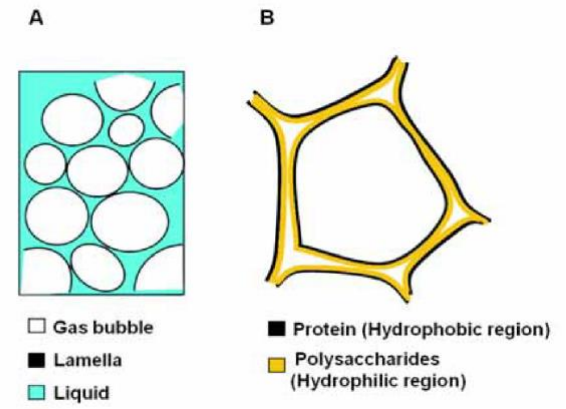

Figure 6: Bubble scheme. A: two phases bubble structure, B: Protein and polysaccharide bubble localization. extracted from (Blasco, Viñas and Villa, 2011)

Some authors have described that foaming formation, stability and duration are related with the chemical composition of sparkling wines (Brissonnet and Maujean, 1991; Pueyo, Martín-Alvarez and Polo, 1995; López-Barajas *et al.*, 1998). On the one hand, the film surrounding bubbles has tensioactive molecules which reduce the surface tension. On the other hand, other compounds enhance the film viscosity. The compounds reducing surface tension and increasing viscosity are very important for the formation and stability of bubbles (López-Barajas *et al.*, 1997; Moreno-Arribas *et al.*, 2000). The chemical composition of sparkling wines depends on several factors such as varietal origin, grape maturity, vintage and winemaking conditions (López-Barajas *et al.*, 1998; Vanrell *et al.*, 2007; Esteruelas *et al.*, 2015; Jégou *et al.*, 2017).

2.3.1. Impact of wine composition on foam quality

Proteins present in wines are some of the molecules that improve the foam quality (Moreno-Arribas and Polo, 2009; Kemp *et al.*, 2019). Proteins, in general, show good correlations between concentration and foam formation, but not all of them help foam stability (López-Barajas *et al.*, 1998; Senée *et al.*, 1998; Moreno-Arribas *et al.*, 2000; Blasco, Viñas and Villa, 2011; Esteruelas *et al.*, 2015; Martínez-Lapuente *et al.*, 2015;

Condé *et al.*, 2017). This behavior is due to the surfactant property of proteins that allows them to fit the hydrophobic domain achieving an interaction with the gas phase, and turning the hydrophilic domain interacting to liquid phase (Brissonnet and Maujean, 1991). Some studies report that high molecular weight protein and glycoproteins have the best correlation with foaming properties (Vincenzi, Crapisi and Curioni, 2014). It has also been reported that peptides released by lees during ageing could have an impact in foam quality (Moreno-Arribas, Pueyo and Polo, 1996; Nunez *et al.*, 2005). Besides, treatments with riddling agents as bentonite that absorb proteins, negatively affect the foam quality of sparkling wines (Senée *et al.*, 1998; Vanrell *et al.*, 2007; Ubeda *et al.*, 2021).

Wine polysaccharides seem to be more related to foam stability than the ability to foam (López-Barajas *et al.*, 1998; Moreno-Arribas *et al.*, 2000). PRAGs and MPs have hydrophobic and hydrophilic domains that could be absorbed at the lamellar phase of the bubble film (Kemp *et al.*, 2019). These polysaccharides increase the viscosity of the bubble's film and reduce the velocity of liquid drainage, enhancing the bubble stability. In addition, the protein fraction of these Polysaccharides can interact with other proteins present in the gas/liquid film generating a more viscoelastic film, which becomes more resistant to tension (Martínez-Lapuente *et al.*, 2015; Martínez-Lapuente, Ayestarán and Guadalupe, 2018). Polysaccharide composition and their molecular weight could also have a relation with foaming properties, being the high molecular weight fraction the one with higher correlation (Moreno-Arribas *et al.*, 2000; López-Barajas *et al.*, 2001; Coelho *et al.*, 2011). Given the positive effect of both macromolecules, proteins and polysaccharides, on foaming properties, wine industry is very concerned in how winemaking processes affect the concentration of these macromolecules and consequently the quality of the foam (Núñez *et al.*, 2006).

Contradictory data exists about the influence of phenolic compounds on the foaming properties. In that sense, it has been described that different forms of anthocyanins have ability to foam and to stabilize bubbles (Kemp *et al.*, 2019). This fact could be explained because proteins can interact with other molecules as anthocyanins generating new amphiphilic molecules enhancing the foamability (Sánchez and Patino, 2005; Martínez-Lapuente *et al.*, 2015). In contrast, total proanthocyanins affect negatively the foamability of wine. This could be due to their ability to precipitate with proteins (Martínez-Lapuente *et al.*, 2015).

High concentrations of ethanol also show a negative affect against foamability in sparkling wines. (Dussaud *et al.*, 1994; Girbau-Solà *et al.*, 2002; Medina-Trujillo *et al.*, 2017). Titratable acidity and viscosity also shows negative correlation against foamability and foam stability (Medina-Trujillo *et al.*, 2017; Guadalupe and Ayestarán, 2007). Sugar added with the expedition liquor seems to negatively affect to foamability and foam stability (Kemp *et al.*, 2017).

2.3.2. Mechanisms of bubbles genesis

Bubbles genesis in sparkling wine works as a constant cycle located in a nucleation site where bubbles are generated. It is known that for a spontaneous or homogeneous nucleation, a CO_2 supersaturating ratio is needed over 1000. In the case of sparkling wine, which CO_2 supersaturating is much lower, pre-existing gas cavities are required for nucleation (Wilt, 1986). The nucleation sites are irregular cavities, for example: glass small break, cellulose fibers or dust attached on the glass surface. These nucleation sites are full of gas being precursors for the bubble formation. Due to the difference of CO_2 concentration between liquid and the gas contained in the nucleation sites, the CO_2 molecules diffuse from liquid to the gas making that the bubble size grows. When the bubble reaches a certain size and Archimedean thrust exceeds surface tension, a bubble is detached and the cycle starts again (Jones *et al.*, 1998). This constant bubbling formation creates trains of bubbles from the bottom of the glass until the liquid surface (Figure 7).

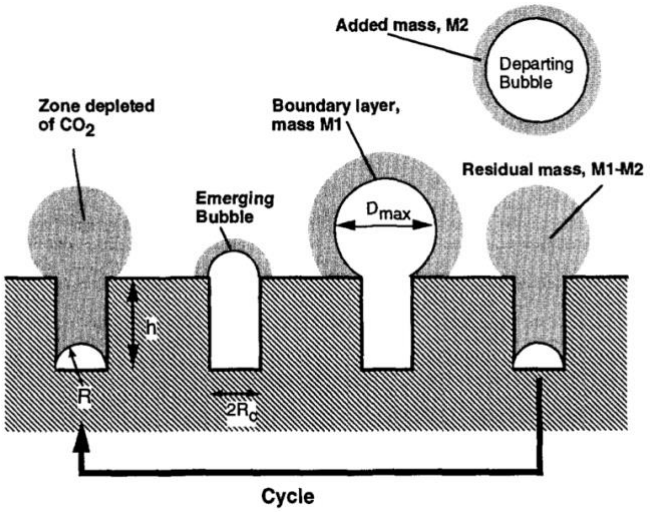

Figure 7: Representation of the cycle of bubble production: bubble nucleation, growth and Detachment (Jones *et al.*, 1998).

After each new cycle of bubble formation, just after bubble detachment, the liquid around the bubble falls into the nucleation site, being stopped by the gas remaining in the nucleation site. When the CO_2 pressure of the liquid declines until an equilibrium between liquid and gas, the gas is unable to emerge from the cavity, so the bubble formation is ended from this site (Jones *et al.*, 1998).

The frequency of the cycle (from 1 to 25 Hz) depends on the size of the cavity. Specifically, the bigger the cavity the lower the frequency of bubble formation, because the amount of CO_2 required for the bubble formation and detachment is higher (Lea & Piggott, 2003; Liger-Belair et al., 1999). After detachment, the bubble growth continues until the bubble reaches the superficial layer. This growth occurs because the gas continue to diffuse inside the bubble while traveling through the liquid. This growth rate is described as constant (Shafer and Zare, 1991).

Once the bubbles reach the surface of the glass, they move towards the glass edges due to the slight convex shape of the surface of the liquid. These rings of bubbles on the glass edge are called crown, being its permanence a main criterion for appreciation of the quality of the foam (Buxaderas and López-Tamames, 2012).

2.3.3. Mechanisms of bubbles destruction

When a bottle of sparkling wine is opened, the internal pressure drastically decreases and consequently the over saturated CO_2 dissolved in wine starts to be released. This gas liberation is done by diffusion from the surface to the air and by bubble formation and their subsequent destruction on the surface by explosion (Buxaderas and López-Tamames, 2012). The stability of the crown will depend on the balance between the bubble formation and destruction rates. The different mechanisms of bubble destruction are described below.

2.3.3.1. Ostwald ripening

The Ostwald ripening is based on the different size of bubbles found in foam. The smaller the bubble size the higher its internal pressure. This different internal pressure leads to

gas migration from the smaller to bigger neighbour bubbles following the pressure gradient. This way, the smaller bubbles disappear and the mean bubble size gradually increases. When the size of the bubbles increases, the thickness of the bubbles film is reduced which favors its subsequent explosion. The presence of surfactant molecules located in bubble film decreases its gas permeability, reducing the Ostwald ripening rate (Tcholakova *et al.*, 2011, 2017).

2.3.3.2. Coalescence

Bubble coalescence occurs when two or more bubbles collapse to form only one of higher size. The process of new bubble formation by coalescence follows the next steps: first, collision between bubbles. Then, a drainage of the liquid films and finally, film rupture causing the formation of a larger particle. These bigger bubbles formed are less stable and their duration is shorter (Chanson, 1996)

2.3.3.3. Drainage and explosion

When bubbles reach the surface, the film coating (lamellar layer) loses liquid, due to drainage. This drainage causes a progressive film wastage which increases its fragility and when the film is thin enough leads to the bubble explosion (Buxaderas and López-Tamames, 2012). When a bubble explodes, a whole in the surface of the liquid is formed and the surrounding liquid in a few milliseconds fills the vacuum generated by the bubble explosion causing several micro-droplets which are projected a few centimeters above the surface of the liquid (Figure 8). The number of projected droplets, their velocity and the height of the ejection depend on the size of the bubble. This projection of droplets is certainly a vector that enhances the aroma that favors its sensory perception (Liger-Belair, Seon and Antkowiak, 2012).

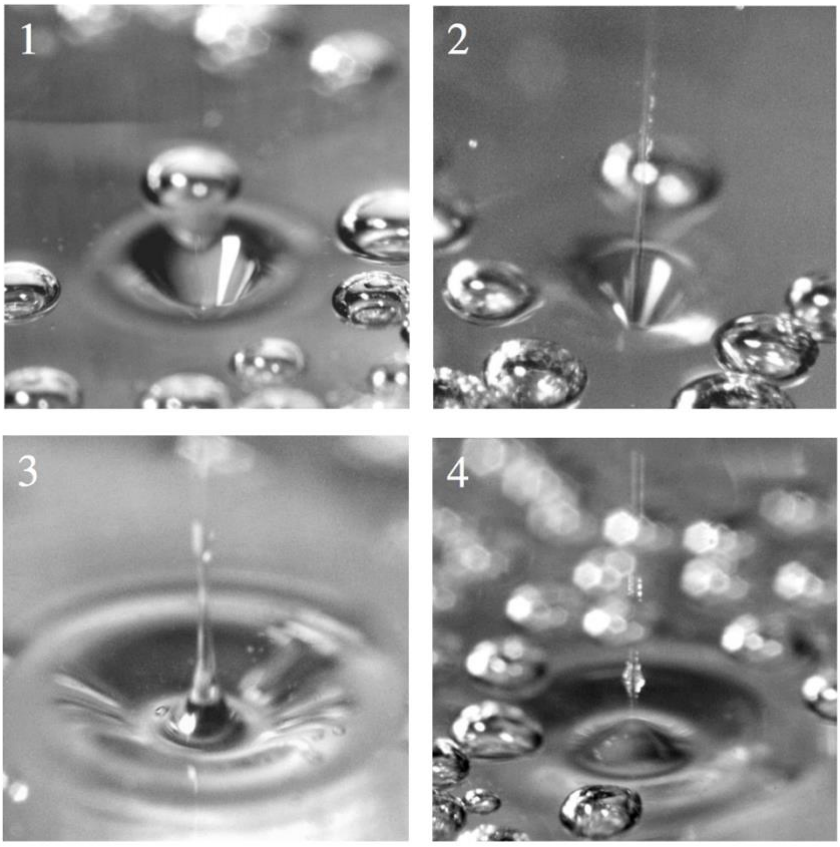

Figure 8: Droplets formation in the wine surface after bubble explosion. 1: Vacuum generated, 2, 3, 4: Dropplet formation and projection (Liger-Belair, Seon and Antkowiak, 2012).

There are two types of film drainage: gravitational and capillary. The foam stability is greatly affected by bubble drainage. It has been described that high viscosity and high surface elasticity diminish the effects of drainage and consequently lengthen the half-life of the bubbles. Viscosity and surface elasticity depends on the wine composition and are especially conditioned by the presence of surfactant molecules and protein interactions in the lamellar layer (Karakashev *et al.*, 2008; Sett, Sinha-Ray and Yarin, 2013).

2.4. Methods for foam characterization

Foam is considered a physical property of a liquid. However, the results of analyzing the foam parameters and characteristics by tasting are difficult to evaluate by a taster and sometimes the reproductively of the achieved results are poor. Since 1938, when Bikerman established the first method to measure the foam properties, several methods for foam characterization were launched to achieve more objective and reproducible analysis. These techniques allow quantifying and also comparing different sparkling

wines. The majority of these techniques are based on the measurement of height reached by the foam and its durability to fulfill the characterization of the ability of the wine to foam (foamability) and its stability (persistence of the foam) respectively.

2.4.1. Bikerman method

In 1938, Bikerman published the first method to measure foam properties of liquids. This method was based on a glass tube calibrated where a constant volume of air was introduced from the bottom of the tube throughout a porous glass membrane below the liquid tested. Consequently, a foam is generated from de liquid and travels up inside the calibrated tube, then bursting, and starting the cycle again. Noting periodic measures of the foam height the foam volume could be calculated. In this model, the foam height showed more erratic values but the foam volume showed a good correlation with the air volume injected, being this parameter a base for foam formation comparison between different liquids. Bikerman also studied the foam volume across time being correlated with the foam stability. This method settled down the basis of the foam quantification and also the apparatus system technology used in future methodologies (Bikerman, 1938).

2.4.1. Rudin method

The Rudin method was launched in 1957 to measure beer foam properties. This apparatus is based on the Bikerman method: a calibrated glass tube where a standardized volume of sample is poured on a porous plate. Then, a constant and uniform flow of CO_2 is injected causing foam formation by bubbling. Specifically, the Rubin method focuses on foam to liquid transformation. For that reason, this methodology studies the foaming collapsing and the consequent amount of liquid separation. So, when this foam reaches the requisite foam height, the CO_2 flow is decreased to avoid seeping through porous plate and foam starts collapsing. When the interface between liquid separated and foam reaches 7.5 cm the procedure is finished. The time needed to reach this 7.5 cm of liquid in the tube since the foam reaches the requisite amount of foam, is considered a good measure of foam stability (Rudin, 1957).

2.4.2. Mosalux method

The Mosalux methodology apparatus uses a Rudin tube and records the height of the foam using infrared light that is intercepted by the foam (Figure 9a). Nowadays, Mosalux is probably the most widely used method for foam characterization by wineries and wine laboratories. As in the Rudin method, CO_2 is injected from the base through a porous plate on which the sample is poured and avoids the sample from falling under the air entrance. Thanks to the constant and uniform gas flow, foam is formed and from the beginning of foaming until the end of bubble collapsing, parameters of foamability and persistence of the foam could be registered (Maujean *et al.*, 1990).

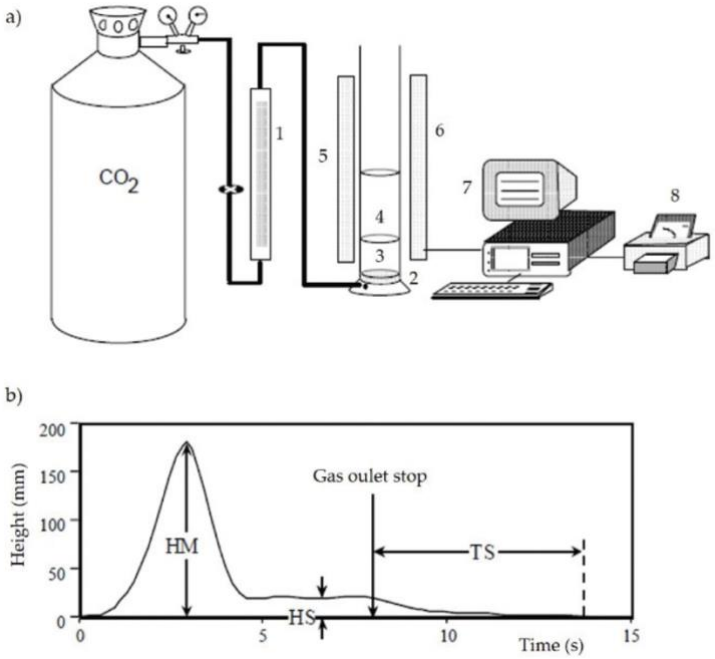

Figure 9: (a) Diagram of the Mosalux apparatus. 1-Flowmeter, 2-Test tube, 3-Sample, 4-Foam, 5-Infrared emitter, 6-Infrared receiver, 7-Personal computer, 8-Printer (Vanrell *et al.*, 2002). (b) example of a foam profile of a sparkling wine. (Martínez-Lapuente, Ayestarán and Guadalupe, 2018)

When a foam parametrization is done, a common profile is observed (Figure 9b). First, when gas flow is started, foam rises until its maximum height. Then, bubble collapsing makes foam decrease until a height whose value is stand until gas flow is interrupted. Finally, foam disappears. In order to explain this trend three parameters were measured:

- **HM or Foamability**, which refers to the maximum height reached by the foam during the CO_2 injection. Related with the ability of the wine to foam.
- **HS or Persistence of the foam**, which refers to the stable height during the analysis during the CO_2 injection. Related with the wine foam persistence.

- **TS or foam stability**, which refers to the time until all bubbles collapse when CO_2 injection is interrupted. Related with foam stability.

2.4.3. Methods based on image analysis

Methods based on image analysis are more focused on describing the effervescence and bubble nucleation and formation, growth rates and velocity of bubbles rising through the liquid until surface. The methodology is based on a photo camera focused on a flute glass where sparkling wine is poured. Behind the glass a graduated paper is used as scale for metric data. For time measures, photographs are taken with an established frequency, so the time between a bubble and the next one could be estimated. Thanks to this photography application, several effervescence characteristics can be established for sparkling wines. Frequency of bubble formation is related with the nucleation site. In addition, the growth rate, the radius of sparkling wine bubbles and the stokes velocity applied to bubbles rising can also be estimated (Liger-Belair et al., 1999).

2.5. Yeasts autolysis

2.5.1. *Saccharomyces cerevisiae* for second fermentation by traditional method

Saccharomyces Cerevisiae is a unicellular eukaryote organism widely used in different food processing sectors. For sparkling wine production some yeast strains are selected based on their characteristics and ability to ferment, grow and tolerate the sparkling wine stressful conditions as the low temperatures, high pressures, high ethanol concentration, low nutrient sources and the presence of accumulation of toxic fermentation sub-products from first fermentation (Alexandre, 2019).

There are other desirable characteristics for these sparkling wine yeast. For example, their ability to flocculate to facilitate the riddling process (Pozo-Bayón *et al.*, 2009). Another characteristic for yeast strain selection is its ability to influence foam quality (Alexandre & Guilloux-Benatier, 2006; A. Martínez-Rodriguez *et al.*, 2001b). Besides, the production of extracellular enzymes related with all the autolysis process such as

pectinases, amylases, lipases, proteases and glycosidases (Torresi, Frangipane and Anelli, 2011), and their autolytic capacity (A. Martínez-Rodriguez *et al.*, 2001a; Nunez *et al.*, 2005).

2.5.1.1. Cell wall Structure

The cell wall is an essential structure for holding the shape and integrity of the cell. Moreover, it plays an important role during all the cell cycle achieving around 10% to 25% of the total dry cell weight (Aguilar-Uscanga and François, 2003) and having a 70-100 nm of thickness (Cappellaro *et al.*, 1994). Three main functions are attributed to the cell wall: Osmotic shock and mechanical stress protection, bud formation for cell division and scaffold for cell-surface proteins that limit the permeability through the cell wall for macromolecules (Klis, Boorsma and De Groot, 2006; Levin, 2011).

The cell wall is composed of polysaccharides and proteins. The organization is based on an inner layer of β-1,3-glucan, β-1,6-glucan and chitin (β-1,4-*N*-acetylglucosamine polymers), and an outer layer containing mannoproteins (Cabib and Arroyo, 2013). These molecules can be covalently bound creating bigger structures (Figure 10). This cell wall composition changes over the time and is strongly dependent of the environmental condition (Klis *et al.*, 2002).

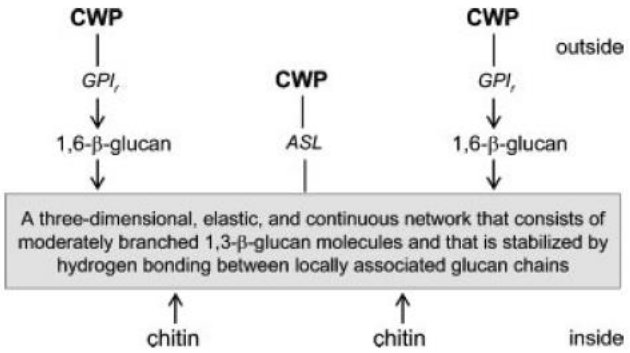

Figure 10: Representation of cell wall organization (Klis, Boorsma and De Groot, 2006).

The inner layer organization is based mainly on β-1,3-glucan chains. β-1,3-glucan, whose weight is 85% of the inner layer composition, which is a chain composed with D-glucose residues. These chains are branched through β-1,6 linkages (Manners, Masson and Patterson, 1973). This layer provides to the cell wall the mechanical strength and elasticity necessary due to the helical nature of β-1,3-glucan chains, and also attachment sites for

proteins that form the outer layer of the wall (Manners, Masson and Patterson, 1973; Klis *et al.*, 2002; Klis, Boorsma and De Groot, 2006; Levin, 2011). The non-reducing ends of the β-1,3-glucan molecules may stand covalent attachment to other polysaccharides such as chitin and β-1,6-glucan. β-1,6-glucan creates cross linking bounds generating a highly complex network (Kollár *et al.*, 1997) as well as their may play an important role attaching mannoproteins to the cell wall by GPI (glycosyl phosphatidylinositol) linkage (Kapteyn *et al.*, 1996). The smallest fraction are chitin (1% to 2% of the dry weight of wall cell) chains located in the base of the cell wall and related to the gemmation structures and stress response (Shaw *et al.*, 1991).

