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RELAÇÃO DO AROMA E DA ESPUMA DOS VINHOS ESPUMANTES COM O POTENCIAL ENOLÓGICO DAS UVAS E DOS VINHOS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Sílvia Maria da Rocha Simões Carriço, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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"A imaginação é mais importante que o conhecimento. O conhecimento é limitado. A imaginação envolve o mundo."

Albert Einstein

"As invenções são, sobretudo, o resultado de um trabalho teimoso"

Santos Dumont

"*Gignit et humores melinus vinum meliores*" (O melhor homem vem do melhor vinho).
In "Regimen Sanitatis. Flos Medicinae Scholae Salerni, século XI"

À minha família

o júri

presidente

Professor Doutor Domingos Moreira Cardoso
professor catedrático da Universidade de Aveiro

vogais

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palavras-chave

Vinho espumante, *Vitis vinifera* L., Região Demarcada da Bairrada, Fernão-Pires, Maria Gomes, Baga, compostos voláteis, polissacarídeos, espuma, microextracção em fase sólida, SPME, extracção soptiva em barra de agitação, SBSE

resumo

Os espumantes produzidos segundo o método Champanhês são obtidos após uma segunda fermentação em garrafa. Quando o vinho é vertido no copo, o CO_2 produzido é libertado, sendo a espuma formada o resultado da sua interacção com os constituintes do vinho. A quantidade e a estabilidade da espuma do vinho espumante estão relacionadas com a sua composição química. Para além da espuma, o aroma é também um parâmetro importante de qualidade na apreciação geral de um vinho espumante. O aroma de um vinho espumante provém do contributo das uvas assim como do processo fermentativo. Dependendo do estado de maturação da uva, o contributo dos compostos voláteis para o aroma é diferente. Em virtude da vindima para os vinhos espumantes ser realizada antes da vindima para os vinhos maduros, dependendo da variedade, as uvas poderão não ser colhidas na expressão máxima do seu aroma, podendo verificar-se uma perda significativa do seu potencial varietal volátil.

O objectivo desta dissertação é relacionar o aroma e a espuma dos vinhos espumantes com o potencial enológico das uvas e dos vinhos. Para isso, foi estudada a composição volátil das duas castas principais da Bairrada, a casta branca Fernão-Pires (FP) e a casta tinta Baga (BG), sendo estas duas das castas usadas para a produção de espumante.

Para estudar a composição volátil das uvas durante a maturação, com vista a avaliar este efeito na expressão máxima de compostos voláteis, foi optimizada para este propósito a metodologia de microextracção em fase sólida em espaço de cabeça (HS-SPME). As uvas foram colhidas semanalmente, em duas vinhas, do pintor à pós-maturidade sendo posteriormente analisadas pela metodologia de HS-SPME seguida de cromatografia de gás acoplada à espectrometria de massa com quadrupolo (GC–qMS). No caso das uvas BG, observou-se um aumento acentuado na expressão máxima de compostos voláteis próximo da maturidade da uva determinada pelo teor em açúcar e acidez titulável, mantendo-se constante durante a pós-maturidade. Na determinação do perfil volátil das uvas ao longo da maturação foram identificados 66 compostos varietais nas uvas provenientes de uma vinha (Pedralvites) e 45 da outra vinha (Colégio). Em ambas as vinhas foram identificados 23 sesquiterpenóides, 13 monoterpenóides, 6 norisoprenóides, 2 álcoois aromáticos e 1 diterpenóide. Os sesquiterpenóides, devido à sua abundância em número e em área cromatográfica, podem ser considerados marcadores da casta BG. As uvas FP apresentaram um comportamento diferente do das uvas BG, sendo a expressão máxima de compostos voláteis expressa durante um curto período de tempo (1 semana), que coincide com a maturidade da uva. Depois de atingido este pico, observa-se uma diminuição drástica logo na semana seguinte. Este comportamento foi observado em ambas as vinhas, onde foram identificados 20 compostos voláteis varietais e 5 pré-fermentativos (álcoois e aldeídos em C_6). Estes resultados mostram que quando estas castas são colhidas precocemente (1 semana antes da maturidade) para a produção de

espumante, é observada uma redução significativa do potencial volátil que é expresso na maturidade.

Para a análise da composição volátil dos vinhos espumantes foi optimizada uma metodologia de microextracção que permite usar uma maior quantidade de fase estacionária, a extracção sorptiva em barra de agitação (SBSE). O método foi optimizado usando 10 padrões de compostos voláteis representativos das principais famílias químicas presentes no vinho, nomeadamente, ésteres, monoterpenóides, sesquiterpenóides, norisoprenóides em C_{13} e álcoois. O método proposto apresenta uma boa linearidade (r^2 > 0,982) e a reprodutibilidade varia entre 8,9 e 17,8%. Os limites de detecção para a maioria dos compostos é bastante baixo, entre 0,05 e 9,09 µg L^{-1}. O método foi aplicado para a análise da composição volátil dos vinhos espumantes. Dentro dos vinhos espumantes analisados, foi estudada a influência da casta, do tipo de solo e do estado de maturação das uvas na sua composição volátil. A casta FP pode dar origem a vinhos com maior potencial de aroma do que a casta BG. Relativamente à avaliação dos diferentes estados de maturação, verificou-se que as uvas da maturidade e as da colheita tardia (uma semana depois da maturidade) deram origem aos vinhos com maior quantidade de compostos voláteis. Para os três tipos de solo estudados (arenoso, argiloso e argilo-calcário), o vinho obtido a partir de uvas colhidas no solo argilo-calcário foi o que mostrou a maior concentração de compostos voláteis varietais. A espuma destes vinhos espumantes foi também avaliada quanto à sua quantidade máxima (HM) e tempo de estabilidade (TS). O vinho espumante que apresentou um maior TS foi o vinho produzido a partir da casta FP proveniente de uma colheita tardia e solo argiloso. Os vinhos provenientes dos solos arenosos e argilo-calcários são os que apresentaram valores mais baixos de TS.

Com vista a avaliar quais os conjuntos de moléculas do vinho que estão relacionados com as propriedades da espuma e possíveis sinergismos entre eles, para cada vinho espumante foi separada a fracção hidrofóbica de baixo peso molecular (MeLMW), a fracção de elevado peso molecular (HMW) e duas fracções de peso molecular intermédio (AqIMW e MeIMW). As propriedades da espuma dos vinhos modelo, reconstituídos com estas fracções e suas misturas, foram avaliadas. A combinação da fracção HMW com a MeLMW aumentou o TS 2,7 vezes quando comparado com o observado para a fracção HMW isoladamente, produzindo um efeito sinergético. Este aumento do TS ainda foi maior quando se combinou a fracção HMW com as subfracções obtidas a partir da fracção MeLMW, principalmente para as fracções menos apolares. A subfracção hidrofóbica menos apolar foi caracterizada por espectrometria de massa de ionização por *electrospray* (ESI-MS/MS) tendo sido identificada uma série de oligómeros de polietileno glicol e um potencial composto tensioactivo, o 8-hidroxi-tridecanoato de dietilenoglicolglicerilacetato.

A fracção MeLMW foi também isolada da espuma do vinho espumante e caracterizada por ESI-MS/MS, permitindo identificar vários compostos potenciais tensioactivos, nomeadamente, dois monoacilgliceróis e quatro derivados de ácidos gordos com glicerieletilenoglicol. Estes resultados confirmam que estes compostos relacionados com a estabilidade da espuma existem em maior número na espuma do que no vinho.

O vinho foi ainda fraccionado em 12 grupos de moléculas: 3 fracções de manoproteínas, 3 de arabinogalactanas, 3 de misturas de polissacarídeos, proteínas e compostos fenólicos e 3 fracções de peso molecular intermédio e baixo, compostas por uma mistura de hidratos de carbono, peptídeos e compostos fenólicos. Foram usados vinhos modelo reconstituídos com cada uma das fracções isoladas na concentração em que estas se encontraram no vinho. Foram também efectuados ensaios com soluções modelo dez vezes mais concentradas e com misturas de algumas das fracções. Todas as soluções formadas foram avaliadas quanto às propriedades da espuma. O aumento da concentração para dez vezes faz com que a solução contendo a fracção rica em manoproteínas (MP1) aumente para mais do dobro a HM e 7,4 vezes mais o TS. A combinação entre a fracção MP1 e a MeLMW produziu um aumento significativo nos parâmetros de HM e TS.

A combinação da fracção HMW (manoproteínas com baixo teor em proteína) com a MeLMW (tensioactivos derivados de ácidos gordos com glicerieletilenoglicol) contém os compostos chave de um vinho espumante para se obter uma maior quantidade e estabilidade da espuma.

keywords

Sparkling wine, *Vitis vinifera* L., Bairrada Appellation, Fernão-Pires, Maria Gomes, Baga, volatile compounds, polysaccharides, foam, solid phase microextraction, SPME, Stir bar sorptive extraction, SBSE

abstract

Sparkling wines produced according to the *Champenoise* methodology are obtained by a second fermentation inside the bottle. When poured from the bottle into a glass, the CO_2 produced is released, and consistent foam is formed as a result of its interaction with wine constituents. Foamability and foam stability are related to the chemical composition of sparkling wines. Additionally to foam, the aroma are an important quality parameter of a sparkling wine. The sparkling wine aroma is determined by the grapes contribution as well as from the fermentative process. Depending on the grape maturity state the contribution of volatile compounds to the wine aroma can change. Grapes for the sparkling wine production are usually harvested earlier than those for table wine purposes, depending on variety. A non coincident harvest with the maximum grape aroma expression may lead to a significant loss of the varietal volatile potential.

The aim of this thesis was to relate the aroma and foaming of sparkling wines to the oenological potential of grapes and wines. The two main varieties of Bairrada Appellation, the Fernão-Pires (FP) white grapes and the Baga (BG) red grapes were used for the production of sparkling wine.

The evaluation of grapes volatile composition was made in order to study the effect of ripening on the maximum volatile compounds expression. For this purpose, the methodology of head space solid phase micro extraction (HS-SPME) was optimized. The volatile composition of grape from two vineyards was weekly harvested from *véraison* to post-maturity and then analysed by HS-SPME methodology followed by gas chromatography coupled to quadrupole mass spectrometry (GC-qMS). For BG, the maximum volatile expression was observed at the grape maturity established according to sugar and titratable acidity contents, and remained constant until post-maturity. In the volatile profile were identified 66 varietal compounds from grapes grown in one vineyard (Pedralvites) and 45 in the other vineyard (Colégio). In both vineyards were identified 23 sesquiterpenoids, 13 monoterpenoids, 6 norisoprenoids, 2 aromatic alcohols, and 1 diterpenoid. From these, due to the abundance in number and chromatographic area of sesquiterpenoids, they can be considered varietal markers of Baga variety. The FP showed a different behaviour of BG, as the maximum volatile expression occurs only in a very short period (one week) that is coincident with grape maturity. After that, it sharply decreases in the following week. This behaviour was observed in both vineyards, where 20 variety- and 5 pre-fermentative (C_6 alcohols and aldehydes) volatile compounds were identified.

These results showed that if these two varieties are harvested earlier (one week before maturity), as for sparkling wine production, their potential aroma compounds decrease significantly compared to the amount that can be found at maturity.

For the sparkling wines volatile characterisation a methodology based on microextraction using a higher amount of stationary phase, the stir bar sorptive extraction (SBSE) was developed.

The optimisation was carried out by using 10 standards representative of the main chemical families of wine, namely esters, monoterpenoids, sesquiterpenoids, C_{13} norisoprenoids and alcohols. The methodology proposed had a good linearity (r^2 >0.982) and reproducibility, between 8.9 and 17.8%. Low detection limits were achieved for the majority of compounds, between 0.05 and 9.09 µg L^{-1}. The methodology was applied for the analysis of the sparkling wine volatile composition, studying the influence of grape variety, soil type, and ripening stage. FP variety can provide sparkling wines with higher aroma potential than BG variety. In what concerns the ripening stage, the grapes from maturity and late maturity states (1 week after maturity) provided wines with the highest content of volatile compounds, including the varietal ones. For the three types of soils (sandy, clayey, and clay-calcareous), the wines produced from the clay-calcareous soil presented the highest content of varietal volatile compounds. Concerning the foaming properties, the sparkling wine produced with grapes FP from late maturity state and clayey soil presents the highest foam stability (TS). The sparkling wines from sandy and clay-calcareous soils presented the lowest TS values.

In order to evaluate the wine molecular fractions that were related to foam properties and possible synergistic effects between them, each sparkling wine was fractioned into four fractions: one fraction of hydrophobic low molecular weight (MeLMW) material, one fraction of high molecular weight (HMW) material, and two fractions of intermediate molecular weight material (AqIMW and MeIMW). The foam properties of reconstituted wine model solutions with these fractions and its mixtures were evaluated. The combination between HMW and MeLMW showed an increase in TS of 2.7 fold when compared with the values observed for the HMW fraction alone, allowing to state a synergistic effect between them. The TS increase was even larger when HMW was combined with the less hydrophobic subfractions obtained from MeLMW. The less hydrophobic subfraction was characterized by electrospray tandem mass spectrometry (ESI-MS/MS) allowing to assign the presence of a series of polyethylene glycol oligomers and diethyleneglycol 8-hydroxy-tridecanoate glycerylacetate, a potential tensioactive compound. In order to confirm the occurrence of these compounds as components of sparkling wine foam, a MeLMW fraction was obtained from the foam and was structurally analysed by ESI-MS/MS. Several possible tensioactive compounds, namely, two monoacylglicerols as well as four glycerylethyleneglycol fatty acid derivatives were identified as foam components. One of these compounds has also been identified as foam promoter and stabilizer of wine model solutions.

The wine was also fractionated in 12 groups of molecules: 3 fractions were composed by mannoproteins, 3 fractions were arabinogalactans, 3 fractions were a mixture of polysaccharides, proteins, and phenolic compounds and 3 fractions were intermediate and low molecular weight compounds composed by a mixture of carbohydrates, peptides, and phenolic compounds. The foam properties of reconstituted wine model solutions with these fractions at the concentration that was found in wine were evaluated. It was also performed wine model solutions at ten folds the wine concentration of 11 fractions and mixtures with selected fractions. The solution containing the fraction rich in mannoproteins (MP1) at ten folds its wine concentration showed an increase of more than two folds the maximum foamability (HM) and 7.4 fold in TS. The combination of MP1 fraction with MeLMW increases significantly the foam parameters HM and TS.

In conclusion, the combination of fraction HMW (mannoproteins with low protein content) with MeLMW (tensioactives of glycerylethyleneglycol fatty acid derivatives) contains the key compounds to promote sparkling wines foam volume and foam stability.

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CAPÍTULO I – INTRODUÇÃO TEÓRICA

1. A Região Demarcada da Bairrada

A Região Demarcada da Bairrada foi criada em 1979, estando localizada entre o rio Vouga e Mondego, a oeste do Buçaco e do Caramulo, cujo nome deriva da natureza dos solos – os barros. Nesta região estão incluídos os concelhos de Anadia, Mealhada, Oliveira do Bairro, 18 freguesias do concelho de Cantanhede, 11 de Águeda, 5 de Coimbra, 4 de Vagos e uma de Aveiro (Decreto-Lei n.º 301/2003 de 4 de Dezembro). Estima-se que na Região Demarcada da Bairrada exista uma área de vinha de cerca de 12000 ha, sendo o encepamento de castas tintas cerca de 70% e 30% de castas brancas.

Os principais tipos de solos da Região Demarcada da Bairrada são argilo-calcários (para pequenas percentagens de calcário são denominados por argilosos) de origem jurássica e triássica, arenosos oriundos do plio-plistocénico e solos de aluvião. Na **figura 1** está o mapa geológico da Região Demarcada da Bairrada, mostrando a distribuição dos diferentes tipos de solo. No Decreto-Lei n.º 301/2003 de 4 de Dezembro é aconselhado que as vinhas destinadas à produção dos vinhos DOC Bairrada devem estar instalados em: a) solos calcários pardos ou vermelhos; b) solos litólicos húmicos ou não húmicos e c) podzóis de materiais arenáceos pouco consolidados.

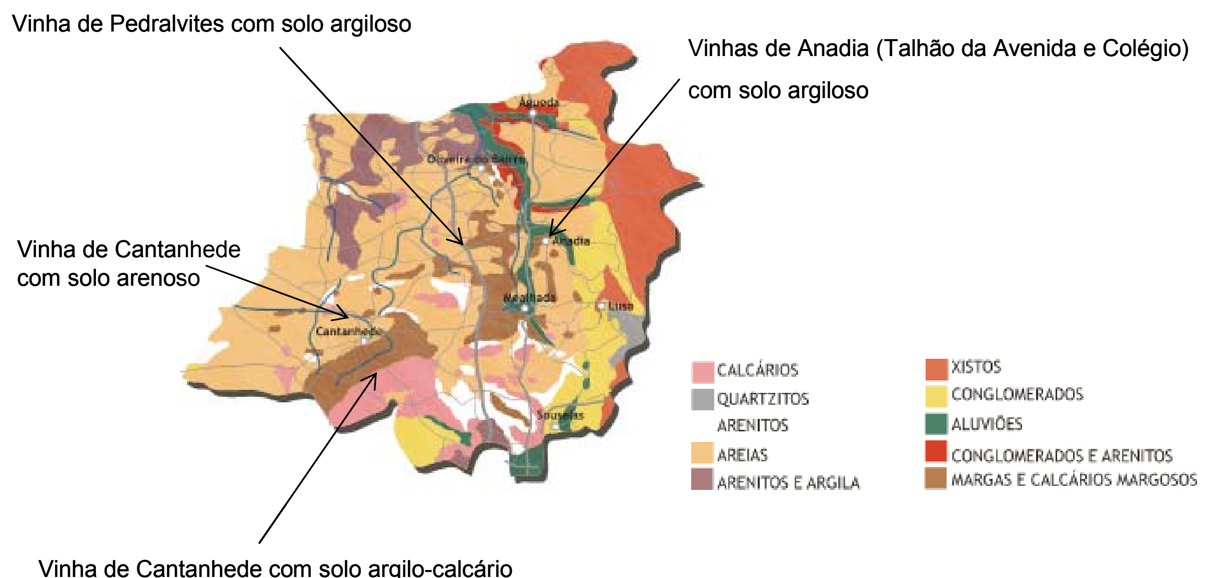

Figura 1 – Mapa geológico da Região Demarcada da Bairrada com a localização aproximada das vinhas de onde foram recolhidas as uvas utilizadas nesta dissertação.

São 10 as castas brancas autorizadas na Região Demarcada da Bairrada: Arinto, Bical, Cercial, Chardonnay, Fernão-Pires (Maria Gomes), Pinot-Blanc, Rabo-de-Ovelha, Sauvignon, Sercialinho e Verdelho. Quanto às castas tintas autorizadas o número é de 18: Alfrocheiro, Aragonez, Tinta-Roriz, Baga, Bastardo, Cabernet-Sauvignon, Camarate, Castelão, Periquita, Jaen, Merlot, Pinot-Noir, Rufete, Syrah, Tinta-Barroca, Tinto-Cão, Touriga-Franca e Touriga-Nacional (Decreto-Lei n.º 301/2003 de 4 de Dezembro). Dentro das castas brancas cultivadas na Bairrada, a casta Fernão-Pires (FP, **fig. 2a**) representa 80% do encepamento. No caso das castas tintas, a Baga (BG, **fig. 2b**) representa 92% das vinhas e 80% do encepamento de toda a região demarcada.

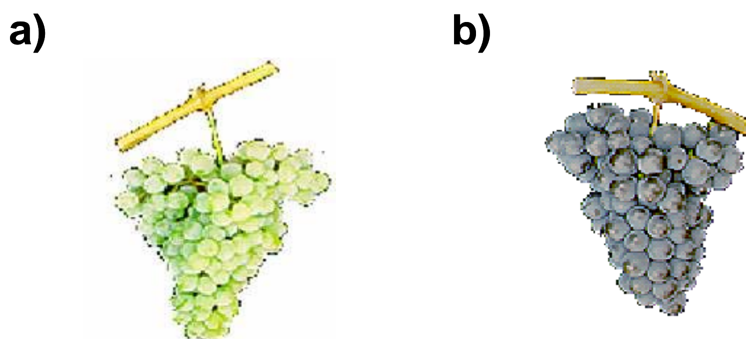

Figura 2 – *Vitis vinifera* L. da casta a) Fernão-Pires e b) Baga.

2. Composição volátil das uvas

A composição volátil das uvas é um dos factores mais importantes que determina o carácter e a qualidade do vinho. Existem vários estudos no âmbito da caracterização das uvas que reconhecem que existe uma relação entre o carácter varietal do vinho e a composição volátil e semi-volátil dos mostos e das uvas, nomeadamente, a composição em monoterpenóides, norisoprenóides em C_{13} e álcoois aromáticos (*1-4*). A composição volátil varietal de uma grande maioria das castas brancas é determinada principalmente por monoterpenóides (*1-7*). Os principais monoterpenóides descritos na bibliográfica como componentes da composição volátil das uvas brancas encontram-se representados na **figura 3.**

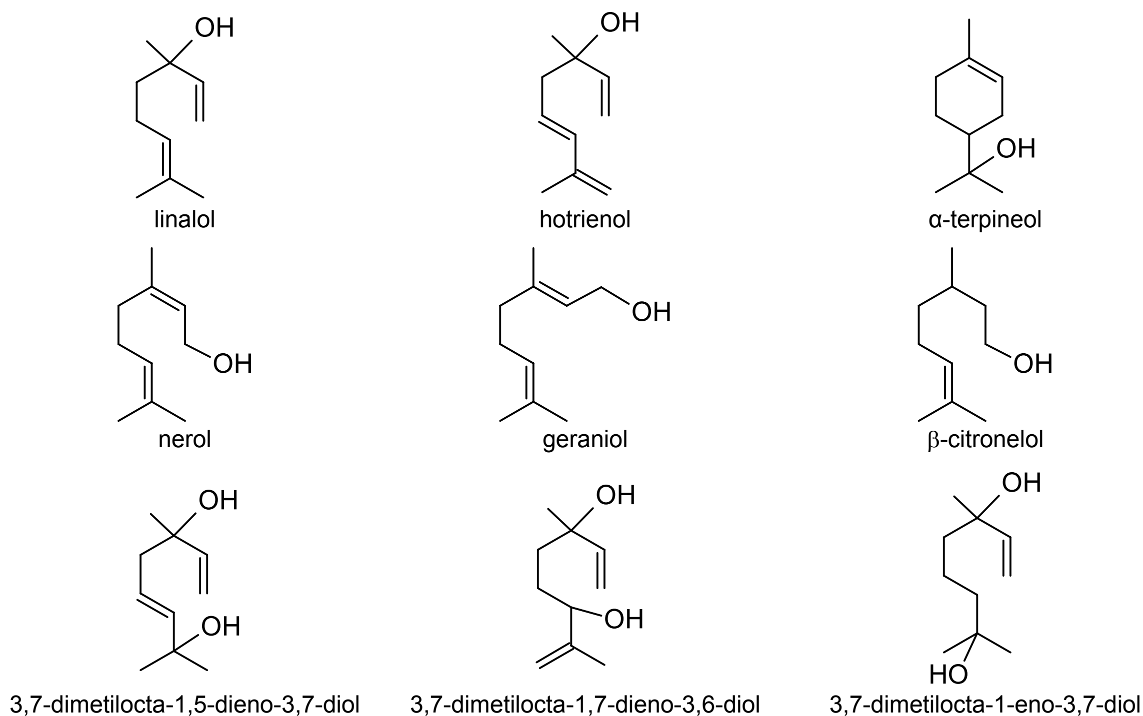

Figura 3 – Exemplos dos principais monoterpenóis descritos como componentes voláteis de uvas brancas (*1-7*).

No caso da maioria das uvas tintas a sua composição volátil é caracterizada pela presença predominante de álcoois aromáticos e norisoprenóides em C_{13} (*8-11*). Os principais álcoois aromáticos descritos na bibliográfica como componentes da composição volátil das uvas tintas são o álcool 2-feniletílico e o álcool benzílico (**fig. 4**) e os norisoprenóides em C_{13} são a β-damascenona e a β-ionona (**fig. 5).**

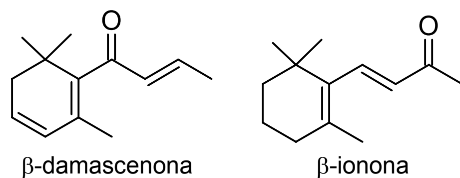

Figura 4 – Exemplos dos principais norisoprenóides em C_{13} descritos como componentes voláteis de uvas tintas (*8-11*).

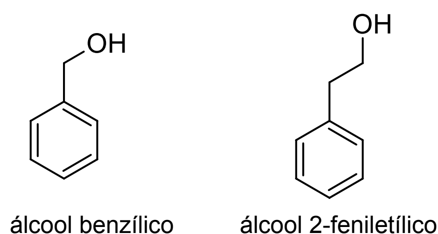

Figura 5 – Exemplos dos principais álcoois aromáticos descritos como componentes voláteis de uvas tintas (*8-11*).

Apesar dos sesquiterpenóides terem sido descritos por Schreier *et al.* (*12*) em 1976 como constituintes de algumas variedades de uvas, nomeadamente Riesling, Traminer, Ruländer, Müller Thurgau, Scheurebe, Optima e Rieslaner, este grupo de compostos só raramente foi considerado como componente varietal das uvas. Em 1994, Versini *et al.* (*13*) descreve a presença de isómeros de α-farneseno e farnesoato de metilo em destilados de películas de uvas. Os poucos estudos que referem a presença de sesquiterpenóides em uvas somente identificaram o farnesol (*3, 9, 14*). Mais recentemente, em bagaços da casta Frappato, cultivada na Sicília (*15*), e bagaceiras de seis castas cultivadas na Croácia (*16*) foram identificadas composições voláteis ricas em sesquiterpenóides. Os principais sesquiterpenóides descritos na bibliografia (*12, 14-16*) como componentes da composição volátil das uvas encontram-se representados na **figura 6.**

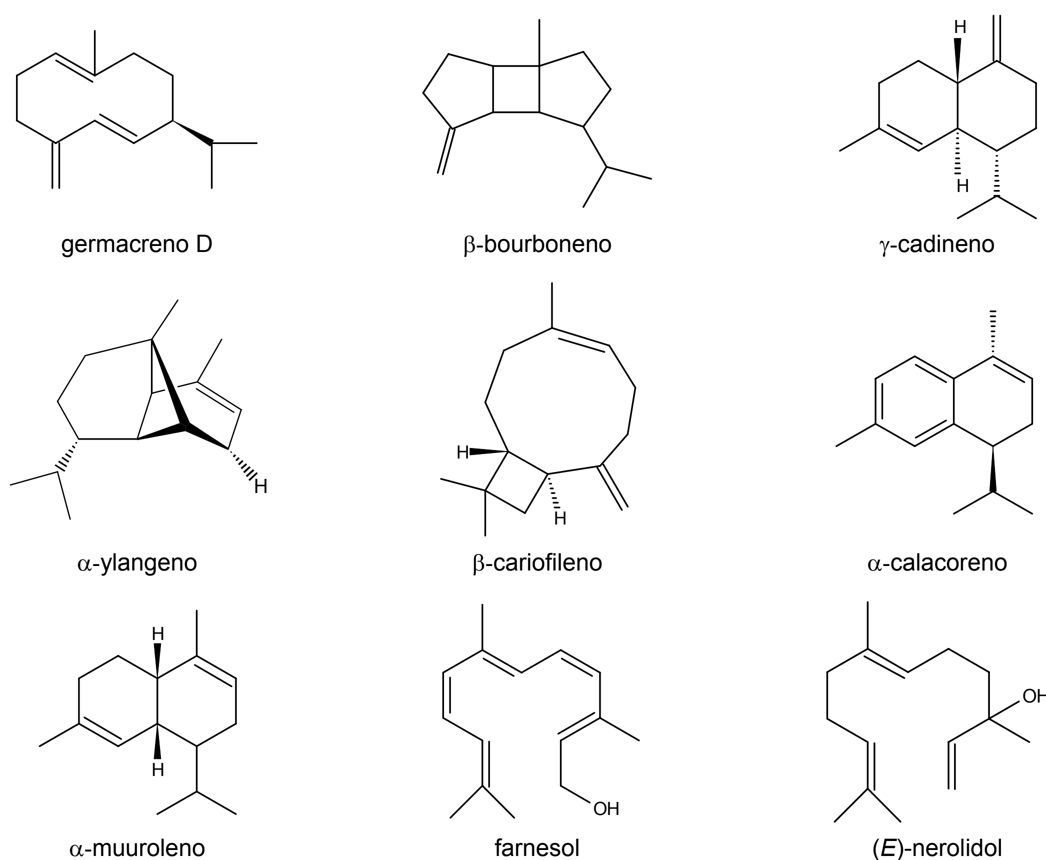

Figura 6 – Exemplos de alguns sesquiterpenóides descritos na composição volátil das uvas (*12, 14-16*).

Os monoterpenóides estão associados a aromas florais e frutados. Por exemplo, o linalol apresenta notas cítricas, doces e florais e o hotrienol, o α-terpineol e o geraniol

exibem aromas florais e doces (*17-19*). Os sesquiterpenóides têm descritores de aroma associados a madeira, especiarias, doce, floral, cravinho, óleo e a frescura (*20-23*). Dentro dos norisoprenóides em C_{13}, a β-damascenona pode potencialmente contribuir com os aromas a doce e a maçã devido ao seu limite de percepção sensorial muito baixo (0,05 µg/L) (*18*). Os álcoois aromáticos, álcool benzílico e álcool 2-feniletílico estão associados a notas doces e florais (*18, 24*). Para além dos compostos varietais, os pré-fermentativos têm um contributo significativo para o aroma do vinho, nomeadamente com odores herbáceos e a gordura (*18, 25*). Os compostos relacionados com estes odores são os álcoois e os aldeídos em C_6 provenientes da actividade da lipoxigenase das uvas (*26*).

No caso das uvas FP, a análise dos compostos voláteis que contribuem para o carácter varietal desta casta permitiu a identificação de 13 monoterpenóides, 3 norisoprenóides em C_{13} e 2 álcoois aromáticos (*3, 27*). Os monoterpenóides mais representativos são os seguintes: terpendiol I (237 – 622 µ/L), 3,7-dimetilocta-1-eno-3,7-diol (94 – 234 µ/L), α-terpineol (22 – 149 µ/L), hotrienol (57 – 153 µ/L) e linalol (30 – 133 µ/L). No caso dos norisoprenóides em C_{13} foram identificados dihidro-β-ionona (24 – 93 µ/L), *trans*-β-damascenona (9 – 95 µ/L) e vitispirano (10 – 12 µ/L). Os álcoois aromáticos presentes são o 2-feniletanol (26 – 268 µ/L) e o benzil álcool (30 – 142 µ/L) (*3, 27*).

O grau de maturação da uva é muito importante e decisivo para a qualidade do vinho. A maturação da uva é o resultado de todos os fenómenos fisiológicos e bioquímicos que ocorrem na videira e está intrinsecamente relacionada com as condições ambientais, variedade, solo e clima (*28-30*).

2.1. Maturação

O crescimento e o desenvolvimento da uva é o resultado de um longo e complexo ciclo de reprodução (*28*). O desenvolvimento da uva pode ser dividido em três fases, tendo em consideração o diâmetro, o peso e o volume do bago:

1ª Fase – Crescimento inicial rápido, designado por crescimento herbáceo. Esta fase dura em média 45 a 65 dias, dependendo da variedade e condições ambientais. Nesta fase a clorofila é o pigmento predominante e as uvas têm uma actividade

metabólica muito elevada, caracterizada por uma grande actividade respiratória e uma rápida acumulação de ácidos.

2ª Fase – Esta fase apresenta um crescimento muito lento. É nesta fase que ocorre o pintor, caracterizado pelo aparecimento de cor nas variedades tintas e por uma película translúcida nas variedades brancas.

3ª Fase – Esta fase corresponde à maturação, que tem início no pintor e se prolonga até à maturidade. A intensidade respiratória diminui e a actividade enzimática aumenta abruptamente. Nesta fase, a uva acumula açúcares livres, catiões como o potássio, aminoácidos e compostos fenólicos enquanto a concentração de ácido málico e amónia diminui.

Dependendo do tipo de vinho que se pretende produzir, são usadas uvas em estados de maturação diferentes. Por exemplo, a produção de vinho branco seco requer uvas que tenham uma concentração máxima de compostos voláteis e pouca acidez. Para o caso de um vinho espumante, uma colheita precoce é importante para obter um produto final ligeiramente acídulo, mas para elaborar um vinho tinto, o desenvolvimento da uva tem de ser tal que se obtenha os compostos fenólicos de uma forma facilmente extractável (*28*). Tendo em conta a especificidade do vinho que se pretende produzir, com o objectivo de obter um produto com uma melhor qualidade, são seguidas diferentes características varietais da uva ao longo da maturação, nomeadamente compostos fenólicos (*31-33*), carotenóides (*31, 34, 35*) e compostos voláteis (*14, 29, 32, 36*). O seguimento das características varietais das uvas ao longo da maturação complementa os métodos clássicos, baseados na percentagem de sólidos solúveis, açúcar, acidez titulável, pH e coloração (*37*).

Os compostos voláteis podem estar na uva em duas formas: na forma glicosilada e na forma não glicosilada vulgarmente designada por forma livre. Na forma glicosilada, os compostos não contribuem para o aroma, podendo transformar-se em compostos odoríferos por hidrólise da ligação entre a aglícona e o açúcar.

Dentro dos compostos voláteis, os compostos terpénicos são os mais estudados e, dentro destes, os monoterpenóis. Os monoterpenóis, quer na forma livre quer glicosilada, aumentam a sua concentração durante o desenvolvimento da uva. Os compostos terpénicos glicosilados são abundantes desde o início do desenvolvimento da uva, quando a uva ainda

está verde, enquanto que os terpenóis livres existem numa pequena proporção (*36*). Durante a maturação, a fracção glicosilada é usualmente muito maior do que a fracção livre e aumenta até à maturidade (*36*). Na maturidade, a concentração da fracção livre mantém-se ou pode mesmo diminuir, nomeadamente o linalol e o α-terpineol (*28, 36, 38*). Esta evolução parece indicar que o armazenamento dos terpenóis na uva ocorre geralmente na forma glicosilada, sendo o linalol muitas vezes uma excepção dado que a sua fracção livre pode ser maior do que a fracção glicosilada (*28, 36*).

Geralmente, o teor das várias classes de compostos voláteis varietais aumenta ao longo da maturação (*14, 36*). Para as castas tintas, nomeadamente a casta Monastrell, este aumento é mais evidente a partir da maturidade. Para esta casta, os norisoprenóides e os terpenóides (monoterpenóides e farnesol) foram identificados como compostos favoráveis para o aroma, verificando-se um aumento destes compostos a partir da maturidade. Contrariamente, verifica-se a diminuição dos álcoois e aldeídos compostos por 6 átomos de carbono (compostos em C_6), que são considerados desfavoráveis para o aroma devido aos seus odores herbáceos (**Fig. 7**) (*14*).

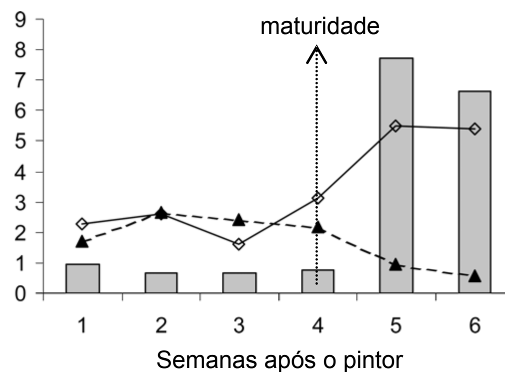

Figura 7 - Evolução dos terpenóides + norisoprenóides (◊) e compostos em C6 (▲) ao longo da maturação (valores expressos em µg/kg de uvas, sendo a maturidade atingida na 4ª semana após o pintor) e razão entre a concentração dos compostos voláteis favoráveis/desfavoráveis (barras) (*14*).

Os carotenóides são precursores importantes de compostos voláteis. Durante a maturação há uma diminuição da concentração de carotenóides e um aumento da concentração de algumas moléculas derivadas dos carotenóides, como os norisoprenóides (*34*) (**Fig. 8**).

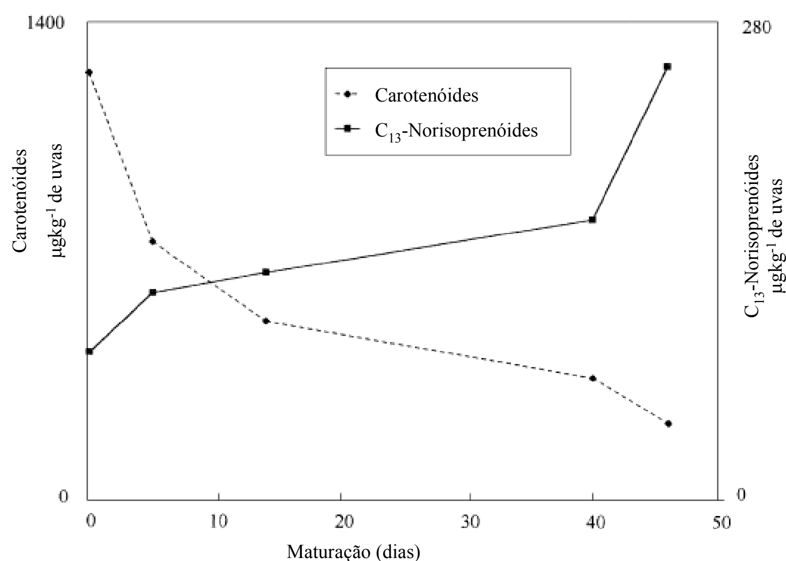

Figura 8 – Evolução dos carotenóides e norisoprenóides em C_{13} glicosilados durante a maturação, desde o pintor (dia 0) até à colheita (maturidade, dia 49) das uvas Moscatel (*34*).

As prirazinas são outro grupo de compostos voláteis presentes nas uvas em que a sua concentração é muito dependente do estado de maturação. Em variedades como a Cabernet Sauvignon, a concentração de metoxipirazinas é elevada quando as uvas estão verdes. A concentração destes compostos decresce bastante ao longo da maturação (*28*).

2.2. Factores que influencim a composição volátil das uvas

A composição volátil das uvas, que inclui a fracção volátil e os seus precursores, está condicionada por numerosos factores para além do estado de maturação e variedade, nomeadamente, exposição solar (*37, 39*), clima, solo, práticas agrícolas, produtividade da videira (*40*) e condições fito-sanitárias (*41*).

No caso das condições fito-sanitárias, a presença de *Botrytis cinerea* contribui para a alteração do perfil volátil dos vinhos, uma vez que as glicosidases produzidas pela *B. cinerea* hidrolisam os compostos terpénicos glicosilados. Há também uma acumulação de furfural, benzaldeído, fenilacetaldeído e de álcool feniletílico. A infecção contribui com defeitos no aroma, nomeadamente o aroma a mofo, devido principalmente à presença dos compostos 1-octeno-3-ona e 1-octeno-3-ol (*28, 42, 43*).

A produtividade de uma vinha é um factor importante para os atributos sensoriais das uvas. A carga da videira deve ser calculada de forma a que exista sustentabilidade para todos os cachos dado que um número elevado de cachos faz com que haja um défice de nutrientes e limitações ao nível da biossíntese de metabolitos secundários, nomeadamente os compostos voláteis e, por outro lado, um reduzido número de cachos faz com que o crescimento vegetativo se torne demasiado grande (*40*).

Há outros factores que variam continuamente, em que a planta tem que se adaptar a algumas práticas viticulturais, tais como a poda, monda de cachos, desparra, tratamentos fitossanitários e fertilização (*28*).

Existem outros parâmetros que são fixos de ano para ano e que exercem uma influência constante e permanente: a variedade usada, a densidade de plantação, o espaçamento entre linhas, a condução da vinha (sistema de prender a videira) e também a idade da vinha e a natureza do solo. Dada a importância da exposição solar e do solo para a composição volátil das uvas estes parâmetros serão seguidamente apresentados com maior detalhe.

Exposição solar

A exposição solar é um factor que influência muito a maturação da uva, porque a radiação e o aquecimento solar excessivos podem influenciar as taxas das reacções metabólicas causando *stress* tanto por desidratação como por aumento da temperatura (*37, 39*). O efeito da luz solar na composição da uva é bastante complexo, pois é a partir da luz que se obtém a energia necessária para a fotossíntese e outros processos metabólicos dependentes da luz, nomeadamente a biossíntese de compostos fenólicos promovida pela fenilalanina amonialiase. O efeito radiante não aquece só a superfície mas também o ar que rodeia os tecidos vegetais. Os cachos de uva pouco expostos ao sol contêm sempre menos quantidade de açúcar, um pH menor e uma acidez total e concentração de ácido málico mais elevada que as uvas que estão mais expostas ao sol (*28*).

A exposição directa à luz pode levar a um aumento do conteúdo em monoterpenóides. Os teores de monoterpenóides glicosilados são muito superiores nas uvas expostas ao sol quando comparados com os das uvas que permanecem na sombra ou sombra parcial. No caso dos terpenóides livres, os teores mais elevados são atingidos para as uvas completamente expostas ao sol e também nas de sombra parcial (*44*). Modificações

no ambiente em redor do fruto, tais como a remoção das folhas basais ou a redução da copa da videira por desparra, são também factores que aumentam a quantidade de terpenóides livres e glicosilados (*45, 46*). A luz também favorece a biossíntese de carotenóides desde a primeira fase do desenvolvimento do fruto até ao pintor, diminuindo depois entre o pintor e a maturidade, dando origem aos norisoprenóides em C_{13} glicosilados (*34, 35*).

A temperatura do meio é um dos factores mais importantes na maturação das uvas. A temperatura afecta a actividade fotossintética, o metabolismo e a intensidade de migração dos compostos na videira. As temperaturas elevadas são desfavoráveis para a multiplicação celular durante a fase de crescimento herbáceo. Durante a maturação, a temperatura afecta a intensidade de migração dos compostos e, indirectamente, o crescimento celular. Uma temperatura demasiada elevada nesta fase, mesmo por um pequeno período de tempo, pode alterar irreversivelmente a acumulação de açúcares. Nestes casos, os açúcares acumulam-se noutras zonas da videira em detrimento das uvas, que apenas recebem uma pequena percentagem. A temperatura influencia muitos mecanismos bioquímicos envolvidos na maturação, nomeadamente, a degradação do ácido málico é acelerada com a temperatura mas esta não tem influência na concentração do ácido tartárico. Temperaturas superiores a 35ºC podem desencadear quocientes respiratórios muito elevados, que normalmente correspondem a um fenómeno fermentativo da polpa da uva, que actua essencialmente sobre o ácido málico. As situações extremas levam a que exista uma deficiência na alimentação da uva, aumentando a competição entre o metabolismo primário (crescimento) e o metabolismo secundário (acumulação). A combinação temperatura e exposição solar determina a acumulação dos compostos associados ao metabolismo secundário (*28*).

Solo

Os factores topográficos, agro-pedológicos e climáticos, que são normalmente descritos usando o termo francês *terroir,* influenciam a composição e a qualidade das uvas (*47*). Apesar do clima ou da variedade da uva serem considerados os parâmetros mais importantes para o aroma, as características de profundidade, capacidade de retenção de água e de drenagem dos solos são parâmetros que também têm influência na composição volátil das uvas (*47, 48*).

A disponibilidade de água condiciona o desenvolvimento da planta e da maturação do fruto (*30*). A natureza do solo influencia o microclima pela sua capacidade de reter calor e reflectir a luz, podendo também afectar o crescimento das raízes devido à sua penetrabilidade (*47*). A capacidade de atenuar o efeito das chuvas fortes num solo profundo, depende de uma boa drenagem. Mesmo num solo argiloso pesado, que absorve a água lentamente após a chuva forte e onde o excesso irá escorrer, um solo profundo vai permitir que as raízes atinjam grande profundidade, onde a água não evapora, permitindo uma hidratação mais prolongada da planta, mesmo em período de seca. A capacidade da planta sobreviver à seca, para além de se relacionar com a existência de raízes profundas, relaciona-se também com uma boa capacidade de retenção de humidade no solo (*47*).

Embora o *stress* hídrico não impeça que o fruto atinja a maturidade em relação à concentração de açúcares e ácidos, já um excesso de água retarda o processo de maturação e altera a composição química da uva. Um fornecimento insuficiente, mas moderado, de água leva a uma concentração mais elevada em compostos terpénicos. Por outro lado, um fornecimento elevado de água resulta num aumento do volume do bago, apresentando as uvas notas herbáceas muito fortes (*28*). O défice de água causado por alguns tipos de solo e clima mostram um aumento do potencial enológico das uvas tintas Agiorgitiko (*48*). Estudos desenvolvidos na região do Douro sobre o efeito da disponibilidade de água e o conteúdo em carotenóides mostrou que o tipo de solo exerce uma influência maior que a irrigação; ou seja, a concentração de carotenóides depende mais da capacidade do solo reter a água do que se o solo é irrigado ou não (*49*). Em geral, os solos argilosos têm uma melhor capacidade de reter a água e maior humidade volúmica do que os solos arenosos. Em oposição, a drenagem é maior em solos arenosos do que em argilosos (*50, 51*).

Uvas provenientes de solos calcários dão origem a vinhos brancos com atributos florais, doces e frutados, enquanto que uvas provenientes de solos arenosos originam vinhos brancos mais relacionados com notas herbáceas (*52*).

3. Produção de vinho espumante

Para a elaboração de um vinho espumante primeiro é produzido um vinho, que se designa por vinho base, e a partir do qual se realiza uma segunda fermentação pela adição de uma suspensão de leveduras e açúcar. Existem quatro métodos principais para produzir

um vinho espumante: método tradicional (Champanhês), método de transferência, *cuvée* fechado e o método usado na produção do espumante Asti. O que distingue o método Champanhês dos restantes é o facto da segunda fermentação ocorrer na garrafa que o consumidor compra, depois de uma eliminação prévia do depósito de leveduras por *dégorgement*. No método de transferência, o vinho espumante é transferido da garrafa onde decorreu a segunda fermentação e o envelhecimento para outra garrafa, sendo filtrado antes desta trasfega. No método do *cuvée* fechado, a segunda fermentação decorre num tanque. No caso da produção de um espumante Asti, a segunda fermentação também decorre num tanque, mas a fermentação é interrompida regularmente, sempre que esta comece a acelerar. Os passos de deposição das borras, clarificação e centrifugação são repetidos as vezes que forem necessárias para separar o vinho das leveduras.

A explicação dos vários passos da produção de vinho espumante encontra-se detalhadamente descrita por Ribéreau-Gayon *et al.* (*28*). Atendendo a que nesta dissertação se usaram somente vinhos espumantes produzidos pelo método tradicional, os passos essenciais para a produção de vinhos espumantes pelo método Champanhês serão seguidamente descritos utilizando a informação disponibilizada por estes autores.

3.1. Produção de vinho base

A produção de um vinho base de espumante sofre o mesmo processo de vinificação que um vinho branco, diferindo apenas no facto do vinho base não ser sulfitado no final, pois ainda vai sofrer uma segunda fermentação. Além disso, o vinho base não deve ter um teor alcoólico muito elevado, dado que vai haver ainda formação de etanol na segunda fermentação. As uvas são colhidas menos maduras comparativamente às uvas utilizadas para a produção de vinho maduro, com o objectivo de produzir um vinho base com maior acidez. O sumo deve ser extraído cuidadosamente, evitando a maceração pelicular, para evitar o sabor amargo, o carácter vegetal conferido pela película e grainha e a cor. No entanto, há vinhos espumantes em que é promovida a extracção de compostos da película de uvas tintas, conferindo aos vinhos uma tonalidade rosé ou mesmo tinta.

Para limitar o fenómeno de maceração, as uvas, brancas ou tintas, são sempre prensadas de modo a que o esmagamento seja o mais suave possível, sendo o sumo resultante dividido em lotes. As primeiras duas ou três prensagens rápidas e sucessivas

produzem o sumo de primeira qualidade, denominado de *cuvée*. Uma prensagem adicional produz os vinhos de "segunda prensagem" (*taille*). Só o *cuvée* é usado para produzir o espumante de qualidade, uma vez que a segunda prensagem produz um vinho mais denso. Existem também diferenças significativas da composição das fracções sucessivas de sumo recolhidas ao longo da prensagem. A acidez total, o ácido tartárico e o ácido málico diminuem, a concentração de minerais e o pH aumentam, a concentração dos compostos fenólicos e a cor aumentam e a concentração de açúcar mantém-se relativamente constante. Sob o ponto de vista sensorial é observada uma diminuição na intensidade de aroma. Na **figura 9** é mostrado um esquema das principais etapas de elaboração de um vinho espumante.

