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(Programa de Doctorado: Biociencias y Ciencias Agroalimentarias)

Título de la Tesis: "Contribución de las levaduras al aroma de los vinos espumosos durante la segunda fermentación y la lisis celular"

Thesis Title: "Contribution of yeasts to the aroma of sparkling wines during second fermentation and cell lysis"

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TITULO: *Contribution of yeasts to the aroma of sparkling wines during second fermentation and cell lysis*

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TÍTULO DE LA TESIS:

CONTRIBUCIÓN DE LAS LEVADURAS AL AROMA DE LOS VINOS ESPUMOSOS DURANTE LA SEGUNDA FERMENTACIÓN Y LA LISIS CELULAR

THESIS TITLE:

CONTRIBUTION OF YEASTS TO THE AROMA OF SPARKLING WINES DURING SECOND FERMENTATION AND CELL LYSIS

DOCTORANDO: RAFAEL MARTÍNEZ GARCÍA

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

Los doctores **JUAN JOSÉ MORENO VIGARA**, Catedrático de Universidad y **MARÍA TERESA GARCÍA MARTÍNEZ**, Profesora Contratada Doctora del Área de Química Agrícola y de Microbiología respectivamente, ambos pertenecientes al Departamento de Química Agrícola, Edafología y Microbiología de la Universidad de Córdoba (UCO).

INFORMAN:

Que el trabajo de investigación presentado por Don **Rafael Martínez García**, titulado "CONTRIBUCIÓN DE LAS LEVADURAS AL AROMA DE LOS VINOS ESPUMOSOS DURANTE LA SEGUNDA FERMENTACIÓN Y LA LISIS CELULAR" se ha realizado bajo la dirección y supervisión de ambos directores en los laboratorios de las Área de Química Agrícola y de Microbiología de la UCO y del Departamento para la Innovación en los Sistemas Biológicos, Agroalimentarios y Forestales de la Universidad de Tucsia (Italia) y reúne las condiciones exigidas para su presentación y defensa pública como Tesis Doctoral con mención internacional y en la modalidad de compendio de publicaciones.

El trabajo presentado por D. **Rafael Martínez García**, Licenciado en Química, en Ciencia y Tecnología de los alimentos y en Enología por la Universidad de Córdoba, se encuadra en la línea de investigación "Proteómica y Metabolómica de levaduras" de reciente implantación en el grupo de investigación "Vitenol" (http://www.uco.es/grupos/vitenol/) que está formado por algunos miembros del profesorado del Departamento de Química Agrícola, Edafología y Microbiología de la UCO. Esta línea de investigación tiene como objetivo principal estudiar las relaciones de proteínas y metabolitos en condiciones enológicas, particularmente, de aquellos relacionados con la calidad organoléptica de los vinos.

El doctorando **Rafael Martínez García** ha desarrollado, desde su incorporación al programa de doctorado Biociencias y Ciencias Agroalimentarias, una intensa y excelente actividad investigadora, cuya novedad e interés para la comunidad científica internacional está avalada por las publicaciones incorporadas a la Tesis Doctoral como primer autor de cuatro artículos de investigación en una revista internacional de un elevado índice de impacto indexada en el primer decil del Journal Citation Reports (JCR) en las categorías Food Science and Technology, Multidisciplinary Chemistry y Applied Chemistry.

El doctorando ha realizado una extensa revisión bibliográfica de las publicaciones más relevantes y actuales sobre su tema de investigación, que le ha permitido elaborar una importante introducción de la Tesis Doctoral (Capítulo 2). Básicamente, esta introducción consiste en una descripción de la situación del mercado mundial de los vinos espumosos, de los métodos de producción y clasificación y de los factores que influyen en la calidad y el aroma de estos vinos especiales.

La originalidad de dicha Tesis Doctoral estriba en el estudio de los metabolitos volátiles con capacidad odorante procedentes tanto de las etapas de fermentación como de crianza sobre lías con dos cepas de levadura *Saccharomyces cerevisiae*, P29, típica de laregión del cava y G1, típica de los vinos de crianza bajo velo de flor andaluces. Esta última cepa ha sido elegida por su alta tolerancia a elevadas concentraciones de etanol y sus propiedades de floculación, y ha resultado ser una candidata idónea para acortar el tiempo de la etapa de removido de lías.

Las investigaciones realizadas en la presente Tesis se han dirigido, fundamentalmente, a dilucidar el efecto de factores claves, como la presión de CO_2, temperatura, tiempo de crianza y formato de inoculación de la levadura, durante las etapas de fermentación en botella y crianza, sobre la calidad aromática de los vinos espumosos y las situaciones de estrés a que están sometidas las levaduras. Estas investigaciones sobre el volatiloma de las levaduras y su estrés forman parte y complementan otros estudios llevados a cabo durante los últimos años por miembros del grupo Vitenol de la Universidad de Córdoba sobre el perfil proteómico y metabolómico de las levaduras usadas para la elaboración de vinos espumosos.

Los objetivos planteados tienen una sólida coherencia y han facilitado la obtención de unos resultados importantes e innovadores dentro del área de Ciencias de los Alimentos, particularmente en Enología. La colaboración interdisciplinar Microbiología - Química de los Alimentos en el ámbito de la Enología ha hecho posible la consecución de los objetivos propuestos en la Tesis, de manera que cada uno forma un capítulo de esta memoria y, a su vez, cada capítulo se corresponde con cada artículo publicado.

El conocimiento generado en esta Tesis Doctoral y su transferencia al sector vinícola permitirá un mejor control de calidad del proceso de elaboración de los vinos espumosos, una diversificación de los productos elaborados y, por tanto, un mayor grado de innovación en esta industria agroalimentaria tan importante en Andalucía.

El doctorando ha realizado una estancia de tres meses de duración en la Universidad de Tuscia, (Viterbo, Italia), financiada por el IDEP de la UCO, que ha sido tutorizada por el Dr. Andrea Bellincontro. Durante esta estancia ha contribuido a mejorar sus conocimientos sobre el uso de la nariz electrónica y a ampliar las aplicaciones de esta

metodología al control objetivo de la calidad de los vinos. Además, esta estancia le permite solicitar la mención de Doctorado Internacional.

Por todo lo expuesto, se considera que la investigación desarrollada y recogida en la presente memoria reúne los requisitos de interés, originalidad, novedad y calidad científica exigidos para una Tesis Doctoral por la UCO, con mención de Doctorado Internacional y por compendios de artículos, y se emite este informe favorable para la presentación de la Tesis Doctoral de Rafael Martínez García.

Este trabajo se ha realizado gracias a los siguientes proyectos:

- "XXIII Programa Propio de Fomento de la Investigación 2018" (MOD 4.2.SINERGIAS, Ref XXIII. PP Mod 4.2) de la Universidad de Córdoba.
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Córdoba, 29 de abril de 2021

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Habida cuenta de que el doctorando reúne los requisitos exigidos (expuestos a continuación), podrá optar a la obtención de la mención de "Doctorado Internacional" a través de la defensa y exposición de la presente memoria.

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“Contribución de las levaduras al aroma de los vinos espumosos durante la segunda fermentación y la lisis celular”

Trabajo presentado para aspirar al grado de Doctor por:

Rafael Martínez García

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Contribución de las levaduras al aroma de los vinos espumosos durante la segunda fermentación y la lisis celular

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17 DE MAYO DE 2021

UNIVERSIDAD DE CÓRDOBA
DEPARTMENT OF AGRICULTURAL CHEMISTRY AND MICROBIOLOGY
VITENOL RESEARCH GROUP

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Para exponer a todas las personas que, directa o indirectamente, me han ayudado a lo largo de estos años y a las que agradezco poder completar esta etapa me harían falta nombres y espacios, aunque su recuerdo siempre me perdurará conmigo.

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Resumen

- **Introducción y motivación de la tesis**

Los vinos espumosos pertenecen a la categoría de vinos especiales y se obtienen mediante una segunda fermentación de un vino base previamente elaborado y con unas características enológicas adecuadas. Las metodologías utilizadas para tal fin pueden ser muy variadas, siendo la más conocida el método tradicional o *Champenoise*, que consiste en realizar la segunda fermentación en una botella cerrada, previa adición de levaduras y azúcar, seguida de un periodo mínimo de contacto con las lías de levadura donde tiene lugar su autolisis. Ambas etapas, fermentación y crianza, constituyen lo que se denomina 'toma de espuma'.

En las últimas décadas el consumo de los vinos espumosos ha aumentado notablemente, lo que ha impulsado a productores y comunidad científica a importantes esfuerzos para introducir mejoras en los procesos de elaboración con objeto de innovar, mantener la calidad y la demanda de estos vinos o captar consumidores emergentes [1]. De este modo, las propuestas presentadas en la literatura científica abarcan tres áreas de mejora: calidad, tecnología/biotecnología y salud [2], lo que permite avanzar en los objetivos de la industria enológica relacionados con la identidad, mejora de la producción y con la calidad, salud y seguridad alimentaria.

La calidad sensorial es un factor determinante para la elección de un producto alimenticio por los consumidores, destacando la calidad visual y del aroma como las primeras características que ellos valoran en la selección de un determinado vino. Varios factores influyen en las propiedades sensoriales de los vinos espumosos (espuma, color, aroma y gusto) entre los que caben destacar: aspectos vitícolas y condiciones edafoclimáticas, variedad de uva, estado sanitario y de maduración, metodología utilizada en la elaboración del vino base y la toma de espuma [2-4]. Sin embargo, el aroma característico de los espumosos está muy influido por la levadura que interviene en las dos fases del proceso de toma de espuma (fermentación y crianza sobre lías). Por un lado, durante la fermentación, las levaduras vivas están sometidas a condiciones de estrés muy severas impuestas por el elevado contenido en etanol y la sobrepresión del dióxido de carbono, que afectan a su metabolismo y, por tanto, a la producción de compuestos del aroma. En una segunda fase (crianza sobre lías), cuando la levadura está muerta, se produce la lisis celular y se liberan algunos metabolitos endo-celulares, proteínas y enzimas que contribuyen también al aroma de estos vinos [5, 6].

Las tendencias actuales de investigación e innovación en la elaboración de vinos espumosos se centran en la elección de cepas de levadura no convencionales y variedades de uva

tradicionalmente usadas para la elaboración de vinos tranquilos. Los estudios realizados a este respecto han permitido la elección de cepas de levadura por su tolerancia a las condiciones de estrés, por la producción de enzimas extracelulares (pectinasas, amilasas, lipasas, proteasas y glucosidasas) y también por su capacidad de lisis y floculación [2, 4, 5, 7, 8]. Sin embargo, son pocas las investigaciones sobre la contribución de las levaduras a la composición del aroma durante la segunda fermentación en botella, ya que los estudios existentes no suelen diferenciar entre esta fase y la de crianza sobre lías [9, 10]. Generalmente, los estudios realizados hasta ahora abordan el efecto de algunos factores implicados en el proceso de elaboración, siendo casi inexistentes las investigaciones dedicadas al análisis de su interacción. También, son muy escasos los estudios sobre la aplicación de nuevas biotecnologías, como los sistemas de inmovilización de levaduras emergentes.

- **Contenido de la investigación**

En esta Tesis Doctoral se han estudiado los metabolitos volátiles con capacidad odorante procedentes de las etapas de fermentación y crianza sobre lías de vinos espumosos obtenidos con dos cepas de levadura *Saccharomyces cerevisiae,* P29 y G1. La primera es una levadura habitualmente usada en la elaboración de vinos espumosos de la región vitícola de D.O. Penedès, mientras que la segunda es una levadura formadora de velo sobre la superficie del vino, típica en la elaboración de vinos de crianza biológica tipo fino y usada por primera vez para elaborar vinos espumosos. Esta levadura fue aislada de la zona vitivinícola de Montilla-Moriles y se ha propuesto para realizar la segunda fermentación de vinos base por el método tradicional debido a su alta tolerancia a elevadas concentraciones de etanol, y sus propiedades de floculación; que la convierten en una candidata idónea para acortar el tiempo de la etapa de removido de lías. Las investigaciones realizadas en la presente Tesis sobre el volatiloma de las levaduras forman parte y completan el estudio llevado a cabo durante los últimos años por miembros del grupo de Vitenol de la Universidad de Córdoba sobre el perfil proteómico y metabolómico de las levaduras usadas para la elaboración de vinos espumosos [10, 11].

El diseño experimental aplicado en cada etapa de elaboración de esta tesis permitió estudiar la influencia de factores claves en el proceso productivo, relacionados con el estrés de las levaduras y, consecuentemente, con la calidad aromática de los vinos espumosos. De este modo, durante la etapa de fermentación en botella, se estudió la presión endógena de CO_2 y, junto a la de crianza, el efecto combinado de la temperatura de fermentación, el tiempo de crianza y el formato de inoculación de levadura. También, se ha evaluado el potencial de técnicas no destructivas para el

análisis rápido de la fracción del aroma, como es la nariz electrónica basada en microbalanzas de cuarzo.

Finalmente, los estudios estadísticos realizados sobre matrices de datos organolépticos y analíticos han permitido contribuir al esclarecimiento de las asociaciones existentes entre la composición de la fracción volátil y la calidad sensorial de los vinos espumosos.

- **Conclusiones**

El estudio realizado en esta Tesis Doctoral permite establecer los efectos de la temperatura, cepa de levadura y tiempo de crianza sobre la calidad analítica y sensorial de los vinos espumosos durante las dos fases del proceso de toma de espuma.

Durante la segunda fermentación en botella se observa una menor influencia de la sobrepresión de CO_2 en la viabilidad de las células de la levadura propias de la elaboración de vinos espumosos, cuya cinética de formación es más rápida comparada con la cepa de levadura de velo, que se justifica por ser una cepa poco adaptada a este tipo de vinificación. Sin embargo, aunque la viabilidad la levadura de flor es menor con respecto a la levadura convencional, parece ser una buena candidata para la clarificación del vino durante la fase de removido, por su capacidad de formación de flóculos gruesos de rápida sedimentación y menor adherencia a la pared de la botella. Este resultado se refuerza también con los obtenidos en el análisis de la fracción volátil, alcanzándose variaciones significativas en los contenidos de las familias químicas del aroma, dependiendo de la cepa de levadura y de la presión de gas CO_2 generado. La sobrepresión de CO_2 provoca una disminución de las series odorantes química y afrutada en los vinos obtenidos por las levaduras ensayadas.

Durante el periodo de crianza estudiado se advierte un mayor efecto del factor tiempo que de la cepa de levadura sobre la fracción volátil. Mediante análisis sensorial se obtiene mayor puntuación de los vinos elaborados con la levadura de flor, que está relacionada con la mayor limpidez de los vinos obtenidos y, probablemente, con las interacciones de compuestos odoríferos que son sólo detectables mediante cata.

Durante el estudio de la etapa de toma de espuma, que contempla el análisis de la influencia de los restantes factores (temperatura, formato y tiempo de crianza), se percibe una ralentización de la cinética fermentativa a bajas temperaturas de las levaduras inmovilizadas como biocápsulas que se justifica por el estrés que sufre la levadura en el proceso de inmovilización, induciendo a un mayor periodo de adaptación al vino base. El estudio llevado a cabo ha permitido establecer compuestos volátiles clave como marcadores de la influencia de cada factor estudiado. Los buenos

resultados obtenidos en la clasificación de los vinos mediante el uso de la nariz electrónica hacen evidente su utilidad como herramienta destinada al control de calidad y la detección de fraudes en los vinos espumosos.

Como conclusión general se establece que el conocimiento de la influencia de estos factores sobre el volatiloma y su relación con los atributos sensoriales permitirá un mejor control de calidad del proceso de elaboración de los vinos espumosos, una diversificación de los productos elaborados y, por tanto, un mayor grado de innovación en esta industria agroalimentaria.

- **Bibliografía**

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Abstract

- **Introduction**

The sparkling wines belong to the category of special wines and are obtained through the second fermentation of a base wine with adequate oenological characteristics. The methodologies used for this purpose are diverse, the best known being the traditional or *Champenoise* method, which consists of a second fermentation in a closed bottle, after adding yeast and sugar, followed by a minimum period of contact with the yeast lees, where its autolysis takes place. Both stages, fermentation and ageing, constitute what is called 'prise de mousse'.

In recent decades, the consumption of sparkling wines has increased notably, which has motivated producers and the scientific community to make significant efforts to introduce improvements in production processes to innovate, maintain quality and demand for these wines or attract emerging consumers [1]. Thus, the proposals presented in the scientific literature cover three fundamental areas: quality, technology / biotechnology and health [2], which allows to achieve the main goals of the oenological industry regarding the identity, better production and quality, health and food safety.

Sensory quality is a determining factor for the selection of a food product by consumers, highlighting the visual and aromatic quality as the first characteristics that they value in the acceptance of a certain wine. Several factors influence the sensory properties of sparkling wines (foam, colour, aroma and taste), among which stand out: viticultural aspects and edaphoclimatic conditions, grape variety, health and maturation status, methodology used in the elaboration of the base wine and the 'prise de mousse' phase [2-4]. However, the characteristic aroma of sparkling wines is greatly influenced by the yeast involved in the two phases of the foam making process (fermentation and aging on lees). On the one hand, during fermentation, yeasts are alive and subjected to harsh conditions imposed by the high ethanol content and the overpressure of carbon dioxide, which are related to their metabolism and therefore to the production of aroma compounds. In a second phase (aging on lees), when the yeast is dead, cell lysis occurs and during it, some intracellular metabolites, proteins and enzymes are released and contribute to the aroma of these wines [5, 6].

Current research and innovation trends in sparkling winemaking focus on choosing unconventional yeast strains and grape varieties traditionally used for making still wines. Studies carried out in this regard have allowed the selection of yeast strains for their tolerance to stress conditions, for the production of extracellular enzymes (pectinases, amylases, lipases, proteases

and glucosidases) and also for their capacity for lysis and flocculation [2, 4, 5, 7, 8]. Nevertheless, there is little research on the contribution of yeasts to the composition of the aroma during the in - bottle second fermentation, since existing studies do not usually differentiate between this stage and the ageing on lees [9, 10]. Generally, the studies carried out address the effect of some factors involved in the production process, but the interaction effect between them is hardly considered. Furthermore, there are very few studies on the application of new biotechnologies, such as emerging yeast immobilization systems.

- **Content of the thesis**

In this Doctoral Thesis, the volatile metabolites with odorant activity have been studied during the fermentation and ageing stages of sparkling wines obtained with two strains of yeast Saccharomyces cerevisiae, P29 and G1. The first yeast is commonly used in the production of sparkling wines from the Penedès wine region, while the second is a veil-forming yeast isolated from the surface of the wine, typical in the production of fine-type biological ageing wines and used for the first time to make sparkling wines. This yeast was isolated from the Montilla-Moriles wine-growing area and has been proposed to carry out the second fermentation of base wines by the traditional method due to its high tolerance to ethanol, and its flocculation properties; which make it an ideal candidate to shorten the time of riddling stage. The research carried out in this Thesis on the yeasts' volatilome completes the study carried out in recent years by members of the Vitenol group of the University of Córdoba on the proteomic and metabolomic profile of the yeasts used for the production of sparkling wines [10, 11].

The experimental design applied in each stage of this thesis allowed to study the influence of key factors in the production process, related to yeast stress and, consequently, with the aromatic quality of sparkling wines. Thus, during the bottle fermentation stage, the endogenous CO_2 pressure was studied and, together with the ageing stage, the combined effect of the fermentation temperature, the ageing time and the yeast inoculation format. Furthermore, the potential of fast and non-destructive techniques for the analysis of the aroma fraction has been also evaluated, such as the electronic nose based on quartz microbalances.

Finally, the statistical studies carried out on organoleptic and analytical data matrices have made it possible to clarify the existing associations between the composition of the volatile fraction and the sensory quality of sparkling wines.

- **Conclusions**

The study carried out in this doctoral thesis makes it possible to establish the effects of temperature, yeast strain and ageing time on the analytical and sensory quality of sparkling wines during the two phases of the 'prise de mousse'.

During the in -bottle second fermentation, a lesser influence of the CO_2 overpressure is observed on the viability of the typical yeast cells for the production of sparkling wines, whose formation kinetics is faster compared to the veil yeast strain; which is justified for being a strain that is not adapted to this type of winemaking. Although the viability of the flor-yeast is lower compared to the conventional one, it appears to be a good candidate for wine clarification in the riddling phase, due to its ability to form thick flocs with rapid sedimentation and less adherence to the walls of the bottle. This observation is also reinforced with the results of the volatile fraction, obtaining significant differences in the contents of the chemical families of the aroma that depend on the yeast strain and the pressure of the CO_2 gas generated. The CO_2 overpressure causes a decrease in the chemical and fruity odorants series in the wines obtained by the tested yeasts.

During the ageing period, a greater effect of the time factor is observed on the volatile fraction, compared to the yeast strain. Through sensory analysis, the wines made with flor yeast obtained higher scores, which is related to the greater limpidity of the wines obtained and, probably, to the interactions of odorant compounds that are only detectable by tasting. During the 'prize de mousse' stage, which involved the analysis of the remaining factors (temperature, format and ageing time), a slowdown in fermentation kinetics is observed when using immobilized yeasts at low temperatures, mainly caused by the stress suffered during the immobilization process. The study carried out allowed the establishment of key volatile compounds as markers of the influence of each factor studied. The good results obtained in the classification of wines through the use of the electronic nose make evident its usefulness as a tool for quality control and fraud detection in sparkling wines.

In general terms, the influence of the studied factors on volatilome and its relationship with sensory attributes will allow better quality control of the sparkling wine making process, product diversification and greater innovation in this sector of the agri-food industry.

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Chapter 1

General overview and objectives of the these

1.1. **Background**

Sparkling wines made according to the traditional method consist in the fermentation of a base wine in closed bottles, previously added with sugar and yeasts, followed by a minimum ageing on yeast lees for nine months. Severe stress conditions affect the yeast during the process (high ethanol content, nitrogen deficiency, acid pH, low temperature and high pressure of the CO_2 generated), which affect its metabolism and consequently modify the organoleptic properties of the final product.

Current innovation strategies for the production of sparkling wines focus on the use of unconventional yeasts to obtain new types of wine, as well as accelerating autolysis and simplifying the process. Until now, countless studies have been carried out focused on the selection of yeast strains for their autolysis and flocculation capacity, their tolerance to ethanol and low temperatures, the release of mannoproteins and the production of extracellular enzymes. Nevertheless, research on the contribution of yeasts to the composition of aromas during the fermentation stage is scarce, making it difficult to know clearly whether the aromas produced are due to this stage or derived from ageing.

In light of the above mentioned, the knowledge of the response mechanisms of wine yeasts to stress conditions, such as composition and external conditions of the environment, is the aim of the -omics sciences. Metabolic studies applied to oenology address the analysis of a large number of metabolites, in order to objectively classify and / or discriminate according to different criteria (geographical origin, the edaphoclimatic conditions of the vine cultivation, the grape variety, the process fermentation and harvest). This enables the integration of metabolic changes from the raw material to the consumption phase and ensure traceability of the product. Special interest in wine science and technology has the study of those volatile metabolites with odorant activity, since they are responsible for organoleptic properties. In this sense, the study of aromatic compounds will allow to differentiate the contribution of yeast by stages and study the effect on the metabolic activity of yeast regarding the aforementioned stress factors. Furthermore, this will contribute to greater control of the process, product quality, the development of new products and the detection of fraud in these types of wines currently in high demand.

1.2. **Starting hypothesis**

The intracellular metabolites excreted by the yeasts during fermentation and ageing are related to the yeast strain used, the way in which its applied and also the physical and chemical conditions that characterized the medium in which they grow. This causes changes in the organoleptic characteristics of the final product that can be detected by different techniques. Therefore, as starting hypothesis, two contributions of yeasts to the aroma of sparkling wines can be established during their elaboration by the traditional method. Firstly, live yeasts are subjected to stress conditions and produce an increase in the ethanol content, an overpressure of carbon dioxide and aroma compounds. Secondly, when the yeasts die, cell lysis occurs and proteins, some endo-cellular metabolites and enzymes are released that add to the aroma of sparkling wines.

1.3. **These Objectives**

In view of the foregoing, the following generic objective has been set:

Differentiate the contribution that the yeast strain has on the aroma produced in the fermentative stage with respect to autolysis and study the effect of other biotechnological factors (CO_2 overpressure, temperature, yeast strain and immobilization systems) that influence the quality and price of sparkling wines.

This main objective encompasses the following specific aims:

- To study the effect of CO_2 overpressure stress on aroma of sparkling wines obtained with conventional and non-conventional *Saccharomyces cerevisiae* yeast strains during the second fermentation.
- To know the effect that temperature, ageing time, and yeast format have on the aroma of sparkling wines and evaluate the potential of new technologies of analysis such as E-nose to discriminate the wines obtained.
- To analyse the contribution of a flor yeast to the sensory quality of sparkling wines obtained with autochthonous grape varieties and different ageing on lees periods.

1.4. **These structure**

This dissertation is organised in 7 chapters:

The first chapter (the present one) introduces the nuclei of development of the dissertation and the objectives that were pursued.

Chapter two deals with the background of sparkling wines, indicating trends in consumption, production methodologies as well as identifying the oenological factors that affect their quality, predominantly the aroma, flavour, and quality of the foam.

The third and fourth chapters address specific objective 1, and include the work carried out by the PhD student to establish the effect of CO_2 pressure on the aroma of sparkling wines obtained by traditional method.

Chapter 5 analyses the specifications made in objective 2 and establishes a comparative study between the usual methodology applied in this dissertation for the determination of key role aroma compounds and other more innovative techniques for these special wines.

The sixth chapter addresses the specific objective number 3. In addition, this chapter establishes, through multivariate analysis, the strength and direction of influence that the compounds identified in the wines have on the sensory attributes previously evaluated by a panel of experts.

The seventh chapter contains the conclusions of the these, as well as suggestions for future research in sparkling wines based either on biotechnology or new analysis techniques.

The last pages contain annexes with the abbreviations, acronyms and scientific contributions made by the PhD student.

1.5. **Antecedentes**

El proceso de elaboración de los vinos espumosos según el método tradicional consiste en la fermentación de un vino base en botellas cerradas herméticamente, previa adición de azúcares y levadura, seguida de un período mínimo de crianza de nueve meses. Durante el proceso fermentativo la levadura está sometida a condiciones de estrés muy severas (elevado contenido en etanol, déficit de nitrógeno, pH ácido, baja temperatura y alta presión del CO_2 generado), que afectan a su metabolismo y consecuentemente modifican las propiedades organolépticas del producto final.