The external layer is mainly based on mannoproteins (Zlotnik *et al.*, 1984). These are around 90% (w/w) glycosylated with a carbohydrate fraction (Klis *et al.*, 2002). Moreover, the cell wall protein composition of yeast is highly depending on the developmental stage of the cell cycle (Caro *et al.*, 1998; Rodríguez-Peña *et al.*, 2000) and the environmental conditions such as temperature, pH and nutrient and oxygen availability (Abramova *et al.*, 2001; Kapteyn *et al.*, 2001; De Groot, Ram and Klis, 2005). GPI modified proteins are the predominant, they are normally covalently linked to the polysaccharide scaffold by their C-Terminus. This layer is less permeable to macromolecules than the polysaccharide internal layer (Zlotnik *et al.*, 1984) because of the presence of disulfide bridges that limits the porosity (De Nobel, Klis, Munnik, *et al.*, 1990) and highly branched carbohydrate side chains (De Nobel, Klis, Priem, *et al.*, 1990). Proteins located in the external layer play important functions such as: cell-cell interaction, adhesive functions, water retention, cell wall porosity, cell maintenance and cell protection against stress (De Groot, Ram and Klis, 2005).

2.5.2. Mechanism of yeast autolysis

Sparkling wine production by traditional method is mandatory done inside the bottle. This fact consequently generates an interaction between wine and yeast. This interaction increases wine complexity, being the ageing time an established parameter of quality for sparkling wines. When the fermentation ends, yeasts also called lees, start to release molecules to wine by two different processes: excretion (or passive exorption) and autolysis. Excretion takes place immediately after fermentation (between third and sixth

month) (Morfaux and Dupuy, 1966) and Autolysis starts later and is done over some years (Alexandre, 2019).

Autolysis is a eukaryote cell process of cytoplasm and organelles degradation (Cebollero, Rejas and González, 2008). Autolysis involves hydrolytic enzymes that leads to self-degradation of cell components, consequently releasing into wine, peptides, fatty acids, nucleotides and amino acids from cytoplasm and polysaccharides and mannoproteins from cell wall (Feuillat and Charpentier, 1982; Alexandre and Guilloux-Benatier, 2006; Buxaderas and López-Tamames, 2012). Autophagy, a degradation pathway activated by carbon or nitrogen starvation, is also related with sparkling wine yeast autolysis (Cebollero, Carrascosa and Gonzalez, 2005).

Yeast autolysis is caused by intracellular enzymes and is an irreversible process favored by adverse conditions (Cebollero, Rejas and González, 2008; Buxaderas and López-Tamames, 2012). Cell degradation starts at the end of the stationary growth phase of alcoholic fermentation and is induced by cell death. The intracellular enzymes start degrading cell endo-structures like vacuoles causing the release of proteases to cytoplasm. First, these proteases are inhibited, but once all the cytoplasmic inhibitors are degraded, some intracellular polymers are hydrolyzed and their byproducts are accumulated inside the cell wall. Finally, when the hydrolytic products are small enough, they are able to pass through the pores of the cell wall and be released to wine (Babayan *et al.*, 1981). This process could be induced by a physical, chemical or biological inductor and the perfect condition are at pH 5 and 45 ºC. Whereas, in wine matrix (pH 3-4) and in cellars whose temperature is relatively low (15 ºC), the slowness of the enzymatic reactions and the whole process is slowed down. In sparkling wines, yeast autolysis begins from 3 to 9 month after the completion of secondary fermentation depending on biological (yeast strain), chemical (wine composition) and physical (temperature) conditions of ageing (Feuillat and Charpentier, 1982; Charpentier and Feuillat, 1993; Todd, Fleet and Henschke, 2000; Alexandre and Guilloux-Benatier, 2006; Alexandre, 2019).

Protease A plays an important endopeptidase that has a relevant role in autolysis activity. It is in fact responsible for around 60% to 80% of total nitrogen released (Alexandre *et al.,* 2001; Lurton *et al.,* 1989). It was reported by Feuillat and Charpentier in 1982 that the proteolytic activity is low during the first 8 month, then at the 9th month this activity

increases gradually and could stand for several years (Moreno-Arribas, Pueyo and Polo, 1996). Cell wall, rich in mannoproteins embedded in a β-glucan layer (Cabib and Arroyo, 2013), is degraded by endo and exo β-glucanases which hydrolyze the β-*O*-glycoside link of β-glucan chains. This cell wall degradation causes the release of glucose, oligosaccharides and mannoproteins covalently bound to the glucan layer easing their proteolysis. Both enzyme families, proteases and glucanases, are active during autolysis (Klis *et al.*, 2002; Buxaderas and López-Tamames, 2012; Alexandre, 2019).

Morphologically, during the autolysis process, yeast experiment changes in their shape and fitness. Some studies have followed changes of yeast morphology by microscopy images. (Hernawan and Fleet, 1995). After alcoholic fermentation, yeast has an ovoid shape with a thick and smooth cell wall, cell organelles are structurally intact having the normal functionality as a living cell (Figure 11a). After 6 months of ageing, vacuole size has diminished and there are some little bodies distributed surrounding the vacuole, about the cell wall, some folds can be observed giving a rough aspect to the wall shape (Figure 11b and Figure 12a). After 12 months of ageing, the cell has collapsed, losing part of the cytoplasm. At that time, even if the cell wall has more folds, it remains unbroken. (Alexandre, 2019; Alexandre & Guilloux-Benatier, 2006; A. Martínez-Rodriguez et al., 2001c).

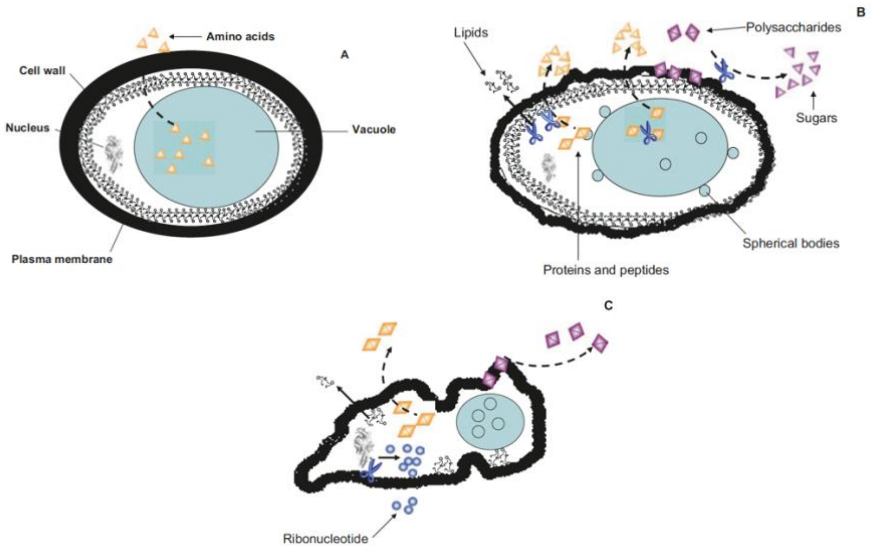

Figure 11: Schematic representation of morphological and biochemical changes in yeast during autolysis in sparkling wine. (A) After the second alcoholic fermentation, (B) After 6 months, (C) After 12 months. Figure extracted from (Alexandre and Guilloux-Benatier, 2006).

It has been reported that the glucan layer, responsible for wall rigidity, is not totally degraded by hydrolytic enzymes. Besides, it seems that mannoprotein depletion does not

affect cell wall integrity although it could increase cell wall porosity. Nevertheless, some intracellular structures are lost over the time generating macromolecules which could be released thorough new created porous (Figure 11c) (Babayan *et al.*, 1981; Charpentier and Feuillat, 1993; Hernawan and Fleet, 1995). Electron Microscopy observations (Figure 12b) showed images of empty cells who had lost most of their cytoplasmic content due to the autolysis process but preserving the cell wall structure (A. Martínez-Rodriguez et al., 2001c).

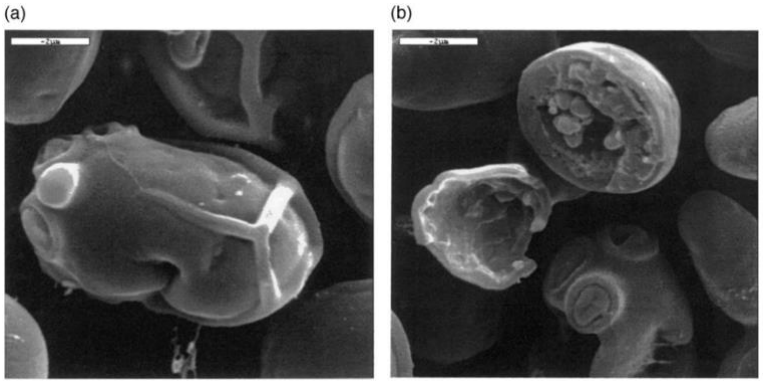

Figure 12: Low Temperature Scanning Electron Microscopy images of yeast cells after 24 h of induced autolysis in a model wine system. (a) Superficial ultrastructure of a yeast cell, (b) Image of fractured empty yeast cells. Figure adapted from (A. Martínez-Rodriguez et al., 2001c).

2.5.3. Compounds released by yeast autolysis and its sensory impact

During the ageing time, yeast autolysis influences and modifies sparkling wine molecules and macromolecules composition and concentration. These changes are led by yeast macromolecules degradation and their leaking to wine matrix, increasing the concentration of that and their relevance into the organoleptic characteristics of sparkling wine as foam properties and aromatic profile. Moreover, these released molecules can also interact generating new compounds influencing wine flavor. These changes and interactions caused by yeast autolysis, confer the wine its final complexity and their typical characteristics of aroma and flavor (Alexandre and Guilloux-Benatier, 2006; Alexandre, 2019).

2.5.3.1. Amino acids

The amino acids concentration in wine changes constantly since the beginning of the second fermentation. Amino acids concentration in base wines for traditional Cava

varieties is around 400-500 mg/L (V. Moreno-Arribas *et al.*, 1998). During second fermentation, amino acids decrease because they are assimilated and consumed by yeast. Once alcoholic fermentation is finished, but before yeast autolysis begins, amino acids are released by passive exorption (excretion) by yeasts increasing their concentration during the first six month (Morfaux and Dupuy, 1966; Feuillat and Charpentier, 1982; Dizy and Polo, 1996). Amino acid concentration also increases due to peptides liberation by yeast and their later hydrolysis (Moreno-Arribas, Pueyo and Polo, 1996; Nunez *et al.*, 2005; Alexandre, 2019). In the late ageing period, the amino acid concentration decreases because of ester formation, decarboxylation or deamination reactions. The changes on the wine amino acid composition during ageing depends on peptide released, being L-proline predominant from base wine to aged sparkling wines (V. Moreno-Arribas *et al.*, 1998).

Amino acids are aroma precursors, so the enrichment of these molecules during ageing enhances the aroma profile of sparkling wines (Alexandre, 2019). Higher alcohols and their related esters and volatile acids (Table 3) are considered the most relevant aroma compounds formed from amino acids, mainly valine, leucine, and isoleucine (Styger, Prior and Bauer, 2011; Lambrechts and Pretorius, 2019). These are formed after a transamination reaction and metabolized to keto acids that yeast convert to high alcohols by Ehrlich pathway (Hazelwood *et al.*, 2008). Besides, threonine deamination and their consequent reaction with acetaldehyde form sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) which increases during ageing on lees giving curry (or walnuts) flavor (Pham *et al.*, 1995; Alexandre, 2019). Vitispiranes, synthesized from methionine are detected in aged Sparkling wines giving eucalyptus odors (Francioli *et al.*, 2003). It has also been reported that amino acids and peptides can participate in the umami taste playing a role of flavor enhancers (Vilela, Inês and Cosme, 2017). Amino acids also show positive correlation with foamability properties (Moreno-Arribas *et al.*, 2000; Martínez-Lapuente *et al.*, 2015).

Compound	Amino acid	Concentration in wine (mg/l)	Odor
Isovaleraldehyde	Leucine	Traces	Fruity, nut-like
Isobutyraldehyde	Valine	Traces	Slightly apple-like
2-Methylbutyraldehyde	Isoleucine	NR	Green (herbaceous), malty
Isobutyric acid	Valine	Traces	Sweet, apple-like
Isovaleric acid	Leucine	<3	Rancid, cheese, rotten fruit
2-Methylbutanoic acid	Isoleucine	NR	Fruity, waxy, sweaty fatty acid
Isoamyl alcohol	Leucine	45–490	Alcohol
Isobutanol	Valine	40–140	Fruity, alcohol, solvent-like
Amyl alcohol (active)	Isoleucine	15–150	Marzipan (almond)
Isoamyl acetate	Leucine	0.03–8.1	Banana, pear
2-Phenyl acetate	Phenylalanine	0.01–4.5	Rose, honey, flowery
Ethyl isovalerate	Leucine	0–0.7	Apple, fruity
Isobutyl acetate	Valine	0.01–0.8	Banana
Ethyl 2-methylbutanoate	Isoleucine	0–0.9	Strawberry, pineapple

Table 3: Alcohols and their associated esters and volatile acids formed from amino acids (Styger, Prior and Bauer, 2011).

2.5.3.2. Peptides and proteins

Peptides concentration in base wines for Cava is around 33 mg/L being approximately 80% lower than in their corresponding musts (Moreno-Arribas, Pueyo and Polo, 1996). As ageing advances, peptides are released having a peak between 12 to 15 months. Later, there is a decrease attributed to peptide hydrolysis. Threonine and serine have a major presence in these peptides because they are involved in glycosidic linkages between mannans and proteins in cell walls (Klis *et al.*, 2002). Peptides structural characteristics vary through aging time, decreasing length and having more hydrophobic profile (Moreno-Arribas, Pueyo and Polo, 1996; Martínez-Rodríguez and Polo, 2000).

There are few works about protein concentration and their evolution. Some studies described an increase of protein concentration during fermentation (Todd, Fleet and Henschke, 2000; Nunez *et al.*, 2005; Rowe *et al.*, 2010). These proteins released by yeast have been described to have molecular weights from 24 to 137 kDa and an acidic Isoelectric point (pI). Some examples of yeast release protein are glycosidase, lysophospholipase or cell wall proteins (Cilindre *et al.*, 2014). After 9 months of ageing, the protein concentration follows a decreasing trend due to the proteolytic activity (Moreno-Arribas, Pueyo and Polo, 1996), that reaches the high level at around five years of ageing (Leroy *et al.*, 1990). Furthermore, it seems that the concentration of some proteins decreases whereas that of others, increase changing the protein composition on sparkling wines through ageing time (Pegg *et al.*, 2021).

Wine peptides have been related to bitter and sweet tastes (Marchal *et al.*, 2011; Alexandre, 2019). Consequently, some of these sensory attributes could be increased during ageing because of the peptide changes during this period of time. Wine peptides have shown contradictory results or even no correlation with foaming properties (Moreno-Arribas *et al.*, 2000). However, their further hydrolysis increases amino acid content, which shows a positive correlation with foamability of sparkling wines (Martínez-Lapuente *et al.*, 2015). The increasing hydrophobic profile of peptides could also modify surfactant properties and therefore enhance foam properties (Brissonnet and Maujean, 1993; M. V. Moreno-Arribas *et al.*, 1998; Martínez-Rodríguez and Polo, 2000; Martínez-Lapuente *et al.*, 2015). Proteins have great impact in foam properties because of their surface properties. In that sense, many authors have described a positive correlation between concentration and foam quality (Pueyo, Martín-Alvarez and Polo, 1995; Moreno-Arribas *et al.*, 2000; Vanrell *et al.*, 2007).

Botrytis cinerea, an important vineyard fungal pathogen, releases enzymes with protease activity to media leading protein degradation. This protein degradation can reduce the wine protein concentration approximately a 50% in two weeks, consequently foamability is also negatively affected showing a decrease around 60% (Marchal *et al.*, 2006).

2.5.3.3. Mannoproteins and polysaccharides

Yeast cell wall is rich in polysaccharides and mannoproteins being the principal responsible of the release of these macromolecules into the sparkling wines. Cell wall is mainly composed of a layer β-1,3-glucan and β-1,6-glucan. Proteases and β-glucanases are important enzymes which cause cell wall skeleton degradation, thus enhancing the liberation of polysaccharides and also proteins anchored to this β-glucan layer (Alexandre, 2019). Different trends were established for polysaccharide fractions behavior in sparkling wine during ageing, On one hand, Martínez-Lapuente et al. (2013) and (2018) showed that mannoproteins and polysaccharides were released to wine during the first six month of ageing. Then a decrease of these two fractions is observed until reaching concentrations lower than their corresponding base wines. This decrease is thought to be related with polysaccharide precipitation due to alcohol rising during second fermentation. Charpentier (2000) also observed a substantial increase of polysaccharides during the first 9 month of ageing (from 366 mg/L in Base wines to 603 mg/L in sparkling

wines). Other authors pointed to the beginning of polysaccharide release after 18 months of ageing (Moreno-Arribas *et al.*, 2000). On the other hand, Esteruelas et al. (2015) did not show any trend between base wine and sparkling wine of nine-month ageing. In addition, different yeast strains yield a no homogeneous polysaccharides evolution during ageing (Nunez *et al.*, 2005). Probably the erratic behavior on the polysaccharide fraction found when comparing different author references can be related to the fact that they used different analytical methods and with some methods that are used no more nowadays. Furthermore, most of the wine polysaccharides come from the grape juice and consequently great differences can be found depending on the grape variety, maturity level, vintage and winemaking process.

Several studies have found a correlation between polysaccharide concentration and foaming properties, especially, with foam stability (López-Barajas *et al.*, 1998). Coelho et al. (2011) observed that a high molecular weight fraction of Polysaccharides from sparkling wines, with an elevated concentration of mannose residues, had a significant positive effect with foaming properties. In contrast, Esteruelas *et al*., 2015 have reported a negative correlation between polysaccharides and foam properties. Authors associated this negative effect to the presence of β-glucans released by *Botrytis cinerea*.

Núñez *et al.*, 2006, have reported that yeast extracts seem to have a positive correlation with foaming properties. For that reason, it is thought that mannoproteins play an important role in the quality of sparkling wines foam. This is due to the ability of this macromolecule to be absorbed at the lamellar layer of bubbles thanks to their amphiphilic character. Consequently, mannoproteins enhances the viscoelastic film formation and increase the viscosity of liquid stabilizing bubbles over time (Blasco, Viñas and Villa, 2011; Martínez-Lapuente *et al.*, 2015).

Mannoproteins also contribute to the flocculation of yeast and improve the riddling process (Caridi, 2006). They also prevents potassium bitartrate precipitation (Moine-Ledoux and Dubourdieu, 2002; Caridi, 2006; Bowyer and Moine-Ledoux, 2007), protein haze (Caridi, 2006) and can interact with phenolic compounds stabilizing red wine color and reducing astringency of wine (Escot *et al.*, 2001). In addition, mannoproteins could be also related with aroma substances retention like terpenes, β-ionone, ethyl hexanoate

and octanal enhancing wine aroma intensity and persistence (Lubbers *et al.*, 1994; Juega *et al.*, 2012; Alexandre, 2019).

2.5.3.4. Lipids

Lipids are compounds related with foam stability and it has been reported that they can act as flavor precursors (Fross, 1969). Their concentration in sparkling wines depends on the lipid type. Thus, free fatty acids are present at concentrations of around 270 µg/L and total fatty acids at around 1500 µg/L whereas sterol esters and triacylglycerols are present in higher concentrations (Pueyo, Martín-Alvarez and Polo, 1995). Some lipids such as 1,3-diacylglycerols, 2-monoacylglycerols, free fatty acids and sterols have shown good correlation with autolysis process (Pueyo *et al.*, 2000). Lipid concentration rises while fermentation occurs, being triacylglycerols the once with more impact. The free fatty acids released in wine are rapidly degraded (Troton *et al.*, 1989). The contact of the wine with lees implies the release of some lipids which increase their concentration throughout ageing (Piton, Charpentier and Troton, 1988).

Nowadays the implication of lipids in foam is not entirely clear. Some studies have not shown any relation between lipids and foam quality (Dussaud *et al.*, 1994). On the one hand, palmitic acid and linoleic acid were positively correlated with foamability and foam stability (Pueyo, Martín-Alvarez and Polo, 1995) and esters of hexanoic, octanoic and decanoic acids only with the ability to foam (Gallart *et al.*, 2002). However, on the other hand, octanoic, decanoic and dodecanoic fatty acids showed negative correlations with foam stability (Maujean *et al.*, 1990; Gallart *et al.*, 2002; Vanrell *et al.*, 2002). Lipids are also related with some aromatic esters that are synthetized from products of lipids metabolism (Styger, Prior and Bauer, 2011).

2.5.3.5. Nucleic acids

There are few studies about nucleic acids evolution and composition in sparkling wine ageing. It is known that RNA is around 5 to 15% and DNA 0.1 to 1.5% of the yeast dry weight (Nagodawithana, 1992; Alexandre, 2019). Some studies indicate that nucleic acid concentration increases over time in wines aged in contact with lees and also that RNAse activity lasts for all the autolysis process (Leroy *et al.*, 1990; Zhao and Fleet, 2005). Some studies carried out in synthetic media reported that DNA could be degraded up to 55%

and afterwards be leaked into the extracellular environment. In that sense, it has been shown that DNAse enzymes are active during the first steps of autolysis which would generate the appearance of nucleotides and nucleosides in the media (Zhao and Fleet, 2003). Nucleotides and nucleosides are flavor enhancers widely used in food industry (Abbas, 2006; Pozo-Bayón *et al.*, 2009) and could therefore work as a flavor enhancer in sparkling wine (Charpentier *et al.*, 2005).

2.5.3.6. Volatile substances

The origin of sparkling wine volatile compounds is diverse. They could be originated in grapes, produced during first and/or second fermentation by yeast activity as well as during aging in contact with lees (Styger, Prior and Bauer, 2011). Lactic acid bacteria could also contribute to the synthesis of some volatile compounds, in the case that base wines develop malolactic fermentation.

As in the case of nucleic acids, there is no clear trend about their evolution during ageing in sparkling wines. It is thought that the degradation, cell wall and bentonite absorption and synthesis of these compounds occurs simultaneously during ageing, so depending on the predominant process their presence can increase or decrease and consequently generate different volatile profiles. In fact, there are some studies describing trends and changes of esters, acetates or higher alcohols throughout ageing (Pozo-Bayón *et al.*, 2009; Alexandre, 2019). Grape variety also plays an important role in the volatile profile of sparkling wines (de Souza Nascimento *et al.*, 2018).