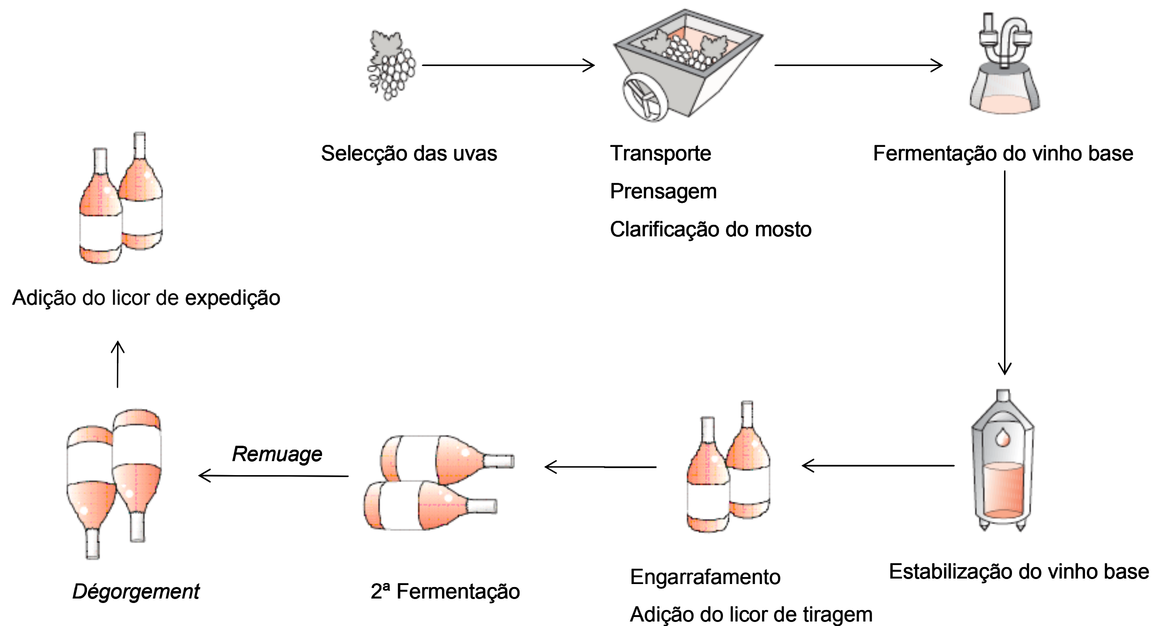

Figura 9 – Esquema das principais etapas de elaboração de um vinho espumante de acordo com o método Champanhês.

3.2. Segunda fermentação

Método tradicional (método Champanhês)

O licor de tiragem (suspensão de açúcares e leveduras) é adicionado depois do engarrafamento do vinho base. A concentração de sacarose adicionada é calculada para produzir 5 a 6 bar de pressão exercida pelo CO_2 depois da fermentação. Teoricamente, 20 g de sacarose por litro é suficiente para obter 5 bar de pressão. Na realidade, este valor só é

verdadeiro para um vinho com 10% de álcool. O aumento da capacidade de dissolução do dióxido de carbono originada pelo aumento da percentagem de álcool tem de ser tomada em consideração a concentrações de álcool mais elevadas. Simultaneamente à adição do licor de tiragem é adicionado um *starter* de leveduras activas assegurando uma fermentação completa e em estado estacionário na garrafa.

Depois das garrafas serem vedadas com uma cápsula, estas são empilhadas horizontalmente, devendo a garrafa estar na posição horizontal enquanto a fermentação ocorre, fermentando o vinho pelo menos dois meses à temperatura constante de 11-12ºC. O facto da fermentação decorrer a baixa temperatura, lentamente e estaticamente, é condição para produzir vinhos espumantes de qualidade, em particular no que respeita à persistência e ao tamanho das bolhas produzidas após a abertura da garrafa. A pressão exercida pelo dióxido de carbono vai aumentando progressivamente, sendo um efeito inibidor da velocidade de fermentação e do crescimento das leveduras, particularmente a pH baixo e elevado teor alcoólico.

No final da fermentação em garrafa, os vinhos permanecem em contacto com o depósito de leveduras que vai sendo depositado ao longo do tempo. As garrafas devem estar deitadas para aumentar a superfície de contacto entre o vinho e o depósito de leveduras. Nesta fase, por excreção ou simples difusão depois da morte celular são transferidos para o vinho vários compostos, em particular aminoácidos. O acréscimo de qualidade do vinho espumante durante este passo depende da composição do vinho base. O vinho espumante é maturado nestas condições pelo menos nove meses, podendo este envelhecimento ser prolongado por 2-8 anos, ou até mais, para vinhos espumantes de elevada qualidade, quando os vinhos base assim permitem.

Para separação do depósito de leveduras, também denominado por borras, as garrafas são submetidas a um processo de *remuage*. Tradicionalmente, a *remuage* é efectuada manualmente por rotação das garrafas, em que estas se encontram inclinadas com o gargalo voltado para baixo, sendo as garrafas rodadas com pequenas rotações várias vezes por mês. As garrafas são progressivamente rodadas com ângulos menores e aproximadas da posição vertical invertida. O processo tradicional envolve muita mão-de-obra e o espaço ocupado durante um período de tempo relativamente longo. Surgiram alternativas a este processo, como a adição de adjuvantes de sedimentação, centrifugação e máquinas que reproduzem os movimentos manuais em grande escala. Outra alternativa

para facilitar a *remuage* é a adição de leveduras imobilizadas em pequenas esferas de alginato de cálcio reticulado. Quando as garrafas são colocadas com o gargalo para baixo o depósito assenta imediatamente contra a cápsula, tornando o processo da *remuage* muito mais rápido.

Quando o depósito está concentrado junto da cápsula, a garrafa está pronta para se efectuar a remoção do depósito de leveduras, designado por *dégorgement*. O processo de *dégorgement* é efectuado por congelamento do gargalo, com as garrafas mantidas na posição invertida e parcialmente imersas num líquido refrigerante a -20ºC. Esta solução congela alguns mililitros de vinho abaixo da cápsula, incorporando o depósito de leveduras, no gelo. A garrafa é voltada para cima, a cápsula é removida e o depósito é expelido na forma de gelo. O *dégorgement* causa um choque oxidativo, podendo durante esta operação o potencial de redução aumentar cerca de 400 mV, podendo a partir desta fase ocorrer defeitos geralmente relacionados com fenómenos de oxidação.

Antes da rolhagem e museletagem (introdução do dispositivo metálico de amarração designado por *muselet*) do produto final, é adicionado o *licor de expedição* ou dosagem. Este xarope contém aproximadamente 600 g/L de açúcar e é usado para ajustar a concentração de açúcar do vinho espumante, geralmente entre 10 g/L (1%, dosagem para bruto) e 40 g/L (4%, dosagem para semi-seco).

4. Composição química do vinho espumante

O vinho espumante é maioritariamente constituído por água, etanol e CO_2. As proteínas solúveis (*41, 53-55*), os polissacarídeos (*41, 54, 56*), os compostos fenólicos (*41, 54*), os ácidos gordos e os lípidos (*57*) fazem parte dos principais grupos de moléculas que também estão presentes num vinho espumante. Os compostos voláteis são outro grupo de moléculas constituintes de um vinho espumante e desempenham um papel importante na percepção do aroma.

4.1. Compostos voláteis

Nos vinhos, a concentração de compostos voláteis e semi-voláteis está compreendida entre 0,8 e 1,3 g/L, que representa 1% da concentração de etanol. A composição volátil e semi-volátil compreende um vasto número de compostos, mais de

800, pertencentes a diferentes famílias químicas, nomeadamente terpenóides, álcoois aromáticos e alifáticos, ésteres, aldeídos, cetonas, lactonas, ácidos e fenóis (*2*).

O aroma de um vinho está directamente relacionado com a matéria-prima de origem, as uvas, e com o processo de vinificação (*58*), sendo constituído por compostos varietais e fermentativos, tais como álcoois, ésteres, terpenóides, compostos sulfurados e ácidos. A composição volátil dos vinhos espumantes Cava foi caracterizada por HS-SPME/GC-MS, para a avaliação da evolução dos compostos voláteis durante a segunda fermentação e envelhecimento, tendo-se concluído que a composição volátil destes vinhos é descrita principalmente por compostos fermentativos da família dos ésteres, álcoois e ácidos (*59-61*). Os compostos voláteis do vinho Cava incluem também monoterpenóides como o limoneno, o α-terpineol e o lilial (*62*).

As maiores diferenças ao nível do aroma entre o vinho base produzido com as castas brancas Chardonnay e Pinot Blanc e com as castas tintas Pinot Meunier e Pinot Noir é que o vinho base de castas brancas é principalmente caracterizado por uma elevada intensidade floral, cítrica e notas de maçã, enquanto o vinho base de castas tintas é caracterizado por aroma a frutos vermelhos e baunilha/manteiga. O contributo para o aroma proveniente da segunda fermentação sobrepõe-se às características varietais do vinho pelo que, ao contrário do verificado para os vinhos base, torna-se mais difícil ou mesmo impossível discriminar os vinhos espumantes por casta usando a análise sensorial (*63*).

O aroma característico do vinho espumante é principalmente adquirido durante o processo de envelhecimento, que é quando ocorre a autólise das leveduras (*59*). As alterações provenientes da autólise das leveduras decorrem mais rapidamente nos vinhos obtidos com castas brancas, sendo necessário mais de três anos para se desenvolver as mesmas alterações nos vinhos obtidos com castas tintas (*63*). O envelhecimento de um espumante é talvez um dos factores que maior influencia as suas características, principalmente ao nível dos compostos voláteis. Um vinho espumante que envelhece durante nove meses (tempo mínimo necessário à elaboração de um espumante) tem um perfil ao nível dos compostos voláteis muito diferente de um vinho que é envelhecido vinte meses ou mais. Para o primeiro caso, o hexanoato de etilo, octanoato de etilo, decanoato de etilo e 2-decenoato de etilo estão presentes em quantidades elevadas, quando comparadas com um vinho com vinte meses de envelhecimento (*59*). Contrariamente, os acetatos e o

decanoato de etilo apresentam os valores mínimos para as amostras mais envelhecidas (vinte meses). Para os compostos succinato de dietilo, vitispiranos e 1,1,6-trimetil-1,2-di-hidronaftaleno (TDN), estes vão aumentando ao longo do envelhecimento (*59*). A quantidade de decanoato de etilo, 2-decenoato de etilo, succinato de dietilo, vitispirano, hexanoato de isoamilo, octanoato de isoamilo e octanoato de etilo pode ser usada como marcador da idade limite legal para o espumante, e os últimos três compostos podem mesmo ser usados para determinar a idade aproximada de um vinho espumante (*64*).

O vinho espumante envelhecido é muitas vezes caracterizado por um carácter empireumático (odor e sabor acre produzido pela combustão), parecido com grãos de café torrados e tostados. Os compostos que parecem estar relacionados com este aroma são tióis: benzenometanotiol, 2-furanometanotiol e 3-mercaptopropionato de etilo, aumentando a concentração destes compostos gradualmente com o tempo de envelhecimento. O impacto do *dégorgement* nestes compostos é muito pronunciado, sendo a concentração dos tióis significativamente superior nos vinhos que sofreram o *dégorgement.* Aparentemente, estes tióis voláteis desenvolvem-se mais rapidamente depois do *dégorgement*, na ausência do depósito de leveduras *(65).*

O *dégorgement* é um processo crítico no que se relaciona com a perda de compostos voláteis e CO_2, dado que as paredes celulares das leveduras também têm a capacidade de se ligar aos compostos voláteis, contribuindo para a perda de aroma. A percentagem de retenção aumenta com a hidrofobicidade dos compostos voláteis (*66, 67*). Os compostos voláteis adsorvidos à superfície das borras são essencialmente compostos da família dos ésteres, aldeídos, norisoprenóides e terpenos (*68*).

O rompimento das bolhas de CO_2 à superfície do líquido é o principal responsável pela libertação de aromas. Estas moléculas parecem ser transportadas ao longo do percurso da bolha, desde o local de nucleação até à superfície, até serem ejectadas para o ar, participando na percepção sensorial global de um vinho espumante (*69*). Álcoois (etanol, butanol, pentanol e 2-feniletanol), alguns aldeídos (butanal, hexanal e hexenais) e ácidos voláteis (ácido propiónico e butírico) são compostos voláteis encontrados no aerossol e que poderão contribuir para o aroma (*69*). Outro estudo aos compostos presentes nos aerossóis que se formam após o colapso das bolhas de CO_2, permitiu a detecção de 163 analitos com massas moleculares entre 150-1000 Da. Destes, 45 foram encontrados no aerossol em concentração superior à do vinho, nomeadamente ácidos gordos e norisoprenóides (*70*).

4.2. Compostos azotados

Na sua maioria, os compostos azotados que compõem o vinho espumante são peptídeos e aminoácidos livres. No início da fermentação os peptídeos são assimilados pelas leveduras juntamente com os aminoácidos livres. No entanto, no final da fermentação há uma libertação para o vinho de aminoácidos livres e de pequenos peptídeos por parte das leveduras. Este processo ocorre de novo com o decorrer da segunda fermentação (*71*). Os peptídeos são importantes para o vinho pelas suas propriedades surfactantes, sendo também responsáveis tanto pelo gosto doce como pelo amargo. A concentração total de azoto num vinho espumante varia entre 150 e 600 mg/L. A maioria das proteínas têm pesos moleculares entre 20 e 30 kDa, mas há proteínas que apresentam 62 kDa. O ponto isoeléctrico das proteínas encontra-se entre 2,5 e 6,5 (*28*). No entanto, a maioria das proteínas dos vinhos espumantes apresenta pontos isoeléctricos entre 3,5 e 4,4, próximos do pH do vinho e, por isso, em condições de solubilidade mínima (*72*). Para muitos autores este fenómeno é considerado um factor positivo pelo facto da espumabilidade e estabilidade da espuma de uma solução de proteínas aumentar para pHs próximos do ponto isoeléctrico.

Tanto o vinho base como o espumante apresentam como α-amino ácidos livres maioritários a prolina, a alanina, a arginina e o ácido glutámico, que ocorrem por ordem decrescente entre 400 e 35 mg/L (**Tabela 1**) (*56*). Dos amino ácidos livres, a glutamina e a fenilalanina são referidas como podendo estar relacionadas com o favorecimento da formação de espuma mas não com a sua estabilidade (*56*).

Tabela 1 – Concentração de α-amino ácidos livres, β-alanina e GABA (ácido γ-aminobutírico) no vinho base e no espumante (*56*).

Amino ácidos	Vinho base (mg/L)	Vinho espumante (mg/L)
Asp	15.00±6.45	12.6±12.8
Glu	34.4±19.6	19.5±15.8
Asn	15.7±10.0	25.2±20.8
Ser	8.45±6.52	7.74±10.0
Gln	4.56±3.95	0.66±0.60
His	13.8±6.27	12.0±11.6
Gly	9.72±4.11	11.6±7.17
Thr	5.21±3.94	8.68±8.42
Arg	38.2±41.4	49.3±72.4
β-Ala	3.30±2.58	3.76±4.30
α-Ala	47.3±58.5	52.8±80.6
GABA	40.0±44.7	45.0±64.0
Tyr	12.3±6.27	12.1±10.7
Met	3.44±1.45	1.41±1.76
Val	6.60±4.47	6.83±7.42
Trp	0.00±0.00	0.00±0.00
Phe	11.2±4.72	9.29±7.28
Ile	3.06±1.55	2.09±2.28
Leu	14.7±6.46	12.2±9.05
Orn	11.4±6.14	9.74±0.74
Lys	17.3±7.29	23.3±12.6
Pro	389±306	324±250

4.3. Polissacarídeos

Os polissacarídeos presentes nos vinhos podem ter origem em duas fontes: as uvas ou as leveduras. Os principais polissacarídeos presentes nos vinhos são as arabinogalactanas ligadas a proteínas (AGP), que possuem características neutras, fracamente acídicas ou mesmo acídicas, e as ramnogalacturonanas do tipo II (RG-II), que apresentam características acídicas. Quer as AGPs quer as RG-II são polissacarídeos provenientes das uvas. As manoproteínas são polissacarídeos neutros provenientes das paredes celulares das leveduras (*73*). Tanto nos vinhos base como nos vinhos espumantes, a concentração de polissacarídeos neutros é superior à concentração de polissacarídeos ácidos. Para os polissacarídeos neutros a gama situa-se entre 107,5 e 736,3 mg/L. No caso dos polissacarídeos ácidos estes tanto podem não ser detectados como atingir a concentração de 91,6 mg/L (*56*). Por vezes os vinhos provenientes de uvas infectadas por

Botrytis cinerea podem também apresentar glucanas (*28*), apesar das glucanas também terem origem na autólise das leveduras (*74*).

As arabinogalactanas (AGs) e as AGPs são compostas por uma cadeia principal de galactose em ligação β-(1→3), ramificada com cadeias laterais de galactose em ligação β-(1→6) substituídas por resíduos de α-arabinofuranose (**Fig. 10**). O ácido glucurónico também pode ser encontrado como terminal não redutor e em ligação (1→4). As AGPs têm uma estrutura similar às AGs, possuindo uma parte proteica (< 10%) ligada covalentemente à parte glicosídica. Existe uma grande heterogeneidade nas AGPs, tanto nas percentagens de proteína (tipicamente 1-4%) como nas de ácido urónico (3-20%) (*73, 75*).

→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*-(4←1)-α-L-Ara*f*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*-(4←1)-α-L-Ara*f*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*
6 ↑ 1
β-D-Gal*p*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*-(4←1)-α-L-Ara*f*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*-(4←1)-α-L-Ara*f*
6 ↑ 1
α-L-Ara*f*-(1→3)-β-D-Gal*p*
6 ↑ 1
α-L-Ara*f*

Figura 11 – Representação da estrutura de uma arabinogalactana do tipo II isolada do vinho (*76*).

As RG-II pertencem ao grupo definido como polissacarídeos pécticos, que são polissacarídeos constituídos por resíduos de ácido galacturónico em ligação α-(1→4). Nas moléculas de polissacarídeos pécticos é possível identificar zonas designadas por homogalacturonanas (compostas apenas por resíduos de ácido galacturónico), ramnogalacturonanas do tipo I (RG-I, em que os resíduos de ácido galacturónico estão intercalados com resíduos de ramnose em ligação α-(1→2) e em que parte deste resíduos de ramnose são pontos de ramificação de cadeias de resíduos de galactose e/ou arabinose) e RG-II, como esquematiza na **figura 11** (*77*).

Figura 11 – Representação esquemática da estrutura primária de um polissacarídeo péctico (*77*).

As RG-II são um polissacarídeo de baixo peso molecular (5-10 kDa), que ocorrem no vinho na forma de monómeros (mRG-II) ou na forma de dímeros (dRG-II) e estão ligados pelo diéster 1:2 borato-diol pela apiose. São compostos por uma cadeia principal similar às homogalacturonanas, mas com várias cadeias laterais (A-D) (**Fig. 12**). As RG-II são compostas por 12 resíduos de açúcares diferentes, incluindo alguns açúcares raros: 2-*O*-metil-D-xilose, D-apiose, ácido acérico, ácido 3-desoxi-D-*lixo*-2-heptulosárico (DHA), ácido 3-desoxi-D-*mano*-2-octulosónico (KDO) (*77, 78*). As RG-II são conservadas no vinho pelo facto destas ligações glicosídicas não serem hidrolisáveis pelas enzimas da uva e das leveduras.

E D C
β-L-Ara*f* D2
α-L-Rha*p* C2
α-L-Ara*f* E1
β-D-Dha*p* D1
α-Kdo*p* C1
[α-D-Gal*p*A(1→4)]-α-D-Gal*p*A(1→4)-α-D-Gal*p*A(1→4)-[α-D-Gal*p*A(1→4)]-α-D-Gal*p*A(1→4)-α-D-Gal*p*A(1→4)-{-α-D-Gal*p*A(1→4)-}$_3$-α-D-Gal*p*A
β-D-Api*f* A1
β-D-Api*f* B1
α-D-Gal*p*A(1→2)-β-L-Rha*p*-(3←1)-β-D-Gal*p*A
A2' A2 A2''
β-L-Rha*p* B2
2-*O*-Me-α-D-Xyl*p*(1→3)-α-L-Fuc*p* A3
A3'
α-L-Ace*f*A B3
2-*O*-Me-α-L-Fuc*p*(1→2)-β-D-Gal*p* B4
B4'
β-D-Glc*p*A A4
α-L-Rha*p*(1→3)-α-L-Ara*p* B5
B5' 50%
α-D-Gal*p* A5
A
α-L-Rha*p* B6
β-L-Ara*f* B7 50%
B

Figura 12 – Representação esquemática da estrutura primária de uma mRG-II, indicando as possíveis cadeias de ramificação, de A a E (*77*).

As manoproteínas são polímeros produzidos pelas leveduras durante a fermentação alcoólica, sendo constituídas por resíduos de manose ligados a uma parte proteica que pode variar entre 2-36% (*73, 79-81*). A componente polissacarídica tem uma estrutura bastante ramificada com uma cadeia principal de resíduos de manose em ligação α-(1→6) ramificada na posição 2 e 3 (*73, 79*).

Os polissacarídeos contribuem para as propriedades organolépticas e para a formação da espuma (*82*). Os polissacarídeos são também considerados “colóides protectores” dos vinhos, responsáveis por prevenir ou limitar fenómenos de agregação e floculação, ou seja, a turvação dos vinhos e/ou precipitação das proteínas (*83*). Pensa-se que esta acção protectora se deve em parte à presença de polissacarídeos pécticos e arabinogalactanas, que aumentam a intensidade da turbidez e favorecem a clarificação, enquanto que os polissacarídeos neutros não apresentam este efeito. Estas mesmas substâncias também parecem ter um efeito positivo nas qualidades organolépticas dos

vinhos, sendo demonstrado pelas diferenças observadas antes e depois de uma filtração fina (*84*).

Em geral, os polissacarídeos inibem o aumento do tamanho dos agregados de taninos (*83, 85*), nomeadamente, as manoproteínas e as AGPs ácidas. No entanto, as dRG-II promovem a agregação, possivelmente por co-agregação das dRG-II com as partículas de taninos (*83*).

4.4. Lípidos

Os vinhos espumantes sofrem grandes alterações no conteúdo lipídico devido à libertação de triacilgliceróis pelas leveduras durante a segunda fermentação. Estes são subsequentemente transformados em diacilgliceróis, e estes em ácidos gordos livres (*86*). Outra fase em que há libertação de lípidos é durante a autólise das leveduras. Nesta fase, os lípidos que são libertados são nomeadamente triacilgliceróis, 1,3-diacilgliceróis, 2-monoacilgliceróis, ácidos gordos livres, ésteres de esteróis e esteróis livres (*57*). A concentração destas diferentes classes de lípidos varia consideravelmente no autolisato, sendo os ésteres de esteróis e os triacilgliceróis as maiores fracções de lípidos presentes (1-5 mg/L, cada). Os valores para os ácidos gordos livres, esteróis livres, diacilgliceróis e monoacilgliceróis são bastante inferiores (entre 100-600 μg/L, cada) (*57*). Nos vinhos base, a concentração de cada um dos ácidos gordos livres presentes (C_6-C_{16}) pode variar entre 0,024 e 10,3 mg/L, sendo o ácido octanóico o maioritário, e o tetradecanóico o minoritário. Os ácidos gordos também se encontram esterificados com etanol, variando a sua concentração individual entre 0,021 e 2,0 mg/L (*87*). Estudos em vinhos *Champagne* mostraram que os ácidos gordos livres e respectivos ésteres etílicos existem em maior concentração na interface gás-líquido do que na fase líquida, sendo libertados nos aerossóis provenientes do colapso das bolhas, nomeadamente os ácidos gordos saturados (C10:0-C24:0) e insaturados (C14:1, C16:1, C18:1 e C18:2) (*70*).

4.5. Compostos fenólicos

Os tipos de compostos fenólicos presentes no vinho podem-se dividir em quatro grupos: ácidos fenólicos e seus derivados, flavonóides, antocianinas e taninos. Dentro do primeiro grupo encontram-se os ácidos benzóicos e cinâmicos, fenóis voláteis, álcoois fenólicos, cumarinas e estilbenos; dentro do quarto grupo, os taninos, subdividem-se em

taninos hidrolisáveis e taninos condensados *(84)*. A composição dos compostos fenólicos no vinho varia de acordo com a variedade das uvas usadas no processo de vinificação, com o próprio processo e com as reacções que ocorrem durante o envelhecimento do vinho *(84)*. No entanto, o efeito variedade sobrepõe-se ao efeito envelhecimento (*88*).

A composição em compostos fenólicos pode também ser alterada pelas condições fito-sanitárias das uvas. Por exemplo, a presença de *Botrytis cinerea* produz lacase, uma enzima responsável pela oxidação de compostos fenólicos a quinonas (*28*).

O conteúdo em cinamatos e estilbenos pode ser usado para caracterizar varietalmente os vinhos espumantes Cava. O conteúdo em hidroxicinamatos e a razão entre os ác. *trans*-coutárico / ác. *trans*-caftárico caracteriza varietalmente os vinhos Cava, das variedades Macabeo, Xarel. lo, Parellada e Chardonnay (*89*).

Os polifenóis, em especial os taninos, têm a capacidade de formar precipitados por interacção com as proteínas e, consequentemente, turvar os vinhos (**Fig. 13**) (*84*).

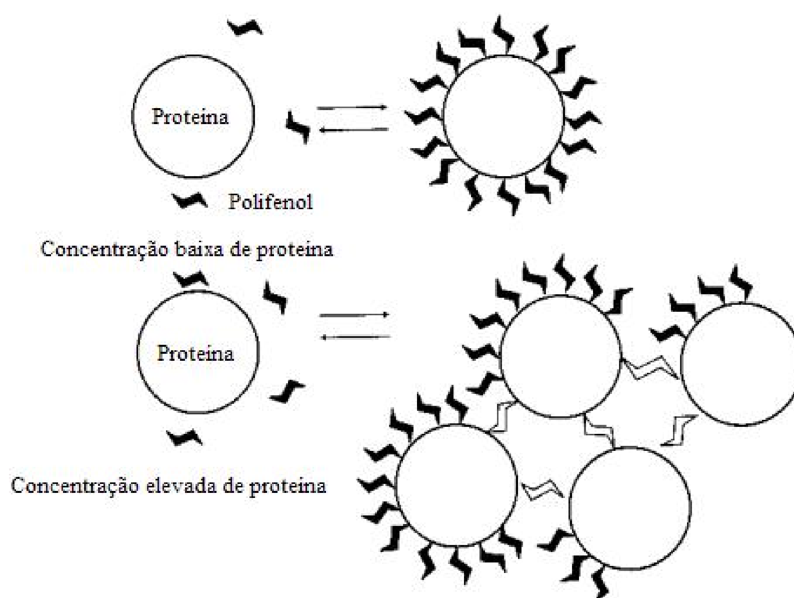

Figura 13 – Modelo da precipitação de proteínas por polifenóis (Haslam, 1981 *in* Ribéreau-Gayon *et al*., 2000) (*84*).

Numerosos factores como pH, tempo de reacção, temperatura, solventes e força iónica, têm influência na formação dos complexos tanino-proteína. As características das proteínas (peso molecular, composição em aminoácidos, estrutura, tamanho, carga, etc.) parecem ter o papel mais importante na formação de complexos insolúveis. Proteínas com elevado conteúdo em prolina têm uma afinidade muito grande para taninos condensados. A

importância da prolina é provavelmente devida à sua forma cíclica, que não pode estabelecer ligações por pontes de hidrogénio pela ligação peptídica, permanecendo a proteína aberta e acessível aos taninos. Por outro lado, as proteínas compactas têm pouca afinidade para os taninos. Devido a todas estas propriedades, proteínas como a gelatina, rica em prolina, são usadas como agentes de clarificação (*84*).

5. Características da espuma do vinho espumante

A espuma do vinho espumante é produzida na segunda fermentação, sendo resultado da libertação do CO_2 proveniente do metabolismo das leveduras. Apenas nos vinhos espumantes em que a pressão do CO_2 atinge 3,5 bar ou mais é que existe formação de espuma quando o vinho é vertido no copo.

As propriedades da espuma dependem principalmente da composição química do vinho, nomeadamente em partículas coloidais, que podem influenciar a estabilidade da espuma dos seguintes modos (*90*):

a) Podem diminuir a taxa de drenagem da espuma por razões hidrodinâmicas, sendo a viscosidade dinâmica de uma suspensão coloidal normalmente maior que a do líquido suspendido, dependendo da fracção de partículas sólidas. Desde que a taxa de drenagem seja sempre inversamente proporcional à viscosidade dinâmica efectiva, a estabilidade da espuma é aumentada pelo abrandamento da drenagem.

b) As partículas coloidais podem também aumentar a estabilidade dos filmes da espuma, impedindo que os filmes fiquem excessivamente finos, tanto por repulsão electrostática como por impedimento estérico.

c) As partículas com um tamanho adequado e com propriedades hidratantes podem também diminuir a estabilidade do filme por um fenómeno de ligação entre as faces de bolhas contíguas promovendo a coalescência.

d) Podem existir materiais que ao desorverem da superfície das partículas coloidais ficam dispersos pela superfície do filme causando o colapso do filme.

As características da espuma estão condicionadas pelas propriedades da bolha, sendo estas as responsáveis pela aparência do anel de espuma na superfície do líquido.

Usualmente o consumidor associa as bolhas pequenas à qualidade do espumante, sendo esta uma observação empírica, pois as bolhas pequenas tornam-se mais estáveis que as maiores, promovendo maior doseamento à superfície da libertação dos compostos voláteis do vinho espumante (*69*).

As propriedades da espuma (quantidade e estabilidade) têm sido referidas como estando relacionadas com a composição química dos espumantes, nomeadamente, proteínas solúveis (*41, 53-55*) polissacarídeos (*41, 54, 56*), compostos fenólicos (*41, 54*), ferro (*91*) e ácidos gordos (*57*). As proteínas foram as primeiras moléculas a ser descritas como responsáveis pela formação da espuma dos vinhos (*55, 92*). Devido à sua influência na viscosidade dos vinhos, as glicoproteínas e os polissacarídeos também parecem ter um papel importante nas propriedades da espuma (*56, 72*). A quantidade de espuma formada nos espumantes é, em alguns casos, inversamente proporcional à sua estabilidade, tendo-se verificado que a estabilidade da espuma aumenta com a clarificação dos vinhos (*93*).

5.1. Bolha

O ciclo de vida de uma bolha é composto por três fases principais: o nascimento, a ascensão e o colapso. Quando o recipiente é aberto, a pressão do dióxido de carbono no líquido diminui abruptamente, quebrando o equilíbrio termodinâmico devido à supersaturação em moléculas de CO_2. Para voltar a existir estabilidade termodinâmica, à pressão atmosférica, as moléculas de CO_2 terão de abandonar o líquido supersaturado (*94*). Este processo foi estudado por Liger-Belair *et al.* (*69, 94*), do qual se faz aqui um breve resumo:

Nascimento

A formação das bolhas é limitada por uma barreira energética, pois as bolhas têm de passar pelas moléculas do líquido que estão agregadas por ligações de Van der Waals. Para isso, é necessário uma razão de supersaturação mais elevada do que a que ocorre em bebidas carbonatadas. Em líquidos pouco supersaturados, em que se incluem o vinho espumante, a cerveja e os refrigerantes carbonatados, a formação de bolhas requer a existência de cavidades de gás com um raio de curvatura suficientemente largo para superar a barreira energética da nucleação e permitir que estas cresçam livremente. A curvatura da interface da bolha leva a um excesso de pressão dentro da bolsa de gás que é

inversamente proporcional ao raio (lei de Laplace). Abaixo do raio crítico, a pressão é excessiva dentro da bolsa de gás impedindo o dióxido de carbono dissolvido de se difundir. Num vinho espumante acabado de abrir o raio crítico é cerca de 0,2 μm.

O ciclo de produção de uma bolha no seu local de nucleação é conhecido pela frequência de borbulhamento, que é o número de bolhas produzidas por segundo. Como a cinética do crescimento da bolha depende da quantidade de dióxido de carbono dissolvido, a frequência da formação de bolhas varia de bebida para bebida. Por exemplo, no vinho espumante, em que a quantidade de gás é aproximadamente três vezes maior que na cerveja, os locais de nucleação activos emitem cerca de 30 bolhas por segundo enquanto na cerveja a emissão é de 10 bolhas por segundo.

Ascensão

Depois da bolha ser libertada do local de nucleação, vai crescendo à medida que vai a caminho da superfície, designando-se esta fase como fase de ascensão. A bolha vai aumentando de tamanho durante a sua ascensão, provocado pela contínua difusão de dióxido de carbono dissolvido na interface gás/líquido das bolhas. A flutuabilidade aumenta com a expansão das bolhas, havendo uma aceleração contínua que provoca uma separação entre as bolhas à medida que vão subindo.

Tanto a cerveja como os vinhos espumantes não são verdadeiras soluções, dado que para além do álcool e do dióxido de carbono dissolvido, contêm muitos outros compostos orgânicos com possível actividade surfactante. Estes surfactantes são principalmente proteínas e glicoproteínas, contendo uma parte solúvel em água e outra insolúvel. Os surfactantes não ficam dissolvidos no meio líquido, ligando-se à superfície das bolhas, com o seu terminal hidrofóbico dentro do gás e o terminal hidrofílico no líquido. A camada de surfactante à volta da bolha é crucial para o seu comportamento uma vez que esta camada endurece a bolha formando uma espécie de protecção à sua superfície. De acordo com a teoria dinâmica dos fluidos, uma esfera rígida ascendendo através de um fluido avança com mais resistência do que uma esfera mais flexível com a superfície livre de surfactantes. A velocidade da bolha é mínima quando a interface gás/líquido está totalmente coberta de surfactantes.

Os coeficientes de arraste das bolhas do espumante e da cerveja durante a ascensão à superfície, quando comparados com os coeficientes teóricos da dinâmica das bolhas,

permite verificar que as bolhas de cerveja agem como esferas rígidas, enquanto as bolhas de espumante e refrigerantes apresentam mais flexibilidade na interface durante a ascensão. Este resultado é de esperar porque a cerveja contém muito mais quantidade de macromoléculas surfactantes (na ordem das centenas de miligramas por litro) do que o vinho espumante (apenas algumas mg/L).

Colapso

Alguns segundos depois do nascimento e da libertação, a bolha percorre alguns centímetros até à superfície da bebida, podendo atingir cerca de um milímetro de diâmetro. A bolha de gás na superfície da bebida emerge apenas ligeiramente acima do líquido, mantendo a maior parte do seu volume abaixo da superfície do líquido, como um *iceberg*. Na parte emergida, a bolha é constituída por uma capa, que é um filme líquido hemisférico, que se vai tornando cada vez mais fino devido à drenagem lateral. Quando a capa da bolha atinge uma espessura crítica, esta torna-se sensível às vibrações e a gradientes térmicos, até que finalmente rompe. Com o rompimento da capa da bolha resulta um processo hidrodinâmico complexo, causando o colapso da parte submergida da bolha e, por instantes, permanece uma cavidade aberta à superfície do líquido. As partes lateralmente opostas da cavidade encontram-se e ejectam um jacto de líquido acima da superfície. Devido à elevada velocidade deste jacto (vários m/s), este torna-se instável, fragmentando em gotas (jacto de gotas). O efeito combinado entre a inércia e a tensão superficial faz com que o jacto de gotas se separe, obtendo-se diferentes formas, que evoluem para a forma quase esférica (**Fig. 14**).

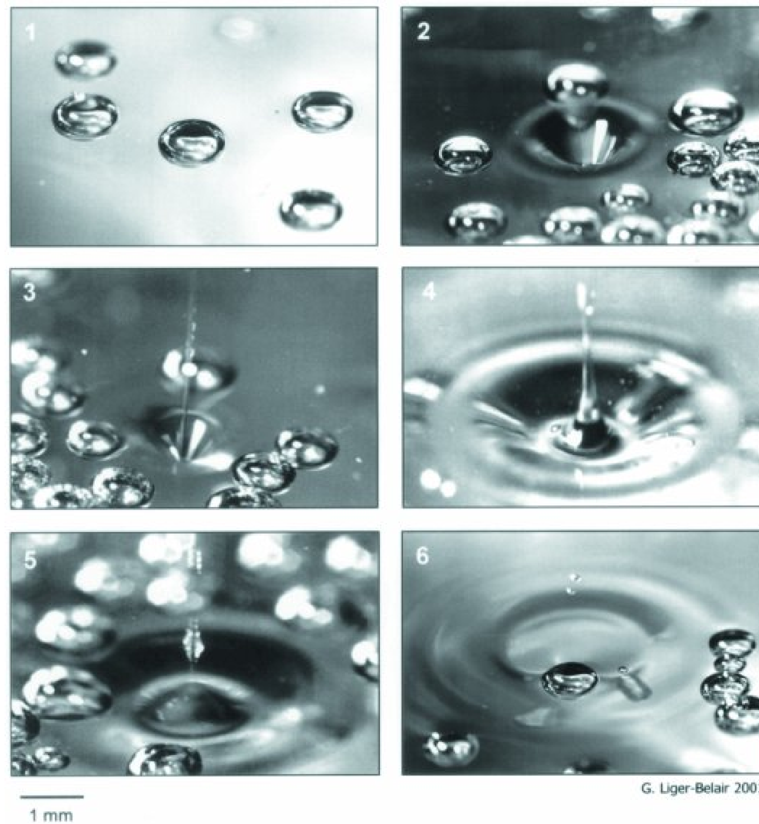

Figura 14 – Reconstituição da sequência do colapso de uma única bolha à superfície do vinho espumante, ilustrada em seis passos. O tempo de intervalo entre cada imagem é de cerca de 1 ms (*69*).

5.2. Factores que influenciam as características da espuma do vinho espumante

Tendo em conta que a composição química do vinho é decisiva para as características da espuma, encontram-se na bibliografia várias referências a factores como a variedade, o ano de colheita e o processo de vinificação, factores estes que serão discutidos seguidamente de forma mais detalhada.

Influência da casta, ano de vindima e parâmetros de vinificação

Das variedades brancas mais usadas para a produção de vinhos Cava (Macabeo, Xarel.lo, Parellada e Chardonnay), são as castas Xarel.lo (*95*) e Chardonnay (*96*) as que produzem vinhos espumantes com melhores atributos de espuma. Quando os parâmetros casta e ano da colheita são considerados, há diferenças significativas entre os anos na estabilidade da espuma (TS) (*95*). A mistura destas castas promove um aumento da altura máxima da espuma (HM), comparativamente a cada um dos vinhos individualmente. O incremento na HM foi igualmente observado mesmo nas misturas onde as HM dos vinhos

espumantes monovarietais apresentavam valores semelhantes entre si, permitindo inferir o envolvimento de um efeito sinergético (*96*).

Adicionalmente à variedade e ano de colheita, os parâmetros de vinificação (filtração e clarificação) condicionam a HM e o TS (*95*). O parâmetro de vinificação que mais condiciona a formação da espuma é a filtração, tal como mostra a **figura 15**. A filtração do vinho exerce uma grande influência na HM e no TS, verificando-se que quanto menor é a porosidade do filtro maior é a sua influência (*97*).

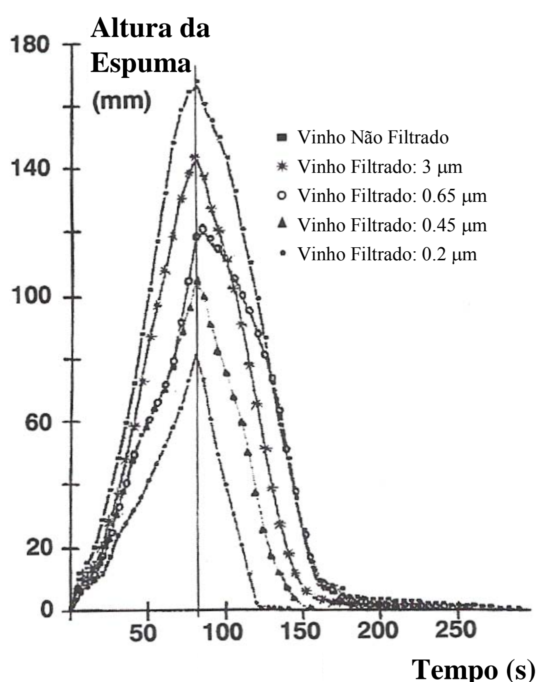

Figura 15 – Curvas típicas da evolução da espuma em função do poro de filtração (*97*).

Em relação ao agente de clarificação, verifica-se uma relação inversa entre a quantidade do agente de clarificação e os parâmetros HM, TS e espumabilidade na estabilidade (HS), talvez porque uma quantidade elevada pode adsorver compostos como proteínas e polissacarídeos, que podem estar afectar a qualidade da espuma (*95*).

Girbau-Solà *et al. (98, 99)* avaliaram a aptidão das castas tintas Trepat e Monastrell na elaboração de espumante, sendo a casta Trepat a que obteve valores mais elevados de HM. Os valores de HM obtidos para os vinhos base da casta Trepat são significativamente superiores aos da Monastrell, assim como são superiores aos valores obtidos para misturas efectuadas com a casta Monastrell e as castas brancas Macabeo, Xarel.lo e Parellada (*99*).

Quando se comparam as características da espuma do vinho base com as do espumante final (2ª fermentação em garrafa e 9 meses de contacto com as leveduras) são observadas variações muito grandes. Em todas as castas e suas misturas há uma redução dos valores de HM em mais de 50% do vinho base para o vinho espumante. No entanto, o TS tem um aumento significativo para a variedade Trepat e suas misturas, entre 1,2 e 14 vezes (*98*).

Para a mesma casta e ano de colheita, como também para o mesmo processo enológico, a variável estado sanitário das uvas é muito importante (*97*). Para diferentes graus de infecção da *B. cinerea* (0, 20 e 40%) há uma diminuição drástica na estabilidade da espuma, dependente do grau de infecção e da casta, sendo a Pinot Noir a mais afectada. Quanto à HM, a sua diminuição drástica ocorre também logo para 20% de infecção, e é semelhante para todas as castas, não se alterando para 40% de infecção (*97*).

Influência do envelhecimento

O envelhecimento na garrafa em contacto com as leveduras tem uma influência positiva no TS, mas exerce uma influência negativa na HM (*96*). Para a maioria das variedades usadas para elaborar os vinhos Cava *(96)*, o aumento do TS com o envelhecimento acontece até aos 15-18 meses de envelhecimento. A excepção é a casta Chardonnay, em que o incremento no TS apenas se verifica para tempos de envelhecimento superiores. Este facto pode estar relacionado com os valores mais elevados de polissacarídeos totais e neutros, proteínas, compostos fenólicos, valores de absorvância a 280, 365 e 420 nm, acidez titulável, teor alcoólico, condutividade e ácido málico que este vinho apresenta, sendo os valores da tensão superficial mais baixos (*96*).

A concentração de glucose e frutose aumenta consideravelmente depois da autólise das leveduras. A glucose poderá ter origem na actividade hidrolítica das enzimas nos polissacarídeos das leveduras. A diminuição da HM observada a partir dos 18 meses poderá estar relacionada com uma diminuição da quantidade de polissacarídeos devido à hidrólise dos polissacarídeos pécticos (*96*).

5.3. Influência de diferentes constituintes do vinho nas características da espuma

Composição química do vinho em geral

Os parâmetros, teor alcoólico, acidez titulável, ácido málico, frutose, proteínas e glutamina apresentam uma correlação positiva com os valores de HM. Por outro lado,

vinhos com um elevado conteúdo em glucose ou ácido láctico formam menos quantidade de espuma (*95*). Para o TS, há vários parâmetros que estão negativamente correlacionados, nomeadamente, pH, absorvância a 520 nm (antocianinas), ácido cítrico, ácido galacturónico, acetaldeído, acetato de etilo, diacetal e os álcoois isoamílicos. Os vinhos que contêm menor quantidade de ácido galacturónico são aqueles que têm melhor TS. O aumento da quantidade de ácido galacturónico nos vinhos, proveniente de um aumento da hidrólise de polissacarídeos pécticos, leva a uma diminuição da HM e TS (*95*). Das castas que foram estudadas por Andrés-Lacueva *et al.* (*95*), a que apresentou vinhos com teores mais elevados em proteínas, ácido málico, prolina e álcool foi a casta Xarel.lo, mostrando ser a casta com melhores propriedades de espuma (*95*).

Polissacarídeos

Os polissacarídeos do vinho de massas moleculares entre 62 e 48, 13 e 11, e entre 3 e 2 kDa estão relacionados positivamente com a espumabilidade, apesar dos polissacarídeos de massas moleculares entre 3-2 kDa também estarem relacionados com o TS (*82*). Estes polissacarídeos estão envolvidos como agentes produtores do filme da bolha, contribuindo para o aumento da viscosidade por dissolução nas paredes das bolhas. Há autores que defendem que os polissacarídeos facilitam a formação de espuma por conterem uma fracção rica em proteína (proteoglicanas) (*82*). No entanto, os polissacarídeos podem também interagir com superfícies activas, sendo adsorvidos na interface gás/líquido tal como as proteínas. Esta pode ser a razão pela qual os polissacarídeos estabilizam o filme, e o aumento da sua concentração no filme produz um efeito de gel que leva à redução da ruptura da estrutura da espuma (*82*).

A HM e HS estão correlacionadas com os polissacarídeos neutros e o conteúdo em polissacarídeos totais, com coeficientes de correlação de 0,82 e 0,80 para a HM e 0,71 e 0,68 para a HS, respectivamente. No entanto, os polissacarídeos ácidos não estão correlacionados nem com a HM nem com a HS (*56*). A HM de um vinho pode ser explicado em 76% pelo conteúdo em polissacarídeos neutros e proteínas e o HS pode ser explicado em 70% pelo conteúdo em polissacarídeos neutros juntamente com a fenilalanina (*56*).

Dentro dos polissacarídeos neutros, o conteúdo em xilose é o que parece estar mais correlacionado com o TS. A correlação da primeira variável canónica com o conteúdo em xilose dos polissacarídeos é 0,810 e com a estabilidade da espuma é de 0,866 (*100*).

Também para o caso da cerveja, alguns açúcares parecem estar envolvidos na espuma, nomeadamente, xilose, glucose e arabinose (*101*).

Compostos azotados

Vários autores encontram correlações entre a concentração de proteínas e a HM (*55, 91, 92, 97*). No entanto, a participação das proteínas no TS é um assunto controverso, dado que há autores que não encontraram nenhuma relação (*92*), outros encontram uma correlação positiva (*55, 100*) e outros uma correlação negativa (*95*). Esta diversidade de resultados leva a concluir que o comportamento das proteínas na espuma possa estar dependente de vários factores tais como hidrofobicidade, solubilidade (depende do ponto isoeléctrico e do pH do vinho) e peso molecular.

Os agentes surfactantes, como as proteínas, podem estabilizar a espuma por se ligarem à periferia das bolhas: o seu lado hidrofóbico coloca-se virado para o gás, e o seu lado hidrofílico coloca-se virado para a fase aquosa; estando de acordo com o modelo da dupla camada de Gibbs (**Fig. 16**) (*91*).

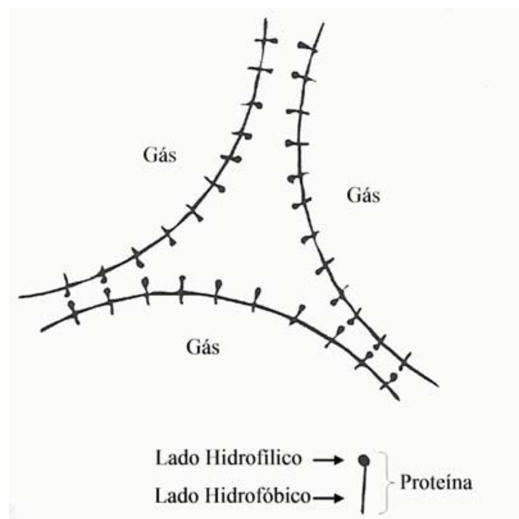

Figura 16 – Modelo da dupla camada de Gibbs (*91*).

Malvy *et al. (55)* descrevem uma correlação positiva entre a concentração de proteínas presentes no vinho e o TS. O vinho que foi ultrafiltrado (poro de 10 kDa) apresenta uma altura de espuma muito baixa (10 mm) (**Fig. 17**) enquanto que o vinho base apresenta valores de HM de 120 mm e o vinho base diluído com o ultrafiltrado apresenta valores decrescentes com a diluição. Os vinhos obtidos por diluição contêm entre 20 a 70%

de vinho base. Estes vinhos, durante os primeiros segundos depois da injecção de gás, mostram que o filme que separa as bolhas é estabilizado por macromoléculas tensioactivas. A amostra que não contém macromoléculas de massa molecular superior a 10 kDa mostra um perfil diferente, mostrando que é necessária uma quantidade mínima de macromoléculas para a estabilização das interfaces (**Fig. 17**). As bolhas que são formadas nos primeiros 40 segundos são pouco estáveis. Quando os fenómenos de difusão e convecção permitem a migração de proteínas para a interface líquido/gás, verifica-se uma forte taxa de coalescência que afecta a espuma quantitativamente (*55*).

