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Monitoring yeast autolysis in sparkling wines from nine consecutive vintages produced by the traditional method

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Abstract

Background and Aims: Yeast autolysis is a key step in the production of sparkling wines using the traditional method and a source of polysaccharides, mannoproteins and proteins. The evidence, however, of the enrichment of these macromolecules during ageing is not evident in the literature. In this study, autolysis of yeast lees in sparkling wines (Cava) was monitored over nine consecutive vintages.

Methods and Results: The concentration of polysaccharides and proteins did not clearly increase over time, probably because these macromolecules are simultaneously released from lees and removed by other processes. In a new approach, the autolytic process was reproduced, and involved recovering the lees and maintaining them in a model wine solution for 1 year. The lees released polysaccharides and proteins, but only a small proportion of that found in the sparkling wines, especially in younger wines.

Conclusions: This study confirmed that yeast enrich sparkling wines in key macromolecules but in low proportion in comparison with the usual concentration present in sparkling wine. This aspect should be considered by wineries that aim to achieve autolysis attributes, since the majority of sparkling wines are commercialised after short periods of ageing.

Significance of the Study: This study determined for the first time the true contribution of yeast autolysis to the total polysaccharide and protein concentration of sparkling wines from nine consecutive vintages.

Keywords: *ageing, Cava, polysaccharides, proteins, sparkling wines, yeast autolysis*

Introduction

Cava is a sparkling wine produced by the traditional method, which involves two alcoholic fermentations (Maujean 1989). In the first fermentation, grape juice is transformed into a base wine according to a standard winemaking process. This base wine then undergoes a second fermentation in the bottle called the *prise de mousse*. The base wines are supplemented with a sugar solution (approximately 22 g of sucrose/L) called the *liqueur de tirage* (Kemp et al. 2015), which provides the necessary sugar for the second fermentation, as well as processing aids such as bentonite and/or alginates, to facilitate the riddling process (Vanrell et al. 2007). Finally, a starter culture of pre-adapted yeast is added to ensure that the second fermentation achieves successful completion. 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). The lees enable longer ageing times for sparkling wines because they protect the wine against oxidation by consuming the small amounts of oxygen that permeate the crown cap (Pons-Mercadé et al. 2021). The extended contact with lees is one of the main differences 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 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 (Martínez-Lapuente et al. 2013) and mannoproteins, into the wine (Martínez-Lapuente et al. 2015). This process alters the final composition, and therefore, the sensory attributes, of the sparkling wine (Kemp et al. 2015).

The release of peptides, proteins, polysaccharides and mannoproteins favours the integration of carbon dioxide, which enhances both effervescence quality and foam stability (Martínez-Rodriguez et al. 2001a, 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 influence the umami taste (Vilela et al. 2016) and to enhance flavour. Finally, amino acids and lipids have been described as aroma precursors (Styger et al. 2011) that contribute to the aromatic complexity of sparkling wines.

Natural autolysis is a slow process that requires several months to achieve a significant sensory effect. It begins with passive excretion of amino acids and other small molecules,

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a process called exorption, which takes 1–2 months in still wines and 3–6 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 hydrolyse the intracellular macromolecules (proteins and polysaccharides) and their hydrolysed products accumulate in the periplasmic space. When hydrolysis byproducts become small enough, they can diffuse through the cell wall to enrich the extracellular environment that is the sparkling wine (Fornairon-Bonnefond et al. 2002).

Protease A is said to play an important role in autolysis activity, and is responsible for 60–80% of the total nitrogen released (Lurton et al. 1989). Proteolytic activity appears to be low during the first 8 months of bottle ageing, before gradually increasing in subsequent months and continuing 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 hydrolyse the β-glucan chains that link mannoproteins (Moreno-Arribas et al. 1996), facilitating the subsequent release of glucose, oligosaccharides, polysaccharides and mannoproteins from cell walls.

Yeast autolysis completely modifies the composition of sparkling wine, and therefore, its sensory attributes (Kemp et al. 2015). For these reasons, the most important Appellation d'Origine Contrôlée (AOC) regulations for sparkling wines have established minimum ageing times to ensure that autolysis imparts an effect on wine composition and quality. For Cava, the minimum ageing time stipulated by the AOC is 9 months, though premium Cava is usually aged for a longer period. In fact, the AOC Cava comprises two other categories of sparkling wine with extended ageing times, *Reserva* and *Gran Reserva*, whose minimum ageing time is 15 and 30 months, respectively. Certain prestigious wineries produce Cavas with an even longer ageing time.

While autolysis apparently enhances the quality of sparkling wines, other phenomena take place in parallel—such as aromatic and colour oxidation or development of excessive lees flavour, which can negatively impact wine sensory attributes. Several studies have, therefore, sought to establish the optimal duration of bottle ageing to enhance product quality. Most studies of sparkling wine autolysis have involved relatively short ageing times (Moreno-Arribas et al. 1996, 1998, Riu-Aumatell et al. 2006, Vanrell et al. 2007, Cilindre et al. 2010, Vichi et al. 2010, Martínez-Lapuente et al. 2013, 2015, 2017, Esteruelas et al. 2015) and, to our knowledge, only a few have comprised longer periods of ageing (Loyaux et al. 1981, Leroy et al. 1990, Charpentier et al. 2005, Pons-Mercadé et al. 2021). In this study, we analysed changes in the colour, polysaccharides, proteins, foaming properties and sensory quality of AOC Cava sparkling wines with ageing times ranging from 1 to 9 years, which we believe to be the longest duration of bottle age studied in relation to AOC Cava sparkling wines.

Materials and methods

Chemicals and equipment

Absolute ethanol (96%), 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 analysed with a Helios Alpha UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All HPLC analyses were performed with 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 with a Bacchus 3 MultiSpec equipment (TDI, Gavà, Barcelona, Spain). Vacuum concentration was carried out with a Univap 148100ECH vacuum evaporator (Progen Scientific, London, England) and lyophilisations were conducted using a Telstar lyophiliser (Telstar LyoQuest HT40, Barcelona, Spain). Scanning electron microscopy (SEM) was performed using A Hitachi S4800 microscope. Finally, centrifugation was performed using a Biofuge Primo centrifuge (Heraeus, Hanau, Germany).

Sparkling wine production

Sparkling wines from nine consecutive vintages (2008–2016) were sourced from the Juve & Camps winery (AOC Cava, Sant Sadurní d'Anoia, Barcelona, Spain) for the experiments. The base wines were produced in the most similar way possible, though some differences between vintages were inevitable. Monovarietal base wines were produced in each vintage from 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 originated from Juvé & Camps vineyards in Sant Sadurní d'Anoia (Barcelona, Spain; 41°26′47.42″N, 1°49′0.63″E), which is 165 masl, while Parellada grapes were from Juvé & Camps vineyards in Mediona (Barcelona, Spain; 41°29′48.6″N, 1°39′56.9″E), which is 506 masl.