Pozo-Bayón *et al.,* (2003) analyzed several volatile compounds during one year and reported different trends in every group of volatile compounds reaching the conclusion that five volatile substances (hexyl and isopentyl acetate, ethyl butyrate and octanoate, and diethyl succinate) were useful to distinguish different aged Cavas. Moreover, other studies showed some representative volatile compounds for young Cavas, such as acetate esters and ethyl esters are present at high concentration in the first stages of ageing and subsequently decrease until being practically no detectable. These changes cause the loss of freshness and fruitiness descriptors in long aged sparkling wines. In contrast, the more distinctive volatile compounds in long aged Cavas are mainly vitispiranes and TDN (1,1,6-trimethyl-1,2-dihydronapthalene). In fact, these compounds together with diethyl

succinate are considered as good markers of the aging time, whereas ethyl lactate is only present when malolactic fermentation occurs (Francioli *et al.*, 2003; Riu-Aumatell *et al.*, 2006; Bosch-Fusté *et al.*, 2007; Torrens *et al.*, 2010).

It has also been described that ageing on lees has an impact on total esters. Specifically, it has been observed a decrease of their concentration during the first three months and a stabilization until nine months of ageing. However, this trend cannot be generally applied to all esters since for example: ethyl esters of fatty acids, cinnamate esters, isoamyl esters of fatty acids and ethyl esters of odd carbon number fatty acids did not follow this general behavior, showing higher correlation with the concentration of their respective acids and alcohols present in wine (Ruiz-Moreno *et al.*, 2017).

Furans, associated with caramel, toasty, or dried fruits flavors, are more present in sparkling wine than base wine (Torrens *et al.*, 2010).

In a wine model system, it has been reported a rapid formation during autolysis of some higher alcohols such as phenyl ethyl alcohol (rose odor) and isoamyl alcohol (Chung, 1986). Moreover, some terpenes such as geraniol, α-terpineol, citronellol and farnesol are also released during autolysis which could impact the sparkling wine aroma if their concentration overcomes its detection threshold (Alexandre, 2019). Furthermore, branched ethyl esters and γ-butyrolactone concentrations tend to increase over time (Pérez-Magariño *et al.*, 2013). Isoamyl alcohol oxidation can also lead to the formation of methyl 3-butanal, reported as the aldehyde with major presence (40%) in long aged sparkling wines. This aldehyde contributes to grassy odor, which is classified as a negative quality (Chung, 1986).

2.6. Evolution of foaming properties throughout the aging process

The evolution of the properties of Sparkling wines' foaming through ageing are important because of their relevance in sparkling wine quality. The transformation of base wine to sparkling wine implies a significant decrease in the foamability and foam stability. This

change is due to the addition of bentonite as a riddling agent that eliminates an important proportion of the wine proteins (Vanrell *et al.*, 2007) and also to higher ethanol concentration of the sparkling wine (Esteruelas *et al.*, 2015). However, autolysis that occurs during ageing seems to exert positive effects for the foam characteristics (Nunez *et al.*, 2005; Cilindre *et al.*, 2010). It has been reported that the autolysis enhances more foam stability than foamability (Pueyo, Martín-Alvarez and Polo, 1995). This fact has been related with the increase of polysaccharide concentration during ageing (Charpentier *et al.*, 2000; Martínez-Lapuente *et al.*, 2013; Martínez-Lapuente *et al.*, 2018) and their role as foam stabilizers, due to their ability of increasing the liquid viscosity and also the viscoelastic film properties (López-Barajas *et al.*, 1998; Blasco, Viñas and Villa, 2011; Martínez-Lapuente *et al.*, 2015).

As it was commented previously, the evolution of proteins doesn't have a clear trend since a complex balance between their release from lees and their proteolysis (Leroy *et al.*, 1990; Moreno-Arribas, Pueyo and Polo, 1996). However, some authors have described a positive correlation between these nitrogenous compounds and foaming properties (Pueyo, Martín-Alvarez and Polo, 1995; Moreno-Arribas *et al.*, 2000; Vanrell *et al.*, 2007). Proteins shows better correlations with foamability than with foam stability (López-Barajas *et al.*, 1998; Senée *et al.*, 1998; Moreno-Arribas *et al.*, 2000; Blasco, Viñas and Villa, 2011; Esteruelas *et al.*, 2015; Martínez-Lapuente *et al.*, 2015; Condé *et al.*, 2017). It could therefore be thought that proteins released during ageing have a few impact with foam stability of sparkling wines, but the subsequent proteolysis to peptides and amino acids could be positive (Martínez-Lapuente *et al.*, 2015).

2.7. Influence of oxygen and seal permeability

Sparkling wine bottles have a high internal carbon dioxide pressure. This characteristic makes sparkling wines better protected than still wines against oxidation because of the high carbon dioxide pressure on the head space and also by the oversaturation of carbon dioxide dissolved in wine. Nevertheless, some carbon dioxide can be leaked (Liger-Belair and Villaume, 2011) and therefore some oxygen can enter inside the bottle (Valade *et al.*, 2011) although the closures used (mainly crown cup and crock) are designed to avoid gas exchange. It has been reported that oxygen permeability ranges from 0.35 to 2.5 mg of

oxygen/L/year depending on the used closure (Valade *et al.*, 2007; Kemp *et al.*, 2015) (Godden *et al.*, 2001; Mas Barón *et al.*, 2001). The progressive oxygen intake can cause some drawbacks among which the appearance of oxidized characters that would denote a quick evolution should be highlighted (Riu-Aumatell *et al.*, 2006; Pons *et al.*, 2015).

Although carbon dioxide oversaturation plays an important role in wine preventing oxygen spoilage, the yeast lees kept inside the bottle also seems to play a relevant role protecting wine against oxidation. Some studies have reported antioxidant activity of yeast lees (Gallardo-Chacón *et al.*, 2010) and oxygen consumption (Salmon *et al.*, 2000; Fornairon-Bonnefond *et al.*, 2002). Besides, some studies have reported that inactivated dry yeast can also consume oxygen (Sieczkowski *et al.*, 2016a; Sieczkowski *et al.*, 2016b). Even though consumption activity by yeast lees is proved, nowadays the mechanism by which the oxygen is consumed is still not clear. Some authors pointed out that the amount of glutathione released by yeast during ageing could be key (Kritzinger, Bauer and Du Toit, 2013). However, other authors asserted that sterol fractions from yeast membranes could be related to oxygen consumption by lees (Fornairon-Bonnefond *et al.*, 2002; Fornairon-Bonnefond and Salmon, 2003).

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3. OBJECTIVES

The aim of this thesis was to fulfill these following objectives:

Objective 1. Characterization of yeast autolysis in sparkling wine produced by traditional method (Cava) over long aging time inside the bottle.

Objective 1.A. Study of the evolution of the sensory characteristics, color and foaming properties over long aging time.

Objective 1.B. Monitoring yeast autolysis over long aging time using Scanning Electron Microscopy (SEM).

Objective 1.C. Study of the evolution of the main macromolecules, polysaccharides and proteins of sparkling wines over long aging time.

Objective 1.D. Quantification of the main macromolecules (polysaccharides and proteins) released by the isolated lees from sparkling wines of different aging time in a wine model solution in order to establish their relative impact on the total sparkling wine composition.

Objective 2. Evaluation of the oxygen consumption capacity of lees of sparkling wine elaborated by traditional method and its variation over long aging time.

Objective 3. Quantification of the oxygen consumption capacity of inactivated dry yeasts in comparison with the main used wine antioxidants: sulphur dioxide, ascorbic acid and glutathione.

4. RESULTS

4.1. Chapter I: Monitoring yeast autolysis in sparkling wines produced by the traditional method over nine years of ageing

The aim of this chapter is to study what occurs inside a bottle of sparkling wine elaborated by the traditional method during nine years old. A new approach reproducing the autolytic process was performed by recovering the lees and maintaining them in a model wine solution for one year in order to be able to quantify and characterize the releasing macromolecules tendency during ageing on lees. Furthermore, a sensory analysis performed by a trained panel were performed to determine the time when wines began to be affected by excess ageing. These results have been sent to the *Australian Journal of Grape and Wine Research* for its consideration and eventual publication.

4.1.1. Introduction

Cava is a sparkling wine produced by the traditional method, which involves two fermentations (Maujean 1989). In the first fermentation the grape juice is transformed into a base wine according to a standard winemaking process. This base wine then undergoes a second fermentation, also called the *prise de mousse*, which takes place inside the bottle. For this second fermentation, the base wines are enriched with a sugar solution (roughly 22 g of sucrose/L) called the *liqueur de tirage* (Kemp et al. 2015), which provides the necessary sugar for the second fermentation. *Liqueur de tirage* also includes certain processing aids, such as bentonite and/or alginates, to boost the riddling process (Vanrell et al. 2007). Finally, a starter culture of previously adapted yeast is added to ensure that the second fermentation reaches a successful end. When the second fermentation is complete, the sparkling wines are aged in the bottle in contact with yeast (also called lees) for a certain length of time, during which the process of autolysis takes place (Alexandre and Guilloux 2006). It is also reported that the lees enable longer ageing times for sparkling wines because they protect the wine against oxidation by consuming small amounts of oxygen that can permeate the crown cap (Pons-Mercadé et al. 2021). This long contact with the lees is said to be the main difference between sparkling wines produced by the traditional method (or *méthode champenoise*) and those produced by other procedures (Ribéreau-Gayon et al. 2006).

Autolysis is the degradation process of the eukaryote cell structures (Cebollero et al. 2008). Yeast autolysis involves hydrolytic enzymes that degrade cell components, causing the release of substances such as amino acids (Martinez-Rodriguez et al. 2002), peptides (Moreno-Arribas et al. 1996), lipids (Pueyo et al. 2000), nucleotides (Charpentier et al. 2005), polysaccharides (Martinez-Lapuente et al. 2013) and mannoproteins into the wine (Martinez-Lapuente et al. 2015). This process completely transforms the composition, and therefore the sensory attributes, of the sparkling wine (Kemp et al. 2015).

The release of peptides, proteins, polysaccharides and mannoproteins favors the integration of carbon dioxide, which enhances both effervescence quality and foam stability (Martinez-Rodriguez et al. 2001, Kemp et al. 2019). Polysaccharides and

mannoproteins also play a positive sensory role by improving mouthfeel (Gawel et al. 2018), while some peptides and proteins can contribute to wine sweetness (Marchal et al. 2011). Certain nucleotides, amino acids and peptides are also reported to participate in the umami taste (Vilela et al. 2016) and to be flavor enhancers. Finally, amino acids and lipids have been described as aroma precursors (Styger et al. 2011) that contribute to the aromatic complexity of sparkling wines.

However, natural autolysis is a slow process that requires several months to achieve a real organoleptic effect. Yeast autolysis begins with a passive excretion of amino acids and other small molecules. This process, called exorption, takes one to two months in still wines and three to six months in sparkling wines (Morfaux and Dupuy 1966). Later, true autolysis begins with the degradation of cell and vacuolar membranes, which causes the release of proteases and other hydrolytic enzymes (Babayan et al. 1981, Lurton et al. 1989). Once these enzymes are free in the cytoplasm, they begin to hydrolyze the intracellular macromolecules (proteins and polysaccharides), which leads to the accumulation of their hydrolyzed products in the periplasmic space. When these hydrolyzed compounds become small enough, they can diffuse through the cell wall to enrich the extracellular environment, the wine (Fornairon-Bonnefond et al. 2002).

Protease A is said to play an important role in autolysis activity, where it is responsible for 60%-80% of the total nitrogen released (Lurton et al. 1989). Proteolytic activity appears to be low in the first eight months before gradually increasing after the ninth month and remaining for several years (Cabib and Arroyo 2013). Yeast autolysis progresses with the degradation of the cell wall structures, a process enabled by the action of endo- and exo-β-glucanases. These enzymes hydrolyze the β-glucan chains that link the mannoproteins (Moreno-Arribas et al. 1996), thus favoring the subsequent release of glucose, oligosaccharides, polysaccharides and mannoproteins from the cell wall.

In synthesis, yeast autolysis completely modifies the composition of the sparkling wine and therefore also its sensory quality (Kemp et al. 2015). For all these reasons, the most important AOC (*Appellation d'Origine Contrôlée*) for sparkling wines have established minimum ageing times to endure that autolysis exerts an effect on their composition and quality. For the AOC Cava, the minimum ageing time is 9 months, though its premium sparkling wines are usually aged for longer. In fact, the AOC Cava contains two other

categories of sparkling wines with extended ageing times. These are the *Reserva* and *Gran Reserva*, whose minimum ageing times are 15 and 30 months, respectively. Certain prestigious wineries produce Cavas with an even longer ageing time.

It appears, therefore, that autolysis favors the quality of sparkling wines, at least during the first few years. However, other phenomena take place in parallel – such as aromatic and color oxidation or an excessive lees flavor – which can damage the sensory qualities of these wines. We may therefore wonder until what ageing time truly favors the quality of the product. Most studies of sparkling wine autolysis have focused on relatively short ageing times (Vanrell et al. 2007, Moreno-Arribas et al. 1996, Martínez-Lapuente et al. 2013, Martínez-Lapuente et al. 2015, Riu-Aumatell et al. 2006, Moreno-Arribas et al. 1998, Vichi et al. 2010, Cilindre et al. 2010, Martínez-Lapuente et al. 2017, Esteruelas et al. 2015) and, to our knowledge, few of them have focused on longer periods (Pons-Mercadé et al. 2021, Charpentier et al. 2005, Loyaux et al. 1981, Leroy et al. 1990). In this paper we analyze changes in the color, polysaccharides, proteins, foaming properties and sensory quality of AOC Cava sparkling wines with ageing times ranging from one to nine years. To our knowledge, this is the longest time ever studied in relation to sparkling wines from the AOC Cava.

4.1.2. Materials and methods

4.1.2.1. Chemicals and Equipment.

Absolute ethanol (96% vol.), L-(+)-tartaric acid (≥99.5%), sodium hydroxide (≥98%), ammonium formate (≥99.0%), ammonium acetate (≥98.0%) and sulfuric acid (≥96.0%) were purchased from Panreac (Barcelona, Spain). Mannose (≥99.0%) was purchased from Sigma-Aldrich (Madrid, Spain). Water was ultrapure Milli-Q quality (Millipore, Bedford, MA, USA). All spectrophotometric measures were analyzed with a Helios Alpha UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Waltman, MA, USA). HPLC analyses were performed using an Agilent 1200 series liquid chromatograph equipped with a G1362A refractive index detector (RID), a G1315D diode array detector (DAD), a G1311A quaternary pump, a G1316A column oven, and a G1329A autosampler (Agilent Technologies, Santa Clara, CA, USA). Ethanol was measured using Bacchus 3

MultiSpec equipment (TDI, Gavà, Barcelona, Spain). Vacuum concentrations were carried out with Vacuum evaporator equipment (Univap 148 100ECH; Progen Scientific, London, UK) and lyophilizations were conducted using a Telstar lyophilizer (Telstar LyoQuest HT40, Barcelona, Spain). SEM study was performed using a Hitachi S4800 microscope. Finally, centrifugations were performed using a Biofuge Primo centrifuge (Heraeus, Hanau, Germany).

4.1.2.2. Sparkling wine production.

The experiments were conducted using sparkling wines from nine consecutive vintages (2008-2016) from the Juve & Camps winery (AOC Cava, Sant Sadurní d'Anoia, Barcelona, Spain). The base wines were produced in the most similar way possible, though some differences between each vintage were inevitable. Monovarietal base wines in all the vintages were produced with three autochthonous cultivars: Xarel·lo (VIVC Prime name: Xarello; VIVC Variety number: 13270), Macabeo (VIVC Prime name: Viura; VIVC Variety number: 13127), and Parellada (VIVC Prime name: Parellada; Variety number VIVC: 8938). The Xarel·lo and Macabeo grapes were from the Juvé & Camps vineyards in Sant Sadurní d'Anoia (Barcelona, Spain; 41°26'47.42" N and 1°49'0.63" E), which is located 165 meters above sea level, while the Parellada grapes were from the Juvé & Camps vineyards in Mediona (Barcelona, Spain; 41°29'48.6" N and 1°39'56.9"E), which is located 506 meters above sea level.

For each vintage, the grapes were harvested at the suitable maturity level for obtaining base wines for sparkling wine production. More specifically, the total soluble solid content (°Brix) ranged from 17.3 to 19.5 for Xarel·lo, from 16.2 to 18.3 for Macabeo, and from 15.2 to 16.5 for Parellada. Titratable acidity (g of tartaric acid/L) ranged from 5.5 to 8.0 for Xarel·lo, from 4.6 to 7.2 for Macabeo, and from 5.1 to 7.8 for Parellada. The grapes were immediately pressed in a pneumatic press to obtain a yield of 0.6 L/kg of grape juice. The grape juices were immediately sulfited with 100 mg/L of $K_2S_2O_5$, and pectinolytic enzymes (20 mg/L) were added to facilitate settling. After 24 h, clean grape juices were racked into the fermentation stainless steel tanks and immediately inoculated with 200 mg/L of selected yeasts (Saccharomyces cerevisiae; - IOC 18-2007; Institut Œnologique de Champagne, Epernay, France). When necessary, titratable acidity was corrected with tartaric acid up to 6.0 g of tartaric acid/L. All alcoholic fermentations were

performed at 16-18ºC. Once alcoholic fermentation was finished, base wines were racked and kept at 18-20ºC until spontaneous malolactic fermentation was completed. Base wines were then racked again, sulfited (40 mg/L of $K_2S_2O_5$) and cold stabilized.

Once the monovarietal base wines were considered stable, they were used to obtain a classic blend of AOC Cava in all vintages. Each vintage, a blend of 50% Xarel·lo, 30% Macabeo and 20% Parellada, was used to produce sparkling wines (Cava) using the traditional method and standard bottles (750 mL). Briefly, all the base wines were supplemented with 22 g/L of sucrose, 40 mg/L of a mixture of bentonite and alginates as the riddling agent (Adjuvant MO; Station Œnotechnique du Champagne, Epernay, France), and 2×10^6 cells/mL of a pre-adapted yeast culture (Saccharomyces cerevisiae - IOC 18-2007; Institut Œnologique de Champagne, Epernay, France). The wines were then bottled, crown corked and stocked at 12-15ºC until disgorgement. This procedure was employed for the nine consecutive vintages in order to reproduce as far as possible the same conditions of production for all the vintages studied.

4.1.2.3. Sparkling wine disgorging and lees recovery.

Three months after the *tirage* of the youngest sparkling wine (vintage 2016), when the second fermentation had completely finished, three bottles were placed in a *pupitre* and the *remuage* process was performed manually. Once all the lees sediment was in the neck of the bottle, disgorging was performed without freezing to facilitate the recuperation of the lees. The sparkling wines from the other vintages were also disgorged in triplicate three months after completing one to eight years of ageing, respectively. We therefore worked with lees from the first to the ninth years of ageing.

The lees sediment was recovered in a plastic bucket and immediately transferred to a centrifuge tube. The tubes were then centrifuged for 5 minutes at 10,000 rpm. The pellet was then washed with 10 mL of saline solution (NaCl 0.9% v/v) and centrifuged again. This operation was repeated twice.

The bottles of sparkling wines were immediately corked without adding any expedition liqueur and stocked at room temperature until the moment of analysis. All samples were

centrifuged (8,500 rpm; 15 minutes) to eliminate carbon dioxide and any small amounts of remaining lees before analysis.

4.1.2.4. Monitoring of the autolysis process.

The recovered lees were placed in clear glass 66 mL flasks with a model wine solution made up of ethanol (12% v/v) and tartaric acid (4 g/L) adjusted to pH = 3.5 with sodium hydroxide. The lees were therefore present in a proportion 11.36 times higher than in the original bottles. This higher proportion of lees was selected to enhance the concentration of polysaccharides and proteins and thus favor their analytical determination. We worked with this model wine to determine the real amount of macromolecules released during the autolysis process. All analytical results were corrected to refer to the original volume (750 mL) while taking into account the higher proportion of lees. The flasks were kept at 20 ± 2ºC for exactly one year, after which the samples were centrifuged (8,500 rpm; 15 minutes) and analyzed.

4.1.2.5. Ultrastructural observation of yeasts using scanning electron microscopy.

The yeasts of the starter culture used for the second fermentation of the last vintage (2016) as well as the lees from the sparkling wines from the first, third, fifth, seventh and ninth vintage were recovered and washed in a similar way as described above. After centrifugation the yeast were resuspended in 0.1 M phosphate buffer (pH 7.2), centrifuged again and fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 °C with gentle shaking. After three washes in 0.1 M phosphate buffer (pH 7.2), 10 min each, cells where dehydrated in increasing ethanol percentages (30, 50, 70, 90 and 100 %), 15 min each. After mounting in filters and supports, critical point was performed before SEM study in a Hitachi S4800 microscope.

4.1.2.6. Standard wine analysis.

Titratable acidity (TA) was measured by acid-base titration using 0.1N NaOH and bromothymol blue as indicator. The pH values were determined by a pH-meter Basic-20

(CRISON, Barcelona, Spain). The CIELab coordinates were determined following the study by Ayala (Ayala et al. 1997) and using a Helios Alpha UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Waltman, MA, USA). The data were processed using MSCV® software. Ethanol contents (% v/v) were measured by FTIR (Kupina and Shrikhande 2003) using Bacchus 3 MultiSpec equipment (TDI, Gavà, Barcelona, Spain).

4.1.2.7. Measurement of the foaming properties.

The sparkling wine samples were tempered at 18°C for 24 h before analysis. The foam properties were measured using the Mosalux method (Maujean et al. 1990). A glass cylinder placed on a glass frit was filled with 100 mL of the sample. CO2 was then injected into the glass cylinder through the glass frit, with a constant gas flow of 115 mL/min under a constant pressure of 1 bar. A Mosalux device (Station Oenotechnique de Champagne) was used to measure HM, the maximum height of the foam after CO_2 injection through the glass frit, and HS, the stable height during CO_2 injection. HM represents foamability (the wine's ability to foam), while HS represents foam stability (the persistence of the foam collar, or the wine's ability to produce a stable foam). HM and HS are expressed in millimeters. All measures were determined in triplicate.