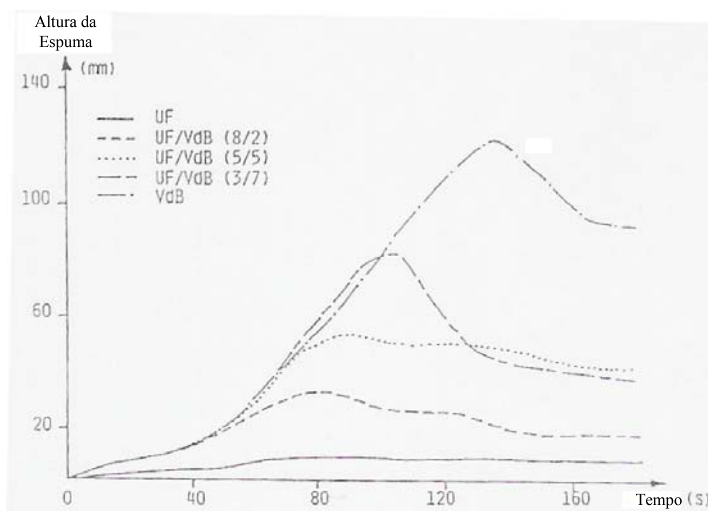

Figura 17 – Evolução da altura da espuma (HM) em relação a diferentes misturas de vinho base (VdB) e ultrafiltrado (UF) (*55*).

Os parâmetros HS e TS estão correlacionados com a concentração de proteínas, r = 0,89 e r = 0,95, respectivamente. Uma pequena diminuição na concentração de proteína faz diminuir algumas características importantes da espuma. Por exemplo, a diminuição em 1 mg/L resulta na diminuição da HM para metade, e a diminuição em 2 mg/L de proteínas resulta numa diminuição entre 20-50% do TS (*55*). Esta boa correlação que existe entre a concentração de proteínas e a estabilidade da espuma, quer na fase estacionária (HS) quer na ausência de efervescência (TS), também leva a concluir que os filmes líquidos que separam as bolhas não estão saturados em tensioactivos e as proteínas estão longe de estar em excesso, como é o caso da cerveja (*55*). Visto que a técnica usada neste estudo ter sido a ultrafiltração, é provável que outras moléculas tensioactivas, como os polissacarídeos, estejam implicadas nos fenómenos estudados. Contudo, fica demonstrado que a quantidade de macromoléculas de um vinho é um factor limitante e muito importante para o

comportamento da espuma (*55*).

Estes trabalhos mostram que a formação de espuma é muito complexa e depende do equilíbrio entre muitos compostos e não de um só isoladamente. As correlações entre as propriedades da espuma e os compostos podem ser válida apenas num intervalo de concentrações muito limitado.

Lípidos

Foi observada uma correlação positiva entre a constituição em ácidos gordos do vinho espumante e as características da espuma, nomeadamente com o conteúdo total em ácido palmítico (0,819) e uma óptima correlação com a altura da espuma (1,000), ou seja, o conteúdo total em ácido palmítico está fortemente correlacionado com a altura da espuma (*100*). No entanto, a espumabilidade está negativamente correlacionada com o teor de ácidos gordos livres de cadeia intermédia, nomeadamente, C_8, C_{10} e C_{12}. Em contraste, existe uma correlação positiva da espumabilidade com estes ácidos gordos quando esterificados como ésteres etílicos. A influência destes ácidos gordos com a espumabilidade parece estar relacionada com a forma química em que se encontram (livre ou esterificada) (*87*). É possível que a influência dos ácidos gordos na espuma dependa de diferentes interacções estabelecidas pelo grupo carboxílico do ácido gordo livre, ou do grupo carboxílico do éster etílico com outros compostos do vinho. O valor da espumabilidade aumenta quando a proporção do ácido gordo esterificado é maior. Todos os procedimentos de vinificação de um vinho que incrementem a síntese de ésteres etílicos a partir dos ácidos gordos livres são benéficos para o vinho em termos de espumabilidade (*87*).

Mobilidade electroforética das partículas endógenas do vinho

Um agente estabilizante eficiente deve ter uma cinética de adsorção rápida para rapidamente restaurar a concentração de equilíbrio da superfície e estabilizar os filmes da espuma pelo efeito de Gibbs. Os peptídeos que são pequenos, são certamente muito mais eficientes como agentes estabilizantes que as moléculas maiores, como proteínas ou glicoproteínas (*102*).

Partindo da premissa de que as moléculas que interagem com a espuma têm de ter mobilidade electroforética, dentro dos constituintes do vinho base, os ácidos tartárico e láctico, e os lípidos não podem ser candidatos a estabilizantes da espuma, pelo que os seus

grupos carboxílicos são fracamente ionizados a pH 3 (pH do vinho), logo apresentarão fraca mobilidade electroforética no vinho. As proteínas e os polissacarídeos até poderiam ser bons candidatos, mas é muito improvável que sejam porque o seu peso molecular está muito longe do peso molecular das substâncias que apresentam mobilidade electroforética no vinho filtrado, que apresentam um peso molecular entre 200-300 Da. Os aminoácidos têm carga a pH ácido, mas o seu tamanho típico é 120 Da; os oligossacarídeos na sua maioria são neutros a pH 3, pelo que nenhum destes dois constituintes poderá ser um bom candidato. Contrariamente, os peptídeos preenchem os dois requisitos, tamanho e carga. Estes encontram-se nos vinhos na ordem das dezenas de mg/L, o seu tamanho tem uma gama entre 250 Da e 10 kDa. Os peptídeos podem ter carga neutra, positiva ou negativa de acordo com o pK_a dos seus aminoácidos, os seus grupos carboxílicos e amina estão em equilíbrio com os seus sais ($R\text{-}COO^-$ e $R'\text{-}NH_3^+$). A pH 3 as cargas positivas são predominantes, os peptídeos ficam totalmente carregados positivamente, podendo ser adsorvidos nos locais carregados negativamente por interacções electrostáticas criando uma barreira para as cargas (*102*).

6. Metodologias usadas neste trabalho

6.1 Análise de compostos voláteis

Os procedimentos de extracção dos compostos voláteis de uvas e vinhos são baseados nas propriedades físico-químicas dos compostos, tais como volatilidade e solubilidade numa fase orgânica imiscível com a matriz aquosa, e na capacidade de serem sorvidos selectivamente por determinados materiais. Usualmente, os compostos voláteis estão presentes em pequenas quantidades na amostra e necessitam de serem extraídos e concentrados antes da fase de análise instrumental. Para a análise de compostos voláteis são necessários dois passos: extracção, onde os compostos são removidos da amostra, e separação e identificação, por métodos cromatográficos.

6.1.1 Extracção

Os métodos de extracção tradicionais, normalmente, usam solventes orgânicos, os mais usados são a extracção líquido – líquido (*3, 27*) e a destilação-extracção simultânea (SDE) (*62*). Estes métodos requerem o uso de solventes, pré-concentração e intenso

trabalho laboratorial, o que os torna morosos. Nas últimas décadas, o desenvolvimento de métodos de extracção nos quais o consumo de solvente orgânico é mínimo ou nenhum, tem-se revelado uma tendência. Estes métodos alternativos incluem a microextracção em fase sólida (SPME) e a extracção sorptiva em barra de agitação (SBSE). Estes métodos apresentam vantagens comparativamente aos métodos tradicionais, nomeadamente, 1) eliminam o uso de solventes orgânicos, 2) permitem a quantificação com baixos limites de detecção uma diversidade de compostos voláteis, 3) têm reduzida manipulação/preparação da amostra e 4) são técnicas relativamente simples e rápidas.

Microextracção em fase sólida

A microextracção em fase sólida (SPME) é uma técnica desenvolvida no início da década de 90 por Janusz Pawliszyn e colaboradores (*103*). O SPME é uma técnica de preparação da amostra baseada na sorção (absorção e/ou adsorção), dependendo do tipo de revestimento da fibra. Esta técnica é usada para a extracção e concentração dos analitos, tanto por imersão numa fase líquida como por exposição a uma fase gasosa. Depois da fibra exposta em contacto com a amostra, os analitos nela sorvidos podem ser termicamente desorvidos no injector de um cromatógrafo de gás (**Fig. 19**).

O princípio da SPME para matrizes líquidas pode ser descrito por um processo de partição dos compostos voláteis entre duas ou três fases. Um sistema de duas fases é considerado quando uma amostra líquida ocupa o volume total do frasco. As duas fases envolvidas neste caso são a matriz líquida e a fibra. Quando a matriz líquida não ocupa a totalidade do frasco, o sistema é composto por três fases: a matriz líquida, o gás que compõe o espaço de cabeça da amostra e a fibra (*103*). O mesmo processo de partição pode ser descrito para uma amostra sólida ou gasosa. No caso de matrizes sólidas estamos perante um sistema de três fases e no caso de uma amostra gasosa estamos perante um sistema de duas fases.

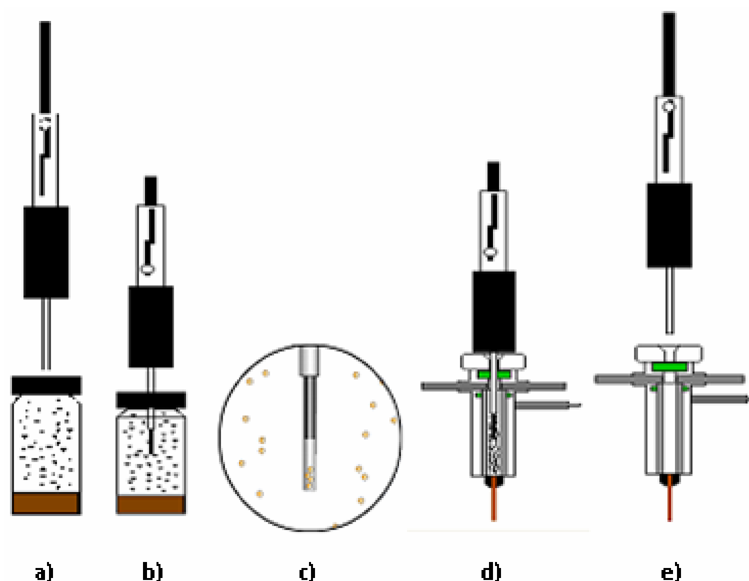

Figura 19 – Sequência experimental do procedimento de SPME: a) Amostra; b) extracção em modo de espaço de cabeça; c) pormenor da sorção dos analitos pela fase estacionária; d) desorção dos analitos no injector do cromatógrafo de gás; e) remoção da fase estacionária do injector (adaptado da Supelco).

Há dois tipos de revestimentos de fibras, os absorventes e os adsorventes. Além destes, são também usados revestimentos com estes dois tipos (revestimentos mistos). Os revestimentos do tipo adsorvente são formados por sólidos porosos ou com elevada área superficial, onde a extracção é efectuada através da sorção dos analitos nos poros internos (microporos, 2-20 Å e mesoporos 20-500 Å), ou externos (macroporos, > 500 Å), que se encontram principalmente na superfície do material. A vantagem dos revestimentos adsorventes está relacionada com a maior selectividade, maior limite de detecção e maior capacidade de retenção de compostos polares. Os revestimentos absorventes são formados por polímeros líquidos. Este tipo apresenta uma vantagem importante relativamente aos adsorventes, pois apresenta uma resposta linear para uma gama de concentrações superior (*104*). As fibras disponíveis apresentam diferentes combinações de revestimento, compostas apenas por um polímero, por misturas de polímeros ou por copolímeros. Actualmente, as fibras comercialmente disponíveis compostas por um só polímero são constituídas por polidimetilsiloxano (PDMS) ou por poliacrilato (PA). Existe uma variedade de fibras compostas por misturas de polímeros: carboxen-polidimetilsiloxano (CAR/PDMS), carbowax-divinilbezeno (CW/DVB), divinilbenzeno-carboxen-polidimetilsiloxano (DVB/CAR/PDMS), polidimetilsiloxano-divilbenzeno (PDMS/DVB).

Os polímeros podem ser agrupados por polaridade: o único revestimento não polar é o PDMS, os polímeros polares são o PA e CW/DVB, os restantes revestimentos são bipolares, ou seja compostos por uma parte polar e outra não polar (PDMS/DVB, CAR/PDMS e DVB/CAR/PDMS) (*105*). A fibra CW/DVB está indicada para a análise de álcoois e compostos polares, com massas moleculares compreendidas entre 40 e 275 (*105*). Desta forma, é uma fibra adequada para a análise de amostras complexas, com compostos voláteis de uma vasta gama de massas moleculares. Sendo uma fibra adsorvente, é também adequada para extracção de analitos em quantidades vestigiais. Por estas razões, esta fibra pode ser utilizada para a análise de uvas e vinhos.

Na análise por SPME em modo de espaço de cabeça (HS – SPME), a fibra é colocada no espaço de cabeça até que o equilíbrio seja estabelecido, apesar de se poder também trabalhar em situações de não equilíbrio. Há dois tipos de equilíbrio que são estabelecidos: $K_{\text{amostra-gás}}$ e $K_{\text{gás-fibra}}$. A quantidade de analito sorvido na fibra pode ser determinada a partir da seguinte equação matemática:

$$n = \frac{C_0 \times V_1 \times V_2 \times K_{gás-fibra} \times K_{amostra-gás}}{K_{gás-fibra} \times K_{amostra-gás} \times V_1 + K_{amostra-gás} \times V_3 + V_2} \quad (1)$$

em que n é a massa do analito absorvido pela fibra, C_0 é a concentração inicial do analito na amostra, V_1, V_2 e V_3 representam o volume da fibra, volume de amostra e volume de espaço de cabeça, respectivamente, $K_{\text{gás-fibra}}$ é o coeficiente de partição dos analitos entre a fibra de SPME e o espaço de cabeça; $K_{\text{amostra-gás}}$ é o coeficiente partição entre o espaço de cabeça e a amostra.

Extracção sorptiva em barra de agitação

A extracção sorptiva em barra de agitação (SBSE) foi introduzida nos finais da década de 90 por Pat Sandra e colaboradores (*106*). A barra de agitação é constituída por três partes essenciais, nomeadamente, um magnete envolvido por um tubo de vidro, o qual é revestido por um filme de polidimetilsiloxano (PDMS), podendo este variar entre 24 μL a 126 μL (**Fig. 20**).

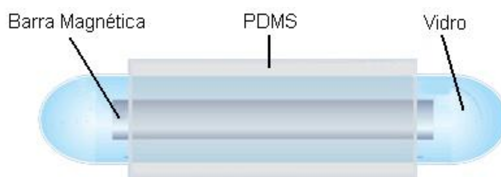

Figura 20 – Esquema de uma barra de agitação usada para a extracção sorptiva (adaptado de Gerstel GmbH & Co. KG).

Na prática, os analitos são extraídos por agitação magnética ao introduzir a barra na amostra, geralmente aquosa, sendo esta posteriormente removida para se realizar a desorção. Este passo pode utilizar uma desorção térmica, através de uma unidade de desorção térmica (TDS) integrante do injector do cromatógrafo. Em alternativa, os analitos podem ser recuperados por desorção líquida (LD), com recurso a pequenos volume de solvente orgânico adequado para a retroextracção. As vantagens de utilizar a desorção líquida são o custo da unidade de TDS e a aplicação a analitos termicamente instáveis.

A extracção sorptiva é, por natureza uma técnica de equilíbrio. Para uma solução aquosa, a extracção dos solutos é controlada pelo coeficiente de partição entre a fase orgânica e a fase aquosa ($K_{PDMS/W}$). Este coeficiente está correlacionado com o coeficiente de distribuição octanol – água ($K_{O/W}$). Além disso, é muito importante salientar que o equilíbrio de sorção é dependente da quantidade de PDMS. A equação matemática (2) ilustra esta correlação.

$$K_{O/W} \approx K_{PDMS/W} = \frac{C_{PDMS}}{C_w} = \frac{m_{PDMS}}{m_w}\frac{V_w}{V_{PDMS}} = \frac{m_{PDMS}}{V_{PDMS}}\beta \quad (2)$$

O coeficiente de distribuição entre o PDMS e a água ($K_{PDMS/W}$) é definido como a razão entre a concentração do soluto na fase PDMS (C_{PDMS}) e a água (C_W), em equilíbrio. Esta razão é igual ao produto da razão da massa do soluto na fase PDMS (m_{PDMS}) e da massa do soluto na fase aquosa (m_W), pela razão das fases envolvidas descrita por β (com $\beta = V_W/V_{PDMS}$).

6.1.2 Separação e detecção

Cromatografia de gás acoplada à espectrometria de massa (GC-MS)

Nesta técnica, a amostra é injectada num cromatógrafo de gás e arrastada pela fase móvel através de uma coluna capilar que contém a fase estacionária onde ocorre a separação dos constituintes da mistura. À medida que são eluídos da coluna, os constituintes da amostra entram na câmara de ionização. A fonte de iões (**Fig. 21**) tem como função a formação de iões sem discriminação de massa. Estes iões têm origem na fragmentação das moléculas quando incididas por um feixe de electrões. Para a análise de compostos voláteis usa-se normalmente um feixe de 70 eV.

O conjunto de iões formados previamente é desviado e acelerado, na direcção do analisador, por acção de um campo eléctrico de fraca intensidade onde os iões são separados de acordo com a razão massa/carga do ião (*m/z)*. Os analisadores geralmente utilizados são o *Ion trap* (armadilha de iões) e o quadrupolo (**Fig. 22**).

Os iões armazenados são ejectados para um detector amplificador electrónico (*electron multiplier*). As substâncias separadas passam por um detector que gera um sinal eléctrico proporcional à quantidade de material separado. O registo deste sinal em função do tempo é o cromatograma, em que as substâncias são representadas por picos com área proporcional à sua massa, que possibilita a análise quantitativa. Dos diversos detectores disponíveis comercialmente, o *electron multiplier* é o mais utilizado.

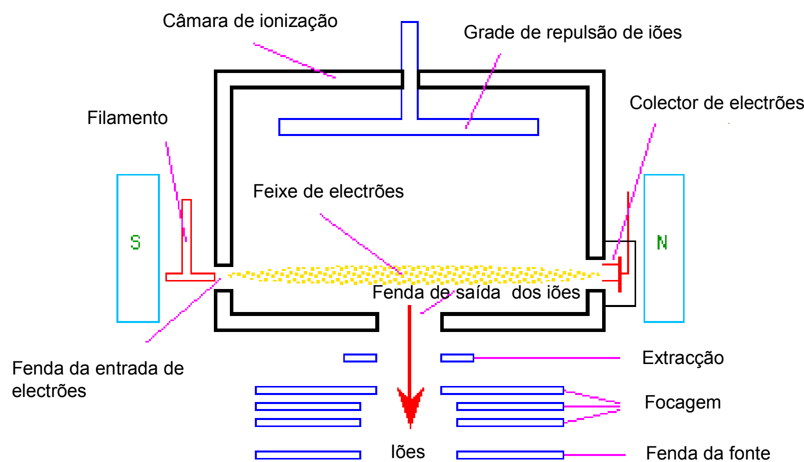

Figura 21 – Representação esquemática de uma fonte de iões (reproduzida de (*107*)).

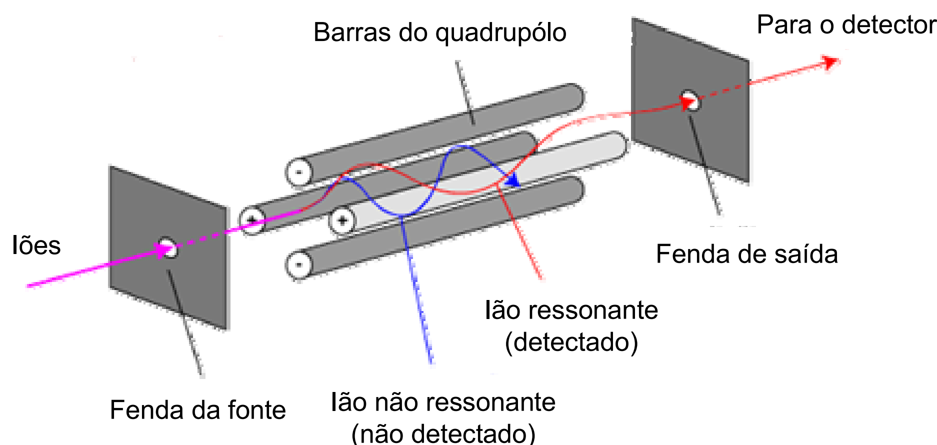

Figura 22 – Representação esquemática de um analisador quadrupolo (reproduzida de (*107*)).

A qualidade do espectrómetro de massa depende de um sistema de vácuo eficiente de modo a criar um ambiente de baixa pressão para evitar a neutralização dos iões que pode ocorrer por colisão. Este tipo de sistema requer condições de vácuo elevadas na câmara de ionização (10^{-5} a 10^{-7} Torr) (*108, 109*).

6.2 Análise das propriedades da espuma

Mosalux

A metodologia do Mosalux pode ser realizada em vinhos base ou espumantes, a partir da qual se pode estimar as propriedades da espuma, nomeadamente o volume de espuma formada e a sua estabilidade.

O aparelho de Mosalux é basicamente composto por quatro partes: uma garrafa de CO_2, um fluxímetro, uma proveta que contém na sua base um filtro de placa porosa e um sistema emissor e receptor de luz infravermelha (série de células fotoeléctricas). O CO_2 é injectado em 100 mL de vinho através de um filtro de placa porosa com uma porosidade compreendida entre 16 a 40 μm, constituindo o fundo da proveta de 4 cm de diâmetro e 57 cm de altura. O fluxo de CO_2 é de 7 L / hora a uma pressão de 1 bar (100 kPa). A altura da espuma é medida em contínuo pelo facto de existir um sistema de células fotoeléctricas que são iluminadas por uma fonte de luz infravermelha. O sinal de saída do receptor é inversamente proporcional à altura da espuma que interrompe o feixe de luz. O sinal de saída é adquirido de 5 em 5 segundos, permitindo depois traçar a curva representativa da amostra (*92*).

A partir do perfil geral da curva representativa das propriedades da espuma do vinho (**Fig. 23**), retiram-se três parâmetros principais:

i) HM, que corresponde à altura máxima da espuma observada nos primeiros minutos depois de iniciada a efervescência artificial de 100 kPa – 7 L/h. Esta altura é medida em mm e é designada por espumabilidade da amostra.

ii) HS, que corresponde à altura constante da espuma em regime estacionário de efervescência.

iii) TS, que representa o tempo necessário para o desaparecimento total da espuma depois de cessada a efervescência. Este tempo mede-se em s e é uma medida da estabilidade da espuma.

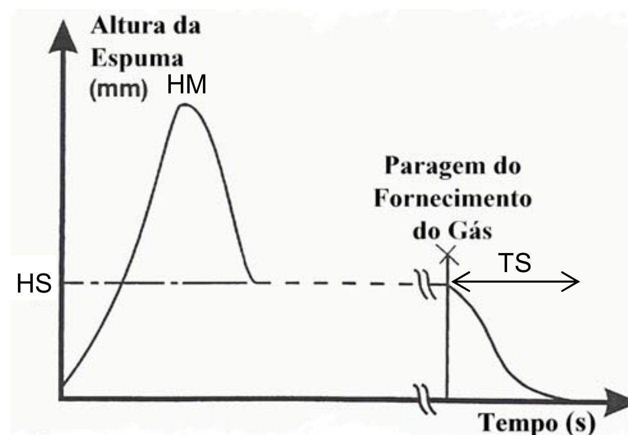

Figura 23 – Evolução da espuma durante o procedimento do Mosalux. A linha a tracejado corresponde à altura da espuma na estabilidade (*110*).

Existem diferenças entre a espuma formada nos primeiros minutos, dada pela HM, e a espuma formada ao fim de alguns minutos, dada pelo HS. A primeira é uma espuma mais seca, onde as bolhas coalescem mais rapidamente, porque nesta fase são as moléculas mais tensioactivas, as mais hidrofóbicas e as mais solúveis no vinho que migram para a espuma. A espuma formada posteriormente é mais húmida. Nesta fase, as bolhas são mais esféricas e finas porque as moléculas tensioactivas que migram para a espuma têm um carácter mais hidrofóbico. Depois de cessada a efervescência a coalescência das bolhas volta a formar-se espuma seca, resultante de um fenómeno de drenagem, contribuindo para o efeito de *Plateau* de fase líquida entre as bolhas (*92*).

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CAPÍTULO II – OBJECTIVOS

O objectivo desta dissertação é relacionar o aroma e a espuma dos vinhos espumantes com o potencial enológico das uvas e dos vinhos, como também relacionar a composição química do vinho espumante com as propriedades da espuma. O aroma do vinho espumante está intimamente relacionado com a sua composição volátil que tem origem nas uvas e no processo fermentativo. Dos compostos voláteis do vinho espumante são os varietais que têm uma maior relevância para o aroma pois são estes que fazem a diferenciação entre castas. Como os compostos varietais têm origem nas uvas, a composição volátil das uvas foi estudada para as duas castas principais da Bairrada, as uvas brancas Fernão-Pires (FP) e as uvas tintas Baga (BG). Se as uvas não forem colhidas na expressão máxima do seu aroma, poderá verificar-se uma perda significativa do seu potencial varietal volátil. Por isso, a composição volátil destas duas castas foi estudada semanalmente ao longo da maturação (do pintor à pós-maturidade) para ambas as castas. Foi optimizada a metodologia de análise dos compostos voláteis das uvas por microextracção em fase sólida em espaço de cabeça (HS-SPME) seguida de cromatografia de gás acoplada à espectrometria de massa com quadrupolo (GC–qMS). A composição volátil dos vinhos foi analisada por extracção sorptiva em barra de agitação seguida de desorção líquida combinada com injecção de grandes volumes e GC–qMS (SBSE–LD/LVI-GC–qMS). Esta metodologia foi optimizada previamente ao nível da extracção (SBSE), da desorção líquida (LD) e de instrumentação (LVI). Foram elaborados vinhos espumantes a partir das castas FP e BG produzidos com uvas em diferentes estados de maturação e para o mesmo estado de maturação provenientes de diferentes tipos de solo. A composição volátil dos vinhos espumantes produzidos foi quantificada por SBSE–LD/LVI-GC–qMS, para conhecer em que condições pedológicas e de maturação as castas maioritárias da Bairrada produzem uma melhoria em termos de compostos voláteis varietais. Para estes mesmos vinhos foi avaliada a sua aptidão para a formação de espuma, em termos de espumabilidade e estabilidade.

Para melhor entender os factores que influenciam a espuma de um vinho espumante é necessário ter noção dos principais constituintes do vinho espumante e de como estes se podem relacionar com as propriedades da espuma. Há diferentes factores que foram descritos que podem influenciar a composição do vinho e, consequentemente, as propriedades da espuma, como sejam o estado de maturação das uvas, a variedade da uva e o tipo de solo. Para conhecer os parâmetros de espuma dos vinhos da Bairrada, vinhos

espumantes das castas FP e BG, crescidas em diferentes solos e colhidas em diferentes estados de maturação, foram avaliados quanto à quantidade e estabilidade da espuma.

O recurso ao uso de vinhos modelo sistematiza o estudo de muitas variáveis. Assim sendo, o vinho foi separado nas suas diferentes biomoléculas para posteriormente ser reconstituído. Foram usados vinhos modelo reconstituídos com cada uma das fracções isoladas e com misturas de algumas das fracções com o objectivo de avaliar quais as biomoléculas com maior influência na espumabilidade e estabilidade da espuma. Depois de identificada a fracção com maior relevância nas propriedades da espuma, procedeu-se à sua caracterização química e estrutural por espectrometria de massa com ionização por *electrospray*, tendo os potenciais compostos tensioactivos identificados sido confirmados como constituintes da espuma do vinho espumante.

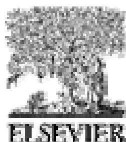
CAPÍTULO III – RESULTADOS E DISCUSSÃO

1. Análise da composição volátil das uvas ao longo da maturação

As uvas são a matéria-prima na elaboração de um vinho espumante. O aroma varietal de um vinho espumante depende do potencial volátil que as uvas exibem no momento da colheita. Sendo o momento da colheita decisivo para a elaboração de um vinho espumante com melhores atributos de aroma, a composição volátil varietal das uvas foi estudada ao longo da maturação para as duas castas principais da Bairrada, a casta tinta Baga (BG) e a branca Fernão-Pires (FP). Para se conhecer o perfil varietal volátil destas duas castas, as uvas foram colhidas semanalmente do pintor à pós-maturidade em duas vinhas. Uma das vinhas localizava-se em Anadia (BG-Colégio e FP-Talhão da Avenida) e a outra em Pedralvites, onde foram colhidas uvas de ambas as castas. A metodologia de análise dos compostos voláteis das uvas foi optimizada usando a microextracção em fase sólida em espaço de cabeça (HS-SPME) seguida de cromatografia de gás acoplada à espectrometria de massa com quadrupolo (GC–qMS).

O capítulo referente à análise da composição volátil das uvas ao longo da maturação está dividido em dois artigos, ambos publicados na *Analytica Chimica Acta*. No primeiro artigo estudam-se as uvas Baga e no segundo estudam-se as uvas Fernão-Pires.

1.1. Headspace SPME applied to varietal volatile components evolution during *Vitis vinifera* L. cv. 'Baga' ripening



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Headspace-SPME applied to varietal volatile components evolution during *Vitis vinifera* L. cv. 'Baga' ripening

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Abstract

Grape berries of *Vitis vinifera* L. cv. Baga were collected during 7 weeks since half-*véraison* to a post-maturation stage. Two vineyards in different locations in Bairrada Appellation, Pedralvites (Ped) and Colégio (Col) were used. The free varietal and pre-fermentative related volatile compounds that arise in the first 2 h after crushing the grapes were followed by Headspace-Solid Phase Microextraction (HS-SPME). Twenty-three sesquiterpenoids, 13 monoterpenoids, 6 norisoprenoids, 2 aromatic alcohols, and 1 diterpenoid were detected in both vineyards. However, 40 sesquiterpenoids and 10 norisoprenoids were detected in Ped. The maximum gas chromatographic peak area was reached at the maturity, and remained constant until post-maturation. At maturity sesquiterpenoids represented 56% and 80% of the total varietal GC peak area in Col and Ped, respectively, which was the group with higher number of constituent varietal compounds. The results obtained indicated a high number of sesquiterpenoid compounds, namely (+)-cycloisosativene, γ-elemene, α-ylangene, β-bourbonene, β-cubenene, β-caryophyllene, 3,7-guaiadiene, (−)-isoledene, (+)-aromadendrene, α-amorphene, (−)-δ-selinene, germacrene D, epizonarene, β-cadinene, γ-cadinene, δ-cadinene, α-muurolene, α-calacorene in 'Baga' ripe grapes, which allows to suggest that this variety is a potential source of sesquiterpenoids.
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Keywords: Sesquiterpenoids; *Vitis vinifera* L.; 'Baga'; Varietal volatiles; Ripening; Headspace-Solid Phase Microextraction

1. Introduction

Several studies carried out on grapes characterization recognized a relationship between the wine varietal character and the grape and musts volatile and semi-volatile compounds, namely monoterpenoids, C_{13} norisoprenoids, and aromatic alcohols. However, few studies report the occurrence of sesquiterpenoids as components of grapes [1,2]. The lack of information available on the sesquiterpenoids from grapes of *Vitis vinifera* L. may be due to the extraction methodologies used or absence of these compounds in the varieties studied. The volatile composition of the grape is one of the most important factors to determine the wine character and quality. Although varietal grape composition is determined, in white grapes, mainly by monoterpenoids [3–11] and, in red grapes, by aromatic alcohols and norisoprenoids [12–17], sesquiterpenoids have also been described as grape constituents of *V. vinifera* L. cv. Riesling, Traminer, Rulânder, Müller Thurgau, Scheurebe, Optima, and Rieslaner [1]. Furthermore, farnesol was detected in grapes of cv. Monastrell [16], Tempranillo and Grenache [17], musts of cv. Maria Gomes and Bical [7], and berry skin distillates of cv. Müller Thurgau, Traminer, Yellow Muscat, and Rose Muscat [2]. Two α-farnesene isomers and methyl farnesoate were also identified in berry skin distillates [2]. As far as we know, sesquiterpenoids were not yet found in table wines, although the sweet Madeira wine is known to contain farnesol [18], nerolidol [18,19], γ-eudesmol [19], α-cadinol [19], and τ-muurolol [19]. These studies do not specify their contribution for the aroma of wine. However, considering the role of the sesquiterpenoids to the spicy hop character of beer [20], it is also expected their importance for the wine aroma characteristics. Furthermore, sesquiterpenoids have been related with medicinal plants with different health applications [21,22], mainly anti-inflammatory [23,24], anti-HIV [25], antibacterial [26], and antitumor activity [25,27–29]. By the moment, no studies were carried out covering the biological activity of sesquiterpenoids from grapes and wine of *V. vinifera* L. However, the biological activity of sesquiterpenoids from many plants and fruits has been already

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reported. Sesquiterpenoids such as farnesol and nerolidol have been reported to have the ability to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds. These compounds increase the susceptibility of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by disrupting the normal barrier function of the bacterial cell membrane, allowing the permeation into the cell of exogenous solutes such as antibiotics [30]. The antioxidant activity of essential oils of *Teucrium orientale* L. is reported to be related with the β-caryophyllene [31].

Grape ripening is a physiological period that starts in the moment of *véraison* and lasts until the fruit is fully ripe, when the maturity is reached. This is a very important period that influences the composition of the grapes and, consequently, the wine, allowing grapes to develop their varietal characteristics [32]. To identify the optimal maturation stage, classical parameters based on percentage of soluble solids (sugar), titratable acidity, pH, and colour are used [33]. The sugar content and acidity are the most used parameters. However, in order to trace more specifically the varietal grape characteristics, to obtain a better wine quality, analysis of phenolics [34], carotenoids [35], and volatile compounds [32,36,37] have been also proposed. The knowledge of the grape varietal volatile composition offers a means of evaluating the aroma potential, and the period of time that the maximum potential is exhibited.

In this work, the varietal volatile compounds of *V. vinifera* L. cv. 'Baga' grape berries were followed during ripening by Headspace-Solid Phase Microextraction (HS-SPME). A preliminary step, in which the grapes were crushed and macerated before HS-SPME analyses, is proposed. The aptness of this methodology for the characterization of grape varietal volatiles and for the determination of its volatile maturity is discussed. Grapes were collected during 7 weeks from two vineyards within different locations. Monoterpenoids, norisoprenoids, aromatic alcohols, as well as sesquiterpenoids, were followed. A special relevance has been given to the sesquiterpenoid compounds due to their number and their higher gas chromatographic (GC) peak area in 'Baga' grapes.

2. Experimental

2.1. Samples

Healthy-state *V. vinifera* L. cv. 'Baga' grapes from the 2002 harvest were collected in Bairrada Appellation, from two experimental vineyards: Colégio (Col), latitude 40º26'N, longitude 8º26'W, 46 m of elevation and rows guided South–North direction, and Pedralvites (Ped), latitude 40º26'N, longitude 8º30'W, 89 m of elevation and rows guided in the West–East direction. Col and Ped vineyards are proprieties of Estação Vitivinícola da Bairrada (EVB), the Vine and Wine Research Institute of Bairrada Appellation. To better characterize the 'Baga' variety, a sampling strategy was defined including eight sampling moments, each one with four replicates ($n = 4$), in two different vineyards. This strategy contributed to the understanding of the intrinsic and natural variability of the fruit and allowed to validate the data obtained.

The grapes were collected during 7 weeks, from August 13, 2002 (half-*véraison*—day 0), to October 1, 2002 (day 49), in a total of eight sampling moments. Half-*véraison* was defined according to blossom, bloom, and berry texture and colour fenological parameters, as the moment, during ripening, where 50% of the grapes are in the turning colour stage. Weight, sugar, and titratable acidity of 'Baga' berries in the sampling period is shown in Table 1. In day 42, grapes in a starting rotting stage were observed in both vineyards. However, visual healthy-state samples were collected. For each sampling, 450 grape berries were picked randomly throughout the vine, taking into consideration the number of berries per bunch, and the balance between shadow and sun exposure in the different vineyard locations. Samples were transported immediately under refrigeration (ca. 2–5 ºC) to the laboratory and were stored at −80 ºC until analysis.

2.2. Chemical analyses of grapes

The chemical analyses: titratable acidity, pH, malic acid, tartaric acid, and sugar concentration were performed by Fourier transform infrared spectroscopy (FTIR). The FTIR spectrometer, WineScan FT120, was calibrated using the standard methods: titration with NaOH (range 5.0–10.0 g l^{-1} tartaric acid), and aerometry (range 150.0–230.0 g $l^{-1}$of sugar). The grape berries were crushed without breaking the seeds, filtered off the skins, and seeds and the juice was finally centrifuged at 3000 rpm for 10 min, at room temperature. The analyses were made in duplicate, the sample was thermostatted at 40 ºC, and 3.0 ml of grape juice were sucked into an IR transparent cell.

2.3. HS-SPME methodology

The SPME holder for manual sampling and fibre used in the analyses were purchased from Supelco (Aldrich,

Table 1
Weight and titratable acidity of 'Baga' berries in the sampling period (data obtained from EVB)

Sampling time	Colégio (Col)			Pedralvites (Ped)		
	Berry weight (g)	Sugar (g l^{-1})	Titratable acidity (g l^{-1})	Berry weight (g)	Sugar (g l^{-1})	Titratable acidity (g l^{-1})
Half-*véraison* (2nd week of August)	1.32	119.2	15.1	0.94	105.5	23.4
Véraison (4th week of August)	1.46	150.5	9.5	1.46	137.4	11.7
Maturity (3rd week of September)	1.62	161.9	6.6	1.47	137.4	7.7
Post-maturity (1st week of October)	1.67	170.2	7.2	1.64	139.5	7.5

Bellefonte, PA). The SPME device included a fused silica fibre, partially cross-linked with 65 μm Carbowax–divinylbenzene (CW–DVB). According to the producer's recommendations, the 65 μm Carbowax/divinylbenzene coating is indicated for analysis of alcohols and polar compounds (MW 40–275). This adsorbent fibre, containing macro- (>500 Å), meso- (20–500 Å), and microporous (2–20 Å), seems to be adequate for the analysis of complex matrix such as grapes. Furthermore, adsorbent fibres are good for trace level extractions.

The SPME fibre was conditioned at 250 ºC for 30 min in the GC injector, according to the manufacturer's recommendations. For headspace sampling, 50.0 g of grapes were crushed manually in a plastic bag and inserted into a 120 ml glass vial, which corresponds to a ratio of the volume of the liquid phase to the headspace volume ($1/\beta$) of 0.5. The vial was capped with a Teflon septum and an aluminium cap (Chromacol Ltd.) after the addition of 8.0 g of NaCl and a 25 mm stirring bar (1000 rpm), and was placed in a thermostatted bath adjusted to 40 ºC for 60 min to promote the transference of the compounds from the sample to the headspace. After this step, the SPME fibre was manually inserted into the sample vial headspace for 60 min. Since headspace volume can be a critical factor determining the precision of the results in three-phase systems—liquid sample-headspace-fibre coating [38], vials from the same producer and lot were used.

2.4. GC–MS analysis

The SPME coating fibre containing the headspace volatile compounds was inserted into the GC injection port at 250 ºC and kept for 15 min for the desorption. The injection port was lined with a 0.75 mm i.d. splitless glass liner. The desorbed volatile compounds were separated in a GC–MS Agilent Technologies 6890 N Network gas chromatograph, equipped with a 30 m × 0.32 mm i.d., 0.25 μm film thickness DB-FFAP fused silica capillary column, connected to an Agilent 5973 mass selective detector (mass spectrometer—MS). Splitless injection mode was used (5 min). The oven temperature was programmed from 35 ºC (3 min) to 65 ºC at 2 ºC $\min^{-1}$, followed by 65 to 90 ºC at 1 ºC $\min^{-1}$, 90 ºC (3 min) to 150 ºC at 2 ºC $\min^{-1}$, and finally 150 to 220 ºC at 10 ºC $\min^{-1}$. The injector and the transfer line were heated at 250 ºC and the He carrier gas had a flow of 1.7 ml $\min^{-1}$. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 30–300 *m/z* in a 1-s cycle. Identification of volatile compounds was achieved comparing the GC retention times and mass spectra with those, when available, of the pure standard compounds. All mass spectra were also compared with the data system library (Wiley 275) and other published spectra [39]. All measurements were made with, at least, four replicates and the reproducibility was expressed as coefficient of variation (CV) in the tables. Blanks were run in between each quadruplicate set. Statistical significant differences among sampling times were evaluated using a Student's *t*-test at the $p < 0.05$ level.

In order to validate the analytical methodology, the repeatability of the HS-SPME–GC–MS method, expressed as relative standard deviation (R.S.D.), was estimated. Three standards representative of the major chemical families understudy were used: geraniol, β-ionone, and β-caryophyllene. The repeatability of the method was estimated for five consecutive analyses of aqueous solutions containing 20 μg l^{-1} of each standard. The analyses were carried out keeping the SPME parameters defined to the grapes study. The results gave R.S.D. values of 3% for the three standards, indicating a good repeatability of the HS-SPME–GC–MS method.

3. Results and discussion

3.1. HS-SPME–GC–MS methodology

Due to the presence of low concentrations of free volatile compounds, the majority of grapes destined for winemaking do not exhibit an intense aroma. Instead, a considerable amount of the volatile compounds responsible for the wine aroma quality is originated from the non-volatile precursors, which are transformed into odourant compounds during winemaking and/or aging [4,40]. Thus, in the present study, a preliminary step in which the grapes were crushed and macerated for 60 min before HS-SPME analyses was considered. This procedure allows to obtain in the grapes headspace a fraction of the free volatile components from the skin and pulp plus the compounds arising from the pre-fermentative reactions. These reactions take place in the crushed grapes promoted by the acidic conditions (pH of the grapes ca. 3.8) and by the endogenous enzymatic activity during 120 min (60 min without fibre + 60 min with the fibre). Under these conditions it was obtained the varietal plus the pre-fermentative volatile components of the grapes. After this first step, the samples were analyzed by headspace-SPME–GC–MS as it allows the characterization of the headspace in contact with the sample by a simple to use, rapid, and not requiring any solvent extraction methodology.

The present study was focused on the monoterpenoids, norisoprenoids, aromatic alcohols, and sesquiterpenoids, named as "varietal and pre-fermentative related volatile compounds". The proposed methodology also allows the detection of the C_6 aldehydes and alcohols, known as pre-fermentative compounds. Considering that these compounds do not have a particular contribution to the 'Baga' wine aroma [41], they were not considered in the present manuscript.

3.2. Headspace varietal volatile composition of grape at maturity (day 35)

The evolution of the chemical parameters during grape ripening from half-*véraison* (day 0) fenological state, to *véraison* (day 14), maturity (day 35), and post-maturity (day 49) is presented in Table 1 for both vineyards. The data obtained show that the sugar content was attained its maximum and stabilized (ca. 140–160 g l^{-1}) at day 35 after half-*véraison*. Furthermore, as in both vineyards the acidity level stabilized after day 35 (ca. 7 g l^{-1} tartaric acid), this day was considered to be the nearest to the maturity state.