Las estrategias actuales de innovación en la elaboración de vinos espumosos se centran en el uso de levaduras no convencionales para obtener nuevos tipos de vinos, así como acelerar el proceso de autolisis y simplificar determinadas etapas del proceso productivo. Hasta el presente, se han realizado numerosos estudios centrados en la selección de cepas de levaduras por su capacidad de autolisis y floculación, su tolerancia al etanol y a bajas temperaturas, la liberación de manoproteínas y la producción de enzimas extracelulares. Sin embargo, son pocas las investigaciones sobre la contribución de las levaduras a la composición de aromas durante la etapa fermentativa, no quedando claro si los aromas producidos se deben a esta etapa o derivan de la etapa de crianza.

En relación a lo mencionado, el conocimiento de la respuesta de las levaduras vínicas a las situaciones de estrés impuestas por la composición del medio y las condiciones externas es objeto de estudio de las ciencias -ómicas. Los estudios del metaboloma aplicados a las ciencias del vino abordan el análisis de tantos metabolitos como sea posible, con objeto de clasificar y/o discriminar objetivamente de acuerdo a distintos criterios (origen geográfico, las condiciones edafo-climáticas del cultivo de la vid, la variedad de uva, el proceso de fermentación y la añada). Permitiendo integrar los cambios metabólicos que ocurren desde el origen al consumo y asegurar la trazabilidad. La importancia del análisis de metabolitos volátiles con actividad odorante radica en su relación con la calidad sensorial del vino. Además, el estudio de los compuestos del aroma facilitará diferenciar la aportación de la levadura por etapas y estudiar el efecto sobre su actividad metabólica de los factores de estrés anteriormente comentados. Lo que contribuirá a un mayor control del proceso, de la calidad del producto, a la elaboración de nuevos productos y detección de fraude en este tipo de vinos actualmente muy demandados.

1.6. **Hipótesis**

Los metabolitos intracelulares excretados por las levaduras durante la fermentación y crianza están relacionados con la cepa de levadura, la forma en que es aplicada y las condiciones físico-químicas que caracterizan al medio en que crecen. Esto causa cambios en las características organolépticas del

producto final que pueden detectarse por diferentes técnicas. De este modo, como hipótesis de partida, se establecen dos contribuciones de la levadura al aroma de los vinos espumosos elaborados por el método tradicional. En primer lugar, las levaduras vivas están sometidas a condiciones de estrés y producen un incremento en etanol, la sobrepresión de dióxido de carbono y compuestos del aroma. En segundo lugar, cuando las levaduras mueren se produce la lisis, de modo que las proteínas, así como metabolitos endo-celulares y enzimas son liberados, formando parte del aroma de los vinos espumosos.

1.7. **Objetivos**

En relación a lo mencionado, se establece el siguiente objetivo general:

Diferenciar la contribución que tiene la cepa de levadura sobre el aroma producido en la etapa fermentativa con respecto a la autólisis, y estudiar el efecto de otros factores biotecnológicos (sobrepresión de CO_2, temperatura, cepa de levadura y sistemas de inmovilización) que influyen en la calidad y precio de los vinos espumosos.

Este objetivo engloba los siguientes objetivos específicos:

- Estudiar el efecto de la sobrepresión de CO_2 en el aroma de los vinos espumosos obtenidos con una cepa *Saccharomyces cerevisiae* convencional y otra no convencional durante la segunda fermentación.
- Conocer el efecto que la temperatura, tiempo de crianza y formato de levadura ejerce sobre el aroma de vinos espumosos y evaluar el potencial de nuevas técnicas de análisis como la nariz electrónica para discriminar los vinos obtenidos.
- Analizar la contribución de una levadura de flor a la calidad organoléptica de los vinos espumosos obtenidos con variedades de uva autóctonas a diferentes periodos de crianza sobre lías.

1.8. **Estructura de la tesis**

Esta tesis está organizada en 7 capítulos:

El primer capítulo (el presente) introduce los núcleos de desarrollo de la tesis y los objetivos que se pretenden conseguir.

El segundo capítulo trata sobre los antecedentes de los vinos espumosos, indicando tendencias en el consumo, metodologías de producción, así como identificando los factores enológicos que inciden en su calidad, predominantemente el aroma, sabor y calidad de la espuma.

Los capítulos tercero y cuarto abordan el objetivo específico 1, e incluyen el trabajo realizado por el doctorando para determinar el efecto de la presión del CO_2 sobre el aroma de los vinos espumosos obtenidos por el método tradicional.

El capítulo cinco estudia las especificaciones realizadas en el objetivo específico 2 y establece un estudio comparativo entre la metodología habitual utilizada en esta tesis para la determinación de los compuestos aromáticos de papel clave y otras técnicas más innovadoras para estos vinos especiales.

El capítulo sexto presenta el objetivo específico 3. Además, este capítulo especifica, mediante análisis multivariante, la fuerza y sentido que los compuestos identificados en los vinos tienen sobre los atributos sensoriales evaluados previamente por un panel de expertos.

El capítulo séptimo contiene las conclusiones de la tesis, así como sugerencias para futuras investigaciones en vinos espumosos basadas en biotecnología o nuevas técnicas de análisis.

Las últimas páginas comprenden anexos con las abreviaturas, siglas y aportaciones científicas realizadas por el estudiante de doctorado.

Chapter 2

Introduction: The sparkling wines aroma

This dissertation offers results related to the traditional method of sparkling wine production. Therefore, in this review, more emphasis will be placed on the results obtained by other authors regarding this method and only when considered relevant, other production methods will be explained.

2.1. The global sparkling wine market

The international trade of sparkling wine has steadily increased both in volume and in value (with an average annual growth rate of 6 % and 8 %, respectively) since the end of the last century. The global success of sparkling wine is directly connected with the changes in consumers' preferences and tastes that are mainly caused by the off-season consumption together with a more diverse supply.

The sparkling wine production has increased around the world in an average of 3 % per year since 2002, reaching a volume of 20 10^6 hL in the most recent years [1]. The production of these special wines is distributed all over the world as shown in ***Figure 1***. However, the highest concentration of producers is within European Union (EU), where the top 5 production countries account the 80 % of the global sparkling wine production.

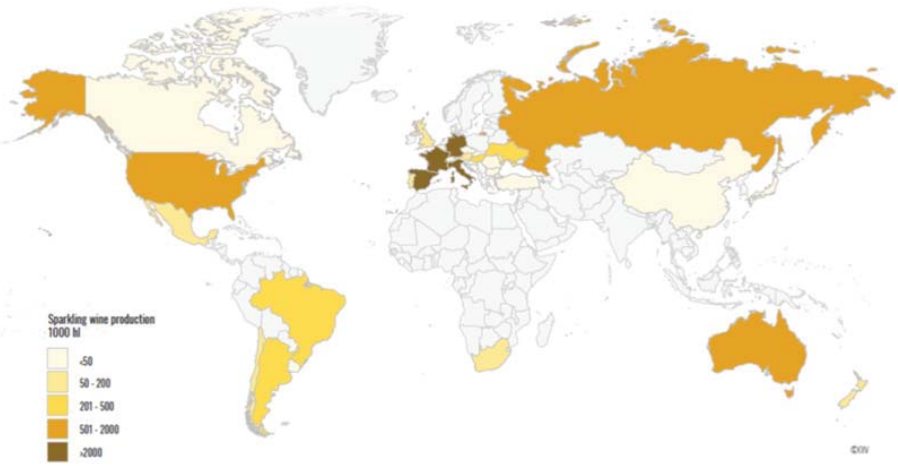

Figure 1*.The sparkling wine production in 2018.* ***Source****: OIV, 2020.*

Italy is the first producing country due to the high demand of both closed tank sparkling wines (e.g., Prosecco) and to a lesser extend bottle fermented wines (e.g., Franciacorta and Trento). The second and third place in this ranking is respectively occupied by France (with Champagne) and Germany (e.g., Schaumwein and Qualitätsschaumwein 'Sekt'). In relation to Spain, which ranks fourth, its production volume has almost doubled since 2000 with a 4 % annual growth rate, where the

market is 89 % occupied by Cava. Outside the EU and sharing the same annual growth rate than this country, highlights USA followed by new producing countries, such as UK and Brazil.

Italy, France, and Spain also represent 85 % worldwide export of sparkling wines in last years, meanwhile Germany, France, USA, Russia, and Italy represent 62 % global consumption.

Although the consumption of sparkling wines in Spain is not outstanding, it should be noted that the rise in demand of Cava wines doubled those of its exports in the recent years. According to the latest report available from the regulatory council, cava has grown in the foreign market, placing sales at 165 million bottles [2]. Exports to third countries (115 countries) maintain the positive trend and stand at 51.4 million bottles. This represents 20.6 % of the total exported, being 3.05 % more than in previous years. Shipments to Europe continue to lead the export of cava with a total of 113.5 million bottles and represents 45.5 % of the total cava exported. Germany, Belgium, USA, UK, Japan and France are the most consuming countries of this product. In addition, the sale of cava has increased in the domestic market, placing it at 84.5 million bottles (+ 6.45 % compared to previous years).

The evolution in the consumption of these special wines has promoted some interesting innovations in their production process, such as the use of new grape varieties and new selected wine yeasts for the second fermentation, which are aimed to maintain or improve these results and achieve a greater number of consumers.

2.2. Production methods and classification of sparkling wines

Sparkling wines belong to the category of 'special wine' and are mainly obtained by a second fermentation of a base wine. According to the technology used for this process, these wines are classified as sparkling wines obtained by the traditional method or 'Champenoise method', when the second fermentation is carried out in closed bottles, and sparkling wines made in pressurized stainless-steel tanks or 'Charmat method' (***Figure 2***). A modified version of this latter technology is the so called 'Asti method' or 'Moscatel Espumante', when it is made in Brazil, and consists of fermenting a must in pressurized stainless-steel tanks, to incorporate the CO_2 gas and produce the desired alcohol level (7-10 % v/v) with a minimum residual sugar concentration of 20.0 g/L [3].

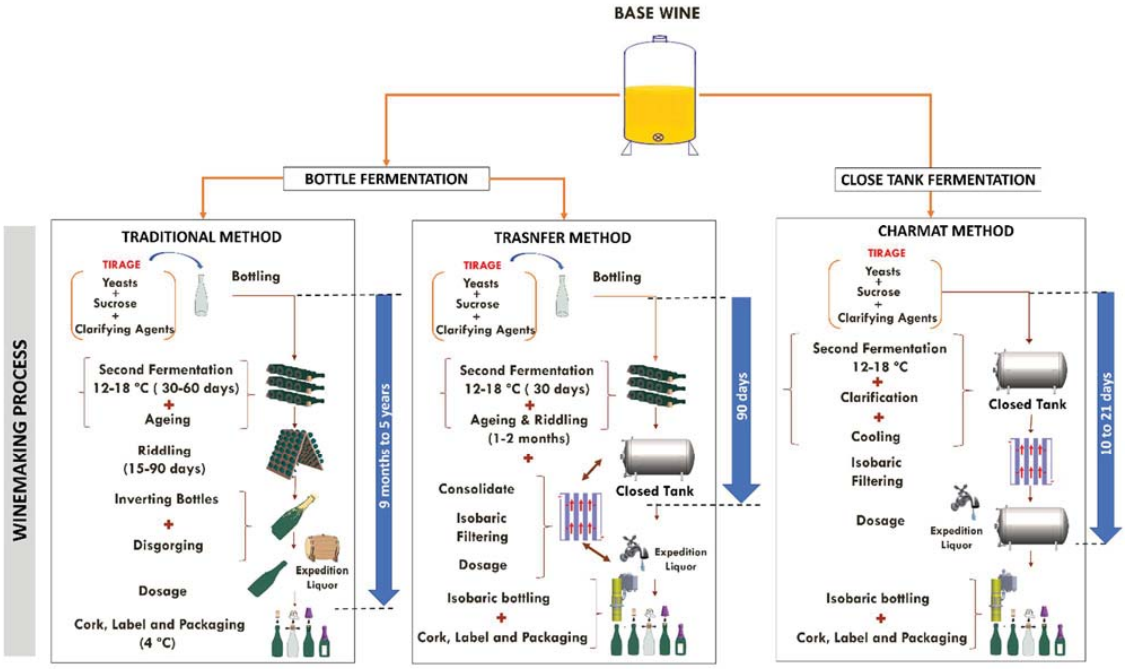

***Figure 2**. Production methods and classifications of sparkling wines. **Source**: own elaboration.*

Another fundamental difference in the traditional method, when compared to Charmat, is the long ageing time in contact with yeast lees, from 9 months to 5 years, after the second fermentation. As a consequence, the autolysis process takes place, releasing a high number of intracellular compounds to the wine that enhance its sensorial profile and resulting a high quality product [4]. In contrast, Charmat method involves a faster production technique where no more than 10-21 days are counted from the start of fermentation until the product is marketed. The performance of second fermentation in sealed tanks has as advantage the preservation of varietal aromas when aromatic grape varieties are used, which is especially remarkable in Asti method [3]. A strategy to preserve all the advantages derived from both traditional and Charmat technologies is called 'Transfer method'. This procedure consists of a second fermentation in the bottle of the base wine and two months of ageing on lees; then it is transferred to a pressurized tank and filtered (both steps under isobaric conditions), before being bottled [5]. In this way, it allows reducing costs without affecting the quality or loss of wine due to disgorging operations [6].

Differences in sensorial properties (foamability, colour, aroma and flavour) can be observed in the sparkling wines regarding the production method used [3, 7]. Although second fermentation and the period in contact with lees have been described as having a major influence on the composition of sparkling wines, other factors are also involved such as: viticultural aspects and edaphoclimatic conditions, healthy and grape berry ripeness, the production of base wine, the choice of yeast strain,

among others [8-11]. Therefore, this review has as main objective to analyse and identify all those factors previously researched at every stage of sparkling wine production by the traditional method that influence their organoleptic properties, with special emphasis in the volatile fraction as the fundamental axis of this thesis. In relation to this latter, there will be also a brief review of the analytical methodologies used, as well as an attempt to identify future research lines to improve the diversification and quality of sparkling wines.

2.3. Factors influencing the quality of bottle-fermented wines

This section reviews all the factors described in current research that affect the quality at each stage of the in-bottle fermentation sparkling winemaking, with special emphasis on the aroma fraction. For this the production phases have been grouped in three main parts (***Figure 3***): (i) pre-fermentative phase that covers all the stages from grape cultivar to must production; (ii) fermentative phase that includes all the aspects related to base wine production, its adjustments and the second fermentation in bottle; (iii) post-fermentative stage that describes the stage prior to commercialization of the product (lees ageing, disgorging, dosage and others).

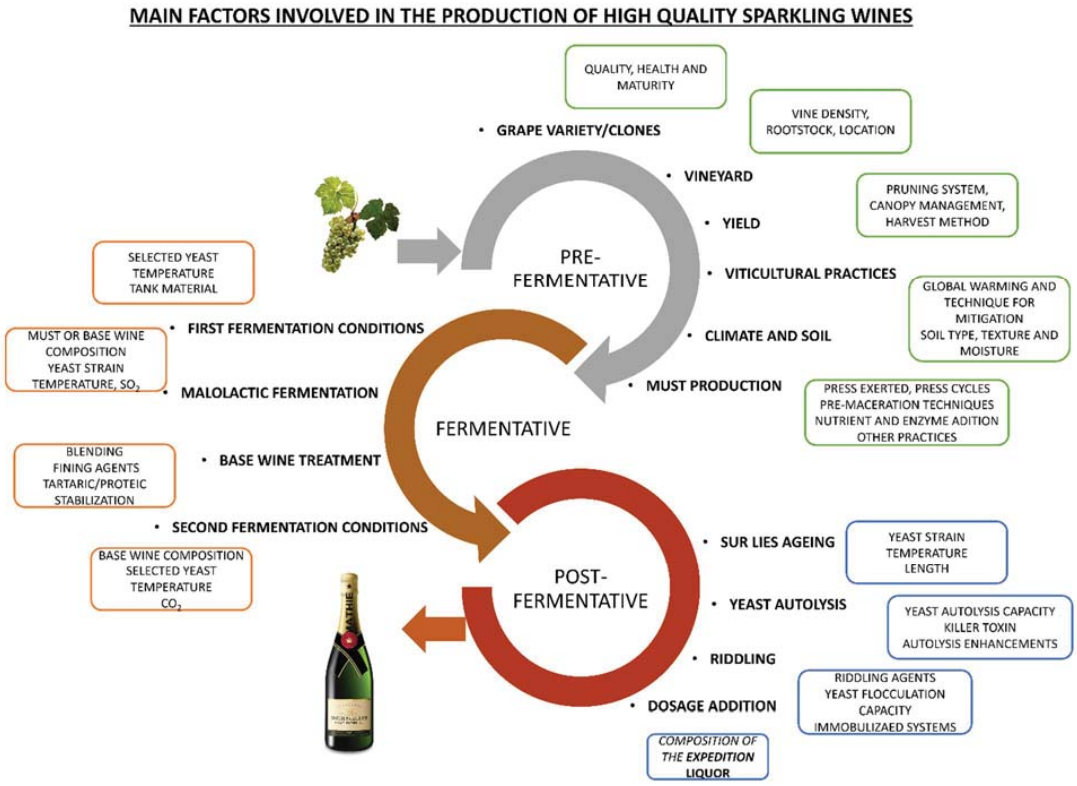

Figure 3. *Factors involved in sparkling wine quality.* ***Source****: own elaboration.*

2.3.1. Pre-fermentative stage: from the vineyard to the grape-must

2.3.1.1. Viticultural and technological aspects related to sparkling wine quality

De la Presa-Owens, stablished that grape variety, vineyard location and yeast autolysis are the tree most influencing factors in the character of wines fermented in bottle [12]. The importance of the grape is such that only because of its colour, the way to name the sparkling wine differ as "*blanc de blancs*" or "*blanc de noirs*", when obtained from varieties (Chardonnay) or dark-skinned grapes (Pinot Noir/Pinot Meunier), respectively [11, 13]. Such is the print that the grape variety gives, that some authors use the adjectives "finesse and elegance" to define Chardonnay wines, "body" for Pinot Noir, and "fruity and roundness" for Pinot Meunier grapes [11]. The aroma of Chardonnay and Pinot Blanc is also characterized by being more citric, floral and with apple notes, while berry fruit, vanilla and butter notes are typical in sparkling wines from red varieties as Pinot Noir and Pinot Meunier [12].

Climate and viticultural practices directly influence on grape cultivar and hence the quality of the final product derived from it [8]. In this context, the selection of the raw material, its adaptation to the edaphoclimatic conditions and the wine-growing practices should have as its main objective the production of sparkling wines with a unique and distinctive sensory profile [11]. For example, Chardonnay is the most extended cultivar over the world, being found either in warm regions or, together with Pinot Noir, in cool-climate regions [11, 13]. In contrast, Parellada, Xarel-lo, Macabeo, Chenin blanc, and Semillon are the most used varieties in warm and semi-arid regions [14]. Differences related to geographical origin were also described by Muñoz- Redondo et al., (2020). These authors determined 26 terpenoids of commercial sparkling wines from different geographical regions (Cava, Champagne and Andalusia), grape varieties and ageing time, and found that only six ((-) - β-citronellol, β-cyclocitral, cis-nerolidol, geranyl acetate, TDN and megastrigmatrienone 6Z8E) were responsible for the changes caused by geographical origin, as a consequence of the adaption of grape varieties to special edaphoclimatic conditions of the area.

Authorized grape varieties and vineyard yield for sparkling wine production are legislated by the Designations of Origin Protected (DOP) and cultivated in their respective registered production areas (***Figure 4***) [6, 15].

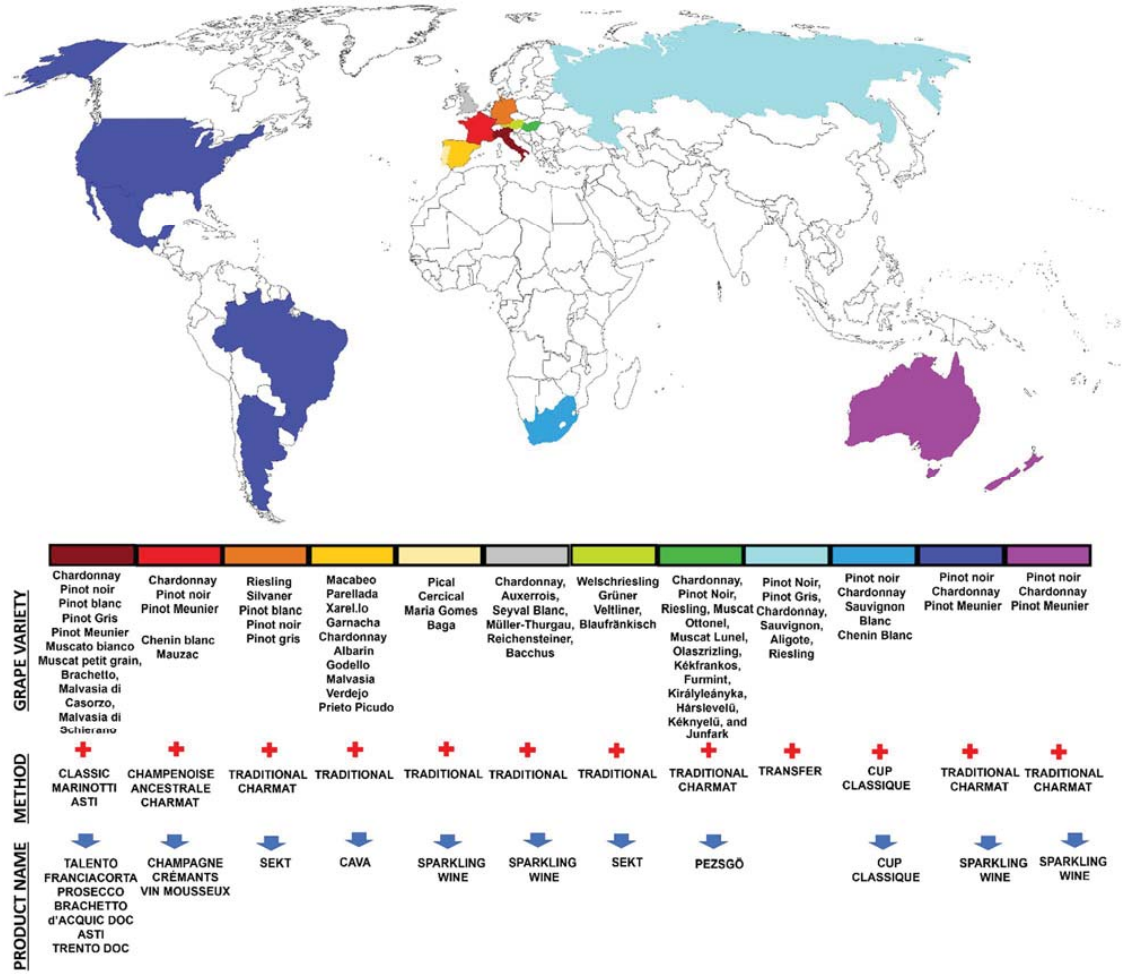

***Figure 4**. Main sparkling wines produced, categorized by their country of origin and varieties used.*
***Source**: own elaboration.*

The search for products linked to regional identity, such as grape varieties, has been proven as a new market strategy in the oenological industry to diversify the wine production [16]. Studies based on the use of autochthonous grapes from a production area are numerous, which is considered an excellent strategy to improve the quality of base wines and sparkling wines [16-27]. Their selection for sparkling wine production is generally based on targeted parameters that are remarkably similar among those regions dedicated to produce these special wines. Low sugar concentration, high total acidity, and low pH are the principal fruit quality criteria used for determining harvest dates for grapes and, hence, wine quality [6, 11], being all the yield management strategies developed for the production of grapes mainly destinated to reach this values. In addition, other important parameters that add complexity to sparkling wine must be considered, such as fruit flavour, 10-11 % (v / v) of potential alcohol content, colour and aroma [6, 11, 28, 29] (***Figure 5***).

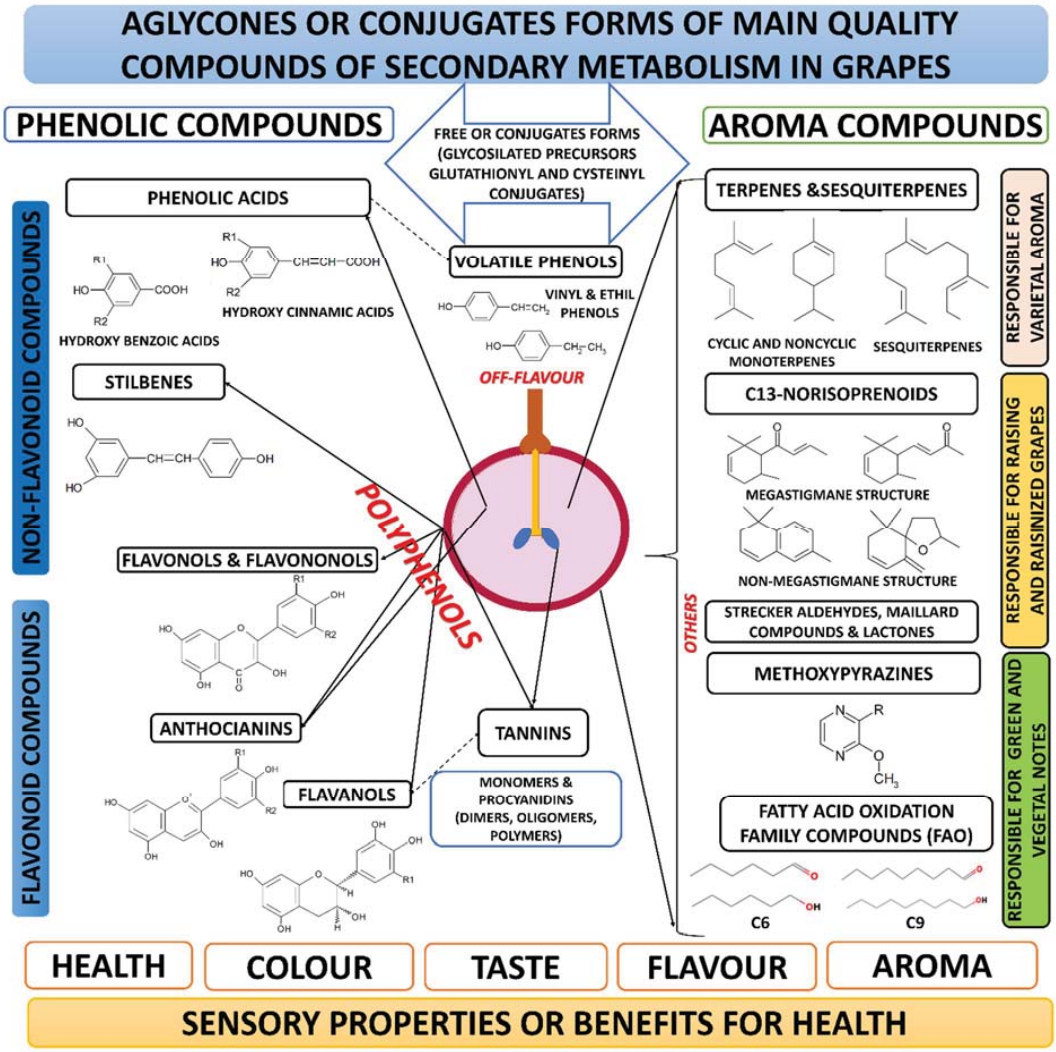

***Figure 5**. Other parameters from grape fruit involved in sparkling wine complexity. **Source**: own elaboration.*

In this context, Ferreira et al., (2019) [30] stated that grape aroma integrates those aroma molecules and chemical systems responsible for the aromatic sensory properties (odour and flavour) of grapes and grape juices, while potential grape aroma are specific precursors of relevant wine odorants; being established a classification of grape aroma regarding their origin. Thereby, the monoterpenoids, sesquiterpenoids, and C13-norisoprenoids play an important role contributing to the differentiation of the wine varietal character and are considered as key aroma compounds of aromatic grapes [17, 30]. C13-norisoprenoides, along with aldehydes derived from Strecker degradation of amino acids and some compounds derived from Maillard reaction are mainly found in raisins or wines produced from raisined grapes [30]. Finally, two groups of compounds are the main responsible from

vegetal and green aroma flavour: methoxypyrazines and compounds derived from the oxidation of fatty acids through lipoxygenase activity.