In each vintage, grapes were harvested at a maturity level suitable for obtaining base wines for sparkling wine production; the TSS ranged from 17.3 to 19.5°Brix for Xarel·lo, from 16.2 to 18.3°Brix for Macabeo and from 15.2 to 16.5°Brix for Parellada. Titratable acidity (TA, as tartaric acid equivalents) ranged from 5.5 to 8.0 g/L for Xarel·lo, from 4.6 to 7.2 g/L for Macabeo and from 5.1 to 7.8 g/L for Parellada. Grapes were immediately pressed in a pneumatic press to obtain a yield of 0.6 L/kg of grape juice. An addition of 100 mg/L of potassium metabisulfite (PMS) and of 20 mg/L of pectinolytic enzymes (Enovin Clar, Agrovin, Alcázar de San Juan, Ciudad Real, Spain) was immediately made to grape juice to facilitate settling. After 24 h, clarified grape juice was racked into stainless steel fermentation tanks and immediately inoculated with 200 mg/L of selected yeasts (*Saccharomyces cerevisiae*; IOC 18–2007; Institut Œnologique de Champagne, Epernay, France). When necessary, TA was corrected by addition of tartaric acid to 6.0 g of tartaric acid/L. Juices were fermented at 16–18°C. Once alcoholic fermentation was finished, base wines were racked and held at 18–20°C until spontaneous MLF was completed. Base wines were then racked again, sulfited (40 mg/L of PMS) and cold stabilised.

Once the monovarietal base wines were considered stable, they were blended to obtain a classic AOC Cava. For each vintage, sparkling wines (Cava) were produced from a blend of 50% Xarel·lo, 30% Macabeo and 20% Parellada,

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using the traditional method and standard bottles (750 mL). Briefly, 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 (*S. cerevisiae*; IOC 18-2007; Institut Œnologique de Champagne, Epernay, France). Wines were then bottled, crown sealed and stored at 12–15°C until disgorgement. This procedure was employed for each of the nine consecutive vintages in order to reproduce, as far as possible, the same production conditions for each vintage studied.

Disgorging of sparkling wine and lees recovery

Three months after *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 performed manually. Once all the lees sediment was in the neck of the bottle, the contents of the bottle were disgorged without freezing to facilitate collection of the lees. The sparkling wines from other vintages were also disgorged in triplicate 3 months after completing 1 to 9 years of ageing. This enabled isolation of lees from the first to the ninth years of ageing, and subsequently, determination of the metabolites released from lees of different ages (1–9 years) over a 1-year duration.

The lees sediment was recovered in a plastic bucket and immediately transferred to a centrifuge tube. The tubes were then centrifuged for 5 min at 7840 *g*. The resulting pellet was washed with 10 mL of saline solution (NaCl, 0.9% v/v) and centrifuged again. This operation was repeated twice.

Meanwhile, the bottles of sparkling wines were immediately corked without addition of any expedition liqueur and stored at ambient temperature prior to analysis. Samples were centrifuged for 15 min at 5664*g* to eliminate carbon dioxide and any remaining lees before analysis.

Monitoring the autolysis process

The recovered lees were placed in clear glass 66 mL flasks together with a model wine solution comprising ethanol (12% v/v) and tartaric acid (4 g/L) adjusted to a pH of 3.5 with sodium hydroxide. Lees were, therefore, present at a concentration approximately 11 times higher than in their original bottles. The higher lees dosage was selected to enhance the concentration of polysaccharides and proteins, thereby facilitating their analytical determination. This enabled model wines to be used to determine the quantity of macromolecules released during the autolysis process; analytical results were corrected to the original sparkling wine volume (750 mL), while taking into account the higher lees dose rate. The flasks were kept motionless at 20 ± 2°C for exactly 1 year, after which samples were centrifuged for 15 min at 5664 *g* and analysed.

The dry mass of the lees from each sparkling wine sample was determined at the end of the experiment. The dry mass, which comprised dead yeasts, bentonite and alginates and other absorbed or precipitated substances, averaged 51.5 ± 19.6 mg/bottle, with no significant difference observed among the different vintages.

Ultrastructural observation of yeasts with SEM

Yeast from the starter culture used for the second fermentation of the final vintage (2016), along with lees from the sparkling wines from the first, third, fifth, seventh and ninth vintages, were recovered and washed as described above. After centrifugation, the yeast/lees were resuspended in 0.1 mol/L phosphate buffer (pH 7.2), centrifuged again and fixed in 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 2 h at 4°C with gentle shaking. After three further washes in 0.1 mol/L phosphate buffer (pH 7.2) for 10 min each, cells where dehydrated in ethanol of increasing proportion (30, 50, 70, 90 and 100%) for 15 min each. After mounting on filters and supports, critical point drying was performed for SEM analysis.

Standard wine analysis

Titratable acidity (TA) was measured by titration against 0.1 N NaOH using bromothymol blue as the indicator and pH was measured with a Basic-20 pH meter (CRISON, Barcelona, Spain). The CIELab coordinates were determined as described previously (Ayala et al. 1997) using a Helios Alpha UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and data processed using MSCV software (Ayala et al. 2001). Ethanol concentration (% v/v) was measured by FTIR (Kupina and Shrikhande 2003) using a Bacchus 3 MultiSpec instrument (TDI, Gavà, Barcelona, Spain).

Measurement of foaming properties

Sparkling wines were held at 18°C for 24 h before foaming properties were determined using the Mosalux method (Maujean et al. 1990). A glass cylinder placed on a glass frit was filled with 100 mL of sample, then CO_2 was injected into the cylinder via the glass frit, with a constant gas flow of 115 mL/min and constant pressure of 100 kPa. A Mosalux device (Station Oenotechnique de Champagne, Epernay, France) was used to measure the maximum height (H_M) of the foam after CO_2 injection through the glass frit, and the stable height (H_S) during CO_2 injection, where H_M represents foamability (the wine's ability to foam), while H_S represents foam stability (the persistence of the foam collar, or the wine's ability to produce a stable foam). They were measured (in mm) in triplicate for each sparkling wine.