Tables 2 and 3 show the varietal and pre-fermentative related volatile compounds found in 'Baga' ripe grapes from Colégio

Table 2
GC–MS peak area (×10^{-5}) and coefficient of variation (CV% in parentheses) of the varietal and pre-fermentative related volatile compounds found in Colégio vineyard, during ripening (from half-*véraison*—day 0 to post-maturation—from day 35 to day 49)

Peak number	Compound	Identification[a]	Days (GC peak area × 10^{-5})															
			0 $\bar{x}$ (n = 4)	(CV)	7 $\bar{x}$ (n = 4)	(CV)	14 $\bar{x}$ (n = 4)	(CV)	20 $\bar{x}$ (n = 4)	(CV)	28 $\bar{x}$ (n = 4)	(CV)	35 $\bar{x}$ (n = 4)	(CV)	42 $\bar{x}$ (n = 4)	(CV)	49 $\bar{x}$ (n = 4)	(CV)
	Monoterpenoids																	
1	Limonene	A, B, C	3.86	(23)	3.70	(10)	2.10	(50)	3.65	(50)	2.41	(29)	5.92	(64)	4.82	(61)	4.16	(11)
2	n.i. (*m/z* 136, 107, 79, 68, 94, 77, 81, 91, 121)	B	3.72	(23)	5.85	(9)	7.92	(20)	4.13	(20)	3.52	(36)	3.85	(23)	5.36	(21)	9.91	(9)
3	n.i. (*m/z* 69, 41, 95, 57, 82, 55, 67, 138, 109, 123)	B	6.52	(25)	5.56	(48)	5.62	(22)	6.45	(45)	3.85	(20)	3.26	(5)	6.17	(15)	6.20	(8)
18	Linalool	A, B, C	6.05	(9)	3.82	(16)	28.49	(87)	2.36	(55)	1.44	(10)	2.93[b]	–	5.31[b]	–	5.89[c]	(9)
28	Hotrienol	B, C	2.54	(12)	1.29	(61)	6.58	(67)		–	–	–	–	–	–	–	–	–
38	α-Terpineol	A, B, C	1.26	(12)	1.34	(9)	7.35	(17)	–	–	–	–	–	–	–	–	–	–
48	Citronellol	A, B, C	12.68	(11)	8.96	(18)	6.56	(62)	6.22	(40)	1.40	(39)	2.01	(46)	2.14	(52)	3.31	(15)
51	γ-Isogeraniol	B	2.42	(9)	1.95	(8)	1.82	(60)	–	–	–	–	6.51[c]	(16)	–	–	–	–
54	n.i. + Nerol	A, B, C	2.45	(9)	3.50	(8)	3.98	(47)	3.32	(17)	2.12	(25)	4.50	(57)	4.15	(42)	6.05	(54)
55	n.i. Terpenol (*m/z* 69, 121, 105, 160, 41)	B	2.36	(9)	1.92	(14)	2.72	(71)	–	–	–	–	–	–	–	–	–	–
56	Geranyl acetone	A, B, C	3.79	(8)	3.20	(28)	5.19	(62)	4.37	(55)	0.93	(73)	1.47	(57)	1.52	(36)	2.55	(50)
57	*E*-Geraniol	A, B, C	23.60	(5)	23.51	(6)	23.10	(36)	36.43[c]	(34)	19.58	(6)	19.40[d]	(13)	22.36[d]	(11)	37.10[d]	(51)
66	Geranic acid	A, B, C	1.41	(35)	0.60	(64)	0.77	(31)	–	–	–	–	–	–	–	–	–	–
	Subtotal (GC peak area)		72.65	(5)	65.19	(8)	102.21*	(28)	66.93	(19)	38.39	(20)	49.86	(37)	51.82	(9)	75.16	(32)
	Subtotal (%)		38		37		22		22		16		12		9		15	
	Sesquiterpenoids																	
4	(+)-Cycloisosativene	B	–	–	–	–	–	–	–	–	–	–	3.45	(47)	5.79	(35)	4.76	(29)
5	δ-Elememne	B	–	–	–	–	–	–	–	–	–	–	0.64[b]	–	1.26	(68)	–	–
6	α-Ylangene	B	–	–	–	–	40.47	(98)	28.07	(45)	43.15	(48)	99.37	(49)	160.30	(29)	108.51	(30)
9	β-Bourbonene (isomer 2)	B, C	–	–	–	–	13.92	(106)	9.27[c]	(113)	4.18	(52)	27.27	(45)	53.68	(35)	16.70	(31)
15	n.i. (*m/z* 161, 120, 105, 91, 119, 93, 133, 204, 147)	B	–	–	–	–	–	–	–	–	–	–	–	–	4.65	(38)	3.48	(34)
16	β-Cubebene	A, B, C	–	–	–	–	9.73	(97)	1.88	(62)	1.38	(65)	9.99[b]	–	8.69	(39)	4.73	(45)
19	β-Caryophyllene	A, B, C	–	–	–	–	–	–	–	–	–	–	–	–	1.35[b]	–	5.54	(42)
22	3,7-Guaiadiene	B	–	–	–	–	8.70	(33)	4.11[b]	–	4.05	(89)	10.00	(57)	15.94	(28)	12.04	(46)
23	(−)-Isoledene	B	–	–	–	–	19.87[b]	–	–	–	–	–	2.10[b]	–	2.06	(30)	–	–
24	(+)-Aromadendrene	A, B, C	–	–	–	–	7.21	(68)	–	–	2.47	(25)	4.26	(59)	7.47	(37)	4.57	(53)
30	α-Amorphene	B	–	–	–	–	11.62	(67)	2.58	(55)	2.03	(47)	3.76	(68)	5.43	(43)	4.16	(21)
31	n.i. (*m/z* 161, 73, 105, 92, 204, 133, 119, 189, 147)	B	–	–	–	–	–	–	–	–	–	–	2.72[b]	–	3.18[c]	(12)	1.35[b]	–
32	(−)-δ-Selinene	B	–	–	–	–	4.68	(84)	3.82[b]	–	1.62[b]	–	3.49[c]	(57)	1.98	(13)	1.68	(13)
33	Germacrene D	B, C	–	–	–	–	30.53	(103)	4.57[c]	(42)	17.84	(51)	24.55	(46)	47.60	(43)	18.99	(47)
34	n.i. (*m/z* 161, 105, 91, 119, 204, 133, 81, 189, 147)	B	–	–	–	–	9.82	(97)	–	–	2.83	(77)	9.66	(61)	18.15	(29)	4.14	(42)
35	Epizonarene	B	–	–	–	–	7.73	(71)	1.24[b]	–	2.43	(69)	3.50	(65)	7.13	(32)	3.98	(37)
36	β-Cadinene	B	–	–	–	–	11.02	(65)	3.21[b]	–	3.25	(55)	5.80	(59)	10.54	(26)	7.40	(32)
37	n.i. (*m/z* 161, 204, 105, 119, 134, 91, 81, 189)	B	–	–	–	–	3.22	(74)	1.26[b]	–	1.87[b]	–	2.33	(41)	3.57	(20)	2.57	(18)
42	γ-Cadinene	A, B, C	–	–	–	–	14.88	(73)	3.23	(50)	4.42	(54)	16.71	(63)	15.98	(30)	10.76	(34)
43	n.i. (*m/z* 161, 105, 119, 91, 204, 93, 79, 81, 77)	B	–	–	–	–	–	–	–	–	–	–	–	–	9.93	(31)	4.97	(37)

Table 2 (*Continued*)

Peak number	Compound	Identification[a]	Days (GC peak area × 10^{-5}) 0 $\bar{x}$ (n = 4)	(CV)	7 $\bar{x}$ (n = 4)	(CV)	14 $\bar{x}$ (n = 4)	(CV)	20 $\bar{x}$ (n = 4)	(CV)	28 $\bar{x}$ (n = 4)	(CV)	35 $\bar{x}$ (n = 4)	(CV)	42 $\bar{x}$ (n = 4)	(CV)	49 $\bar{x}$ (n = 4)	(CV)
45	δ-Cadinene	A, B, C	–	–	–	–	–	–	–	–	–	–	2.86[b]	–	0.92	(117)	1.51	(43)
46	α-Muurolene	B, C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.68[b]	–
59	α-Calacorene	A, B, C	–	–	–	–	–	–	–	–	–	–	2.98	(88)	4.22	(49)	4.57	(37)
	Subtotal (GC peak area)		0.00		0.00		193.42	(118)	63.24	(62)	91.51	(54)	235.44	(51)	389.81	(28)	227.07*	(25)
	Subtotal (%)		0		0		41		21		39		56		67		46	
	Diterpenoid																	
65	Manoyl oxide	B	–	–	–	–	8.48	(95)	34.57	(103)	7.91	(95)	11.03	(52)	5.57	(13)	15.61	(61)
	Subtotal (GC peak area)		0		0		8.48	(95)	34.57	(103)	7.91	(95)	11.03	(52)	5.57	(13)	15.61	(61)
	Subtotal (%)		0		0		2		11		3		3		1		3	
	Norisoprenoids																	
7	Theaspirane A (2*R*,5*R*)	A, B, C	18.11	(11)	14.37	(17)	65.07	(35)	32.75	(34)	45.34	(30)	27.16	(68)	38.98	(12)	52.98	(17)
14	Theaspirane B (2*R*,5*S*)	A, B, C	18.93	(6)	14.12	(13)	7.95	(68)	9.63	(41)	3.83	(59)	4.63	(26)	4.60	(22)	3.27	(31)
25	β-Ciclocitral	B, C	8.09	(25)	9.57	(9)	8.55	(37)	6.74	(42)	2.43	(57)	3.63	(44)	4.82	(34)	4.53	(16)
40	Naphthalene	B, C	3.83	(17)	2.33	(6)	2.49	(34)	1.89	(26)	1.10	(22)	2.10	(20)	2.60	(18)	3.98	(20)
52	*E*-β-Damascenone	A, B, C	18.19	(19)	16.17	(25)	13.55	(14)	14.86	(64)	2.81	(54)	4.95	(38)	5.05	(18)	8.94	(31)
61	β-Ionone	A, B, C	7.34	(54)	12.79	(23)	9.29	(64)	8.57	(49)	1.84	(54)	3.11	(51)	2.26	(13)	4.46	(27)
	Subtotal (GC peak area)		74.49	(11)	69.34	(12)	106.89	(36)	74.44	(37)	57.34	(20)	45.58	(46)	58.31	(5)	78.16*	(17)
	Subtotal (%)		39		40		23		24		25		11		10		16	
	Aromatic alcohols																	
58	Benzyl alcohol	A, B, C	26.86	(13)	22.51	(23)	38.70	(45)	57.91	(21)	32.94	(44)	70.42	(30)	58.56	(21)	74.20	(17)
60	2-Phenylethanol	A, B, C	16.11	(12)	17.41	(20)	18.85	(18)	10.68	(23)	4.95	(48)	11.24	(56)	15.65	(37)	24.00	(41)
	Subtotal (GC peak area)		42.96	(10)	39.92	(17)	57.55	(30)	68.60	(21)	37.89	(44)	81.66*	(32)	74.21	(25)	98.20	(21)
	Subtotal (%)		23		23		12		22		16		19		13		20	
	Total varietal volatile compounds (GC peak area)		190.10	(7)	174.45	(7)	468.55	(53)	307.77	(37)	233.04	(26)	423.57	(36)	579.72	(20)	494.20	(16)

[a] The reliability of the identification or structural proposal is indicated by the following: A—mass spectrum and retention time consistent with those of an authentic standard; B—structural proposals given on the basis of mass spectral data (Wiley 275); C—mass spectrum consistent with spectra found in literature.
[b] The compound was detected only in one replicate.
[c] The compound was detected only in two replicates.
[d] Geraniol was co-eluted with hexanoic acid.
* Mean, within the same group of compounds, is significantly different from the week before ($p = 0.05$).

Table 3
GC–MS peak area (×10^{-5}) and coefficient of variation (CV% in parentheses) of the varietal and pre-fermentative related volatile compounds found in Pedralvites vineyard, ripening (from half-*véraison*—day 0 to post-maturation—from day 35 to day 49)

Peak number	Compound	Identification[a]	Days (GC peak area ×10^{-5}) 0 $\bar{x}$ (n = 4)	(CV)	7 $\bar{x}$ (n = 4)	(CV)	14 $\bar{x}$ (n = 4)	(CV)	20 $\bar{x}$ (n = 4)	(CV)	28 $\bar{x}$ (n = 4)	(CV)	35 $\bar{x}$ (n = 4)	(CV)	42 $\bar{x}$ (n = 4)	(CV)	49 $\bar{x}$ (n = 4)	(CV)
	Monoterpenoids																	
1	Limonene	A, B, C	3.44	(147)	0.45	(23)	0.64	(24)	0.57	(16)	4.06	(45)	3.92	(45)	14.07	(14)	2.96	(24)
2	n.i. (m/z 136, 107, 79, 68, 94, 77, 81, 91, 121)	B	5.87	(26)	7.19	(29)	7.45	(14)	5.37	(22)	9.39	(25)	12.07	(16)	19.08	(8)	11.95	(15)
3	n.i. (m/z 69, 41, 95, 57, 82, 55, 67, 138, 109, 123)	B	5.35	(34)	3.24	(22)	3.30	(20)	2.83	(17)	12.15	(12)	8.51	(17)	14.57	(10)	8.46	(13)
18	Linalool	A, B, C	5.84	(15)	3.10	(32)	2.90	(26)	2.84	(15)	3.24[b]	–	–	–	–	–	–	–
28	Hotrienol	B, C	1.59	(14)	0.71	(18)	0.82	(26)	0.76	(14)	–	–	–	–	–	–	–	–
38	α-Terpineol	A, B, C	0.28	(68)	v	–	0.39[b]	–	–	–	–	–	–	–	–	–	–	–
48	Citronellol	A, B, C	3.32	(6)	2.53	(34)	2.78	(30)	3.44	(21)	9.51	(26)	13.95	(31)	8.13	(18)	14.17	(18)
51	γ-Isogeraniol	B	–	–	–	–	–	–	–	–	3.47	(14)	3.68	(30)	1.31	(67)	4.34	(31)
54	n.i. + Nerol	A, B, C	–	–	0.85	(4)	1.19	(17)	1.08	–	6.29	(18)	5.83	(16)	2.86	(23)	4.25	(20)
55	n.i. Terpenol (m/z 69, 121, 105, 160, 41)	B	–	–	–	–	–	–	–	–	3.29[b]	–	3.14	(69)	0.92[b]	–	3.40	(30)
56	Geranyl acetone	A, B, C	3.35	(19)	2.03	(4)	1.92	(21)	1.20	(29)	7.41	(18)	6.85	(31)	4.22	(77)	6.81[d]	(23)
57	*E*-Geraniol	A, B, C	11.27	(13)	22.87[c]	(21)	25.84[c]	(22)	33.45[c]	(5)	112.53[c]	(31)	32.75	(37)	22.06	(22)	54.26[d]	(11)
66	Geranic acid	A, B, C	–	–	–	–	–	–	–	–	3.12	(73)	4.97[b]	–	–	–	6.77[b]	–
	Subtotal (GC peak area)		40.32	(12)	42.97	(15)	47.23	(16)	51.56	(6)	174.46*	(41)	95.66*	(28)	87.22	(15)	117.37	(34)
	Subtotal (%)		35		39		29		26		11		3		3		4	
	Sesquiterpenoids																	
4	(+)-Cycloisosativene	B	–	–	–	–	–	–	–	–	12.72	(60)	27.54	(18)	29.54	(20)	27.75	(17)
5	δ-Elememne	B	–	–	–	–	–	–	–	–	9.60[d]	(29)	12.40	(23)	12.21	(23)	12.60	(24)
6	α-Ylangene	B	–	–	–	–	9.36	(80)	9.67	(72)	234.02	(59)	616.25	(22)	581.09	(20)	608.83	(23)
8	β-Bourbonene (isomer 1)	B, C	–	–	–	–	–	–	–	–	–	–	70.50	(23)	61.10	(14)	52.66	(21)
9	β-Bourbonene (isomer 2)	B, C	–	–	–	–	11.19[d]	(28)	3.46	(12)	109.89	(60)	234.81	(29)	225.69	(23)	225.08	(19)
11	β-Bourbonene (isomer 3)	B, C	–	–	–	–	–	–	–	–	22.55	(77)	32.55	(34)	26.29	(20)	28.75	(25)
15	n.i. (m/z 161, 120, 105, 91, 119, 93, 133, 204, 147)	B	–	–	–	–	–	–	–	–	19.29	(60)	41.50	(22)	34.28	(11)	42.05	(19)
16	β-Cubebene	A, B, C	–	–	–	–	0.66[d]	(20)	v	–	14.16[d]	(10)	7.35	(17)	3.86	(16)	6.31	(26)
17	Epi-bicyclosesquiphellandrene	B	–	–	–	–	–	–	–	–	–	–	60.87	(26)	40.85	(18)	63.26	(29)
19	β-Caryophyllene	A, B, C	–	–	–	–	–	–	–	–	–	–	–	–	33.60	(11)	33.55	(16)
20	α-Guaiene	B, C	–	–	–	–	–	–	–	–	11.93[d]	(1)	20.33	(53)	13.13	(23)	13.63	(2)
21	n.i. (m/z 93, 81, 107, 161, 147, 119, 133, 121, 189)	B	–	–	–	–	–	–	–	–	10.11[b]	–	15.77	(31)	11.24	(14)	9.96	(30)
22	3,7-Guaiadiene	B	–	–	–	–	1.08[d]	(63)	v	–	43.93	(29)	101.95	(21)	80.61	(19)	101.62	(22)
23	(−)-Isoledene	B	–	–	–	–	–	–	–	–	11.63	(42)	21.61	(24)	15.41	(27)	18.70	(11)
24	(+)-Aromadendrene	A, B, C	–	–	–	–	0.61	–	–	–	30.96	(39)	68.08	(22)	56.82	(23)	67.28	(26)
26	Valencene	B	–	–	–	–	–	–	–	–	6.06	(47)	11.95	(40)	9.23	(22)	9.60	(24)
27	γ-Elemene	B	–	–	–	–	–	–	–	–	–	–	9.29	(14)	10.67	(13)	9.77	(15)
29	γ-Cadinene	B	–	–	–	–	–	–	–	–	16.64[b]	–	25.71	(29)	31.92	(11)	22.97	(31)
30	α-Amorphene	B	–	–	–	–	–	–	–	–	16.31	(60)	58.34	(23)	27.53	(22)	58.61	(23)
31	n.i. (m/z 161, 73, 105, 92, 204, 133, 119, 189, 147)	B	–	–	–	–	1.25[b]	–	v	–	9.87[d]	(17)	19.81[d]	(69)	30.05[e]	(18)	18.76[d]	(75)
32	(−)-δ-Selinene	B	–	–	–	–	v	–	v	–	9.32	(71)	20.41[d]	(37)			21.03[d]	(40)
33	Germacrene D	A, B, C	–	–	–	–	6.87	(45)	4.82	(20)	135.98	(49)	286.74	(23)	277.38	(26)	285.29	(22)
34	n.i. (m/z 161, 105, 91, 119, 204, 133, 81, 189, 147)	B	–	–	–	–	1.39[d]	(88)	–	–	85.02	(14)	114.88	(30)	75.50[d]	(1)	98.68	(14)
35	Epizonarene	B	–	–	–	–	0.58[b]	–	–	–	32.29	(32)	76.14	(30)	62.07	(25)	66.63	(21)
36	β-Cadinene	B	–	–	–	–	0.98[d]	(73)	–	–	41.52	(53)	86.71	(25)	71.08	(25)	76.01	(9)
37	n.i. (m/z 161, 204, 105, 119, 134, 91, 81, 189)	B	–	–	–	–	v	–	v	–	15.59	(51)	28.42	(33)	20.78	(22)	22.97	(10)
39	n.i. (m/z 161, 105, 204, 91, 119, 133, 79, 189, 147)	B	–	–	–	–	–	–	–	–	11.19	(28)	18.36	(12)	27.51	(20)	18.37	(15)

Table 3 (*Continued*)

Peak number	Compound	Identification[a]	Days (GC peak area × 10^{-5}) 0 $\bar{x}$ (n = 4)	(CV)	7 $\bar{x}$ (n = 4)	(CV)	14 $\bar{x}$ (n = 4)	(CV)	20 $\bar{x}$ (n = 4)	(CV)	28 $\bar{x}$ (n = 4)	(CV)	35 $\bar{x}$ (n = 4)	(CV)	42 $\bar{x}$ (n = 4)	(CV)	49 $\bar{x}$ (n = 4)	(CV)
41	2-Isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-en	B	–	–	–	–	–	–	–	–	7.95	(36)	10.95	(29)	11.80	(16)	11.64	(29)
42	γ-Cadinene	A, B, C	–	–	–	–	–	–	–	–	49.07	(43)	130.92	(25)	95.46	(21)	129.60	(19)
43	n.i. (m/z 161, 105, 119, 91, 204, 93, 79, 81, 77)	B	–	–	–	–	–	–	–	–	34.98	(29)	63.49	(38)	51.55	(19)	82.39	(28)
44	n.i. (m/z 161, 204, 119, 134, 105, 91, 189, 81, 133)	B	–	–	–	–	–	–	–	–	10.16	(86)	15.06	(46)	16.85	(14)	19.85	(22)
45	δ-Cadinene	A, B, C	–	–	–	–	–	–	–	–	–	–	15.46	(46)	7.50	(32)	15.45	(26)
46	α-Muurolene	B, C	–	–	–	–	–	–	–	–	–	–	7.89	(21)	4.90	(23)	7.87	(10)
47	α-Cadinene	B	–	–	–	–	–	–	0.55[b]	–	8.45	(40)	9.22	(20)	4.94	(31)	7.32	(33)
49	γ-Elemene	B	–	–	–	–	–	–	–	–	11.56	(44)	10.03	(71)	8.36	(7)	12.35	(16)
50	Germacrene B	B	–	–	–	–	–	–	–	–	5.55[b]	–	16.70	(30)	12.20	(12)	16.85	(31)
53	Calamenene	A, B, C	–	–	–	–	–	–	–	–	–	–	13.36[b]	–	7.27	(25)	14.90	(12)
59	α-Calacorene	A, B, C	–	–	–	–	1.40[b]	–	–	–	17.46	(26)	31.69	(19)	22.04	(20)	29.56	(20)
63	n.i. (m/z 161, 105, 79, 119, 204, 133, 107, 81, 191)	B	–	–	–	–	–	–	–	–	–	–	3.40	(31)	1.36	(14)	2.25	(22)
64	Cadalene	B	–	–	–	–	–	–	–	–	–	–	2.25	(14)	1.49	(23)	3.24	(24)
	Subtotal (GC peak area)		0.00		0.00		35.37*	(5)	18.50*	(38)	945.79*	(53)	2418.69*	(22)	2115.17	(13)	2373.97	(15)
	Subtotal (%)		0		0		22		9		65		80		79		79	
	Diterpenoid																	
65	Manoyl oxide	B	–	–	–	–	1.76	(110)	17.04[d]	(13)	63.89	(15)	48.64	(10)	73.35	(46)	48.76	(10)
	Subtotal (GC peak area)		0.00		0.00		1.76	(110)	17.04*	(13)	63.89*	(15)	48.64*	(10)	73.35	(46)	48.76	(10)
	Subtotal (%)		0		0		1		9		4		2		3		2	
	Norisoprenoids																	
7	Theaspirane A (2*R*,5*R*)	A, B, C	19.03	(14)	15.08	(9)	12.86	(14)	37.70	(6)	35.50	(91)	99.84	(27)	95.30	(16)	90.22	(25)
10	Dihydroedulan I	A, B, C	5.14	(7)	4.46	(24)	4.27	(23)	3.00	(23)	34.71	(70)	44.91	(25)	47.33	(19)	44.16	(16)
12	Vitispirane isomer 1	B, C	3.04	(7)	1.76	(29)	2.12	(42)	2.37	(18)	v	–	v	–	–	–	–	–
13	Vitispirane isomer 2	B, C	2.02	(10)	1.37	(21)	1.01	(18)	0.96[d]	(10)	–	–	–	–	–	–	–	–
14	Theaspirane B (2*R*,5*S*)	A, B, C	18.76	(8)	14.99	(13)	13.09	(14)	9.86	(5)	25.13	(23)	31.98	(12)	18.97	(15)	31.94	(11)
25	β-Ciclocitral	B, C	3.99	(17)	4.32	(21)	4.35	(10)	3.97	(12)	14.47	(12)	16.39	(18)	15.39	(14)	16.41	(14)
40	Naphthalene	B, C	1.26	(33)	0.92	(17)	0.94	(34)	0.91[d]	(5)	–	–	–	–	–	–	–	–
52	*E*-β-Damascenone	B, C	4.58	(23)	7.77	(26)	9.80	(14)	10.59	(27)	34.87	(26)	27.37	(22)	20.27	(10)	26.36	(22)
61	β-Ionone	A, B, C	2.28	(27)	3.22	(39)	2.87	(18)	2.69	(8)	12.60	(25)	19.25	(18)	12.23	(22)	18.67	(18)
62	1,1,6-Trimethyl-1,2-dihydronaphthalene	B	–	–	–	–	–	–	–	–	–	–	5.39	(21)	2.98	(18)	5.14	(20)
	Subtotal (GC peak area)		60.10	(5)	53.89	(10)	51.31	(6)	72.04*	(5)	157.28	(46)	245.15	(4)	212.47	(12)	232.91	(18)
	Subtotal (%)		52		48		32		36		10		8		8		8	
	Aromatic alcohols																	
58	Benzyl alcohol	A, B, C	11.69	(31)	9.45	(43)	20.08	(17)	33.20	(20)	161.03	(49)	200.56	(13)	165.40	(33)	198.19	(14)
60	2-Phenylethanol	A, B, C	3.72	(10)	5.22	(41)	6.38	(8)	7.37	(23)	21.64	(35)	25.79	(24)	19.86	(23)	25.69	(24)
	Subtotal (GC peak area)		15.41	(26)	14.67	(43)	26.46*	(13)	40.57*	(20)	182.67*	(47)	226.35	(14)	185.26	(26)	223.88	(14)
	Subtotal (%)		13		13		16		20		11		7		7		7	
	Total varietal volatile compounds (GC peak area)		115.83	(2)	111.52	(13)	162.12	(6)	199.72	(5)	1629.95	(14)	3034.49	(2)	2673.47	(2)	2996.90	(3)

v: Vestigial.

[a] The reliability of the identification or structural proposal is indicated by the following: A—mass spectrum and retention time consistent with those of an authentic standard; B—structural proposals given on the basis of mass spectral data (Wiley 275); C—mass spectrum consistent with spectra found in literature.

[b] The compound was detected only in one replicate.

[c] Geraniol was co-eluted with hexanoic acid.

[d] The compound was detected only in two replicates.

[e] The compounds were co eluted.

* Mean, within the same group of compounds, is significantly different from the week before ($p = 0.05$).

(Col) and Pedralvites (Ped) vineyards, respectively, grouped by chemical groups: monoterpenoids, sesquiterpenoids, diterpenoid, norisoprenoids, and aromatic alcohols. To better characterize the 'Baga' variety, including the variability associated to the environment, agricultural practices, geographical location, and vines from different clones, grapes from two experimental vineyards from Bairrada Appellation were analyzed (Col and Ped). At maturity, 45 compounds were common to the grapes from the two vineyards, as 19 compounds present in Ped were not detected in Col under the same experimental conditions (Tables 2 and 3). Of these common compounds, 23 were sesquiterpenoids, 13 monoterpenoids, 6 norisoprenoids, 2 aromatic alcohols, and 1 diterpenoid. Furthermore, the GC peak area of these common varietal and pre-fermentative related volatile compounds of the grapes at maturity was 6.3 times higher in Ped vineyard than in Col. This tendency was followed by all chemical families of compounds: sesquiterpenoids were 8.8 times higher in Ped, 2.1 for monoterpenoids, 4.3 for norisoprenoids, 2.8 for aromatic alcohols, and 4.4 for the diterpenoid (Tables 2 and 3), indicating that the grapes from Ped vineyard contained a higher number and amount of volatile compounds than Col. These observations should be associated to the different characteristics of the two vineyards, described in Section 2. Differences in sunlight exposure may be considered as a consequence of rows orientation and geographic localization. Sun exposure is reported as a factor that greatly influences grape ripening, as radiation and heat from sunlight can influence metabolic reaction rates and cause stress, either by dehydration and by direct temperature increase [42].

In both vineyards, sesquiterpenoids were the compounds that contribute, at maturity, with more area to the varietal volatile composition, 56% in Col and 80% in Ped (taking only into account the 45 compounds in common, this percentage was 78% in Ped). Monoterpenoids, norisoprenoids, aromatic alcohols, and the diterpenoid accounted for 12%, 11%, 19%, and 3% in Col total GC peak area, and 3%, 8%, 7%, and 2% in Ped, respectively. Beyond the observed differences between the two vineyards, a common sesquiterpenoid profile can be identified. The major sesquiterpenoids were α-ylangene (42% and 25% in Col and Ped, respectively), β-bourbonene (12% and 10%), germacrene D (10% and 12%), and γ-cadinene (7% and 5%), whose structures are represented in Fig. 1. Although these four sesquiterpenoids were already reported as grape constituents [1], the high number of different sesquiterpenoids in 'Baga' ripe grapes suggests that this variety is a potential source of sesquiterpenoids. These sesquiterpenoids were present in similar relative amounts in both vineyards, which show that their presence and their relative proportion may be varietal markers of this variety. However, in a previous work where the volatile composition of 'Baga' monovarietal wine was studied by liquid–liquid dichloromethane continuous extraction, the sesquiterpenoids were not detected [41]. Aliphatic and aromatic alcohols, aliphatic acids, esters, phenols, lactones, and amides were the volatile compounds reported. Beyond the fact that these observations could be explained by the use of different volatile extraction methodologies, it is also possible that the lack of sesquiterpenoids in the liquid–liquid continuous extract may be explained by the inclusion of the sesquiterpenoids in the wax layers of berry skins, which would prevent their extraction to the must in quantitative amounts to be detected in the wine [2]. The methodology here proposed seems to be adequate to detect the grape berries sesquiterpenoids.

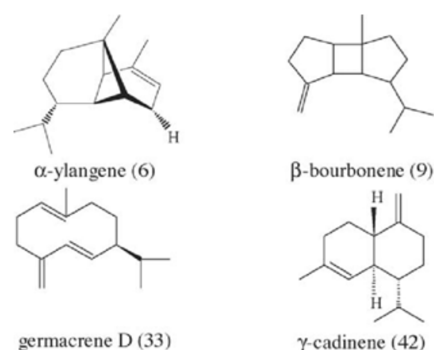

Fig. 1. Major sesquiterpenoids present in grapes from both vineyards (peak number in parenthesis as in Tables 2 and 3).

To foresee the potential contribution of the compounds to the fruit aroma characteristics it was considered the odour descriptors of the different components. To study the evolution during ripening it was established that the variation of the GC peak area for each compound is directly related with the variation of its concentration. This assumption allowed to study the evolution of each compound during ripening only by comparison of its chromatographic areas.

The monoterpenoids, if present in amounts above their sensorial perception limits, can contribute with characteristic notes: linalool has a citrus-like, sweet and flowery notes, and hotrienol, α-terpineol, and geraniol exhibit flowery and sweet aromas [5,7]. The sesquiterpenoids have odour descriptors associated to woody, spicy, sweet, floral, clove, oily, musty and fresh [43–46], and the spicy hop character reported in beer [20]. The norisoprenoids, theaspirane A (2*R*,5*R*) and theaspirane B (2*R*,5*S*) may potentially contribute with weak camphoraceous note and with an intense fresh–fruity (black currant or cassis) odour, respectively [47]. β-Damascenone, due to its very low sensorial threshold (0.002 mg l^{-1} in water) may potentially contribute to honey-like odour [48]. The aromatic alcohols, benzyl alcohol, and 2-phenylethanol are associated to sweet and flowery notes [48] and the 2-phenylethanol was already reported as a 'Baga' would-be impact odourant [41].

3.3. Development of headspace varietal volatile composition during ripening (days 0–49)

3.3.1. Monoterpenoids, norisoprenoids, and aromatic alcohols

At half-*véraison* (day 0) monoterpenoids accounted for 38% and 35% of the GC peak area of varietal compounds in Col and Ped vineyards, respectively (Tables 2 and 3). The total area of monoterpenoids had an irregular behaviour during ripening. Except for day 14 in Col, geraniol was the monoterpenoid with higher GC peak area in all sampling points.

At day 0, norisoprenoids accounted for 74% and 52% of the GC peak area of varietal compounds in Col and Ped vineyards, respectively (Tables 2 and 3). In Col vineyard the norisoprenoids were almost constant during ripening. In Ped vineyard the GC peak area of norisoprenoids was almost constant in the first 2 weeks, but it was observed a continuous increase in the following 3 weeks until maturity. After day 35 the GC peak area of norisoprenoids was almost constant. Due to the fact that between *véraison* and maturity the glycosylation of norisoprenoids may occur, a decrease of these compounds in free form was expected [35,49]. However, as in this work the experiments were done with crushed grapes, it is possible that pre-fermentative hydrolysis reactions, catalyzed by the endogenous enzymes and by the acidic medium, took place in a large extent, releasing the norisoprenoids to the headspace phase. The norisoprenoids detected under the conditions of the analysis have no hydroxyl functional groups for the glycosylation reaction, their generation requires several steps (hydrolysis of the glycosidic precursor, reduction of Grasshopper ketone, dehydration, . . .), and all these reactions are influenced by the composition of the crushed grapes and assay conditions.

At half-*véraison* (day 0) aromatic alcohols accounted for 23% and 13% of the GC peak area of varietal compounds in Col and Ped vineyards, respectively. In both vineyards, the GC peak area of the aromatic alcohols showed a tendency to increase along ripening. The ratio of the areas of benzyl alcohol to 2-phenylethanol was 2:1 and 3:1 at day 0—half-*véraison*, in Col and Ped, respectively. From half-*véraison* to day 35 this ratio increased to 6:1 and 8:1 in Col and Ped, respectively. The ratios of the areas of aromatic alcohols observed at maturity in Col vineyard tend to decrease to 4:1 and 3:1 at days 42 and 49 due to the increase of the GC peak area of 2-phenylethanol [50].

3.3.2. Sesquiterpenoids and diterpenoid

Sesquiterpenoids are biosynthesized by plants as a defence mechanism against fungal [51] and an attractant of insects [52], and they are also biosynthesized by grapevine flowers and berries [53]. The presence of sesquiterpenoids in flower scent was already described in both white and red grapevine varieties, where valencene was the major component of vine blossoms [54–56]. The GC–FTIR–MS and GC-sniffing technique allowed the identification of sesquiterpenoids as odour dominating and monoterpenoids as odour assistant of blossoms headspace [55,56]. Terpenoid volatiles in flowers of grapevines could attract insects' pollinators or provide protection of reproductive tissues against pathogens or small herbivores [53]. In this work sesquiterpenoids and the diterpenoid were only detected at day 14 (*véraison*). Fourteen sesquiterpenoids were detected at *véraison* in Col and 11 in Ped vineyards, respectively, and manoyl oxide was detected in both vineyards (Tables 2 and 3).

At day 14, sesquiterpenoids accounted for 41% and 22% of total varietal GC area, in Col and Ped vineyards, respectively, and the diterpenoid accounted for 2% and 1% of total varietal GC peak area. The maximum GC peak area and the maximum number of sesquiterpenoids were reached at maturity (day 35), and remained constant until post-maturation (from day 35 to day 49). These observations are in accordance with the expression of sesquiterpene synthase transcripts of grapevine of *V. vinifera* L. that were reported to be only detected during late ripening of the berries [53]. The expression of this enzyme during fruit development seems to be closely related to the level of sesquiterpenoids. Sesquiterpene synthase is reported to be expressed by grapevine in two different stages, in flowering and 2 months later, during grapes development, increasing to a maximum that is reached at maturity, presenting an evolution coincident with the stabilization of acid levels in the grapes [53]. The fact that the maximum of sesquiterpenoids was attained from day 35 is also coincident with the acidity stabilization shown in Table 1.

According to the data of Tables 2 and 3, high CVs were obtained for the majority of the compounds, namely the sesquiterpenoids and manoyl oxide. This variability is due to the intrinsic and natural variability of the fruit and not of the analytical methodology, as observed by the 3% R.S.D. of its repeatability, as described in Section 2. In some cases, compounds were only detected in one or two replicates. Among the replicates of the same sampling day, some replicates presented higher GC peak areas while others showed smaller areas. This behaviour, leading to the increase in the variability of these compounds, showed that the heterogeneity observed in this period was due to biosynthetic differences in the ripening stages of the grapes used in the sampling.

Despite the variability of sesquiterpenoids in both vineyards during the first weeks of their detection (days 14–20), they increased slowly. However, in days 28 and 35, it was observed a sharply increase of 1.4 and 2.6 times more, respectively, in Col vineyard. In Ped, the GC peak area of sesquiterpenoids also increases sharply in days 28 and 35, 51 and 2.6 times more, respectively. Although α-ylangene, due to its higher area, was the leading compound, the majority of all other sesquiterpenoids followed the same tendency. The compounds that showed the small GC peak area were detected for the first time at day 35.

The diterpenoid in Col showed a high variability during all sampling points, and in Ped vineyard, it increased since day 14, reaching its maximum at day 28, and remaining almost constant after that day.

4. Concluding remarks

The methodology used in the present manuscript allows to follow the varietal and pre-fermentative related volatile compounds of 'Baga' red grape berries during ripening. Twenty-three sesquiterpenoids, 13 monoterpenoids, 6 norisoprenoids, 2 aromatic alcohols, and 1 diterpenoid were detected in both vineyards. The maximum GC peak area was reached at the maturity, and remained constant until the post-maturation state. According to the chemical parameters determined for the grapes (Table 1), the maximum amount of varietal volatile compounds was coincident with the harvesting day (day 35) for red wine production. This allows to conclude that for 'Baga' variety, its volatile maturity, correspondent to its maximum GC peak area, is coincident with the maturity state defined by the ratio sugar/acid content.

At maturity sesquiterpenoids represented 56–80% of the total varietal GC peak area in both vineyards, which was the group with higher number of constituent varietal compounds.

Sesquiterpenoids can be detected at *véraison* and had their maximum expression at grape maturity, remaining constant until post-maturation. Thus, 'Baga' grapes in a state of maturity related with a sugar content ca. 140–160 g l^{-1} and an acidity level stabilized ca. 7 g l^{-1} tartaric acid are a potential source of sesquiterpenoids. Future studies must be done to confirm this tendency in other vintages.

The 23 sesquiterpenoids detected in common in the ripe grapes from the two vineyards are also detected in the post-maturation states (from day 35 to day 49). Due to the lack of availability of commercial standards, from these, the 18 were identified and allows to suggest 'Baga' ripe grapes as a potential source of sesquiterpenoids: (+)-cycloisosativene, γ-elemene, α-ylangene, β-bourbonene, β-cubenene, β-caryophyllene, 3,7-guaiadiene, (−)-isoledene, (+)-aromadendrene, α-amorphene, (−)--selinene, germacrene D, epizonarene, β-cadinene, γ-cadinene, δ-cadinene, α-muurolene, and α-calacorene. The most abundant ones were the α-ylangene, germacrene D, β-bourbonene, and γ-cadinene. α-Ylangene was reported as exhibiting a precious woody aroma [57], germacrene D was associated to wood, spice, and warm-spicy–wood notes [46,57] and γ-cadinene present a wood aroma [57]. These compounds associated to the spicy and woody aromas can contribute favourably to the wine aroma characteristics.

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1.2. Screening of variety- and pre-fermentation-related volatile compounds during ripening of white grapes to define their evolution profile

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Screening of variety- and pre-fermentation-related volatile compounds during ripening of white grapes to define their evolution profile

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Abstract

The variety- and pre-fermentation-related volatile compounds of 'Fernão-Pires' (FP) white grape berries were analyzed during ripening by headspace-solid phase microextraction (HS-SPME) coupled to gas chromatography-quadrupole mass spectrometry (GC-qMS). A preliminary step, in which the grapes were crushed and macerated before HS-SPME analyses, was used. The sampling started at *véraison* (beginning of berry ripening) and was carried on during 5 weeks in two different vineyards. Sixteen terpenoids, two C_{13} norisoprenoids, two aromatic alcohols, two C_6 aldehydes, and three C_6 alcohols were identified. The amount of all volatiles increased since *véraison* towards day 20. A sharp decrease was observed after this day. The maximum amount of varietal volatile compounds was coincident with the harvesting day for white table wine production defined by the ratio sugar/acid content. The varietal volatile evolution observed for FP grapes shows that the maximum amount of volatiles occurs only in a very short period. As a consequence, the establishment of the optimum moment for harvesting of FP white variety, based on its volatile content, deserves higher accuracy than that necessary for all other already studied grapes. This work also evidences that the analysis of the evolution of the terpenoids with higher GC peak area can represent the evolution of all varietal compounds. For FP grapes, this work shows that the screening of linalool, α-terpineol, and geraniol during ripening can be used to define the evolution profile of the varietal volatile compounds.
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Keywords: *Vitis vinifera* L.; Fernão-Pires; Varietal volatiles; Pre-fermentation-related volatiles; Ripening; Maturity; Headspace-solid phase microextraction

1. Introduction

Grape ripening is a physiological period that starts in the moment of *véraison* and lasts about 40 days, depending on the variety, environment and agricultural practices. This is a very important period that influences the composition of the grapes and, consequently, the wine, allowing grapes to develop their varietal characteristics [1]. The changes undergone by the grapes are physical (weight, volume, rigidity, and colour), and chemical (pH, acidity, sugars, phenolics, and volatile composition).

To follow grape ripening, classical parameters based on percentage of soluble solids, sugar, titratable acidity, pH, and colour are used [2]. However, in order to trace more specifically the varietal grape characteristics to achieve a better product quality, analysis of phenolics [3], carotenoids [4] and volatile compounds [1,5–9] have been used. The knowledge of the grape varietal volatile composition offers means of evaluating the aroma potential, and the period of time that the maximum potential is exhibited [9]. Furthermore, if a balanced sugar/acidity ratio is accompanied by the maximum amount of volatile components, especially those that contribute to the varietal character, it is possible to determine the most suitable time for harvesting using parameters that should be more closely related with the wine quality. Although, in some white wines, a small herbaceous perception is appreciated by some consumers [10], the herbaceous and greasy odours of C_6 alcohols and aldehydes are related to detrimental effects in the wine if present in concentrations above their sensorial perception limits [11,12]. This is the reason why, to determine the best moment for harvesting, the balance between the concentration of varietal volatiles (terpenoids and norisoprenoids) and C_6 compounds (alcohols and aldehydes) has also been proposed [7] as a criterion to define the harvesting moment.

The varietal aroma composition of grapes has been established by the use of liquid extraction methodologies [1,5,6,13]. More recently, the stir bar sorptive extraction (SBSE) [7] and

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headspace-solid phase microextraction (HS-SPME) [8,9] were used. Although HS-SPME methodology allows evaluating only the volatiles present in free form, it has been shown to be an easy to use and suitable methodology to follow the evolution of grape volatile compounds during ripening and to establish its volatile maturity, correspondent to its maximum varietal volatile content [9].

'Fernão-Pires' (FP) is the main white grape variety harvested in Portugal, representing more than 70% of the white vineyard. This variety is used mainly to produce table wine and, in a few cases, sparkling wine. The volatile composition of FP grapes has not been established yet. However, the volatile composition of the musts and monovarietal wines showed that monoterpenoids, aromatic alcohols, and C_{13} norisoprenoids were the chemical compounds that contribute to the varietal volatile composition of FP variety [14–16].

In red grapes, the maximum of varietal volatile compounds is coincident with the maturity established by the ratio sugar/acidity and remains constant in the following weeks [7,9]. However, in white varieties, changes in the concentration of the volatile compounds during ripening are not uniform, which yield some difficulties for the determination of the maturity on the basis of the content of varietal volatile compounds [17]. As the aroma is an extremely important characteristic in wines, the establishment of the optimum moment for harvesting based on its volatile content is of utmost relevance.

The aim of this work is the screening of the varietal volatile compounds of the FP white variety in order to know their evolution during ripening. The varietal volatile compounds of FP grape berries, grown in Bairrada Appellation using the same vineyard fields used for the establishment of the volatile maturity of a red grape variety [9], was followed during ripening by HS-SPME coupled to gas chromatography-quadrupole mass spectrometry (HS-SPME/GC-qMS). A preliminary step, in which the grapes were crushed and macerated before HS-SPME analyses, was used according to the methodology previously developed for the analysis of grapes [9]. The sampling started at *véraison*, as most of the volatile flavour compounds are produced after this period [5,7], and was carried on for 5 weeks in two different vineyards.

2. Experimental

2.1. Samples

Healthy-state *Vitis vinifera* L. cv 'Fernão-Pires' grapes from the 2002 harvest were collected in Bairrada Appellation, Portugal, from two experimental vineyards: Talhão da Avenida (TAv), latitude 40º26'N, longitude 8º26'W, 46 m of elevation and rows guided south-north direction, and Pedralvites (Ped), latitude 40º26'N, longitude 8º30'W, 89 m of elevation and rows guided in the west–east direction. TAv and Ped vineyards are proprieties of Estação Vitivinícola da Bairrada (EVB), the Vine and Wine Research Institute of Bairrada Appellation. TAv vineyard is surrounded by an urban open space and Ped is bordered at east and south by a pine forest. TAv vineyard has a less dense canopy than Ped, which is much more plentiful, creating shadowed bunches. To better characterize the FP variety, a sampling strategy was defined including 6 sampling times, each one with five replicates (n = 5), in two different vineyards. This strategy contributed to the understanding of the intrinsic and natural variability of the fruit and allowed to validate the data obtained.

The grapes were collected from August 13 (*véraison* – day 0), to September 17 (day 35), in a total of 6 sampling times. The berry weight of grapes from Ped was 10–36% higher than that from TAv (Table 1). *Véraison* was defined according to blossom, bloom, and berry texture and colour fenological parameters. For each sampling, 450 grape berries were picked randomly throughout the vine, taking into consideration the number of berries per bunch, and the balance between shadow and sun exposure in the different vineyard locations. Samples were transported immediately in refrigerated conditions (*ca*. 2–5 ºC) to the laboratory and were stored in a freezer at −80 ºC until analysis.

Table 1
Weight, sugar and titratable acidity of 'Fernão-Pires' grapes during ripening in Talhão da Avenida (TAv) and Pedralvites (Ped) vineyards (data obtained from EVB, with a minimum of three experiments with less than 5% error)

Days after *véraison*	Sampling day	Berry weight (g)	Sugar (g L^{-1})	Titratable acidity (g tartaric acid L^{-1})	Sugar/titratable acidity
Talhão da Avenida					
0	August 13	1.30	147.5	9.4	15.7
7	August 20	1.61	174.0	7.1	24.5
14	August 27	1.36	186.8	6.3	29.7
20	September 02	1.26	203.7	4.9	41.6
28	September 10	1.46	190.6	4.5	42.4
35	September 17	1.29	190.2	3.8	50.1
Pedralvites					
0	August 13	1.52	131.3	15.5	8.5
7	August 20	1.78	169.5	10.2	16.6
14	August 27	1.85	175.8	8.1	21.7
20	September 02	1.70	189.6	6.9	27.5
28	September 10	1.61	196.4	6.2	31.7
35	September 17	1.72	207.9	5.2	40.0

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2.2. *Chemical analyses of grapes*

The chemical analyses: sugar content and titratable acidity were performed by FTIR spectroscopy. The FTIR spectrometer, WineScan FT120, was calibrated using the standard methods: aerometry (range 150.0–230.0 g of sugar L^{-1}) and titration with NaOH (range 5.0–10.0 g tartaric acid L^{-1}). The grape berries were crushed without breaking the seeds, filtered off the skins and seeds, and then the juice was centrifuged at 3000 rpm for 10 min, at room temperature. The analyses were made in duplicate, the sample was thermostatted at 40 ºC (*ca*. 5 min), and 3 mL of grape juice was sucked and pressed by a pump into an IR transparent cell.

2.3. *HS-SPME methodology*

The HS-SPME methodology was used as previously described for red grape analysis [9]. The SPME holder for manual sampling and fibre used were purchased from Supelco (Aldrich, Bellefonte, PA). The SPME device included a fused silica fibre, partially cross-linked Carbowax-divinylbenzene (CW-DVB) with 65 µm film thickness. The SPME fibre was conditioned at 250 ºC for 30 min in the GC injector port, according to the manufacturer's recommendations. For headspace sampling, 50 g of grapes were crushed manually in a plastic bag and introduced into a 120 mL glass vial, which correspond to a ratio of the volume of the sample to the headspace ($1/\beta$) of 0.5. Because the mean weight of each grape berry from Ped was higher than that from TAv (Table 1), the amount of berries necessary to reach 50 g was always higher in TAv than in Ped. The vial was capped with a PTFE septum and an aluminium cap (Chromacol Ltd., Welwyn Garden City, UK) after the addition of 8 g of NaCl and a 25 mm stirring bar (1000 rpm), and was placed in a thermostatted bath adjusted to 40 ºC for 60 min to transfer the compounds from the sample to the headspace. Following this step, the SPME fibre was manually inserted into the sample vial headspace for 60 min. This procedure allows to obtain, in the grapes headspace, a fraction of the free volatile components from the skin and pulp plus the compounds arising from the reactions that may take place by the acidic conditions used (pH of the grapes *ca*. 3.8) and by the endogenous enzymatic activity during 120 min (60 min without fibre + 60 min with the fibre). The compounds detected under these conditions were named "variety- and pre-fermentation-related volatile compounds" [9]. Since headspace volume can be a critical factor that needs to be controlled [18], vials of the same lot and from the same producer were used.

2.4. *GC-qMS analysis*

The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250 ºC and kept for 15 min for the thermal desorption. The injection port was lined with a 0.75 mm ID splitless glass liner. The desorbed volatile compounds were separated in a GC-qMS Agilent Technologies 6890 N Network gas chromatograph, equipped with a 30 m × 0.32 mm I.D., 0.25 µm film thickness DB-FFAP fused silica capillary column (J&W Scientific Inc., Folsom, CA, USA), connected to an Agilent 5973 quadrupole mass selective detector. Splitless injection mode was used (5 min). The oven temperature was programmed from 35 to 220 ºC at 2 ºC min^{-1}, and the transfer line was heated at 250 ºC. Helium carrier gas had a flow of 1.7 mL min^{-1}. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 33–300 m/z in a 1 s cycle, in a full scan acquisition mode. Identification of volatile compounds was achieved comparing the GC retention times and mass spectra, with those, when available, of the pure standard compounds. All mass spectra were also compared with the data system library (Wiley 275), other published spectra [19], and according with the compounds described for the musts and wines of this variety [14–16]. All measurements were made with, at least, five replicates, each replicate representing the analysis of one different aliquot (50 g) of crushed grapes under analysis. The reproducibility is expressed with error bars in the figures. The GC peak area data were used as an indirect approach to estimate the relative content of each volatile compound and to follow their evolution during ripening. Blanks, corresponding to the analysis of the coating fibre not subjected to any extraction procedure, were run between sets of three analyses. Statistical significant differences among sampling times were evaluated using Student's *t*-test at the $p < 0.05$ level.

2.5. *Principal component analysis (PCA)*

A PCA was applied to the normalised areas of the 25 compounds identified by HS-SPME/GC-qMS present in 12 samples (from two different vineyards, in six different stages of ripening), each with five replicates. The initial projection of the samples on the first two principal components space (scores) was not easy to interpret due to distribution of the samples along the two main axes which also hindered the interpretation of the corresponding loadings. Hence, a rotation of 45º was applied to the plane defined by the first two principal components in order to uncover the characteristics under study.

3. Results and discussion

3.1. *Variety- and pre-fermentation-related volatile compounds*

Table 2 shows the variety- and pre-fermentation-related volatile compounds found in FP grapes from TAv and Ped vineyards grouped by chemical classes: five C_6 compounds (alcohols and aldehydes), 16 terpenoids, two C_{13} norisoprenoids, and two aromatic alcohols. All these compounds occur in both vineyards. A total ion chromatogram of the HS-SPME/GC-qMS analysis of grapes with the indication of the varietal volatile compounds under study is shown in Fig. 1. As expected, the majority of these compounds were previously found as components of FP musts obtained by liquid–liquid dichloromethane continuous extraction [14,16]. The differences observed may be due to: (i) the application of different volatile extraction methodologies, (ii) the use of different vintage, and (iii) the higher extent of pre-fermentative phenomena in musts, due to the higher

Table 2
Varietal and pre-fermentative related volatile compounds found in Talhão da Avenida (TAv) and Pedralvites (Ped) vineyards, by HS-SPME/GC-qMS

Retention time (min)	Peak number	Compound	Identification[a]
C_6 compounds			
3.80	1	*n*-Hexanal	A, B, C
7.57	3	(*E*)-2-hexenal	A, B, C
14.08	5	1-Hexanol	A, B, C
15.51	6	(*Z*)-3-hexenol	A, B, C
16.77	7	(*E*)-2-hexenol	B, C
Monoterpenoids			
5.91	2	Limonene	A, B, C
8.99	4	α-Terpinolene	B, C
17.98	8	Linalool *Z*-furanic oxide	B, C
24.55	9	Linalool	A, B, C
27.84	10	Hotrienol	B, C
30.66	11	*Z*-citral	A, B, C
31.87	12	α-Terpineol	A, B, C
33.51	13	*E*-citral	A, B, C
34.15	14	Linalool *E*-pyranic oxide	B, C
35.76	15	Linalool *Z*-pyranic oxide	B, C
36.11	16	Citronellol	A, B, C
36.92	17	γ-Isogeraniol	B
37.57	19	Nerol	A, B, C
40.23	20	Geraniol	A, B, C
45.58	24	Terpendiol I	B, C
62.32	25	Geranic acid	A, B, C
C_{13} norisoprenoids			
37.46	18	*E*-β-damascenone	A, B, C
43.26	23	β-Ionone	A, B, C
Aromatic alcohols			
41.09	21	Benzyl alcohol	A, B, C
42.59	22	2-Phenylethanol	A, B, C

[a] The reliability of the identification or structural proposal is indicated by the following: A – mass spectrum and retention time consistent with those of an authentic standard; B – structural proposals given on the basis of mass spectral data (Wiley 275); C – mass spectrum consistent with spectra found in literature.

time of contact between the must and the solid fraction of the grape, when compared with the 120 min used in the present study. Sixteen of the 25 varietal volatile compounds found in grapes were also detected in musts, namely, the three C_6 alcohols; 10 terpenoids: linalool *Z*-furanic oxide, linalool, hotrienol, α-terpineol, linalool *E*- and *Z*-pyranic oxide, nerol, geraniol, 3,7-dimethyl-1,5-octadien-3,7-diol (terpendiol I), and geranic acid; the two aromatic alcohols, and one C_{13}-norisoprenoid:

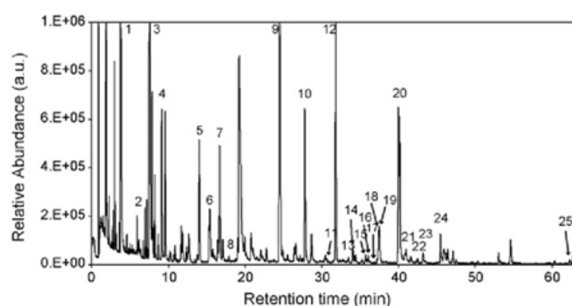

Fig. 1. Total ion chromatogram of the HS-SPME/GC-qMS analysis of 'Fernão-Pires' grape at day 20 after *véraison*, with the indication of the varietal and pre-fermentation-related volatile compounds (attribution of peak numbers shown in Table 2). a.u. – arbitrary units.