The establishment of these quality parameters for the selection of fruits used in the production of high-quality sparkling wines facilitate the work of winegrowers when determining specific management strategies applied by blocks [11]. Martinez-Lapuente et al., (2013) [19] examined the sensory attributes and phenolic profile to produce varietal high-quality sparkling wines manufactures from different *Vitis vinifera cv*. (Verdejo, Viura, Malvasia, Albarin, Godello, Garnacha, and Prieto Picudo) and concluded that Prieto Picudo, Albarin, and Verdejo were the most promising varieties for this purpose. Perez-Margariño et al., (2013) [21] found that base and sparkling wines from Albarin, Verdejo, Godello and Prieto Picudo were the richest in most of the volatile compounds analysed, especially of ethyl esters and the acetates of higher alcohols, compounds that contribute to the fruity aroma of wines.

Clonal evaluation has been also reported by research teams worldwide [31, 32]. The different studies have been focused on clonal selection for adequate yield and on the basis of obtaining optimal technological ripeness parameters (sugar, acidity and pH), although phenolic and aromatic ripeness have been overlooked, which supposes a significant knowledge gap, crucial for the production of high-quality fruits and premium sparkling wines.

In addition to the fruit quality and grape variety, considerations related to canopy management, climate and soil, vine density and yield vineyard are also necessary [8, 11, 15, 17, 33], most of them interrelated. For this, soil fertility determines the density planting, bud production and ripening rate. The grape harvest yield affects to the soluble solids, organic acids, pH, phenolic compounds, and anthocyanins; although scarce studies have been done regarding these aspects and sparkling wine quality [15, 34, 35]. As mentioned above, this parameter is legislated by appellations of origin and can be focused either on producing quality fruit or limiting production costs and wine volume. However, some authors defend that the ideal harvesting level may be dependent on the desired style of sparkling wine to make [11]. Pozo-Bayon et al., (2004) [15] studied the influence that yield vineyard has on other quality parameters such as the phenolic, volatile and nitrogen compounds, the foam characteristics, as well as the sensory quality. Although no conclusions could be drawn from the volatile fraction related to yield vineyard since the malolactic fermentation took place, the wines obtained from harvest yield lower than 10,500 kg/ha were characterized by a higher concentration in phenolic compounds and the best tasting scores, while no differences were found for nitrogen compounds and foam properties.

Changes in production terms (yield, berries per clusters, yield per pruning weight, cluster size, etc.) have been found when using different clones in the same geographic area, being dependent on the grape variety, climate and soil studied [31, 32]. These changes were also observed in each country and region, when using different rootstocks or cultural practices, but its impact on the quality of the final product was not studied [8].

The rate of ripening is also closely related to vineyard yield [34]. Regarding this, grape varieties can be classified as early (Pinot Meunier), intermediate (Pinot Noir) or late maturating variety (Chardonnay), which has a direct influence on the composition of the wines obtained from them [11, 36]. The global warming constitutes a significant challenge to grape production since the rise in temperature has a direct effect on their ripening process rate and an inverse effect on their acidity, besides being the cause of compositional changes between vintages [37]. The warmer the sparkling wine producing region, the earlier the grapes harvest is to ensure low pH and high acidity levels [11]. Some studies show that elevated temperatures lead to a lag between technological and phenolic ripeness and the obtained wines were unbalanced in colour and ethanol contents, causing the increase in grape sugars and alcohol, with fatal consequences for the sparkling wine quality [38]. Furthermore, the unwanted increase in overripe grapes will be an emerging problem in many vine-growing areas since they give raisin odorant notes to the wines. Norisoprenoids (β-damascenone), Strecker aldehydes (phenylacetaldehyde, methional), lactones (γ-nonalactone and C10 massoia lactone) and some pyrazines derived from Maillard reaction, have been described in wines obtained with overripe grapes, although their effect on sparkling wine quality is still unknown [30]. This raises the need to use new grape varieties or clones that are better adapted to the climatic change or move the vineyards to cooler regions.

The vine canopy management, commonly used in cool climate regions, is also related to grape ripening. The exposed leaf area/grape ratio affects the rate of berry maturation and consequently the wine quality [39, 40]. Alkyl methoxypyrazines are powerful aroma molecules with vegetable and green aroma and flavours. These compounds accumulate in fruit harvested from cool climates whose levels decrease during ripening, although the vine genotype, altitude, temperature, light exposure and nitrogen fertilization also influence [30]. The leaves, shoots and fruit removal from the winter pruning time are the most important practices to lead a faster berry maturation as well as for modifying vine microclimate, the grape yield and its composition [41-43]. Jones et al., (2018) [41] analysed the effect of cane and spur pruning on harvest yield, grape and base wine composition of Chardonnay and Pinot Noir cultivars, and found significant differences on the phenolic profile when applied both treatments, without affecting total soluble solids (TSS) and titratable acidity.

Early leaf removal, when properly apply, results in an increase of tartaric acid and sugar concentration at harvest as well as a strong effect on glycoside aroma precursors, in particular by increasing glycoside terpinols and norisoprenoids; but a wrong use should overexpose the clusters and lead to a decrease in malic acid and/or hydroxycinnamates [42, 44]. An innovative variant of this practice is the pre-bloom leaf removal which has a strong effect on glycoside aroma precursors, in particular by increasing glycoside terpinols and norisoprenoids [42]. Grape bunch removal could also increase the potential alcohol from 0.5-1.5 % when the 30-50 % of unwanted berry-clusters are eliminated at the veraison stage [11]. Likewise, cluster thinning has been shown to increase total anthocyanins and total phenolics [34]. Either leaf or fruit removal are cultivar-specific practices that must be locally applied in the production area, where the balance between production costs *vs.* the potential improvement of the final wine quality must be also considered. In this context, the mechanization of these practices and the grape-harvest is believed an alternative to reduce costs. Nevertheless, the adverse consequences derived from its use on the quality of the final wine should be considered, since the damage it causes in the vineyard or in the oxidation of the must may be irreversible [45, 46].

Soil type constitutes another important factor, since its composition, texture and depth directly affect soil temperature and moisture content which influence vine vigour, berry composition and wine quality [11]. Compared with table wine varieties, there is higher flexibility in soil type specifications for sparkling wine, given lower sugar concentration is required at harvest. Coelho et al., (2009) [17] studied the effect that grape variety, soil composition and ripening stage have on the volatile fraction of sparkling wines, and reported that the first two were the most influential factors. In addition, an interaction factor is described since higher content in clay-calcareous soils affect more the varietal component of wines than clay or sandy soils. In relation to ripening stage, those wines made of grapes with optimal or late ripening stage offer highest content in volatile compounds, while those obtained with early harvest were richer in varietal compounds (sesquiterpenes and norisoprenoids) and hexan-1-ol. Adversely, some authors affirm that if grapes selected for sparkling wines are harvested earlier, the wines obtained will show low aromatic intensity and less varietal character. This is in accordance with the results of Herrero et al., (2016) [13], when compared sensory profile between early harvesting Chardonnay and Pinot Noir grapes to produce white base wines, attributing the low intensities observed in white fruit, tropical and citric to the early stage of ripening of the grape used. Nevertheless, a delay in harvest date is an oenological decision capable of improving base wine protein content and foamability [47, 48].

Considering the imminent need to adapt the vine crop to the environmental changes or to mitigate the negative impact caused when the ripening rate is out of control, trends in current research in this area of knowledge try to cover two targets. Firstly, knowing the effect that global warming has on the production of these special wines and secondly, to determine if the quality of grape and sparkling wine will be affected by changes in the viticultural practices applied to the terroir [11, 36].

2.3.1.2. Must production

After harvesting, grapes are subjected to pressing and the obtained must is transferred to a tank in which the first fermentation takes place. Pressing process provides two must fractions: the first and highest quality fraction is called "*cuvée*", while the second pressing ("*tailles*" or tails) has a lesser quality [6, 8]. An optimal pressing procedure should avoid must oxidation by aeration and minimizes the transfer of insoluble solids that reduce the quality of the final product [49]. In this regard, the most important factors affecting the aroma, flavour and foaming properties of base wine and sparkling wines are the grape variety, the type of press, the pressure exerted and the length of cycles [8, 49]. From the research on this topic, a decrease in foamability during pressing is observed as a consequence of the loss in proteins when binding to phenolic compounds, which increase with the length and the number of pressing cycles. In addition, a decrease in acidity and an increase in pH is also noted [50]. However, some typical must processing compounds such as products of enzymatic fatty acid oxidation (FAO), mainly the C6 and C9 aldehydes and alcohols, are not mentioned in these studies [30].

Some sparkling wine-making regions, located in cold-climate zones, often apply pre-fermentative corrections of sugar and acidity contents to the musts, which are controlled by the regulatory council in each wine region. The changes derived from its use are related to changes in wine body, sweetness and balance acid-flavour [8]. However, to the knowledge of the author of this thesis, complete studies on the effect that these treatments have on the aroma, flavour and sparkling wine quality have not yet determined.

Other oenological techniques to obtain a suitable base wine, in terms of its final composition, have also been the subject of scientific literature [26, 51]. Thus, Perez-Margariño et al., (2019) [26, 51] studied the effect on base wine composition of four winemaking techniques (pre-fermentative cold maceration, 'delestage', sugar reduction and partial dealcoholisation) and observed that sugar reduction and partial dealcoholisation with mature grapes allowed to obtain base wines with more adequate alcohol content. In addition, this latter along with pre-fermentative cold maceration had a greater influence on the volatile composition of the base and red sparkling wines, while sparkling

wines from delestage of premature grapes showed higher vegetal aromas. Despite no differences were observed for instrumental foam parameters, pre-fermentative cold maceration displayed the best foam sensory descriptors. Pre-fermentative maceration has been also used to improve the characteristics of sparkling wine made with neutral grapes varieties. Ruiz-Moreno et al., (2017) investigated the influence of skin-maceration (10 ºC for 6 h) on the ester profile of Pedro Ximenez sparkling wines and found that wines subjected to this technique displayed higher contents in ethyl esters of branched acids (EBBAs) and cinnamic acid derivatives. Furthermore, these authors described an interaction effect between skin-maceration and ageing factor that increases the content of alcohol acetates (HAAs) and EBBAs, although only ethyl heptanoate was proposed as skin-maceration marker.

2.3.2. Fermentative stage: Elaboration of the base wine and the 'in bottle' second fermentation

2.3.2.1. The base wine

The base wine used in these methodologies should meet some characteristics such as: pale colour, fruity aroma, moderate alcohol, low residual sugar and volatile acidity, as well as tartaric stabilization [4, 52]. Vigentini et al., (2017) [53] stated that the base wine composition, the yeast strain used and the temperature at which the second fermentation takes place are relevant to define the fermentation rate of bottle-fermented wines. As mentioned above, the most influential factors for base wine composition (considered in relation to its flavour, aroma and foaming potential) are grape variety and clone cultivated, rootstock, yield, ripeness degree, pressing fraction and technological parameters (sugar and acid content) as well as the ethanol content [5, 47, 48, 54-56]. The use of botrytized grapes also leads to changes in composition of the wine, affecting its quality [5, 8]. A delay in fermentation kinetics, a destabilization of foam properties and changes in wine aroma and flavour are an example of the main changes resulting from the use of infected grapes by this fungal pathogen [55, 57]. Lower levels of glycerol, 2,3-butanediol, succinate, tyrosine, valine derivatives, and phenylpropanoids and higher levels of oligosaccharides along with new proteins (fungal proteins, plant proteins as a response to the infection, or fragments resulting from partial proteolysis) have been detected in the botrytized wines [55, 56]. Strecker aldehydes derived from a chemical reaction between amino acids precursors with quinone or α-dicarbonyl (from oxidizing polyphenols) can be also produced through the laccase enzymatic activity from *Botrytis* [30]. A common treatment to prevent base wine oxidation and biological degradation is the addition sulphur dioxide. However, this type of infection leads to an increase in the dose of SO_2 during pressing, which have a negative influence in fermentation rate and foaming properties [8, 10], without considering that it can be harmful to health. Alternatives to the use

of SO_2 to prevent oxidation and microbial instability have been also described in literature, being needed to determine the changes in the aroma and flavour of sparkling wines derived from their use [8, 58].

The must fermentation is usually carried out in stainless steel tanks, although other materials (oak, cement) can be used, leading to differences in the sensory properties of the obtained wines and personalising the winery style. [8, 59]. The usual temperatures range between 15 and 18 ºC, but in the production of high quality base wine the fermentation temperature is lower (13 ºC) [5, 6] and implies a high energy consumption, since grape must is cooled during all the process. In this sense, the selection of yeast (mainly *Saccharomyces bayanus*) for primary fermentation is based on their capacity to ferment at these temperatures as well as low pH, sulphur dioxide and the absence of grape skins [6]. Some studies are based on the use of *S. cerevisiae* wine strains, selected for their sensorial performances and low SO_2 production, at higher temperatures than the standard ones [58]. The use of these yeasts in base wine production had positive effects on energy saving without compromising sensory, chemical, and aromatic profiles. Nevertheless, other factors are involved in the yeast selection process for the first and second fermentation and will therefore be addressed more fully in a later section.

The most important changes caused by alcoholic fermentation are directly related with yeast metabolism. The key metabolic process is the conversion of sugar into ethanol, carbon dioxide glycerine, and a large number of additional by-products that contribute to the final aroma and flavour profile of the wine (***Figure 6***). Alcohols, acids, esters, carbonyl compounds, sulphur compounds, and volatile phenols are the main chemical families into which yeast metabolites can be grouped.

Higher alcohols (so called fusel alcohols) can be synthesized by yeast through the anabolic pathway from glucose or the catabolic pathway from their corresponding amino acids (valine, leucine, iso-leucine and phenylalanine), also called Ehrlich pathway [16]. In general terms, the process involves a succession of steps that begins with decarboxylation of amino acids into an α-ketoacids, followed by its conversion into a branched-chain aldehyde, and finally its reduction to a fusel alcohol through a concatenated action of yeast enzymes (pyruvate decarboxylase and alcohol dehydrogenase, respectively) [60]. Regarding their concentration, fusel alcohols contribute with a positive complexity to the wine aroma when they are lower than 400 mg/L [18].

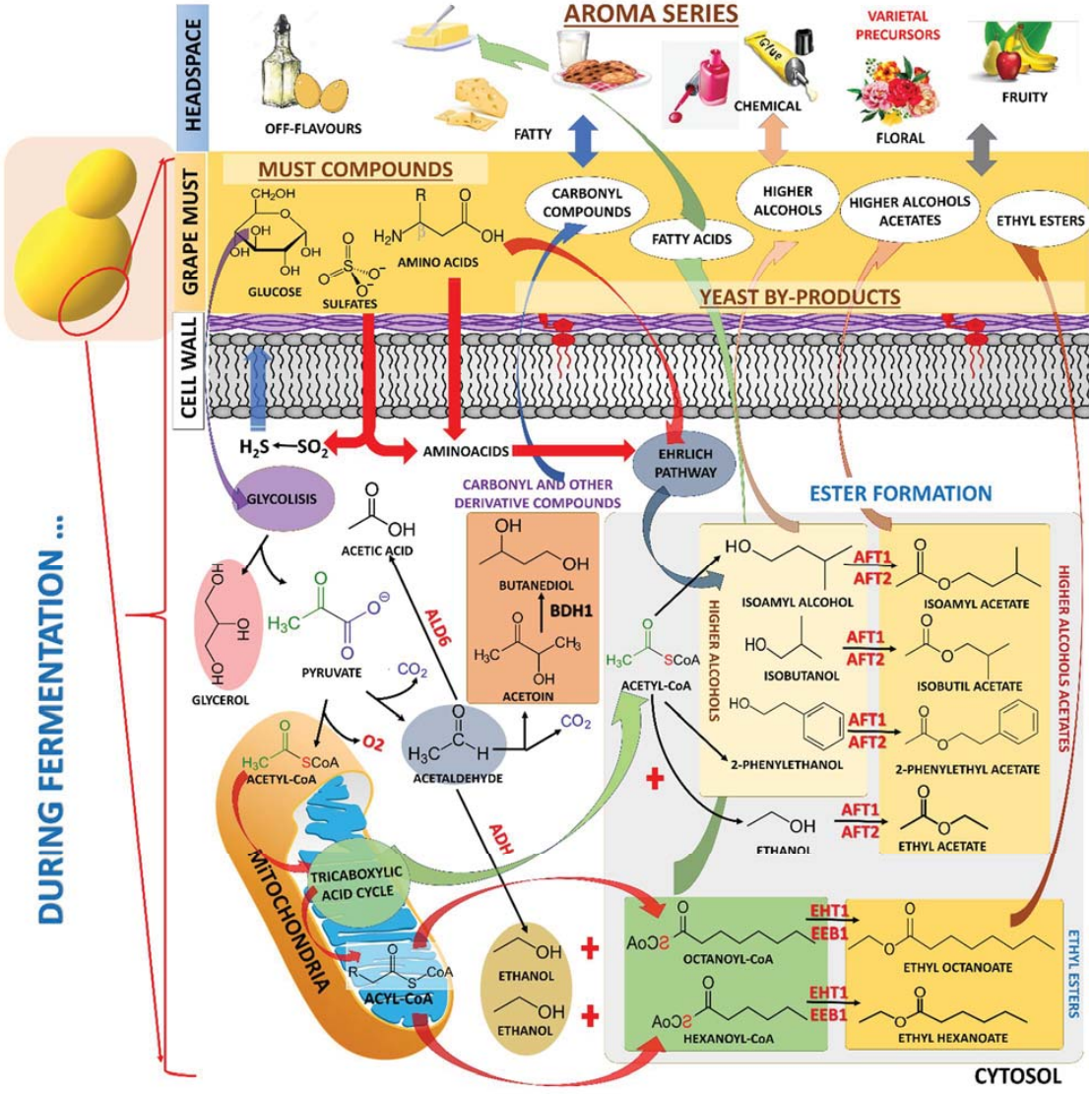

***Figure 6**. Volatile fraction during fermentation process. **Source**: Own elaboration.*

Additionally, higher alcohols are precursors of some esters, such as higher alcohols acetates (HAAs) which comprise, along with ethyl esters of fatty acids (EEFAs), the most important set of yeast-derived aroma-active compounds. Both fractions are responsible for fruity and candy-like aroma character of wine [61]. HAAs are formed when reaction between alcohols and acetyl-CoA occurs through the action of alcohol acetyltransferase, while EEFAs are a product derived from the esterification reaction between ethanol and medium chain fatty acids released during exponential growth phase through cytoplasmic fatty acid synthetase complex [3, 26, 61, 62]. On the other hand, lactones are intramolecular esters characterized by contributing to candy floss, sweet fruits and coconut

odour and derive from hydroxy acids as results of yeast metabolism during the fermentation stage or by chemical processes during the ageing stage [61]. As stated before, carbonyl compounds (aldehydes and ketones) can be formed from Ehrlich pathway although the main source of these compounds is the metabolism of sugars by yeasts [61, 63]. Vinylphenols and ethylphenols respectively derive from yeast metabolism of *Saccharomyces* and *Brettanomyces* genus via enzymatic decarboxylation and vinylphenol reductase of the corresponding hydroxycinnamic acids [30]. The importance of these compounds lies in their contribution to flavour, colour and smell, which could be detrimental to quality if they are found in high concentration [61]. Finally, thiols or mercaptans are also considered as off-flavour released during fermentation from odourless precursor molecules, whose content is mainly influenced by grape variety, yeast strain, ageing and other oenological practices [64].

The role that plays each volatile compound on the aroma depends on the wine matrix analysed. In this context, significant positive correlations were observed by some authors between esters and their precursors such as ethyl hexanoate-hexanoic acid (R = 0.92) and ethyl octanoate-decanoic acid (R = 0.93); or between compounds in which this relation is not expected such as isoamyl acetate and ethyl octanoate (R= 0.73), ethyl hexanoate (R = 0.88), citronellol (R = 0.73) and geraniol (R = 0.81) [3].

Herrero et al., (2016) [13], when studying the aroma fraction of varietal base wines, stated that constitutive molecules are the group of compounds belonging to the main common aromatic core of a group of wines, while discriminant compounds are more responsible for the aromatic differences between samples. In this study the authors observed that Pinot Noir wines had more constitutive compounds while Chardonnay wines had more discriminant compounds. Furthermore, some compounds could play a dual-role (constitutive-discriminant), being only four compounds predominated in Chardonnay wines: 4-vinylphenol, guaiacol, sotolon and 4-methyl-4-mercapto-2-pentanone. Notwithstanding, caution must be taken when trying to predict the organoleptic profile of sparkling wine through the flavour and aroma that characterized the base wine used to its production [11]. Sensory properties of the base wine changes as a consequence of the amplification of aromas during the second fermentation [8, 25], modification during autolysis [65] or loss during ageing stage by their retention on yeast lees [66, 67].

Malolactic fermentation (MLF) is a common process in cool-climate regions, such as Champagne area, when the grape must has high organic acid (tartaric and malic acid) content and low pH [5]. Frequently it is induced by addition of *Oenococcus oeni* in wines, either after or during the first fermentation, when yeast and lactic acid bacteria (LAB) co-ferment in the same medium [6], that prevents from undesirable deposits when bacterial growth takes place after bottling. Nevertheless, this

is not a spontaneous process, since low pH, high alcohol and SO_2 contents (8 to 10 g/hl), the deficiency or the imbalance in some nutrients of wines inhibit the LAB growth. Properly done, it changes favourably the flavour of wine since some compounds derived from bacteria metabolism go into the wine [5]. In contrast, some authors [5, 10] described positive or negative contributions of the MLF on the sparkling wines quality, given that malic and lactic acids endow different effects to the foam properties and highlight the knowledge to be covered about this process.

After completion of first alcoholic fermentation and according to winery style, several still wines with different characteristics are blended (*coupage*). This allows to compensate the differences between vintages and vineyards regarding right balance of sugar, flavour and acidity suitable for sparkling winemaking or to produce multivarietal sparkling wines in which the best characteristics of each variety is combined [5, 6]. This practise constitutes a key step to produce a unique profile, being sometimes mixed more than 80 different wines from different grape varieties, vineyards and vintages [6, 54]. Once blending is finished, the wines are subjected to tartaric and protein stability treatments prior to fining or filtration process, for which the most important methods have been recently revised [8]. Notwithstanding, all the methods proposed in the current research mainly affect tartaric acid, mineral content, phenolic and chromatic and sensory properties, while the effect on the aromatic fraction is not yet determined or null [28, 29].

The fining agents added in the pre-fermentative treatments also influence quality of base and sparkling wines [68, 69]. Puig-Deu et al., (1999) [68] described more complete fermentation and foam stability, lower content of nitrogenous fraction, polyphenols (less browning ability) and volatile compounds of grape juice treated with a fining mixture (potassium caseinate, bentonite and microcrystalline cellulose) than the ones treated with bentonite. Garcia et al., (2009) [18] working with other fining agents (albumin, bentonite, bentonite-gelatine and bentonite-albumin) to produce Bobal base wine, described best foaming properties when albumin is used alone or jointly with bentonite, while most important changes in volatile fraction are observed in presence of bentonite. Thus, an increase in ethyl butyrate, ethyl lactate, ethyl decanoate, diethyl succinate, nerol, 2-phenylethanol, methyl acetate, methanol is obtained when using bentonite-gelatine, whereas the content in isobutyl acetate, n-amyl alcohol, γ-butyrolactone, geraniol, ethyl acetate, isoamyl alcohols increase with bentonite addition. Lambri et al., (2012) [69] studied the effect of bentonite at different stages (in must, in wine or double bentonite treatment on must and wine) and found best protein removal when added to must only, while lower terpinols removal was observed with double treatment. Other treatments to avoid the adverse effects of bentonite are based on the use of lysozyme, a highly ordered, rigid, hydrophilic and positively charged protein which confers a protective effect on wine foaming

properties when used before bentonite [5]. Notwithstanding, since prion diseases were described, the substitution of animal based-products by other plant-based compounds has been carried out in the wine industry [8].

After cold stabilization and fining treatments are done, a filtration process is carried out before bottling to reduce the turbidity of the base wine and meet the legal requirements [5]. Most of the studies carried out on this topic are based on the effect that different filtration methods have on those foam-active compounds, reaching the conclusion that this fraction is mostly removed when smaller the filter pore size is. Nevertheless, comparative studies about the effects of the different filtration methods on the aroma, flavour and foaming properties of sparkling wines, has not yet been carried out [8].