Polysaccharide extraction and determination by HRSEC-RID

Polysaccharides were measured in each sparkling wine and model wine solution (in triplicate) using methodology described previously (Ayestarán et al. 2004). Briefly, 10 mL of each sample was concentrated to a final volume of 2 mL using a vacuum evaporator (Univap 148100ECH; Progen Scientific, London, England). Total soluble polysaccharides were precipitated by adding 10 mL of cold acidified ethanol (hydrochloric acid 0.3 mol/L in absolute ethanol) and held at 4°C for 24 h. Samples were then centrifuged at 10 000 *g* for 15 min and the supernatant discarded. The remaining precipitates were dissolved in 1 mL of ultra-pure water, frozen at −20°C, and freeze-dried using a lyophiliser (Telstar LyoQuest HT40, Barcelona, Spain). The lyophilised samples were analysed by high-resolution size-exclusion chromatography (HRSEC) to quantify the polysaccharides and determine their molecular distribution in each sample. Lyophilised samples were re-suspended in 1 mL of 50 mmol/L ammonium formate and filtered through 0.22 µm acetate cellulose filters (Merck Millipore, Darmstadt, Germany). Then 100 µL were injected into the chromatographic system. Analyses were carried out with an HPLC Agilent 1200 Series system (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID). Separation was carried out at 20°C using two Shodex

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gel permeation HPLC columns (OHpak SB-186803 HQ and SB-804 HQ, 300 mm × 8 mm I.D.; Showa Denko, Japan). The mobile phase consisted of an aqueous solution of 50 mmol/L ammonium formate applied with a constant flow of 0.6 mL/min for 60 min, and a cell RID temperature of 35°C. The molecular mass (MM) distribution of the wine fractions was followed by calibration with a Shodex P-82 pullulan calibration kit (P-5, MM = 5.9 kDa; P-10, MM = 11.8 kDa; P-20, MM = 22.8 kDa; P-50, MM = 47.5 kDa; P-100, MM = 112 kDa; P-200, MM = 212 kDa; P-400, MM = 404 kDa and P-800, MM = 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 standards (Sigma-Aldrich) ranging from 0 to 2 g/L ($r^2 > 0.99$).

Sample preparation for analysis

Aliquots (15 mL) of each sparkling wine and model wine solution were concentrated in triplicate following two-step dialysis in tubes with a MM cut-off of 3.5 kDa (Membrane Filtration Products, San Antonio, TX, USA). The first step was completed over 48 h with 0.3 mol/L ammonium acetate solution at a sample-to-solution ratio of 1:10, with constant agitation, while the second step was completed with water over a further 48 h. The dialysed samples were then lyophilised and frozen at −20°C.

Determination of proteins by HRSEC-DAD

The soluble fractions were analysed by HRSEC to quantify proteins and determine their molecular distribution in each sample (Canals et al. 1998). The lyophilised samples were re-suspended in 0.6 µL of ammonium acetate solution (300 mmol/L) and centrifuged at 12 000 *g* for 5 min. The supernatant was filtered through 0.22 µm acetate cellulose filters (Merck Millipore) and 100 µL of supernatant was then injected into the chromatographic system. Analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies, Barcelona, Spain) with a DAD to monitor output at 230 and 320 nm. Separation was achieved at 20°C using an S 165 Shodex gel permeation HPLC column 210 (OHpak 166 SB-803 HQ, 300 mm × 8 mm i.d.; Showa Denko). The mobile phase comprised an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The eluting proteins were quantified according to the peak area for each fraction using the external standard method with a bovine serum albumin standard (Sigma-Aldrich, Madrid, Spain) ranging from 0 to 1 mg/mL ($r^2 > 0.99$).

Acid hydrolysis for mannose quantification

Acid hydrolysis was performed following published methodology (Quirós et al. 2012). The samples corresponding to lees maceration were processed according to the polysaccharide extraction process described above, up to and including the lyophilisation step. The lyophilised samples were dissolved in 3 mL of ultra-pure water, passed through a 30 × 10 mm Econo-Pac 10 DG desalting column (Bio-Rad, Hercules, CA, USA) and eluted with 4 mL of distilled water. Aliquots (1.9 mL) of eluent were evaporated to dryness under vacuum (Univap 148100ECH; Progen Scientific, London, England). The residue was then suspended in 100 µL of 1 mol/L H_2SO_4 and incubated at 100°C for 4 h in an oven. After hydrolysis, samples were diluted to 1 mL with ultra-pure water, before 1 mL of diluted hydrolysate was passed through a Strata NH2 500 mg/3 mL column (Phenomenex, Torrance, CA, USA) to remove any residual sulphuric acid. Ultra-pure water (1 mL) was immediately eluted to recover a final sample volume of 2 mL.

Quantification of mannose by HRSEC-RID

The samples (2 mL) recovered from the Strata NH2 column were filtered through 0.22 µL pore size nylon filters (Phenomenex) before 25 µL aliquots were injected into the chromatographic system. Analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies) with a RID. The column used was a 300 × 7.7 mm Hi-Plex Pb 8 µm (Agilent Technologies). Ultra-pure water was used as the mobile phase at a flux of 0.6 mL/min and a column temperature of 70°C (Quirós et al. 2012). A calibration curve covering a range between 50 and 2000 mg/mL ($r^2 > 0.99$) was prepared for HRSEC-RID quantification.

Sensory analyses

Sensory analyses were performed in a purpose-built tasting room located in the Faculty of Enology, Rovira i Virgili University, Tarragona, designed in accordance with UNE 87004.197) (AENOR 2010). Samples were tasted by 10 panellists (six males and four females, aged between 22 and 62) using ISO tasting glasses (International Organization for Standardization 1997). The panellists, all of whom were experts in sparkling wine tasting, were asked to score the age of the sparkling wines from the nine consecutive vintages, from youngest (1 point) to the oldest (9 points). They were also requested to consider whether any of the sparkling wines were 'too old' by classifying them as 'acceptable' or 'not acceptable'.

Statistical analyses

All chemical and physical properties are expressed as means ± SD of three replicates. The sensory panel's appraisal of the chronological order of the sparkling wines for the nine consecutive vintages was considered the mean score ± SD given by the ten panellists. One-factor ANOVA was conducted using SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results and discussion

The ethanol concentration of the Cavas produced from nine consecutive vintages ranged from 12.0 to 12.6% (v/v), TA from 5.70 to 6.75 g/L and pH from 3.05 to 3.23. Such ranges were considered typical of sparkling wines of AOC Cava. Moreover, no clear trends were observed among vintages that were expected to influence the results.

Table 1 shows the CIELab coordinates of the nine consecutive vintages of sparkling wine. 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 colour.

Table 1 also shows the foaming properties of the sparkling wines. The maximal height of the foam (H_M, foamability) and the stable height of the foam (H_S, foam stability) followed a similar trend, that is they increased significantly between the first and second year of ageing, and progressively decreased thereafter; although 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 (Andrés-

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Table 1. CIELAB coordinates and foaming properties of sparkling wines from nine consecutive vintages.