4.1.2.8. Polysaccharide extraction and determination by HRSEC-RID.

The samples were processed using the methodology described by Ayestarán (Ayestarán et al. 2004). Briefly, 10 mL of sample in triplicate were concentrated to a final volume of 2 mL using a vacuum evaporator (Univap 148 100ECH; Progen Scientific, London, UK). Total soluble polysaccharides were precipitated by adding 10 mL of cold acidified ethanol (hydrochloric acid 0.3 M in absolute ethanol) and kept for 24 h at 4°C. The samples were then centrifuged (10,000 × g for 15 min) and the supernatants discarded. Finally, the precipitates were dissolved in 1 mL of ultra-pure water, frozen to −20°C, and freeze-dried using a lyophilizer (Telstar LyoQuest HT40, Barcelona, Spain). The soluble fractions were analyzed by high-resolution size-exclusion chromatography (HRSEC) to determine the molecular distribution and to quantify the polysaccharides obtained from the samples (Canals et al. 1998). The lyophilized samples were resuspended in 1 mL of 50 mM ammonium formate and filtered through 0.22 μm acetate cellulose filters (Merck

Millipore, Darmstadt, Germany). Then 100 µL were injected into the chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies Inc., Santa Clara, USA) with a refractive index detector. Separation was carried out at 20°C using two Shodex gel permeation HPLC columns (OHpak SB-186 803 HQ and SB-804 HQ, 300 mm × 8 mm I.D.; Showa Denko, Japan). The mobile phase consisted of an aqueous solution of 50 mM ammonium formate applied with a constant flow of 0.6 mL/min for 60 min, and the cell RID temperature was 35°C. The molecular weight distribution of the wine fractions was followed by calibration with a Shodex P-82 pullulan calibration kit (P-5, MW = 5.9 kDa; P-10, MW = 11.8 kDa; P-20, MW = 22.8 kDa; P-50, MW = 47.5 kDa; P-100, MW = 112 kDa; P-200, MW = 212 kDa; P-400, MW = 404 kDa; and P-800, MW = 788 kDa) purchased from Waters (Barcelona, Spain) and four dextrans (BioChemika; 12, 25, 50 and 80 kDa) purchased from Fluka (St. Louis, MO, USA). The polysaccharides were quantified according to the peak area for each fraction using the external standard method with pectin and dextran commercial standards (Sigma-Aldrich, Saint Louis, MO, USA) ranging from 0 to 2 g/L (r2 > 0.99).

4.1.2.9. Preparation of samples for protein analysis.

Fifteen mL of each sample were concentrated in triplicate following two-step dialysis in tubes with a molecular weight cut-off of 3.5 kDa (Membrane Filtration Products Inc., San Antonio, TX, USA). The first step underwent 48h with 0.3 M ammonium acetate solution at a sample-to-solution ratio of 1:10 and constant agitation. The second step was completed with water for 48h more. The dialyzed samples were then lyophilized and preserved at −20°C.

4.1.2.10. Determination of proteins by HRSEC-DAD.

The soluble fractions were analyzed by high-resolution size-exclusion chromatography (HRSEC) to determine the molecular distribution and to quantify the proteins obtained from the samples (Canals et al. 1998). The lyophilized samples were resuspended in 0.6 µL of ammonium acetate solution (300 mM) and centrifuged (12 000 × g for 5 min). The supernatant was filtered through 0.22 µm acetate cellulose filters (Merck Millipore, Darmstadt, Germany) and 100 µL of supernatant was then injected into the

chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies, Barcelona, Spain) with a diode array detector (DAD) to monitor output at 230 and 320 nm. Separation was carried out at 20°C using an S 165 Shodex gel permeation HPLC column 210 (OHpak 166 SB-803 HQ, 300mm× 8mm i.d.; Showa Denko, Tokyo, Japan). The mobile phase consisted of an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The proteins were quantified according to the peak area for each fraction using the external standard method with bovine serum albumin (Sigma-Aldrich, Madrid, Spain) ranging from 0 to 1 mg/mL ($r^2 > 0.99$).

4.1.2.11. Acidic hydrolysis for mannose quantifications.

An acidic hydrolysis was performed following the methodology described by Quirós (Quirós et al. 2012). The samples corresponding to lees macerations were processed after the polysaccharides extraction process described above until the lyophilization step. The lyophilized samples were redissolved with 3 mL of ultra-pure water. These samples were passed through a 30 x 10 mm Econo-Pac® 10 DG desalting column (Bio-Rad, Hercules, CA) and eluted with 4 ml distilled water. An aliquot of 1.9 ml was completely vacuum evaporated (Univap 148 100ECH; Progen Scientific, London, UK). The dried material was suspended in 100 μL of 1M H_2SO_4 and incubated at 100ºC for 4h in an oven to undergo acidic hydrolysis. After this process, the samples were filled up to 1 mL with ultra-pure water. One milliliter of the hydrolyzed diluted sample was passed through a Strata NH2 500 mg/3 mL column (Phenomenex, Torrance, CA, USA) to remove sulfuric acid before HPLC injection. Ultra-pure water (1 mL) was immediately eluted to recover a final sample volume of 2 mL.

4.1.2.12. Mannose Quantification by HRSEC-RID.

The samples (2 mL) recovered from the Strata NH2 column were filtered through 0.22 μL pore size nylon filters (Phenomonex, Torrance, CA, USA). Then, 25 μL were injected into the chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies Inc., Santa Clara, USA) with a refractive index detector. The column used was a 300 x 7.7 mm Hi-Plex Pb 8 μm (Agilent Technologies

Inc., Santa Clara, USA). Ultra-pure water was used as the mobile phase at a flux of 0.6 ml/min and a column temperature of 70ºC (Quirós et al. 2012). A calibration curve for HRSEC-RID quantification was done in a range between 50 and 2000 mg/mL ($r^2 > 0.99$).

4.1.2.13. Sensory analyses.

All sensory analyses were performed in the tasting room of the Faculty of Enology in Tarragona (Rovira i Virgili University), which was designed in accordance with UNE 87004.197 (AENOR, 2010). Tasting was conducted using ISO official tasting glasses (ISO-3591, 1997). All the samples were tasted by 10 trained panelists (six males and four females aged between 22 and 62). The tasters were asked to score the sparkling wines of the nine consecutive vintages from the youngest (1 point) to the oldest (9 points). They also were asked to consider whether some of the sparkling wines were too old under the qualitative sensory criterion by classifying them as ‘acceptable’ or ‘not acceptable’.

4.1.2.14. Statistics.

All chemical and physical properties are expressed as arithmetic average ± standard deviation of three replicates. The panel’s appraisal of the chronological order of the sparkling wines for the nine consecutive vintages was considered as the arithmetic average ± standard deviation of the points suggested by the ten panelists. One-factor analysis of variance (ANOVA) was conducted using SPSS 15.0 software (SPSS Inc., Chicago, IL).

4.1.3. Results and discussion

The nine consecutive vintage Cavas presented ethanol strengths ranging from 12.0 to 12.6% (v/v), titratable acidities ranging from 5.70 to 6.75 g/L (expressed as tartaric acid), and pH values ranging from 3.05 to 3.23. These ranges may be considered normal.

Moreover, no clear tendency was observed between these vintages that could influence the results.

Aging time (years)	L*	a*	b*	H_M (mm)	Hs (mm)
1st	98.00 ± 0.17 **C**	-0.88 ± 0.06 **CD**	6.88 ± 0.12 **A**	87 ± 1 **C**	71 ± 3 **C**
2nd	98.07 ± 0.06 **C**	-1.02 ± 0.04 **BCD**	7.69 ± 0.08 **B**	170 ± 1 **E**	93 ± 3 **E**
3rd	97.93 ± 0.06 **C**	-1.11 ± 0.02 **AB**	8.78 ± 0.07 **C**	110 ± 5 **D**	81 ± 1 **D**
4th	97.67 ± 0.15 **BC**	-1.11 ± 0.07 **AB**	8.66 ± 0.21 **C**	80 ± 4 **BC**	72 ± 2 **C**
5th	97.40 ± 0.00 **B**	-1.09 ± 0.08 **AB**	9.87 ± 0.13 **E**	76 ± 4 **BC**	68 ± 1 **BC**
6th	97.57 ± 0.06 **BC**	-1.25 ± 0.08 **A**	9.59 ± 0.07 **DE**	61 ± 1 **A**	53 ± 3 **A**
7th	96.77 ± 0.46 **A**	-1.05 ± 0.03 **ABC**	9.39 ± 0.02 **DE**	73 ± 1 **ABC**	58 ± 2 **A**
8th	97.60 ± 0.10 **BC**	-1.00 ± 0.10 **BCD**	10.77 ± 0.19 **F**	71 ± 4 **AB**	54 ± 1 **A**
9th	97.37 ± 0.15 **B**	-0.92 ± 0.10 **BCD**	10.99 ± 0.34 **F**	74 ± 1 **ABC**	59 ± 1 **AB**

Table 1. CIELAB coordinates and foaming properties of the sparkling wines of different aging time

Table 1 shows the CIELab coordinates of these nine consecutive vintages of sparkling wines. As expected, the blue-yellow component (b*) clearly increased as the ageing time increased. These data confirm a fact that is well known by winemakers: the longer the ageing time, the more intense the yellow color.

Table 1 also shows the foaming properties of these sparkling wines. Both the maximal height of the foam (Foamability – H_M) and the stable height of the foam (Foam stability - H_S) showed a similar tendency, they increased between the first and second year of ageing and decreased progressively thereafter. This was a general trend as some vintages did not follow a perfect chronological order. In general, the values of H_M and H_S were in agreement with those reported by other authors for AOC Cava sparkling wines (Esteruelas et al. 2015, Andrés-Lacueva et al. 1996, Gallart et al. 2004).

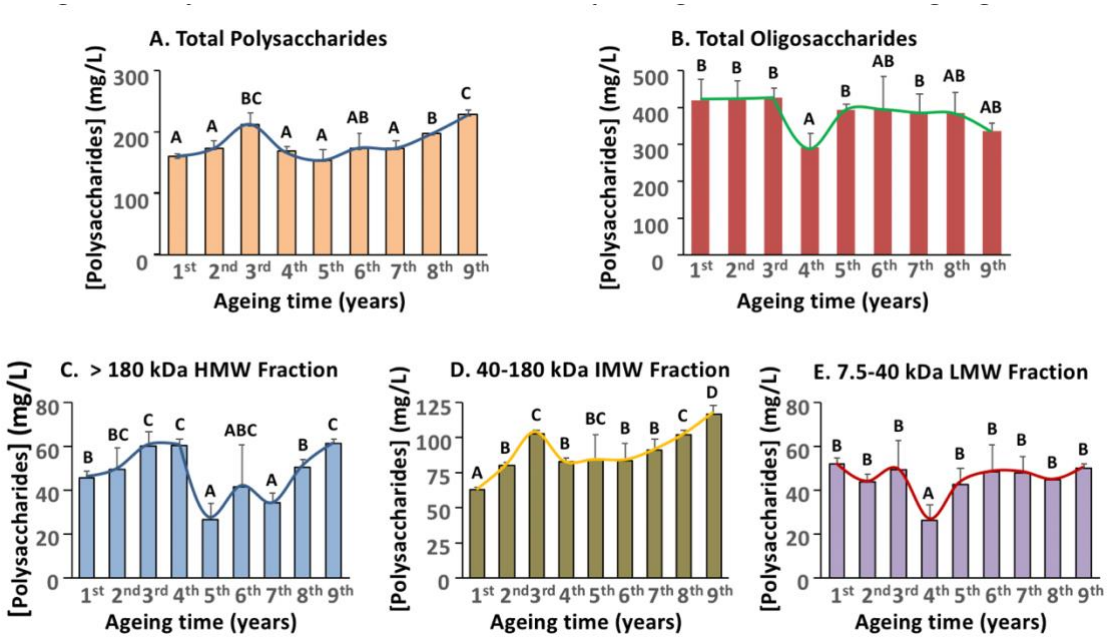

Figure 1. Polysaccharide concentration of the sparkling wines of different ageing time.

Figure 1 shows the polysaccharide and oligosaccharide fractions of the sparkling wines for these vintages. The total polysaccharide concentration ranged from a minimal value of 153 mg/L to a maximal value of 228 mg/L. Other authors have previously reported similar concentrations of polysaccharides in sparkling wines (Martínez-Lapuente et al. 2013, Esteruelas et al. 2015, Andrés-Lacueva et al. 1997). Some significant differences were found between the vintages but no clear trend was observed over the ageing time. This erratic behavior was also observed in the High Molecular Weight (HMW: Molecular weight > 180 kDa), Low Molecular Weight (LMW: Molecular weight between 7.5 and 40 kDa), and oligosaccharide (Molecular weight <7.5 kDa) fractions. However, the Intermediate Molecular Weight (IMW: Molecular weight between 40 and 180 kDa) fraction showed a certain tendency to increase over time, though this was not true for all vintages. The lack of tendency to increase over time observed in the polysaccharide fraction seems to contradict what should be expect from yeast autolysis. However, other authors also found no increase (Martínez-Lapuente et al. 2016) or even reported a decrease in polysaccharide fraction throughout the ageing of sparkling wines (Martínez-Lapuente et al. 2013, Moreno-Arribas et al. 2000).

The reason for this lack of clear tendency in relation to the polysaccharide fraction over time may be that polysaccharides are simultaneously released and removed from the media. Yeast autolysis may be a source of polysaccharides and mannoproteins (Kemp et

al. 2019). However, depending on the nature of the polysaccharides of grape origin, some can precipitate (Martínez-Lapuente et al. 2013), some can be absorbed by the riddling agents (Catarino et al. 2008), and some can be enzymatically degraded (Moreno-Arribas et al. 2000). Moreover, as this work was comparing nine consecutive years, the variability among vintages may have been much greater than the possible release, which makes it impossible to identify any clear trends.

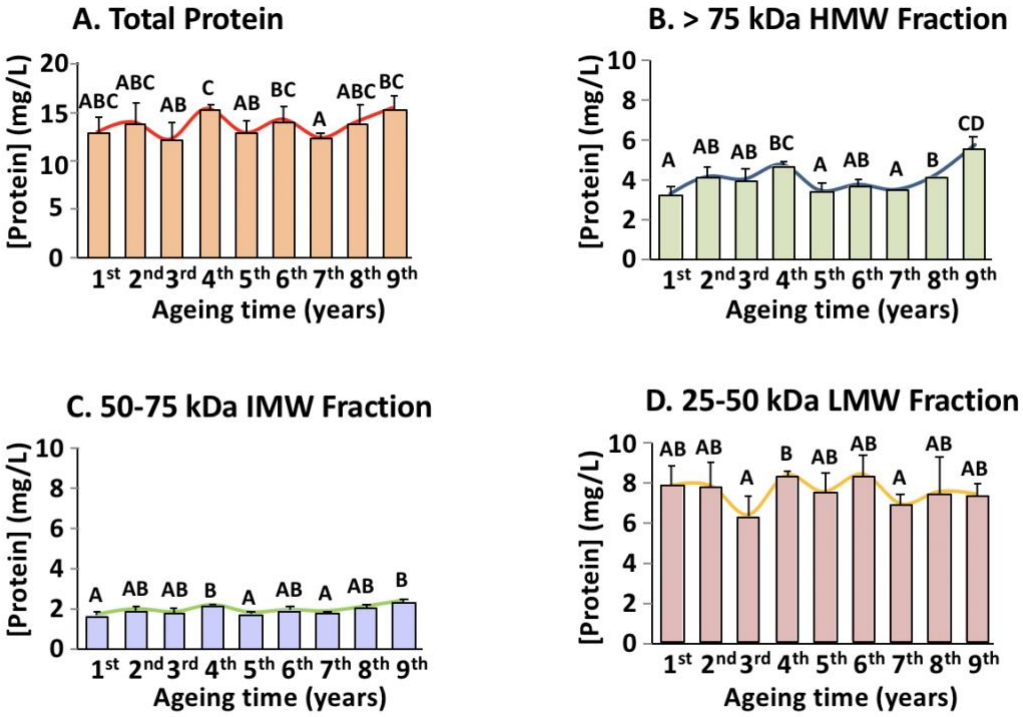

Figure 2. Protein concentration of the sparkling wines of different ageing time.

Figure 2 shows the protein fraction of the sparkling wines of the various vintages. Total protein concentration ranged from 11.0 mg/L to 15.2 mg/L. These concentrations, which are in agreement with those reported in the literature (Esteruelas et al. 2015, Martínez-Rodríguez et al. 2002), are much lower than the normal ones found in base wines because using bentonite as the riddling agent significantly reduces their concentration (Vanrell et al. 2007). As with polysaccharides, several significant differences were found among the protein concentrations of sparkling wines from these vintages, but no significant tendency was observed throughout the ageing time. This lack of specific tendency was also observed in all molecular weight fractions: High Molecular Weight (HMW: Molecular

weight > 75 kDa), Intermediate Molecular Weight (IMW: Molecular weight between 50 and 75 kDa), Low Molecular Weight (LMW: Molecular weight between 25 and 50 kDa).

Once again, these results may appear to contradict what is expected from yeast autolysis. However, other authors also reported erratic behavior from the protein fraction over the ageing time (Esteruelas et al. 2015, Martínez-Rodríguez and Polo 2003, Nunez et al. 2005). This lack of clear trend may also be related to a balance between the proteins released from yeast autolysis and those that disappear due to bentonite absorption (Moreno-Arribas et al. 1996, Vanrell et al. 2007, Nunez et al 2005) and enzymatic degradation (Moreno-Arribas et al. 1996, Martínez-Rodríguez et al. 2003). Moreover, the variability in the original protein concentrations of each vintage of sparkling wine can make it difficult to draw conclusions from comparisons.

Since we observed no tendency for either the polysaccharide fraction or the protein fraction of the sparkling wines to increase as a function of their ageing time, it was decided to study the release of these macromolecules from the lees using a different approach. This involved placing the lees recovered from the sparkling wines of the nine consecutive vintages in a model wine solution and analyzing the polysaccharides and proteins after exactly one year.

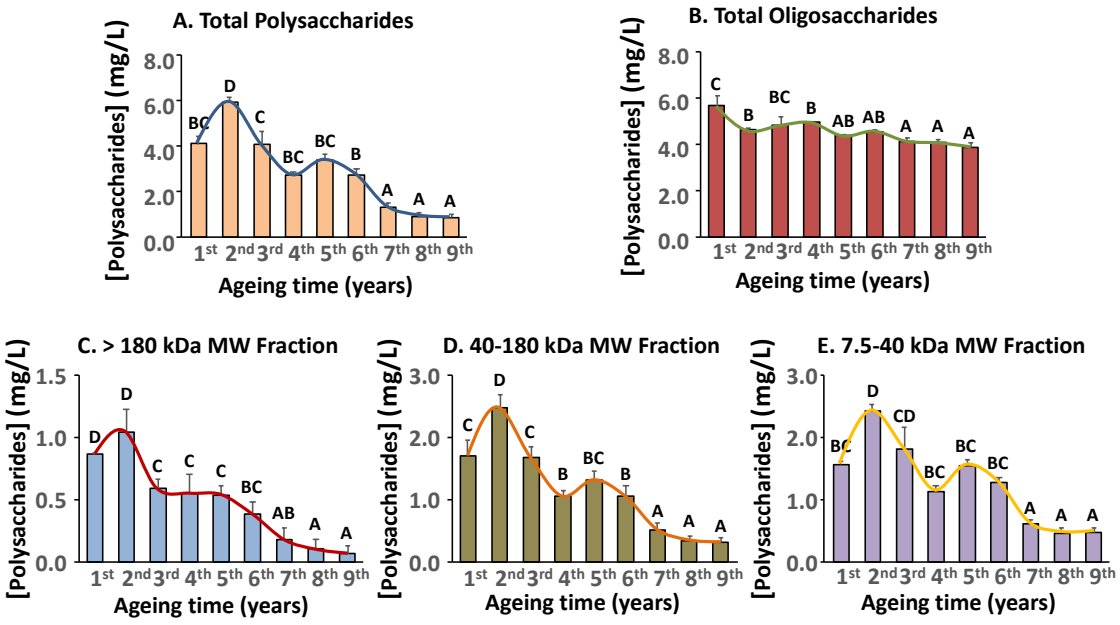

Figure 3. Polysaccharide release from lees of different ageing time in a model wine solution.

Figure 3 shows the polysaccharide release from the lees of the various vintages in a model wine solution after one year of contact. The lees from the first-year of ageing released roughly 4 mg/L of total polysaccharides in this model wine solution. The lees from the second-year reached a significant maximal amount of roughly 6 mg/L, while the amounts of lees from the later vintages decreased progressively, reaching a minimal value in the ninth year of ageing of roughly 0.90 mg/L. This behavior was similar for all polysaccharide molecular weight fractions.

The oligosaccharide fraction released by the lees also tended to decrease as the age of the lees increased. In this case, however, the maximum value was observed in the lees from the first-year vintage (around 5.6 mg/L). This decrease was also less clear than it was for polysaccharides, with a minimal value of roughly 3.8 mg/L observed in the lees from the ninth-year of ageing. This steady decrease may be related to a hydrolysis of the polysaccharides, which would reduce their average degree of polymerization.

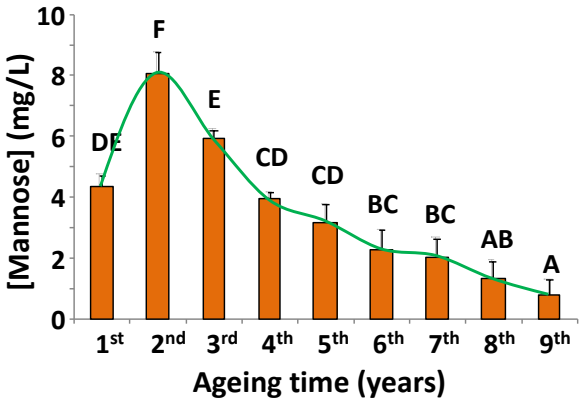

Figure 4. Mannose present in the polysaccharide released from lees of different aging time in a model wine solution.

To evaluate the release of mannoproteins from yeast autolysis, the same polysaccharide samples as were used for HPLC analysis were hydrolyzed in acidic media to determine mannose concentration. Figure 4 shows the mannose present in the polysaccharide released from the lees of different ageing time in a model wine solution. The mannose concentrations from polysaccharide hydrolysis showed very similar patterns to the total polysaccharides released by the lees, they increased from the first to the second year but decreased progressively in later vintages. Moreover, their concentrations were very

similar. These data confirm that the polysaccharides released by the lees were mainly mannoproteins.