2.3.2.2. The in-bottle second fermentation

Once base wine has been prepared, a 'tirage' solution is added, which contains sucrose and a selected yeast population enough to reach a content close to 24 g/L and 10^6 CFU/mL, respectively. Bentonite is usually added as clarifying agent, before closing the bottle or stainless steel tank [5]. The fermentation of sugar by yeasts results in the CO_2 gas, ethanol and other by-products, with 4.00-4.30 g/L of sucrose being necessary to obtain one atmosphere of CO_2 pressure [4, 70]. This pressure is usually monitored by an external afrometer previously coupled to the bottle used as control. On the other hand, riddling agents and co-adjuvants allow lees removal, avoid sluggish of the fermentation and provide structure to the sparkling wine. The material where the second fermentation takes place is also important to produce high-quality sparkling wines and depends on the winery style. Thus, although bottles are arranged in areas protected from light at low temperatures (from 10 ºC to 15 ºC) during the second fermentation, the selection of bottle colour is crucial to avoid the degradation of some photosensitive wines, such as rosé sparkling wines [71]. Furthermore, bottles are closed with two types of caps: a polyethylene cap or 'bidule' and a metal crown cap seal, made either steel or aluminium with plastic backing. Bidule is mainly used to collect lees when disgorging process and also to prevent wine leakages and their contact with the crown metal cap, while this latter ensures the hermetic closure of the bottle [4]. Despite this, the crown cap permeability has a CO_2 loss rate range from 0.12 to 0.68 mL/day, while the O_2 going into bottle ranges from 500 to 3000 ppb each two years [8].

Several factors are involved in fermentation rate such as yeast strain, temperature and base wine composition, but the interaction of the three factors is considered the most important [4, 53, 72-74]. Yeast cell viability depends on their adaptation capacity to the stress factors that modify the environment in which they grow [75, 76]. In this context, yeasts should be able to detect and respond

to stress conditions without significant losses of their viability and therefore fermentation rates. The response mechanisms encompass detection systems and transcription pathways that activate transcription factors, which in turn are involved in the gene expression regulation [77, 78]. This will lead to changes in the protein level that will allow the adaptation of the yeast and its growth to the new conditions [79-82]. Several stress factors affect yeast viability and fermentation rates [78], some of them are common to other winemaking techniques (high ethanol concentration, low pH, nitrogen starvation) or are specific from the in bottle second fermentation (low temperature and CO_2 overpressure) (***Figure 7***).

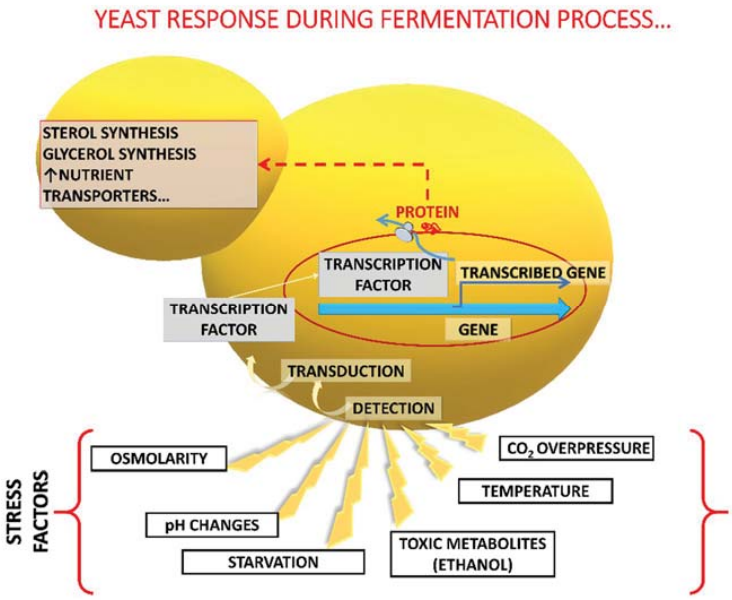

***Figure 7**. Stress factors during fermentation process. Source: Thesis Zuzuarregui-Miró A. (2005) (https://www.academia.edu. Last date: 14/10/2020).*

Penacho et al., (2012) [52] described a parallelism between transcriptomic profile of wine yeast during the first and second fermentation that included the expressions of genes involved in aerobic respiration, oxidative stress response, autophagy and peroxisomal function. However, these authors considered only ethanol and temperature as main factors driving gene transcription during second fermentation, not being observed changes related to CO_2 overpressure and pH. The same authors explained that the strong transcriptional response elicited by ethanol may masks specific responses derived from any other stress factors. Morphological changes related to yeast viability (small cell size and granular cytoplasm) were also observed by some authors in relation to temperature of second

fermentation, being demonstrated that high temperatures (16 ºC), compared with low temperatures (11 ºC), cause a dramatic decrease of yeast viability after day 20 of fermentation in bottle until total disappearance when reaching 90 days [52].

Marti-Raga et al., (2017)[83], working with interspecific hybrids from *S. cerevisiae*, identified four genes involved in the genetic determinism of second fermentation kinetics, which are characterized by playing a central role in maintaining cell homeostasis, such as intracellular pH regulation, yeast cell detoxification, control of plasma membrane composition, and the response to cold stress. The same authors observed that the heterozygous status of the hybrid for *PMA1* and *VMA13* genes provides more phenotypic robustness due to genetic-environment interactions between these genes with the pH of base wine, which evidences the effect of this factor in a non-genetically modified strain.

Studies conducted by our research group, regarding yeast proteome during second fermentation, showed an important effect of endogenous CO_2 overpressure on the cell viability and metabolites such as glycerol, reducing sugars and ethanol; and stress proteome. This overpressure increased the content of glycerol biosynthesis-related proteins and decreased those involved in the response to toxic metabolites like ROS, ethanol, acetaldehyde and acetic acid [84]. Furthermore, by using Gene Ontology (GO) analysis, it is observed that a high number of biological process are repressed under overpressure conditions, the most representatives being: the translation as tRNA metabolic process, chromosome organization, mRNA processing, ribosome biogenesis, and the ribonucleoprotein complex assembly [85, 86]. Additionally, the effect of CO_2 overpressure on the cell proteome is also yeast strain dependent, being observed that some cell death and cell wall integrity related proteins increase in number and content, regarding yeast strain used for the second fermentation [80, 86]. The effect of pressure is also observed in the yeast exo-metabolome, the results are exposed in chapters 3 and 4 of this thesis.

In light of the above mentioned, before tirage solution, the yeasts population must be adapted to overcome these stresses and therefore to ensure the completion of the second fermentation. Regarding this, the preparation of a yeast starter culture (a *pied-de-cuve*) is a sequential process carried out to adapt and grow the cells under the same stress factors as those involved during fermentation stage. For this, either active dry yeast (previously rehydrated) or a culture of the yeast selected (previously grown in sterilized must or complete medium with sucrose) is used, the initial population being of 10^8-10^9 colony-forming units (CFU)/mL. Although there is not a common protocol for this process, it is usually conducted with a progressive increase in sugar and ethanol contents of the culture broth [6, 52, 53, 87-91]. An example of this process is shown in ***Figure 8***. Nitrogen intake,

fermentation temperature as well as yeast strain and its type of metabolism are the main factors to be considered during yeast acclimation [6, 92]. In this context, some authors [73] establish that the fermentation kinetics is determined by the moment in which the nitrogen source is added and its organic or inorganic nature, having a strong impact on yeast growth during the preparation of starter culture. Furthermore, yeast viability and fitness will be maintained during second fermentation for a long time if amino acids, as nitrogen source, are added during acclimation phase [6, 92].

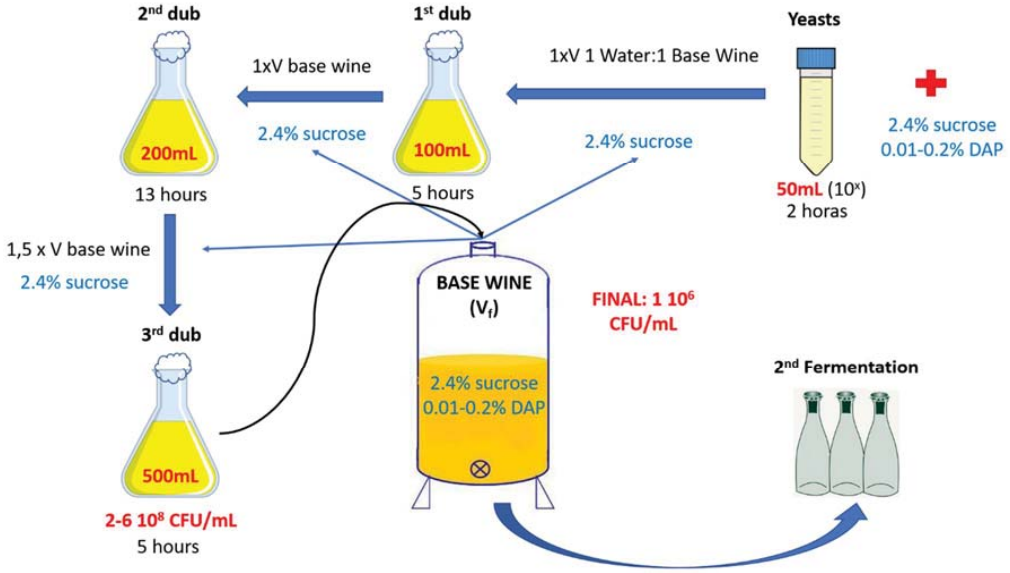

Figure 8. *Yeast acclimatation process for the second fermentation. Adopted from: Velazquez (2019).*

Gianvito et al., (2019) [6] recommend the addition of a sufficient volume of inoculum to provide 10^6 CFU/mL in the final blend, in order to avoid the production of sulphur compounds and other off-flavour compounds. Despite the adaptation stage, there is a lag phase followed by a short proliferation stage that increases the yeast population to 10^7 CFU/mL, after tirage [6].

Changes observed at the highest levels of the biological information system are also related to changes in the yeast metabolome, which in turn is directly related to variations in the sensory profile of the final product. Thereby, low temperature to which yeast is subjected during second fermentation, increases the rate of fatty acid unsaturation and decreases the carbon chain length to obtain medium-chain fatty acids [93]. Esteruelas et al., (2011) [94], studying second fermentations conducted at 12 ºC and 16 ºC, described better foaming properties for sparkling wines obtained at low temperature, probably caused by an increase in protein and oligosaccharide content.

Compared to other wine types, there is limited literature about the second fermentation effect on the aroma fraction of sparkling wines, despite the important yeasts contribution during this stage [23, 25, 81, 93, 95, 96]. This is mainly because these studies have been focused on the ageing period of sparkling wine-making [3, 16, 20-22, 24, 25, 72, 97-99]. In this regard, some published articles have established differences between aged sparkling wines and their respective base wines, and other reported changes in volatile profiles related to the second fermentation followed by an ageing time [26, 100-103]. Thereby, in these reports, changes caused by second fermentation are modified during ageing on lees, not making possible to establish the specific origin of each aroma fraction. In addition, those works done in this stage are only focused on targeted compounds belonging to some specific chemical families [21, 25, 104].

Perez-Magarino et al., (2013) [21] described an increase in esters, alcohols and some varietal compounds when compared wines after 3, 6 and 9 months of ageing on yeast lees to base wines; whereas the amount of acetates and fatty acids decreases due to their adsorption to the yeast cell walls. Similar trend is described by Verzeletti et al., (2016) [104] who defined three volatile profiles: (i) fermentation profile (0-30 days), (ii) post-fermentation profile (30-90 days) and (iii) mature profile (90-365 days). Although the same chemical families of compounds have been determined in all of them, a generalized decrease in high alcohol acetates (HAAs) and an increase in fusel alcohols, ethyl ester of fatty acids (EEFAs) and fatty acids were observed when going through fermentation to mature profile.

According the aforementioned, the key-volatile markers of the second fermentation can be established. Muñoz-Redondo et al., (2017) [26] when working with the champenoise method (11-12 weeks of fermentation), suggested ethyl-2-methylpropanoate, 3-methylbutyl hexanoate, ethyl-3-methylbutyrate, 2-methylpropyl hexanoate and ethyl valerate as potential volatile markers of this stage. The same authors also proposed ethyl hydroxycinnamate as volatile marker in previous studies [26]. However, not only this trend is observed during this stage of the traditional method, but also in the Charmat and Asti method, where there is no ageing stage. Thereby, propan-1-ol, 3-methyl-1-pentanol, 3-ethyoxypropanol and short chain acids have been proposed as potential volatile markers of second fermentation of varietal semi-sweet '*frizzante'* wines [93], while hexan-1-ol and ho-trienol and oxide forms of linalool have been proposed as markers of the same stage for Moscato Giallo sparkling wine obtained with Charmat and Asti method, respectively [3]. The studies carried out by Soares et al., (2015) [23] about the evolution of varietal compounds during the second fermentation in stainless steel tanks of Asti Spumante at 0, 6, 12 and 20 days, established an increase in HAAs (terpenyl acetate, hexyl acetate), EEFAs (ethyl octanoate, ethyl decanoate) and nonan-1-ol, and a decrease in

monoterpenes (limonene, 4-terpineol, terpinolene, citronellol, α-terpineol, linalool, hotrienol and nerol oxide).

2.3.2.3. Yeast strain selection

Yeasts are the protagonists in this special kind of winemaking, as different authors have related [4, 53, 87-90, 105]. Considering that nowadays consumers force new market trends and this special wine plays an increasing role in our daily life, some traditional winegrowing areas have adopted strategies to satisfy this demand. These areas can produce high-quality sparkling wines using new yeast starter cultures and local resources, obtaining cost-competitive wines in a sustainable way and avoiding high environmental impact [58, 106]. It is well known the importance that yeast strain has in the sparkling winemaking, due to its metabolite production, which may improve their organoleptic profile, even if it is obtained from non-aromatic base wines [4]. In this sense, a good starter selection is considered crucial in the production of high-quality sparkling wines [107]. However, second fermentation in closed recipients is a challenge for the yeasts, due to the hostile conditions to which they are subjected. Hence, additional technological features are required when used as first fermentation starter cultures (***Figure 9***).

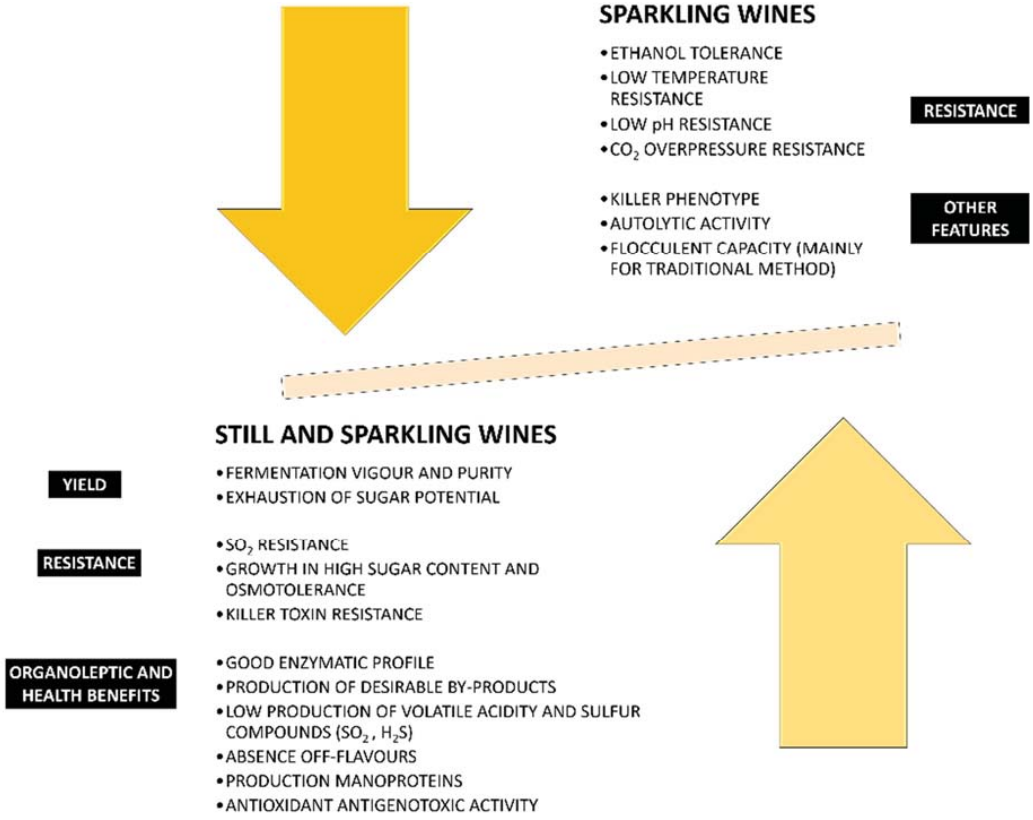

Figure 9. *Technological features of strains for the first and the second fermentation. Adopted from: Di Gianvito (2019).*

Those yeasts that meet the requirements exposed in ***Figure 9***, will be able to produce the base wine, grow and carry out the second fermentation, as well as infer on the organoleptic profile of the obtained wine types. Nevertheless, this does not always happen, since not all stress factors are equally relevant and therefore the action strategies are quite diverse. The lack of nutrients can be avoided by adding supplements during tirage, while the impact caused by ethanol, low temperature and pH, as well as CO_2 overpressure or SO_2 resistance, must be addressed through a previous acclimation process of the yeast [6, 108]. In addition, other properties are strain-dependent and determining in its selection, such as the autolysis and flocculation abilities and killer phenotype [4, 6, 88, 108, 109].

Even considering that the selected yeast feasibly meets all these requirements, a subsequent evaluation process about their contribution to the organoleptic properties is necessary, aimed to differentiate the new wines from those already in the market. In this sense, the strain selection is a long testing process where the interaction between environmental and technological factors is difficult to elucidate [53]. Some authors propose yeast selection protocols for this species in which a consecutive

screening steps based on technological and qualitative criteria are established to face uniformity in sparkling wine production [53]. Vigentini et al., (2017) suggested three stages for yeast screening: (i) the first screening based on resistance to alcohol and SO_2, (ii) the second selection attending to quality parameters (production of acetic acid, glycerol and hydrogen sulfide), and the third (iii) limited to those strains with a flocculant property and capacity to grow in ethanol. Other selection protocols are based on a preliminary tailored genotype and technological screening, followed by a study of their performance during first fermentation as well as their contribution into second fermentation aroma profile [90].

In light of the above mentioned, selected yeasts belonging to genus *Saccharomyces* generally conduct both the first and second fermentation [52, 107, 109, 110], but *S. bayanus* is the most used yeast, commercially available, for the traditional method [4, 20-24, 26, 74, 98, 111]. The current trend focused on the use of *S. cerevisiae* for this winemaking method is based on studying in depth those characteristics above described that technologically facilitate the sparkling wine making [6, 107, 112]. This section will only address flocculating *S. cerevisiae* yeasts, while the remaining properties (autolysis and killer phenotype) will be discussed in a later section.

Yeast flocculation is an asexual and reversible process in which thousands of cells adhere to each other to form flocs and sediments [6, 113]. This process is influenced by three categories of factors according to their mode of action: ethanol, temperature and nutrients that affect the gene activity; the genetic activity background of the specific yeast strain, and lastly the factors affecting cell to cell interactions, such as the ionic strength, the calcium contents and agitation. Regarding this, the most important genes involved in flocculation are the FLO family that comprise 13 genes with different characteristics [6, 113, 114]. From a technological point of view, the use of flocculent yeasts allow diminishing costs in sparkling wine production, since a faster cell removing during the disgorging phase is possible [114]. In addition, yeasts showing this phenotype have greater resistance to stressful environments, either due to the physical structure formed from their association or because of their cooperation to face stress factors. In this context, several mechanisms are invoked to explain this behaviour, such as physical protection, altruistic suicide, inhibitors degradation, deficit of ergosterol, extracellular matrix production and sporulation induction, which have been recently reviewed [6]. Regarding sparkling wine quality, there are scarce studies about the impact of flocculent yeasts in the organoleptic properties during the second fermentation [4, 109]. Nevertheless, the flocculent phenotype seems to be associate to an enhancement in floral and fruity aroma as a consequence of the higher content in esters and 2-phenylethanol in wines obtained with these yeasts [6, 107]. Coloretti et al., (2006)[112] studied interspecific hybrids generated from flocculent *S. cerevisiae* and non-

flocculent *S. uvarum,* being characterized by a higher degree of flocculation as well as resistance to a wide range of temperatures (from 6 ºC to 36 ºC), and the sparkling wines produced from them showed similar quality to those obtained by the parent strains. Similarly, the quality of sparkling wines obtained from *S. cerevisiae* with different flocculation degree was assessed by Gianvito et al., (2018) through the analysis of free amino acids, high molecular weight nitrogen release as well as physico-chemical composition [107]. Despite oenological performance (in terms of fermentation rates, maximum pressure reached, amino acids and other nitrogenous compounds) was similar for these yeasts to the commercially available yeasts, higher content in alcohols and esters were obtained in sparkling wines after 3 months fermentation.

The use of selected flor yeast strains in the Champenoise method is an innovative proposal for the sparkling wine industry, since their resistance to high ethanol content and surface adhesion properties has been demonstrated [115, 116]. Several -omics studies carried out about this topic have compared its proteomic and metabolomic profiles with those of other conventional *S. cerevisiae* yeasts [80-82, 85, 86, 117]. In this regard, Gonzalez-Jimenez et al., (2020a) found that flor yeasts displayed higher levels in the proteins Adh1p, Fba1p, Tdh1p, Tdh2p, Tdh3p, and Pgk1p by compared to the conventional ones, which is directly related to the differences in ester profiles and in the body of the sparkling wines obtained from them [81, 82]. The composition in the volatile metabolites of the obtained wines during the second fermentation and ageing stage was also studied and will be displayed in the chapters 4 and 6 of this dissertation.

While extend literature can be found related to *Saccharomyces* genus, scarce researches about the use of non-*Saccharomyces* for the 'prise de mousse' stage has been done [89, 105], compared to the production of base wine where is becoming widely diffused [87, 118, 119]. Furthermore, these yeasts are usually not recommended as single inoculum, since high-pressure conditions or alcohol content affect their ability to complete the wine fermentation. Mixed or sequential cultures along with the use of killer strains to improve this species' prevalence during must fermentation, has been also proposed [87, 88]. Despite this, some studies have used single inoculums during second fermentation but, unfortunately, neither the proportion of inoculated yeast during this stage nor the possible contamination by yeast *S. cerevisiae* has been determined [89, 105].

High amount of polysaccharides and proteins have been described, during the first fermentation, when using sequential inoculation of *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* together with *S. cerevisiae* [118, 119]. These increases were related to the faster autolysis of non-*Saccharomyces* in the presence of *Saccharomyces* yeasts and to an improvement of the base wine foamability. Furthermore, base wines of *M. pulcherrima* are characterized by smoky and flowery

nuances, while those obtained with *T. delbrueckii* display a high content in glycerol and low content in volatile acidity, both considered high quality indicators for sparkling wine production [53]. This is in accordance with other authors results who state that mixed *T. delbrueckii* + *S. cerevisiae* inoculation cause a decrease in acetic acid, acetaldehyde and acetoin contents and an increase in 2-phenylethanol, terpenols, and lactones [120-123]. In contrast, Velazquez et al., (2019), when producing base wine with killer strains of *T. delbrueckii,* found that neither foaming capacity nor organoleptic quality were considered appropriate for the base wine production, compared to those obtained with *S. cerevisiae* in pure culture [87, 88]. Furthermore, no evidence is found by the authors related to a positive correlation between polysaccharides and foam properties.

T. delbrueckii has also been used during second fermentation in pure or mixed cultures with *S. cerevisiae,* being demonstrated a positive impact for esters production and a different aroma profile of sparkling wines compared to those obtained only with *S. cerevisiae* [89]. Other studies in which killer strains of *T. delbrueckii* were used in pure culture suggest that CO_2 overpressure induces the death of these yeasts, being unfeasible for them to complete the fermentation, leaving wines with a high residual sugar contents and low CO_2 pressure [87]. However, this yeast, in mixed culture with *S. cerevisiae*, improves the quality of the final wine, which is mainly attributed to an increase in the contents of ethyl propanoate and some acids (e.g., isobutyric and butanoic), alcohols (e.g., 3-ethoxy-1-propanol), and phenols (e.g., 4-vinylguaiacol).

Saccharomycodes ludwigii and *Schizosaccharomyces pombe* have been also tested by Ivit et al., (2018) [105], in pure cultures during second fermentation of 4 months aged sparkling wines at 12 ºC, being possible for these yeasts to ferment the base wine to dryness. Although these microorganisms are considered as spoilage yeasts, regarding the change in acidity, colour, the content in biogenic amines, aroma compounds and sensory properties that they produce, no significant changes in the overall quality of sparkling wines were observed when compared to the obtained with *S. cerevisiae.* In addition, some authors find in *S. pombe* a promising innovation resource since it exhibits a malic dehydrogenase activity and an enhancement in mannoproteins and polysaccharides released during ageing [8, 109]. Nevertheless, deepen the use of these yeasts is still necessary when they are used to improve other technological and organoleptic properties of long-aged sparkling wine.

2.3.2.4. Riddling agents/Co-adjuvants

A common practice carried out during the *tirage* liquor preparation is the addition of riddling agents, being bentonite the most used in doses ranging from 0.1 to 10 g/L, either pure [16, 20-22, 124, 125], or combined with other organic compounds [18]. Two class of bentonite are commercially available, calcium bentonite and sodium bentonite, but only the former is characterized by producing more compact lees [8]. As a montmorillonite clay mineral, bentonite has a negative electric charge and attracts compounds positively charged, having a different cation exchange capacity each one. In this way, sodium bentonite has more absorption swelling ability, while calcium bentonite has more adsorption ability and best exchange capacity, being preferred in the production of Champagnes [8].

Although the use of bentonite is beneficial for removing the yeast lees, other components directly related to the organoleptic properties of the wine may be affected. Thereby, some studies describe that foaming properties are affected when using this co-adjuvant in bottle-fermented wines, compared with other co-adjuvants [18, 126-128]. Regarding this, Vanrell et al., (2007) [129], reported that the 80 % of soluble proteins (protein fraction of 20-30 kDa and 60kDa) is removed, causing a significant diminution of foam properties. This agrees with the results obtained by Martinez-Rodriguez and Polo (2003) [128], who explain that the addition of bentonite significantly modified protein and peptide composition of wines, while amino acid concentration is not affected. These authors showed that the wines made without bentonite have a greater foam formation, surface coverage and smaller bubble size than those obtained with bentonite addition.

Aroma compounds are also affected by the type of co-adjuvant used during tirage, being observed a significant effect when using pure bentonite, compared to its joint use with other organic compounds. In that way, bentonite addition causes a decrease in the content of some alcohols (methanol, isobutanol, n-amyl alcohol, isoamyl alcohol and geraniol), carbonyl compounds (acetaldehyde), EEFAs (ethyl acetate, ethyl propionate, ethyl butyrate, ethyl lactate, ethyl octanoate and ethyl decanoate), MEFAs (methyl acetate) and γ-butyrolactone [18]. In contrast, Pozo-Bayon et al., (2003) [127] verified that neither yeast strain nor addition of bentonite to the tirage solution, greatly influence the volatile composition of the wines compared to ageing factor. Notwithstanding, none of these authors reported information on the changes in the sensory profile of the wines. Other studies carried out on sparkling wines 20, 40, 90, 180, 270 and 365 days aged, show that the bentonite addition results in a decrease in sensory quality (visual aspect, aroma intensity, aroma quality, taste quality and harmony) of the wines evaluated [128].