Ageing time (years)	L*	a*	b*	H_M (mm)	H_S (mm)
1	98.00 ± 0.17 c	−0.88 ± 0.06 cd	6.88 ± 0.12 a	87 ± 1 c	71 ± 3 c
2	98.07 ± 0.06 c	−1.02 ± 0.04 bcd	7.69 ± 0.08 b	170 ± 1 e	93 ± 3 e
3	97.93 ± 0.06 c	−1.11 ± 0.02 ab	8.78 ± 0.07 c	110 ± 5 d	81 ± 1 d
4	97.67 ± 0.15 bc	−1.11 ± 0.07 ab	8.66 ± 0.21 c	80 ± 4 bc	72 ± 2 c
5	97.40 ± 0.00 b	−1.09 ± 0.08 ab	9.87 ± 0.13 e	76 ± 4 bc	68 ± 1 bc
6	97.57 ± 0.06 bc	−1.25 ± 0.08 a	9.59 ± 0.07 de	61 ± 1 a	53 ± 3 a
7	96.77 ± 0.46 a	−1.05 ± 0.03 abc	9.39 ± 0.02 de	73 ± 1 abc	58 ± 2 a
8	97.60 ± 0.10 bc	−1.00 ± 0.10 bcd	10.77 ± 0.19 f	71 ± 4 ab	54 ± 1 a
9	97.37 ± 0.15 b	−0.92 ± 0.10 bcd	10.99 ± 0.34 f	74 ± 1 abc	59 ± 1 ab

Results are means ± SD from three replicates. Different letters (within columns) indicate a statistical significance ($P < 0.05$). a*, green−red component; b*, blue−yellow component; H_M, foamability; H_S, foam stability; L*, lightness.

Lacueva et al. 1996, Gallart et al. 2004, Esteruelas et al. 2015).

Figure 1 shows the polysaccharide and oligosaccharide fractions of the sparkling wines produced from nine consecutive vintages. The total polysaccharide concentration (expressed as pectin and dextran equivalents) ranged from 153 to 228 mg/L. Previous studies have reported a similar concentration of polysaccharides in sparkling wines (Andrés-Lacueva et al. 1997, Martínez-Lapuente et al. 2013, Esteruelas et al. 2015). Some significant differences were found between the vintages but no clear trends were observed with ageing time. This erratic behaviour was also observed in the high molecular mass (HMM, molecular mass > 180 kDa), low molecular mass (LMM, molecular mass between 7.5 and 40 kDa) and oligosaccharide (molecular mass < 7.5 kDa) fractions. However, the intermediate molecular mass (IMM, molecular mass between 40 and 180 kDa) fraction tended to increase over time, though this was not true for all vintages. That polysaccharides did not tend to increase over time appears to contradict the outcomes expected of yeast autolysis. Other authors, however, also found polysaccharides did not increase (Martínez-Lapuente et al. 2016) or they reported a decrease in polysaccharides with ageing of sparkling wines (Moreno-Arribas et al. 2000, Martínez-Lapuente et al. 2013).

One reason for the lack of any trend in polysaccharide composition over time might be explained by their simultaneous release and removal during bottle ageing. Yeast autolysis may be a source of polysaccharides and mannoproteins (Kemp et al. 2019), however, depending on the nature of polysaccharides of grape origin, some will precipitate (Martínez-Lapuente et al. 2013), some will be absorbed by riddling agents (Catarino et al. 2008) and some will be enzymatically degraded (Moreno-Arribas et al. 2000). The current study compared sparkling wines produced in nine consecutive years and the variability in polysaccharide concentration among vintages may have been greater than the quantity released from autolysis, making it impossible to identify any clear compositional trends.

The total protein concentration of the different wines (expressed as BSA equivalents) ranged from 11.0 to 15.2 mg/L (Figure 2). This range of concentration, which agrees with those reported in the literature (Martinez-Rodriguez et al. 2002, Esteruelas et al. 2015), was much lower than the average concentration found in base wines due to removal of protein through binding interactions with the bentonite used as the riddling agent (Vanrell et al. 2007). As with polysaccharides, a significant difference was observed among the protein concentration of sparkling wines from the different vintages, but no compositional trends were attributable to the ageing time, irrespective of protein molecular mass: high molecular mass (HMM, molecular mass > 75 kDa), intermediate molecular mass (IMW, molecular mass between 50 and 75 kDa) and low molecular mass (LMM: molecular mass between 25 and 50 kDa). Again, such results apparently contradict what is expected from yeast autolysis. Other authors have also reported erratic accumulation of proteins during ageing of sparkling wines (Martinez-Rodriguez and Polo 2003, Nunez et al. 2005, Esteruelas et al. 2015).

This lack of any clear trend may also be related to a balance between protein released from yeast autolysis and protein loss due to bentonite adsorption (Moreno-Arribas et al. 1996, Nunez et al. 2005, Vanrell et al. 2007) or enzymatic degradation (Moreno-Arribas et al. 1996, Martinez-Rodriguez and Polo 2003). Again, variability among the original protein concentration of the different vintages of sparkling wine make it difficult to draw conclusions from compositional comparisons.

Since no trends were observed for either the accumulation of polysaccharides or proteins in sparkling wines as a function of their ageing time, the release of these macromolecules from lees was studied using a different approach. This involved placing the lees recovered from the nine consecutive vintages of sparkling wine into model wine solutions and analysing polysaccharide and protein composition after 1 year.

Figure 3 shows the polysaccharides released from the different lees samples after 1 year. The lees corresponding to 1 year of bottle ageing released ~4 mg/L of total polysaccharides. The lees from 2 years of bottle ageing gave a significantly higher amount, at ~6 mg/L, while the polysaccharide concentration from lees from later vintages decreased progressively, reaching the lowest levels (< 1 g/L) in the lees from sparkling wines aged for 8 and 9 years. This pattern of polysaccharide release was similar for all molecular mass fractions (Figure 3).

The oligosaccharide fraction released by the lees also decreased as the age of the lees increased. In this case, however, the maximum value was observed in the lees from the youngest vintage (~5.6 mg/L). A slower decline in oligosaccharides release was observed than for polysaccharides, with a minimum value of ~3.8 mg/L in the lees corresponding to 9 years of bottle ageing. This may reflect hydrolysis of polysaccharides, resulting in a decrease in their average degree of polymerisation.