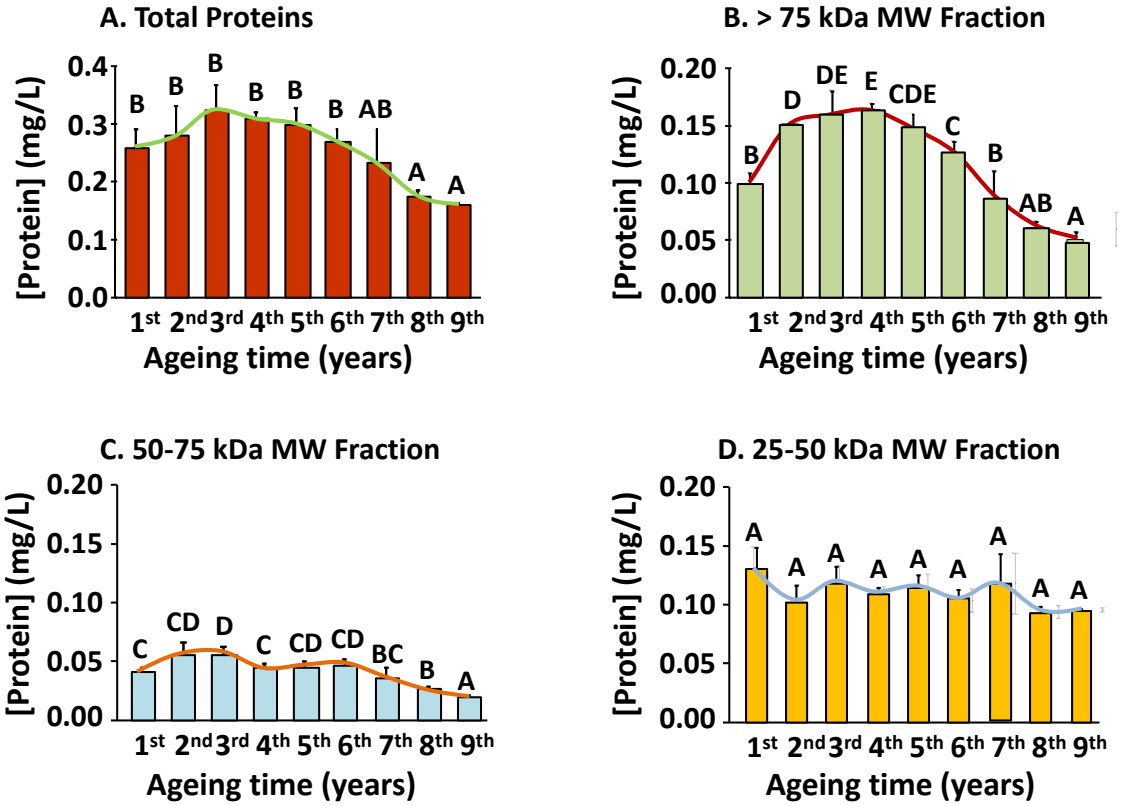

Figure 5. Protein release from lees of different ageing time in a model wine solution.

Figure 5 shows the protein release from the lees of different ageing time in a model wine solution after one year of contact. Total protein concentration generally showed a similar pattern to that of the polysaccharides but reached a maximal value in the lees of the third-year ageing (roughly 0.32 mg/L) and a minimal value in the lees of the ninth-year of ageing (roughly 0.17 mg/L). This behavior was even clearer in the HMW protein fraction. In contrast, it was hardly observed in the IMW fraction and not observed at all in the LMW fraction.

Using a simple approach, we added the concentrations of both macromolecules released from the first-year lees to the ninth-year lees to reproduce the cumulative effect on their release over the lees ageing time (see Figure 6). In both cases, a clear increase was observed in the accumulated concentrations over the ageing time. Moreover, the younger the lees, the greater the increase (especially in the first five years when the differences

were statistically significant). The total accumulation of polysaccharides at the end of the nine-year period was 26.6 mg/L, while that of proteins was 2.4 mg/L.

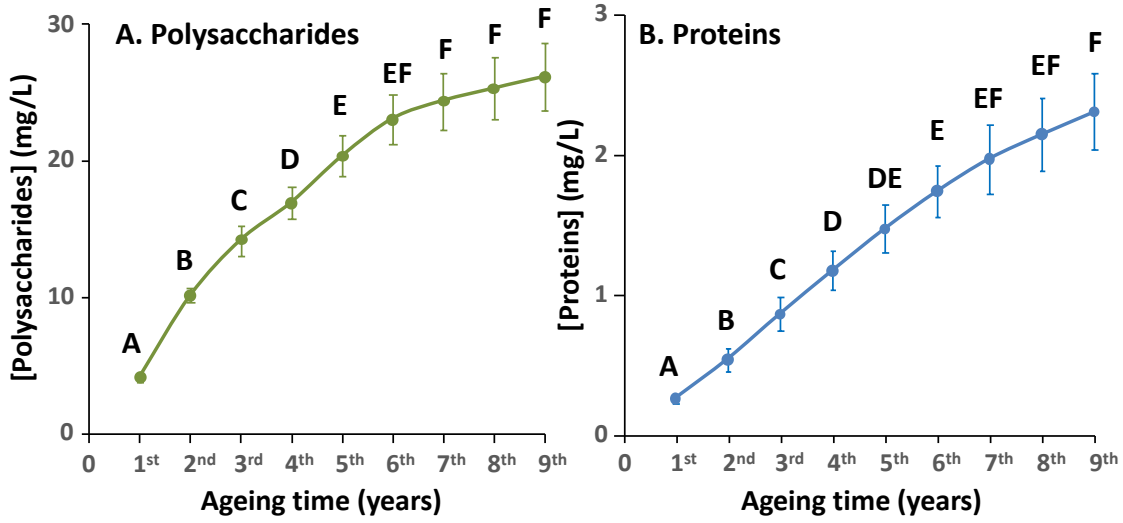

Figure 6. Accumulation of proteins and polysaccharides from yeast autolysis.

These values should be taken with caution since they reflect just one approach. However, they do seem to indicate that the release of polysaccharides and proteins from the lees during the ageing process of these sparkling wines was much lower than the regular concentrations present in them.

We applied another simple approach to determine the distribution of polysaccharides and proteins in function of their origin (from lees autolysis or from the base wine). Figure 7 shows the percentage of polysaccharides (A) and proteins (B) from lees autolysis or base wines with respect to total concentration. This figure clearly shows that the percentage of polysaccharides and proteins from lees autolysis was extremely low in the young sparkling wines. That means that during the first year of ageing, sparkling wine had only 2% of proteins and 3% of polysaccharides from the lees. These percentages increased as the ageing time increased and reached maximal values in the seventh year of ageing (14% for polysaccharides and 16% for proteins).

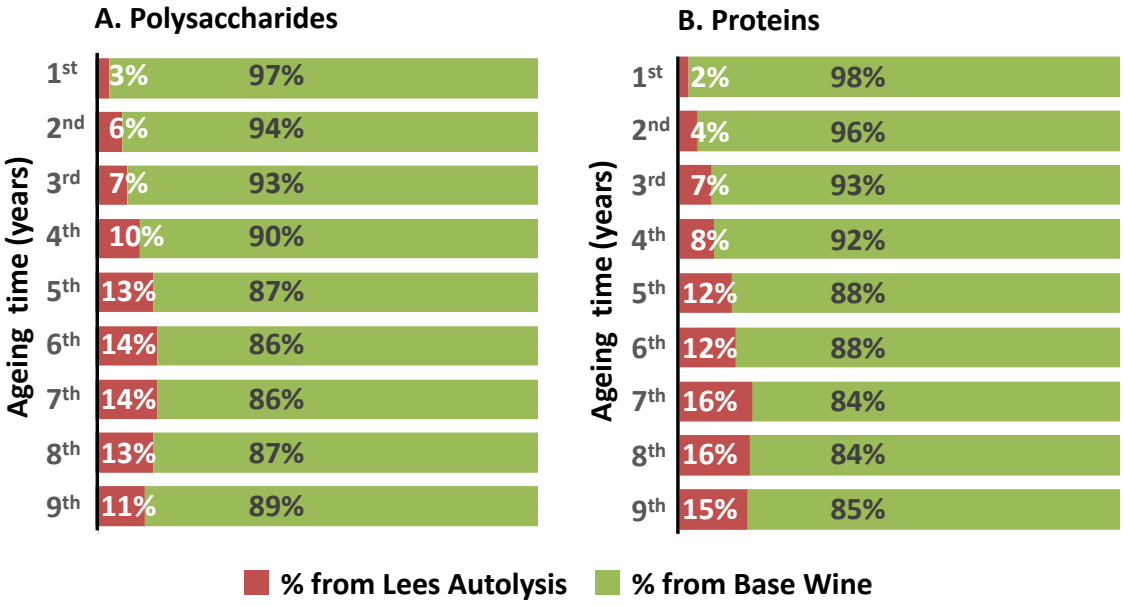

Figure 7. Distribution of proteins and polysaccharides of sparkling wine according to their origin.

Note that, according to the information provided by the Regulatory councils, 88% of sparkling wines from AOC Cava are aged between 9 and 15 months and 78% of Champagnes are aged between 12 and 36 months. This means that only a small proportion of sparkling wines, even those produced by the traditional method, are aged for a long time. Given these data, the vast majority of sparkling wines produced by the traditional method have percentages of polysaccharides and proteins from lees autolysis below 7%, and this value should even be lower in the youngest sparkling wines – especially those not produced by the traditional method.

Figure 8 shows the results of the sensory analysis performed by the trained panel. Statistical comparison of the nine consecutive vintages established four significant groups: group A, which the panel considered the youngest, comprised only the sparkling wine from the first year; group B comprised sparkling wines from the second and third years; group C, which was considered significant older than group B, comprised wines from the fourth to the sixth years; and finally group D, which was classified as being significantly the oldest, comprised sparkling wines from the seventh to the ninth years. We can see, therefore, that the panel successfully appreciated the chronological order of these sparkling wines, though the differences between some consecutive vintages were not always significant.

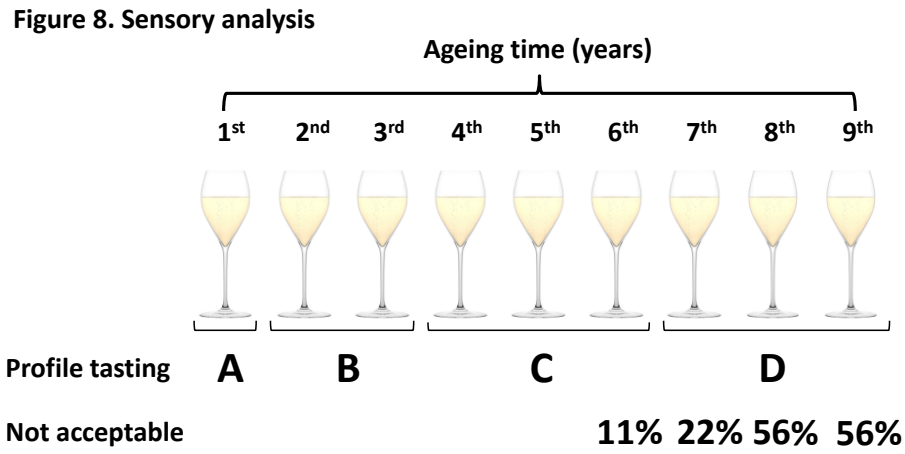

Figure 8. Sensory analysis results scheme

All panelists considered the five youngest vintages of sparkling wines as 'acceptable' for the market under their qualitative sensory criterion. On the other hand, to varying degrees depending on the vintage, the older vintages of sparkling wines were considered 'not acceptable' by some tasters. The percentages of 'not acceptable' evaluations increased as the ageing time increased, being 11% for the sixth year, 22% for the seventh year, and 56% for the eighth and ninth years. These data indicate that after five years the sparkling wines began to be affected by excess ageing. These results quite closely match those of our previous study in which we measured and compared the changes in oxygen consumption by the lees in the same nine consecutive vintages to the theoretical oxygen permeability through the crown cap (Pons-Mercadé et al. 2021). According with this work, the oxygen consumed by the lees is not enough to compensate for oxygen intake through the crown cap after roughly four years of ageing. After this time, the sparkling wine does not have enough defense against oxidation. Under these conditions, its sensory quality may begin to deteriorate, though the effects of this oxidation will depend on its composition and storage conditions. In the present study, sensory deterioration seems to begin from the 6th year onwards.

Finally, to better illustrate the process of yeast autolysis throughout the ageing period in the bottle, some photographs of the yeasts were taken using a scanning electron microscope (SEM) in order to visualize, by comparing the lees of the sparkling wines, how the structure of the yeasts is degraded when the wines are aged by up to nine years. Although similar studies have already been reported (Charpentier et al. 1986, Cebollero and Gonzalez 2006, Tudela et al. 2012, Martínez-Rodríguez et al. 2001), to our

knowledge none have sequentially analyzed such a long period. Figure 9 shows several images that clearly illustrate how the structures of the yeast cells are progressively degraded, folded and deflated. In the first image, which shows the yeast of the starter culture used for the second fermentation of the last vintage (2016), the yeast cell seems very healthy since it is elongated, ovoid and turgid without any wrinkle or fold. Several bud scars can even be identified. The second image shows what happens after the second fermentation (three months later): the yeast cell has lost some turgor and is beginning to display wrinkles and folds. Two years later, in the third year of ageing, the yeast cell is even more degraded and wrinkled and begins to deflate. At the fifth year of ageing, the yeast cell is completely flattened at the edges and retains only a little turgor in the middle, which is full of wrinkles and folds. In the seventh year of ageing, the yeast cell is even more degraded and deflated and the center of the cell has crumbled, wrinkled and flattened. Finally, in the ninth year, the yeast cell has completely collapsed and some of its structures are broken.

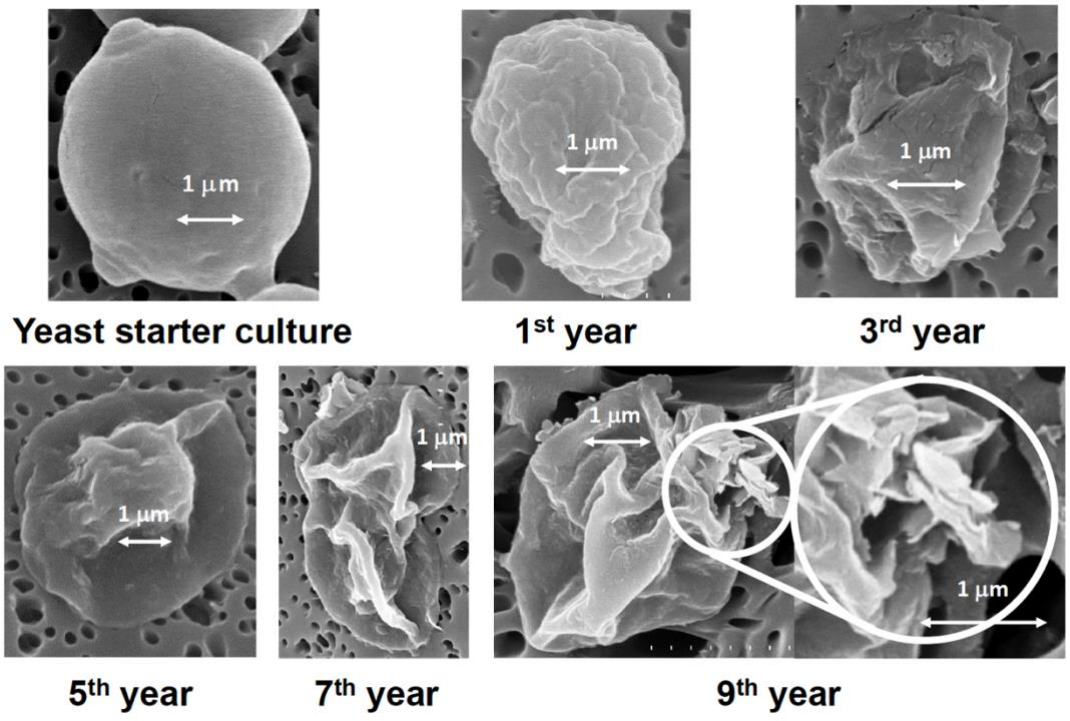

Figure 9. Monitoring yeast autolysis using Scanning Electron Microscopy.

In summary, comparisons of the polysaccharide and protein fractions of sparkling wines from nine consecutive vintages elaborated by the traditional method showed no clear trends over ageing time in contact with the lees. This absence of trend seems to contradict

the expected release of these macromolecules by the autolysis of the lees. However, as discussed above, other authors reported similar results. The lack of trend may be due to the fact that both types of macromolecules are simultaneously released from the lees and removed from the media by precipitation, absorption or enzymatic degradation. This creates a complicated balance that becomes even more complex considering the inevitable variations in the original concentrations of polysaccharide and protein fractions in each vintage. It is understandable, therefore, that this and other studies have found such erratic behavior.

To verify whether polysaccharides and proteins are indeed released from the lees, in this study we introduced a new approach that involved recovering lees from the sparkling wines of nine consecutive vintages and reproducing the autolytic process in synthetic wine media. Our results clearly showed that both macromolecules are indeed released from the lees. This enabled us to compare the theoretical proportion of polysaccharides and proteins from yeast autolysis to their corresponding total concentrations in the sparkling wines. According with these results, the proportion of polysaccharides and proteins was very low in the young sparkling wines, roughly only 2-3% in the first year of ageing and around 7% in the third. This suggests that the real impact of polysaccharides and proteins from lees autolysis in the sparkling wines disgorged before the end of the first year should be very low. Wine producers should bear this conclusion in mind, since most sparkling wines elaborated by the traditional method are aged for less than one year and those made by other methods are aged even less. Consequently, only sparkling wines aged for longer would therefore benefit from a greater presence of polysaccharides and proteins from yeast autolysis. Producers of sparkling wines should also bear in mind that, as the tasting panel stated, too long an ageing time can negatively affect wine quality due to excess oxidation.

Funding

This research was funded by the Spanish Ministry of Science and Innovation, Centre for the Development of Industrial Technology (CDTI) (Program CIEN, Project GLOBALVITI: Global solution to improve wine production against climate change based on robotics, IT technology and biotechnological strategies and vineyard management;

Program CIEN, Project CAVAWINNER: Study and technological improvement of the traditional processes of Cava elaboration).

Declaration of Competing Interest

The authors declare they have no known competing financial interests or personal relationships that may have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to Juvé & Camps (Sant Sadurní d'Anoia, Barcelona, Spain) for providing the sparkling wines with different ageing times.

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4.2. Chapter II: Oxygen consumption rate of lees during sparkling wine (Cava) aging; Influence of the aging time

Food Chemistry 342 (2021) 128238

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Oxygen consumption rate of lees during sparkling wine (Cava) aging; influence of the aging time

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One of the main problems of sparkling wine elaborated by the traditional method is the oxidation suffered during the prolonged period of ageing that can significantly affect the sparkling wine quality. Even if after second fermentation inside the bottle the sparkling wine is oversaturated with carbon dioxide, oxygen can permeate across the crown cap over time. However, it is generally considered by winemakers that lees protect the sparkling wine against oxidation consuming the small amounts of oxygen that can permeate across the crown cap. This chapter focus on the confirmation of the oxygen consumption by lees, to quantify these oxygen consumptions by lees, to study their evolution over time and to determine the time where these oxygen consumptions by lees is not enough to protect sparkling wine from oxidation. This work was published in Food Chemistry.

4.2.1. Introduction

Sparkling wines made using the traditional method follow an elaboration process that involves two fermentations (Maujean, 1989). The first fermentation transforms the grape juice into base wine following a conventional winemaking process. In contrast, the second fermentation, also named *prise de mousse* occurs inside a bottle after the wine is enriched with a solution called *liqueur de tirage*, which provides the necessary sugar for the second fermentation (around 22 g of sucrose/L) (Kemp, Alexandre, Robillard, & Marchal, 2015). This *liqueur de tirage* also contains some co-adjuvants, such as bentonite and/or alginates, to favor the riddling process (Vanrell, Canals, Esteruelas, Fort, Canals, & Zamora, 2007), and yeasts, which have previously been acclimatized to ensure that the second fermentation comes to a successful end. Once the second fermentation has finished, which can last between three and six weeks, the sparkling wines remain in contact with the yeasts, called lees, for a long time so that the autolysis process takes place (Alexandre, & Guilloux-Benatier, 2006).

During this ageing period, the yeast cells release many parietal and cytoplasmic compounds that have a large influence on the sparkling wine's quality (Feuillat, & Charpentier, 1982; Pozo-Bayón, Martínez-Rodríguez, Pueyo, & Moreno-Arribas, 2009). Among the various substances released during the autolysis process, the following substances can be highlighted due to their effects on the sensory attributes of sparkling wine: proteins (Luguera, Moreno-Arribas, Pueyo, Bartolome, & Polo, 1998), mannoproteins (Martínez-Lapuente, Guadalupe, Ayestarán, & Pérez-Magariño, 2015), peptides (Moreno-Arribas, Pueyo, & Polo, 1996), amino acids (Martinez-Rodriguez, Carrascosa, Martin-Alvarez, Moreno-Arribas, & Polo, 2002), polysaccharides (Martınez-Lapuente, Guadalupe, Ayestaran, Ortega-Heras, & Perez-Magariño, 2013), lipids (Pueyo, Martínez-Rodríguez, Polo, Santa-María, Bartolomé, 2000) and nucleotides (Charpentier, Aussenac, Charpentier, Prome, Duteurtre, & Feuillat, 2005).

Proteins, mannoproteins, peptides and polysaccharides play an important role in favoring the integration of carbon dioxide, which improves the quality of the effervescence and increases the foam stability (Martínez-Rodríguez, Carrascosa, Barcenilla, Pozo-Bayón, Polo, 2001; Kemp, Conde, Jegoud, Howell, Vasserot, & Marchal, 2019). It has also been reported that polysaccharides and mannoproteins play a positive role in improving

mouthfeel (Gawel, Smith, Cicerale, & Keast, 2018), and that some proteins and peptides released by yeasts can contribute to wine sweetness (Marchal, Marullo, Moine, & Dubourdieu, 2011). In addition, some amino acids, peptides and nucleotides can contribute to the umami taste (Vilela, Ines, & Cosme, 2016) and have been described as flavor enhancers. Finally, it has also been reported that amino acids and lipids are aroma precursors (Styger, Prior, & Bauer, 2011) and consequently their release from yeast cells can also contribute to the aromatic complexity of sparkling wines.

However, natural autolysis is a slow process that takes a long time to achieve a real organoleptic effect. This means that great sparkling wines require a long aging time (several years) in order to acquire all the richness and complexity that autolysis provides. In sparkling wines, yeast autolysis does not begin until 2−4 months after the completion of secondary fermentation (Todd, Fleet, & Henschke, 2000). Autolysis starts with a passive excretion of amino acids and other small molecules. This process, called exorption, lasts between 3 and 6 months (Morfaux, & Dupuy, 1966). Later, true autolysis begins with degradation of membranes (cell and vacuolar), causing the progressive hydrolytic degradation of all cell structures (Alexandre, & Guilloux-Benatier, 2006), whose fragments can be released into the wine. Once autolysis has begun, cell wall polysaccharides and mannoproteins, proteins, lipids and nucleotides increase very slowly, completely transforming the composition of the sparkling wine and therefore its sensory attributes (Kemp et al., 2015).

For these reasons, most of the sparkling wines made with the traditional method have established minimum aging times that guarantee that autolysis has had an effect on the composition and quality of the product. In the particular case of AOC Cava a minimum aging time of 9 months has been established. However, the finest sparkling wines from this AOC are usually aged for a much longer time. In fact, AOC Cava distinguishes between two other qualities of sparkling wine with a longer aging time: *Reserva* and *Gran Reserva* with a minimum aging time of 15 and 30 months respectively. Some prestigious wineries even produce Cavas with much a longer aging time, despite the fact that there is no specific category to distinguish them.