Alternatives to the use of bentonite have been published such as: potassium alginate [70] or gelatine [3], but to the author knowledge no studies have been published of its effect on the sensory profiles of wines obtained by the traditional method.

2.3.2.5. Immobilized yeasts used during second fermentation

Considering that bentonite is not a specific adsorbent and some important fractions for sparkling wine sensorial quality can be lost, the use of other alternatives have been proposed in literature [4, 6]. From a technological point of view, yeast immobilization facilitates higher cell densities than traditional fermentation methods, improves yield, allows the reuse of the biocatalyst as well as diminishes and simplifies or eliminates the riddling and disgorging procedures [6, 115]. In addition, the wines obtained with this biotechnology have similar contents in ethanol, organic acids and volatiles to those obtained by using free cells and also organoleptic properties [6, 99, 126, 130, 131].

An example of a natural way of immobilization during in-bottle fermentation was previously described by using yeasts with flocculating phenotype, a relevant technological feature for the sparkling wine production [6]. In this context, taking advance of yeast cells capacity to adhere each other specific by cell-surface molecular interactions (flocculation) or to attach to surfaces (biofilm formation), various supports and immobilization techniques have been proposed and tested in beverage production [132] and a summary of the different systems is exposed in ***Figure 10***. It should be noted that supports used in wine industry must meet certain requirements related to food-grade purity, low cost, abundance, nature, stability and suitability to low-temperature fermentation [4].

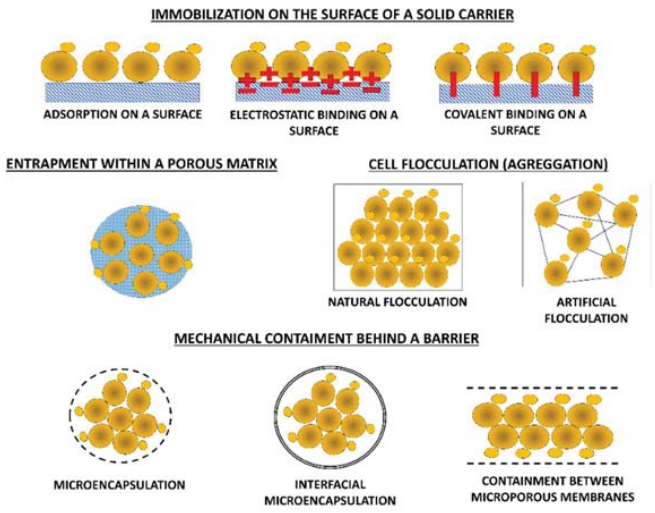

Figure 10. *Yeast Immobilization Systems. Adopted from Kourkoutas, 2004.*

Different immobilization techniques were tested for sparkling wine production such as carrageenan entrapping, collagen casting, chitosan/glutaraldehyde moulding, and most notably calcium-alginate gel or films entrapping [6, 99, 126, 133, 134]. Nevertheless, some of them have as disadvantage the detachment of cells from the support [4], the possible transfer of calcium or sodium ions to the wines if calcium alginate gels are used [99, 126], or the adsorption of volatile compounds [134]. Thus, current trends are focused on using new materials for yeast confinement. Recently, Berbegal et al., (2019) [130] studied the effect of two compatible supports (oak chips and cellulose power) in bottle-fermented sparkling wines and found technological differences, such as a faster sugar depletion and lees deposition, including differences in volatile composition, compared to those obtained with free cells.

Furthermore, the immobilization procedure is stressful for the yeasts, so that when used under the harsh conditions of second fermentation, unexpected results could be obtained [4, 126]. The efficiency of these systems is influenced by culture conditions used for the biomass accumulation and therefore the physiological state of yeasts before their immobilization [4]. In this context, some authors apply a stepwise adaptation of yeasts to ethanol before their entrapment in an adequate support [131]. These authors compared the aroma fraction of 6 months aged sparkling wines when using free cells of *S. bayanus* or entrapped into chitosan-calcium alginate double layer microcapsules (previously adapted or not to ethanol), obtaining a significant decrease in alcohol content when using ethanol-adapted yeasts in both forms. Additionally, lower total content of carbonyl compounds, phenols and higher in acids was found for encapsulated yeasts, although the sensory properties (aroma, flavour and mouthfeel) for the latter were similar to those obtained with free cells when ethanol tolerant yeast was used.

A novel method of yeast immobilization developed in our laboratory is called ‘biocapsules’, in which cells of the yeast *S. cerevisiae* remain attached naturally and spontaneously to the hyphae of the fungus *Penicillium chrysogenum* (with status GRAS) without the loss of viability [135]. This co-immobilization system is dependent on the properties to flocculate and to form bio-film of yeast strain used, showing higher immobilization rate, higher size and more consistent biocapsules to those strains with bio-film forming ability [115]. Some applications of the biocapsules are explored in a recent review [136], highlighting their importance to facilitate relevant phases of sparkling wine making technology, without affecting the quality of the final product [99, 126]. However, considering the importance of temperature on cell viability during the fermentation process, its study combined with this new immobilization system has not yet been carried out until the present thesis work.

Finally, there are other alternatives for yeast immobilization, such as the magnetization procedure, which consists in the adsorption of magnetic nanoparticles of iron-oxide maghemite (γ-Fe_2O_3) onto the yeasts cell surface [137]. This system allows to remove yeasts in an easy and fast way when a magnetic force is applied, increasing the metabolic activity of yeast during fermentation process. Nevertheless, their effect on sparkling wine quality is still unclear [8].

2.3.3. Post-fermentative stage: From the bottle to the tasting glass

2.3.3.1. On lees ageing

After the second fermentation, sparkling wines are subjected to an '*on lees'* ageing period that ranges from a few months to several years, according to the quality of the product and the production area. Thus, the Appellation of Origin Cava allows to sale only four categories, regarding the ageing time: cava (9 months), 'cava reserva' (15 months), 'cava gran reserva' (30 months), and ' cava paraje calificado' (36 months) [2]. During this stage, important changes in composition take place, contributing to develop some organoleptic properties that make of these products unique. The changes in wine composition caused through this phase override the effects derived from other technological or biotechnological factors [124]. Compared to still wines, this period is generally longer in sparkling wines when produced by traditional method. In addition, still wine lees are mainly composed by tartaric acid salts, organic residues, cells of yeasts and bacteria, while sparkling wine lees are mainly composed by cells from a single species of yeast and technological co-adjuvants [8].

This phase encompasses simultaneous oxidation-reduction and hydrolysis reactions, which generate organoleptic changes in the wines obtained by the traditional method, compared to the Charmat method [3, 7]. Amino acids, peptides, proteins, polysaccharides, nucleic acid derivatives and lipids are the main compounds released and therefore the greatest contributors to the quality of the final product [4, 138, 139]. Furthermore, during this period the yeast autolysis takes place, a process considered by some authors to be specific to each yeast strain used for the second fermentation (see **section 2.3.3.1**).

According to the scientific literature [24, 70, 103], during on lees ageing there is an increase in pH and a decrease in total acidity, total and free SO_2, not being observed changes in volatile acidity or ethanol content, although some authors have described an increase for this latter [103]. These changes can be explained either by consumption of acids by yeasts (pyruvic and malic acid) or by their own precipitation as potassium hydrogen tartrate salt. Rodriguez-Nogales et al., (2012) [140] described a decrease in neutral polysaccharides and total protein content (0.25 g/L and 27.8 mg/L respectively)

after 3 months of ageing, when compared to the base wine and a subsequent increase of 40-45 mg/L of protein at 9 months. In contrast, Martinez-Lapuente et al., (2013) [140] described that the highest content of mannoproteins and polysaccharides rich in arabinose and galactose were obtained after six months of ageing. In addition, Torrens et al., (2010) [103] showed a decrease in protein content when comparing the base wine with wines aged for 14-24 months. These changes are respectively a consequence of their intake by the yeasts, as well as the precipitation and/or partial enzymatic hydrolysis in the acidic hydro-alcoholic medium, after releasing from yeast cells. Opposite trend is described by the same authors for yeast assimilable nitrogen (YAN), attributing the increase up to 3 months to the passive release of amino acids from yeast to wine and the decrease between 3-9 months to autolysis of yeast. Pozo-Bayon et al., (2009) [141] established four main phases during ageing on lees, regarding nitrogen compounds. Thus, during second fermentation a decrease in amino acids and proteins as well as an increase in peptides occurs. Then, when viable and dead cells coexist, the peptides are degraded to amino acids, until there are no longer viable cells in the medium. At this time, it is where a release of peptides and proteins occurs again, followed by a decrease in amino acids after 9 months of ageing. In contrast, other authors tried to stablish a correlation between free l- and d-enantiomer amino acids and the storage time in bottle, not finding any evidence in this relationship [142].

Amino acids released from yeast lees during ageing could contribute to changes in volatile profile. In this regard, it is well known that fusel alcohols such as propan-1-ol, isobutanol, phenyl ethanol and isoamyl alcohols and their acetate esters are strongly correlated with this fraction through the Ehrlich pathway [60] in live yeasts. Some norisoprenoids are synthesized from methionine, such as vitispirane, which is considered as potential markers of wine ageing [143]. In addition, ethyl esters (ethyl vanillate), fatty acids (isobutyric acid) and some lactones (γ-butyrolactone), were found in wines elaborated from musts richer in amino acids [8, 21, 141]. In addition, Pérez-Margariño et al., (2013) [21] reported that total biogenic amines increased in wines with an ageing period higher than 3 months if high content of amino acids and decarboxylase positive microorganisms concur in the same medium.

The same trend described for YAN is observed in colour intensity (CI) or absorbance at 420 nm (for white wines). This parameter is directly related to the total polyphenol index (TPI or A_{280}) and the content of hydroxycinnamic esters (A_{320}). These latter compounds can be chemically or enzymatically oxidized resulting yellowish-brown products. Martinez-Lapuente et al., (2013)[19], when studying sparkling wines manufactured from different *Vitis vinifera,* described that during the initial months of ageing on yeast lees all the types of polyphenols decreased, while some were released to the wines during final months of ageing. A decrease in polyphenol content, and consequently in CI,

should be caused by their adsorption on yeast lees or by polymerisation and precipitation processes [70]. Sartor et al., (2019) [144, 145] stated that the ageing led to an increase in the individual phenolic compounds, especially caffeic, gallic, and ellagic acids, with an increase in the browning index. Furthermore, the authors found that caffeic acid was significantly correlated with browning and ageing period in all sparkling wines, being proposed as a quality marker to monitor the profile of white sparkling wines aged. Other authors describe the opposite trend between colour and polyphenol content during ageing [103], which could be explained by the formation of other browning compounds in wines such as furanic compounds or other compounds that are formed by sugar degradation during cava ageing [146, 147].

Foaming properties of sparkling wines are also influenced by compositional changes during ageing and an important review has been recently published [10]. Some authors found a positive correlation between these properties and the release of amino acids and proteins from the yeasts, not being found any evidence related to peptides [4, 10, 148, 149]. Kupfer et al., (2017a) [150] suggested that the protein seripauperin 5 (PAU5) from *S. cerevisiae* has foam stabilizing properties in sparkling wine. Likewise, those compounds released during yeasts autolysis (mannoproteins, polysaccharides rich in arabinose and galactose, homogalacturons, glucans and rhamnogalacturons) have a positive influence in foam stability, reaching a maximum at 18 months of ageing, that agree with an increase in monomeric compounds derived from enzymatic hydrolysis [151]. However, no correlation has been found between this fraction and foamability. On the other hand, lipids are also important contributors to the flavour along foam properties, although the effect on this latter is not still clear. Coelho et al., (2011) [152] studied the effect of some surface active compounds (monoacylglycerols of palmitic and stearic acids, glyceryl ethylene glycol fatty acid derivatives, among others) on the foam properties and found synergic effect when high molecular weight material (> 12kDa) is combined with hydrophobic material of lower molecular weight (1 kDa), resulting in an increase in the foam stability. This is in accordance with the results of Voce et al., (2019) [95], who found that high contents in palmitic and linolenic acids have a positive influence on foam properties. In contrast, other authors found that C8, C10 and C12 acids had a negative effect on foam, being observe opposite trend with their ethyl esters [8, 153].

Torrens et al., (2010) [103], when comparing base wine with aged sparkling wines (14-24 months), described a more complex profile for cava reserve wine, with toasty, lactic, sweet and yeasty the most characteristic nuances. Gallardo-Chacon et al., (2010) [67] stablished that the length of ageing on lees determines the type and amount of wine volatiles removed with lees. Regarding this, the sorption of most hydrophobic volatiles increases until 18 months and decreases thereafter, while those

less hydrophobic are mostly adsorbed until 2 months. The same authors described [66] that most positive flavour and aroma impact compounds (esters, aldehydes, norisoprenoids and terpenes) have been determined in lees headspace, while not evidence is provided for the effect of yeast contact on the level of those considered as off-flavour (sulphur compounds) [64]. Perez-Magarino et al., (2015) [124] compared changes in volatile fraction during 9, 18 and 30 months of ageing with lees and after 12 months of the disgorgement, and found a higher content in ethyl esters of fatty acid (EEFAs) and lower content in acetates (HAAs) and terpenoids at 9 months, compared with 18- 30 months. Additionally, wines at 9 and 18 months showed higher contents in isoamyl alcohols and ethyl lactate, while lower contents in EEFAs, C6 alcohols and terpenes and higher in ethyl ester of branched acids (EBBAs) and vainillin were found at 12 months after disgorging. Apart from the adsorption–desorption processes that occur in the cell walls, complex balances of intracellular synthesis and extracellular hydrolysis also determine the esters content [22]. Despite this, potential volatile markers of this stage have been suggested, such as hexenol, ethyl lactate, ethyl isovalerate, ethyl isobutyrate, ethyl 2-methylbutyrate, diethyl succinate, vitispirane or TDN (1,2-dihydro-1,1,6-trimethylnaphthalene) [21, 24, 26, 100, 141]. Some thiols and mercaptans have been also found in high concentration in aged Champagne wines [8], while phenylethyl acetate, isoamyl acetate, propyl acetate and hexyl acetate show high contents in young wines and are considered as youth markers [26].

Finally, monophosphate nucleotides are also released during this stage [65, 138, 139]. Despite being used as flavourings in the food industry, its effect on the quality of sparkling wine has not yet been determined, since other wine components released during autolysis can interfere with its determination [4, 8].

2.3.3.1. Yeast autolysis

Changes described above during ageing on lees are also related to yeast autolysis, since the compounds released from the yeast cells or autolysates to the wine affect its analytical and sensorial characteristics [138]. Yeast autolysis is a slow natural process that takes place before cell death, at the end of the stationary phase of growth (2-4 months after second fermentation), and it is characterized by the hydrolysis of intracellular polymers carried out by yeast enzymes, resulting in the gradual release of compounds from the cytoplasm or yeast cell walls during wine ageing [20, 65, 138] (***Figure 11***). This process has been subject of many researches mainly focussed on: (i) studying structural changes of yeast cell walls during this process or, (ii) studying product released to the medium (nitrogen compounds, polysaccharides, glycoproteins, lipids and others) [4, 65, 138, 139].

Depending on the authors, yeast autolysis mechanism takes place in four-five steps [65, 138, 139]: First, degradation of cells endo-structures and release of hydrolytic enzymes; second, inactivation of specific inhibitors of these enzymes located in the cytoplasm; third, enzymatic degradation of intracellular molecules followed by their accumulation on the cytosol; fourth, increase in the porosity of cell walls and release of autolytic products to the environment; and last, their degradation by yeast enzymes in extracellular environment.

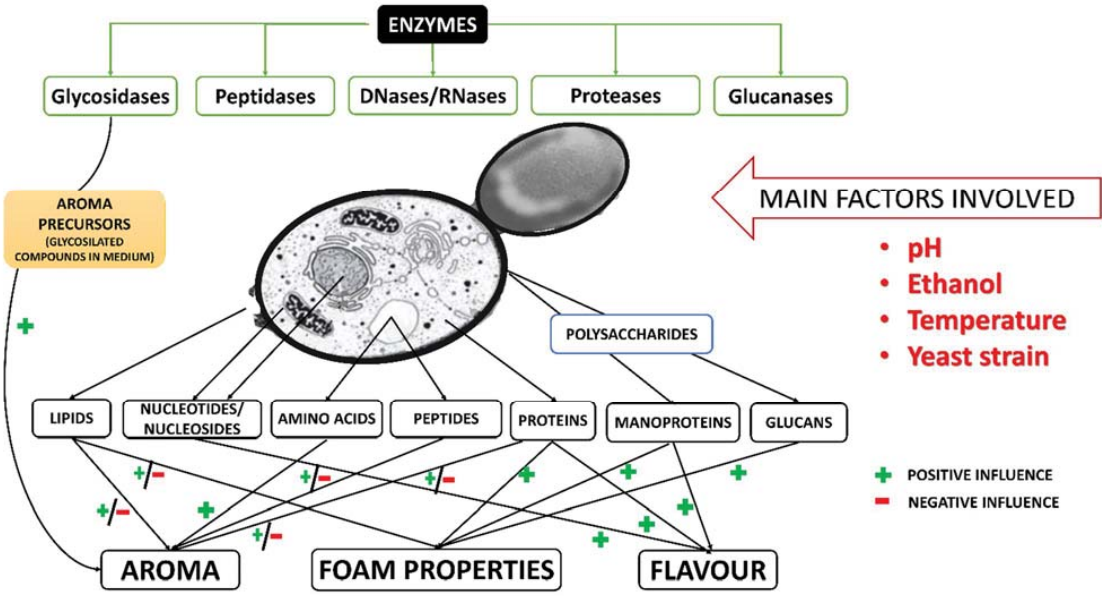

***Figure 11**. Yeast Autolysis in sparkling wine. **Source**: own elaboration.*

Autolysis development, the quality and quantity of auto-lysates are mainly dependent on yeast strain used, although other factors are involved, such as growing conditions and population, base wine composition (ethanol content and pH), temperature and ageing time [65, 138]. Therefore, since this is a process where enzymes play an important role, the enzymatic activity may be affected by these mentioned factors. Among the hydrolytic enzymes that take place during autolysis, proteases have been extensively studied [8]. The activity of its group of acidic enzymes (mainly led by Protease A) is responsible for the release nitrogen compounds, and it is especially influenced by the lack of sugar, pH, temperature and yeast strain, that increases drastically after 9 months of ageing [65, 138].

Studies focused on morphological changes and cell-wall compounds released into the environment, evidence degradation of the yeast cell wall [138]. Notwithstanding, few studies report the measurement of the enzymes involved for still wines, even less those carried out under sparkling

wine making conditions [8, 80, 86]. It has been demonstrated that the degradation of cell walls is carried out by glucanases in still wines with a consequent release of its main components, glucans and mannoproteins, in a process that can be summarized in three steps [8, 138, 139]. Firstly, the glucans hydrolysis, releasing the mannoproteins entrapped on them; secondly, a release of glucans either by residual glucanases on cell walls or solubilized in the medium; and finally, the mannoproteins degradation due to the proteases activity. Despite this enzymatic degradation, no cell breakdown occurs [65]. The studies carried out show that after autolysis, yeast cells are much smaller and show wrinkles or folds and ridges [138]. Furthermore, morphological changes are time-dependent, being observed a reduction of cytoplasm and vacuole contents at higher ageing times.

A process closely related to the autolysis is the autophagy [4] that consists of a catabolic process in which the cytoplasm is encapsulated in a double membrane structure to be subsequently released to the lysosome/vacuole and degraded to low molecular weight compounds. Inhibition of this process has been shown to accelerate autolysis [154], making it the subject of recent research. Recently, changes in the autophagy-related proteins under second fermentation conditions have been demonstrated for different *S. cerevisiae* strains by Porras-Agüera et al., (2020c) [117], being also noted that the regulators and proteins related to autophagosome formation, the vesicle nucleation and expansion, are strain-dependent. Furthermore, the same authors proposed the proteins Bcy1p, Sec2p, Sec13p, Sec18p, Shp1p and Vps15p as potential biomarker for accelerating the autolysis during ageing period in sparkling wine production.

2.3.3.2. Enzyme preparation and yeast autolysis enhancement

As mentioned at the beginning of ***section 2.3.3***, the ageing on lees is a slow process that implies a high cost of production until the product is marketed [102]. Given its positive influence on the roundness, aroma, flavour and foam properties of sparkling wines, suggesting the elimination of this stage would not be understandable [70] and most of the efforts developed in the recent decades are aimed to shorten this stage, specially based on the induction of autolysis, without affecting the quality of the final product.

At industrial scale, when it is sought to extract products derived from microorganisms, the usual processes for inducing autolysis range from the use of physical, chemical or biological inducers and depending on which one is used, it could last from 48 to 72 h [138]. However, when it comes of the oenological industry and more specifically that focused on bottle-fermented sparkling wine, any method is not always efficient. This is explained because the low pH and high ethanol content of

sparkling wines, along with the temperature conditions used during ageing (from 10 ºC to 12 ºC), are far from the ideal conditions in which autolysis takes place (absence of ethanol, pH 5 and 45 ºC) [6, 65]. In this sense, the current processes proposed to induce autolysis during this winemaking process can be grouped into two categories: physical treatments and biotechnological treatments (***Figure 12)***.

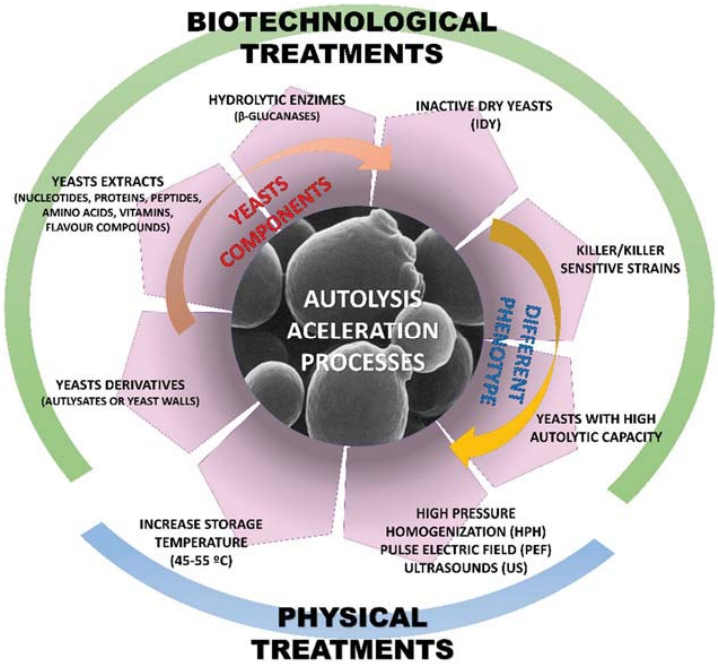

Figure 12*. Autolysis acceleration processes.* ***Source****: own elaboration.*

Within the physical treatments, the first attempts to induce the acceleration of autolysis were based on the temperature rise, which allows a fast release of high content of nitrogen compounds into the wine. However, the high temperatures required for an effective autolysis (from 45 ºC to 55 ºC) also lead to a destruction of the intracellular proteases, resulting in a slowdown of the process, or a reduction of the wine quality by the production of off-flavour compounds [6]. High-pressure homogenization (HPH), pulse electric fields (PEF) or ultrasounds (US) are nonthermal alternatives for a large-scale microbial cell disruption, intracellular bioproduct recovery, or enzyme activity modulation as well as relatively low-cost and environmentally friendly techniques [6]. From them, only HPH has been used for sparkling wine causing an improvement of the ester profile [111], although further research is needed to understand their effect on other sensory attributes as well as foaming properties [6, 8].

From a biotechnological point of view, various attempts to induce autolysis can be found in the scientific literature: (i) the use of yeasts components or enzymes additions, (ii) the use of killer/killer

sensitive yeast and (iii) the use of yeasts with autolytic capacity [141]. The addition of yeast components to base wine during tirage is the most common practice [8, 70, 124]. Regarding the manufacture process used in their production, four commercial products can be distinguished: inactive dry yeasts (IDY), yeasts autolysates, yeast walls and yeasts extracts [4, 6, 155].

It has been shown that the use of inactive dry yeast during tirage improve the glycerol content and provides the wines with proteins, mannoproteins and polysaccharides [6]. In fact, the results obtained depend on the yeast genus (*Saccharomyces* or non-*Saccharomyces*) used, being demonstrated that the better results are obtained with IDY from *T. delbrueckii* [119, 140]. Nevertheless the thermal or enzymatic manufacturing process carried out to obtain these products, negatively affects the sensory properties, since off-flavours may release into the wine [6, 156]. Mannoproteins positively influence the contents in tyrosol, *trans*-resveratrol, gallic acid, catechin and hydroxycinnamic acids, mainly observed at the end of the on-lees ageing [145]. Foaming properties and volatile profile could be also enhanced with mannoproteins or yeasts lees [124, 157]. The combined use of mannoproteins or lees with immobilized yeasts during tirage for the production of aged sparkling wines has been demonstrated a promising strategy to enhance the aroma quality of sparkling wines [134]. Fruity esters, nerolidol and β-damascenone increase in wines treated with mannoproteins compared to those untreated that are characterized by fatty/metallic nuances due to the higher levels in long-chain fatty acids. Furthermore, modulating the volume of lees added to the tirage liqueur is considered relevant to report an economic benefit, since it allows to obtain the desired flavour in a short time [158].

Other alternative to autolysis is the addition of enzymes, that induces the release of components with positive impact on the quality of the wine are released [4, 6, 8]. Rodriguez-Nogales et al., (2012) [158] studied the effect that different co-adjuvants had on the chemical characteristics and sensory properties of sparkling wines (3, 6, 9 months aged) and concluded that only β-glucanases increase the ageing characteristics, while yeast derivatives (yeast cell walls and yeast autolysates) improve their fruity and flowery character. Nevertheless, its use would also increase the cost of the final product [6].

Killer and killer sensitive yeasts strains, used in co-culture, is another strategy recently applied to promote autolysis [87, 88, 159]. Higher amount in total proteins content, amino acids and polysaccharides are the main changes described in the literature, in which the positive influence on the foam properties without affecting fermentation kinetics. However, these results would not occur if the sensitive killer / killer coupling were not effective, since it is a strain-dependent procedure, in which the amount of toxin an the dissimilar degree of sensitivity must be considered [6].

Since alternatives described above are not free of drawbacks, and therefore the final quality of the wine could be affected, the use of yeasts with high autolytic capacity has also been proposed.