To evaluate the release of mannoproteins due to yeast autolysis, the polysaccharide samples used for HPLC analysis were hydrolysed in acidic media to determine mannose

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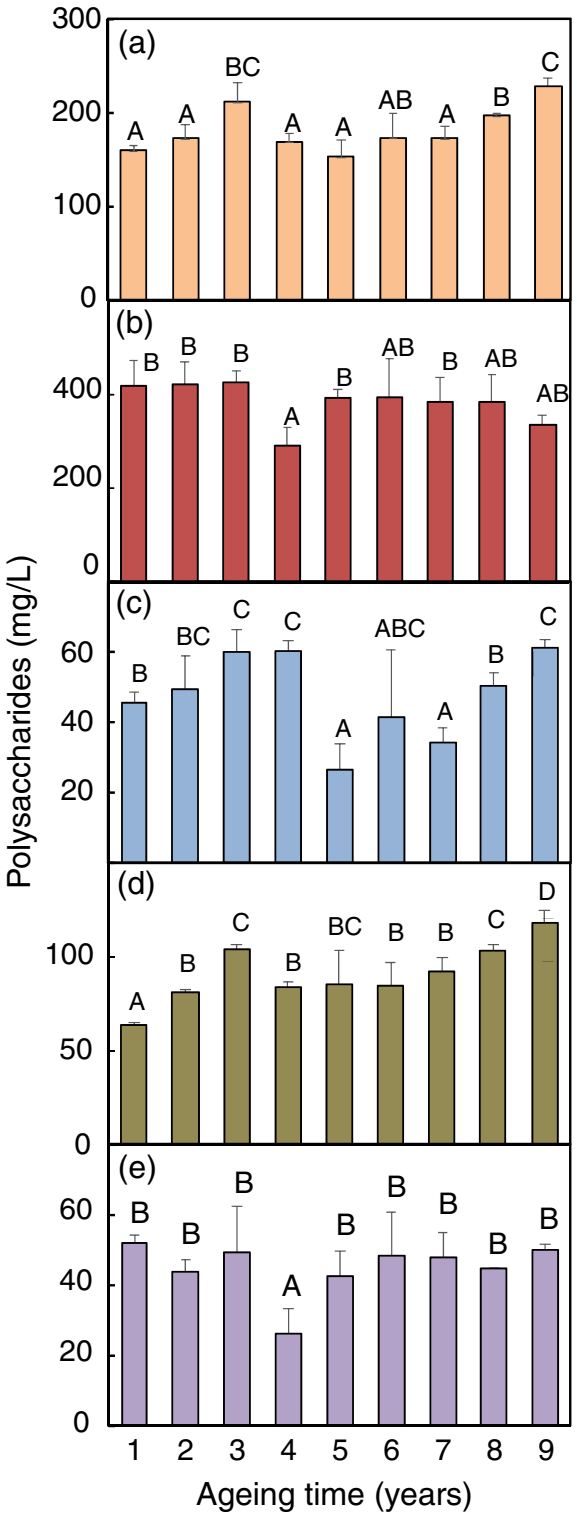


Figure 1. Effect of ageing time on the polysaccharide composition of sparkling wines from nine consecutive vintages: (a) total polysaccharides, (b) total oligosaccharides, (c) >180 kDa high molecular mass fraction, (d) 40–180 kDa intermediate molecular mass fraction and (e) 7.5–40 kDa fraction. Results are expressed and as mean ± SE of three replicates. The concentration of polysaccharides is expressed as pectin and dextran equivalents. Different letters indicate the existence of a statistical difference ($P < 0.05$).

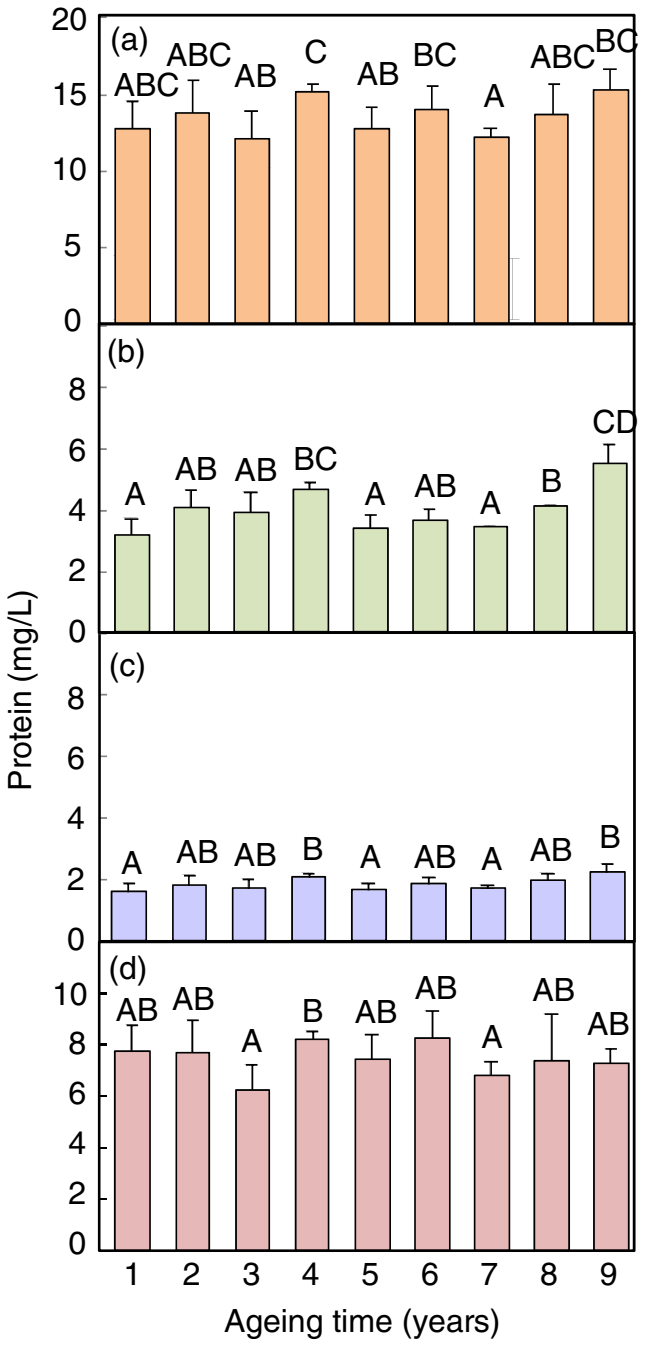


Figure 2. Effect of ageing time on the protein concentration of sparkling wines from nine consecutive vintages: (a) total protein, (b) >75 kDa = high molecular mass fraction, (c) 50–75 kDa fraction and 25–50 kDa fraction. Results are expressed as the mean ± SE of three replicates. The concentration of protein is expressed as bovine serum albumin equivalents. Different letters indicate the existence of a statistical difference ($P < 0.05$).

concentration. Figure 4 shows the mannose present in the polysaccharides released from the different lees samples; the mannose concentration showed a similar pattern to that of total polysaccharides released by the lees (Figure 3a), that is, an initial increase followed by a progressive decline in later vintages. Moreover, the concentration of mannose and polysaccharide was similar, confirming that the polysaccharides released from the lees were mainly mannoproteins.