Nevertheless, a long aging time can sometimes have some drawbacks, the clearest of which is a very rapid evolution (Riu-Aumatell, Bosch-Fuste, López-Tamames, &

Buxaderas, 2006) that causes the premature appearance of oxidized characters (Pons, Nikolantonaki, Lavigne, Shinoda, Dubourdieu, & Darriet, 2015). Sparkling wines are better protected against oxidation than still wines due to their higher internal pressure in carbon dioxide, which makes it difficult for oxygen to enter through the crown cap. However, gas exchange takes place through the crown cap so that carbon dioxide can exit (Gerard Liger-Belair, & Villaume, 2011) and oxygen from the air can enter inside the bottle even under these conditions (Valade, Bunner, Tribaut-Sohier, Tusseau, & Moncomble, 2011). There is some variability in the literature about the oxygen permeability of the different crown caps used for the second fermentation of sparkling wine. Valade, Tribaut-Sohier, Bunner, Laurent, Moncomble, & Tusseau (2007) reported an oxygen permeability ranging from 0.35 to 2.5 mg of O_2/L/year depending on the cap liner, and other authors have reported similar values (Kemp et al., 2014). Evidently the higher the oxygen transfer rate the faster the wine oxidation. Different levels of oxidation have been reported in still wines (Godden et al., 2001; Mas, Puig, Lladó, & Zamora, 2002) and sparkling wines (Mas, Puig, Lladó, & Zamora, 2001) depending on the permeability of the stopper used.

Another reason why sparkling wines are more protected against oxidation than still wines is the presence yeast lees inside the bottle. It has been reported that yeast lees exert antioxidant activity (Gallardo-Chacón, Vichi, Urpí, López-Tamames, & Buxaderas, 2010) and consume oxygen (Salmon, Fornairon-Bonnefond, Mazauric, Moutounet, 2000; Fornairon-Bonnefond, Camarasa, Moutounet, & Salmon, 2002; Fornairon-Bonnefond, & Salmon, 2003). Consequently, the presence of lees slows down the evolution of the wine by consuming the oxygen that permeates the crown cap. The mechanism by which the lees consume oxygen is not clear, but some authors associate it with the oxidation of membrane lipids, sterol fractions in particular (Fornairon-Bonnefond, 2000; Fornairon-Bonnefond, & Salmon, 2003); however, it may also be due to the fact that lees can release glutathione (Kritzinger, Bauer, & du Toit, 2013). Regardless of the mechanism by which the lees consume oxygen, it is clear that this oxygen consumption is probably the main reason why sparkling wines can usually age for a far longer time than still white wines.

There is no doubt that lees can consume the small amounts of oxygen that can permeate the stopper protecting the wine against oxidation. However, it would be interesting to determine whether this ability to consume oxygen is very long lasting and therefore can

protect the sparkling wine during its entire shelf life or rather it decreases or even disappears after a certain aging time. In fact, it has been reported that the antioxidant capacity (FRAP/DPPH) as well as the glutathione content of sparkling wine lees decrease progressively over the aging time (Gallardo-Chacón et al., 2010). It has also been reported that the ability of lees to consume oxygen in a model wine solution decreases over time (Salmon et al., 2000; Fornairon-Bonnefond et al., 2002; Fornairon-Bonnefond et al., 2003). This is, in our opinion, a key question since it determines the maximum aging time that can be applied to a sparkling wine without it deteriorating.

The aim of this work was to study the oxygen consumption rate (OCR) of the sparkling wine lees of AOC Cava with an aging time from 1 to 9 years. To our knowledge there is no specific study on the capacity of the lees present in a bottle of sparkling wine to consume oxygen or on how this capacity evolves over a long aging time.

4.2.2. Materials and methods

4.2.2.1. Chemicals

Absolute ethanol, L-(+)-tartaric acid and sodium hydroxide pellets were purchased from Panreac (Barcelona, Spain). Copper (II) sulfate pentahydrate and iron (III) chloride hexahydrate were purchased from Sigma–Aldrich (Madrid, Spain).

4.2.2.2. Base wine elaboration

The experiment was carried out during nine consecutive vintages (2008-2016) in Juve & Camps winery (AOC Cava, Sant Sadurní d'Anoia, Barcelona, Spain). The base wines were made in the most similar way possible, although some differences between each vintage are inevitable. Monovarietal base wines in all the vintages were elaborated with three autochthonous cultivars: Xarel.lo (VIVC Prime name: Xarello; VIVC Variety number: 13270), Macabeo (VIVC Prime name: Viura; VIVC Variety number: 13127) and Parellada (VIVC Prime name: Parellada; Variety number VIVC: 8938). Xarel.lo and Macabeo grapes came from the Juvé & Camps vineyards in Sant Sadurní d'Anoia (Barcelona, Spain; 41°26'47.42" N and 1°49'0.63" E) at 165 meters above sea level and

Parellada grapes from the Juvé & Camps vineyards in Mediona (Barcelona, Spain; 41°29'48.6" N and 1°39'56.9"E) at 506 meters above sea level.

For each vintage the grapes were harvested at the appropriate maturity level for obtaining base wines for sparkling wine production. More specifically, the total soluble solid content (°Brix) varied between 17.3 and 19.5 for Xarel.lo, between 16.2 and 18.3 for Macabeo and between 16.2 and 18.3 for Parellada. The titratable acidity (g of tartaric acid/L) varied between 5.5 and 8.0 for Xarel.lo, between 4.6 and 7.2 for Macabeo and between 5.1 and 7.8 for Parellada. The grapes were immediately pressed in a pneumatic press to obtain a yield of 0.6 L/kg of grape juice. The grape juices were immediately sulfited with 100 mg/L of $K_2S_2O_5$ and pectinolytic enzymes (20 mg/L) were added to facilitate settling. After 24 h, clean grape juices were racked into the fermentation stainless steel tanks and were immediately inoculated with 200 mg/L of selected yeasts (Saccharomyces cerevisiae - IOC 18-2007; Institut Œnologique de Champagne, Epernay, France). When necessary, titratable acidity was corrected with tartaric acid up to 6.0 g of tartaric acid/L. All alcoholic fermentations were performed at 16-18 ºC. Once alcoholic fermentation was finished, base wines were racked and kept at 18-20 ºC until spontaneous malolactic fermentation was completed. Base wines were then racked again, sulfited (40 mg/L of $K_2S_2O_5$) and cold stabilized.

4.2.2.3. Sparkling wine elaboration

Once the monovarietal base wines were considered to be stable, they were used to obtain a classic blend of the AOC Cava in all the vintages. Each vintage, a blend of 50 % of Xarel.lo, 30 % of Macabeo and 20 % of Parellada, was used to produce sparkling wines (Cava) with the traditional method. Briefly, all the base wines were supplemented with 22 g/L of sucrose, 40 mg/L of a mixture of bentonite and alginates as riddling agent (Adjuvant MO; Station OEnotechnique du Champagne, Epernay, France) and 2×10^6 cells/mL of a pre-adapted yeast culture (Saccharomyces cerevisiae - IOC 18-2007; Institut Œnologique de Champagne, Epernay, France). The wines were then bottled, crown corked and stocked at 12-15 ºC until disgorgement. This procedure was employed during the nine consecutive vintages with the aim of reproducing as much as possible the same elaboration conditions in all the vintages studied.

4.2.2.4. Experimental design

The experimental design used for determining the lees' oxygen consumption rate was an adaptation of those previously described by Navarro et al. (2016) and Pascual et al. (2017) for determining the oxygen consumption rate of oak chips and oenological tannins respectively.

Three weeks before fulfilling the required aging time, three bottles of each vintage were placed in a "pupitre" and the "remuage" process was performed manually. Once all the lees sediment was in the neck of the bottle, disgorging was performed without freezing to facilitate the recuperation of the lees. The lees sediment was recovered in a plastic bucket and immediately transferred to a centrifuge tube. The tubes were centrifuged (5 minutes at 10,000 rpm). Then the pellet was washed with 10 mL of saline solution (NaCl 0.9% v/v) and centrifuged again. This operation was repeated twice. The lees were then ready for measuring their oxygen consumption rate.

We used a model wine solution composed of ethanol (12% v/v) and tartaric acid (4 g/L) adjusted to pH = 3.5 with sodium hydroxide. This solution was enriched with 3 mg of Iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L, in the form of copper (II) sulfate pentahydrate. We worked with this model wine solution and not with real wine because the naturally occurring phenolic compounds would compete with the lees in oxygen consumption, making it impossible to determine their real oxygen consumption rate. This model wine solution was saturated in oxygen by bubbling with air for 10 min immediately before the beginning of the experiment.

The sparkling wine lees of 1 to 9 years of aging time were placed in clear glass flasks (66 mL) into which a pill had previously been inserted (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203-01_PSt3-0828-01, Regensburg, Germany) for measuring dissolved oxygen noninvasively by luminescence (Nomasense TM O2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium). We used flasks with a very small volume to concentrate the lees and reduce the time of the experiment. The bottles were completely filled with the model wine solution and closed immediately after with a shutter and a crown cap to minimize the volume of headspace. The bottles were then gently shaken to resuspend the lees. The

flasks were kept at 20±2 ºC during the entire time. Oxygen was measured (Diéval, Vidal, & Aagaard, 2011) every day during the first month and then measurements were taken at increasing intervals until the end of the experiment (365 days). Figure 1 shows a schematic representation of the experimental design. Two types of control were prepared. Control-A bottles were filled with the oxygen-saturated model wine solution without adding lees, and Control-B bottles were filled with the same model wine solution but after bubbling for 10 minutes with nitrogen to eliminate oxygen. Control A was used to verify that the model wine solution did not consume oxygen and Control B was used to verify the tightness of the closure system. All assays were performed in triplicate.

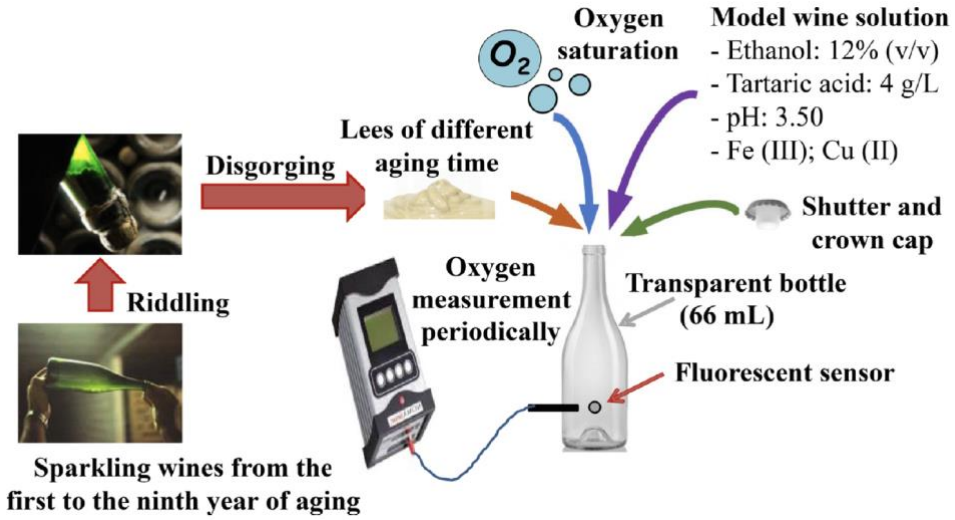

Figure 1: Experimental design for mesasuring oxygen consumption.

4.2.2.5. Statistics

All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA) was carried out using the SPSS 15.0 software (SPSS Inc., Chicago, IL).

4.2.3. Results and discussion

Figure 2 shows the oxygen consumption kinetics of the lees from sparkling wines from 1 to 9 years of aging time. The oxygen consumption of the Control-A model wine solution (without adding lees) and the oxygen intake in Control-B (solutions without oxygen) were very low and can be considered negligible (data not shown). In contrast, the oxygen consumption of all the samples containing lees increased over time, demonstrating that the lees really have the ability to consume oxygen. All samples showed similar behavior

with higher oxygen consumption during the first 30 days, which later smoothed out, reaching an asymptotic profile in some cases. Moreover, the lees were observed to have a different oxygen consumption capacity depending on the aging time of the sparkling wine from which they were extracted. Looking at the graph it can be clearly seen that the lees of the 1, 2 and 3 year wines show higher initial slopes than the older lees. It can be seen that the lees of the first three years, especially those of the second year, consume much more oxygen than the lees of later years. It therefore seems clear that the ability of the lees of sparkling wines to consume oxygen tends to decrease throughout the aging period.

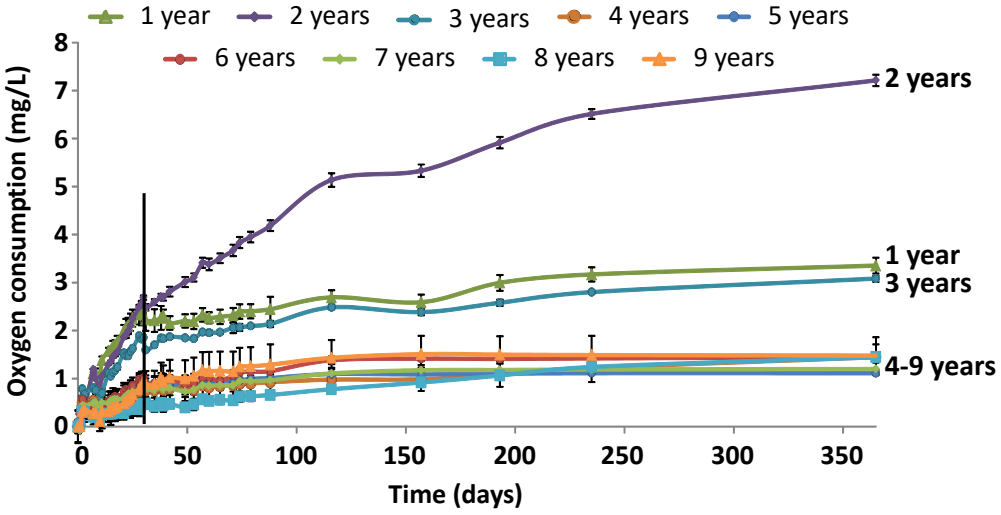

Figure 2: Oxygen consumption by lees extracted from sparkling wines of different ageing time. All data are expressed as the average of 3 replicates ± standard deviation.

Fornairon-Bonnefond, & Salmon, (2003) reported that the oxygen consumption of lees in a model solution was between 1 and 4 μmol of O_2/h.10^{10} cells from the second month to the third year of wine aging. This means a total oxygen consumption of between 125 and 500 mg of oxygen/L.year, considering that the total population of yeast inside a sparkling wine bottle is around 6 x 10^6 cells/mL (Martinez-Rodriguez et al., 2002). Evidently these values are too high and certainly much greater than our results. However, these studies were not performed with lees from sparkling wines and they used a less precise and invasive method for measuring oxygen (Clark electrode). In any case, the decrease in the ability of lees to consume oxygen over time has been previously reported (Salmon et al., 2000; Fornairon-Bonnefond et al., 2002; Fornairon-Bonnefond et al., 2003). Moreover, other authors have measured the antioxidant capacity of lees of

sparkling wines and their results agree with our data as they reported a progressive decrease over time during aging (Gallardo-Chacón et al., 2010).

However, the quantitative comparison of the oxygen consumption kinetics of the different lees is not evident from looking at these figures. To better quantify the real ability of lees to consume oxygen, we applied the kinetic model proposed by Pascual et al. (2017) to determine more precisely how the oxygen consumption capacity of the lees of sparkling wines decreases according to their aging time. This model consists in displaying the inverse of consumed oxygen versus the inverse of time.

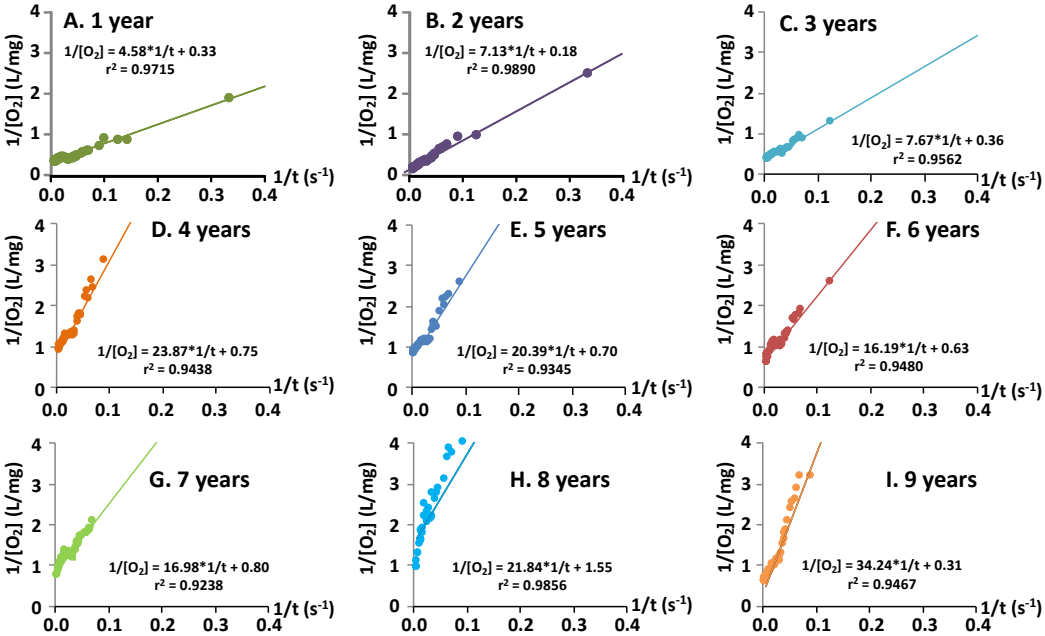

Figure 3: Application of the oxygen consumption model to the lees of different ageing time

Figure 3 shows the results obtained for the sparkling wine lees of 1 to 9 years of aging time. It can be seen that satisfactory linear regression coefficients were obtained in all cases. This confirms that this mathematical model works quite well. According to this mathematical model, the following equation can be established: 1/[O2] = A/t + B. This equation describes the relationship between the consumed oxygen versus time and is shown in Figure 4A, which also shows how the consumed oxygen can be cleared up, how the first derivative is obtained, and finally how the oxygen consumption rate at time zero (OCRto) can be determined, which corresponds to the inverse of the slope of the initial equation.

This modelling can also be used to compare the experimental data (Figure 4B) with the theoretical data (Figure 4C) obtained after applying this model. As it can be seen in these figures, the mathematical model reproduces quite well the experimental kinetics of oxygen consumption by the lees from the sparkling wines of different aging time. These results demonstrate the validity of the modelling.

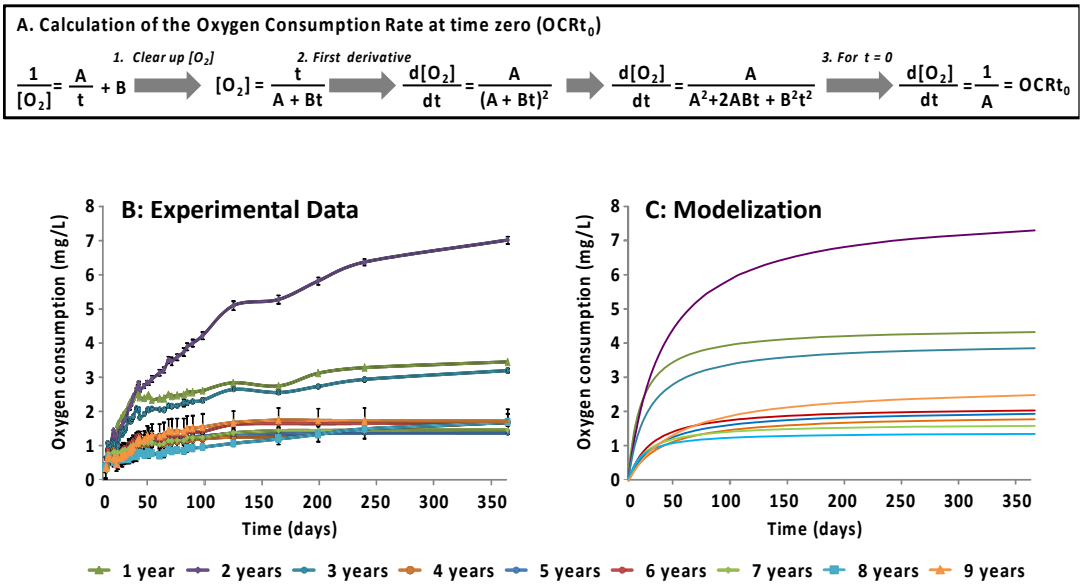

Figure 4: Modelization of the oxygen consumption by lees.

Figure 5A shows the OCRto of the sparkling wine lees of 1 to 9 years of aging time calculated using the model described above. These OCRto values refer to the volume of the original sparkling wine bottles (750 mL), considering the volume of the flasks (66 mL) in which the oxygen measurements were performed. According to these data, OCRto was highest in the first year lees, then decreased significantly in the second and third year lees, and finally decreased significantly again in the older lees. These results confirm more accurately that the capacity of the lees to consume oxygen decreases with the aging time. Nevertheless, the initial oxygen consumption at time zero does not match the total capacity of oxygen consumption exactly, as can be seen in Figure 5B. Considering the total oxygen consumption in 365 days, the second year lees are capable of consuming slightly more than double the oxygen than the lees of the first or second years. Subsequently, the total oxygen consumption decreases drastically in the older lees. The surprising behavior of the second year lees could be related to the described progress of the autolysis process which, according to some authors, starts slightly after 4 months and is more intense during the second year (Todd et al., 2000: Fornairon-Bonnefond et al.,

2002; Alexandre, & Guilloux-Benatier, 2006; Kemp et al., 2015). Some authors have related the oxygen consumption to the lipids from the cell membranes (Salmon et al., 2000; Fornairon-Bonnefond, & Salmon, 2003), especially to certain sterol fractions (Fornairon-Bonnefond, 2000). Since autolysis is more intense during the second year, it is logical that there is more availability of these lipids and there may be other substances capable of consuming oxygen. In any case, it is clear that the lees' oxygen consumption capability decreases drastically after the first three years of aging since older lees (4 to 9 years) have much lower OCR values than young lees (1 to 3 years).