Nonetheless, natural selection of yeasts with autolytic phenotype is not an easy way, since they must also meet other non-binding features, such as flocculation capacity. Some authors have demonstrated the independence of both phenotypes as well as have associated autolytic capacity to the expression of the genes ATG1, ATG17 and ATG29 [160]. Compared to the natural selection of yeasts with high autolytic capacity, there are other possibilities based on yeast mutagenesis or genetic engineering which, not being accepted by the consumer and their use is restricted in some production areas, are promising for quality improvement of wine [154, 157]. Additionally, the importance of these studies lies in the fact that they have been able to elucidate the relationships between autolysis and autophagy, a field that needs to be explored for an efficient yeast selection process [4, 6].

2.3.3.3. Riddling (*Remuage*) and Disgorging (*Dégorgement*)

The last step of the Champenoise elaboration method is the 'riddling' (*remouage* in French). Broadly speaking, this procedure is a bottle handling that consists of a rotation, followed by an increase in the angle of inclination of the bottle, which is carried out manually or mechanically over a period of 10-15 days, until placing them neck-down. This allows to remove the yeast lees by gravity, carrying it to the bottle neck, while the sparkling wine remains completely clear [4]. Afterwards, bottles are ready to removal of sediments or 'disgorging' (*dégorgement*), so the neck of the bottle is frozen at –20 ºC with a glycol solution, which facilitates the sediment being trapped in the plastic cap (*bidule*) and its rapid ejection when the crown cap is removed at the disgorging operation.[4, 8].

During disgorging, the loss of CO_2, intake of oxygen as well as gushing can occur [9, 150, 161]. While the first two factors are more related to the loss of quality of sparkling wine, the last causes a financial loss due to the reduction in wine volume, the decrease in bottle-line speed and efficiency; not having yet determined its impact on foam, volatile compounds and flavours [8, 161]. The loss of carbon dioxide can be prevented by and efficient bottle-closure, meanwhile different strategies has been developed to avoid of post-bottling oxidation such as ascorbic and SO_2 additions or jetting, which consists in the insertion of 100 µL of wine into the bottle neck to induce foaming and avoid the ingress of oxygen in the bottle-neck [8].

2.3.3.4. Expedition Liquor and Dosage

Immediately after disgorging, a dosage solution (*expedition liquor*) is added to replace the wine losses caused by gushing at disgorging phase. The composition of this liquor is specific to each winery

and generally consists of a blend made with sparkling aged wines, still wines aged in barrels or brandy, to which sugar, citric acid and SO_2 are added. Taking into account the sugar content in the expedition liquor, several wine types can be made according to sweetness degree in the final product: brut, 0-12 g/L; extra-dry, 12-17 g/L; dry, 17-32 g/L, demi-sec, 32-50 g/L and sweet, > 50 g/L [1].

Some researchers have studied the effect of expedition liquor on the volatile compounds of sparkling wines made according the traditional method. Thus, Kemp et al., (2017) [9] investigated sensorial and chemical changes at 5, 10 and 15 weeks after disgorging, added with different dosage styles: zero dosage or Brut nature (ZD); multivarietal sparkling wine + sugar (BS); unoaked still Chardonnay wine + sugar (UC); Pinot noir sparkling wine + sugar (PN); Brandy + sugar (B) and Icewine (IW); all of them with a sugar level of 300 g/L in the expedition liquor. Their results showed that dosage solutions made from sparkling wines showed higher pH values than dosage solutions of still wines, not finding differences for titratable acidity. In addition, higher levels of dissolved oxygen were found for dosages ZD (6.6 mg/L) and BS (5.0 mg/L), while IW displayed the lowest level (3.1 mg/L). Differences in foam properties are also observed due to the different content in sugar, ethanol, phenolic compounds, of dosages. The volatile fraction was also affected, being observed that the type of wines used has more influence than sugar added, which is attributed to the different wine styles, production techniques, ageing as well as grape variety used. Another important factor analysed is the time elapsed between disgorging and the sale of sparkling wine, which depends on the producer and can last several months [8]. Thus, the authors stated that at the beginning of the dosage, sugar addition mainly affects the higher alcohols contents than esters, although such differences remit at 15 weeks of disgorging. ZD, BS dosages were similar after 15 weeks and along with PN were characterized by higher levels in ethyl isovalerate. Likewise, IW and UC and were most influenced by 2-phenylethanol while 1-hexanol, ethyl octanoate, ethyl hexanoate and ethyl butanoate characterised most B dosage. This suggests the need to prevent sparkling wines from being placed on the market without reaching a prior chemical equilibrium. Nevertheless, the study does not consider the effect of other factors such as CO_2, the concentration of sugar during dosage or the enzymatic activity, as other authors do [162].

Recently, Benucci et al., (2020) [71] studied the impact of post-bottling storage conditions (9 months post disgorging) on colour and sensory profile of a rose sparkling wine at different conditions of temperature (5 ºC and 30 ºC) and darkness/UV radiation; revealing a considerable decrease in colour intensity (CI) and an increase in hue (H) as well as the worst sensory score when radiation was used at 5 ºC. Furthermore, the storage at 30 ºC in darkness increases CI and H, being the wines characterized by burnt notes.

Summarizing, ***Figure 13*** shows a summary of the different factors involved in the quality of sparkling wines obtained by the traditional method.

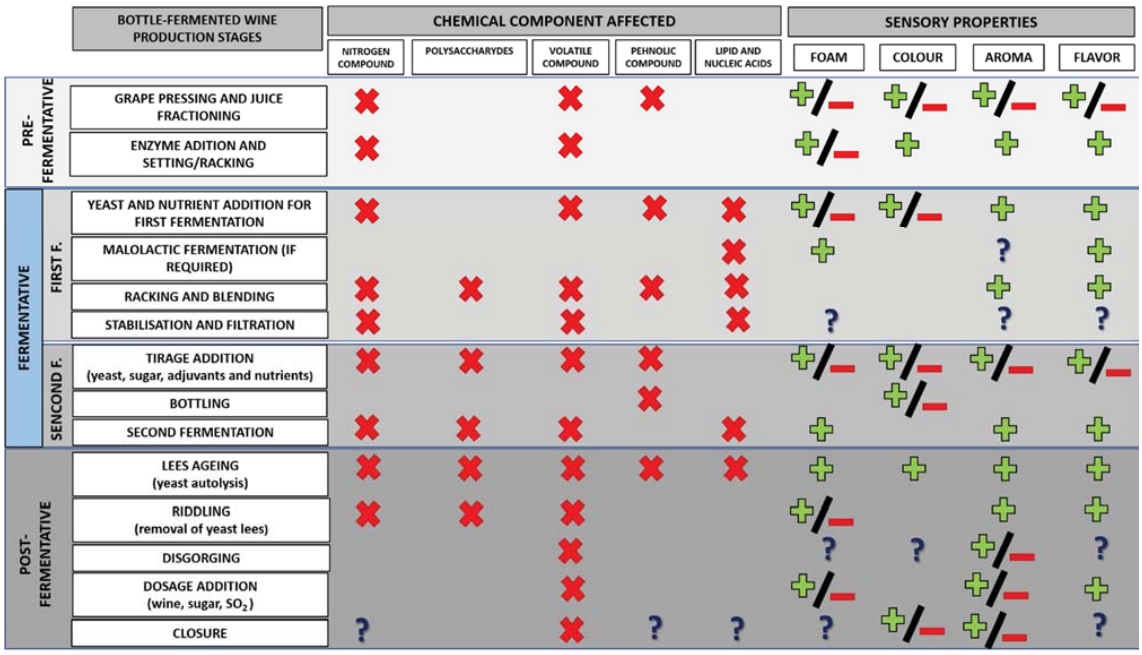

Figure 13. *Summary of main factors involved in sparkling wine quality. Symbols: "x": indicates the affected parameter. "?": object of future research. "+": Positive influence. "-": negative influence. Source: own elaboration.*

2.4. Aroma of sparkling wines

Aroma is one of the most important quality indicators of sparkling wine [8], and considering their relevance in the acceptability by consumers, it is very interesting to know the volatile compounds involved in it. In this context, the volatile organic compounds (VOCs) constituting the aroma fraction, can be classified into four groups in correspondence with each sparkling wine production stage [4, 95]. The first group (i) is constituted by the VOCs from grape cells; the second (ii) are those secondary grape aromas from pre-fermentative phases or by thermal, chemical and enzymatic reactions in must, which are positively or negatively related with wine quality. Group (iii) are the fermentative aromas, from the first and secondary fermentation, which are mostly influenced by must and base wine composition, fermentation temperature and yeast strain. Lastly, group (iv), also called maturation or ageing aroma, encompasses those volatile compounds with low molecular weight that are released

from yeast autolysis along wine ageing. However, this classification is not necessarily indicative that an aroma compound originates solely and exclusively in one phase of the winemaking process.

Free aroma fraction of grapes is very small, since they are hydrophobic molecules, which are stored into grape pulp and skin as bound forms with sugars or amino acids, but not always with the same chemical structure. Ferreira et al., (2019) [30] established seven pools of aroma precursors in grapes, regarding the structures of their bound forms, their ability to be released in the wine and their impact on the aroma perception. According these authors, only five groups play an essential role in the development of varietal wine aroma during ageing, but not all the analytical strategies permits to analyse all the compounds belonging to each defined pool, being subjected to their constant review [30]. In addition, the number and distribution of volatile compounds, involved in the complexity of wine aroma regarding each production stage, constitutes a hard challenge from an analytical point of view, mainly when compounds from different chemical families showing low concentration levels, must be identified and quantified [97, 163].

2.4.1. Analytical techniques

Given the low concentration of these compounds in wine matrix, their analysis frequently requires an extraction-concentration step before determination and quantification. Extraction techniques of volatiles from aqueous matrices have extensively evolved in the recent decades and all of them being applied to different wine types. In this sense, less environmentally friendly techniques, organic solvents based, such as the liquid-liquid extraction (LLE) has been substituted by solvent-less procedures, which are more selective, sensitive, reproducible and less time consuming [18, 20, 21, 95]. Simultaneous distillation extraction method (SDE) combines advantages of LLE and steam distillation extraction [164, 165]; while solid phase extraction (SPE) [3, 16, 72, 93, 95]; stir bar sorptive extraction (SBSE) with thermal [82, 99] or liquid desorption [17]; and head space phase microextraction (HS-SPME), are an example of the different solvent-free extraction techniques described in the literature for sparkling wines ***(Figure 14)*** [166]. From them, HS-SPME has become the most widely used extraction technique in wine aroma analysis because of the high number of adsorbent fibers commercially available, being highly satisfactory for the varietal aroma analysis [14, 23-26, 30, 95, 97, 100, 101, 164, 165, 167, 168]. However, SPME has a lower extraction capacity and sensitivity than SBSE, being two variants used for this latter technique: (i) direct immersion of the twister (SBSE); (ii) placing the stir bar into the head space (HSSE) [7, 163]. Compared to SBSE method, HSSE increases

the lifetime of the stir bar and shows more efficient extraction for those compounds more volatile, while the former extracts a large amount of aroma compounds (volatile and semi-volatile). Different strategies based on this extraction procedure have been tested to improve its sensitivity, increase the number of volatile compounds to be extracted and the recovery percentages, through the combined use of twister coated with different adsorbents and/or using combined extraction variants (by immersion or in the wine headspace).

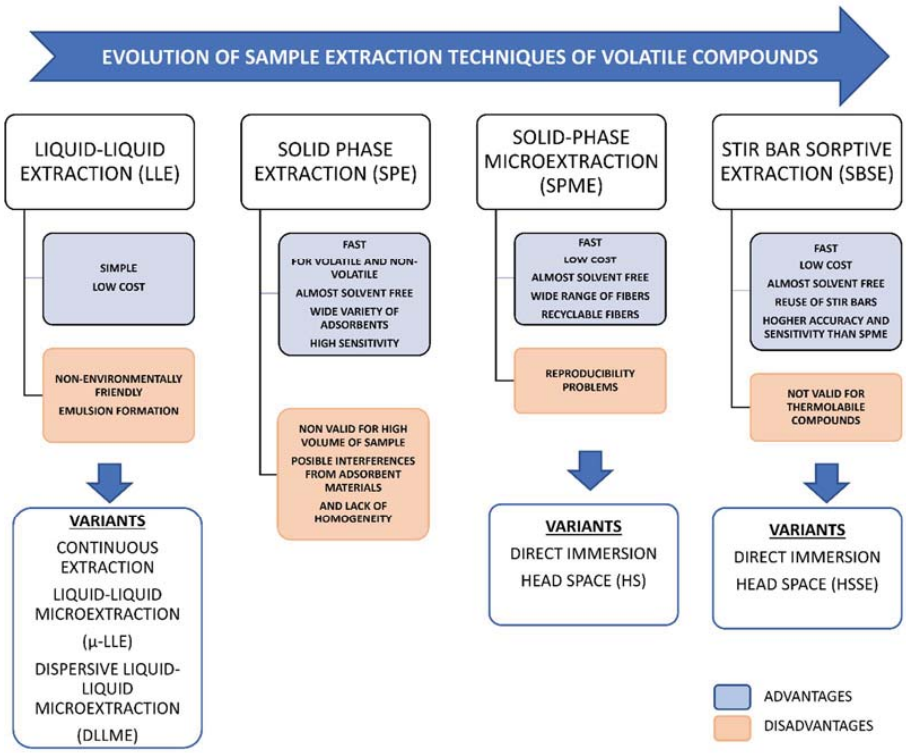

***Figure 14**. Extraction techniques for volatile compounds. **Source**: own elaboration.*

Some advanced polymeric adsorbents provide a wider extraction of aroma precursors and aglycones, but they are not effective for all the glycosides [30, 101]. These fractions should be subjected to acidic or enzymatic hydrolysis prior extraction. In this regard, the use of enzymes, in comparison to acids, provides a relatively unbiased composition of the aglycone present in the matrix when the appropriate enzyme is used. Nevertheless, this is only useful to estimate direct precursors of terpinols, but not for some important compounds derived from nor-isoprenoids, which require acidic hydrolysis at high temperatures (from 45 to 100 ºC), to the detriment of other aroma molecules [63, 169]. Another important aspect to take into account in the hydrolysis process is the oxidation of some

aroma compounds and to avoid it, new extraction techniques based on a reducing environment in presence of grape polyphenols has been recently proposed [30].

Some extraction techniques, such as LLE and SPE, require a post-treatment consisting on an evaporation or concentration step to remove the solvent, once the extraction procedure is finished and previously to the chromatographic analysis [163, 166]. Other techniques, such as SBSE, require a desorption process which is carried out in a thermal desorption unit (TDU), coupled online to a GC-MS system [99]. TDU has been described in scientific literature as a device with a remarkable sensitivity but at the same time, destructive and expensive [7]. In addition, the high desorption temperatures used in the operating conditions (> 200 ºC), make it unsuitable for analysing thermo-labile compounds, contributing at the same time to the appearance of artefacts [17]. Alternatives found in literature substitute the thermal desorption system with liquid desorption (LD), based on the injection of analytes with a small volume (200 µL) of an organic solvent, combined with large volume injection (LVI), leaving be solvent-free techniques.

Physicochemical properties and compound concentration of the analytes in the matrix are the main factors involved in the choice of the analytical technique [163]. Furthermore, there are two approaches to determine volatile metabolites: profiling (targeted) or fingerprinting (untargeted) analysis. While the former allows to identify and quantify pre-selected metabolites, fingerprinting is based on determining as many compounds as possible with a minimal pre-treatment of sample [163]. The importance of untargeted analysis lies in obtaining more information to generate the molecular fingerprint of a complex matrix, such as wine. Nevertheless, metabolite identification is very difficult, since it is based on matching the linear retention index and mass spectrum of sample peak of a pure compounds previously analysed under identical instrumental conditions [23, 102, 166]. Additionally, although today there are increasingly complete spectral libraries (NIST library, Wiley Library, among others), they do not always contain all the expected compounds when studying the volatile metabolites, given that some are not commercially available [163, 166].

The total number of aroma compounds in the sparkling wine matrix can be grouped in major volatile compounds (≥ 10 mg/L) and minor volatile compounds (< 10 mg/L). Thus, in the current study, major volatile compounds have been usually identified and quantified with one-dimensional gas chromatography (1D-GC) using a flame ionization detector (FID) or a mass spectrometer detector (MSD). This last technique (GC-MSD) along with two-dimensional gas chromatography (GCxGC) and MS detector have been mainly used in the last decade to characterize the minority volatile profile of sparkling wines [102, 168, 171, 172]. The number of compounds positively and/or tentatively

identified through GCxGC-MS as well as by the separation of co-eluted compounds is higher than in 1D-GC.

Statistical tools such as the univariate analysis of variance (ANOVA) and multivariate analysis have been widely used for wine volatilome dataset analysis. Regarding multivariate data analysis two approaches can be established: unsupervised or supervised methods [166]. While the former are descriptive models in which intrinsic structures/patterns and relations are fixed into the data set; the latter allows to build classification or predictive models based on the previously known information [166]. For this, the predictive properties of these models are previously tested and internally or externally validated (training set), before their use in unknown samples. Regarding sparkling wine aroma, an example of unsupervised methods are: the principal component analysis (PCA) [7, 22, 24, 70, 99, 173, 174], factor analysis (FA) [124] and cluster analysis (CA) [102]. On the other hand, linear discriminant analysis (LDA) [7, 100, 126], partial least squares (PLS) [13, 25, 26, 98], and artificial neural networks (ANN) [166], among others, are used as supervised methods.

For the selection of possible key-markers, related to the factor or stage under study, some authors take advantage from the information provided by the loading plot of PCA model, for the cluster formation of a given dataset [99, 166]. Thus, the numerical value gives information about the contribution of this variable to the score plot, as well as how much they have in common with a specific component. Likewise, those authors who use supervised recognition techniques, such as partial-least - squares discriminant analysis (PLS-DA) or orthogonal projections to latent structures discriminant analysis (OLPS-DA), use in combination the variable importance in projection (VIP) values and the category α in the least significant differences (LSD, Fishers' test) [25, 26, 98].

Table 1 shows some selected bibliographic references, related to the determination of the different chemical families of volatile compounds, extraction techniques, determination methods and factors involved in each stage of sparkling wine making.

Table 1. Application of GC in metabolomic studies carried out in sparkling wines

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
VOLATILE COMPOUNDS RELATED TO PRE-FERMENTATIVE STAGES									
LD/LV-GC-qMS	Varietal, soil and ripening influence in volatile fraction	T (tentatively quantified with structurally related standards)	71	Monoterpenoids (16), sesquiterpenoids (13), norisoprenoids (4), esters (36), alcohols (2) (7.59 to 11.44 mg/L).	SBSE	Basic statistic (RSD)	**Varietal compounds**: *monoterpenols*: linalool, hotrienol, α-terpineol, geraniol, nerol *sesquiterpenoids*: _-Chamigrene, nerolidol, and (*E,Z*)-α-farnesene *norisporenoids*: Vitispirane, TDN *esters:* butyl acetate, phenylethyl acetate **Varietal components are more affected by soil**: Higher content in clay-calcareous soil than clayey or sandy soil **Stage of ripening:** Early harvest: ↑sesquiterpenes and norisoprenoids and hexan-1-ol Highest content of volatiles when grapes are in optimal or late ripening stage Changes related to grape variety used are also observed and FP variety can provide sparkling wines with higher aroma potential than BG variety	**Two grape varieties and its mixture:** Baga (BG) Fernão-Pires (FP) with ripening stages and harvested in different soil **Yeast strain and 2nd fermentation conditions:** NOT REPORTED **Ageing on lees:** 12 months	[17]
GC/MS	Influence of pre-fermentative maceration and ageing	T (quantitative)	24	Esters (1.6-2.2 mg/L) EEFAs (3), HAAs (5), EEBAs (4), cinnamates (2), MEFAs (3), IEFAs (3), EEOCNFAs (2), miscellaneous (2)	HS-SPME (PDMS)	*ANOVA (LSD-Fisher) PLS-DA*	**Pre-maceration markers:** ethyl heptanoate **Ageing markers:** ethyl isovalerate, ethyl isobutyrate, ethyl 2-methylbutyrate **Youth markers:** phenylethyl acetate, isoamyl acetate, propyl acetate, hexyl acetate	Varietal Traditional method, (0, 3, 6, 9 months)/ *S. cerevisiae var. bayanus* (Viniferm PDM, Agrovin) + Enozym AROME (Agrovin)	[26]
GC/MS	Varietal characterization of Maresco sparkling wine	T (quantitative)	13	Alcohols (4), HAAs (3), EEFAs (4), Acid (1) and a Terpinol (0.3-28 mg/L)	HS-SPME (PDMS, PDMS/DVB, CAR/PDMS; DVB/CAR/PDM) SPE	ANOVA (Tuckey test)	2-metyl-butanol, phenyl-ethanol, linalool, isoamyl acetate, phenyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, octanoic acid	Varietal Traditional (9 months) /autochthonous yeast strain	[97]
GC/FID GC/MS	Varietal Characterization	T(quantitative)	25	alcohols (5), acids (3), esters (5), terpenes (10), thiols (1) (0.001-19.2 mg/L)	SPE (C18-reverse phase)	ANOVA (Tuckey test) Cluster analysis, PCA	Higher content in ethyl esters and monoterpenes for Villenave and Moscato Emgrapa Niagara grapes with high content in linalool oxide (A, B, D)	5 classical (*V. vinifera)* and 5 innovative grape varieties (*V. labrusca)* Traditional method (18 months ageing)// *S. cerevisiae PB2002*	[16]
Volatile compounds (Free and	Varietal characterization	U (Semi-quantified) IS: ethyl heptanoate, 1-	58	acids (12), alcohols (5), C6 compounds (6), Diols (2), esters (26), carbonyl	**LLE:** non-varietal aromas **SPE (C18)**:	Basic statistic (SD and CV)	2-methyl propanoic acid, 3-methylbutanoic acid, hexanoic acid, decanoic acid, isoamyl alcohols, 2-phenylethanol, isoamyl acetate, 2-	Varietal, white (Ribolla Gialla) Commercial sparkling wine from different wine regions	[95]

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
bounds): GC-MS, GC-MS/MS		heptanol, 2-octanol		compounds (3), thiols (1), lactones (2) **Free compounds:** Terpenes (11), Norisoprenoids (6) **Bound compounds:** Terpenes (4), Norisoprenoids (5)	free and bound terpenes and norisopreoind **SPME** (DVB/CAR/PDMS): varietal and non-varietal compounds		phenylethyl acetate, hexyl acetate, ethyl lactate, linalool, geraniol, TDN, β-damascenone Off-flavour precursors: 2-aminoacetophenone (2-AAP) High content in palmitic and linolenic acid: positive influence in foam properties	Mainly produced by Charmat method without lees contact	
1DxGC/MS GCxGC/TOF MS	Varietal characterization	U (qualitative, semi-quantitative)	70 compounds tentatively identified with 1DxGC and 173 with GCxGC (21 positively identified)	**1Dx GC:** esters (27), terpenes (18), alcohols (11), acids (7), norisoprenoids (2), aldehydes (2), phenols (2) and pyran (1) **GCxGC:** esters (50), alcohols (21), acids (10), aldehydes (8), ketones (8), lactones (6), phenols (6), sulphur compounds (5), norisoprenoids (2), pyrans (3) (expressed as chromatographic area)	HS-SPME (DVB/PDMS)	*Fisher ratio* *PCA*	2-phenylethanol, propyl octanoate, propyl decanote, 2-phenylethyl acetate, α-terpineol, linalool, nerol, menthol, geraniol, limonene, linalool acetate	Varietal Traditional, Charmat, Asti methods//commercial sparkling wines	[168]
VOLATILE COMPOUNDS RELATED TO FERMENTATIVE STAGES (FIRST AND SECOND FERMENTATION) AND ELABORATION METHOD									
GC/FID GC/MS	Varietal characterization	T(quantified)	48	monoterpenes (6), alcohols and C6 alcohols (14), fatty acids (8), EEFAs (9), HAAs (5), ketones (2), polyols (1), volatile phenols (2), lactones (2) (253-309 mg/L)	LLE (Na_2SO_4, anhydrous) and elution with dichloromethane	PCA	**First fermentation:** long chain acids, EEFAs and C6-alcohols **Second fermentation:** propan-1-ol, 1-butanol, 3-methyl-1-pentanol, 3-ethoxypropanol, short chain acids	Charmat Varietal (white) Sweet-semi-sparkling wine (frizzante)// (Zymaflore X5, Laffort OEnologie, Bordeaux, France)	[93]
GC/MS	Influence of base wine composition and second fermentation	T (quantitative)	25	Esters (1.3-2.2 mg/L) EEFAs (3), HAAs (5), EEBAs (4), cinnamates (2), MEFAs (3), IEFAs (3), EEOCNFAs (3), miscellaneous (2)	HS-SPME (PDMS)	*ANOVA (LSD-Fisher)* *PLS-DA*	**Second fermentation markers:** ethyl 2-methylpropanoate, 3-methylbutyl hexanoate, ethyl 3-methylbutyrate, 2-methylpropyl hexanoate, ethyl valerate	Varietal Traditional method (11-12 weeks)/ *S.cerevisiae var. bayanus* (Viniferm PDM, Agrovin)	[25]
GC-MS	Varietal characterization (aroma precursors)	T (quantitative)	37	EEFAs (9), HAAs (2), alcohols (10), acids (3), terpenes (5), norisoprenoids (1)	HS-SPME Extraction of aroma precursors CW/DVB	LSD test	**2nd Fermentation** ↑short-chain aliphatic acids and ethyl esters ↓medium-chain aliphatic acids and ethyl esters ↑more linear and branched alkanols	Varietal (Chardonnay, Riesling) **Base wine:** *S. cerevisiae cerevisiae* **2nd Fermentation:**	[101]