Figure 5 shows the protein released from the different lees samples after 1 year. Total protein concentration followed a similar pattern to that of total polysaccharides, but the maximum value of 0.32 mg/L corresponded to lees from 3 years of bottle ageing, and the minimum value for lees from 9 years of bottle ageing was ~0.17 mg/L. This pattern was observed for the HMM protein fraction, and to a

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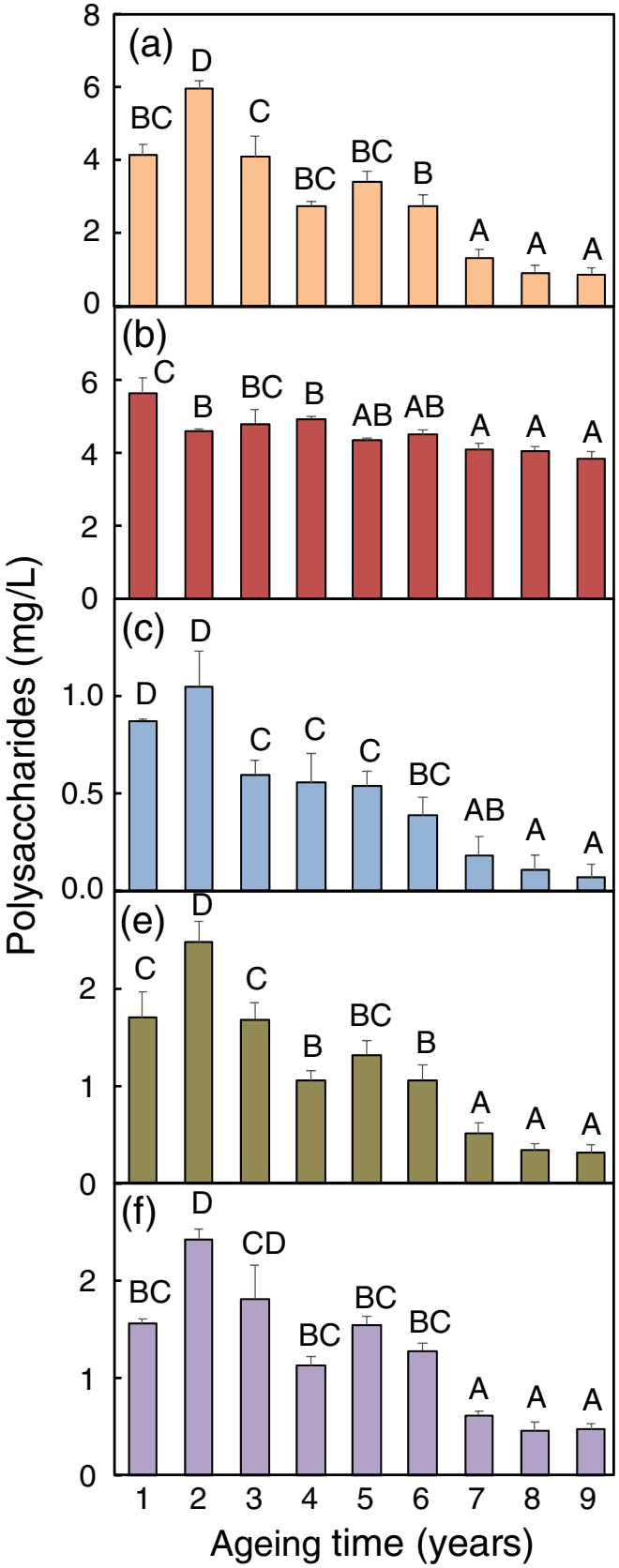


Figure 3. Effect of ageing time on the polysaccharide release in model wine solutions of lees isolated from sparkling wines from nine consecutive vintages: (a) total polysaccharides; (b) total oligosaccharides; (c) >180 kDa molecular mass fraction; (d) 40–180 kDa molecular mass fraction and (e) 7.5–40 kDa molecular mass fraction. Results are expressed and as mean ± SD of three replicates. The concentration of polysaccharides is expressed as pectin and dextran equivalents. Different letters indicate the existence of a statistical difference ($P < 0.05$).

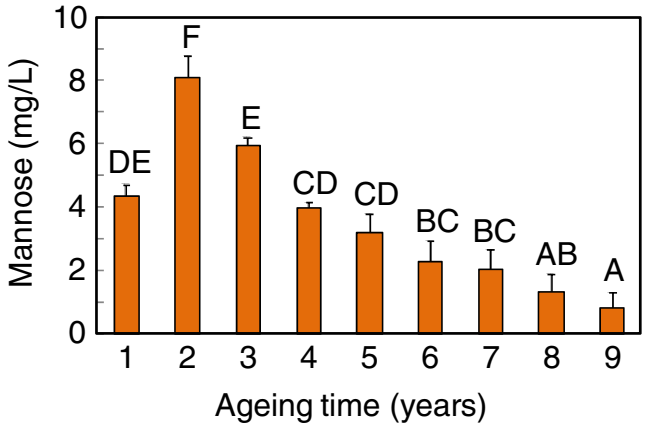


Figure 4. Effect of ageing time on the mannose present in the polysaccharides released from lees in model wine solutions of lees isolated from sparkling wines. Results are expressed and as mean ± SD of three replicates. Different letters indicate the existence of a statistical difference ($P < 0.05$).

lesser extent, the IMM fraction, but not for the LMM fraction.

When the concentration of polysaccharides and proteins released from the different lees samples was summed, the cumulative effect of their release with ageing time was observed (Figure 6). In each case, a clear increase was observed in the accumulated concentration over time. Moreover, the younger the lees, the greater the increase in macromolecule concentration (especially in the first 5 years, when compositional differences were statistically significant). The total accumulation of polysaccharides at the end of the 9-year period was 26.6 mg/L, while that of proteins was 2.4 mg/L.

These values should be taken with caution since they reflect just one approach. They do appear to indicate, however, that the release of polysaccharides and proteins from lees during bottle ageing of sparkling wines is much lower than the concentration already present in the wines.

The distribution of polysaccharides and proteins as a function of their origin (i.e. from lees autolysis or from the base wine) is shown in Figure 7. The proportion of polysaccharides and proteins clearly derived from lees autolysis was low, especially in the young sparkling wines; during the first year of ageing, only 2% of proteins and 3% of polysaccharides present in sparkling wine came from lees. These proportions increased as the duration of bottle ageing increased and achieved maximum values after 7 years of ageing (14% for polysaccharides and 16% for proteins).

According to information provided by the Regulatory councils, 88% of sparkling wines from AOC Cava are aged between nine 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 bottle aged for extended periods of time. Data from this study suggests that for the vast majority of sparkling wines produced by the traditional method, less than 7% of the polysaccharides and proteins present arise from lees autolysis, and this contribution is likely to be even lower for younger sparkling wines, especially those produced by alternate production methods.