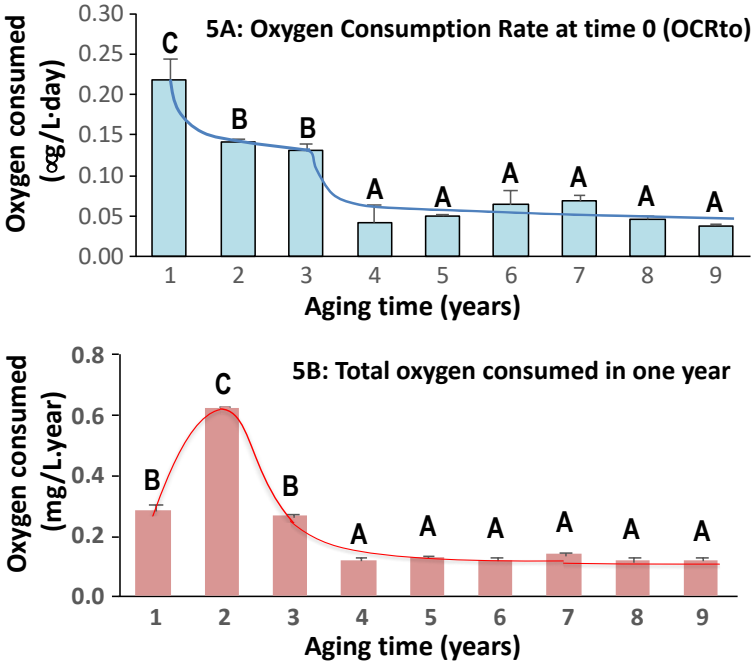

Figure 5: Oxygen Consumption Rate (OCR) of the different aged lees from 1 to 9 years old

Therefore, it is logical to consider that the balance between the entrance of oxygen through the crown cap and the consumption of oxygen by the lees must determine the evolution of the sparkling wine. As long as the lees' oxygen consumption capacity is greater than the oxygen intake, the sparkling wine will be protected against oxidation. However, what would happen if the lees stop consuming enough oxygen. To better illustrate this balance Figure 6 shows the accumulated oxygen consumption by the lees, considering the addition of their annual oxygen consumption over the aging time. This curve fits reasonably well with a logarithmic equation ($[O_2] = 0.7078 \times \ln(t) + 0.351$; $r^2 = 0.9895$). Figure 6 also shows what the oxygen intake across the crown cap would be considering the minimal value of oxygen permeability reported by Valade et al. (2007).

The comparison of the two curves is just a theoretical approximation, but even so it provides very interesting information.

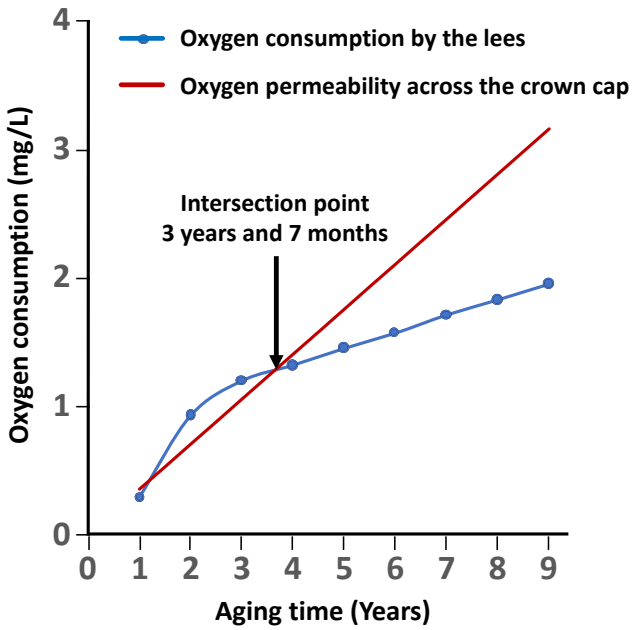

Figure 6: accumulation of oxygen consumed by the lees in comparison with the oxygen permeability of the crown cap.

According to this approach the oxygen intake across a crown cap of low permeability remains below the accumulated oxygen consumed by the lees during the first three years of aging time and exceeds it at around three and a half years. More precisely the exact interception point is at 3 years and 7 months. This means that after this aging time, the oxygen consumed by the lees would not be high enough to compensate the oxygen intake across the crown cap. This would leave the sparkling wine without a sufficient defense against the oxidation that oxygen intake could cause. It should be taken into account that the considered permeability was very low and that any increase in this permeability would entail an earlier point of intersection in time. As an example, with a 20% higher permeability the intersection would take place just after two years. As mentioned above, this is only a theoretical approach based on our results; however, it illustrates what may occur during sparkling wine aging.

4.2.4. Conclusions

It can be concluded that lees of sparkling wines elaborated using the traditional method have a real capacity to consume oxygen and consequently to protect sparkling wines

against oxidation. However, the lees' capacity to consume oxygen decreases drastically after three years of aging, which could seriously affect the ability of sparkling wines to age properly. These data also explain what AOC Cava winemakers know empirically. Only very well selected sparkling wines made using the traditional method can age more than three years without being affected by oxidation.

Funding

This research was funded by the Spanish Ministry of Science and Innovation, *Centro para el Desarrollo Tecnológico Industrial* (CDTI) (Programa CIEN, Project GLOBALVITI: Global solution to improve wine production against climate change based on robotics, IT technology and biotechnological strategies and vineyard management).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to Juvé & Camps (Sant Sadurní d'Anoia, Barcelona, Spain) for providing the sparkling wines of different aging times.

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4.3. Chapter III: Measuring the oxygen consumption rate of some inactivated dry yeasts: comparison with other common wine antioxidants.

Received: 17 February 2021 | Accepted: 9 March 2021 | Published: 21 April 2021
DOI:10.20870/oeno-one.2021.55.2.4618

Measuring the oxygen consumption rate of some inactivated dry yeasts: comparison with other common wine antioxidants

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After confirming that lees really consume oxygen (chapter II) the aim of this new study was to determine if inactivated dry yeasts (IDY) could also do it. For that reason, the oxygen consumption rate of three inactivated dry yeasts was determined in a model wine solution in comparison with the other more usual wine antioxidants: Sulfur dioxide, ascorbic acid and glutathione.

4.3.1. Introduction

Wine aging on lees is currently a widespread practice in oenology, especially for white wines (Dubourdieu, 1992; Feuillat, 1994). Due to the phenomenon of autolysis, the presence of lees enriches the wine with many compounds that improve its quality (Fornairon-Bonnefond *et al*., 2002; Alexandre and Guilloux-Benatier, 2006). It has been reported that lees release many components of yeast membranes and cytoplasm into wine, such as polysaccharides (Martínez-Lapuente *et al*., 2013), mannoproteins (Martínez-Lapuente *et al*., 2015) peptides and proteins (Moreno-Arribas *et al*., 1996; Luguera *et al*., 1998), amino acids (Martinez-Rodriguez *et al*., 2002), lipids (Pueyo *et al*., 2000) and nucleotides (Charpentier *et al*., 2005). It therefore seems clear that the process of yeast autolysis completely transforms the composition of wine, improving some of its sensory attributes (Kemp *et al*., 2015).

Yeast autolysis has been reported to have different sensory effects on wine. Polysaccharides and mannoproteins have been found to improve wine mouthfeel (Gawel *et al*., 2018) and, in combination with some peptides and proteins, to contribute to wine sweetness, smoothing the perception of acidity and astringency (Marchal *et al*., 2011). Furthermore, some amino acids, peptides and nucleotides can contribute to creating an umami taste (Vilela *et al*., 2016) and have been described as flavour enhancers, while lipids and amino acids have been described as aroma precursors (Styger *et al*., 2011). Additionally, mannoproteins seem to exert a protective colloid effect, increasing the stability of proteins (Waters *et al*., 1994) and tartaric acid salts (Gerbaud *et al*., 1997).

Lees has also been reported to consume oxygen, slowing down the oxidation of the wine (Salmon *et al*., 2000; Pons-Mercadé *et al*., 2021). The mechanism by which this occurs is not known. Some authors associate it with the oxidation of membrane lipids (Fornairon-Bonnefond and Salmon, 2003), but it may also be related to the release of glutathione by yeast (Kritzinger *et al*., 2013). Whatever the mechanism, oxygen consumption by lees appears to be the main factor involved in white wines aged on lees being able to age for longer than other white wines.

In short, wines aged on lees are generally richer in mouthfeel and have greater depth and flavour complexity, and white wines on lees tend to age better. The practice, however, is

laborious and entails certain risks, such as the appearance of reductive characters (Dubourdieu, 1995) and *Brettanomyces* taints (Renouf *et al*., 2007). For this reason, inactivated dry yeasts (IDY) are now used, because in theory they have the same effect as lees, but without any of the drawbacks (Pozo-Bayón *et al*., 2009; Del Barrio-Galán *et al*., 2011).

Several studies have examined how IDYs can contribute to wine composition and quality. Many positive effects have been described, such as improving mouthfeel and reducing the perception of acidity (Charpentier, 2010; Del Barrio-Galán *et al*., 2012), smoothing of astringency (Mekoue-Nguela *et al*., 2015; González-Royo *et al*., 2016; Del Barrio-Galán *et al*., 2019) and bitterness (Pozo-Bayón *et al*., 2009a; González-Royo *et al*., 2013; Del Barrio-Galán *et al*., 2019), improving the foaming properties of sparkling wines (Vanrell *et al*., 2005; Marti-Raga *et al*., 2016; Medina-Trujillo *et al*., 2017), eliminating the presence of ochratoxin (Piotrowska *et al*., 2013; Petruzzi *et al*., 2015), preventing browning (Comuzzo and Zironi, 2013; Comuzzo *et al*., 2015) and ensuring oxidation does not affect aroma (Pozo-Bayón *et al*., 2009b; Rodríguez-Bencomo *et al*., 2014) due to the ability of IDYs to release glutathione and other antioxidant compounds (Gabrielli *et al*., 2017; Bahut *et al*., 2020).

It has also been recently reported that some IDYs consume oxygen in a similar way to other common antioxidants used in wine, such as sulfur dioxide (Sieczkowski et al., 2016a, 2016b; Pons *et al*., 2019). However, little is known about using IDY to protect wine against oxidation. Therefore, the aim of this study was to measure the kinetics of oxygen consumption using different IDYs and to compare them with those of other common wine antioxidants (sulfur dioxide, ascorbic acid and glutathione) in order to determine their real antioxidant capacity for protecting wine from the detrimental effects of oxygen.

4.3.2. Materials and methods

4.3.2.1. Chemicals

Absolute ethanol, L-(+)-tartaric acid, sodium hydroxide, formic acid, hydrogen chloride and potassium metabisulfite were purchased from Panreac (Barcelona, Spain). Copper

(II) sulfate pentahydrate, iron (III) chloride hexahydrate, ascorbic acid, disodium ethylenediaminetetraacetate dihydrate (EDTA), 5,5´-dithiobis-2-nitrobenzoicacid (DTNB), tris(hydroxymethyl)aminomethane (TE8), cysteine, γ-L-glutamyl-L-cysteine and glutathione were purchased from Sigma Aldrich (St Louis, USA). Two different commercial inactivated yeasts (IDY-1: Pure-Lees™ Longevity and IDY-2: Noblesse®) and an experimental (IDY-3: Antiox-1), all provided by Lallemand Inc (Montreal, Canada), were used. IDY-1 and IDY-3 were specifically selected for their ability to consume oxygen, whereas IDY-2 was developed as a substitute for lees for its capacity to release polysaccharides and mannoproteins. Pure water was obtained from a Milli-Q purification system (Millipore, USA).

4.3.2.2. Experimental design

The experimental design for determining the oxygen consumption rate of IDYs and other antioxidants was an adaptation of those previously described by Navarro *et al*. (2016) and Pascual *et al*. (2017) for the oxygen consumption rate of oak chips and oenological tannins respectively. A model wine solution composed of ethanol (12 % v/v) and L-(+)-tartaric acid (4 g/L) adjusted at pH = 3.5 with sodium hydroxide was used. This solution was enriched with 3 mg of iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L in the form of copper (II) sulfate pentahydrate. We worked with this model wine solution rather than with real wine, because the naturally occurring phenolic compounds would have competed with the IDY or other antioxidants, making it impossible to determine their oxygen consumption kinetics. This model wine solution was saturated in oxygen (around 8.0 mg/L) by bubbling with air for 10 min just before beginning the experiment.

The usual doses of IDY (400 mg/L), potassium metabisulfite (30 mg/L), ascorbic acid (100 mg/L) or glutathione (20 mg/L) were placed in clear glass bottles (750 mL) into which a pill had previously been inserted (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203-01_PSt3-0828-01, Regensburg, Germany) for the noninvasive measurement of dissolved oxygen by luminescence (Nomasense TM O2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium). The bottles were completely filled with the model wine solution and then immediately closed with a crown cap and bedule so as to minimize the headspace volume. The bottles were

then gently shaken to resuspend the IDYs or to dissolve the different antioxidants: sulfur dioxide (added as potassium metabisulfite), ascorbic acid or glutathione. The bottles were maintained at 20 ± 2 ºC during all this time. Oxygen was measured (Diéval *et al*., 2011) periodically to determine the oxygen consumption rate: oxygen measurements were made every day for the assays containing IDYs, sulfur dioxide and glutathione over two weeks, and every hour for the assay containing ascorbic acid over one day. Figure 1 is a schematic representation of the experimental design.

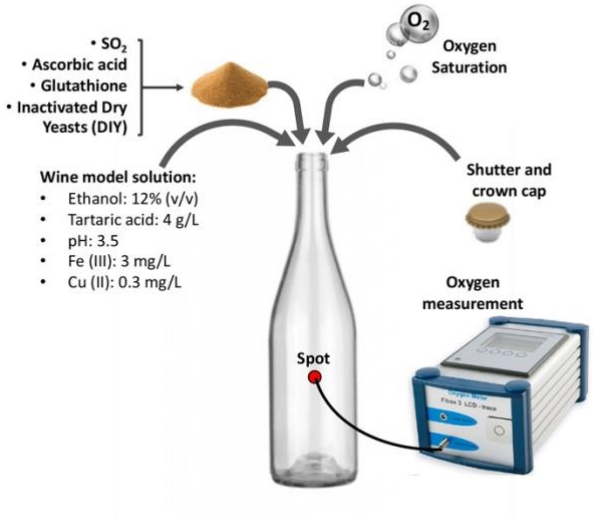

Figure 1. Experimental design for measuring oxygen consumption.

Two types of control were prepared. Control-A bottles were filled with the oxygen-saturated model wine solution without adding any antioxidant, and Control-B bottles were filled with the same model wine solution, but after bubbling for 10 min with nitrogen to eliminate the oxygen. Control A was used to verify that the model wine solution did not consume oxygen, and Control B was used to verify the tightness of the closure system. All assays were performed in triplicate.

4.3.2.3. Modelisation of the oxygen consumption kinetics

In order to quantify the real oxygen consumption ability of the IDY in comparison with that of the other common antioxidants, we applied the kinetic model proposed by Pascual *et al*. (2017) for estimating the oxygen consumption kinetics of oenological tannins. This model consists in displaying the inverse of consumed oxygen versus the inverse of time.

According to this mathematical model, the following equation can be established: 1/[O2] = A/t + B. This equation, which describes the relationship between oxygen consumed and time, can be used to determine the oxygen consumption rate at time zero (OCRto) and also the total oxygen consumption capacity (TOCC). Figure 2A shows how the consumed oxygen can be cleared up. Figure 2B shows how the OCRto can be determined after calculating the first derivative and considering time equal to zero. Finally, Figure 2C shows how the TOCC is obtained by calculating the limit when time tends towards infinity.

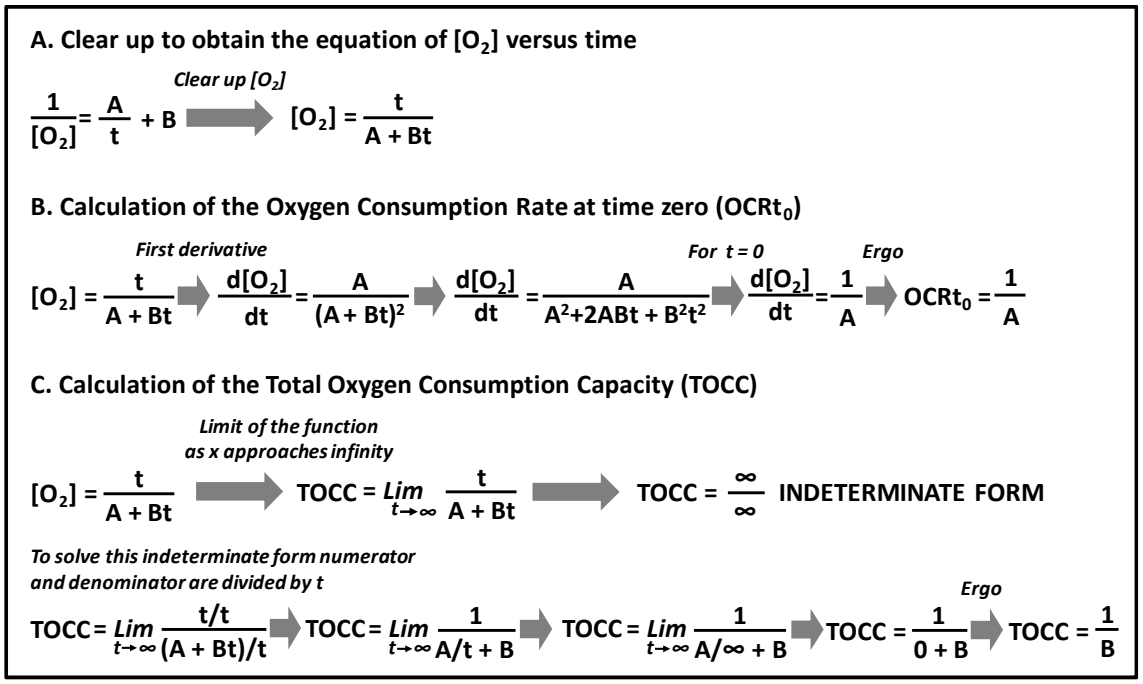

Figure 2. Modeling the Oxygen consumption by the different antioxidant products.

According to this model, the $OCRt_o$ corresponds to the inverse of the slope of the initial equation and the TOCC to the inverse of the y-intercept of the initial equation.

4.3.2.4. Determination of the glutathione content of inactivated dry yeasts

Glutathione extraction and analysis were performed according to an adaptation of the method reported by Nisamedtinov *et al*., (2010).

4.3.2.5. Reagents

TE8 solution: 6.05 g/L of tris(hydroxymethyl)aminomethane and 1.12 g/L of EDTA disodium salt dehydrate adjusted at pH = 8.00 with HCl 1N. This solution was stored at 4 °C. DTNB stock-solution: 39.6 mg of DTNB + 8 ml of TE8 solution + 2 ml of NaOH 0.1 N. This solution is stable for at least one month when protected from light. DTNB working-solution: 8 ml of DTNB stock-solution + 92 ml of TE8 solution; fresh solution was prepared and used daily.

4.3.2.6. Glutathione extraction

Two grams (dry weight) of each inactivated dry yeast was placed into 15 ml centrifuge tubes to which 5 ml of 0.1 N formic acid solution was immediately added. The samples were maintained at room temperature for one hour with frequent vortex shaking. After one hour had elapsed, the samples were centrifuged (Sorval RC5C) at 11.500 rpm for 10 min at 10 ºC. One hundred □L of the supernatant were added to 4.9 mL of DNTB working solution. The sample was immediately vortexed and kept for 10 min at room temperature. The samples were used immediately for UPLC analysis.

4.3.2.7. Glutathione UPLC Analysis

An ACQUITY UPLC system (Waters, USA), equipped with a C18 column (AccQ Tag Ultra Column, 100 × 2.1 mm, 1.7 □m, Waters, USA) and a photo diode array (PDA) detector ACQUITY PDA 2996 was used for the chromatographic determination of reduced glutathione. A two-gradient mobile phase (A: water + 0.1 % formic acid and B: acetonitrile + 0.1 % formic acid) and a flow rate of 0.3 mL min-1 was used. The standards (cysteine, γ-L-glutamyl-L-cysteine and reduced glutathione) were used for external calibration after derivatisation with DTNB. Results are expressed as mg of reduced glutathione by gram of dry weight.

4.3.2.8. Statistics

All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA) was carried out using the SPSS 15.0 software (SPSS Inc., Chicago, IL).

4.3.3. Results and discussion

4.3.3.1. Evolution of oxygen concentration over time.

Figure 3 shows the oxygen concentration evolution over time of the initially oxygen-saturated model wine solutions with supplements (the different IDYs and the other common antioxidants) or without supplements.

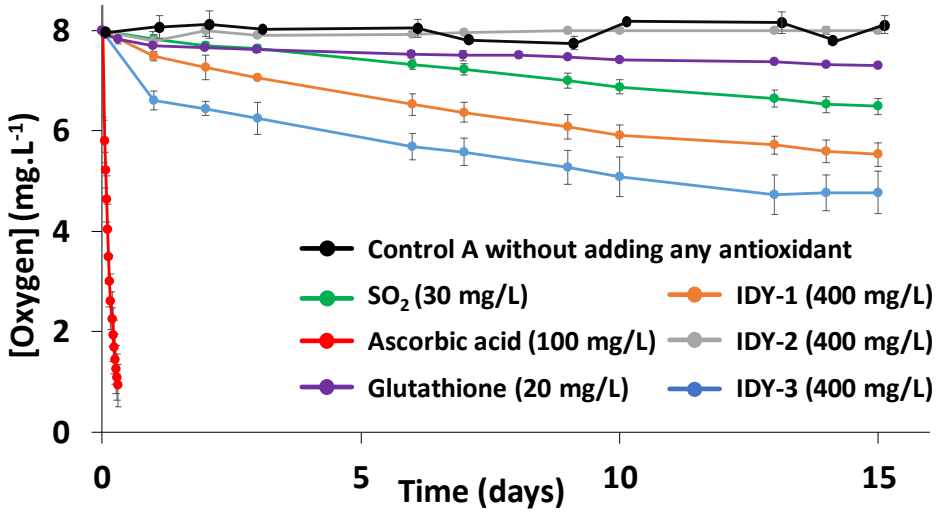

Figure 3. Oxygen concentration evolution in the model wine solution supplemented with the different antioxidant products. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated Dry Yeasts.

The oxygen concentration of Control-A (no antioxidant added) remained stable with only slight, erratic variations that are probably linked to the inherent variability of the analytical procedure. The oxygen intake in Control-B (no oxygen) was very low and can be considered negligible (data not shown). By contrast, the oxygen concentration of the model wine solutions supplemented with the different antioxidants decreased over time, with the exception of IDY-2, where the concentration remained stable.

As expected, the model wine solution containing ascorbic acid consumed all the oxygen in a few hours, confirming that it is more effective than the other antioxidants (Barril *et al.*, 2012).

The graph clearly shows that, with the sole exception of IDY-2, all the antioxidants consumed oxygen: the most effective was IDY-3, followed in decreasing order by IDY-1, sulfur dioxide and glutathione.

4.3.3.2. Modeling oxygen consumption kinetics.

Since Figure 3 does not provide a clear quantitative comparison of the oxygen consumption kinetics of the IDYs and the other antioxidants, we applied the kinetic model proposed by Pascual *et al*. (2017) to better estimate the real capacity of both IDYs to consume oxygen in comparison to that of the other common antioxidants. This model consists in displaying the inverse of consumed oxygen versus the inverse of time, thus obtaining the following equation: **1/[O2] = A/t + B**. Figure 4 shows the results for ascorbic acid, sulfur dioxide, glutathione and both IDYs. In all cases, satisfactory linear regression coefficients were obtained (between 0.9752 and 0.9921), confirming that this mathematical model works reasonably well.