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
					CW/DVB + DVB/CAR/PDMS		↑norisoprenoids **Riesling vs. Chardonnay** Higher monoterpene alcohols and linalool oxide concentrations	Charmat// *S. cerevisiae var.bayanus (IOC 18-2007) and S. cerevisiae cerevisiae* (Fermicru VB 1) **Ageing with and without lees:** 4 months (on lees) + 10 week (in bottle)	
1DxGC/MS GCxGC/TOF MS	Varietal characterization	U (semi quantified)	Tentatively identified: 42 (1DxGC), 172 (GCxGC) and 31 positively identified	(Results in area percentage: 1.2-39.5 %) acids (10), alcohols (19), aldehydes (5), esters(68), ethers (7), ketones (12), lactones (3), phenols (3), sulphur compounds (5), terpenes (38), norisoprenoids (2)	HS-SPME (PDMS; CAR/PDMS; PDMS/DVB; DVB/CAR/PDMS) **Best Performance:** PDMS/DVB	Not Reported	**Increase during fermentation:** terpenyl acetates, ethyl octanoate, ethyl decanoate, hexyl acetate, 1-nonanol **Decrease during fermentation:** monoterpenes: limonene, 4-terpineol, terpinolene, citronellol, α-terpineol, linalool, hotrienol, nerol oxide	Asti Spumante or Moscatel Sparkling (fermentation of must in closed tank) (0, 6, 12, 20 days)// *S. cerevisiae bayanus*	[23]
GC/FID GC/MS	Changes caused by production method	T (quantitative)	25	alcohols (5), acids (3), esters (5), terpenes (10), thiols (1) (0.02-8.23 mg/L)	SPE (C18-reverse phase)	ANOVA (Tuckey test) PCA	Higher concentration in volatile compounds (ethyl esters, alcohols, terpinols and acids) for Traditional and Charmat since 2ndFermentation occurs **Markers traditional method:** citronellol, linalool, geraniol, octanoic acid, decanoic acid, isoamyl acetate and ethyl octanoate **Markers Charmat:** Hexan-1-ol, hotrienol **Markers Asti:** oxide forms of linalool (A, B and D)	Varietal (Moscato Giallo) **Traditional and Charmat**: Base wine *S. cerevisiae PB2019* 9.4 v/v alcohol; 22.4 mg/L free SO_2; 2.33 g/L residual sugar: 2nd Fermentation: *S. cerevisiae PB2002* 10 months of ageing (only traditional) **Asti method** Must: 56 g/L residual sugar Fermentation: *S. cerevisiae PB2002*	[3]
GC/MS	Changes on related to different commercial yeast autolysates rich in mannoproteins and polysaccharides	T (quantitative)	30	alcohols (8), carbonyl compounds (1), acids (4), esters (11), lactones (2), terpenes (3), phenols (1)	LLE	ANOVA (LSD, test) Factor analysis Stepwise discriminant analysis Generalized Procrustes analysis (GPA)	Stronger effect of ageing factor and grape variety than commercial autolysate used **Ageing time markers:** ↑ EBBAs, ethyl lactate, 2-phenylethanol ↓ EEFAs, HAAs, terpenes (citronellol and linalool) **Greatest discriminant power related to grape varieties**: decanoic acid, ethyl hexanoate, ethyl decanoate, ethyl butyrate, methyl vanillate, citronellol, terpineol **related to treatment**	Traditional method White and Rose Sparkling wine (3, 6, 9 months) *Vitis vinifera* cv. Verdejo,. Godello, Tempranillo and Garnacha **2nd Fermentation/Tirage solution:** Temperature: 11-13 ºC *0.3g/L S. cerevisiae var. bayanus* 23 g/L sucrose 0.10 g/L bentonite Commercial yeast autolysates	[124]

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
							↓ EEFAs when treatment, compared with control DYA-2 treatment: ↓ ethyl decanoate, decanoic acid	(DYA-1, DYA-2, DYA-3, DYA-4)	
AGEING ON LEES									
GC/MS (volatile fraction)	Varietal characterization in a specific geographic area	T (semi-quantitative)	33	Alcohols (8), EEFAs (7), HAAs (3) , acids (4), terpenes (4), lactones (2), volatile phenols (4)	LLE	ANOVA (LSD, test) Factor Analysis (FA) Linear Discriminant Analysis (LDA)	**Old sparkling wine markers:** EEBAs, ethyl lactate, γ-butyrolactone **Young sparkling wine markers:** citronelol and geraniol **Grape Variety** Glycine, trans-3-hexenol, acetovanillone, β-alanine, linalool, γ-nonalactone, cis-3-hexenol, isoamyl alcohols and 1-propanol **Ageing** γ-butyrolactone, ethyl decanoate, decanoic acid, histidine and threonine	Classical and Innovative grape varieties (*V. vinifera)*: Verdejo, Viura, Malvasía, Albarín, Prieto Picudo, Godello and Garnacha **1st Fermentation:** *S. cerevisiae* (IOC 18-2007, *Lallemand)* **2nd Fermentation:** Traditional method (3, 6, 9 months)// PVPP + bentonite *S. cerevisiae var. bayanus* (IOC 18–2007 *Lallemand*)	[21]
GC/MS	Varietal characterization of Pais grape sparkling wine	T (quantitative and semi-quantified)	50	Esters (23), Alcohols (9), Terpenes (7), Norisoprenoids (6), Acids (3), and Aldehydes (2) as most numerical groups (0.1 µg/L-1041 mg/L)	HS-SPME (Carboxen/DVB/PDMS)	ANOVA (LSD-Fisher or Friedman test) PCA	**Ageing markers:** diethyl succinate, ethyl lactate, ethyl isovalerate, vitispirane	Varietal, Traditional (0, 3, 6, 9 and 12 months)/ *S. cerevisiae var. bayanus*	[24]
GC/MS	*Geographical origin and varietal characterization*	*T (quantitative)*	*26*	*Terpenes (0.90-112 µg/L)*	*HS-SPME* (DVD/CAR/PDMS)	*ANOVA (LSD-Fisher)* *PCA* *PLS-DA*	**9-15 months ageing**: α-terpineol, (-)-β-citronellol, β-cyclocitral, geranyl acetate, trans-nerolidol, cis-citral, (±)-camphor, γ-terpinene, TDN, (-)-terpinen-4-ol **>24 months of ageing:** β-damascenone **Varietal sparkling wines:** geranyl acetate, 1,4-cineole, TDN, cis-geraniol **Multivarietal sparkling wine:** cis-citral, 1,4-cineole, cis-geraniol, α-terpineol, geranyl acetate, TDN **Geographical origin (Cavas):** (-)-β-citronellol, β-cyclocitral, cis-nerolidol, geranyl acetate, TDN, megastrigmatrienone 6Z8E	Varietal and Multivarietal commercial sparkling wine Champagnes (24 months) Cavas and 7 Andalusian sparkling wines (9-30 months)	*[98]*
GC/MS	Changes in volatile fraction during ageing at	U (semiquantitative,	32 compounds **16 free** (0.02-	Most of them terpenoids, exception made 1-hexanol,	SPE (C18-reverse phase)	ANOVA	**Ligthly vs. fully sparkling wines:**	red sweet varietal sparkling wine (Brachetto grapes): 120-128 g/Lsugar	[72]

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
	different temperatures	relative area) Internal standard: 1-heptanol	8.71 µg/L) and **16 glycosylate compounds** (0.004-0.5 µg/L)	2-phenylethanol, 2-phenyletyl acetate	For glycoconjugates an enzymatic hydrolysiswith a commercial preparation was carried out previous to SPE extraction procedure	**Two Factors:** ageing and temperature **Regression models** Central Composite Design (CCD) and Response Surface Methodology (RSM)	↓ (trans-pyran linalool oxide, nerol, citronellol, 2,6-dimethyl-3,7-octadien-2,6-diol) ↑ (geraniol, cis-pyran linalool oxide and cis-furan linalool oxide) ↑Temperature + ↑ ageing →↑α-Terpineol, Linalool oxides (more important in 'Spumante') ↓Temperature + ↑ ageing →↑Geraniol and Diol (more important in 'Spumante')	Charmat//Zymaflore VL1 (Laffort, France) + vit.B1 + 20g/hL amonio phosphate and sugar 34 g/L **Two Types:** Lightly or 'Tappo raso' (<1.7 bar) and fully or 'Spumante' (>3.0 bar) sparkling wines **Levels or Factors:** Ageing time: 0, 53, 182, 312 and 365 days Temperature: 5, 8, 15, 22 and 25 ºC	
RIDDLING AGENTS AND YEASTS IMMOBILIZATION SYSTEMS									
GC/MS	Yeat strain and Yeast format (immobilization systems) characterization	T(quantitative)	46	Alcohols (4), carbonyl compounds (6), acids (6), HAAs(5), EEFAs(12), EBBAs (2), miscellaneous esters (4), lactones (4), terpenes (1), norisoprenoids (2) (0.001-29 mg/L)	SBSE (PDMS)	*ANOVA (LSD-Fisher) PCA*	**Immobilization/free cells markers:** ethyl octanoate, hexanol, 2-methoxy-4-vinylphenol, octanoic acid, decanoic acid, TDN	multivaretal Cava Traditional method (32 months)// pure culture of *S. cerevisiae P29 (native) and Enoferm QA23 (commercial)*	[99]
Volatile fraction: GC-FID GC-MS	Changes in volatile fraction and foam properties with fining agents	T (quantitative) IS:2-octanol	23	EEFAs (9), EBBAs (1), HAAs (4), lactones (1), alcohols (5), terpenes (3)	LLE (diethyl ether and *n*-pentane 2:1)	ANOVA (student-t test)	**1st Fermentation (base wine):** bentonite-gelatine: ↑ethyl butyrate, ethyl lactate, ethyl decanoate, diethyl succinate, nerol, 2-phenylethanol, methyl acetate, methanol bentonite: ↑isobutyl acetate, n-amyl alcohol, γ-butyrolactone, geraniol, ethyl acetate, isoamyl alcohols **2nd Fermentation:** Decrease when using during this stage Higher concentration in volatile for Colle 2P than bentonite treatment: ethyl propionate, ethyl butyrate, ethyl lactate, n-amyl alcohol, ethyl octanoate, ethyl decanoate, γ-butyrolactone, geraniol, acetaldehyde, methyl acetate, ethyl acetate, methanol, isobutanol and isoamyl alcohols	Varietal red sparkling wine (Bobal) **Fining agent's previous 2nd fermentation (12 ºC, 10days):** bentonite, albumin, bentonite-gelatine, bentonite-albumin **2nd Fermentation:** two tirage solutions: (I)Bentonite and (II) Colle 2P *Saccharomyces cerevisiae* **Ageing on lees:** 12 months	[18]
POST-DISGORGING									
GC/MS	Varietal characterization	T (semi-quantitative)	31 compounds	EEFAs(8), HAAs(2), MEFAs(1), acids (4),	Liquid-Liquid extraction (LLE)	ANOVA (LSD-test)	**Ageing markers (with lees): 9months vs. 18-30 months**	Varietal (white and rose sparkling wine)	[20]

Analytical technique	Main aim	Type[a]	Number of compounds	Compounds analysed (range of concentrations)	Sample treatment	Chemometric method	Discriminant compounds (OAV>1)	Winemaking process/Yeast strain	Ref.
				alcohols (8), aldehydes (1),ketones (1), terpenes (3), lactones (2), volatile phenols (1) (0.001-30 mg/L)		Factor Analysis	↑EBBAs, ↓HAAs, ↓terpenes (citronellol and linalool) **9-18 months vs. 30 months:** ↑ isoamyl alcohols and ethyl lactate **Varietal markers:** ethyl lactate, ethyl vainillate, higher alcohols (propan-1-ol, isoamyl alcohols), EEFAs, fatty acids, 1-hexanol, trans-3-hexen-1-ol **Ageing in bottle (without lees):** ↓EEFAs, ↓C6 alcohols and ↓terpenes ↑EBBAs and ↑vainillin	Traditional method (9, 18, 30 months on lees + 12 months without lees)/// *S. cerevisiae var. bayanus* (IOC 18-2007, Lallemand, Spain)	

. EEFAs: Ethyl esters of fatty acids: EEBAs: Ethyl esters of branched acids, MEFAs: Methyl esters of fatty acids; HAAs: Higher alcohols acetates: IEFAs: Isoamyl esters of fatty acids. EEOCNFAs: Ethyl esters of odd carbon number fatty acids. [a] type of study: Targeted (T) and un-targeted study (U). LLE: Liquid-Liquid extraction. SPE: Solid phase extraction. SBSE: Stir bar sorption extraction. HS-SPME: Head Space-solid phase microextraction. PCA: Principal Component Analysis. PLS-DA: Partial Least Squares-Discriminant Analysis.

2.4.2. New trends in analysis

The increasing role that sparkling wine plays in our daily life is mainly caused by the control that consumer does over new market trends [6]. The high economic impact of this wine enforced producers and researchers to make a great effort to reply this demand, investing large resources in technology and the development of new products (***Figure 15***). Nevertheless, it is not an easy way given the great variability in consumer tastes [175]. In addition, taking into account what has been said in this review, current studies are focused on three fundamental areas: quality, technology / biotechnology and health [58].

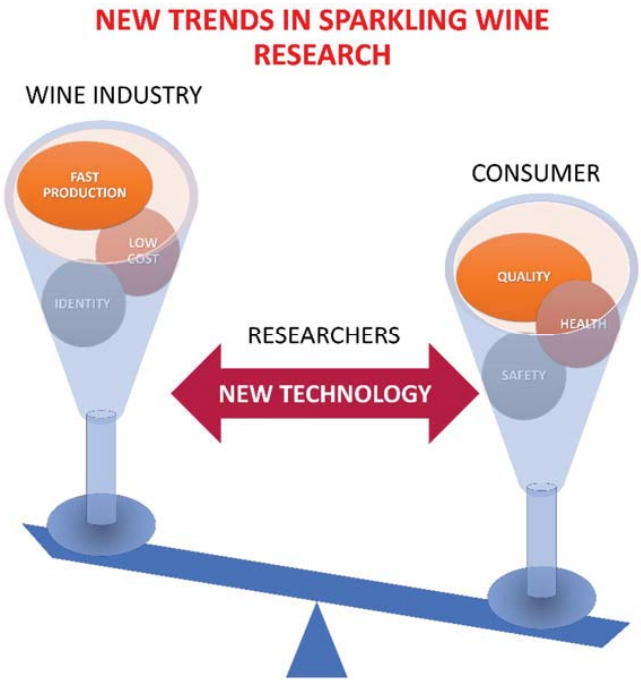

Figure 15. *New trends in sparkling wine research.* ***Source****: own elaboration.*

Traditional methods to assess sparkling wine quality (chemical, bubble and foam-related parameters) are very expensive and time-consuming to small and medium companies. Hence, developing modern techniques based on the use of new and emerging technologies, such as robotics, rapid non-invasive chemometric methods to have more standardized measurements and reduce the human error factor and trial and error process, must also be considered [149]. Novel technologies

introduced over the last decades provide a global information, based in the simultaneous detection of a high number of compounds in the same sample, being near-infrared spectroscopy, affordable electronic noses, and computer vision analysis, an example of them. Considered as holistic methods, since they respond to the changes caused by a large number of molecules belonging to different chemical families, these methodologies have been developed in combination with pattern recognition techniques to obtain unique pattern or 'fingerprint' that allows the discrimination or classification procedure [149, 163, 176].

Fourier Transform Near Infrared spectroscopy (FT-NIR) is a non-invasive and non-destructive methodology that offers a fast analysis for a wide range of food and beverage processes, based on the vibrational modes of molecules with C-H, N-H, O-H, S-H bonds in their structure. This technique is valuable for metabolic fingerprinting that requires a minimum sample preparation, although the main drawback is the intense absorption of water [163]. Regarding sparkling wine, some researchers use FT-NIR to determine general parameters such as reducing sugars, ethanol content, total acidity, volatile acidity, pH, malic, lactic and tartaric acid in wines previously degassed by vacuum filtration [95, 99], or to determine calcium in grape must and base wine, as an alternative to the analysis by atomic absorption spectrometry (AAS) [177].

Another non-destructive and environmentally friendly analysis methodology is fluorescence spectroscopy, with high sensitivity and specificity. The use of this technique in the wine matrix is based on the presence of some substances that exhibit fluorescence properties (stilbenes, anthocyanins, amino acids, vitamins, flavanols and tannins), offering a valuable tool for wine characterization and monitoring. In relation to sparkling wine, fluorescence excitation-emission matrix spectroscopy along with PARAllel FACtor chemometric method of analysis (PARAFAC) have been proposed by some authors to assess the browning in sparkling wine in comparison with other browning indicators such as A_{420} and 5-HMF [178]. The obtained results showed the feasibility of this technique to monitor the fluorophores located at the wave length pairs 465/530 nm and 280/380 nm, closely related with wine browning. Furthermore, the study showed as the third PARAFAC factor (F3), a peak centered around 465 and 530 nm and related to vitamin B2 or riboflavin, has a linear correlation to the browning index (A420) and the 5-HMF content, with determination coefficients (R^2) ranged from 0.87-0.97 and 0.89-0.99, respectively [178].

Other emerging technologies are based on simulating human senses such as vision, smell and taste, combined with chemometric techniques [149, 176, 179]. In addition to the Mosalux methodology, based on measuring the interruption of a beam of ultra-red light to obtain maximum foam height (HM), foam stability height (HS) and foam stability in time [180]; robotics and computer

vision, have been also used to assess bubble and foam-related parameters of sparkling wines [149]. Computerized Assisted Viewing Equipment (CAVE), FIZZeyeRobot are an example of these methods to assess the mentioned parameters as well as velocity and time of collar, drainage, foam expansion, foam velocity, volume of foam, maximum volume time, percentage of wine in the foam, and ratio of small bubbles in the foam [149]. However, to the knowledge of the author, the studies carried out in sparkling wine are still scarce [148, 149, 181].

Regarding the aroma profile, the most widely used methodology for quality control and frauds detection is the electronic nose (e-nose), which is based on multiple sensor array systems [176, 182]. The volatile compounds from the wine headspace are conducted and adsorbed on the sensor surface, causing a physical or chemical changes, which are transduced to signals, providing an aroma profile or pattern of the wine sample [183]. Thus, the use of multivariate analysis allows the differentiation of sample groups or the individual identification of sample components. Doped metal oxides (MOX), MOSFETs, conducing polymers (CP), quartz microbalance (QMBs) sensors, and surface acoustic wave sensors (SAW) are some examples of the different variants of electronic noses applied in wines. Furthermore, given that some components of the matrix (water, ethanol or CO_2) interfered in the determination of the volatile fraction with e-noses, some authors use sampling techniques to avoid or reduce these interferences in the determination [176]. Most of these applications focus their studies at different stages of still wines production, from grapes to the bottled products [183-185], through fermentation [186, 187] and ageing [188, 189]. The use of this technology at different stages of winemaking process provides early detection of non-compliant batches to save production time, as well as to produce a highly standardized product as is required in the international markets [182]. However, less attention has been paid to the application of e-noses in the quality control of sparkling wines, with few studies carried out to monitor the production process [111, 190]. Regarding this, being part of this thesis, the results derived from the use of a QMB e-nose, for sparkling wine quality during ageing, has been exposed in Chapter 5.

Finally, multi-system of low-selective and cross-sensitivity sensors to different species in solution have been also applied to the wine industry [179]. Great varieties of physical and chemical principles (mass, optical or electromechanical) are used to build the different electronic tongues (ET) commercially available. By contrast, the most used are electrochemical sensors (potentiometry, amperometry, voltammetry or impedance based sensors) [176]. Likewise e-noses, the use of ET has been applied for quality control of grapes and wines, monitoring of the second fermentation and ageing, fraud detection, and chemical parameters assessment [176, 179, 191, 192]. Gutierrez-Capitán et al., (2016) combined Linear Discriminant Analysis (LDA) and an array of micro-sensors formed by six

ion-selective field effect transistors sensitive to pH, Na^+, K^+, Ca^{2+}, Cl^-, and CO_3^{2-}ions, one conductivity sensor, one redox potential sensor, and two amperometric gold microelectrodes to classify cava wines regarding to ageing time, with a classification percentage ranged from 80 to 90 %. While other authors apply voltametric ET for the quantification of phenolic indexes (A280, A320, Folin-Ciocalteu index, total tannins and anthocyanins content) through the use of artificial neural networks (ANN) [192].

The use of these systems increase the amount of information extracted from a certain sample, when an electronic panel is formed through them, enhancing the prediction capabilities and recognition of the organoleptic properties [176]. However, considering that sensorial properties of wines change during time, it is very difficult to have a large number of samples for the appropriate training. Thus, to achieve a correct validation of these techniques, the construction of a database is required. Additionally, more researches need to focus on exploring more repeatable, and accurate methods to assess the CO_2 content and bubble size and also on the use of new material to improve sensitivity of e-nose and ET [149].

2.4.3. Sensory Analysis

Among the analytical techniques used for sparkling wine characterization, sensory analysis has been gaining special interest in recent decades (***Figure 16)***. Most of the studies developed in the literature are based on descriptive analysis (hedonic scales) whose terms are previously selected by consensus by panellists training [13, 24, 99, 101, 193-198] difference tests or consumer acceptance testing (paired comparison tests or triangle tests), mainly focused on establishing differential features relative to a given production method [9, 126, 149, 193, 194]. Limpidity, effervescence, visual aspect, olfactory intensity, olfactory quality, aroma frankness, taste intensity, taste quality, persistence, taste frankness, fruity (exotic and citrus fruits), varietal, floral, vegetal, yeasty, fungus, reduced, oxidized notes and harmony, foam collar, bubble size, effervescence are some descriptors used in sparkling wine sensory analysis that require several previous sessions of training [8, 13, 24, 99]. Nevertheless, the methods used are still oriented to the previously set objective, making it necessary to establish a criterion specially designed for the sensory analysis of this type of wine, which in turn can be internationally accepted [8]. This will allow a better understanding of consumer preferences, enabling sparkling winemakers better targeting their products and marketing them within different market segments [175].

According to Viejo et al., (2019) [149], “consumer assessment and acceptability of carbonated beverages are mainly based on carbonation, foam, and bubbles, as a flat carbonated beverage is usually perceived as low quality”. Given the importance of CO_2 in the sparkling wines assessment, certain

evaluation criteria have been established by some authors in relation to time elapsed between opening/pouring/tasting; room and bottle temperature; type of glass used and inclination when pouring [149, 193, 199-201]. Jointly to the number of times to taste the same sample, a consensus related to bubble size and foam properties, visual, aroma and mouth sensation descriptors, must be also considered [8, 149, 202].

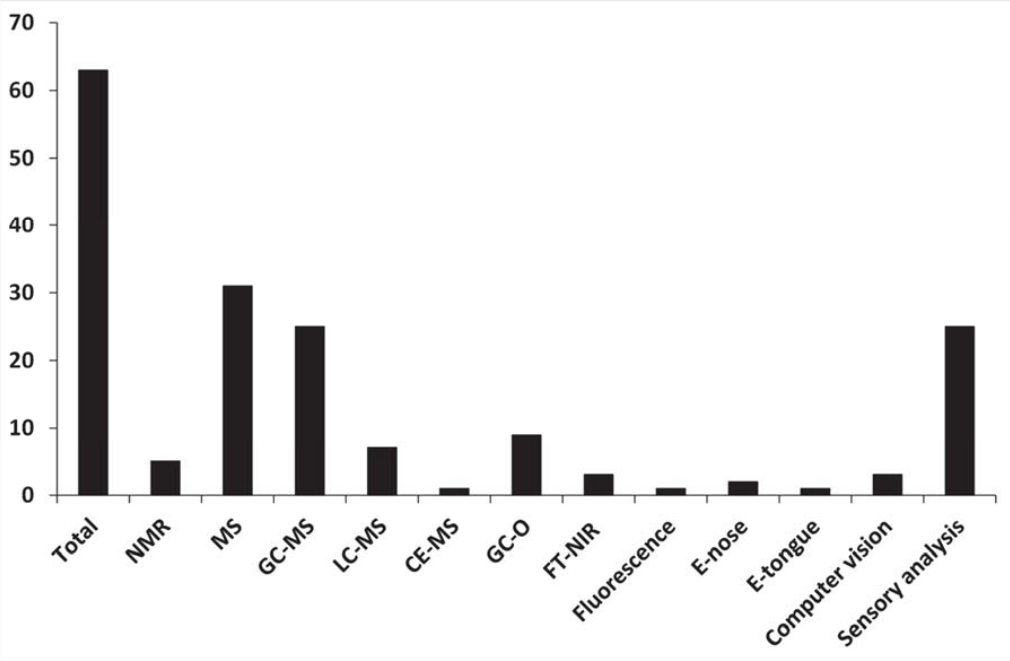

Figure 16. Sparkling wine papers number published during the last decade in the Web of Science (25 October 2020). The used keywords were as follows: (1) Total: "Sparkling wine" AND profiling. ON the basis of research (1), the rest of searches were carried out by using "AND" the following key words: (2)NMR, (3) MS or "mass spectrometry", (4) LC OR HPLC OR UHPLC, OR "liquid chromatography" AND "mass spectrometry" OR MS, (5) GC OR "gas chromatography" AND "mass spectrometry" OR MS, (6) CE OR "capillary electrophoresis" AND "mass spectrometry" OR MS, (7) GC OR Olfactometry, (8) FT OR "Fourier transform", (9) "Fluorescence spectroscopy" OR "Fluorescence excitation-emission matrix", (10) E-nose OR "electronic nose" (11) E-tongue OR "Electronic tongue"(12) "Computer vision", (13) "Sensory analysis".

As set out previously, the characterization of the volatile fraction has significantly evolved with the use of gas chromatography coupled to mass spectrometry (GC-MS), which allows to establish the aroma compound profiling and their contents, but no their impact in the odour perceived by the human nose [203]. In this context, the odour activity value (OAV) of a volatile compound is related to its

influence on the wine aroma [8]. This value results from the quotient between the concentration of the compound and its perception threshold (OPT) and constitutes an approximation to their contribution on the overall aroma of wine, given the impossibility of decoupling sensory interactions with other wine volatile compounds [99]. Considering this, the OAVs calculated for those compounds with the same or similar descriptor can be added together and constitute an odorant series (OS). On the basis of this parameter, some authors find in gas chromatography combined with olfactometry (GC-O) or "sniffing", a major improvement for the identification and quantification of the main compounds responsible for the specific aroma of wines [24, 203-205]. The importance of GC-O associated with descriptive sensory analysis lies in stablishing a comparison between classical sensory analysis and the individual quantification performed by GC-MS [203, 206]. Nevertheless, although it is still an objective method, the information provided by this technique is limited, due to the interactions between volatile compounds or other wine components, such as the CO_2, that enhance or suppress aromas and whose effect is scarcely known or studied. [8, 54, 195, 207]. In contrast, other authors try to establish relevant relationships between the content in volatiles and sensory analysis, through the use of multivariate analysis such as principal component analysis (PCA), factorial analysis (FA), discriminant analysis (DA), or correlation methods (PLS, ANN or Generalized Procrustes Analysis (GPA)). Nevertheless, its application in sparkling wines is also limited [13, 20, 21, 24, 193, 208, 209].

2.5. Conclusions

This review analyses the current knowledge on the sparkling winemaking from the grape harvest to the wine-tasting, highlighting the factors affecting their quality, especially those involved in their aroma profiling. Given the wide range of factors involved, it is not easy for winemakers to find an optimum balance among them, in order to improve quality, accelerate the production and meet the market requests. In this context, research on the mechanisms and reactions that make this wine unique and peculiar is of particular interest.