E-β-damascenone. From these, linalool, hotrienol, α-terpineol, geraniol, and 3,7-dimethyl-1,5-octadien-3,7-diol have recently been reported [15] to contribute to the varietal aroma of FP monovarietal wines. Linalool has characteristic citrus-like, sweet and flowery notes, and hotrienol, α-terpineol and geraniol exhibit flowery and sweet aromas [14,20,21].

The total amount of *variety- and pre-fermentation*-related volatile compounds was, during ripening, always higher in TAv vineyard than in Ped, varying almost twice more at day 0 to 1.5 times at day 35 (Table 3). This trend is followed by monoterpenoids and C_6 compounds, the major contributing groups for the total area of *variety- and pre-fermentation*-related volatile compounds. On the contrary, aromatic alcohols showed always areas twice higher in Ped than in TAv vineyard. The areas of C_{13} norisoprenoids were always similar in both vineyards. As the berries from Ped vineyard were bigger than those from TAv (Table 1), to reach 50 g of sample it was necessary to use more berries from TAv than from Ped vineyard. Also, because the skins are the major source of monoterpenoids [22], it is expected to find higher amount of these volatile compounds in TAv. Furthermore, as the origin of C_6 compounds was related mainly to the lipooxygenase activity of the grape [13,23] and/or must aeration [24], it is expected their formation after the crushing of the skins. The berries from higher sun exposure clusters are known

Table 3
GC peak area of monoterpenoids (MT), C_{13} norisoprenoids, aromatic alcohols and C_6 compounds found in Talhão da Avenida (TAv) and Pedralvites (Ped) vineyards during ripening, by HS-SPME/GC-qMS

Days after *véraison*	GC peak area × 10^{-6} (arbitrary units)									
	Talhão da Avenida					Pedralvites				
	MT	C_{13} norisoprenoids	Aromatic alcohols	C_6 compounds	Total	MT	C_{13} norisoprenoids	Aromatic alcohols	C_6 compounds	Total
0	392.31*	15.91*	3.31	560.17*	971.7*	129.83*	13.46	5.59	353.68*	502.6*
7	519.49*	13.42*	3.95	766.66	1303.5*	210.95	11.36*	5.39*	507.92	735.6
14	598.87*	10.73*	3.48*	758.86*	1371.9*	230.59*	12.20*	5.38*	480.84*	729.0*
20	701.97*	12.63*	8.11*	997.79*	1720.5	381.04*	20.03*	15.98	908.44*	1325.5*
28	449.55	9.92	5.05	694.18	1158.7	247.76	9.17	9.18	511.80	777.9
35	463.66	9.10	4.40	810.98	1288.1*	319.06*	10.32	8.25	547.36	885.0*

* Values significantly different from the week before ($p < 0.05$), $n = 5$, for the same class of compounds.

to be lighter than those less sunlight exposed [2]. The higher amount of volatile compounds of TAv grapes can thus also be related to the higher sunlight exposure [2,25–27], explained by the less dense canopy of the TAv vineyard and its localization in an open space. Sun exposure is reported as a factor that greatly influences grape ripening, as radiation and heat from sunlight can influence metabolic reaction rates and cause stress, either by dehydration or by direct temperature increase [2,28]. Direct exposure of grapes to the sunlight may lead to an increase in monoterpenoid content [29]. Also, modification of fruit environment by hedging and basal leaf removal, or crop level reduction was shown to increase the amount of free and bound terpenoids [30,31].

3.2. Monoterpenoids

Fig. 2 shows the GC peak areas of the monoterpenoids along ripening. Linalool was the monoterpenoid with major area. At *véraison* (day 0), linalool represented 66% of the monoterpenoids in TAv and 47% in Ped. Compounds that contribute with more than 5% for the terpenoids total area were α-terpineol (10%), hotrienol (10%), geraniol (5%), and α-terpinolene (5%) in TAv, and hotrienol (23%), geraniol (8%), and α-terpineol (6%) in Ped (Fig. 2 and Table 3). The relative proportion of the monoterpenol's GC peak areas found in grapes was similar to the proportion of these compounds found in wine [15].

Table 3 shows that total amount of monoterpenoids increased from *véraison* to day 20, where a maximum was reached, decreasing in the following week. Linalool exhibited the highest area, which influence considerably this tendency (Fig. 2). However, the majority of all other monoterpenoids followed the same increasing tendency (Fig. 2). In both vineyards, during the 2 weeks after day 20, the total area of each monoterpenoid was much lower than that of day 20. For the majority of the analyses, in both vineyards, these variations were significantly different ($p < 0.05$) from the week before.

3.3. C_{13} norisoprenoids

Fig. 3 shows the GC peak areas of *E*-β-damascenone and β-ionone along ripening. As observed for monoterpenoids, the total amount of C_{13} norisoprenoids increased since *véraison* to day 20, where a maximum was reached, showing a decrease in the following weeks (Table 3). For the majority of the analyses, in both vineyards, these variations were significantly different ($p < 0.05$) from the week before.

The amount of free β-damascenone in grapes has been reported to be much lower than the levels of hydrolytically liberated β-damascenone [32]. However, in crushed grapes, it seems probable that pre-fermentative hydrolysis reactions, catalysed by the endogenous enzymes and acidic medium, took place releasing the C_{13} norisoprenoids to the headspace phase. Although the generation of β-damascenone requires several steps, namely, hydrolysis of glycosidic precursor, reduction of Grasshopper ketone, and dehydration, as the experimental conditions were mild when compared to the conventional acidic hydrolysis (pH *ca.* 2) and hydrolysis by heat treatment (*ca.* 100 ºC), it is possible that the C_{13} norisoprenoids detected were mostly those that were in the free form in the native grape and only a small portion could have arisen from the catalysis by the endogenous enzymes and by the assay conditions. In both vineyards, the area of *E*-β-damascenone was at least twice higher than that of β-ionone.

3.4. Aromatic alcohols

The profile observed for the total area of monoterpenoids and C_{13} norisoprenoids during ripening can also be observed for benzyl alcohol and 2-phenylethanol (Fig. 3). For the majority of the analyses, these variations were significantly different ($p < 0.05$) from the week before in both vineyards. The aromatic alcohols, even showing a varietal origin, are produced mainly during fermentation [33], which explains their lower percentage (Table 3). In both vineyards, the area of benzyl alcohol was at least three times higher than that of and 2-phenylethanol.

3.5. C_6 compounds

In the present study, the use of crushed grapes, simulating the pre-fermentative step of wine making, promotes the formation of C_6 compounds. Two C_6 aldehydes (hexanal and *E*-2-hexenal) and three C_6 alcohols (1-hexanol, *Z*-3-hexenol and *E*-2-hexenol)

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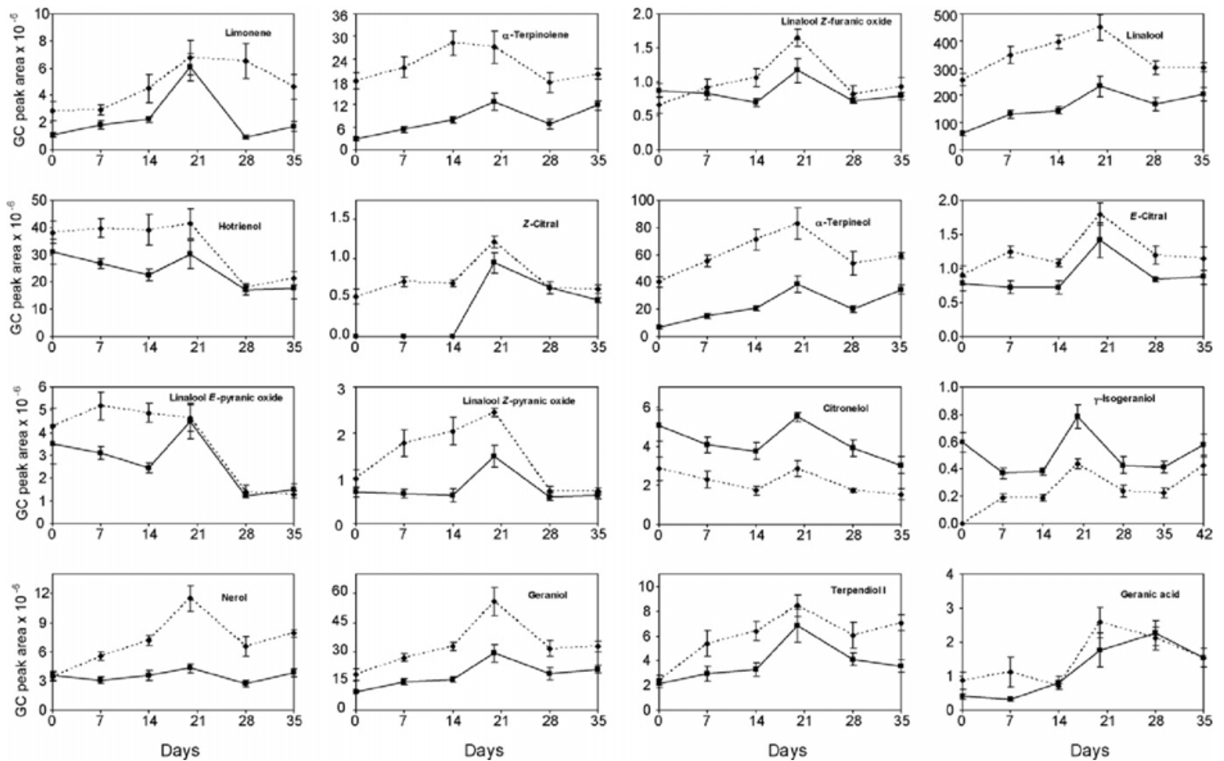

Fig. 2. GC peak area of the monoterpenoids found in Talhão da Avenida (- -♦- -) and Pedralvites (–■–) vineyards during ripening.

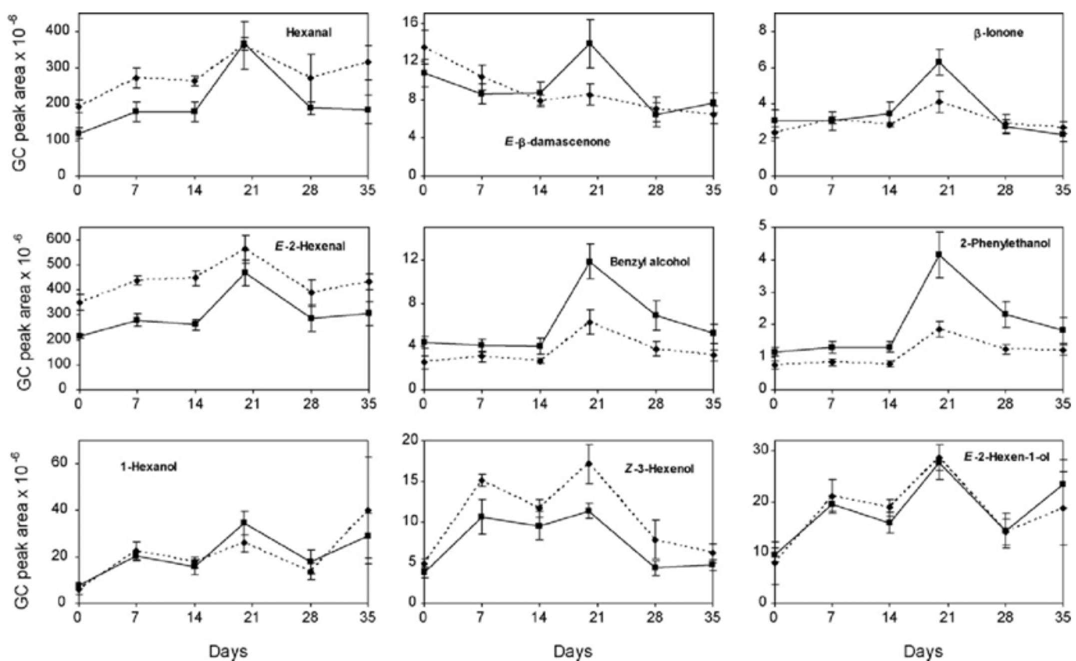

Fig. 3. GC peak area of the C_6 compounds, C_{13}-norisoprenoids and aromatic alcohols found in Talhão da Avenida (- -♦- -) and Pedralvites (–■–) vineyards during ripening.

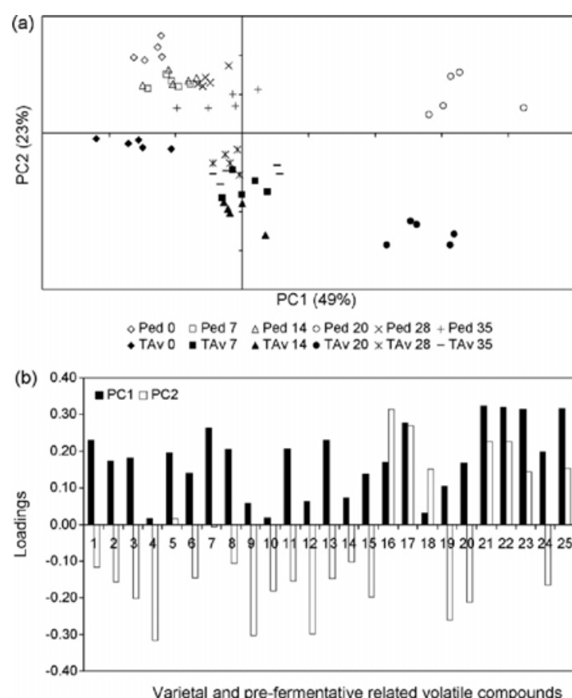

Fig. 4. Rotated PC1 × PC2 of (a) scores scatter plot and (b) loadings plot of the main source of variability between the sampling days in two vineyards and the volatile and pre-fermentation-related compounds; (attribution of peak numbers shown in Table 2).

were found in FP grapes. These compounds, usually related to the lipooxygenase activity of the grapes [13,23], increased, in both vineyards, from day 0 to 7 and from day 14 to 20. The maximum was reached at day 20 (Fig. 3), decreasing in the following weeks to values that approached those observed at *véraison*.

3.6. PCA

The PCA was used to study the main sources of variability between the different sampling days across ripening in the two vineyards, and to establish relationships between the ripening stage (in both vineyards) and variety- and pre-fermentation-related volatile compounds. Fig. 4a shows the rotated scores scatter plot of the two first principal components (that explains 72% of the total variability of the data set) which represents the distinction among the 12 samples. Fig. 4b represents the corresponding loadings plot which establishes the relative importance of each volatile component, and is therefore useful for the study of relations among the volatile compounds and relations between volatile compounds and samples. PC1, which explains 49% of the total variability, distinguishes TAv 20 and Ped 20 samples, placed in PC1 positive, from all other samples, placed in PC1 negative or near origin. According to the loadings plot, the samples are distinguished by the higher GC peak area of all individual volatile compounds under study. The samples with lower GC peak area of volatiles were placed in PC1 negative or near origin, and those with higher GC peak area of volatiles were placed in PC1 positive. The samples with higher positive PC1 values were the grapes collected from both vineyards at day 20. According to the chemical parameters of FP grapes during ripening shown in Table 1, the maximum berry weight was reached, for both vineyards, at days 7–14 after *véraison*, and the maximum sugar content was attained at days 20 and 35 in TAv and Ped, respectively. Furthermore, in both vineyards, from day 20 the acidity level stabilized. Thus, day 20 was considered closer to the harvesting day for white wine production.

PC2, which contains 23% of the total variability, distinguish TAv from Ped vineyards. Ped (PC2 positive), is characterized by higher amounts of the monoterpenoids citronellol, γ-isogeraniol, and geranic acid, the C_{13} norisoprenoids *E*-β-damascenone and β-ionone, and the aromatic alcohols benzyl alcohol and 2-phenylethanol. TAv (PC2 negative), is characterized by higher amounts of all other monoterpenoids and C_6 compounds. The major difference between the two vineyards is associated with the compounds abundance, which is in accordance with the total GC peak area of the different chemical groups of variety- and pre-fermentation-related volatile compounds in the two vineyards (Table 3).

4. Concluding remarks

Sixteen monoterpenoids, two C_{13} norisoprenoids, two aromatic alcohols, two C_6 aldehydes, and three C_6 alcohols were identified as variety- and pre-fermentation-related volatile compounds of FP white grapes provided from two different vineyards in Bairrada Appellation. Only quantitative differences were observed between the two vineyards, linalool being the most abundant monoterpenoid. The amount of variety- and pre-fermentation-related volatile compounds, estimated based on their GC peak area, increased since *véraison* until day 20 and, from that day, a sharp decrease was observed. As the maximum amount of volatile compounds is reached at the same time for the compounds that potentially contribute to the varietal character (fruit, sweet, floral, and citric notes of monoterpenoids, C_{13} norisoprenoids, and aromatic alcohols) and for the compounds that may contribute with herbaceous notes (C_6 compounds), attention should be paid to avoid the deleterious effect associated with the presence of the later ones in FP wines. However, the presence of C_6 compounds in FP musts and wines has been reported to be in concentrations lower than their sensory perception limits [14,15].

According to the ratio sugar/acidity determined for the grapes, the maximum amount of volatile compounds, correspondent to their maximum GC peak area, was coincident with the harvesting day for white table wine production (day 20 after *véraison*). Furthermore, according to the different characteristics of the wine to be produced (for example table or sparkling wine), the winemaker uses grapes with different ratio sugar/acidity, corresponding to different maturation states. Thus, it is of great importance to know the evolution of volatiles during ripening and the correspondent ratio sugar/acidity. This type of information about the raw material of wine represents an additional helpful tool to support the winemaker decision.

As observed for red grapes, the maximum of variety- and pre-fermentation-related volatile compounds of this white variety is coincident with the maturity. However, contrarily to the red ones, it sharply decreases in the weeks following after harvesting. This behaviour of the varietal volatile evolution observed for FP shows that the maximum volatile content occurs only in a very short period. As a consequence, the establishment of the optimum moment for harvesting of FP white variety, based on its volatile content, deserves much more accuracy than that necessary for the already studied red grape varieties.

This work also evidences that the analysis of the evolution of the terpenoids with higher GC peak area can be representative of the evolution of all varietal compounds. For FP grapes, this work shows that the screening of linalool, α-terpineol, and geraniol during ripening can be used to define the evolution profile of the varietal volatile compounds. The identification of only few compounds that can represent the behaviour of them all during ripening (e.g. linalool, α-terpineol, and geraniol in Fernão-Pires) allows to propose a simplified technique based on HS-SPME/GC-qMS as a suitable methodology to define the evolution profile.

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2. Análise da composição volátil dos vinhos espumantes

Para a análise da composição volátil dos vinhos espumantes foi optimizada uma metodologia de microextracção que permite usar uma grande quantidade de fase estacionária, a extracção sorptiva em barra de agitação (SBSE). A composição volátil foi analisada por SBSE seguida de desorção líquida combinada com injecção de grandes volumes e GC–qMS (SBSE–LD/LVI-GC–qMS). Esta metodologia foi optimizada ao nível da extracção (SBSE), da desorção líquida (LD) e da instrumentação (LVI). Para conhecer em que condições pedológicas e de maturação é que as castas maioritárias da Bairrada produzem uma melhoria em termos de compostos voláteis varietais e fermentativos, foram elaborados vinhos espumantes a partir das castas FP e BG em diferentes estados de maturação e solos. Para a casta FP foram produzidos vinhos monovarietais com uvas em três estados de maturação (uma semana antes do dia adequado para a vindima, no dia adequado à vindima e uma semana depois do dia adequado à vindima) e uvas colhidas no dia da vindima provenientes de três tipos de solo (argilo-calcário, argiloso e arenoso). Com a casta BG foram produzidos vinhos monovarietais com uvas colhidas no dia da vindima. Foi também produzido um vinho mistura (50:50) em mosto das castas FP e BG, também do dia da vindima. A composição volátil (monoterpenóides, sesquiterpenóides, norisoprenóides em C_{13}, álcoois e ésteres) das sete modalidades de vinhos espumantes produzidos foi quantificada por SBSE–LD/LVI-GC–qMS.

O capítulo referente à análise da composição volátil dos vinhos espumantes está dividido em dois artigos, ambos publicados na *Analytica Chimica Acta*. No primeiro artigo optimiza-se a metodologia de SBSE–LD/LVI-GC–qMS para a análise de vinhos espumantes e no segundo aplica-se a metodologia desenvolvida à análise dos vinhos espumantes da Bairrada.

2.1. Optimisation of stir bar sorptive extraction with liquid desorption combined with large volume injection-gas chromatography-quadrupole mass spectrometry for the determination of volatile compounds in wines

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Optimisation of stir bar sorptive extraction and liquid desorption combined with large volume injection-gas chromatography–quadrupole mass spectrometry for the determination of volatile compounds in wines

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A B S T R A C T

Stir bar sorptive extraction and liquid desorption followed by large volume injection coupled to gas chromatography–quadrupole mass spectrometry (SBSE–LD/LVI-GC–qMS) had been applied for the determination of volatiles in wines. The methodology was optimised in terms of extraction time and influence of ethanol in the matrix; LD conditions, and instrumental settings. The optimisation was carried out by using 10 standards representative of the main chemical families of wine, *i.e.* guaiazulene, *E,E*-farnesol, β-ionone, geranylacetone, ethyl decanoate, β-citronellol, 2-phenylethanol, linalool, hexyl acetate and hexanol. The methodology shows good linearity over the concentration range tested, with correlation coefficients higher than 0.9821, a good reproducibility was attained (8.9–17.8%), and low detection limits were achieved for nine volatile compounds (0.05–9.09 μg L^{-1}), with the exception of 2-phenylethanol due to low recovery by SBSE. The analytical ability of the SBSE–LD/LVI-GC–qMS methodology was tested in real matrices, such as sparkling and table wines using analytical curves prepared by using the 10 standards where each one was applied to quantify the structurally related compounds. This methodology allowed, in a single run, the quantification of 67 wine volatiles at levels lower than their respective olfactory thresholds. The proposed methodology demonstrated to be easy to work-up, reliable, sensitive and with low sample requirement to monitor the volatile fraction of wine.

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1. Introduction

The aroma is an important parameter of wine quality, in which the bouquet is the product of a biochemical and technological sequence, and it is influenced by the volatile varietal components (monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids), as well as those produced during fermentation. Alcohols, esters, acids, aldehydes, ketones, lactones, terpenoids, and phenols, representing more than 800 volatile compounds, have already been identified in grapes and wines [1]. The volatile composition of the wine is one of the most important factors to determine its aroma character and quality.

The low concentration of the majority of the volatile compounds present in a wine matrix makes enrichment as a basis for identification and quantification. For this purpose, liquid–liquid extraction (LLE) followed by gas chromatography coupled to mass spectrometry using the common quadrupole analyser (GC–MS) has been the analytical method of choice. Nevertheless, LLE is a time consuming and labour intensive technique, involving multi-step procedures and use of toxic solvents [2]. Nowadays, the solventless approaches such as solid phase microextraction (SPME) and, more recently, stir bar sorptive extraction (SBSE), showed to be environmental friendly alternatives due to its easy of use, high selectivity, high sensitivity and reproducibility, and less time consuming than the conventional techniques for which the most important theoretical considerations have been already described [3,4]. Similarly to SPME in SBSE, the efficiency of analyte partitioning between the polymeric phase and water, can be predicted by the octanol–water partitioning coefficients ($K_{PDMS/W} \approx K_{O/W}$) at the equilibrium [5]. Therefore, the recovery of an analyte from the sample can theoretically be calculated through the equation $m_{SBSE}/m_0 = (K_{O/W}/\beta)/(1 + (K_{O/W}/\beta)$, where m_{SBSE} is the amount of analyte in the polydimethylsiloxane (PDMS) phase, m_0 is the total amount of analyte originally present in the sample, $K_{O/W}$ is the octanol–water partition coefficient, β (= V_W/V_{SBSE}) is the phase ratio, V_W is the volume of water and V_{SBSE} is the volume of PDMS phase. Thus, the recovery of an analyte from the sample becomes only dependent of the ratio of partitioning constant ($K_{O/W}$) and the phase ratio (β), between the PDMS volume of the stir bar and the water sample volume [5].

SBSE have been widely used in several type of applications, especially for the association with thermal desorption (TD) systems followed by GC–MS analysis. However, the TD units are expensive devices and, although this approach presents a remarkable sensitivity, it is not the most indicated to analyse thermolabile compounds due the very high desorption temperatures of operation. In addition, TD does not offer the opportunity of reanalysis, which is an important issue in many studies for validation purposes. Alternatively, SBSE with liquid desorption (LD) has shown very interesting features to overcome these limitations and, besides being cost-effective, it has been successfully applied to the analysis of several classes of semi-volatile compounds in many types of matrices prior to GC–MS analysis [6–9]. Meanwhile, SBSE combined with LD as far as we know was never proposed for the enrichment of volatile compounds, which can be easily performed by trapping the analytes followed by removal through back-extraction with a small volume (typically microliters) of a convenient organic solvent. Subsequently, a very small aliquot, *i.e.* 0.5–2 μL, of this extract is injected in the gas chromatograph for analysis. Although this approach works quite successfully, the sensitivity can be highly enhanced through the combination of LD with large volume injection (LVI) [6–9].

Although several studies have been already published using the SBSE–TD/LVI-GC–MS methodology to characterize the volatile fraction of wine [3,4,10,11], the use of liquid desorption was never suggested as an alternative. The aim of the present work is the development of a novel approach for the analysis of volatile compounds using SBSE–LD followed by LVI-GC–MS and the application of the methodology to characterize the volatile components of wine. For this purpose, the most important SBSE–LD parameters were systematically optimised, namely, extraction time, influence of the ethanol matrix, back-extraction time and solvent type, and the main LVI instrumental settings. The performance of the methodology was evaluated in terms of accuracy, precision, limits of detection and linearity, and a lack-of-fit test was also performed, using for such purpose standard compounds representative of the main chemical classes usually found in the wine volatile composition, *i.e.* monoterpenoids, sesquiterpenoids, C_{13} norisoprenoids, esters, and aliphatic and aromatic alcohols. Finally, the optimised methodology was applied in real matrices, such as sparkling and table wines.

2. Experimental

2.1. Samples and reagents

White sparkling wine (WSW), and white table wine (WTW) and red table wine (RTW) were used. Their ethanol content varied between 10 and 14%.

Analytical grade ethanol (99.8%, Riedel-de Haën), methanol (MeOH; 99.9%, Fluka), acetonitrile (ACN; 99.9%, Fluka), diethyl ether (99.5%, Riedel-de Haën), acetone (99.9%, Fluka), 2-propanol (99.5%, Aldrich), *n*-pentane (99%, Riedel-de Haën), tartaric acid (foodstuff grade, José M. Vaz Pereira), and NaOH (98%, AnalaR) were used. Ultra-pure water was obtained from a Milli Q system (Millipore, Bedfords, MA, USA). Hexanol (98%), hexyl acetate (>99%), linalool (98.5%), 2-phenylethanol (99%), β-citronellol (95%), ethyl decanoate (>99%), geranylacetone (98%), β-ionone (97%), and *E*,*E*-farnesol (96%) standards were supplied from Sigma–Aldrich Química S.A. (Madrid, Spain) and guaiazulene (>98%) standard was supplied from TCI Europe N.V. (Zwijndrecht, Belgium). The stock solutions of individual standards were prepared in analytical grade ethanol.

2.2. Optimisation of SBSE conditions

This step was carried out by using wine model solutions fortified with standards, which were prepared from 27 mL of an aqueous solution of 0.5% tartaric acid adjusted with NaOH at pH 3.5, then was added to 2.8 mL of absolute ethanol spiked with 200 μL of a mixture of the 10 standards, in a total of 30 mL. Each wine model solution was introduced into a glass vial (30 mL; Macherey-Nagel, Düren, Germany), a stir bar (Twister;

Gerstel, Müllheim a/d Ruhr; Germany) containing a PDMS coating film (0.5 mm thick; 10 mm long, 24 μL) was immersed, and the vial was closed with a seal (aluminium seals with PTFE septa) using a manual crimper (Agilent Technologies, Little Falls, DE, USA). Assays were performed in a fifteenth agitation point plate (Variomag Multipoint komet, Thermo Fisher Scientific Inc., Waltham, MA, USA) at room temperature (20 ºC), with extraction times of 15, 30 and 60 min and a rotation speed of 800 rpm. The influence of ethanol matrix was tested for three contents, *e.g.* 10, 12, and 14% (v/v).

To evaluate the best LD conditions, several assays using back-extraction solvents (pentane, diethyl ether, MeOH, and azeotropic mixtures constituted by MeOH:acetone, MeOH: ACN, MeOH:pentane, MeOH:diethyl ether, ACN:pentane, acetone:pentane and 2-propanol:pentane) were also performed. For back-extraction purposes, the stir bars were placed into 250-μL glass flat-bottom inserts filled with 200 μL of solvent inside a glass vial, or into 2 mL glass vials filled with 1 mL of solvent for ensuring the total immersion. The back-extraction was performed by using ultrasonic treatment (Branson 3510, Branson Ultrasonic Corporation, Danbury, USA) and desorption time was tested for 15 and 30 min at constant temperature (25 ºC). After back-extraction, the stir bars were removed by means of a magnetic rod and the vials were closed with seals, using a hand crimper, and placed in the automatic liquid sampler tray for LVI-GC–qMS analysis. After each extraction, the stir bars were cleaned to dryness under a stream of purified nitrogen followed by a cleaning with ACN. All the experiments were performed at least in triplicate.

2.3. Instrumental settings

LVI-GC–qMS analysis were performed on an Agilent 6890 Series gas chromatograph equipped with an Agilent 7683 automatic liquid sampler tray (Agilent 7683, Agilent Technologies, Little Falls, DE, USA) coupled to an Agilent 5973 N mass selective detector (Agilent Technologies, Little Falls, DE, USA). A programmed temperature vaporization injector (PTV) with a septumless sampling head having a baffled liner (SLH; Gerstel, Mülheim a/d Ruhr, Germany) was used, operating in the solvent vent mode with liquid nitrogen as inlet cooling. For LVI, the solvent vent injection mode was performed (vent time: 0.30 min; flow rate: 5, 10, 20, and 50 mL min^{-1} ; pressure: 0 psi; purge: 60 mL min^{-1} at 2 min), for which the inlet temperature was programmed from 0, 10, and 20 (0.35 min) to 300 ºC at a rate of 600 ºC min^{-1} and, subsequently, decreased to 200 ºC (held until end) at a rate of 50 ºC min^{-1}. The injection volume and speed were 20 μL and 100 μL min^{-1}, respectively. GC analysis was performed on a TRB-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness) capillary column (5% diphenyl, 95% dimethylpolysiloxane; Teknokroma, Spain). Helium as carrier gas was maintained in the constant pressure mode and the inlet pressure was 21.36 psi with a flow rate of 2.8 mL min^{-1}. The oven temperature was programmed from 40 ºC at 2 ºC min^{-1} to 175 ºC, then at 10 ºC min^{-1} to 220 ºC (5 min) in a 77.00 min running time. The transfer line, ion source, and quadrupole (q) analyser temperatures were maintained at 280, 230, and 150 ºC, respectively. A solvent delay of 4 min was selected. In the full-scan mode, electron ionization mass spectra in the range 35–550 *m*/*z* were recorded at 70 eV electron energy with an ionization current of 34.6 μA. The mass spectra were obtained in full-scan mode and compared with the Wiley's library reference spectral bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA). Data recording and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). For the determination of the retention indices (RI) a C_{10} –C_{24} *n*-alkanes series was used.

2.4. Analytical curves and wine analysis

Wine samples (30 mL) were analysed under the previously optimised experimental conditions: 60 min of extraction time, 10% ethanol content, pentane as the desorption solvent, 15 min of back-extraction time, 10 mL min^{-1} for the solvent vent flow rate, and 10 ºC for the inlet temperature.

For quantification purposes, analytical curves were performed for guaiazulene, *E*,*E*-farnesol, β-ionone, geranylacetone, ethyl decanoate, β-citronellol, 2-phenylethanol, linalool, hexyl acetate and hexanol, under the concentration range shown in Table 1. The analytical plots were made in a wine model solution (30 mL) with 10% ethanol, 0.5% tartaric acid adjusted to pH 3.5 with NaOH fortified with 200 μL of a mixture of all standards. Beyond these 10 standards representing the main chemical families in wine, other volatiles from these chemical families were also detected in wine. For the quantification of these, the structure related standard (functional group and chemical structure) were used: the monoterpenoids were quantified with linalool analytical plot, cyclic sesquiterpenes were quantified with guaiazulene, linear sesquiterpenoids were quantified with *E*,*E*-farnesol, C_{13} norisoprenoids were quantified with β-ionone, esters until eight carbon skeleton were quantified with hexyl acetate, esters with more than nine carbons skeleton were quantified with ethyl decanoate, and aliphatic and aromatic alcohols were quantified with hexanol and 2-phenylethanol, respectively.

Recovery tests comprising the 10 standards were done in sparkling and table wines. The wines were fortified with a mixture of the 10 compounds: guaiazulene (3.5 μg L^{-1}), *E*,*E*-farnesol (93 μg L^{-1}), β-ionone (23 μg L^{-1}), geranylacetone (27 μg L^{-1}), ethyl decanoate (91 μg L^{-1}), β-citronellol (320 μg L^{-1}), 2-phenylethanol (31.8 mg L^{-1}), linalool (282 μg L^{-1}), hexyl acetate (435 μg L^{-1}) and hexanol (378 μg L^{-1}). Although the concentration range reported in Table 1 for citronellol and linalool, linearity was observed until concentrations of 512 μg L^{-1} for both compounds (data not shown). These values were plotted against $K_{O/W}$ values for each standard, which were calculated with the SRC-KOWWIN v1.67 software package, according to a fragment constant estimation method [12].

3. Results and discussion

3.1. Optimisation of the parameters affecting the SBSE–LD/LVI-GC–qMS

3.1.1. Effect of the SBSE conditions

Several SBSE parameters were performed using standard conditions, according to previous work [3]. During our preliminary

Table 1 – Aroma descriptor, odour threshold (OT) [24,28,29], octanol–water partitioning coefficients ($\log K_{O/W}$), and calibration parameters (concentration range, slope, interception, correlation coefficients (r^2), P-value of lack-of-fit test (LOF), limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation (R.S.D.)) for the 10 volatile compounds under study in wine model solutions by SBSE–LD/LVI-GC–qMS, under optimised experimental conditions

Volatile compounds	Aroma descriptor	OT ($\mu g L^{-1}$)	$\log K_{O/W}$	Concentration range ($\mu g L^{-1}$)	Slope (R.S.D.)	Interception (R.S.D.)	r^2 (R.S.D.)	LOF, P-value	LOD ($\mu g L^{-1}$)	LOQ ($\mu g L^{-1}$)	R.S.D. (%)	Recovery (R.S.D.) SW	Recovery (R.S.D.) TW
Guaiazulene	–	–	4.37	0.3–5	1.78E+09 (7)	1.32E+05 (52)	0.9908 (2.8)	0.997	0.05	0.18	13.7	73.5 (9)	88.3 (7)
E,E-farnesol	Lemon, floral, anise, honey	20	5.77	6–113	3.58E+07 (6)	5.80E+05 (25)	0.9897 (0.9)	0.993	1.10	3.71	17.8	65.0 (7)	60.0 (18)
β-Ionone	Violets	0.09	4.29	6–189	7.21E+08 (3)	−7.37E+05 (139)	0.9987 (0.4)	0.999	1.81	6.02	15.2	95.3 (7)	87.3 (17)
Geranylacetone	Magnolia, green	60	4.36	6–174	7.07E+08 (3)	3.50E+04 (43)	0.9997 (0.2)	0.999	1.43	4.77	11.3	90.4 (8)	83.7 (12)
Ethyl decanoate	Grape	200	4.79	0.3–643	1.48E+09 (4)	−3.15E+06 (92)	0.9985 (0.6)	0.951	0.08	0.27	9.6	67.9 (9)	83.9 (10)
β-Citronellol	Rose	100	3.56	55–218	3.00E+07 (6)	−7.67E+05 (37)	0.9821 (0.6)	0.656	9.09	30.3	12.4	87.0 (7)	76.7 (11)
2-Phenylethanol	Roses	14,000	1.57	3,770–120,661	8.71E+05 (2)	1.71E+06 (59)	0.9978 (0.1)	0.429	161	537	12.7	37.7 (8)	32.6 (16)
Linalool	Flowery, muscat	25	3.38	11–113	1.90E+07 (4)	−1.01E+05 (27)	0.9916 (1.6)	0.724	2.56	8.54	14.1	84.3 (12)	60.3 (15)
Hexyl acetate	Fruit, herb	1,500	2.83	2–1,161	3.25E+08 (2)	4.07E+06 (49)	0.9990 (0.4)	0.390	0.63	2.11	8.9	71.2 (6)	79.3 (5)
Hexanol	Herbaceous, greasy	8,000	1.82	67–2,691	4.58E+06 (3)	1.80E+06 (11)	0.9881 (0.7)	0.167	2.21	7.36	13.2	58.9 (8)	72.1 (15)

Recovery was achieved for the 10 volatiles in sparkling (SW) and table wines (TW) obtained by SBSE–LD/LVI-GC–qMS, under optimised experimental conditions.

studies, the same sample matrix, vial volume, and PDMS coating (24 μL) were used in a two-phase system, *i.e.* a liquid sample-stir bar. A PDMS volume of 24 μL was chosen since this polymer is very effective for non-polar volatiles and its low amount used minimizes the size of the stir bar which is a key parameter in the back-extraction assays. Thus, the use of a minimum amount of solvent for LD is very convenient since avoiding the undesirable solvent evaporation step usually performed for sensitivity enhancement; it minimizes the possible volatile losses.

Based on the theory reported by Baltussen et al. [5], the extraction time was evaluated by stirring the sample during three different periods, *i.e.* 15, 30, and 60 min. Fig. 1a shows that for the compounds with higher volatility and functional alcohol groups (*e.g.* hexanol, linalool, 2-phenylethanol, and β-citronellol) no advantages were observed for extraction times higher than 15 min. Nevertheless, for the remaining compounds that present low volatility (*e.g.* ethyl decanoate, geranylacetone, β-ionone, *E,E*-farnesol, guaiazulene, and hexyl acetate) slight signal increments from 15 to 30 min and larger signal increments from 30 to 60 min were noticed. For the case of ethyl decanoate, geranylacetone, β-ionone, *E,E*-farnesol, and guaiazulene, the GC peak area almost double (Fig. 1a). No longer times were evaluated, since several authors had point out that for periods higher than 60 min, no advantages were observed for volatile enrichment purposes [8,9]. Therefore, the extraction time of 60 min was set for further experiments.

The influence of the ethanol matrix was evaluated by using matrices with 10, 12, and 14% ethanol. The data obtained is depicted in Fig. 1b, in which it is shown that, for these three ethanol contents, in general, the GC peak area of the volatile compounds did not differ significantly, with the exception of β-ionone for 14% in ethanol. The non-polar compounds, with low water solubility, are better dissolved in the ethanolic medium. Then, it was expected that for larger amounts of ethanol in the matrix these compounds could be lower recovered. This behaviour will be more effective with higher amounts of ethanol, like in sweet wines and distillate drinks, although for table wines the ethanol content seems to be a negligible parameter, this methodology could be also be applied to spirits if they was diluted to the rage under study. Consequently, the further studies using wine model solutions were performed with 10% ethanol.

3.1.2. Effect of the LD conditions

In a preliminary study, different desorption solvents were tested for the back-extraction process, including MeOH and ACN, since they are the most used for LD of the semi-volatile compounds analysed by SBSE [6–9]. Nevertheless, this analytical approach needs a solvent switch step, *i.e.* evaporation to dryness of the polar solvent used for desorption followed by addition of an organic solvent more suitable for LVI in solvent vent mode during GC–qMS analysis. In our particular case, the solvent switch step should be avoided to prevent possible losses of some volatile compounds during evaporation. Once the volatile fraction of the wine samples present high volatility, it is necessary to ensure that the extraction of these compounds is representative of the real composition. Thereby, by using this approach, it is also important to avoid

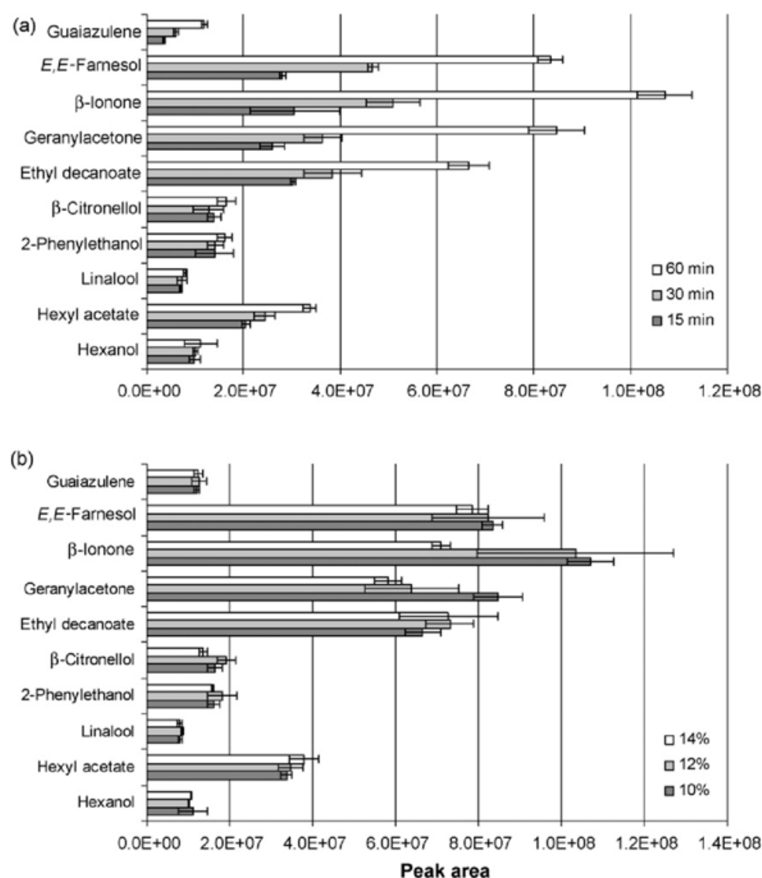

Fig. 1 – Effect of the extraction time (a) and matrix ethanol influence (b), on the peak area of the 10 volatile compounds under study by SBSE–LD/LVI-GC–qMS.

fronting peaks and guarantee precision, excluding ACN as a suitable solvent for LVI in solvent-vent mode. In the first step, He flow rate at 150 mL min^{-1} was used, based on the experimental conditions reported for the analysis of semi-volatile compounds [6–9]. However, this condition was not considered acceptable since from the 10 compounds under study, only five, *i.e.* ethyl decanoate, geranylacetone, β-ionone, *E,E*-farnesol, and guaiazulene were detected. These were the compounds with the higher molecular mass, whereas the most volatile ones were not detected at all. Therefore, instead of 150 mL min^{-1}, it was used 50 mL min^{-1}. However, the chromatographic performance of MeOH to this flow rate was unacceptable, presenting peaks with fronting. The chromatographic performance is very dependent from the He flow rate against solvent used; although it were detected more three volatile compounds (*i.e.* hexyl acetate, linalool and β-citronellol), and the peak areas were, in general, 1000 times lower. As a result, it was decided to test other solvents individually with lower boiling points (bp), such as pentane (bp = 35 ºC) and diethyl ether (bp = 35 ºC), as well as azeotropic mixtures such as MeOH:acetone (χ_{MeOH} = 0.24; bp = 54 ºC), MeOH:diethyl ether (χ_{MeOH} = 0.05; bp = 32 ºC), MeOH:pentane (χ_{MeOH} = 0.19; bp = 30 ºC), ACN:pentane (χ_{ACN} = 0.10; bp = 25 ºC), acetone:pentane ($\chi_{acetone}$ = 0.17; bp = 26 ºC) and 2-propanol:pentane ($\chi_{2\text{-}propanol}$ = 0.14; bp = 26 ºC) [13]. From all azeotropic mixtures tested, MeOH:acetone showed the best chromatographic signal. Additionally, with this particular mixture it was also evaluated the LD performance by desorption the stir bars with MeOH prior the addition of acetone reaching a equimolar proportion, using LVI with a solvent flow vent rate of He of 50 mL min^{-1} (Fig. 2a). In both cases, the reproducibility, expressed as error bars in Fig. 2a, was low, especially in the case of the MeOH:acetone azeotropic mixture. The results obtained with diethyl ether were unacceptable, as they presented peaks with fronting and do not allowing the detection of all standards. All the experiments carried out for selection of the back-extraction solvent indicated that pentane showed higher affinity for the back-extraction of the volatiles under study, showing standard deviation (error bars in Fig. 2a) lower than those obtained with other solvents and presenting superior recovery for the compounds with higher volatility, such as, hexanol, hexyl acetate, linalool, and 2-phenylethanol. Furthermore, no fronting peaks were achieved with pentane. Therefore, pentane was selected as the best back-extraction solvent for the LD process.

The time for the back-extraction process was tested for 15 and 30 min by using 250 μL glass flat-bottom inserts with

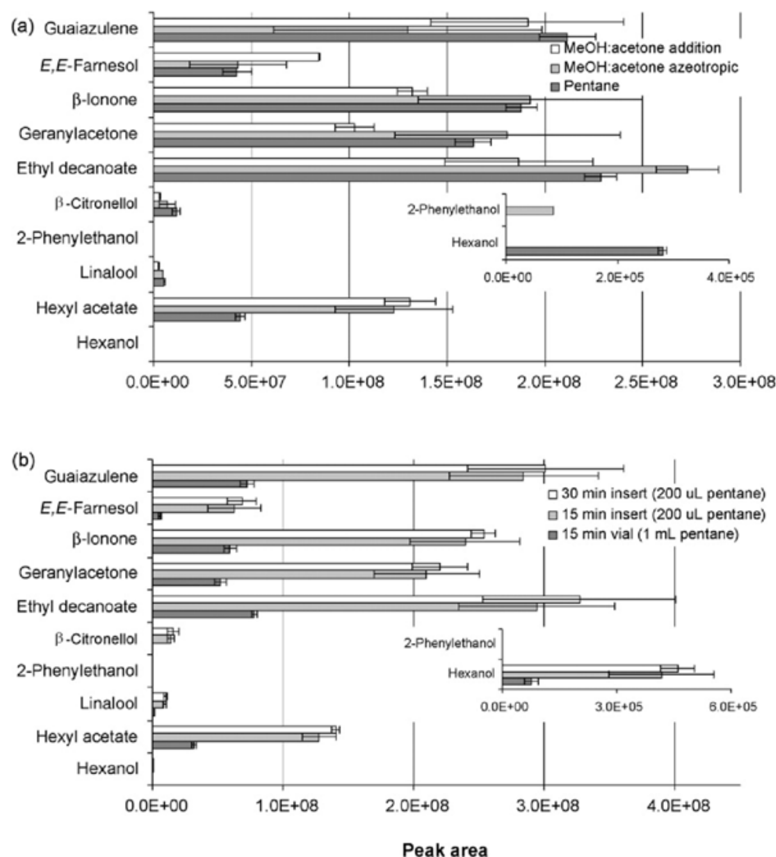

Fig. 2 – Effect of the back-extraction solvent type (a) and desorption time (b), on the peak area of the 10 volatile compounds under study by SBSE–LD/LVI-GC–qMS.

200 μL of pentane, and for 15 min by using 2.0 mL vials having 1 mL of pentane, ensuring the total stir bar immersion in both cases. These assays were performed under ultrasonic treatment at constant temperature (25 ºC) and the results obtained are shown in Fig. 2b. The desorption experiments inside the vials were performed during 15 min and the peak areas were 73 (ethyl decanoate) to 92% (*E,E*-farnesol) lower than with the glass flat-bottom insert alone, which reflects the diluting effect when a larger solvent volume was used. Negligible differences were observed for 15 and 30 min by using the 250 μL glass flat-bottom insert with 200 μL of pentane and, according to previous works [8,9], no advantages were obtained for longer periods than 15 min. The following studies were performed for 15 min by using the 250 μL glass flat-bottom insert with 200 μL of pentane.