Figure 8 shows the results of the sensory analysis performed by the trained panel. Statistical analysis of the ratings given to the nine consecutive vintages of sparkling

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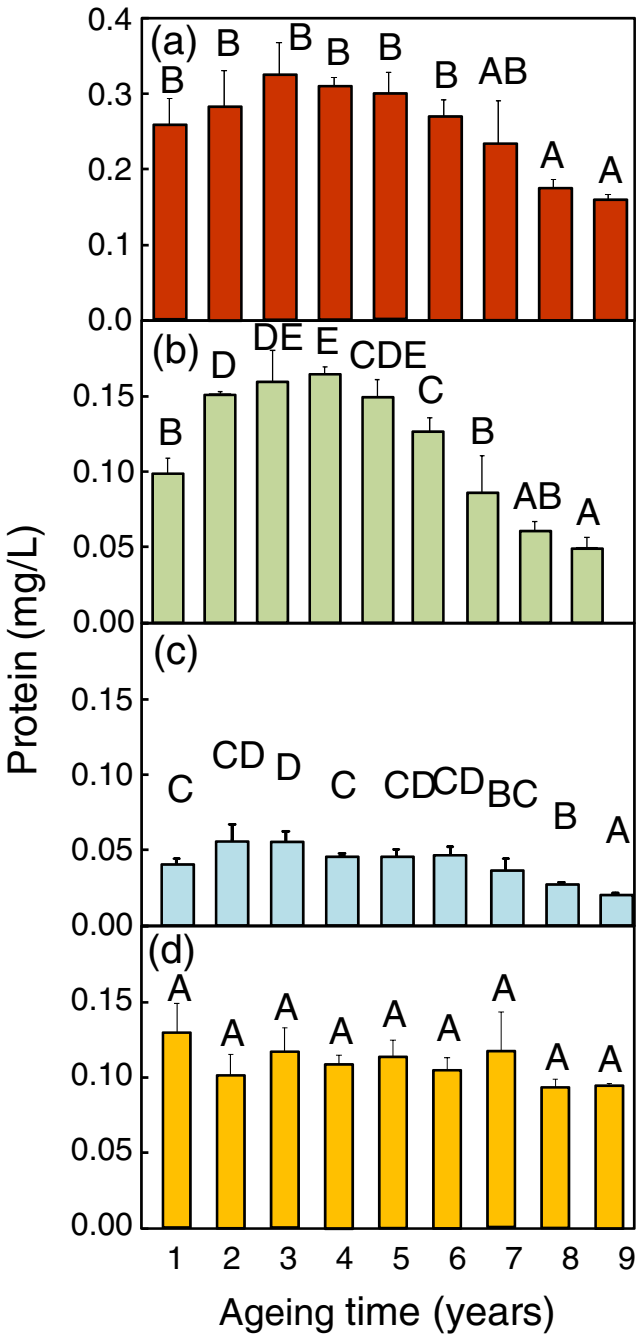


Figure 5. Effect of ageing time on the protein release in model wine solutions of lees isolated from sparkling wines from nine consecutive vintages: (a) total proteins, (b) >75 kDa molecular mass fraction, (c) 50–75 kDa moleculare mass fraction and (d) 25–kDa molecular mass fraction. Results are expressed and as mean ± SD of three replicates. Concentration of proteins is expressed as bovine serum albumin equivalents. Different letters indicate the existence of a statistical difference ($P < 0.05$).

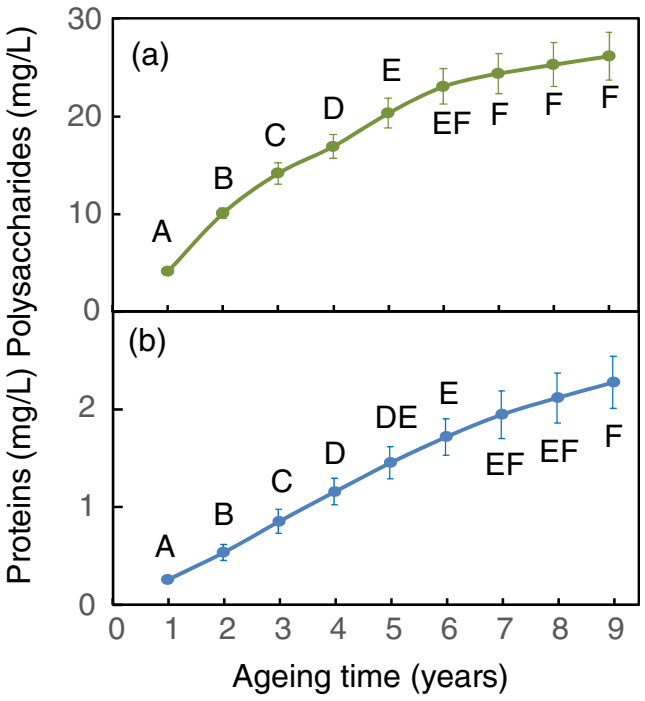


Figure 6. Effect of ageing time on the accumulation of (a) polysaccharides and (b) proteins from yeast autolysis. Results are expressed and as mean ± SD of three replicates. Concentration of polysaccharides is expressed as pectin and dextran equivalents. Concentration of proteins is expressed as bovine serum albumin equivalents. Different letters indicate the existence of a statistical difference ($P < 0.05$).

wine established four significant groups: A, which the panel considered the youngest, comprised only the sparkling wine from the first year; B comprised sparkling wines from the second and third years; C, which was considered significantly older than group B, comprised wines from the fourth to the sixth years and finally group D, which was classified as the oldest, comprised sparkling wines from the seventh to the ninth years. The panel successfully differentiated the sparkling wines on basis of their chronological production, although sensory differences between some consecutive vintages were not always perceptible.

Panellists considered the five youngest sparkling wines as 'acceptable' for the market according to their qualitative sensory criteria. In contrast, the older sparkling wines were considered 'not acceptable' by some panellists, with the number of 'not acceptable' evaluations increasing as the duration of ageing 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 5 years of bottle ageing the sparkling wines began to be affected by excessive ageing. These results closely match those of our previous study in which we compared changes in oxygen consumption by lees for the same set of sparkling wines to the theoretical oxygen permeability of the crown cap (Pons-Mercadé et al. 2021). According to this previous work, after 4 years of ageing, the oxygen consumed by the lees is no longer sufficient to compensate for oxygen intake through the crown cap, such that the sparkling wine becomes susceptible to oxidation. Under these conditions, its sensory quality may begin to deteriorate, though any effects of oxidation will depend on wine composition and storage conditions. In the present study, sensory deterioration appears to have commenced after 6 years of bottle ageing.