Considering the imminent changes derived from the global warming, it is likely that future research will continue to focus on three fundamental areas: vine / grapes / yeast for the production of high-quality sparkling wines. From them, the rational selection of yeast to carry out the second fermentation takes on special importance, as it offers the best way to obtain strains with technological properties that could improve the sensory profile of wines and the production technology. In addition, the implementation of other technological or biotechnological strategies, such as the use of non-destructive and on-line analysis techniques, induced autolysis techniques, or yeast immobilization

systems/flocculent yeasts, are some keys for a fast, sustainable, and low-cost wine production, without compromising the quality of the final product.

Finally, in a global market, it is essential to understand clearly and with greater approximation the consumer preferences, which implies the use of easy and universal tools and methodologies to evaluate the quality of wine.

Publications

Informe de los artículos publicados según el Journal Citation Reports (JCR)

Los artículos que se exponen en la presente memoria han sido publicados en revistas cuyos factores de impacto se encuentran en el Journal Citation Reports (JCR) del ISI web of Knowledge y están recogidos en la **Tabla 2**.

Tabla 2. Informe de las publicaciones científicas derivadas y no derivadas de la tesis.

DERIVADAS DE LA TESIS
Relacionadas con el Objetivo 1
Title: "Changes in sparkling wine aroma during the second fermentation under CO_2 pressure in sealed bottle" **Autores:** Martínez-García R., García-Martínez T., Puig-Pujol A., Mauricio J. C. and Moreno J. **Publicado en:** *Food Chemistry 237 (2017): 1030-1040.* **Categoría**: Food Science and Technology. **JCR:** 6.306. **Rank**: 6/139. **Decil:** D1.
Title: "Use of a flor yeast strain for the second fermentation of sparkling wines: effect of endogenous CO_2 over-pressure on the volatilome" **Autores:** Martínez-García R., Roldán-Romero Y., Moreno J., Puig-Pujol A., Mauricio J. C. and García-Martínez T. **Publicado en:** *Food Chemistry 308 (2020) 125555.* **Categoría**: Food Science and Technology. **JCR:** 6.306. **Rank**: 6/139. **Decil:** D1.
Relacionadas con el Objetivo 2
Title: "Using an electronic nose and volatilome analysis to differentiate sparkling wines obtained under different conditions of temperature, ageing time and yeast formats" **Autores:** Martínez-García R., Moreno J., Bellincontro B., Centioni L., Puig-Pujol A., Peinado R. A., Mauricio J. C. and García-Martínez T. **Publicado en**: *Food Chemistry 334 (2021) 127574.* **Categoría**: Food Science and Technology. **JCR:** 6.306. **Rank**: 6/139. **Decil:** D1.
Relacionadas con el Objetivo 3
Title: "Towards a better understanding of the evolution of odour active compounds and the aroma perception of sparkling wines during ageing" **Autores:** Martínez-García R., Mauricio J. C., García-Martínez T., Peinado R. A. and Moreno J. **Aceptado en** *Food Chemistry 357 (2021) 129784.* **Categoría**: Food Science and Technology. **JCR:** 6.306. **Rank**: 6/139. **Decil:** D1.
NO DERIVADAS DE LA TESIS
Title: "Aroma characterization of grape juice enriched with grapevine by-products using thermomaceration" **Autores:** Martínez-García R., Valderrama N., Moreno J. and de Bruijn J. **Publicado en**: *Chilean Journal of Agricultural Research, 77 (2017):234-242*. **Categoría**: Agriculture, Multidisciplinary. **JCR:** 0.883. **Rank**: 33/58. **Quartil**: Q3.

Food Chemistry 237 (2017) 130-140

Chapter 3

Effect of CO_2 overpressure stress on aroma of sparkling wines obtained with conventional Saccharomyces cerevisiae

Capítulo 3. Estudio del efecto del estrés por sobrepresión de CO_2 en el aroma de los vinos espumosos obtenidos con una cepa convencional de *Saccharomyces cerevisiae.*

Como se ha comentado en la introducción de la presente Tesis Doctoral, la elaboración de vinos espumosos de calidad a través del método tradicional requiere del conocimiento y control de los factores implicados en el proceso. De este modo, durante la etapa de fermentación en botella, la sobrepresión de CO_2 liberada tiene un efecto importante en el metabolismo de la levadura y consecuentemente en las características organolépticas del producto final. Sin embargo, hasta la fecha son escasos las investigaciones que tratan de estudiar el efecto de este factor y si este puede ser generalizado o no para cualquier tipo de cepa seleccionada para la elaboración de este tipo de vinos.

En el presente trabajo se desarrollan los resultados obtenidos con respecto a los cambios de composición química y de producción de compuestos volátiles liberados durante la segunda fermentación de un vino espumoso obtenido por uso de una cepa convencional de *S. cerevisiae.* En este caso se trata de una levadura típica para la producción de este tipo de vinos especiales, P29, a su vez utilizada como referencia para el estudio comparativo con otras cepas no convencionales en la elaboración de estos vinos, que se abordarán en capítulos posteriores.

Para ello, se analizaron los cambios en la composición química y de 43 compuestos del aroma identificados durante la fermentación desarrollada a través de un diseño experimental que contemplaba dos condiciones de fermentación (con y sin presión). La aplicación de varias técnicas estadísticas de análisis permitió seleccionar quince compuestos del aroma como principales marcadores del efecto del factor objeto de estudio. Entre ellos, destacaron el dodecanoato de etilo, tetradecanoato de etilo, acetato de hexilo, butanoato de etilo y isobutanoato de etilo, como los compuestos contribuyentes más importantes en el estudio de la presión de CO_2.

Estos resultados se han publicado en la revista *Food Chemistry* con el siguiente título: “Changes in sparkling wine aroma during the second fermentation under CO_2 pressure in sealed bottle”.

Food Chemistry 237 (2017) 130-140; doi.org/10.1016/j.foodchem.2017.06.066.

Food Chem. 308 (2020) 125555

Chapter 4

Effect of CO_2 overpressure stress on aroma of sparkling wines obtained with non-conventional Saccharomyces cerevisiae

Capítulo 4. Estudio del efecto del estrés por sobrepresión de CO_2 en el aroma de los vinos espumosos obtenidos con una cepa no convencional de *Saccharomyces cerevisiae.*

Una de las posibles estrategias a contemplar para la producción diversificada de vinos espumosos, destinadas a alcanzar un mayor número de consumidores, se basa en el uso de cepas de levadura no convencionales que permitan obtener un producto de características organolépticas singulares que se puedan asociar a la región vitícola donde se producen.

En el presente trabajo se desarrollan los resultados obtenidos con respecto a los cambios de composición química y de producción de compuestos volátiles liberados durante la segunda fermentación de un vino espumoso obtenido por uso de una cepa no convencional de *S. cerevisiae.* En este caso se trata de una levadura, la cepa G1, caracterizada por formar velo de flor en vinos generosos tipo fino de la región vitícola de Montilla-Moriles. La justificación de esta propuesta en la producción de vinos espumosos se basa en la elevada tolerancia que este tipo de levaduras tienen al etanol, así como su capacidad para la adherencia, permitiendo acelerar el proceso de clarificación de estos vinos sin que aumente el coste de producción.

Para ello, se procedió con el mismo diseño experimental empleado para la cepa P29 del estudio anterior y que contemplaba dos condiciones de fermentación (con y sin presión), permitiendo analizar veintiséis variables enológicas y cincuenta y tres metabolitos volátiles. La aplicación de técnicas estadísticas de análisis demostró que el efecto de la presión en el metabolismo de la levadura de velo era mayor que el observado para la cepa convencional P29 del estudio previo. Los vinos obtenidos a elevadas presiones de CO_2 (6 bar) se caracterizaban por verse afectados un mayor número de familias químicas, en comparación con aquellos obtenidos a mitad de la fermentación (3 bar). Además, el estudio propone nueve marcadores del efecto de la presión de CO_2 y cinco de la etapa fermentativa.

De acuerdo a las diferentes características de composición observados en los vinos espumosos para la levadura de velo, se plantea necesario profundizar más en el estudio de esta cepa durante la etapa de crianza y, por consiguiente, en el perfil organoléptico de los vinos espumosos obtenidos a partir de la misma. Siendo este un trabajo abordado en una publicación posterior.

Los resultados para el presente estudio se han publicado en la revista *Food Chemistry* con el siguiente título: "*Use of a flor yeast strain for the second fermentation of sparkling wines: Effect of endogenous CO_2 over-pressure on the volatilome*".

Food Chemistry 308 (2020) 125555; doi.org/10.1016/j.foodchem.2019.125555

Chapter 5

Effect of temperature, ageing time and yeast format on the aroma of sparkling wines

Capítulo 5. Estudio del efecto de la temperatura, tiempo de envejecimiento y formato de inoculación de levadura en el aroma de los vinos espumosos.

Para facilitar y acortar algunas de las etapas de la elaboración de vinos espumosos, se han empleado los denominados sistemas de inmovilización de levaduras. Los estudios realizados hasta la fecha se basan en el uso de materiales artificiales como soporte de inmovilización que facilitan el confinamiento de las células de levadura en un espacio donde es posible la transferencia al medio de los compuestos provenientes de su metabolismo y autolisis. Sin embargo, otros estudios basados en el empleo de estos sistemas han descrito la liberación de compuestos provenientes del propio soporte que pueden afectar la calidad del producto final. Alternativas a estos sistemas artificiales se basan en la bioinmovilización, o co-inmovilización espontánea de un hongo filamentoso (*Penicillium chrysogenum H3*) y una levadura de la especie *Saccharomyces cerevisae.* Las biocápsulas resultantes evitan la interacción artificial y preservan las propiedades biocatalíticas de las células de levadura.

Este sistema se ha utilizado en la producción del cava, mostrando buena efectividad en la etapa fermentativa y el removido, aunque hasta la fecha no se han contemplado el efecto combinado que, sobre la fracción volátil tiene el formato de inoculación, la temperatura de fermentación y el tiempo de crianza.

En el presente trabajo se analiza el efecto de los tres factores implicados en el proceso de elaboración de vinos espumosos, obtenidos con una cepa convencional de *S. cerevisiae*, a través del análisis de la composición química y fracción de compuestos volátiles liberados durante la segunda fermentación y crianza. Además, por primera vez se ha estudiado el potencial discriminante de una nariz electrónica basada en microbalanzas de cuarzo como alternativa a un panel de cata de expertos para el control de calidad del proceso productivo de estos vinos.

Para ello, se elaboraron vinos espumosos según el método de elaboración tradicional con la cepa de levadura P29 aplicada en dos formatos de inoculación (libre y biocápsulas) sometida a dos temperaturas de fermentación (10 y 14 ºC). Los vinos obtenidos se muestrearon transcurridos los 15 y 24 meses de crianza bajo lías y se sometieron al análisis químico y de la fracción volátil por GC/MS y nariz electrónica.

La aplicación de técnicas estadísticas de análisis permitió seleccionar diez compuestos volátiles como marcadores del factor temperatura, once del factor tiempo y doce relacionado con el formato de levadura. Además, se demostró, a través de modelos discriminantes por mínimos cuadrados parciales

(PLSDA), el potencial de la nariz electrónica en la correcta clasificación de los vinos según el tiempo y formato empleados (100 % de correcta clasificación), y en menor medida del factor temperatura (83 %).

Los resultados para el presente estudio se han publicado en la revista *Food Chemistry* con el siguiente título: "*Using an electronic nose and volatilome analysis to differentiate sparkling wines obtained under different conditions of temperature, ageing time and yeast formats*".

Food Chemistry 334 (2021) 125555; doi.org/10.1016/j.foodchem.2020.127574

Chapter 6

Contribution of non-conventional Saccharomyces cerevisiae yeast strains and new grapes varieties to the aroma of sparkling wine

Capítulo 6. Contribución de una cepa no convencional *Saccharomyces cerevisiae* y nuevas variedades de uva al aroma de los vinos espumosos.

En este último capítulo se completan los estudios realizados previamente durante la etapa de fermentación para cepas de levadura no convencionales y convencionales en la elaboración del cava (capítulos 3 y 4). De este modo, se presentan y comparan los resultados derivados del análisis fisicoquímico, de la fracción volátil y análisis sensorial de vinos obtenidos por una cepa de levadura formadora de velo (G1) y una cepa convencional (P29) mediante la aplicación del método tradicional en mosto de uva de la variedad Pedro Ximénez, típica de la elaboración de vinos tranquilos de la región vitícola de Montilla-Moriles. Para ello, se monitorizaron los vinos obtenidos durante la etapa de crianza de 15 meses, estableciendo cinco puntos de muestreo separados en el tiempo cada tres meses a lo largo del proceso productivo.

Los resultados derivados del análisis de 82 compuestos volátiles y de sus familias químicas corroboran afirmaciones hechas previamente por otros autores en relación al mayor efecto que sobre ellos tiene la etapa de crianza sobre lías en comparación con la cepa de levadura usada. Sin embargo, el análisis de las series odorantes obtenidas, así como de test descriptivos aplicados a los vinos mostraron una buena separación de las muestras con respecto a ambos factores. Además, la aplicación de la prueba triangular de evaluación sensorial proporcionó puntuaciones más altas para los vinos obtenidos con la levadura G1 en cualquier etapa de crianza.

De forma adicional, el análisis por regresión con mínimos cuadrados parciales (PLSR) estableció importantes asociaciones entre el contenido de los compuestos volátiles analizados y los atributos sensoriales derivados de la aplicación de test descriptivos. Lo que permitió seleccionar treinta y ocho compuestos como los más influyentes en el aroma de los vinos elaborados con Pedro Ximénez. De ellos, acetaldehído (serie química); butanoato de etilo, acetato de 2-feniletilo e isoamil acetato (serie afrutada); 2-feniletanol (serie floral) y alcoholes isoamílicos (series química y afrutada), se consideraron como compuestos constitutivos de los vinos. Por otro lado, los ácidos octanóico y decanóico (serie grasa), butirolactona (serie empireumática), trans-2-hexenol y limoneno (vegetal), isovalerato de etilo (serie especiada) y guayacol (series balsámica y empireumática), se consideraron discriminantes para ambas levaduras. Por último, también lo fueron el linalol (serie floral) de forma exclusiva para los vinos elaborados con G1 y 4-etil-2-metoxifenol (serie balsámica), furan-2-carbaldehído (series química y empireumática) para vinos elaborados con P29.

Estos resultados se han publicado en la revista *Food Chemistry* con el siguiente título: "Towards a better understanding of the evolution of odour-active compounds and the aroma perception of sparkling wines during ageing".

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Chapter 7

Conclusions

***Differentiate the contribution that the yeast strain has on the aroma produced in the fermentative stage with respect to autolysis and study the effect of other biotechnological factors (temperature, yeast strain and immobilization systems) that influence the quality and price of sparkling wines*.**

Effect of CO_2 overpressure stress on aroma of sparkling wines obtained with conventional and non-conventional *Saccharomyces cerevisiae* yeast strains during the second fermentation.

The results obtained by analysis of the excreted metabolites in sparkling wines obtained during second fermentation by conventional (P29) and non-conventional (G1) *Saccharomyces cerevisiae,* allow the establishment of the following conclusions:

- A more pronounced effect of CO_2 overpressure has been observed on the viability of a yeast strain *S. cerevisiae* flor (G1) compared to the conventional strain (P29), the latter being faster in formation kinetics and that is justified by its greater adaptation to this type of wine-making.
- The flor yeast forms thick floccules with fast sedimentation and less adhesion to the wall, what makes of them a good candidate for wine clarification during riddling stage.
- The number of chemical families affected by CO_2 overpressure is dependent not only on the yeast strain but also on the pressure levels reached. Thus, the number of volatile compounds affected by this factor is higher in G1 sparkling wines obtained at 6 bar.
- The CO_2 overpressure contributes to the decrease in chemical and fruity series of the wines obtained by both yeasts.

Effect that temperature, ageing time and yeast format have on the aroma of sparkling wines and evaluate the potential of new technologies of analysis such as E-nose to discriminate the wines obtained.

The analysis of the aroma metabolites excreted to the media by a *S. cerevisiae,* either as free cells or co-immobilized with the filamentous fungus *P. chrysogenum* to form biocapsules, establishes the following conclusions:

- A delay in CO_2 production kinetics was observed related to temperature and yeast format, being the wines obtained with biocapsules at low temperatures those with longer lag phase. This is mainly caused by the yeast stress during handling in the immobilization process.
- Twelve volatile compounds can be considered key markers of yeast format, ten of temperature and eleven of "on lees" ageing time.

- Sparkling wines obtained with free cells display higher values in chemical, fruity, vegetal and spicy odorant series. Further studies are needed to know if an absorption effect exist on the biocapsules' surface.
- Higher classification rates were obtained through the use of an electronic nose based on QMB sensors related to ageing time and yeast format. This reveals an easy and fast detection tool, either focused on quality control of sparkling wines or to detect frauds and adulterations.

Contribution of a flor yeast to the sensory quality of sparkling wines obtained with autochthonous grape varieties and different ageing on lees periods.

- No differences were observed in the majority of chemical families' content analysed in sparkling wines obtained by both a flor yeast and a conventional *S. cerevisiae*.
- Univariate statistical analysis shows that ageing has a more pronounced effect on the volatilome than the yeast strain used for the second fermentation.
- Compared to the conventional strain, sparkling wines obtained with flor yeast displayed the best scores when judged by panellist at any stage of ageing on lees, which makes it a good candidate for the production of sparkling wines.
- Thirty-eight volatile compounds were selected as the most influential in the aroma of PX-sparkling wines obtained either by the conventional or a flor yeast *S. cerevisiae* strain and out of them, only twenty-seven were unique to certain aroma descriptors.
- These results contribute to the development and implementation of instrumental and chemometric technologies as a way for the complementation of a trained tasting panel.

Final considerations

As a general conclusion, it is established that endo-cellular metabolites released to the medium during 'prise de mousse' stage are related to the yeast strain used and the physicochemical characteristic of the growing environment. The advantages of using volatile markers aimed at improving the quality of a product are well known, as a consequence of their direct relationship with the organoleptic profile of the final product. However, the advantages are even better if its use is focused on the selection of yeasts for the second fermentation, which allows new contributions mainly aimed at diversify the characteristics of the final product. In addition, its study allows the improvement of the added value that some biotechnological applications have, such as the immobilization of yeasts, when their effect on the compounds released from autolysis is clarified.

Moreover, with this, we shed light on the flor yeast abilities to produce sparkling wines, the discriminant power that electronic nose has to the quality control of sparkling wines, as well as clarified

part of the relationships between the volatile fraction and the sensory profile of wines obtained. However, further studies are needed to consolidate the key-markers exposed here to assess the quality of PX sparkling wines in the presence of CO_2.

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Appendix

Abbreviations and Acronyms

ANOVA	Analysis of Variance
AU	Absorbance Units
BIO	Biocapsules
BOE	Boletín Oficial del Estado
BW	Base Wine
CA	Cluster Analysis
CAS	Chemical Abstracts Service Number
CI	Color Intensity
CIELab	International Commission on Illumination
CV	Cross-Validation
EEBA	Ethyl Esters of Branched Acids
EC	European Commission
EEFA	Ethyl Esters of Fatty Acids
E-nose	Electronic Nose
EU	European Union
F	Format factor
FC	Free Cells
FEDER	Fondo Europeo de Desarrollo Regional
FID	Flame Ionization Detector
FT-NIR	Fourier transform near-infrared spectroscopy
GC	Gas Chromatography
GRAS	Generally Recognized as Safe
HAA	Higher Alcohol Acetate
HG	Homogenous Group
HM	Foam Height
HS	Plate Height or Persistence
IEFA	Isoamyl Esters of Fatty Acids
INCAVI	The Catalan Institute of Vines and Wines
INIA	The National Institute for Agricultural and Food Research and Technology
LOOCV	Leave-One-Out Cross-Validation
LRI	Linear Retention Index
LV	Latent Variable
MANOVA	Multivariate Analysis of Variance
MEFA	Methyl Ester of Fatty Acids
MPS	Multipurpose Sampler
MS	Mass Spectrometry
MSC	Multiple Sample Comparison Procedure
MVA	Multiple Variable Analysis
NIST	National Institute of Standards and Technology
NTU	Nephelometric Turbidity Unit
OAV	Odorant Activity Value
OB	Open Bottle
OIV	International Organisation of vine and wine
OPT	Odor Perception Threshold
OS	Odorant Series
PCA	Principal Component Analysis

PDMS	Polydimethylsiloxane
PLSR	Partial Least Squares Regression
PLS-DA	Partial Least Squares Discriminant Analysis
QMB	Quartz Microbalances
RMSEC	Root Mean Squares Error at the calibration step
RMSECV	Root Mean Squares Error at the cross-validation step
S	Sensor
SB	Sealed Bottle
SBSE	Stir Bar Sorptive Extraction
t	Ageing time factor
T	Temperature factor
TDU	Thermal Desorption Unity
TPI	Total Phenol Index
TPP	5,10,15,20- tetraphenylporphyrin
USA	United Stated of America
UV-Vis	Ultraviolet-Visible Spectrophotometry
VIP	Variable Importance Projection
YAN	Yeast Assimilable Nitrogen
YEPD	Yeast Extra Peptone Dextrose
YNB	Yeast Nitrogen Base

Scientific contributions

• Scientific articles

1. Martínez-García R., García-Martínez T., Puig-Pujol A., Mauricio J. C. and Moreno J.
"Changes in sparkling wine aroma during the second fermentation under CO_2 pressure in sealed bottle".
Food Chemistry 237 (2017): 1030-1040.
(Chapter 3)

2. Martínez-García R., Roldán-Romero Y., Moreno J., Puig-Pujol A., Mauricio J. C. and García-Martínez T.
"Use of a flor yeast strain for the second fermentation of sparkling wines: effect of endogenous CO_2 over-pressure on the volatilome"
Food Chemistry 308 (2020) 125555
(Chapter 4)

3. Martínez-García R., Moreno J., Bellincontro B., Centioni L., Puig-Pujol A., Peinado R. A., Mauricio J. C. and García-Martínez T.
"Using an electronic nose and volatilome analysis to differentiate sparkling wines obtained under different conditions of temperature, ageing time and yeast formats"
Food Chemistry 334 (2021) 127574
(Chapter 5)

4. Martínez-García R., Mauricio J. C., García-Martínez T., Peinado R. A. and Moreno J.
"Towards a better understanding of the evolution of odour active compounds and the aroma perception of sparkling wines during ageing"
Food Chemistry 357 (2021) 129784
(Chapter 6)

• Congress Communications

1. Martínez-García R.; Bellido-Agüera E.; García-Martínez T., Puig-Pujol A., Mauricio, J.C. y Moreno J.
"Efecto de la temperatura y tiempo de la fase de toma de la espuma sobre la composición del cava".
XXXXIX Jornadas de Viticultura y Enología de "Tierra de Barros". Centro Universitario Santa Ana, Almendralejo (Badajoz), 2017.
Oral Communication

2. Martínez-García R., García-Martínez T., Mauricio J.C. y Moreno J*.*
"Selección de compuestos del aroma para establecer el efecto de temperatura y tiempo en la etapa de toma de espuma de cavas".
VI Congreso de Investigadores en Formación (Universidad de Córdoba), 2018.
Oral Communication

3. Roldán-Romero Y., Martínez-García R., Martín-García F.J., Moreno J., Mauricio J.C. y García-Martínez T.
"Efecto de la segunda fermentación sobre la composición en familias de compuestos del aroma de los vinos espumosos".
XL Jornadas de Viticultura y Enología de "Tierra de Barros". Centro Universitario Santa Ana, Almendralejo (Badajoz), 2018.
Oral Communication

4. Martínez-García R., García-Martínez T., Mauricio J.C., Puig- Pujol A. y Moreno J.
"Cambios en compuestos volátiles durante la segunda fermentación de vinos espumosos elaborados con dos cepas de levadura".
VII Congreso de Investigadores en Formación (Universidad de Córdoba), 2019.
Oral Communication

5. Martínez-García R., García-Martínez T., Peinado R.A., Puig-Pujol A., Mauricio J.C. y Moreno J.
"Efecto del formato de levadura y la temperatura en compuestos del aroma de cavas sometidos a dos tiempos de crianza".
XLI Jornadas de Viticultura y Enología de "Tierra de Barros y Primer Congreso Agroalimentario de Extremadura". Centro Universitario Santa Ana, Almendralejo (Badajoz), 2019.
Oral Communication

6. Martínez-García R., García-Martínez T., Peinado R. A, Bellincontro A., Mauricio, J.C. y Moreno J.
"Aplicación de sensores no destructivos para la clasificación de vinos".
VIII Congreso de Investigadores en Formación (Universidad de Córdoba), 2020.
Oral Communication

7. Martínez-García R., García-Martínez T., Peinado R. A, Bellincontro A., Mauricio, J.C. y Moreno J.
"Aplicaciones de una nariz electrónica al control de calidad de vinos espumosos durante la segunda fermentación y la crianza sobre lías"
IX Congreso de Investigadores en Formación (Universidad de Córdoba), 2021.
Oral Communication

- **Other Contributions**

1. Martínez-García R., Valderrama N., Moreno J. y de Bruijn J.
"Aroma characterization of grape juice enriched with grapevine by-products using thermomaceration" (2017).
Chilean Journal of Agricultural Research, 77(3):234-242.
Scientific article (Q3)

2. Moreno J., Martínez-García R., López-Muñoz B., García-Martínez T. y Moreno-García J.
"Disminución natural del contenido en etanol de vino tinto joven".
Publicado en SeVi nº 3.491: 390-395. Fecha: 25 de marzo de 2017.
Divulgative article

3. *De Buijn J., Aguilar T., Loyola C., Luis Bustamante L., Vergara J.C., von Baer D., Mardones C., Serra I.J., Martínez R. y Moreno J.*
"Grape must enrichment with winery and grapevine by-products to increase its antioxidant and aroma potential".
40th World Congress of Vine and Wine, Sofía (Bulgaria), 2017.
Proceedings of the 40th World Congress of vine and wine: "Vine & Wine: Science and Economy, Culture and Education". Código: 2017-1483, pp. 148. ISBN: 979-10-91799-77-5.
Oral Communication

4. Martín-García F.J., García-Martínez T., González-Jiménez M.C., Martínez-García R., Porras-Agüera J.A., y Mauricio J.C.
"Estudio del fenotipo killer de levaduras vínicas de la Denominación de Origen Montilla-Moriles".
XXXXIX Jornadas de Viticultura y Enología de "Tierra de Barros". Centro Universitario Santa Ana, Almendralejo (Badajoz), 2017.
Oral Communication