3.1.3. Effect of the LVI conditions

Due to the dependence of the inlet purge flow rate and temperature during LVI in the solvent vent mode, pentane was tested under four flow rates, *i.e.* 5, 10, 20, and 50 mL min^{-1} . Fig. 3a shows that the He flow which allows better recovery for all standards was 5 mL min^{-1} , however unacceptable chromatographic shape resulting in undesirable fronting was observed. The lower recoveries were attainted with 50 mL min^{-1} . He flow rates of 10 and 20 mL min^{-1} showed similar recoveries, with the exception of hexyl acetate, where 10 mL min^{-1} promoted the higher recovery. Thus, 10 mL min^{-1} was chosen as the purge vent for the following experiments.

The initial inlet purge temperature during solvent vent mode is also a critical parameter that should be optimised since lower temperatures allow a better trapping of the volatiles. This has the drawback of decreasing, however, the chromatographic signal of the low molecular weight compounds. For the three temperatures tested, the recovery of the volatile compounds increase from the experiment with inlet temperature of 20 to 10 ºC, with the exception of ethyl decanoate, but decrease when the inlet temperature was 0 ºC (Fig. 3b). Furthermore, the higher reproducibility was observed for 10 ºC. Therefore, no advantages were obtained by using temperatures below 10 ºC, as shown in Fig. 3b. For the 10 volatile compounds under study, the inlet temperature at 0 ºC gave, in general, unexpectedly smaller peak areas compared with those obtained at 10 or 20 ºC. The mass transfer of the trapped analytes from the PTV inlet to the column is less effective at 0 ºC due probably to water condensation and frosting of the injection port, a phenomenon that was also reported by León et al. [14]. Therefore, as the higher reproducibility and recovery were observed for 10 ºC this was the cryofocusing inlet temperature selected for the further experiments.

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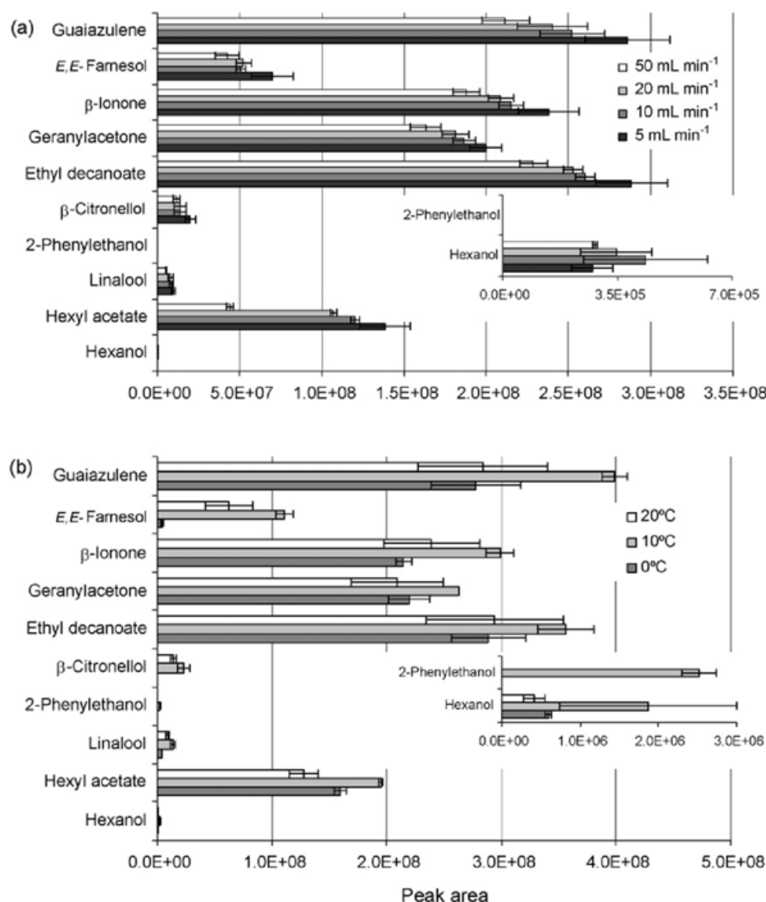

Fig. 3 – Effect of the inlet purge vent flow rate (a) and temperature (b) on the peak area of the 10 volatile compounds under study by SBSE–LD/LVI-GC–qMS.

3.2. Validation of the SBSE–LD/LVI-GC–qMS methodology

The aroma descriptor, odour threshold, octanol-water partitioning coefficients (log $K_{O/W}$), (OT), limits of detection (LOD) and quantification (LOQ), and the calibration parameters of 10 standards under the optimised experimental conditions are shown in Table 1. The analytical plots were constructed with a minimum of five concentration levels and in triplicate. The LODs (*i.e.* the minimum amount of each compound that can be reliably distinguished) of the methodology were achieved as the amount of each compound to provide a signal-to-noise ratio of 3 above the variability; the LOQs were achieved as the amount of each compound to provide a signal-to-noise ratio of 10 above the variability. The lack-of-fit test performed for the analytical plots, showed for all curves no lack of linear fit for a α = 0.05, as all *P*-values were higher than 0.05. Each standard under study may contribute individually to the solution aroma, with several fruity and floral notes, according to its aroma descriptor, if present in concentration equal or higher than its respective OT (Table 1). The exception was the guaiazulene, which aroma descriptor was not found in the literature. The LODs and LOQs obtained by SBSE–LD/LVI-GC–qMS were much lower than their respective OT, which indicates that this methodology, if used for wines, allows inferring the wine aroma properties based on its volatile composition.

Correlation coefficients, higher than 0.9821, were obtained for the 10 standards. The relative standard deviation (R.S.D.) was calculated by performing six consecutive extractions to the lower concentration of each volatile compound, which ranged from 8.9 to 17.8% for hexyl acetate and *E,E*-farnesol, respectively. The recovery ranged from 37.7 to 95.3% in sparkling wine, and from 32.6 to 87.3% in table wine, being 2-phenylethanol the compound that exhibited the lower recovery and β-ionone the higher recovery, in both wines.

Fig. 4 depicts the average recovery for the 10 standards in sparkling and table wines, under the conditions, plotted against their corresponding log $K_{O/W}$ (Table 1). The equilibrium theoretical line was calculated assuming a 30 mL of ultra-pure water (V_W) and a stir bar coated with 24 μL (V_{SBSE}) of PDMS, for which a phase ratio (β = V_W /V_{SBSE}) of 1153.85 was established. The log $K_{O/W}$ values ranged between 1.57 (2-phenylethanol) and 5.77 (*E,E*-farnesol) (Table 1). The recovery values obtained

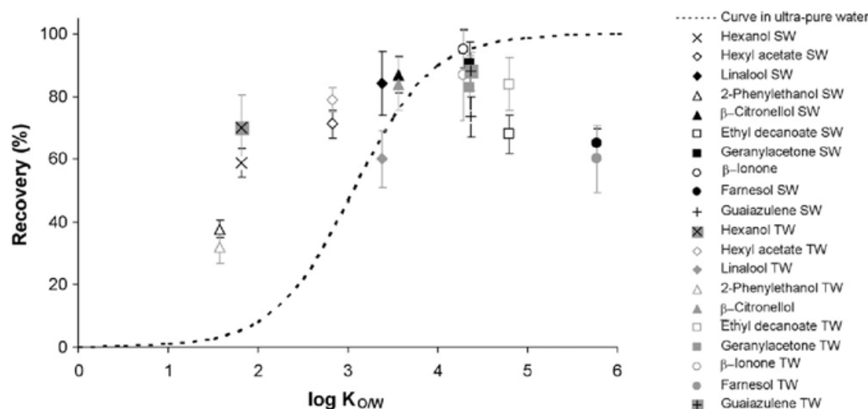

Fig. 4 – Theoretical (ultra-pure water) and experimental (SW-sparkling wine and TW-table wine) recovery plotted against log $K_{O/W}$ for the 10 volatile compounds by SBSE–LD/LVI-GC–qMS, under optimised experimental conditions.

in sparkling and table wines were similar, with the exception of the values obtained for linalool.

According to the SBSE theory, the distribution coefficients of the analytes between the PDMS and the water matrix ($K_{PDMS/W}$) are strongly correlated with the corresponding $K_{O/W}$. It is therefore expected that the non-polar compounds have a log $K_{O/W}$ > 4.5. From the 10 volatiles under study, only *E,E*-farnesol (log $K_{O/W}$ = 5.77) and ethyl decanoate (log $K_{O/W}$ = 4.79) exhibit log $K_{O/W}$ > 4.5; other three compounds, guaiazulene, geranylacetone, and β-ionone presented $K_{O/W}$ close to 4.5. It is therefore expected that the non-polar compounds should have larger affinity for the PDMS polymeric coating of the stir bars, which in the theoretical SBSE curve recovery will be greater than 96%. For instance, for β-ionone, having a log $K_{O/W}$ of 4.29, a theoretical recovery of 94.4% should be expected. Indeed, the experimental average recovery in ultra-pure water obtained for β-ionone was 95.3 and 87.3%, in sparkling and table wines, respectively, and therefore, the agreement among the expected and the experimental data was good. In general, this example with a volatile compound (β-ionone) clearly illustrates the behaviour for the non-polar end of the $K_{O/W}$ scale, where experimental data fit the theoretical line very well. The exception was observed for ethyl decanoate and *E,E*-farnesol, which the hypotheses of ethanol matrix influence, could explain this behaviour. These compounds have low polarity but they are ethanol soluble, and ethanol could act as a co-solvent. Contrarily, 2-phenyletanol and hexanol should present theoretical low recoveries, *i.e. ca*. 3 and 5%. However, the experimental data was 37.7 and 58.9%, respectively, in sparkling wine, and 32.6 and 87.3% in table wine. In this case, the ethanol seems to play a support role acting as an interface to make the sorption process of these compounds more extensive by the PDMS polymer. Therefore, the wine matrix, in a global way, could play particular synergisms, performing an important role on the efficient recovery of the volatile compounds.

3.3. Application to wines

To demonstrate the analytical ability of the SBSE–LD/LVI-GC–qMS to characterise the volatile components of wine, assays were performed in real matrices: a sparkling wine, and a white and a red table wines. Thus, beyond several volatile compounds detected by this methodology, this manuscript was focused only on the analysis of the five most important chemical classes. Table 2 shows the concentration of 67 volatile compounds detected in sparking and/or table wines, distributed as following: monoterpenoids (14), sesquiterpenoids (11), C_{13} norisoprenoids (4), esters (29) and alcohols (9).

The applicability of the SBSE–LD/LVI-GC–qMS methodology for the study of the wine volatile composition may be used for different purposes, such as definition of the varietal volatile profile closely related with grape origin traceability, verification if the volatile compounds are present in concentrations that might contribute to the wine aroma, and/or detection of compounds that may promote off-flavours, such as C_6 alcohols. Examples comprise these applications will be described.

Sparkling wine was produced according to the *Champagnois* methodology that is obtained from a base wine submitted to a second fermentation inside the bottle. During the second fermentation, more esters are produced by the yeast metabolism, and also by the bulk reactions that could take place between alcohols and acids. Consequently, it is expected a large number of fermentative volatile compounds (esters and alcohols) in this wine. The fermentative compounds represent in terms of concentration 99% of volatile compounds under study, where 2-phenylethanol, ethyl octanoate, ethyl hexanoate, diethyl succinate and ethyl decanoate were the major ones, with respectively 41686, 706, 702, 180 and 136 μg L^{-1} . For all types of wines, esters represented the chemical family that included the higher number of compounds; however the WSW presented higher number of esters (25) than the WTW (20) and RTW (20), although not exhibiting the highest ester concentration. These differences are expected as the three types of wines under study were provided from different winemaking processes, as well as from different varieties, geographical origins and harvests.

Beyond the fermentative compounds, the varietal ones are important volatile components of wines due mainly to their

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Table 2 – Concentration of volatile compounds of the main chemical classes detected in white sparkling wine (WSW), white table wine (WTW) and red table wine (RTW) obtained by SBSE–LD/LVI-GC–qMS

RI_{calc} [a]	RI_{lit} [b]	Volatile compounds	Concentration ($\mu g\ L^{-1}$)					
			WSW		WTW		RTW	
Monoterpenoids								
985	–	Geranic oxide (linaloyl oxide)	23.5	(8)[c]	170.0	(10)	37.1	(10)
1002	973	*Z*-Herboxide	16.6	(12)	–	–	–	–
1008	988	*E*-Herboxide	27.6	(6)	<LOD	–	–	–
1012	1033	Limonene	–	–	88.6	(7)	–	–
1021	–	n.i. monoterpene (*m*/*z* 93, 105, 121)	–	–	76.2	(17)	–	–
1024	–	n.i. monoterpene (*m*/*z* 93, 121, 136)	–	–	40.4	(7)	34.8	(9)
1044	1087	α-Terpinolene	10.9	(10)	98.5	(18)	–	–
1053	1098	Linalool	17.6	(14)	–	–	–	–
1081	1131	Nerol oxide	55.2	(7)	144.9	(10)	–	–
1085	–	n.i. monoterpene (*m*/*z* 71, 93, 121)	–	–	49.5	(12)	–	–
1210	–	n.i. monoterpene (*m*/*z* 93, 121, 136)	–	–	63.3	(22)	96.2	(29)
1216	–	n.i. monoterpene (*m*/*z* 93, 69, 136)	–	–	44.2	(23)	66.1	(19)
1233	1189	α-Terpineol	75.6	(11)	<LOD	–	–	–
1252	1260	Geraniol	29.4	(12)	271.8	(12)	–	–
Subtotal ($\mu g\ L^{-1}$)			256.3		1047.6		234.3	
Sesquiterpenoids								
1462	–	n.i. sesquiterpene (*m*/*z* 136, 123, 93)	<LOD	–	–	–	–	–
1467	–	n.i. sesquiterpene (*m*/*z* 136, 121, 93)	–	–	1.4	(17)	1.2	(14)
1476	1475	β-Chamigrene	0.5	(20)	–	–	–	–
1490	1568	Nerolidol	6.7	(6)	–	–	29.2	(15)
1620	–	n.i. sesquiterpene (*m*/*z* 189, 204, 161)	<LOD	–	1.4	(12)	1.2	(14)
1641	–	n.i. sesquiterpene (*m*/*z* 93, 121, 107)	<LOD	–	0.9	(4)	1.1	(17)
1649	–	(*E*,*Z*)-α-Farnesene	4.2	(6)	–	–	–	–
1650	–	n.i. sesquiterpene (*m*/*z* 93, 119, 204)	0.2	(22)	–	–	–	–
1690	–	Guaiazulene	0.2	–	–	–	–	–
1829	1742	(*E*,*Z*)-Farnesol	<LOD	–	–	–	84.1	(16)
1873	–	n.i. sesquiterpene (*m*/*z* 161,189, 204)	<LOD	–	–	–	–	–
Subtotal ($\mu g\ L^{-1}$)			11.7		3.8		116.8	
C_{13} Norisoprenoids								
1242	1286	Vitispirane	12.0	(8)	34.3	(79)	16.4	(3)
1278	–	TDN (1,1,6-trimethyl-1,2-dihydronaphthalene)	11.0	(22)	34.5	(13)	6.8	(4)
1397	1381	*E*-β-Damascenone	3.4	(13)	2.1	(16)	5.9	(52)
1611	–	2,5,8-Trimethyl-1,2-dihydronaphthalene	2.1	(18)	–	–	–	–
Subtotal ($\mu g\ L^{-1}$)			28.5		70.9		29.1	
Esters								
897	–	Ethyl lactate	<LOD	–	–	–	–	–
911	857	Ethyl 2-methyl butanoate	<LOD	–	<LOD	–	14.7	(12)
913	856	Ethyl 3-methyl butanoate	<LOD	–	9.7	(7)	32.5	(11)
924	876	3-Methyl-butylacetate	8.5	(16)	52.6	(9)	198.8	(13)
925	880	2-Methyl-butylacetate	<LOD	–	–	–	–	–
1006	1001	Ethy hexanoate	702.5	(16)	943.7	(9)	203.2	(9)
1007	1014	Hexyl acetate	–	–	2.7	(6)	<LOD	–
1020	–	Ethyl 2-hexenoate	–	–	<LOD	–	–	–
1046	–	Ethy heptadecanoate	–	–	<LOD	–	<LOD	–
1067	1126	Methyl octanoate	<LOD	–	<LOD	–	<LOD	–
1099	1167	Diethyl succinate (diethyl butanedioate)	180.5	(19)	227.7	(10)	536.7	(13)
1209	1196	Ethyl octanoate	706.3	(22)	1092.3	(12)	171.0	(4)
1228	1244	Ethyl phenylacetate	2.4	(43)	3.4	(15)	5.0	(16)
1233	–	3-Methylbutyl octanoate + n.i. (*m*/*z* 87, 59, 104)	3.0	(9)	–	–	3.5	(5)
1234	1256	2-Phenylethylacetate	4.0	(47)	3.9	(23)	7.9	(16)
1242	–	n.i. ester (*m*/*z* 117, 71, 89)	2.5	(50)	–	–	–	–
1250	–	n.i. ester (*m*/*z* 88, 138, 101)	–	–	5.3	(18)	4.1	(6)
1275	–	Di-isobutyl succinate	2.3	(16)	2.5	(12)	5.1	(16)
1282	–	2-Methylpropyl octanoate	2.3	(15)	2.6	(4)	–	–
1403	–	Ethyl 9-decenoate	3.2	(7)	–	–	6.1	(15)
1408	1397	Ethyl decanoate	136.0	(20)	397.5	(16)	36.1	(13)

Table 2 (*Continued*)

RI_{calc} [a]	RI_{lit} [b]	Volatile compounds	Concentration ($\mu g\ L^{-1}$)					
			WSW		WTW		RTW	
1425	–	Ethyl 3-methylbutyl butanedioate	6.2	(26)	12.5	(10)	44.9	(85)
1433	–	2-Methylbutyl octanoate	3.1	(26)	5.7	(23)	3.2	(3)
1608	1597	Ethyl dodecanoate	3.8	(23)	9.0	(16)	4.2	(22)
1628	–	2-Phenylethylbutanoate	2.3	(16)	–	–	–	–
1633	–	2-Methylbutyl decanoate	2.3	(19)	3.8	(4)	–	–
1686	1762	Benzylbenzoate	2.4	(15)	–	–	–	–
1825	–	2-Phenylethyl hexanoate	2.6	(38)	–	–	8.2	(64)
1833	–	2-Phenylethyl decanoate	2.4	(15)	–	–	–	–
Subtotal ($\mu g\ L^{-1}$)			1778.5		2775.1		1285.4	
Alcohols								
920	867	Hexanol	<LOD	–	1101.9	(17)	904.3	(14)
1012	–	2-Ethylhexanol	–	–	–	–	60.8	(6)
1016	–	Benzyl alcohol	–	–	–	–	<LOD	–
1021	–	3,5,5-Trimethyl-1-hexanol	–	–	–	–	251.7	(18)
1031	–	3-Ethyl-2-pentanol	–	–	–	–	462.8	(15)
1034	–	Octanol	–	–	–	–	<LOD	–
1058	1118	2-Phenylethanol	41686.5	(42)	47732.7	(16)	247190.1	(15)
1237	1263	Decanol	–	–	195.0	(30)	–	–
1252	–	2-Undecanol	–	–	–	–	80.3	(10)
Subtotal ($\mu g\ L^{-1}$)			41686.5		49029.6		248950.0	
Total volatile compounds ($\mu g\ L^{-1}$)			43761.4		52926.9		250615.6	

[a] RI: retention index calculated using C_{10}–C_{24} *n*-alkanes series.
[b] RI: retention index reported in the literature for 5% phenyl polysilphenylene-siloxane GC capillary column or equivalents.
[c] R.S.D. (%) in parentheses.

contribution to the varietal aroma peculiarities and biological activities. The optimised methodology allowed to quantify a large number of varietal volatile compounds (monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids), 29 from the total 67 compounds. As expected, the WTW accounted for the higher number (12) and concentration of monoterpenoids (1047.6 $\mu g\ L^{-1}$), followed by the WSW (eight compounds, 256.3 $\mu g\ L^{-1}$) and RTW (four compounds, 234.3 $\mu g\ L^{-1}$). As the monoterpenoids are secondary metabolites whose synthesis is encoded by variety-related genes, the terpenoid profile may be used as a way to trace the varietal origin [15]. The sesquiterpenoids were also reported as an important chemical group present in *Vitis vinifera* L. due to their aroma properties and also bioactive effect as anti-bacterial activity [16] or the ability to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds [17], being present especially in red varieties [18,19]. Table 2 shows that the higher concentration of sesquiterpenoids was observed in the RTW (116.8 $\mu g\ L^{-1}$), followed by WSW (11.7 $\mu g\ L^{-1}$) and WTW (3.8 $\mu g\ L^{-1}$). Nerolidol and (*E,Z*)-α-farnesol were the main sesquiterpenoids quantified. These compounds have been reported to occur as volatile components of some wines, being their origin also resultant of yeast activity during alcoholic fermentation [20]. β-Chamigrene and guaiazulene, detected only in WSW, are described for the first time as wine constituents. Four different C_{13} norisoprenoids were detected, the higher concentration was observed in the WTW (70.9 $\mu g\ L^{-1}$) and similar concentrations were accounted for RTW (29.1 $\mu g\ L^{-1}$) and WSW (28.5 $\mu g\ L^{-1}$).

The monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids represent, quantitatively, minor chemical families, have their origin in the grapes. These compounds have usually low perception limits (few $\mu g\ L^{-1}$), however could have the higher impact on wine aroma properties [21,22]. *E*-β-damascenone has odour descriptors of apple and sweet [23], and backed apple [24] and its OT in wines is 0.05 $\mu g\ L^{-1}$ [25]. All the wines analysed exhibited concentrations above its OT, suggesting the contribution of this compound to the wines aroma. The OT of 2-phenylethanol is 14,000 $\mu g\ L^{-1}$ [26], and all the wines analysed exhibited concentrations above its OT, indicating that this compound contribute with roses note. The esters ethyl hexanoate and ethyl octanoate have fruity and anise as odour descriptors, and their OT are 5–14 $\mu g\ L^{-1}$ and 2–5 $\mu g\ L^{-1}$, respectively [25,26]. All the wines analysed exhibited concentrations higher than their OT, suggesting their individual contribution to the wine aroma. Furthermore, the sensorial contribution to the overall aroma of a substance, when its concentration is at least 20% of the OT should be considered [27].

C_6 alcohols have herbaceous and greasy odours, which have been related to deleterious effects in the wines, although in white wines a small herbaceous perception is appreciated by some consumers. Their origin has been reported to be related mainly to the lipooxygenase activity of the grape and/or must aeration. The limit of perception for hexanol was estimated as 8 mg L^{-1} (Table 1). According to Table 2, hexanol was above its OT in all wines analysed and a contribution to an herbaceous aroma may not be excluded.

4. Conclusions

In the present work, stir bar sorptive extraction with liquid desorption followed by large volume injection and capillary gas chromatography coupled to quadrupole mass spectrometry (SBSE–LD/LVI-GC–qMS) was applied for the first time in the analysis of the volatile fraction of wine samples. The main parameters that are known to influence the methodology are fully discussed, such as liquid desorption and large volume injection parameters. According to the results obtained, the SBSE–LD/LVI-GC–qMS optimised experimental conditions were established: 60 min of extraction time, 10% ethanol content, pentane as desorption solvent, 15 min for the back-extraction period, 10 mL min^{-1} for the solvent vent flow rate and 10 ºC for the inlet temperature. The method proposed showed good linearity over the concentration range tested, with correlation coefficients higher than 0.9821, a good reproducibility was attained (8.9–17.8%) and, additionally, low detection limits were achieved for nine volatile compounds (0.05–9.09 μg L^{-1}), with the exception of 2-phenylethanol due to its low recovery by SBSE.

The SBSE–LD/LVI-GC–qMS methodology allowed, in a single run, the quantification of 67 wine volatiles that can be quantified accurately at levels lower than their respective olfactory thresholds. This type of information can be used to infer the potential contribution of the volatile compounds to the wine's aroma properties. This methodology showed great sensibility for the varietal compounds that only comprised *ca*. 1–2% of volatile fraction of wines. The deep discussion covering the experimental parameters involved in this methodology allows its extension to other types of beverages, as well as other liquid matrices.

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2.2. Quantification approach for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties by stir bar sorptive extraction with liquid desorption

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Quantification approach for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties by stir bar sorptive extraction with liquid desorption

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Stir bar sorptive extraction with liquid desorption followed by large volume injection coupled to gas chromatography–quadrupole mass spectrometry (SBSE-LD/LVI-GC–qMS) was applied for the quantification of varietal and fermentative volatiles in sparkling wines. The analytical data were performed by using suitable standards of monoterpene hydrocarbons (α-pinene), monoterpenols (linalool), sesquiterpenoids (*E*,*E*-farnesol, *Z*-nerolidol, and guaiazulene), C_{13} norisoprenoids (β-ionone), aliphatic and aromatic alcohols (hexanol and 2-phenylethanol), and esters (hexyl acetate and ethyl decanoate) as model compounds. The wine volatiles were quantified using the structurally related standards. The methodology showed good linearity over the concentration range tested, with correlation coefficients ranging from 0.950 to 0.997, and a reproducibility of 9–18%. The SBSE-LD/LVI-GC–qMS methodology allowed, in a single run, the quantification of 71 wine volatiles that can be quantified accurately at levels lower than their respective olfactory thresholds. This methodology was used for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties. The variety and soil influenced significantly the volatile composition of sparkling wines; lower effect was observed for the ripening stage of grapes picked up one week before or after the maturity state.

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1. Introduction

According to the *Champagnois* method, sparkling wine is a double fermented wine. After the first alcoholic fermentation, it is submitted to a second fermentation inside the bottle by addition of the *tirage* liquor (suspension of yeasts and sugar). Thus, its aroma is influenced by the varietal components (monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids), as well as by those produced during the fermentation processes. Alcohols, esters, acids, aldehydes, ketones, lactones, terpenoids, and phenols, representing more than 800 volatile compounds, have already been identified in grapes and wines [1]. The volatile composition of the wine is one of the most important factors to determine its aroma character and quality. The monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids play an important role in the wine varietal character, contributing to their differentiation.

'Baga' (BG) is the main variety in Bairrada Appellation, an ancient winemaking region in Portugal. This variety represents 92% of the red grape vineyard, and 80% of the overall Bairrada vineyard. Volatile composition of BG monovarietal wine studied by liquid–liquid dichloromethane continuous extraction includes aliphatic and aromatic alcohols, aliphatic acids, esters, phenols, lactones, and amides [2]. Sesquiterpenoids, which have been detected for the first time by the use of solid phase microextraction (SPME), represent 56–80% of varietal compounds of BG grapes at maturity [3]. 'Fernão-Pires' (FP) is the main white grape variety harvested in Bairrada Appellation, representing 80% of the white vineyard and 10% of the overall Bairrada vineyard. Studies carried out on grapes and table wine of FP variety showed that monoterpenoids, aromatic alcohols, and C_{13} norisoprenoids were the main chemical compounds that contribute to the varietal volatile characteristics of this variety [4–7].

Environmental factors (topographical, agro-pedological, climatic, etc.), usually described by the French term "terroir", influence grape and wine composition and quality [8]. However, the dependence of grape berry attributes on environmental conditions, as well as the possible effects of the soil types still remains uncertain. Soil may affect water and nutrient availability to the plant by its retaining capacity, may affect the microclimate by its heat-retaining and light reflecting capacity, and may affect the root growth by its penetrability [8]. Deficit water status imposed by same types of soils and climate parameters have been shown

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to increase the oenological potential of Agiorgitiko red grape variety [9].

The low concentration of the majority of the volatile compounds present in a wine matrix makes enrichment as a basis for identification and quantification. Nowadays, the solventless approaches such as SPME and, more recently, stir bar sorptive extraction (SBSE), showed to be environmental friendly alternatives due to its ease of use, high selectivity, high sensitivity and reproducibility, and less time consuming than the usually used solvent extraction techniques [10,11]. SBSE has been widely used in several types of applications, especially associated with thermal desorption (TD) systems on-line with gas chromatography–mass spectrometry (GC–MS). However, the TD units are expensive devices and, although this approach presents a remarkable sensitivity, it is not the most indicated to analyse thermolabile compounds due the very high desorption temperatures of operation (up than 200 ºC) which can contribute to the occurrence of artefacts. In addition, TD does not offer the opportunity of reanalysis, which is an important issue in many studies for validation purposes. Several studies have already been published using the SBSE-TD/LVI-GC–MS methodology to characterize the volatile fraction of wine [10–13].

The volatile composition of sparkling wines has already been characterized by HS-SPME/GC, in order to evaluate the development of volatiles during second fermentation and aging [14–16]. The volatile fraction of these wines was composed mainly by esters, alcohols, and acids. The C_{13} norisoprenoids and terpenoids were absent in all studies. The volatile fraction of Spanish sparkling wine (Cava) has been studied by three different techniques: simultaneous distillation extraction (SDE), closed-loop stripping analysis, and HS-SPME. Beyond the esters, alcohols, and acids, terpenoids such as limonene, α-terpineol, and lilial were also detected by SDE [17]. SBSE combined with LD was recently proposed for the enrichment of volatile compounds in sparkling and table wines, which can be easily performed by trapping the analytes followed by removal through back-extraction with a small volume (200 μL) of a convenient organic solvent such as pentane, combined with large volume injection [18].

The volatile composition of natural products is usually composed by several compounds corresponding to different chemical structures and presenting very different relative amounts. The corresponding chromatograms present several co-elutions and/or interferences, even if it was used relatively long chromatographic programmes. Thus, the aim of this manuscript is to develop a new approach for the determination of volatiles in sparkling wines based on the SBSE-LD/LVI-GC–qMS methodology previously developed [18]. The quantification of individual components was achieved by linear regression using structurally related standards and suitable ion extraction chromatography (IEC). This methodology was applied to BG and FP sparkling wines produced from grapes obtained from different ripening stages and soils.

2. Experimental

2.1. Samples and reagents

Sparkling wines from two grape varieties, *i.e.* 'Fernão-Pires' (FP) and 'Baga' (BG), having different ripening stages and soils were used. To produce FP wines grapes were picked up at three harvest moments: (1) at the adequate harvest maturity to produce sparkling wines, determined by the physical–chemical parameters berry texture and colour, sugar content, and titratable acidity (FP_{HC}); (2) at an early harvest moment, one week before maturity harvest ($FP_{Early\ HC}$); and (3) at a late harvest moment, one week after maturity harvest ($FP_{Late\ HC}$). All samples were obtained from a clayey soil. Samples were also collected in soils presenting different textures: sandy (FP_{HS}) and clay-calcareous soils (FP_{HCC}). BG wine was produced from ripe grapes (adequate harvest moment to produce sparkling wines determined by the physico–chemical parameters) and one soil type, clay, (BG_{HC}). A mixture of musts (50:50) obtained from BG and FP grapes picked up at harvest moment from clayey soil was also used to produce sparkling wines (FP_{HC}+BG_{HC}). The sparkling wines were produced according the *Champagnois* method, and two independent winemaking replications were performed for each type of wine ($FP_{Early\ HC}$, FP_{HC}, $FP_{Late\ HC}$, FP_{HS}, FP_{HCC}, BG_{HC}). The second fermentation was performed inside the bottles, after *tirage* and at least four different bottles were analysed for each type of wine, in a total of 24 bottles. The exception of this strategy was the mixture FP_{HC}+ BG_{HC} for which only two bottles were obtained. The wines were analysed after 24 months of *dégorgement* (removal of yeast sediment from bottles). Each bottle was analysed twice.

Analytical grade ethanol (99.8%, Riedel-de Haën), acetonitrile (ACN; 99.9%, Fluka), *n*-pentane (99%, Riedel-de Haën), tartaric acid (foodstuff grade, José M. Vaz Pereira), and sodium hydroxide (NaOH; 98%, AnalaR) were used. Ultra-pure water was obtained from a Milli Q system (Millipore, Bedford, MA, USA). α-Pinene (97%), hexanol (98%), hexyl acetate (> 99%), linalool (98.5%), 2-phenylethanol (99%), ethyl decanoate (> 99%), β-ionone (97%), *Z*-nerolidol (96%) and *E,E*-farnesol (96%) standards were supplied from Sigma–Aldrich Química S.A. (Madrid, Spain) and guaiazulene (> 98%) standard was supplied from TCI Europe N.V. (Zwijndrecht, Belgium). Stock solutions of individual standards were prepared in analytical grade ethanol.

2.2. SBSE-LD methodology

SBSE-LD experimental parameters were optimized according to a previous work [18]. Thirty mililiters of sparkling wine or wine model solution (used to prepare the analytical plots, see Section 2.4) were introduced into a glass vial (30 mL; Macherey-Nagel, Düren, Germany), a stir bar (Twister; Gerstel, Müllheim a/d Ruhr; Germany) containing a polydimethylsiloxane (PDMS) coating film (0.5 mm thick; 10 mm long, 24 μL) was immersed, and the vial was closed with a seal (aluminium seals with PTFE septa) using a manual crimper (Agilent Technologies, Little Falls, DE, USA). Assays were performed in a fifteenth agitation point plate (Variomag Multipoint Komet, Thermo Fisher Scientific Inc., Waltham, MA, USA) at room temperature (20 ºC), with extraction times of 60 min and a rotation speed of 800 rpm. For back-extraction purposes, the stir bars were placed into 250 μL glass flat-bottom inserts filled with 200 μL of pentane inside a glass vial. The back-extraction was performed by using ultrasonic treatment (Branson 3510, Branson Ultrasonic Corporation, Danbury, USA) and desorption time was 15 min at constant temperature (25 ºC). After back-extraction, the stir bars were removed by means of a magnetic rod and the vials were closed with seals, using a hand crimper, and placed in an automatic liquid sampler for LVI-GC–qMS analysis. After each extraction, the stir bars were dried under a gentle stream of purified nitrogen followed by cleaning with ACN.

2.3. Instrumental settings

LVI-GC–qMS analysis were performed on an Agilent 6890 Series gas chromatograph equipped with an automatic liquid sampler (Agilent 7683, Agilent Technologies, Little Falls, DE, USA) coupled to an Agilent 5973 N mass selective detector (Agilent Technologies, Little Falls, DE, USA). A programmed temperature vaporization injector (PTV) with a septum-less sampling head having a baffled liner (SLH; Gerstel, Mülheim a/d Ruhr, Germany) was used, operating in the solvent vent mode with liquid nitrogen as inlet cooling. For LVI, the solvent vent injection mode was performed

Table 1
Analytical plots used for the quantification of volatile compounds, aroma descriptor, odour threshold (OT), concentration range, recovery, relative standard deviation (R.S.D.) and calibration parameters (selected ion (*m/z*), correlation coefficients (r^2), limit of detection (LOD), and limit of quantification (LOQ)) obtained by SBSE-LD/LVI-GC–qMS.

Volatile compounds	Aroma descriptor	OT (μg L^{-1})	Concentration range (μg L^{-1})	Recovery (%)	R.S.D. (%)	Selected ion (*m/z*)	r^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
α-Pinene	Pine	6	3–67	43	9	93	0.996	0.7	2.2
1-Hexanol	Herbaceous, greasy	8,000	43–532	59	13	56	0.972	7.5	24.9
Hexyl acetate	Fruit	1,500	2–1161	71	9	88	0.997	0.1	0.4
	Herbaceous					73	0.997	0.05	0.2
						101	0.997	0.2	0.7
Linalool	Flowery	25	11–113	84	14	93	0.953	6.1	20.4
	Muscat					71	0.950	8.2	27.2
	Lemon					67	0.956	13.4	44.8
2-Phenylethanol	Rose, sweet	14,000	675–13504	38	13	91	0.956	416.5	1388.2
Ethyl decanoate	Grape	200	0.3–643	68	10	88	0.992	0.2	0.6
						101	0.992	0.2	0.7
β-Ionone	Violet	0.09	6–189	95	15	177	0.997	3.3	10.9
						91	0.995	3.2	10.8
Z-Nerolidol		–	3.33–133	42	17	69	0.988	1.4	4.7
E,E-Farnesol	Lemon	20	6–113	65	18	69	0.970	1.5	5.0
	Floral, anise					93	0.987	1.8	6.1
	Honey					204	0.972	2.2	7.5
Guaiazulene	–	–	0.3–5	74	14	183	0.951	0.4	1.2
						91	0.957	0.2	0.6

according to a previous work [18] (vent time: 0.30 min; flow rate: 10 mL min^{-1}; pressure: 0 psi; purge: 60 mL min^{-1} at 2 min), for which the inlet temperature was programmed from 10 ºC (0.35 min) to 300 ºC at a rate of 600 ºC min^{-1} and, subsequently, decreased to 200 ºC (held until end) at a rate of 50 ºC min^{-1}. The injection volume and speed were 20 and 100 μL min^{-1}, respectively. GC analysis was performed on a TRB-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness) capillary column (5% diphenyl, 95% dimethylpolysiloxane; Teknokroma, Spain). Helium as carrier gas was maintained in the constant pressure mode and the inlet pressure was 21.36 psi with a flow rate of 2.8 mL min^{-1}. The oven temperature was programmed from 40 ºC at 2 ºC min^{-1} to 175 ºC, then at 10 ºC min^{-1} to 220 ºC (5 min) in a 77.00 min running time. The transfer line, ion source, and quadrupole (q) analyser temperatures were maintained at 280, 230, and 150 ºC, respectively. A solvent delay of 4 min was selected. In the full-scan acquisition mode, electron ionization mass spectra in the range 35–550 *m/z* were recorded at 70 eV with an ionization current of 34.6 μA. Ion extraction chromatograms were obtained from the full-scan acquisition mode using the selected ions presented in Tables 1 and 2. The mass spectra data were compared with the Wiley library reference spectral bank (G1035B; Rev D.02.00; Agilent Technologies, USA). Data recording and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies, USA). For the determination of the retention indices (RI) a C_{10} -C_{24} *n*-alkanes series was used.

2.4. Analytical plots

For quantification purposes, analytical plots were performed for α-pinene, hexanol, hexyl acetate, linalool, 2-phenylethanol, ethyl decanoate, β-ionone, *Z*-nerolidol, *E,E*-farnesol, and guaiazulene standards, by using wine model solutions fortified with standards. The wine model solutions were prepared by measuring 27 mL of an aqueous solution of 0.5% tartaric acid adjusted with NaOH at pH 3.5, then adding 2.8 mL of absolute ethanol (10% ethanol–v/v) spiked with 200 μL of a mixture of the 10 standards, performing a total of 30 mL. The concentration ranges of each standard in the wine model solutions are shown in Table 1. A minimum of five concentration levels were used to build the analytical curves. All the experiments were performed at least in triplicate. The calibration curves were carried out by using the IEC mode at the *m/z* characteristic values for each standard (Table 1). Recovery tests comprising the 10 standards were done in wine. The wines were fortified with a single addition of a combined standard solution. The recovery for each compound is shown in the Table 1.

3. Results and discussion

3.1. Quantification approach for assessment of sparkling wine volatiles

As shown in Fig. 1a, the total ion chromatogram (TIC) from the sparkling wines is very complex, combining major compounds, having very high signal intensity, with minor compounds, most of them presenting co-elution. This is a chromatographic profile typical of natural products, corresponding to several chemical structures showing very different relative amounts. Generally, in these cases, the accurate quantification, as well as the corresponding identification, is always a very hard step. The full-scan acquisition mode widely used during GC–MS analysis allows the detection and quantification of a large number of compounds. This approach is particularly interesting for complex matrices such as wine samples. Nevertheless, it usually cannot overcome many co-elutions neither low chromatographic resolution. For these particular cases, single ion monitoring mode, which increases the specificity and sensitivity, can be a possibility. Another way to overcome the lack of resolution is the use of IEC that allows the analysis of a global volatile profile by combining the spectral evidence with the target ion selection and retention time, thus minimizing the co-elution effect. IEC mode is always performed by selecting characteristic target ions from the analytes under study and extracting them from the TIC. By using this approach, the lack of chromatographic resolution can be definitely overcome by the remarkable spectrometric selectivity [10]. In our study, two specific co-elutions were selected to demonstrate the potential application of the IEC mode. In the first case, the very intense peak of diethyl succinate co-elutes with alloocimene, and the detection of the later can only be possible by selecting the ion at *m/z* 93 (Fig. 1b). Similarly, another co-elution was observed with 3-methylbutyl-octanoate, α-terpineol, and 2-phenylethylacetate, where the detection of each compound was

Table 2
Concentration of volatile compounds of the main chemical classes detected in the different types of sparkling wines obtained by SBSE-LD/LVI-GC–qMS.

RI_{cal} [a]	Volatile compounds	Ion (m/z)	Concentration of sparkling wines (μg L^{-1})													
			Clay-Calcar		Sand		Clay									
			FP_{HCC}		FP_{HS}		$FP_{Early\ HC}$		FP_{HC}		$FP_{Late\ HC}$		BG_{HC}		FP_{HC} + BG_{HC}	
			X (n = 8)	(R.S.D.)	X (n = 8)	(R.S.D.)	X (n = 8)	(R.S.D.)	X (n = 8)	(R.S.D.)	X (n = 8)	(R.S.D.)	X (n = 8)	(R.S.D.)	X (n = 3)	(R.S.D.)
	Monoterpenoids															
954	α-Pinene	93	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
985	Geranic oxide (linaloyl oxide)[b]	93	84.72	(15)	54.58	(40)	10.80	(59)	38.16	(70)	32.76	(20)	<LOD		<LOD	–
1002	*Z*-Herboxide[b]	93	30.91	(19)	15.63	(59)	<LOD	–	9.47	(76)	13.96	(38)	–	–	–	–
1008	*E*-Herboxide[b]	93	17.03	(27)	11.86	(39)	6.11	(43)	10.30	(24)	11.48	(28)	<LOD	–	<LOD	–
1015	Limonene[c]	93	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1044	α-Terpinolene[c]	93	1.07	(41)	<LOD	–	<LOD	–	<LOD	–	<LOD	–	–	–	<LOD	–
1053	Linalool	93	53.97	(23)	20.06	(38)	<LOD	–	11.96	(37)	19.63	(43)	–	–	<LOD	–
1055	Hotrienol[b]	71	43.52	(20)	37.01	(33)	24.17	(34)	21.13	(32)	30.75	(14)	<LOD	–	10.85	(39)
1081	Nerol oxide[b]	67	171.69	(16)	149.36	(29)	131.86	(43)	98.89	(34)	120.94	(9)	<LOD	–	40.08	(29)
1100	Alloocimene[c]	93	1.40	(14)	0.98	(21)	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1233	α-Terpineol[b]	93	117.20	(14)	58.99	(42)	12.92	(28)	48.08	(49)	44.17	(33)	8.63	(94)	11.61	(24)
1241	Nerol[b]	93	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1252	Geraniol[b]	93	72.26	(19)	32.38	(43)	<LOD	–	22.09	(46)	26.86	(27)	–	–	<LOD	–
1445	n.i. (m/z 121, 107, 93)[b]	93	29.69	(24)	14.77	(42)	14.30	(24)	17.98	(22)	13.83	(30)	9.27	(41)	11.22	(27)
1446	n.i. (m/z 121, 107, 91)[b]	93	34.80	(22)	17.76	(42)	16.54	(21)	22.61	(26)	15.56	(23)	10.00	(33)	12.29	(33)
1462	n.i. (m/z 136, 121, 123)[b]	93	12.02	(38)	10.52	(74)	10.43	(39)	13.47	(36)	8.70	(38)	16.17	(38)	6.44	(84)
	Subtotal (μg L^{-1})		670.28	(14)	423.9	(35)	227.13	(34)	314.14	(36)	338.64	(11)	44.07	(37)	92.49	(28)
	Sesquiterpenoids															
1476	β-Chamigrene[d]	93	3.18	(25)	2.71	(57)	2.55	(32)	3.10	(31)	1.86	(34)	2.74	(23)	1.94	(56)
1490	Nerolidol	69	3.46	(37)	1.90	(55)	<LOD	–	1.95	(56)	1.97	(33)	<LOD	–	1.64	(41)
1620	n.i. (m/z 189, 204, 161)[e]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1624	Cycloisolongifolene[e]	91	0.84	(22)	<LOD	–	–	–	<LOD	–	–	–	–	–	–	–
1641	n.i. (m/z 93, 121, 107)[d]	93	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1642	Cadalene[e]	183	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1649	(*E*,*Z*)-α-Farnesene[d]	93	2.57	(27)	2.56	(55)	<LOD	–	2.22	(25)	<LOD	–	<LOD	–	<LOD	–
1650	n.i. (m/z 93, 119, 204)[d]	93	2.78	(20)	1.90	(48)	<LOD	–	1.97	(35)	<LOD	–	<LOD	–	<LOD	–
1673	α-Cedrene[e]	91	0.27	(26)	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1690	Guaiazulene	183	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1829	(*E*,*Z*)-Farnesol[d]	69	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	–	–
1873	β-Patchoulene[d]	204	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
2073	α − Gurjunene[d]	204	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
	Subtotal (μg L^{-1})		13.10	(24)	9.07	(53)	2.55	(32)	9.24	(30)	3.38	(29)	2.74	(23)	3.58	(49)
	C_{13} Norisoprenoids															
1242	Vitispirane[f]	177	7.35	(10)	4.61	(39)	3.30	(19)	5.71	(36)	3.54	(21)	<LOD	–	<LOD	–
1278	1,1,6-Trimethyl-1,2-dihydronaphthalene[f]	91	5.12	(16)	<LOD	–	<LOD	–	3.92	(22)	<LOD	–	3.77	(29)	<LOD	–
1297	*E*-β − Damascenone[f]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1611	1,1,6-Trimethyl-1,2-dihydronaphthalene isomer[f]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
	Subtotal (μg L^{-1})		12.47	(12)	4.61	(39)	3.3	(19)	9.63	(29)	3.54	(21)	3.77	(29)	–	–
	Esters															
897	Ethyl lactate[g]	73	19.39	(66)	33.62	(68)	12.29	(23)	11.08	(68)	8.59	(71)	14.29	(81)	11.36	(36)
911	Ethyl 2-methylbutanoate[g]	88	11.80	(36)	15.57	(27)	9.80	(21)	8.77	(35)	10.09	(29)	6.55	(30)	3.10	(23)
913	Ethyl 3-methylbutanoate[g]	88	18.05	(29)	24.33	(26)	17.29	(30)	13.29	(42)	14.25	(9)	10.55	(19)	7.22	(16)
924	3-Methyl-butylacetate[g]	88	50.20	(13)	39.64	(26)	23.29	(20)	31.63	(14)	27.93	(25)	18.56	(38)	19.82	(21)
925	2-Methyl-butylacetate[g]	101	2.54	(21)	2.87	(44)	0.68	(45)	1.33	(41)	0.71	(48)	1.59	(143)	0.28	(126)
1006	Ethyl hexanoate[g]	88	640.27	(15)	279.13	(29)	869.63	(17)	746.78	(15)	716.02	(19)	795.35	(24)	878.09	(19)
1053	Ethyl heptanoate[g]	88	0.52	(15)	0.38	(43)	0.77	(14)	0.78	(36)	1.20	(8)	0.89	(48)	0.77	(24)
1067	Methyl octanoate[g]	101	1.95	(19)	<LOD	(159)	2.55	(64)	2.02	(81)	0.62	(53)	4.16	(43)	3.88	(22)

1099	Diethyl succinate (diethyl butanedioate)[g]	101	366.72	(14)	541.58	(25)	395.23	(20)	448.99	(15)	432.20	(17)	319.99	(22)	268.56	(29)
1209	Ethyl octanoate[g]	88	3223.69	(13)	1337.46	(31)	3747.92	(19)	3433.93	(16)	3057.87	(16)	3552.82	(22)	4049.64	(21)
1228	Ethyl phenylacetate[h]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1233	3-Methylbutyl octanoate[i]	88	3.58	(18)	1.45	(63)	0.53	(84)	1.51	(46)	1.45	(30)	<LOD	–	0.43	(135)
1234	2-Phenylethyl acetate[h]	91	434.57	(13)	<LOD	(32)	<LOD	(38)	425.03	(42)	424.54	(26)	<LOD	–	<LOD	–
1242	Diethyl dl-malate[i]	88	0.62	(119)	0.94	(152)	3.66	(66)	1.25	(53)	1.01	(65)	1.76	(71)	0.92	(29)
1246	Di-isobutyl succinate[i]	101	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1250	Diethyl glutarate[i]	88	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1257	Ethyl nonanoate[i]	88	0.19	(11)	<LOD	–	0.24	(16)	<LOD	–	<LOD	–	0.18	(24)	<LOD	–
1275	Di-isobutyl succinate isomer[i]	101	0.50	(34)	1.30	(40)	0.44	(28)	0.37	(38)	0.36	(27)	0.27	(38)	0.25	(38)
1282	2-Methylpropyl octanoate[i]	101	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1403	Ethyl 9-decenoate[i]	88	1.15	(30)	0.89	(45)	1.45	(19)	2.10	(59)	4.23	(16)	4.39	(87)	1.41	(48)
1408	Ethyl decanoate	88	140.15	(15)	59.49	(41)	116.42	(27)	123.63	(22)	97.35	(23)	145.61	(29)	158.85	(34)
1425	Ethyl 3-methylbutylbutanedioate[i]	101	7.74	(19)	13.56	(31)	6.67	(29)	6.60	(21)	6.20	(18)	3.99	(29)	3.26	(35)
1426	Ethyl 3-methylbutylbutanedioate isomer[i]	101	1.88	(22)	4.48	(52)	1.55	(27)	1.51	(23)	1.49	(18)	0.94	(27)	0.75	(34)
1433	2-Methylbutyl octanoate[i]	101	1.17	(21)	0.58	(77)	1.35	(24)	1.17	(19)	1.05	(17)	1.04	(38)	0.90	(49)
1477	Ethy 3-hydroxyoctanoate[i]	88	0.37	(132)	<LOD	–	0.52	(79)	0.68	(83)	0.31	(101)	0.59	(123)	0.30	(173)
1608	Ethyl dodecanoate[i]	88	0.76	(23)	0.51	(61)	0.79	(71)	1.33	(38)	0.62	(43)	1.68	(21)	1.42	(31)
1628	2-Phenylethyl butanoate[h]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1680	Ethy 3-hydroxydodecanoate[i]	88	0.62	(122)	0.27	(139)	0.45	(93)	1.31	(33)	0.25	(110)	0.77	(87)	0.31	(173)
1686	Benzylbenzoate[h]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	(31)	<LOD	–
1698	Ethyl tetradecanoate[i]	88	<LOD	–	<LOD	–	0.20	(40)	0.23	(42)	0.24	(84)	0.22	(33)	0.19	(13)
1825	n.i. 2-Phenylethyl ester (*m/z* 104, 105, 101)[h]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1833	2-Phenylethyl decanoate[h]	91	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–	<LOD	–
1900	Ethyl 9-hexadecenoate[i]	88	0.23	(15)	<LOD	–	0.35	(47)	0.28	(62)	0.35	(39)	0.24	(37)	0.32	(23)
2028	Ethyl hexadecanoate[i]	88	1.81	(50)	1.29	(61)	2.13	(31)	2.28	(37)	2.30	(23)	1.46	(37)	1.77	(20)
2293	Ethyl *Z*-9-octadecenoate[i]	88	0.25	(79)	0.23	(101)	0.64	(34)	0.62	(45)	0.84	(32)	0.49	(32)	0.52	(18)
2323	Ethyl octadecanoate[i]	88	0.97	(193)	1.18	(202)	0.50	(21)	1.19	(145)	1.48	(106)	0.41	(42)	0.48	(31)
	Subtotal (μg L^{-1})		4931.69	(11)	2360.75	(28)	5217.34	(18)	5269.69	(11)	4813.55	(15)	4888.79	(22)	5414.8	(22)
	Alcohols															
920	1-Hexanol	56	478.78	(42)	540.72	(70)	743.79	(39)	245.22	(38)	279.21	(64)	431.47	(52)	280.27	(27)
1058	2-Phenylethanol	91	4974.31	(29)	4576.78	(33)	4678.87	(38)	5593.98	(33)	5869.87	(20)	2491.87	(61)	1799.60	(17)
	Subtotal (μg L^{-1})		5453.08	(29)	5117.51	(31)	5422.66	(38)	5839.20	(31)	6149.08	(21)	2923.34	(51)	2079.87	(18)
	Total (mg L^{-1})		11.08	(18)	7.92	(29)	10.87	(26)	11.44	(15)	11.31	(16)	7.86	(23)	7.59	(18)

n.i. - not identidied.
[a] RI: retention index calculated using C_{10} –C_{24} ***n***-alkanes series.
[b] Expressed in equivalents of linalool.
[c] Expressed in equivalents of α-pinene.
[d] Expressed in equivalents of *E,E*- farnesol.
[e] Expressed in equivalents of guaiazulene.
[f] Expressed in equivalents of β-ionone.
[g] Expressed in equivalents of hexyl acetate.
[h] Expressed in equivalents of 2-phenylethanol.
[i] Expressed in equivalents of ethyl decanoate.