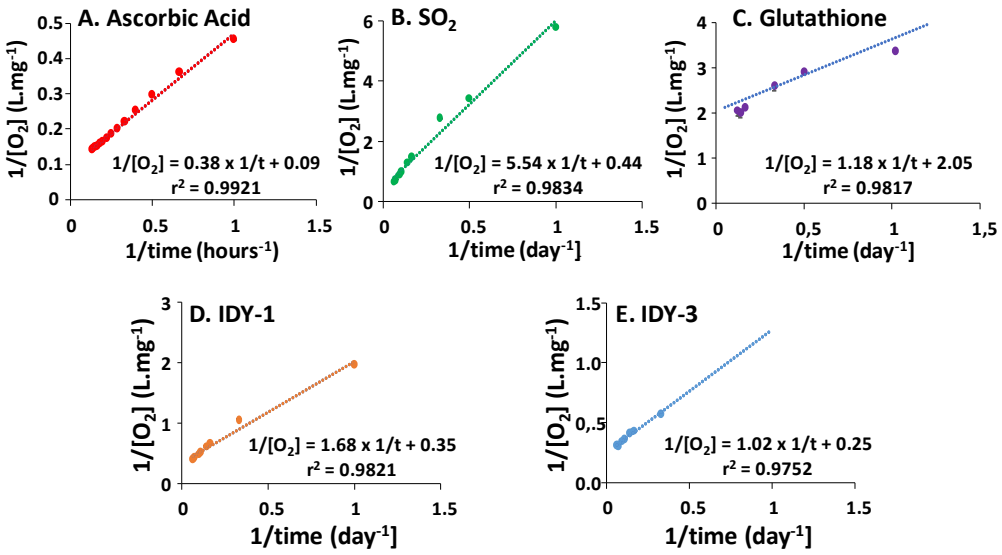

Figure 4. Application of the oxygen consumption model to the different antioxidant products. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated Dried Yeast.

According to the model, the OCRto corresponds to the inverse of the slope and the TOCC corresponds to the inverse of the y-intercept of the above equation (See Figure 2).

After clearing up the oxygen, the equation **[O2] = t/(A + Bt)** was obtained, which can be used to compare the experimental data (Figure 5A) with the theoretical data (Figure 5B) obtained after applying this model.

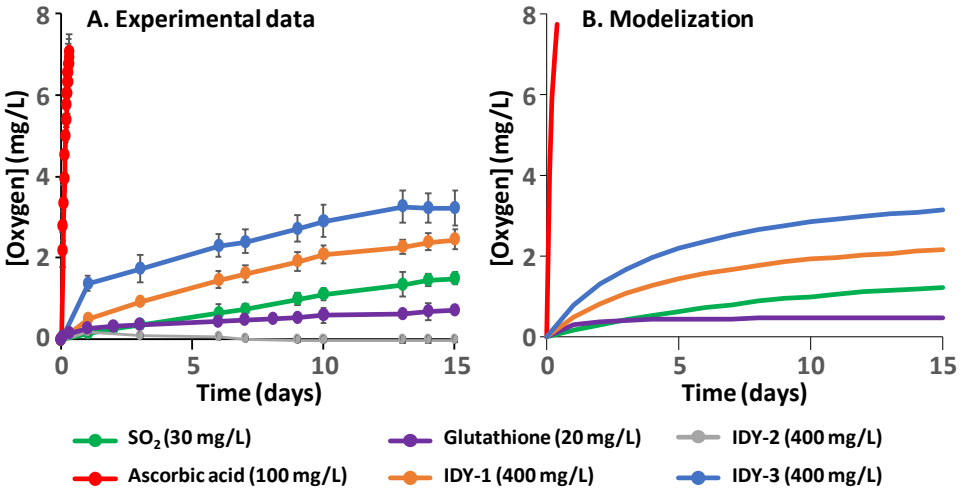

Figure 5. Oxygen consumption of the different antioxidant products; Comparison between experimental and model data. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated dry yeasts.

As the figures show, the mathematical model reproduces the experimental kinetics of oxygen consumption by all the antioxidants quite well. Once again, these results demonstrate the validity of the model.

4.3.3.1. Oxygen consumption rate at time zero and total oxygen consumption capacity.

As explained above, the kinetic model of Pascual *et al.* (2017) can be used to determine two parameters that define the kinetics of oxygen consumption: OCRto and TOCC. Table 1 shows the resulting values for both IDYs and the other tested antioxidants. It also shows the oxygen consumption rate at time zero (% ROCRto) and the total oxygen consumption capacity (% TOCC) of the different antioxidants compared to those of sulfur dioxide at the usual doses.

Sulfur dioxide, which is by far the most widely used antioxidant in wine production, showed the lowest OCRto of all the studied antioxidants, indicating that its direct reaction with oxygen is slow. However, sulfur dioxide is mainly used as an antioxidant because of its ability to react with hydrogen peroxide formed by oxidation of polyphenols

(Danilewicz, 2015), its effectiveness to inhibit polyphenol oxidases (Vignault *et al.*, 2020), and its capacity to react with ethanal and therefore eliminate its unpleasant smell (Sheridan and Elias, 2016). The TOCC obtained for sulfur dioxide using this model was lower than the corresponding stoichiometric value of 30 mg of SO_2/L (2.27 mg/L versus 7.50 mg/L respectively). This difference can probably be attributed to the fact that the reaction is very slow, and that after 15 days not all of the sulfur dioxide had been consumed.

Table 1: Oxygen consumption rate at time 0 and total oxygen consumption capacity. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated Dry Yeasts; OCRto: Oxygen consumption rate at time 0; % ROCRto: Relative OCRto referred to sulfur dioxide; TOCC: Total oxygen consumption capacity; RTOCC: Relative TOCC referred to sulfur dioxide.

Product	Usual dose	OCRto mg/L.day	%ROCRto	TOCC mg/L	%RTOCC
SO_2	30 mg/L	0.18 ± 0.06 A	100	2.27 ± 0.76 A	100
Ascorbic Acid	100 mg/L	63.16 ± 2.34 D	35089	9.52 ± 0.41 D	419
Glutathione	20 mg/L	0.85 ± 0.09 C	472	0.49 ± 0.05 C	22
IDY-1	400 mg/L	0.6 ± 0.01 B	333	2.86 ± 0.05 B	126
IDY-3	400 mg/L	0.98 ± 0.17 C	544	4.02 ± 0.40 C	177

The % ROCRto results indicate that ascorbic acid initially consumes oxygen around 350 times faster than sulfur dioxide, and the % TOCC indicates that it can consume around 4.2 times more oxygen than sulfur dioxide at the usual doses. These data confirm the high effectiveness of ascorbic acid reported in the literature (Barril *et al.*, 2012). However, the fact that ascorbic acid generates hydrogen peroxide after consuming oxygen must be taken into account, as its use in wine may cause subsequent oxidations (Gibson, 2006; Oliveira *et al.*, 2011) which can affect the sensory quality of the wine. For this reason, ascorbic acid must be always used in the presence of sulfur dioxide to prevent wine oxidation (Barril *et al.*, 2016). The TOCC obtained using this model was very close to the corresponding stoichiometric value of 100 mg of ascorbic acid/L (9.52 mg/L versus 9.08 mg/L respectively).

The % ROCRto of glutathione is around 4.7 times greater than that of sulfur dioxide but the % TOCC is around 5 times lower. These data indicate that glutathione reacts faster with oxygen than sulfur dioxide; however, the maximal dose of this antioxidant (20 mg/L) authorised by the International Organization of Vine and Wine (OIV, 2018) can only directly consume small amounts of oxygen. To our knowledge, there are no data on the kinetics of oxygen consumption by glutathione in a medium similar to wine. There is extensive literature, however, on its protective effect against browning (El Hosry *et al.*, 2009; Kritzinger *et al.*, 2013) and the loss of certain aromas (Roussis *et al.*, 2007; Rodríguez-Bencomo *et al.*, 2014), especially volatile thiols (Ugliano *et al.*, 2011; Nikolantonaki *et al.*, 2018). The mechanism by which glutathione manifests these protective effects seems to be more closely related to its ability to block the orthoquinones that form grape reaction product (GRP) than to its direct reactivity to oxygen (Nikolantonaki *et al.*, 2014; Webber *et al.*, 2017). The orthoquinones formed by the reaction of oxygen with orthophenols can subsequently react with volatile thiols to form adducts that are no longer volatile, or with other phenols to form melanins that cause browning. Consequently, its blockage by glutathione prevents the loss of volatile thiols and browning. The TOCC obtained using this model was also very close to the corresponding stoichiometric value of 20 mg of ascorbic acid/L (0.49 mg/L versus 0.52 mg/L).

Table 1 also shows the oxygen consumption kinetic parameters of IDY-1 and IDY-3. No data are shown for IDY-2, because this inactivated dry yeast did not show any effects (see

Figure 3). The % OCRto indicates that oxygen is consumed 3.3 times faster by IDY-1 and 5.4 times faster by IDY-3 than sulfur dioxide. Furthermore, the TOCC values for both IDYs were also higher than those of sulfur dioxide: comparison of the % TOCC values shows that IDY-1 and IDY-3 can consume around 1.2 and 1.8 more oxygen respectively. These TOCC values were similar to those reported by Sieczkowski *et al.*, (2016a, 2016b) for an equivalent dose of IDY.

4.3.3.2. Glutathione content of the inactivated dry yeasts

Table 2 shows the glutathione content of the different IDY. The highest glutathione concentration was found in IDY-3 followed in decreasing order by IDY-1 and IDY-2. Table 2 also shows the corresponding glutathione concentration corresponding to the total glutathione released to the model wine solution by the different IDYs and the corresponding stoichiometric oxygen consumption capacity (SOCC).

Table 2. Glutathione content and oxygen consumption. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated Dry Yeasts.

Inactivated dry yeast	Glutathione (mg/g dry weight)	Glutathione (mg/L)	Stoichiometric oxygen consumption capacity (mg/L)	TOCC (mg/L)
IDY-1	8.87 ± 0.95 B	3.55 ± 0.37 B	0.09 ± 0.01 B	2.86 ± 0.25 B
IDY-2	3.95 ± 0.80 A	1.59 ± 0.32 A	0.04 ± 0.01 A	n.d
IDY-3	13.90 ± 1.41 C	5.56 ± 0.56 C	0.14 ± 0.01 C	4.02 ± 0.40 C
Glutathione	-	20.00	0.52	0.49 ± 0.22 C

Two clear conclusions can be drawn from these results: first, the glutathione concentration released by all the IDYs is much lower than the maximal authorised dose of pure glutathione; second, the IDYs better able to consume oxygen were precisely those that released more glutathione (IDY-1 and especially IDY-3). However, the levels of glutathione released by the different IDY does not justify all the oxygen consumption.

Figure 6 displays the total oxygen consumption capacity (TOCC) of pure glutathione, and the different IDYs versus their corresponding values of the stoichiometric oxygen consumption capacity (SOCC).

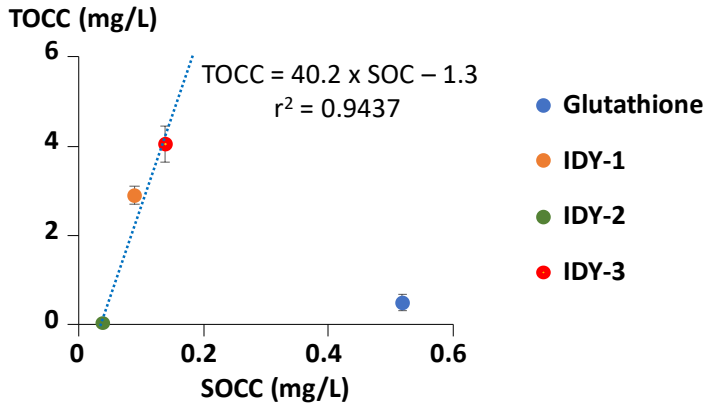

Figure 6. Total oxygen consumption capacity versus stoichiometric oxygen consumption capacity of glutathione and inactivated dry yeasts. All data are expressed as the average of 3 replicates ± standard deviation. IDY = Inactivated Dry Yeasts; TOCC: Total oxygen consumption capacity; SOCC: Stoichiometric oxygen consumption capacity.

This figure indicates a reasonably good correlation between TOCC and SOCC for the three studied IDYs. It also shows that IDY-2 does not consume oxygen and that IDY-1 and IDY-3 consume much more than the corresponding stoichiometric oxygen consumption capacity of the glutathione they are able to release. Therefore, these data indicate that the oxygen consumption by IDYs must be related to mechanisms other than glutathione release.

4.3.4. Conclusion

This work showed that nearly all the studied antioxidants directly consume oxygen in a model wine solution. Ascorbic acid consumed oxygen much faster than the other antioxidants, followed in decreasing order by IDY-3, glutathione, IDY-1 and sulfur dioxide at the usual doses. Ascorbic acid also showed the highest total oxygen consumption capacity, followed in decreasing order by IDY-3, IDY-1, sulfur dioxide and glutathione.

Furthermore, these results confirm that some inactivated dry yeasts are more effective than sulfur dioxide at directly consuming oxygen; they could therefore be a very useful tool for protecting wine against oxidation. However, the direct oxygen consumption by

IDYs cannot be justified by their content in glutathione since the corresponding stoichiometric consumption is much lower than their real oxygen consumption capacity. Further research is needed to investigate the mechanisms by which some inactivated dry yeasts consume oxygen, and to determine whether they can be used to reduce the dose of sulfur dioxide while still preventing spoilage due to oxidation.

Acknowledgments

This research was funded by the Spanish Ministry of Science and Innovation, Interministerial Commission for Science and Technology (CICYT - project RTI2018-095658-B-C33) and Centre for the Development of Industrial Technology (CDTI – CIEN program, projects GLOBALVITI and CAVAWINNER).

4.3.6. References

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5. GENERAL DISCUSSION

The main research line of this thesis was to study the ageing process thorough time in sparkling wine (Cava) produced by the traditional method. During ageing, different processes take place that influence strongly the sparkling wines composition, quality and sensory properties. This investigation is especially focused on the probably main processes: yeast autolysis and oxidation. To our knowledge this is the longest period of time whatever studied in sparkling wines from the AOC Cava.

With the aim of perform this study, sparkling wines from AOC Cava of nine consecutive vintages were used. The sparkling wines of these nine vintages were elaborated as much similarly as possible in order to minimize the possible variation of their highly complex matrix, which is influenced by many environmental conditions.

As expected the intensity of yellow colour tended to be higher when the sparkling wines were older. In a similar way, the perception of sensory oxidation was higher in the older vintages. Being some of them considered as inacceptable by the tasting panel. In contrast, no clear trend was observed in the foaming properties although the sparkling wines from the second and the third vintages seemed to have higher values of foamability (HM) and persistence of the foam (HS). These results correspond to objective 1.A.

Some photographs of the yeasts were taken using a scanning electron microscope (SEM) in order to visualize the lees autolysis in sparkling wines over time. These photographs allow to see how cells structure is degraded over ageing time. First, losing turgor and beginning to show some wrinkles and folds, subsequently being deflated and finally being really very degraded and presenting clearly broken structures. These results correspond to objective 1.B.

Polysaccharide and protein fractions from the nine consecutive vintage sparkling wines were analyzed and no general tendencies were clearly observed in function of the ageing time. This lack of tendency seems to not agree with what should be expected from yeast autolysis. However, this erratic behaviour was also reported previously by other authors and it could be due to complex balance generated by the vintage effect, the different releasing yield of lees, precipitation phenomena, absorption of riddling agents or enzymatic degradation. These results correspond to objective 1.C.

In order to analyze clearly which is the real effect of lees in Sparkling wine, a new approach was proposed placing the recovered lees from the disgorging of the nine consecutive vintages' sparkling wines in a model solution for a year. This model was used for objectives 1.D and 2.

The polysaccharide and protein fractions released from lees of different aging time in a model wine solution after one year of contact were analysed. These results allow to clearly quantify the macromolecules released by lees from each year and also draw a significant tendency throughout the aging time. On the one hand, polysaccharide fraction reached a maximal level at the second year with a value around 6 mg/L to follow later a progressive decrease trend. On the other hand, protein fraction reached a maximal value at the third year aging with a value around 0.32 mg/L to show later a progressive decrease. The total accumulation of polysaccharides at the end of the nine years 'period was 26.6 mg / L whereas that of proteins was 2.4 mg / L. Mannose was also quantified to confirm that the majority of the polysaccharides released by lees were mannoproteins. These results correspond to objective 1.D.

The different release values of polysaccharides and proteins from the lees of the different aging time were used to determine the theoretical accumulation of both macromolecules over time. This strategy was considered with the aim of elucidating the relative weight of the polysaccharides and proteins released from yeast autolysis in comparison with the total concentration present in the corresponding sparkling wines. This approach shows that the percentage of polysaccharide and protein fractions released by lees were really low at the first year of aging (3% and 2% respectively) reaching values of 14% for polysaccharides and 16% in the case of proteins at the seventh year of aging. These data are relevant because the majority of sparkling wines elaborated are commercialized in the world are aged very young, many of them with around of one year of aging, being this time much shorter in the case of sparkling wines elaborated with other methods different from traditional method. These results suggest that the impact of the autolysis process in these products is considerably low. It could be therefore though that longer aging would be necessary if it is wanted to elaborate sparkling wines with a real impact of lees autolysis. However, it must be taken into account that the trained tasting panel considered that sparkling wines after sixth year of ageing started to be non-acceptable probably because oxidation becomes too high.

It has been reported that lees play an active role in wine protection against oxidation. However, no data has been reported previously about the oxygen consumption rate of the lees of the sparkling wines elaborated by the traditional method. For that reason, the same experimental model was used for estimating the oxygen consumption by lees during one year. This research corresponds to objective 2.

The results show that the maximum ability to consume oxygen by lees is reached at second year and subsequently decreases progressively. This behavior agrees with the data reported in the results of the objective 1.D. where the release of macromolecules reached its maximum between second and third year approximately. Hence, these data indicate that the maximal oxygen consumption rate match in time with the maximal release of macromolecules, suggesting that probably some of the released substances might exert an antioxidant effect.

After third year, the oxygen consumption decreases drastically, consequently, sparkling wine are less protected against oxidation. Comparing the oxidation capacity of lees and the oxygen permeability through the crown cap, it can be stablished a point approximately around fourth year where the oxygen consumed by the lees starts to be inferior than the oxygen permeated across the crown cap. Due to this balance, the sparkling wine will not be longer completely protected and consequently oxidation could affect negatively the sensory quality.

The sensory data indicates that sparkling wines begun to be considered as non-acceptable after six years of aging. However, the equivalence point between oxygen consumption and intake was reached around the fourth year of aging. This different time could indicate that the spoilage oxidation process is not immediate, being required some time to significantly affect the sparkling wine quality. It should be noted that these results are obtained from a specific product using the same yeast strain, so varying blend, varieties used and yeast strain could modify the oxidation profile.

As result of collaboration with Lallemand society, we wondered if yeast lees really consume oxygen why not inactivated dry yeasts could do it too. For that reason, the oxygen consumption rate of three inactivated dry yeasts (IDY-1, IDY-2 and IDY-3) was

determined and compared with other common wine antioxidants (sulfur dioxide, ascorbic acid and glutathione) at the usual doses. The results indicate that ascorbic acid consumed oxygen much faster than the other antioxidants. Nevertheless, it must be considered that it generates hydrogen peroxide and it cannot therefore be used without sulfur dioxide. As expected, sulfur dioxide and glutathione also consume oxygen but at much lower rate than ascorbic acid. These results also show that two of the inactivated dry yeasts (IDY-1 and IDY-3) consume oxygen at rates even higher than sulfur dioxide suggesting that some IDY can be useful tools to protect wine against oxidation.

The mechanism by which inactivated dry yeast do not seem to be related with their capacity to release glutathione since the total oxygen consumption of these inactivated dry yeast is much higher than that corresponding to its glutathione content.

Further studies are needed to elucidate the mechanisms by which inactivated dry yeast consume oxygen and to verify if inactivated dry yeasts can be used to reduce or even eliminate the use of sulfur dioxide without affecting the wine composition and quality.

6. CONCLUSIONS

1. As expected the sparkling wine colour tended to be more yellowish as the ageing time was longer.

2. Foamability (HM) and persistence of the foam (HS) reached maximal values during the second and third years of ageing. Subsequently both foam parameters tended to decrease.

3. The concentration of the main macromolecules, polysaccharides and proteins, of sparkling wine produced by traditional method (Cava) did not show a clear tendency during ageing in contact with lees. In general, this erratic behavior was observed in all the molecular weight fractions of polysaccharides and proteins.

4. The maceration for one year of the recovered yeasts from the sparkling wines from the nine consecutive vintages in a model wine solution show that lees release polysaccharides and proteins. In general, the release of both macromolecules reached a maximum at the second-third year to decrease progressively later. This new approach allowed to confirm that yeast autolysis really takes place and exert a role on the composition on polysaccharides and proteins of sparkling wines.

5. The analysis of mannose composition of the polysaccharides released from lees confirm that they are mainly mannoproteins.

6. The proportion of polysaccharides and proteins released from lees represent a small proportion when compared with the regular concentration of these macromolecules in sparkling wines. This may be the reason because some authors have not found any clear releasing tendency over ageing time.

7. During the first year of ageing, the proportion of polysaccharides and proteins coming from yeast represent only 2-3 % of their total content to reach around only 7 % after the third year of ageing.

8. According with these results, the real impact of polysaccharides and proteins from lees autolysis in the sparkling wines disgorged before the end of the first year is very low and in sparkling wines made by other methods is even lower.

Consequently, only sparkling wines aged for longer would therefore benefit from a greater presence of polysaccharides and proteins from yeast autolysis.

9. The images achieved using a Scanning Electron Microscopy (SEM) confirms the degradation process of autolysis that yeast undergo during ageing process and how it affects to their external structure.

10. It is confirmed for the first time that the lees of sparkling wines consume oxygen. The oxygen consumption rate reached a maximal level on the second year to subsequently decrease progressively.

11. Comparing the accumulated oxygen consumption by the lees with the theoretical accumulated oxygen permeation across the crown cap, the intersection would take place around 3 and half to 4 years of ageing. Consequently, the sparkling wines would be not well protected against oxidation after this period of time.

12. This last conclusion was confirmed by the trained panel since they began to consider the sparkling wines as ‘not acceptable’ after the fifth year of ageing.

13. It is confirmed, also for the first time, that some inactivated dry yeasts consume oxygen. They can therefore be considered as possible tool to protect the wine against oxidation.

14. The comparison between the initial oxygen consumption rate and the total oxygen consumption capacity of two of the studied inactivated dry yeast shows that are more effective that sulfur dioxide and glutathione. However ascorbic acid was much more effective.

15. It seems that the mechanism by which inactivated dry yeasts consume oxygen is not completely related with their content in glutathione.

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