Finally, to better illustrate the process of yeast autolysis throughout the ageing period in the bottle, photographs of the yeasts were taken using a SEM in order to visualise how the structures of the different yeasts are degraded over the 9 years. Although similar studies have already been reported (Charpentier et al. 1986, Martínez-Rodríguez et al. 2001b, Cebollero and Gonzalez 2006, Tudela et al. 2012), to our knowledge none have analysed samples from sequential vintages over such a long period. Figure 9 clearly illustrates how the structures of the yeast cells are progressively degraded, folded and deflated. In the first image, which

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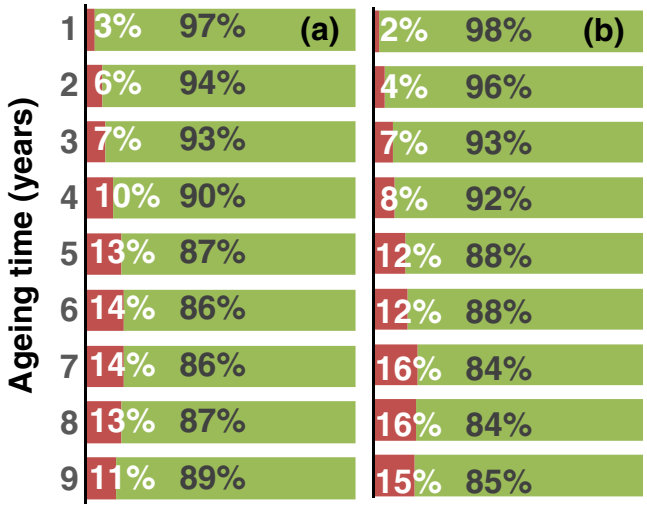


Figure 7. Distribution of the proportion of (a) polysaccharides and (b) proteins in sparkling wine based on their origin from either lees autolysis (■) or the base wine (■).

shows the yeast from the starter culture used for the second fermentation of the last vintage (2016), the yeast cell appears to be healthy since it is elongated, ovoid and turgid, without any wrinkles or folds. Several bud scars can even be identified. The second image shows what happens after the second fermentation (i.e. 3 months later): the yeast cell has lost some turgor and is beginning to display wrinkles and folds. In the third year of ageing, the yeast cell is even more degraded and wrinkled, and has begun to deflate. After 5 years 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. After 7 years of ageing, the yeast cell is even more degraded and deflated and the centre of the cell has crumbled, wrinkled and flattened. Finally, after 9 years, the yeast cell has completely collapsed and some structural parts appear to have broken.

Conclusion

In summary, comparison of the polysaccharide and protein fractions of sparkling wines from nine consecutive vintages produced by the traditional method showed no clear trends attributable to the duration of ageing in contact with lees. This appears to contradict the expected release of macromolecules due to lees autolysis. As discussed above, however, other authors have reported similar results. This might be explained by the fact that these macromolecules are being simultaneously released from lees and removed by precipitation, absorption and/or enzymatic degradation. This complicated

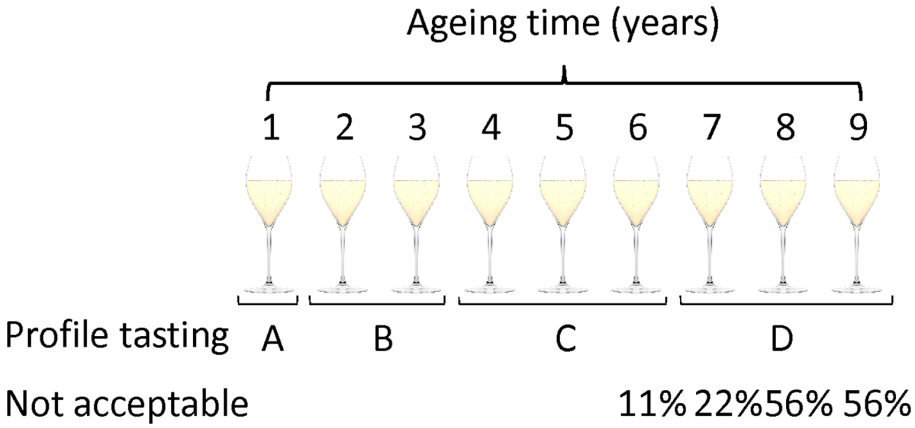


Figure 8. Effect of ageing time on the sensory analysis of sparkling wines from nine consecutive vintages.

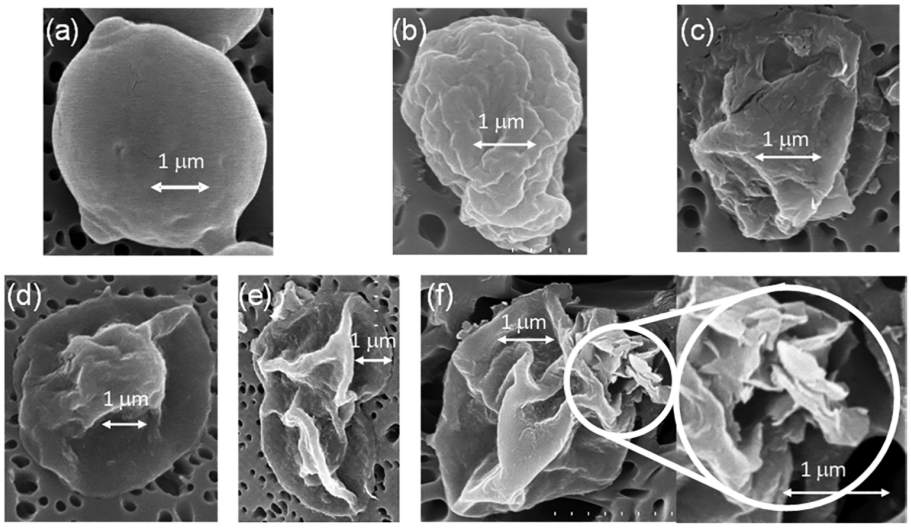


Figure 9. Yeast autolysis over time observed by scanning electron microscopy of the autolysing yeast as affected by ageing time: (a) yeast starter culture, (b) 1 year, (c) 3 years, (d) 5 years, (e) 7 years and (f) 9 years.

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balance becomes even more complex given the inevitable variation in the original concentration of polysaccharide and protein fractions between the different sparkling wine vintages. It is understandable, therefore, that this and other studies have observed inconsistent accumulation patterns.

To verify to what extent polysaccharides and proteins are released from lees, this study introduced a new experimental approach that involved recovering lees from sparkling wines from nine consecutive vintages and reproducing the autolytic process in model wine. Our results demonstrate that both macromolecules are released from lees. This also enabled us to compare the proportion of polysaccharides and proteins derived from yeast autolysis, relative to their corresponding wine concentration. The proportion of lees-derived polysaccharides and proteins was low in the young sparkling wines, that is, 2–3% in the first year of ageing and ~7% after 3 years of ageing. This suggests that the impact of polysaccharides and proteins from lees autolysis in the sparkling wines disgorged before the end of the first year of bottle ageing should be low. Wine producers should bear this conclusion in mind, since most sparkling wines produced by the traditional method are aged for less than 1 year and those made by other methods are aged for an even shorter duration. Consequently, only sparkling wines aged for extended periods of time (i.e. several years) would be expected to benefit from increased polysaccharides and proteins arising from yeast autolysis. Producers of sparkling wines should also bear in mind that if the duration of ageing is too long, wine quality can be negatively affected due to excessive oxidation, as perceived by our tasting panel.

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