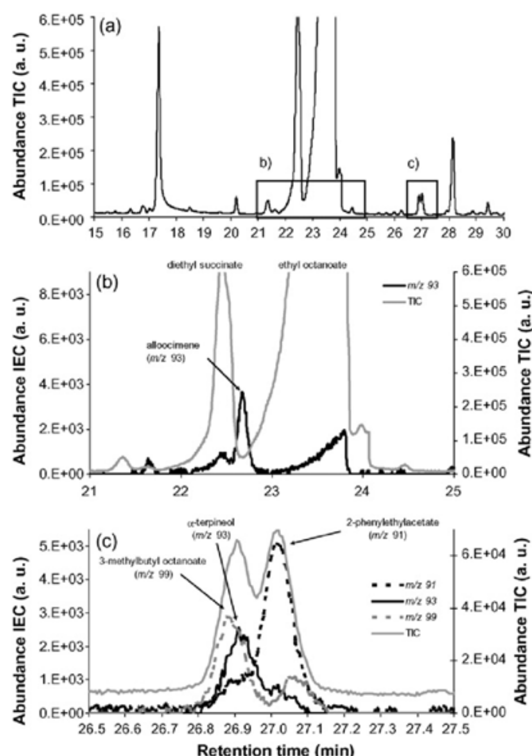

Fig. 1. (a) Total ion current chromatogram (TIC) of FP_{HCC} ; (b) blow-up of part of the TIC and extracted ion chromatogram obtained at *m/z* 93; and (c) blow-up of a part of the TIC and extracted ion chromatogram obtained at *m/z* 91, 93 and 99. (a.u.: arbitrary units).

achieved through IEC mode, by selecting the ions at *m/z* 99, 93, and 91, respectively (Fig. 1c).

For quantification purposes, calibration plots were performed with each standard using the IEC mode by extracting the target ion, usually the peak base of each standard. However, it was observed that some compounds structurally related do not have always a similar fragmentation pattern. Therefore, in the cases where the peak base ion of the standard selected represented less than 10% of spectral abundance of the target analyte, a common ion was chosen, maintaining the same abundance ratio with the more important qualifier ion of the corresponding spectrum. Thus, for some standards, more than one calibration plot was performed (Table 1). It is important to point out that excellent signal-to-noise ratio was observed for the individual ions selected.

Table 1 shows the aroma descriptor, odour threshold (OT), concentration range, recovery, relative standard deviation (R.S.D.) and calibration parameters (selected ion, correlation coefficients, limit of detection (LOD) and limit of quantification (LOQ)) obtained by SBSE-LD/LVI-GC–qMS for the 10 standards used. Five levels of concentration were tested in triplicate and regression lines were calculated for each compound (Table 1). The wine volatiles were quantified using linear regressions of the structurally related standards (similarity of functional group and/or chemical structure) for quantification purposes of the volatiles detected in sparkling wines (Table 2). Calibration plots of α-pinene was used to quantify monoterpenes, linalool was used to quantify monoterpenols, guaiazulene to quantify cyclic sesquiterpenes, *E,E*-farnesol and *Z*-nerolidol for quantifying linear sesquiterpenoids, and β-ionone to determine the concentration of C_{13} norisoprenoids. Hexyl acetate was used to quantify aliphatic esters until eight carbon skeleton, and ethyl decanoate was used to quantify aliphatic esters with more than nine carbons skeleton. 2-Phenylethanol was applied to quantify aromatic esters and alcohols, whereas hexanol was used to quantify aliphatic alcohols. The linear range used covered the concentration of the volatile compounds expected to be present in the sparkling wines. The recovery of each standard ranged from 38 (2-phenylethanol) to 95% (β-ionone), which agree the theoretical values expected from SBSE (recovery of standards plotted against their corresponding log $K_{O/W}$) [18]. The R.S.D. calculated by performing six consecutive extractions to the lower concentration of each volatile compound, ranged from 9 (α-pinene and hexyl acetate) to 18% (*E,E*-farnesol). Good regression coefficients (r^2) were obtained in all cases ranging from 0.950 (linalool) to 0.997 (hexyl acetate and β-ionone) (Table 1). Low detection limits were achieved for the terpenoids guaiazulene (0.36 μg L^{-1}) and α-pinene (0.66 μg L^{-1}), and for the hexyl acetate (0.12 μg L^{-1}) and ethyl decanoate (0.18 μg L^{-1}) esters. With the exception of the C_{13} norisoprenoid β-ionone (3.23 μg L^{-1}), the detection limit values determined for all other standards were very much lower than their respective odour thresholds.

3.2. Application to sparkling wines

Table 2 shows the concentration of 71 volatile compounds detected in the sparking wines studied, grouped by chemical classes: monoterpenoids (16), sesquiterpenoids (13), C_{13} norisoprenoids (4), esters (36), and alcohols (2). The optimized methodology allowed to quantify a large number of varietal volatile compounds (monoterpenoids, sesquiterpenoids, and C_{13} norisoprenoids), 33 from the total of 71 compounds detected. According to Table 2, fermentative compounds represented in terms of concentration 94–99% of total volatile compounds, depending on variety, ripening stage, and/or type of soil. Varietal volatile compounds comprised 1–6% of the volatiles of sparkling wines, however they have an important role in wine aroma, being already proposed as FP varietal markers [19].

From monoterpenoids, the monoterpenols appeared as the major group, especially in white variety (FP), represented by linalool, hotrienol, α-terpineol, geraniol, and nerol [1]. These compounds contribute to the aroma varietal characteristics [3,4,7]. Nerol oxide was the major monoterpenoid of FP sparkling wine, ranging from 98.9 in FP_{HC} to 171.7 μg L^{-1} in FP_{HCC} (Table 1). Sesquiterpenoids have been reported to occur in FP sparkling wines [18], as well as in BG grapes [3,20]. The wine that showed a higher concentration on sesquiterpenoids was FP_{HCC}. β-Chamigrene, nerolidol, and (*E,Z*)-α-farnesene were the major sesquiterpenoids quantified in this sparkling wine. The sesquiterpenoids represent an important chemical group of *Vitis vinifera* L. due to their aroma properties and also bioactive effect as anti-bacterial activity [21] or the ability to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds [22]. From the four C_{13} norisoprenoids detected, vitispirane was the most abundant in all wines, followed by 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). These two compounds have been described as aging markers of sparkling wines [15]. The C_{13} norisoprenoids are very important contributors to the wine aroma due to their pleasant odour descriptors, presenting usually low OT.

Thirty three esters and two alcohols were detected in the sparkling wine studied (Table 2). Esters are mainly produced by yeast during alcoholic fermentation, in reactions between alcohols and acetyl-CoA catalyzed by alcohol acetyltransferase [23]. In general, the esters contribute to the fruity and flowery character of wine. Compounds such as butyl acetate and phenylethyl acetate have been reported as the esters that exhibit the higher contribution to the sparkling wines aroma [23]. FP_{HCC} , FP_{HC} , and $FP_{Late\ HC}$

showed the higher concentration of phenylethyl acetate. The fact that only two alcohols (1-hexanol and 2-phenylethanol) have been detected may be explained mainly by the fact that SBSE has low recoveries for alcohols, which usually showed lower log $K_{O/W}$ [18]. 2-Phenylethanol may contribute with rose and sweet notes, while 1-hexanol may confer herbaceous aroma.

3.2.1. Effect of soil type

Sparkling wines obtained from grapes produced in three types of soils (clay-calcareous, sandy, and clayey) were studied. The soil type is highly related to the water status [8]. The soil has been considered less important for wine aroma than climate or variety, furthermore depth, water-holding and drainage capacity of soils have been more important rather than soil composition [8,9]. In the present study, the wines produced from the clay-calcareous soil (FP_{HCC}) presented the highest content of total volatiles as well as of varietal compounds followed by the wines from sandy soil (FP_{HS}). Similar amount of total volatiles was achieved for wines from clay-calcareous (FP_{HCC}) and clayey soils (FP_{HC}) however the former exhibited lower content of varietal components. Monoterpenoids (670.3 μg L^{-1}), sesquiterpenoids (13.1 μg L^{-1}), and C_{13} norisoprenoids (12.5 μg L^{-1}) concentrations of FP_{HCC} were 2.1, 1.4, and 1.3 times higher than in the clayey soil (FP_{HC}), and 1.6, 1.4, and 2.7 times higher than in the wines from the sandy soil (FP_{HS}). In general, clayey soils have better water retain capacity and volumetric wetness than sandy soils. In opposition, the drainage is larger in sand than in clay [24,25]. Thus, the clay-calcareous and clay soils that have good water-holding and drainage capacities should allow obtaining wines richer in volatiles than sandy soils (Table 2).

3.2.2. Effect of grapes ripening stage

Similar content of total volatiles (*ca.* 11 mg L^{-1}) were found for wines produced with grapes picked up at three ripening stages ($FP_{Early\ HC}$, FP_{HC}, $FP_{Late\ HC}$). Considering the data variability, the monoterpenoids content tends to increase (Table 2) with ripening. The behaviour of the other varietal chemical classes, sesquiterpenoids, and C_{13} norisoprenoids showed an increase from early harvest to the harvest moment and a decrease to the late harvest, which is in accordance to the profile that has been reported for FP grapes along maturity [4]. Similar content of total ester and total alcohols was observed for wines $FP_{Early\ HC}$, FP_{HC}, and $FP_{Late\ HC}$. As expected, 1-hexanol that has its origin in grape lipooxygenase activity and/or must aeration, decreased ca. 60% during ripening. This could be related to the enzymatic activity, as usually an unripe fruit is associated to an herbaceous and a grassy aroma, corresponding to 1-hexanol aroma descriptors. Similar results have been previously reported for white wines produced from grapes at early maturity state, exhibiting more intense herbaceous notes than the ones obtained from ripe grapes [26]. These observations allow concluding that the grapes from maturity and late maturity states (one week after maturity) provided wines (FP_{HC} and $FP_{Late\ HC}$) with the highest content of volatiles, including the varietal ones.

3.2.3. Effect of grape variety

Two varieties were studied: FP and BG. The wines produced from FP variety ($FP_{Early\ HC}$, FP_{HC}, $FP_{Late\ HC}$, FP_{HS}, FP_{HCC}), independently of soil type and ripening stage, presented the highest content of varietal compounds (233.0 to 695.9 μg L^{-1}). Contrarily, sparkling wines of BG variety (BG_{HC}) and the mixture of the two varieties (FP_{HC} + BG_{HC}) contained only 50.6 and 96.1 μg L^{-1}, respectively, which may be explained by the lower concentration in monoterpenoids (44.1 and 92.5 μg L^{-1}, respectively). The lower content of monoterpenoids in these wines is associated to the low amount of monoterpenoids exhibited by BG variety [3]. Previous studies [3,4,19] showed that FP grapes are characterised mainly by the monoterpenoids and BG grapes by the sesquiterpenoids composition. The characteristic FP monoterpenoid profile was observed in FP sparkling wines, but BG sesquiterpenoid profile was not observed. As for both varieties winemaking process did not include skin maceration, the data obtained suggest that the monoterpenoids presented higher extractability to the grape juice (hydrophilic medium) than the sesquiterpenoids. The 50:50 mixture of the two varieties (FP_{HC} + BG_{HC}) showed a volatile composition similar to the BG_{HC} composition, plus the hotrienol and nerol oxide, which were the major components of FP wines. Esters of FP_{HS} were almost a half of alcohols, and for BG_{HC} and FP_{HC} + BG_{HC} alcohols were a half of esters. Considering the FP_{HC} + BG_{HC}, it was observed that its composition was clearly influenced by BG character. Taking into account the wines under study, all FP modalities analysed gave wines with higher content of volatiles, including those that can contribute to varietal character. These data suggest that FP variety can provide sparkling wines with higher aroma potential than BG variety.

4. Conclusion

SBSE-LD/LVI-GC–qMS methodology allowed, in a single run, the quantification of 71 wine volatiles (major compounds – fermentative and minor compounds – varietals) at levels lower than their respective olfactory thresholds. This methodology, in combination with IEC mode, allowed profiling volatiles, with special emphasis to terpenoids that are minor compounds but greatly influence the wine aroma. Fermentative compounds comprise 94–99% of the sparkling wine volatile compounds. Low detection limits have been achieved for terpenoids: guaiazulene (0.36 μg L^{-1}), α-pinene (0.66 μg L^{-1}), *Z*-nerolidol (1.40 μg L^{-1}), *E,E*-farnesol (1.51 μg L^{-1}), and linalool (6.13 μg L^{-1}), and esters: hexyl acetate (0.12 μg L^{-1}) and ethyl decanoate (0.18 μg L^{-1}), all values greatly higher under their respective odour thresholds. The wine volatiles were quantified using the structurally related standards (functional group and chemical structure). The quantification was done always as possible with the peak base ion of each standard. Ion extraction chromatography used to minimize co-elution and interferences proved to be a remarkable choice, since this approach represents a powerful tool for unknown samples when not all standards are commercially available. Within FP variety, the sparkling wine from clay-calcareous soil showed a higher concentration of total varietal volatile compounds. The grapes from maturity and late maturity states (1 week after maturity) provided wines (FP_{HC} and $FP_{Late\ HC}$) with the highest content of volatiles, including the varietal ones. Taking into account the wines under study, all FP modalities analysed gave wines with higher content of volatiles, including those that can contribute to their varietal character. These data suggest that FP variety can provide sparkling wines with higher aroma potential than BG variety. The parameter that greatly influence the volatile composition of sparkling wine is the variety, followed by the soil type, and the ripening stage of grapes picked up close to the maturity state (± one week). From all sparkling wines studied, the wines from 'Fernão-Pires' variety, from a clay-calcareous soil with ripe grapes provided the wines with the highest aroma potential.

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3. Estudo das propriedades da espuma dos vinhos espumantes

Com vista a avaliar quais os conjuntos de moléculas do vinho que estão relacionados com as propriedades da espuma e possíveis sinergismos entre eles, para cada vinho espumante foi separada a fracção hidrofóbica de baixo peso molecular (MeLMW), a fracção de elevado peso molecular (HMW) e duas fracções de peso molecular intermédio (AqIMW e MeIMW). As propriedades da espuma dos vinhos modelo, reconstituídos com estas fracções e com as suas misturas, foram avaliadas. Depois de identificada a fracção com maior relevância nas propriedades da espuma, procedeu-se à sua caracterização química e estrutural por espectrometria de massa com ionização por *electrospray* (ESI-MS e ESI-MS/MS). Para confirmar se os compostos identificados no vinho espumante como sendo importantes para as propriedades da espuma se encontram realmente presentes na espuma do vinho espumante, a espuma foi também caracterizada. Este trabalho foi redigido no formato do *Journal of Agricultural and Food Chemistry*, encontrando-se em fase de revisão e constitui a primeira parte deste capítulo dedicado ao estudo das propriedades da espuma dos vinhos espumantes.

Para melhor se poder relacionar os parâmetros da espuma com os constituintes do vinho, o vinho foi fraccionado em doze grupos de moléculas: três fracções de manoproteínas, três de arabinogalactanas, três de mistura de polissacarídeos, proteínas e compostos fenólicos e três fracções de peso molecular intermediário e baixo, compostas por uma mistura de hidratos de carbono, péptidos e compostos fenólicos. Foram usados vinhos modelo reconstituídos com cada uma das fracções isoladas e com misturas de algumas das fracções com o objectivo de avaliar quais as biomoléculas com maior influência na espumabilidade e estabilidade da espuma. Este trabalho foi redigido no formato do *Journal of Agricultural and Food Chemistry*, encontrando-se em fase de submissão e constituindo a segunda parte deste capítulo.

3.1. Synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines

Synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines

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The foam of sparkling wines is a key parameter of their quality. However, the compounds that are directly involved in foam formation and stabilization are not yet completely established. In this work, seven sparkling wines were produced in Bairrada Appellation (Portugal) under different conditions and their foaming properties evaluated using a Mosalux based device. The fractionation of the sparkling wines in four independent fractions: 1) high molecular weight material, with molecular weight higher than 12 kDa (HMW), 2) hydrophilic material with molecular weigh between 1 and 12 kDa (AqIMW), 3) hydrophobic material with molecular weigh between 1 and 12 kDa (MeIMW), and 4) hydrophobic material with a molecular weight lower than 1 kDa (MeLMW) allowed to observe that the wines presenting the lower foam stability were those that presented lower amount of MeLMW fraction. The fraction that presented the best foam stability was HMW. When HMW is combined with MeLMW fraction, the foam stability largely increased. This increase was even larger, approaching the foam stability of the sparkling wine, when HMW was combined with the less hydrophobic subfraction of MeLMW (F3). Electrospray tandem mass spectrometry (ESI-MS/MS) of F3 allowed the assignment of polyethylene glycol oligomers (n = 5-11) and diethylene glycol 8-hydroxy-tridecanoate glyceryl acetate. In order to observe if these molecules occur in sparkling wine foam, the MeLMW was recovered directly from the sparkling wine foam and was also analyzed by ESI-MS/MS. It was observed the presence of monoacylglycerols of palmitic and stearic acids, as well as four glycerylethylene glycol fatty acid derivatives. These surface active compounds are preferentially partitioned by the sparkling wine foam rather than the liquid phase, allowing to infer their role as key components in promotion and stabilization of sparkling wine foam.

Keywords: *Foam, sparkling wines, mass spectrometry, glycerol derivatives, ethylene glycol, tensioactives, surfactants.*

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INTRODUCTION

According to the C*hampenoise* method, sparkling wine is a double fermented wine. In this method, the wine obtained by the fermentation of the must (base wine) is submitted to a second alcoholic fermentation by addition, in the bottle, of suspension of yeast and fermentiscible sugars. When poured from the bottle into a glass, the carbon dioxide produced during the second fermentation is released from the liquid in the form of bubbles and by diffusion through the free air/liquid interface (*1*). Consistent foam is formed as a result of its interaction with wine constituents.

Foam is the dispersion of a gas in a continuous liquid phase, and thus foam dispersions possess bulk densities closer to those of a gas rather than a liquid. Foam is stable if gas bubbles remain separated by thin liquid walls and do not coalesce. Drainage, the runoff of liquid between bubbles in foam, is dependent on the liquid viscosity and density (*2*). The chemical composition of induced base wine foam is less acidic than the bulk liquid phase due to the lower concentration of organic acids and higher in protein and polysaccharides (*3*), as well as free fatty acids (C6:0-C16:0) and their ethyl esters (C8:0 and C10:0) (*4*).

The foam properties, foamability and foam stability, have been correlated with the sparkling wine chemical composition, namely soluble proteins (*5, 6*), polysaccharides (*7, 8*), polyphenols (*5, 8*), iron (*3*), organic acids, and lipids (*9*). Proteins were the first candidates to be correlated with foam characteristics due to their surfactant properties. Surfactant agents are inferred to stabilize foams by settling at the bubbles edge, with the hydrophobic side interacting with the gas phase and the hydrophilic side interacting with the aqueous liquid phase (*3*). Protein concentration was previously positively correlated with foamability by several authors (*3, 6, 10, 11*) however, correlation with foam stability has presented contradictory results, with both positive (*6*) and negative correlations (*12*). All these data were obtained by measuring the foamability and foam stability of base (*3-12*) or sparkling wines (*7, 10*), and relating these physical characteristics with the chemical characteristics of sparkling wines with different foam properties.

Peptides have also been associated to the foam characteristics, namely the amphiphilic low molecular weight peptides (*13*). The presence of aromatic amino acids (that confer hydrophobicity to the peptides) in Cava sparkling wines have also been shown to improve the quality of the foam of these wines. (*14*). Contrarily, Moreno-Arribas *et al.* (*7*) did not found a relationship between foam characteristics and concentrations of wine peptides.

Additionally to peptides and proteins, some polysaccharide fractions were also correlated with foamability and a fraction with 2-3 kDa was correlated with foam stability (*15*). Neutral polysaccharides were well correlated with foamability in opposition to acidic polysaccharides which did not show any correlation with foamability (*7*). Lipids have also been correlated with foam properties: palmitic acid was positively correlated with foamability (*16*), as well as fatty acids under C_{12} esterified with ethanol, however, a negative correlation with foamability was obtained for these fatty acids in free form (*4*).

Mass spectrometry (MS) techniques, and particularly through the soft ionization methods, allow the analysis of low molecular weight compounds from food matrices. Soft ionization mass spectrometry has been used for analysis of a large number of low molecular compounds, including peptides (*17*), oligosaccharides (*18*), lipids (*19*), and ionic and non-ionic surfactants (*20, 21*). The ultrahigh-resolution MS (FT-ICR-MS) was used to discriminate surface active components from *Champagne* aerosols and bulk (*22*).

In order to study the synergistic effect of high and low molecular weight molecules in the foamability and foam stability of sparkling wines, in this work, the foam aptitude of seven sparkling wines were evaluated. These sparkling wines were then fractionated into four independent fractions according to their molecular weight and hydrofobicity and the amount of material in these fractions was related with their foaming properties. The fractions obtained with highest foam stability were then used to reconstitute wine model solutions to evaluate the individual contribution of each fraction to foam properties. The fraction showing the highest influence on the foam stability was structurally characterized by electrospray tandem mass spectrometry (ESI-MS^n). In order to observe if the molecules identified as major contributors to sparkling wines foam stabilization are really present in sparkling wine foam, the foam was collected and its low molecular weight material was also structurally characterized by ESI-MS and ESI-MS/MS.

MATERIALS AND METHODS

Sparkling wine samples. Sparkling wines were prepared by Estação Vitivinícola da Bairrada (EVB) from two grape varieties, *i.e.* Fernão-Pires (FP) white variety and Baga (BG) red variety, obtained from different ripening stages and soils. To produce FP wines, grapes from a clayey (C) soil were picked up at three harvest moments: 1) at the adequate harvest

maturity (A) to produce sparkling wines (FP_{AC}), determined by berry texture, color, sugar content, and titratable acidity; 2) at an early harvest moment (E), one week before maturity harvest (FP_{EC}); and 3) at a late harvest moment (L), one week after maturity harvest (FP_{LC}). FP wines were also produced from grapes collected in soils presenting different textures: sandy (S) (FP_{AS}) and clay-calcareous (CC) soils (FP_{ACC}). BG sparkling wine was produced from ripe grapes (adequate harvest moment to produce sparkling wines determined by the physico-chemical parameters) and one soil type, clay (BG_{AC}). A mixture of musts (50:50) obtained from BG and FP grapes picked up at the harvest moment from clayey soil was also used to produce sparkling wines (FP_{AC}+BG_{AC}). The sparkling wines were produced according the *Champenoise* method, and two independent winemaking replicates were performed for each type of wine (FP_{EC}, FP_{AC}, FP_{LC}, FP_{AS}, FP_{ACC}, and BG_{AC}). The second fermentation was performed inside the bottles after *tirage* and at least four different bottles were analyzed for each type of wine, in a total of 24 bottles. The exception of this strategy was the mixture FP_{AC}+BG_{AC} for which only 2 bottles were obtained. The wines were analyzed after twenty four months of *dégorgement* (removal of yeast sediment from bottles). Each bottle was analyzed in duplicate.

Extraction of polymeric material from sparkling wines. The sparkling wine samples were rotary-evaporated under reduced pressure at 35ºC to degas and eliminate the ethanol, allowing to concentrate the non-volatile molecules. The material was then dialyzed (12 kDa cut-off membrane, Medicell) in order to remove the tartaric acid and other small molecules. The retentate was concentrated, frozen, and freeze-dried, to give the wine high molecular weight (HMW) material as a powder (**Scheme 1**). The material that diffused through the dialysis membrane (dialyzate) was recovered by concentration under rotary-evaporation and frozen for use in the following isolation step.

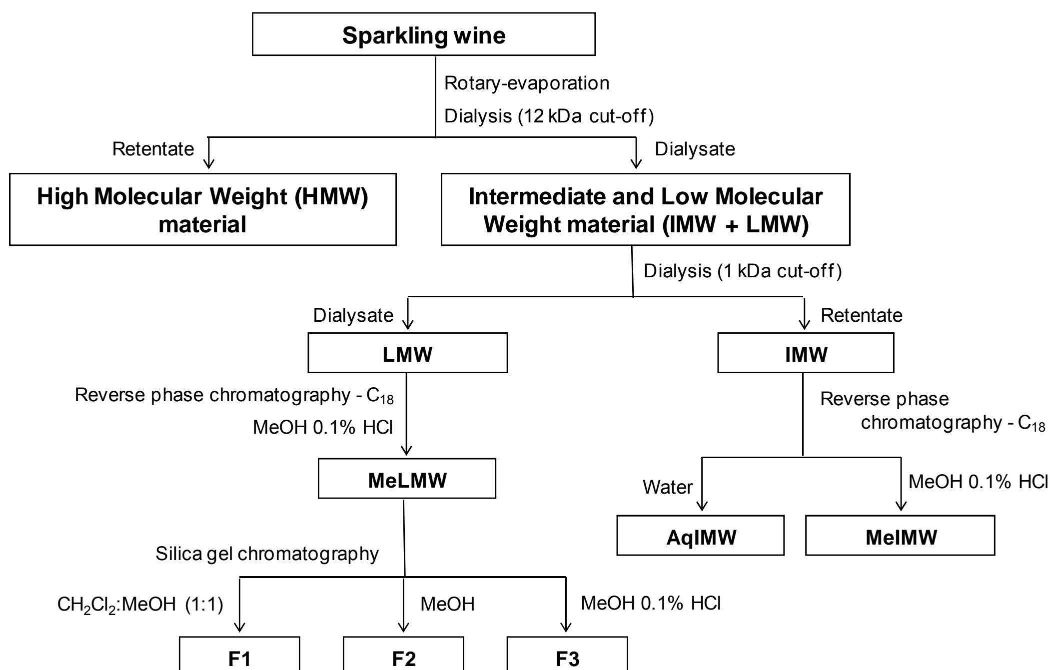
Extraction and isolation of intermediate and low molecular weight material from sparkling wines. The different concentrated 12 kDa dialyzated solutions obtained during the isolation of the polymeric material of each sparkling wine were then submitted to a new dialysis, now with a cut-off of 1 kDa (Spectra/Por®) (**Scheme 1**). Each aqueous solution was added, under stirring, to a batch of a C_{18} resin suspension, during 3 h, for sortion of the hydrophobic material. The resin was recovered by filtration, washed with water until the conductivity of the water is reached, and then was extracted with acidic methanol (MeOH 0.1% v/v HCl). Using this procedure, the retentate, which comprised the

material with molecular weight between 1 and 12 kDa (IMW), gave origin to two fractions, AqIMW, the fraction of material not sorbed to the C_{18}, that remained in the water solution, and MeIMW, the fraction of material retained in the C_{18} resin and recovered with acidic methanol (**Scheme 1**). The dialyzate, which comprised the material with molecular weight lower than 1 kDa, gave origin to fraction MeLMW, extracted with methanol; the fraction not sorbed, containing the salts, was discarded.

The fraction MeLMW was then fractionated by polarity through a silica column using the sequence of the following eluents: CH_2Cl_2:MeOH (1:1), MeOH and acidic MeOH (0.1% HCl, v/v), giving origin to fractions F1, F2, and F3, respectively (**Scheme 1**).

Extraction of foam low molecular weight material. The fraction MeLMW was also extracted from sparkling wine foam of FP_{LC} sample. The foam formed by uncorking the bottle (750 mL) and then by bottle agitation was collected (100 mL of collapsed foam) and dialyzed (1 kDa cut-off membrane, Spectra/Por®) against water (1 L) at 5ºC, under stirring, until the conductivity of the dialysis water became similar to distilled water (one water exchange of 1 L each, during 48 h). The two dialyzates (containing the lower molecular weight material) were combined and eluted by a C_{18} column (SPE-C_{18}, Supelco-Discovery – 10 g). Then, the retained material was washed with ultrapure water, until water conductivity reaches 2.3 μS/cm, and the foam low molecular weight hydrophobic material was recovered with acidic methanol (1.0% v/v acetic acid). The sample was concentrated by rotary-evaporation at 35ºC, and suspended in ultra pure water. The solution was centrifuged and the supernatant was used for ESI-MS and ESI-MS/MS analysis.

A blank to disclose the possible release of compounds from the dialysis membrane was performed by dialysis of 100 mL of distilled water in 1 L of water during 48 h, with 1 L water exchange. The dialyzate was eluted through a C_{18} column and the retained material was washed with ultrapure water and recovered with acidic methanol (1.0% v/v acetic acid) in the same conditions as used from the sparkling wine foam. Solvents used were HPLC grade.



Scheme 1 – Schematic diagram of the isolation and fractionation steps of sparkling wines.

Wine model solutions. Wine model solutions were constructed from a hydroalcoholic base solution with 10% ethanol (v/v), 0.5% tartaric acid (w/v) adjusted at pH 3.5 with NaOH solution. Glycerol and ethyl octanoate were also added to perform the concentrations of 0.7% (w/v) (*16, 23*) and 0.4% (w/v) (*24*), respectively. The fractions obtained from the sparkling wine FP_{LC} were added individually and in combination to the wine model solution for measurement of their foam properties.

Foam properties measurement. Foamability and foam stability were assessed using an adaptation of Mosalux and Bikerman method (*10, 11, 16*). Analytical grade CO_2 from a cylinder flowed through a glass-frit fitted in the bottom of a column (530 × 15 mm i.d.). The gas flow rate was controlled at 10 L/h by a flow meter (Cole-Parmer Instruments Company, IL, USA). Foamability was evaluated as the increase in height of 10 mL of degassed sparkling wine or model wine solutions placed inside the glass column, after CO_2 injection through the glass-frit. Two parameters of foamability were measured: 1) HM (maximum height reached by foam after CO_2 injection through the glass frit, expressed in cm) represents the solution ability to foam. 2) HS (foam stability height during CO_2 injection, expressed in cm) represents the solution ability to produce stable foam persistence of foam collar. Foam stability time (TS) was evaluated as the time elapsed before bubble collapse until the liquid appears after

the interruption of CO_2, and is expressed in s. Each bottle of sparkling wine was analyzed in duplicate, and for each type of wine 8 replicates (4 bottles x 2 replicates per bottle) was obtained. The isolated fractions obtained from the wine were added independently or in mixtures to the wine model solution taking into account their average proportions in these seven sparkling wines. For these solutions, the foam properties measurements were done with 5 replicates.

Chemical analysis. *Sugar analysis.* Monosaccharides were released from cell wall polysaccharides by a pre-hydrolysis in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC. Neutral sugars were analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (*25, 26*). A Perkin Elmer Clarus 400 GC apparatus with split injector and a FID detector was used, equipped with a 30 m column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC and then 220 ºC for 7 min, followed by an increase until 230 ºC at rate of 20 ºC/min, being this temperature maintained for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1 mL/min. Uronic acids (UA) were quantified by a modification (*25*) of the 3-phenylphenol colorimetric method (*27*). Sugar analysis was assayed for the HMW of the seven sparkling wines, and for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine. *Protein analysis.* Protein quantification was based on the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard, using the Bicinchoninic Acid Protein Assay Kit from Sigma (Aldrich-Chemie, Steinheim, Germany) (*28*). Protein analysis was assayed for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine. *Amino acid analysis.* Amino acid quantification was performed for hydrophobic low molecular weight fraction < 1kDa (MeLMW) and for the most acidic subfraction of that, obtained from normal phase purification (F3). The amino acid residues were released by acidic hydrolysis (*29*), derivatized with heptafluorobutyric anhydride, and the N-heptafluorobutyryl isobutyl esters of amino acids were analyzed by GC-FID (*30, 31*). Calibration curves for Ala, Val, Leu, Asx, and Glx were obtained in the concentration range of 0.0–0.2 mg/mL; for all other amino acids, the concentration range was 0.000–0.025 mg/mL. *Determination of total phenolic compounds***.** Total phenolic composition was determined by the Folin-

Ciocalteu colorimetric method (*28, 32*), using gallic acid as standard. The analysis of total phenolic compounds was performed for AqIMW, MeIMW, and MeLMW of FP_{LC} sparkling wine.

Electrospray ionization mass spectrometry conditions. Electrospray ionization (ESI) mass spectrometry analyses were performed on the subfraction of MeLMW recovery with MeOH acidic from silica gel column (F3) and on the hydrophobic low molecular weight material obtain from sparkling wine foam. Prior to MS analysis, the sample F3 was dissolved in water and eluted through a C_{18} column, washed with diethylether, and recovered with MeOH (HPLC grade, Fisher Scientific, UK) with 1.0% (v/v) acetic acid. Both samples were independently concentrated and suspended in ultra pure water and each solution (2 μL) was further diluted 100 fold in MeOH/H_2O (1:1, v/v) solution with 1.0% (v/v) formic acid. The samples were introduced into the mass spectrometer using a flow rate of 8 μL/min. Positive ion mode ESI-MS and MS/MS spectra were acquired in a LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Typical ESI conditions were as follows: electrospray voltage was 5.0 kV; capillary temperature was 275 ºC and the sheath gas flow was 25 units. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized Collision Energy™ (CE) was varied between 15 and 35 (arbitrary units) for both MS^2 and MS^3 according to the ion of interest. Data acquisition was carried out on an Xcalibur data system (V2.0). The water was of MilliQ purity filtered through a 0.22 μm filter (Millipore, USA), and all organic solvents were HPLC grade.

RESULTS AND DISCUSSION

Evaluation of foam aptitude of Bairrada sparkling wines. Seven sparkling wines were produced from two grape varieties (Fernão-Pires and Baga) using grapes from different ripening stages and soils. In order to evaluate the range of their foam aptitude, the maximum height reached by foam after CO_2 injection through the glass frit, expressed in cm (HM), the foam stability height during CO_2 injection, also expressed in cm (HS), and the foam stability time, expressed in s (TS), were measured (**Figure 1**).

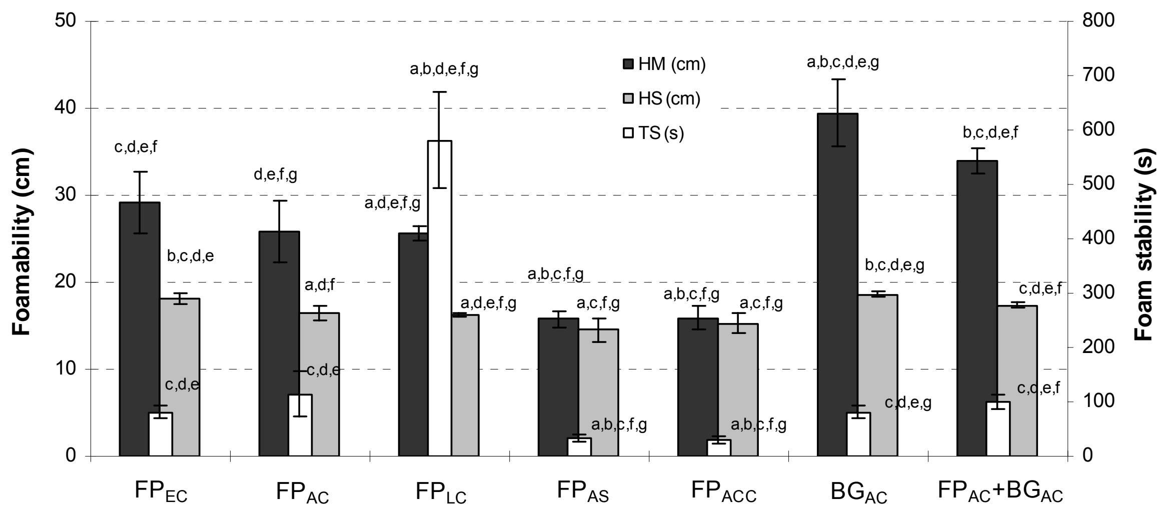


Figure 1- Foamability HM (maximum height reached by foam after CO_2 injection) and HS (foam stability height during CO_2 injection) and stability TS (foam stability time) measured for seven different sparkling wines from Bairrada Appellation.

a- significantly different ($p > 0.05$) from FP_{EC}, **b**- significantly different ($p > 0.05$) from FP_{AC}, **c**- significantly different ($p > 0.05$) from FP_{LC}, **d**- significantly different ($p > 0.05$) from FP_{AS}, **e**- significantly different ($p > 0.05$) from FP_{ACC}, **f**- significantly different ($p > 0.05$) from BG_{AC}, **g**- significantly different ($p > 0.05$) from $FP_{AC} + BG_{AC}$.

The HM ranged from 15.8 to 39.4 cm, the minimum values were observed for FP variety from grapes harvested at the adequate maturity, grown in sandy (FP_{AS}) and clay-calcareous (FP_{ACC}) soils and the maximum was achieved for BG variety from grapes harvested at the adequate maturity, grown in clayey soil (BG_{AC}). For HS, these sparkling wines showed a shorter interval than for HM, from 14.5 to 18.6 cm. The maximum and minimum HS were observed for the same samples as for HM. The range observed for TS varied between a minimum of 31 and 33 s, for FP_{AS} and FP_{ACC}, as observed for the other foam parameters, and a maximum of 582 s, observed for FP variety from grapes from a late harvest, grown in a clayey soil (FP_{LC}). However, the TS observed for FP_{LC} is much higher than the TS observed for all other sparkling wines. The range observed for TS without the contribution of this wine is 31-115 s. These results show that the foam aptitude of different Bairrada sparkling wines can vary considerably, mainly for foam stability time. Although TS seems to be influenced by the soil type (31 s for FP_{ACC}, from a clay-calcareous soil, 33 s for FP_{AS}, from a sandy soil, and 115 s for a clayey soil), the influence of the ripening stage of the grape at harvest showed the highest influence in clayey soil for the FP variety (81 s for early

harvest, FP_{EC}, 115 s for harvest at maturity, FP_{AC}, and 582 s for late harvest, FP_{LC}). Considering the variety, the wines containing BG grapes showed highest HM values but this characteristic is not present in HS or TS. These results are in accordance with the works carried on sparkling wines from other Appellations where the aptitude of some varieties to foamability and foam stability, as well as the impact on foam of harvest and winemaking process, were studied (*12, 33, 34*).

Fractionation of sparkling wine components and relation with foam properties. In order to observe the compounds present in sparkling wine that most influence their foam aptitude, the wines were fractionated according to the molecular weight of their components in high molecular weight (HMW) material, *e.g.* compounds with molecular weight higher than 12 kDa, material with intermediate molecular weight (IMW), *e.g.* compounds that diffused through the pores of the dialysis membrane of 12 kDa but were retained by the dialysis membrane of 1 kDa pores, and material with low molecular weight (LMW), *e.g.* compounds that diffused through the pores of the dialysis membrane of 1 kDa. The IMW fraction was further divided according to its polarity in a hydrophobic fraction, extracted with acidic methanol from a C_{18} resin (MeIMW), and a hydrophilic one, not retained (AqIMW). The hydrophobic compounds were also recovered from LMW fraction by extraction with acidic methanol from a C_{18} resin, giving origin to fraction MeLMW (**Scheme 1**).

Table 1 shows the yield of the four fractions obtained from each one of the seven sparkling wines under study. The wines with lower TS, FP_{AS} and FP_{ACC}, were those with the lower amount of MeLMW, 11.9 and 19.5 g/L respectively. These values are 30 and 18 fold less, respectively, than FP_{LC}, the wine that has showed the highest TS. FP_{LC} wine also showed the higher yield in AqIMW. For the FP variety grown in clayey soil, the amount of uronic acids (UA) present in wines decreased during ripening, from 16 to 11 and to 3 mol% for early, adequate, and late harvest, respectively. The other sparkling wines (FP_{AS}, FP_{ACC}, and BG_{AC}) showed values between 10-12%, corresponding to wines produced with grapes picked up at the adequate harvest moment. This shows that the decrease in polymeric UA in wines seems to be related with the increase of TS value in wines.

Table 1- Sugar composition of high molecular weight material (HMW) and yields of HMW, MeLMW, MeIMW, and AqIMW from sparkling wine.

Wine	Yield (mg/L)				*Mol %*								Total sugars (%, w/w)
	MeLMW	**MeIMW**	**AqIMW**	**HMW***	**Rha**	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**UA**	
FP_{EC}	532.9	25.6	6.4	349.6 (8)	2	0	9	0	39	29	4	16	66
FP_{AC}	412.3	28.5	7.9	448.0 (21)	2	0	10	0	45	24	7	11	63
FP_{LC}	359.5	26.3	19.9	422.8 (5)	2	0	11	1	43	32	8	3	46
FP_{AS}	11.9	1.7	4.7	352.4 (2)	2	1	11	0	39	28	6	12	73
FP_{ACC}	19.5	0.8	7.0	480.8 (3)	4	0	11	0	46	26	3	10	70
BG_{AC}	47.0	1.1	8.1	417.5 (8)	2	0	13	0	39	27	7	11	54
$FP_{AC}+BG_{AC}$	124.3	0.5	7.2	550.6 (3)	2	0	13	0	37	30	8	10	51

* Average of four independent extractions, with exception of the wine FPHC+BGHC that has two replicates, %RSD in parenthesis.

The decrease in UA is related to the degradation of pectic polysaccharides with ripening, which is in accordance to Yakushiji *et al.* (*35*) that reported degradation of cell-wall polysaccharides from the mesocarp of grape berries when comparing *véraison* with maturity. Although the acidic polysaccharides did not show any correlation with foamability (*7*), galacturonic acid content was anti-correlated with TS by Andrés-Lacueva *et al.* (*12*), showing that the wines with the lowest galacturonic content had better TS. The degradation of pectic polysaccharides observed for the late harvest results in the decrease of UA in the HMW fraction with its consequent increase in the AqIMW, as shown in **Table 2**.

Table 2 – Sugar composition, total sugar, total protein and total phenolic content of the intermediate and low molecular weight fractions isolated from sparkling wine (FP_{LC}).

Fraction	*Mol %*								Total Sugars (%, w/w)	Protein (%, w/w)	Phenolic compounds (%, w/w)
	Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**UA**			
AqIMW	7	1	6	3	30	11	10	32	53	19	18
MeIMW	3	1	15	6	10	4	45	18	9	*	*
MeLMW	2	1	10	5	20	4	49	10	7	39	6

* Fractions with high content of protein and phenolic compounds.

Many factors have been correlated with foam properties, namely, *Botrytis cinerea*, wine aging, and bentonite addition. *B. cinerea* infection have a negative influence on foam properties (*36*); wine aging for 18 months was reported to confer the best HM and TS, apparently due to the release of proteins and polysaccharides by yeast autolysis (*37*), and

bentonite addition was reported to promote a decrease of HS and TS, possibly due to the reduction of total soluble protein concentration (*38*). These three studies related foam properties with wine composition modulated by treatments. To understand the influence of the different wine components and their possible synergistic effects in foam behaviour, reconstituted sparkling wine solutions were prepared from the HMW, MeIMW, AqIMW, and MeLMW fractions obtained from FP_{LC}, the sparkling wine that presented the highest TS.

Evaluation of foam properties of individual sparkling wine fractions in model solutions. Figure 2 shows the foam evaluation of the wine model solutions reconstituted from each one of the four fractions previously obtained from FP_{LC} sparkling wine (**Scheme 1**). For the reconstitution, the same amount of material recovered from the wine was used (**Table 1**): 420 mg/L for HWM, 26 mg/L for MeIWM, 20 mg/L for AqIWM, and 360 mg/L for MeLWM.

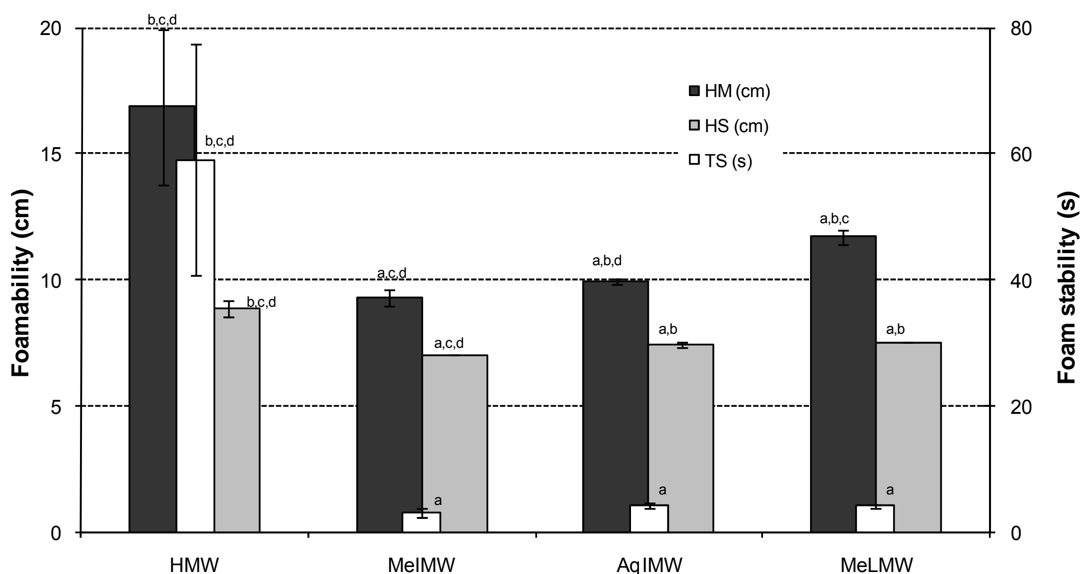


Figure 2 - Foamability, HM and HS, and foam stability, TS, measured for the fractions previously isolated from wine (FP_{LC}). All fractions were in wine concentration in the model solution.

a- significantly different ($p > 0.05$) from HMW, **b**- significantly different ($p > 0.05$) from MeIMW, **c**- significantly different ($p > 0.05$) from AqIMW, **d**- significantly different ($p > 0.05$) from MeLMW.

The better foam properties, *e.g.*, the higher HM, HS, and TS, were observed for HMW. The HM increased in the sequence of MeIMW (9.3 cm), AqIMW (10.0 cm), MeLMW (11.7 cm), and HMW (16.9 cm). For the HS, a slight variation between 7.0 and 8.9 cm was observed for MeIMW and HMW, respectively. Furthermore, TS showed values 14 to 20 fold higher than for the other fractions (59 s for HWM and 3-4 s for the others). These foam measurements showed that the wine model solutions reconstituted with the HMW fraction, for itself, explained 65%, 53%, and 9% of HM, HS, and TS, respectively, of the foam values achieved for the FP_{LC} sparkling wine (**Figures 1 and 2**). This HMW fraction was composed by 46% of sugars (**Table 1**), with a sugar composition mainly constituted by Man (43 mol%), Gal (32 mol%), and Ara (11 mol%). According to the bibliography, these sugar residues are components of mannoproteins from yeasts and arabinogalactans and pectic polysaccharides from grapes (*39, 40*). As the HWM from the different wines has similar content and composition, by itself, it does not explain the differences in foam properties of these wines. In order to observe the possible presence of a synergistic effect between the components of the different fractions, the foam evaluation of wine model solutions containing combined fractions were performed.

Evaluation of foam properties of model solutions of combined wine fractions. The foam parameters of the simultaneous combination of the four fractions: 420 mg HMW + 26 mg MeIMW + 20 mg AqIMW + 360 mg MeLMW in 1 L of 10% alcoholic solution, simulating a total reconstitution of original sparkling wine, when compared with the solution containing the HMW fraction, had similar HM (15.3 cm) and HS (8.6 cm) but the TS was considerably lower (6 s). The total wine reconstitution represented only 1% of the TS achieved for the sparkling wine. It is possible that the mixture of these different molecules has different contributions to the foam aptitude, as some of them could have a positive and others a negative effect on foam. In fact, according to **Table 2**, fraction AqIMW was composed mainly by sugars (53%) followed by proteins (19%) and phenolic compounds (18%). The sugar composition showed 32 mol% of UA and 30 mol% of Man, sugars characteristics of pectic polysaccharides and mannoproteins, respectively (*39, 40*). On the other hand, the fraction MeIMW was composed mostly by phenolic compounds and proteins. The colorimetric methods used for quantification of phenolic compounds and proteins present mutual interferences of these compounds, preventing their realistic quantification. The fraction MeLMW showed to be constituted mainly by peptides (39%),

followed by sugars (7%), and phenolic compounds (6%). The major sugar was glucose, possibly arising from the glycosylation of phenolic compounds (*41, 42*). The amino acid composition of fraction MeLMW (**Table 3**) showed that the major amino acid was Glx (estimated by the sum of Glu and Gln) with 13.12 μg/mg, followed by Asx (estimated by the sum of Asp and Asn), Leu, and Gly with 9.79, 9.03 and 8.59 respectively. The amino acid profile in the free form was quite similar to the total amino acid content profile, with the exception of Pro that was the third major amino acid instead of Leu (**Table 3**).

Table 3 – Total and free amino acid composition of fraction MeLMW and total amino acid content of F3 (the most acidic sub fraction obtained from MeLMW in the normal phase column).

Amino acid	Concentration (μg/mg)		
	MeLMW		F3
	Total	Free	Total
Ala	1.68	0.37	0.07
Gly	8.59	2.40	0.43
Val	6.92	0.46	0.29
Thr	2.37	0.36	0.10
Ser	2.04	0.57	0.08
Leu	9.03	1.73	0.30
Ile	7.12	0.49	0.25
Pro	6.79	2.99	0.23
Hyp	1.72	0.63	0.30
Asx	9.79	7.23	0.08
Phe	2.40	0.61	-
Glx	13.12	5.03	0.45
Lys	2.56	0.48	-
Tyr	2.03	0.57	-
Arg	tr	tr	-
Total	74.65	23.46	2.16

tr- Trace amounts.

When the HMW was combined with MeLMW fraction in the proportions recovered from the sparkling wine (420 and 360 mg/L), it was obtained a wine model solution presenting HM and HS of 16.3 and 8.6 cm, values that are similar to those observed for the solution containing the HMW fraction alone (**Figures 2 and 3**). This combination also showed a TS of 161 s, a value 2.7 times higher than that obtained for the TS of the HMW fraction alone (59 s). This value of TS showed that the wine model solution reconstituted with the HMW + MeLMW fractions explained 24% of the TS measured for the FP_{LC} sparkling wine.

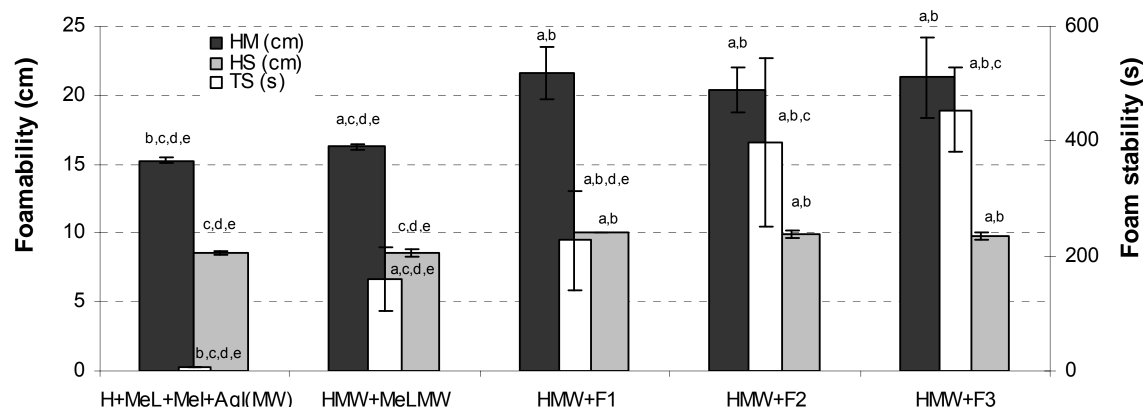


Figure 3 - Foamability, HM and HS, and foam stability, TS, measured for reconstituted wine (FP_{LC}): total wine reconstitution (H+MeL+MeI+AqI(MW)), HMW+MeLMW fraction, and HMW plus the three fractions isolated from MeLMW by a silica gel column (F1, F2, and F3). All fractions were present in the model solution were added in the concentration found in wine.

a- significantly different (p > 0.05) from HMW+MeLMW+MeIMW+AqIMW, **b**- significantly different (p > 0.05) from HMW+MeLMW, **c**- significantly different (p > 0.05) from HMW+F1, **d**- significantly different (p > 0.05) from HMW+F2, **e**- significantly different (p > 0.05) from HMW+F3.

Evaluation of foam properties of the combination of HMW with subfractions of MeLMW material (F1-F3). In order to better understand the foam behavior in relation to the solution composition containing the HMW fraction and the low molecular weight hydrophobic material present in the fraction MeLMW, this later material was further fractioned. The MeLMW material was separated by polarity through a silica gel column into 3 fractions: F1 was the most hydrophobic fraction, F2 had an intermediate hydrophobicity, and F3 was the least hydrophobic. These three fractions were individually added to the model wine solutions containing the HMW material, keeping the relative as amount of their recovery from the wine, *e.g.* 90, 37, and 228 mg/L, respectively, and the foam aptitude of the resultant solutions was measured (**Figure 3**). For all solutions, the HM, HS, and TS measured were significantly higher than for the HMW + MeLMW. The TS value also increased in the following order HMW + F1, HMW + F2, and HMW + F3, from the highest hydrophobic material to the least hydrophobic one. No significant differences were observed for HM, HS, and TS between HMW + F2 and HMW + F3, but the later showed better relative standard deviation, namely in TS. The foam range values

observed for the addition of these three subfractions to the HMW were close to the values observed for the wine, as HM represents 77-85% of sparkling wine HM, HS represents 59% of sparkling wine HS, and TS represents 34-68% of sparkling wine TS. Furthermore, the fractions HMW, HMW + MeLMW, HMW + F1, HMW + F2, and HMW + F3 showed HM and TS in the range of values observed for the sparkling wines, as only HS (8.6-10.0 cm) was under the interval (14.5-18.6 cm). Subfraction F3 seems to be an important fraction to explain the foam behavior, as its presence in solution together with HMW allowed an increase in TS in 8 fold.

Based on the assumption that the stability of *Champagne* bubbles require the presence of an adsorption layer, a recent study was described by Abdallah *et al.* (*43*) in order to evaluate the hypothesis of the significant contribution of macromolecules to the formation of the adsorption layer at the interface with the gases. These authors studied three macromolecular fractions (> 100 kDa, > 30 kDa, > 10 kDa) isolated from native *Champagne* wines. The isolated macromolecules were dissolved in a wine matrix constituted by an ultra filtered wine submitted to a cut-off of 5 kDa and the surface activity was measured by ellipsometry. This study showed that the macromolecules present in *Champagne* allowed the formation of the adsorption layers comparable to those observed at the surface of native wines (*43*). In fact, this study corroborates our findings showing that the use of ultra filtered wine with a cut-off 5 kDa as wine model solution in combination with the wine high molecular weight fractions allowed reconstituting the sparkling wine foam properties.

The literature available regarding the relationship of wine low molecular weight molecules and foam properties proposes peptides of low molecular weight (200-300 Da) as foam stabilizers (*13*). The presence of aromatic amino acids (that confer hydrophobicity to the peptides) in Cava sparkling wines have also been shown to improve the quality of the foam of these wines (*14*). **Table 3** shows the amino acids content of fraction F3. The major amino acids were Glx (0.45 μg/mg) and Gly (0.43 μg/mg), in a total concentration of amino acids of 2.16 μg/mg, which does not explain the chemical composition of this fraction. The sugar analysis was also assessed, showing only 26 μg/mg, where the major sugars were Glc (42 mol%) and UA (34 mol%). In order to assess a detailed composition of F3 that could explain its relevant foam properties, it was analyzed by ESI-MS and MS/MS.

ESI-MS and ESI-MS/MS characterization of fraction F3. Figure 4a shows the ESI-MS spectrum of fraction F3. The ions at *m/z* 305, 349, 393, 437, 481, 525, and 569 show differences of 44 Da. According to the ESI-MS/MS spectra of these ions were observed neutral losses of 44 Da (data not shown), it was possible to assign them to the sodiated adducts of polyethylene glycol ($OH\text{-}CH_2\text{-}(CH_2\text{-}O\text{-}CH_2)_n\text{-}CH_2\text{-}OH$) where n varies from 5 to 11. These molecules could have natural or technological origin. The presence of ethylene glycol in wines has been reported as a native constituent (*44, 45*), produced by yeasts from ethanolamine via glycolaldehyde. The strain *Zygosaccharomyces bailii* 429 (a yeast species that is also found in wine) has been reported as the major ethylene glycol producer, accounting for more than half of the ethanolamine consumed. Under aerobic, as well as under anaerobic conditions, strains of *Saccharomyces cerevisiae* formed only small amounts of ethylene glycol (*45*). Polyethylene glycol could also have a technological origin, as it is used in bioprocessing, promoting the increase of the release of extracellular products through interaction with cell membrane components during the fermentation step (*46*). Also, it is also used to control fermentations foam (*2*). Polyethylene glycol enhances the solubilization of surfactants (*47*), the amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids, increasing the solubility, mobility and bioavailability of immiscible components (*46*). To our knowledge, and according to the wine producers, no additives have been added during the winemaking of the sparkling wines used in this study. A blank to disclose the possible release of compounds from the dialysis membranes, both 1 and 12 kDa, was performed but none of these compounds were identified. The ion at *m/z* 413 is a contaminant, as it was also present in the spectrum of the solvent.

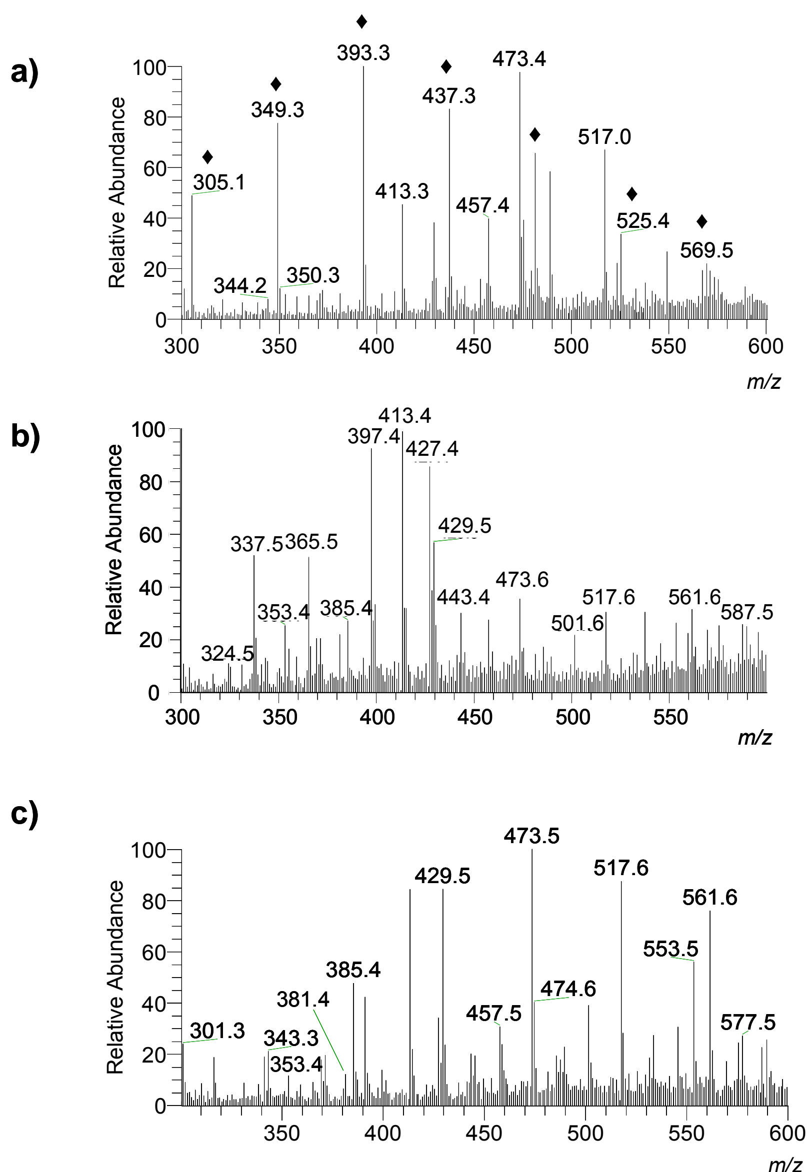


Figure 4 - Mass spectrum of full-MS acquisition by ESI-MS of a) the fraction F3, diamonds (♦) indicate the polyethylene glycol (HO–(–O–)n–OH) series from n= 5 to n= 11, b) low molecular weight hydrophobic compounds of sparkling wine foam after addition of lithium acetate, and c) low molecular weight hydrophobic compounds of sparkling wine foam.

The ESI-MS/MS of the ion at *m/z* 457 (**Figure 5a**) showed the major neutral loss of 60 Da, attributed to an acetic acid molecule, with formation of the ion at *m/z* 397, and the loss of 134 Da attributed to the loss of a glycerol acetate molecule, with formation of the ion at *m/z* 323. The ion at *m/z* 397 showed also by MS^3 the formation of the ion at *m/z* 323 (**Figure 5b**), allowing to infer the presence of a sodiated glyceryl derivative. The MS^4 of

the product ion at *m/z* 323 (**Figure 5c**) showed successive losses of neutral molecules with differences of 14 Da, characteristic of a carbon chain fragmentation profile (*48, 49*). The main neutral loss was 102 Dan that can be attributed to a hydroxylated carbon chain fatty acid, as shown in **Figure 5d**. The successive cleavages of the C-C bonds result in the neutral losses of 116, 130, 144, 158, and 172 Da, giving the ions at *m/z* 207, 193, 179, 165, and 151, respectively. This fragmentation pattern allows proposing the structure shown in **Figure 5d** for the glyceryl acetate diethylene glycol 8-hydroxy-tridecanoate, although the order of the substituents in the glycerol moiety is still uncertain. Ether-containing polar lipids are rare (*50*), being mainly confined to the Archaea domain (*51, 52*). Anyway, ether-containing lipids were described to occur in alkylglycerols, namely 1-*O*-alkyl/alkenyl-2-*O*-acyl-glycero-3-phosphocholine, found in the cell membrane of *Mycoplasma fermentans* (*53*) and also glycerol ethers sugar derivatives in *Propionibacterium propionicum* (*54*).

It is possible that low molecular weight compounds found in these fractions may contribute to Bairrada sparkling wine foaming properties have also a microbial origin, not yet identified.

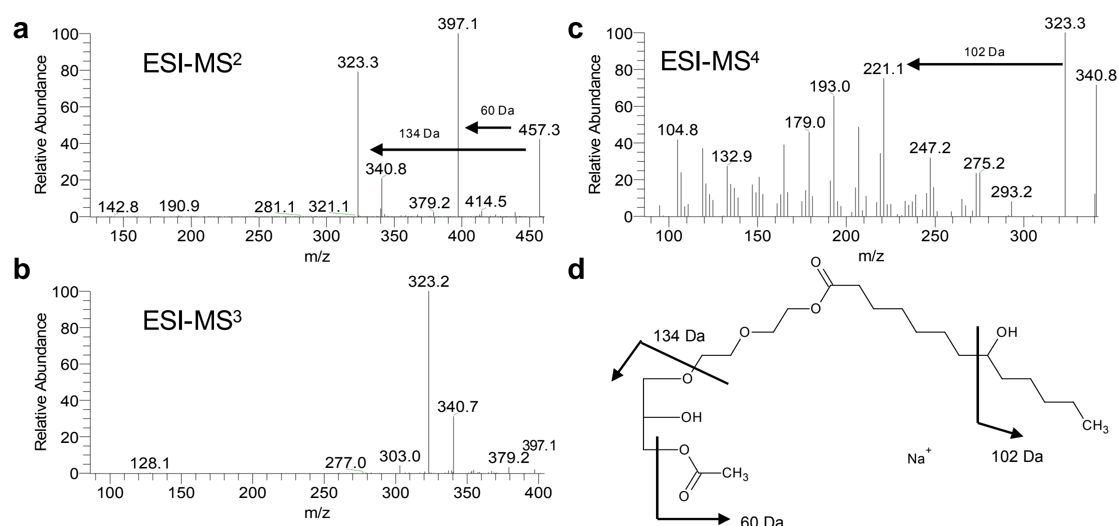


Figure 5 – ESI-MS^n spectra of ion at *m/z* 457 present in fraction MeLMWF3. a) MS^2 of ion at *m/z* 457; b) MS^3 of ion at *m/z* 397; c) MS^4 of ion at *m/z* 323; d) tentative structure assignment.

ESI-MS characterization of hydrophobic low molecular weight material from sparkling wine foam. In order to find potential tensioactive molecules that can be present in the low molecular weight fraction obtained from sparkling wine foam, the compounds present in the foam recovered by dialysis and reverse-phase chromatography were analyzed by ESI-MS. As soft ionization methods usually give different ions dependent on the type of cations involved on the ionization procedure, ESI-MS analysis was performed using lithium and sodium adducts.

Figure 4b shows the full-MS spectrum of the lithium ions of low molecular weight hydrophobic compounds obtained from sparkling wine foam. The most abundant ions were obtained at *m/z* 337, 365, 397, 413, 427, 429, 473, 517, and 561. As the ion at *m/z* 413 was also present in the spectrum of the solvent, it was assumed to be a contaminant. The ions at *m/z* 397, 427, 473, 517, and 561 were not yet possible to assign. **Figure 4c** shows the full-MS spectrum of the sodium ions of low molecular weight hydrophobic compounds obtained from sparkling wine foam. The most abundant ions were obtained at *m/z* 343, 369, 385, 413, 429, 457, 473, 517, 553, and 561. As the ion at *m/z* 413 was also present in the spectrum of the solvent, it was assumed to be a contaminant. The ions at *m/z* 473, 515, 553, and 561 were not yet possible to assign, although the ions at *m/z* 473 and 561 are in common in both spectra (**Figures 4b and 4c**). Tandem mass spectrometry (ESI-MS^n) was performed to identify the ions obtained.

Evidence of the occurrence of monoacyl glycerols in sparkling wine foam. In order to determine the structures of the ions occurring as lithium adducts in the ESI-MS spectrum, they were submitted to tandem MS analysis. The ion at *m/z* 337 showed a loss of 74 Da, attributed to a glyceryl moiety, giving the ion at *m/z* 263 (**Figure 6b**), and a loss of 238 Da that corresponds to the ketene form of palmitic acid ($C_{14}H_{29}$-CH=C=O), at *m/z* 99. This fragmentation allows to infer the presence of [glyceryl palmitate + Li]$^+$. The ion at *m/z* 365 showed also the loss of 74 Da attributed to a glyceryl moiety, giving the ion at *m/z* 291 (**Figure 6c**), allowing to infer the presence of [glyceryl stearate + Li]$^+$.

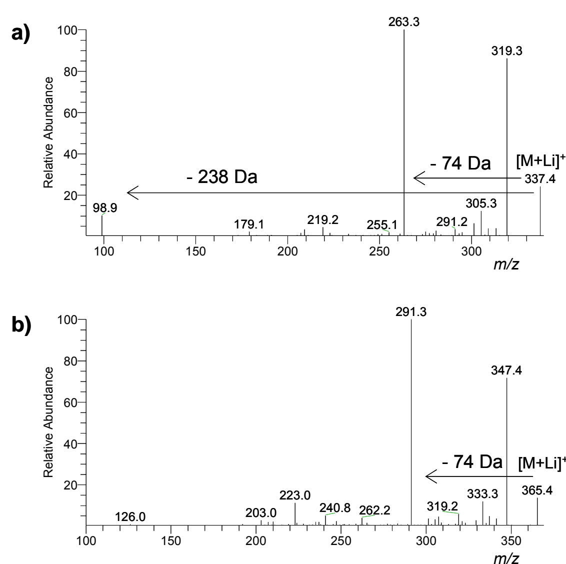


Figure 6 – ESI-MS^2 spectra of lithium adducts of low molecular weight hydrophobic compounds of sparkling wine foam, [M+Li$^+$] ions a) at *m/z* 337 and b) *m/z* 365.

These fragmentation patterns allowed to assign these two ions to two potential tensioactive molecules, glyceryl palmitate and glyceryl palmitate, present in this sparkling wine foam. Monoacylglycerol of fatty acids (C_{14}-C_{18}) belong to the food emulsifiers (E471 series) class. Indeed, they improve the manufacture of products by acting as foam and cream stabilizers, crumb-softeners or reducer staling agents (*55*). Studies on maternal milk have demonstrated that monoacylglycerols also exhibit antibacterial and antiviral properties (*56*). In addition, glyceryl palmitate is a cosmetic ingredient used as emollient and/or surfactant-emulsifying agent (*57*). Concerning these compounds in sparkling wine foam, no reports are yet available. Anyway, the presence of free fatty acids and their ethyl esters are known compounds of base wines induced foam (*4*) and have also been reported to be present in aerosols released by the collapsed bubbles of *Champagne* wine (*22*). Monoacylglycerols have been reported to be released into the wine by yeast autolysis (*9*). The release of fatty acids from hydrolysis of the monoacylglycerols from *Champagne* wines showed the presence of the following fatty acids: 16:0, 16:1, 18:0, 18:1 and oxidized fatty acid (*58*). Although in small relative abundance, the sodium adducts of glyceryl palmitate and glyceryl stearate can also be observed at *m/z* 353 and 381 in **Figure 4c** (fragmentation data not shown).

Evidence of the occurrence of glycerylethylene glycol fatty acid derivatives in sparkling wine foam. In order to observe if other tensioactive molecules can be present as sodium adducts in the sample of sparkling wine foam, all major ions present in ESI-MS spectrum of low molecular weight hydrophobic compounds obtained from sparkling wine foam were studied by tandem MS. The ions at *m/z* 369, 385, 429, and 457 exhibit fragment ions that are consistent with the presence of glyceryl fatty acid derivatives. For the ion at *m/z* 369, the major ion was formed at *m/z* 324, which can be attributed to the loss of a formic acid radical ($HCOOH^{\cdot}$, **Figure 7a**). Also, the MS^2 spectrum shows the ion at *m/z* 251, resultant of a loss of 118 Da, attributed to the loss of glycerylformate. The MS^3 spectra ion at *m/z* 324 shows the ion at *m/z* 97, attributed to the sodiated glyceryl residue, confirming the occurrence of a glyceryl moiety in this molecule and allowing to infer the occurrence of an esterification of glycerol by a formic acid. This product ion spectrum also shows the ions at *m/z* 137, 123, 109, and 95, resultant from successive losses with differences of 14 Da, consistent with a saturated hydrocarbon chain fragmentation profile (*48, 49*). The loss of 184 Da from the ion at *m/z* 369 observed in the MS^2 spectrum can be attributed to a dodecanoic acid residue. The ion at *m/z* 369 can be attributed to a glycerylformate associated to a dodecanoic acid moiety by a 44 Da linker, possibly a monoethylene glycol residue.The MS^2 spectrum also shows the ion at *m/z* 185, attributed to $[glycerylformate\ monoethylene\ glycol+Na]^+$ and the ion at *m/z* 267, MS^3 (324 → 267), showing the loss of 56 Da (glyceryl residue-H_2O), attributed to $[monoethylene\ glycol\ dodecanoate+Na]^+$. Based on these results, one possible assignment for the ion at *m/z* 369 was sodiated glycerylformate monoethylene glycol dodecanoate. The ESI-MS spectrum of the blank sample showed the occurrence of a low intensity ion at *m/z* 369.3. However, its fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing to conclude that this ion is not an artifact of the methodology used.

The ion at *m/z* 385 gave a MS^2 spectrum with the main fragments at *m/z* 367, 324, 281, and 213, due to the neutral loss of 18, 61, 104, and 172 Da, corresponding to the loss of H_2O, acetate radical ($CH_3COOH^{\cdot}$), $C_4H_8O_3$, $C_{10}H_{20}O_2$, respectively (**Figure 7b**). The MS^3 of the ion at *m/z* 324 showed a several product ions consistent with a saturated hydrocarbon chain fragmentation, with cleavages at the C_β, C_γ, and C_δ, leading the formation of the ions at *m/z* 123, 109, and 95, respectively. Based on these results, the ion at *m/z* 385 can be assigned to the sodiated glycerylacetate diethylene glycol nonanoate ion.

The ESI-MS spectrum of the blank sample showed the occurrence of a very low intensity ion at *m/z* 385.1. Its fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing to conclude that it is not an artifact of the methodology used.

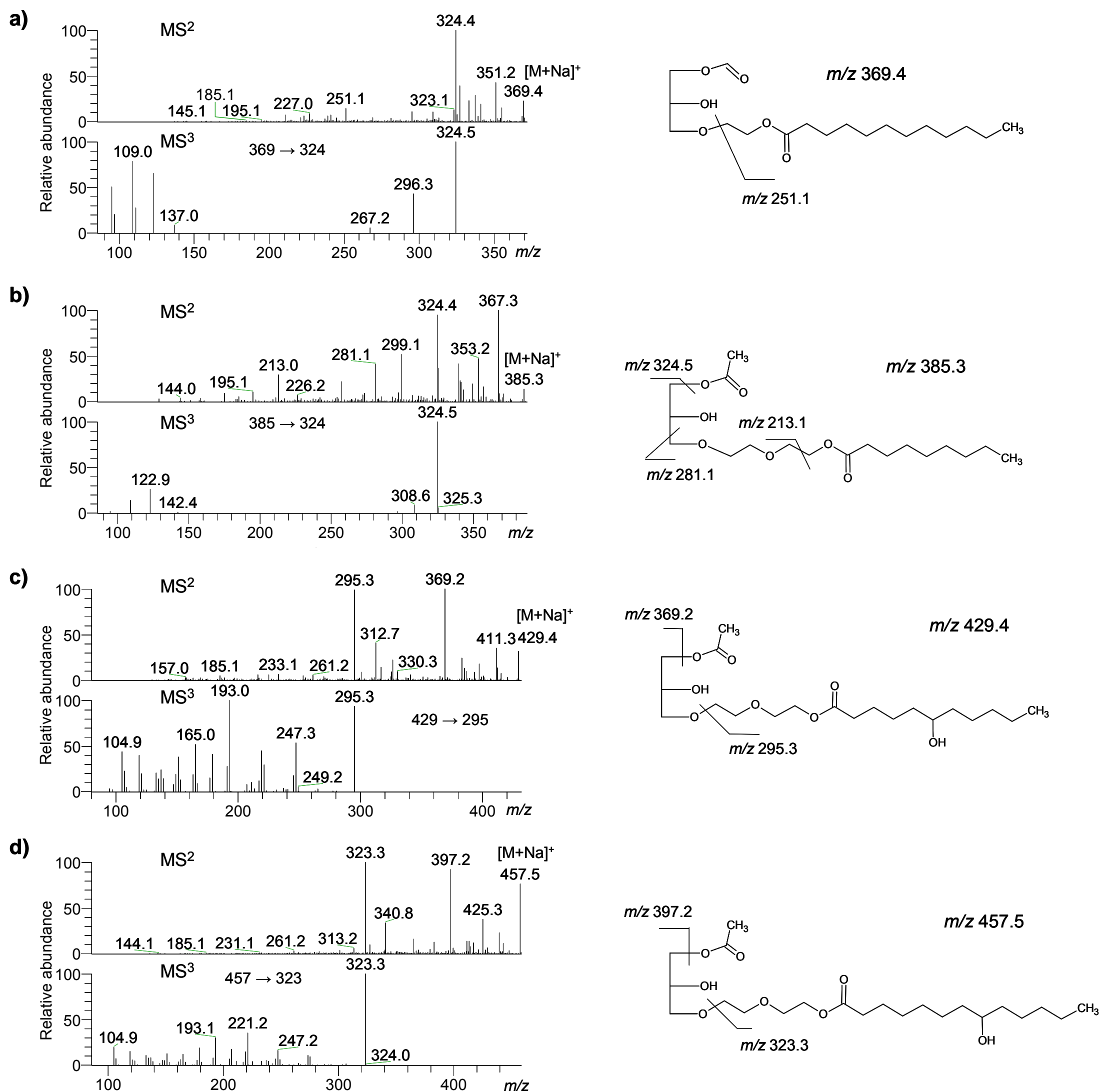


Figure 7 - MS^2 and MS^3 spectra fragmentation of sodium adducts of ions at a) *m/z* 369, b) *m/z* 385, c) *m/z* 429, and d) *m/z* 457, and tentative structure assignment for these ions, present in sparkling wine foam.

The ion at *m/z* 429 showed a MS^2 spectrum with a similar fragmentation pattern to the ion at *m/z* 457 (**Figure 7c and 7d**). In both spectra, the major fragment ion neutral losses corresponds to 60 Da, attributed to an acetic acid molecule, with formation of the ions at *m/z* 369 and 397, respectively, and loss of 134 Da, attributed to a glycerylacetate molecule, with formation of the ions at *m/z* 295 and 323, respectively. The MS^3 spectrum of the ion at *m/z* 295 (**Figure 7d**) showed the ions at *m/z* 193, 179, 165, 151, and 137, resultant from successive losses with differences of 14 Da, consistent with a saturated hydrocarbon chain fragmentation profile. The most intense fragment was the ion at *m/z* 193, with loss of 102 Da that can be attributed to a hydroxylated carbon chain fatty acid, as shown in **Figure 7c**. Based on these results and on the fragmentation consistent with the presence of ethylene glycol in previous structures, the ion at *m/z* 429 can be assigned to the sodium adduct of glycerylacetate diethylene glycol-6-hydroxy-undecanoate. The MS^3 spectrum of the ion at *m/z* 323, from the parent ion at *m/z* 457, showed the ions at *m/z* 221, 207, 193, 179, 165, and 151. These ions and fragmentation profile are similar to those observed for the ion at *m/z* 457 of F3 fraction (**Figures 5a, 5c and 7d**). Based on these results, the ion at *m/z* 457 can be assigned the sodium adduct of glycerylacetate diethylene glycol 8-hydroxy-tridecanoate (**Figure 7d**). The ESI-MS spectrum of the blank sample showed the occurrence of an ion at *m/z* 429.3 and 457.3. Their fragmentation resulted in a very different pattern of different fragment ions (results not shown), allowing to conclude that they are not artifacts of the methodology used.

As observed for monoacylglycerols, the glycerylethylene glycol fatty acyl derivatives here reported to be present in sparkling wine foam have potentially surfactant properties due to their more hydrophilic (glyceryl moiety) and more hydrophobic (fatty acid residue) components. The compounds identified have a similar structure to the synthetic polyethoxylated non-ionic surfactants, glycerol polyoxyethylene (POE) ricinoleates, that are composed by glycerol tri-polyethylene glycol ethers (n=12-38) esterified by one, two, or three molecules of ricinoleic acid (*59*).

In summary, the data obtained allowed concluding that sparkling wine foam presented glycerylethylene glycol fatty acid derivatives. These compounds have been shown to be involved in foam promotion and stabilization of wine model solutions. A higher number of glycerylethylene glycol fatty acid derivatives were found in sparkling wine foam than in the fraction containing the low molecular weight hydrophobic material

recovered from the whole sparkling wine (F3). As the same sparkling wine (FP_{LC}) was used to obtain the two samples, it is possible to infer that these surface active compounds are preferentially partitioned by the sparkling wine foam rather than the liquid phase, as observed for *Champagne* aerosols and bulk by Liger-Belair *et al.* (*22*). Additionally to glycerylethylene glycol fatty acid derivatives, the monoacylglycerols are also surface active compounds present in sparkling wine foam.

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3.2. Foamability and foam stability of molecular reconstructed sparkling wines

Foamability and foam stability of molecular reconstructed sparkling wines

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Sparkling wines foamability and foam stability have been shown to be modulated by the synergistic combination of the high and low molecular weight wine compounds. The present work aims to identify the contribution of the different wine components to the sparkling wines foaming properties. Twelve fractions were isolated: a) three fractions were composed by mannoproteins with different protein content (MP1, snMP2, and ppMP2, with 5, 38, and 64%, respectively), b) three fractions were arabinogalactans, one neutral and two acidic (AG0, AG1, and AG2), c) three fractions were a mixture of polysaccharides, proteins, and phenolic compounds (MeHMW, Aq1HMW, and Aq2HMW), and d) three fractions were intermediate and low molecular weight compounds (AqIMW, MeIMW, and MeLMW) composed by a mixture of carbohydrates, peptides, and phenolic compounds. The foam aptitude of each fraction was measured individually at the average concentration it was recovered from wines, using wine model solutions. Moreover, foam measurements were also performed using 2, 5, and 10 fold their average wine concentration. For the concentration that these fractions occur in wines, the maximum foam height (HM) was 8.4-11.7 cm, for foam height on stability (HS) was 6.9-7.5 cm and foam stability was (TS) 3.0-6.5 s. The increase in 2, 5, and 10 fold their wine concentration showed that HM and TS increased linearly and exponentially, respectively, with the increase of MP1 concentration. Also, the hydrophobic low molecular weight fraction (MeLMW), for 2 fold wine concentration, showed an increase in HM, HS, and TS for 13.8 cm, 12.9 cm, and 11.5 s, respectively. The fractions that individually showed higher foaming properties (MP1, AG0, and MeLMW) were mixed in binary and ternary combinations, showing that the foam of sparkling wines is mainly influenced by mannoproteins with low content of protein when mixed with hydrophobic compounds with less than 1 kDa.

Keywords: Foam, sparkling wines, polysaccharides, phenolic compounds, mannoproteins, arabinogalactans.

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INTRODUCTION

Foam characteristics are one of the most important organoleptic properties of sparkling wines. Several studies have searched to establish a correlation between their chemical composition and foam properties, namely, foamability and foam stability. Proteins were the first group of molecules to be proposed to explain sparkling wines foam properties due to their surfactant characteristics. Some authors have correlated positively protein concentration and foamability (*1-4*) but for foam stability the results are contradictory with both positive (*2*) and negative correlations (*5*). Recent studies have shown that glycoproteins rather than proteins are the most prominent macromolecules responsible for the foam of sparkling wines (*6, 7*). Among the wine glycoproteins, the yeast mannoproteins have been associated with the improvement of the foam properties in sparkling wines (*8*). The use of mannoproteins or cell wall extracts as additives for improving the foam properties of sparkling wines elaborated by the *champenoise* method was also proposed (*6*). Concerning carbohydrates, a oligosaccharide fraction with 2-3 kDa was correlated with foam stability, whereas the polysaccharides were related with foamability (*9*), although this correlation has been only observed for neutral polysaccharides, not for the acidic ones (*10*).

The main polysaccharides that are present in wines are the mannoproteins and the type II arabinogalactans (*11*). Mannoproteins are neutral polysaccharides composed mainly by mannose and small amounts of glucose, associated with 2-36% of protein (*11-14*). Mannoproteins are composed by a highly branched, short chain structure, where most of the mannopyranose residues are terminally-linked and 2,6-linked, together with 2- and 3-linked linear residues (*11, 14*). Type II arabinogalactans are composed mainly by a 3-linked galactopyranose backbone branched at C6 by galactose and arabinose residues. Glucuronic acid is also found as terminal non-reducing and 4-1inked (*16*). Different amounts of uronic acids (3-20%) can also be present (*15, 16*). The different contents in uronic acids confer them characteristics of weak acidic or even acidic polysaccharides.

The influence of polysaccharides on the foam stability of *Champagne* wines was also inferred by the similarity of the adsorption layers of *Champagne* wines with those of reconstituted solutions containing the low molecular weight material and polysaccharide-rich fractions (*17*). Sparkling wines foam behavior results from the synergistic interaction between the different foam active compounds that due to aggregation or complex formation may modify their surface-active properties (*18*). Thus, foaming properties are

not only due to the presence or absence of a specific group of compounds but are also influenced by the net balance of the number and type of compounds ranging different chemical structures (*1, 5, 19*).

In a previous work it was shown that the better foam stability of a reconstituted sparkling wine was achieved by the synergistic effect of the combination of the high molecular weight (HMW) material with the hydrophobic low molecular weight fraction (MeLMW-F3) (*20*). In the present work, the HMW fraction previously isolated was fractioned into nine sub-fractions representing mannoproteins with different amounts of protein (5, 38 and 64%), arabinogalactans (one neutral and 2 acidic fractions), and three fractions with different amounts of polysaccharides, proteins, and phenolic compounds. Also, the lower molecular weight compounds (<12 kDa), composed by different amounts of carbohydrates, peptides, and phenolic compounds, were divided into 3 fractions according to their size and polarity. The foam parameters of the wine model solutions containing each one of these 12 fractions or the combination of selected fractions were evaluated.

MATERIALS AND METHODS

Source of wine fractions. The high molecular weight (HMW) material was obtained from 35 bottles of base wine (26 L), used as a single lot from four different varieties (Bical, Arinto, Fernão-Pires, and Baga). Its isolation sequence is illustrated in **Scheme 1**. The wines were rotary-evaporated under reduced pressure at 35ºC to eliminate the ethanol and concentrate the total solids. The material was then dialyzed (12 kDa cut-off membrane, Medicell) in order to remove tartaric acid and other small molecules. The retentate was concentrated, frozen, and freeze-dried, to give the wine HMW material as a powder.

The intermediate (IMW) and low (LMW) molecular weight material was obtained from 4 bottles of sparkling wine (3 L) produced by Estação Vitivinícola da Bairrada (EVB), from Fernão-Pires (FP) a white variety, from a clayey soil, with grapes harvested at a late harvest moment, in 2002, one week after maturity, produced according to the *Champenoise* method, as described by Coelho *et al.* (*20*). The second fermentation was performed inside the bottles, after *tirage*. The wines were used after twenty four months of *dégorgement* (removal of yeast sediment from bottles). The sparkling wines were rotary-evaporated

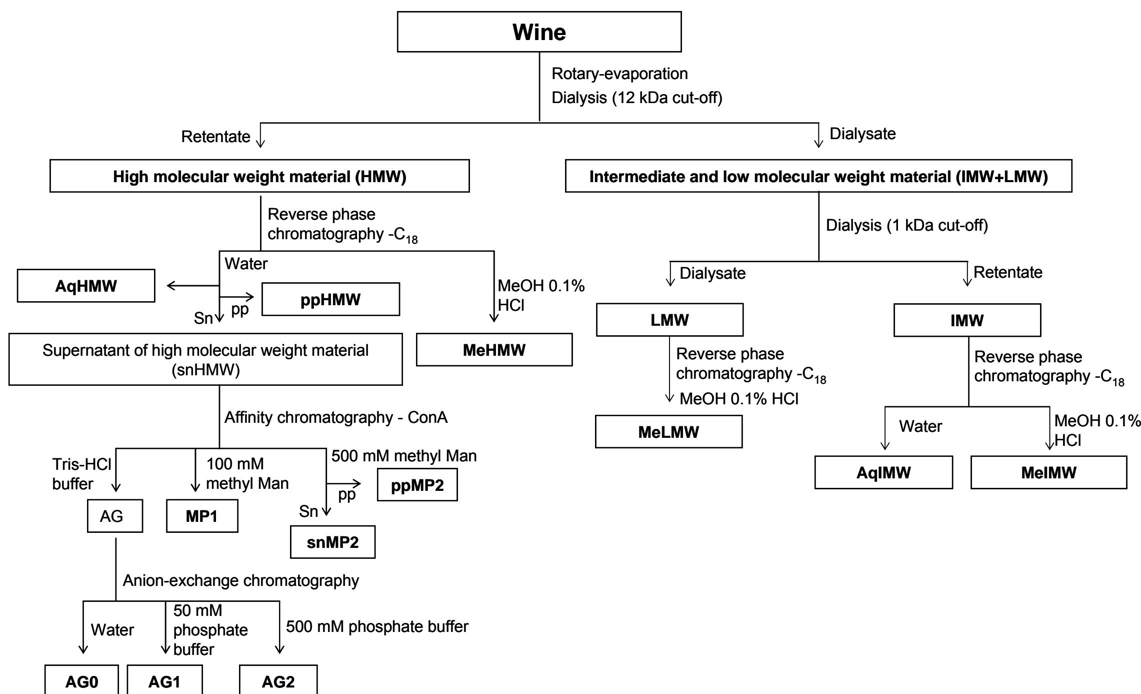
under reduced pressure at 35ºC and dialyzed (12 kDa cut off). The material that diffused through the dialysis membrane (dialyzate) was recovered by concentration under rotary-evaporation and frozen for use in the isolation step described in **Scheme 1**. The fractions were the same used by Coelho *et al.* (*20*).

Fraction of HMW material. The fractions of polysaccharides were obtained by combining the use of different chromatographic supports to allow the separation of the different classes of polysaccharides **(Scheme 1**). The wine polymeric material was fractionated using a C_{18} solid-phase-extraction column (SPE-C_{18}, Supelco-Discovery – 10 g). The material was eluted with water and the uncolored unbound material was recovered and concentrated. Upon concentration, it was observed the occurrence of a precipitate (ppHMW) that was separated from the supernatant (snHMW). During the elution with water a dark red band was observed in the C_{18} column and was recovered separately (AqHMW). The three fractions were frozen and freeze-dried. The bound fraction was recovered with acidic methanol (0.1% v/v HCl in MeOH), concentrated, frozen, and freeze-dried, presenting an intense red color (MeHMW).

The snHMW material was eluted through an affinity medium of Concanavalin A (Con A) Sepharose^{TM} 4B (GE Healthcare, Uppsala, Sweden) in a column with 30 cm length and 2 cm diameter, operated at 5 °C with a constant flow of 1 mL/min. Prior to elution, the column was pre-washed with a solution of 1 M NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 5 mM $CaCl_2$, and equilibrated with a buffer solution of Tris-HCl 20 mM and 0.5 M NaCl at pH 7.4. The arabinogalactans (AG) were eluted with Tris-HCl buffer and mannoproteins fraction (MP1) was desorbed with two bed volumes of the same buffer containing 100 mM of methyl-α-D-mannopyranoside, as described by Vidal *et al.* (*11*). Due to the large amount of material handled, successive batches were done, always after regeneration of Con A resin with 0.1 M Tris buffer, 0.5 M NaCl at pH 8.5 followed by 0.1 M sodium acetate, pH 4.5, containing 1 M NaCl. The Con A resin was also eluted with 500 mM of methyl-α-D-mannopyranoside. Both fractions were dialyzed and freeze-dried. After the dialysis of the fraction recovered with 500 mM of methyl-α-D-mannopyranoside, it was observed the formation of a precipitate inside the dialysis membrane that was separated from the supernatant. The supernatant gave origin to the fraction snMP2 and the precipitate to fraction ppMP2.

Anion-exchange chromatography was performed for the fraction rich in AG using a HyperSep SAX 10 g (Thermo Fisher Scientific, UK). Prior to elution, the column was conditioned with methanol followed by water and MeOH:water (5:95 v/v). The eluted AG rich fraction gave a neutral fraction (unretained) eluted with water (AG0), and two acidic fractions obtained in a stepwise elution using 50 mM (AG1) and 500 mM (AG2) of phosphate buffer pH 6.5. Acidic fractions were dialyzed and all fractions were freeze-dried, as described in **Scheme 1**.

Extraction and isolation of IMW and LMW material. The dialyzate (the molecules that through out of the membrane tube of 12 kDa) from sparkling wine sample were dialyzed with a cut-off of 1 kDa (Spectra/Por®). The 1 kDa dialyzate was added, under stirring, to a batch containing a C_{18} resin suspension, during 3 h, for sorption of the hydrophobic material. The resin was recovered by filtration, washed with water until the conductivity of the water is reached, and extracted with acidic methanol (MeOH 0.1% v/v HCl). This solution, which comprised the material with molecular weight lower than 1 kDa, gave origin to fraction (MeLMW). Using this procedure, the retentate, which comprised the material with intermediate molecular weight (between 1 and 12 kDa), gave origin to two fractions, the fraction of material not sorbed to the C_{18}, that remained in the water solution (AqIMW), and the fraction of material retained in the C_{18} resin and recovered with acidic methanol (MeIMW) (**Scheme 1**).



Scheme 1 – Diagram of the isolation steps of wine biomolecules.

Foam properties measurement. Foamability and foam stability were assessed using an adaptation of Mosalux and Bikerman method (*3, 4, 18*). Analytical grade CO_2 from a cylinder flowed through a glass-frit fitted in the bottom of a column (530 × 15 mm i.d.). The gas flow rate was controlled at 10 L/h by a flow meter (Cole-Parmer Instruments Company, IL, USA). Foamability was evaluated as the increase in height of 10 mL of degassed sparkling wine or model wine solutions placed inside the glass column, after CO_2 injection through the glass-frit. Two parameters of foamability were measured: 1) HM (maximum height reached by foam after CO_2 injection through the glass frit, expressed in cm) represents the solution ability to foam. 2) HS (foam stability height during CO_2 injection, expressed in cm) represents the solution ability to produce stable foam persistence of foam collar. Foam stability time (TS) was evaluated as the time elapsed before bubble collapse until the liquid appears after the interruption of CO_2, and is expressed in s. Each bottle of sparkling wine was analyzed in duplicate, and for each type of wine 8 replicates (4 bottles x 2 replicates per bottle) was obtained. The isolated fractions obtained from the wine were added independently or in mixtures to the wine model solution taking into account their average proportions in these seven sparkling wines. For these solutions, the foam properties measurements were done with 5 replicates.

Wine model solutions. Wine models were constructed from a hydroalcoholic base solution with 10% ethanol (v/v) and 0.5% tartaric acid (w/v) adjusted at pH 3.5 with NaOH solution. Glycerol and ethyl octanoate were added to this previous wine model in the concentrations of 0.7% (w/v) and 0.4% (w/v), respectively. The glycerol concentration used was in the range usually found in wines (*18*), and previously used to prepare wine model solutions (*21*). The ethyl octanoate concentration used was the concentration previously quantified in Bairrada sparkling wines (*22*). The isolated fractions of wine were added individually to the model solutions and in combination with the other fractions. The fractions that individually were more contributive for foam parameters were mixed in binary and ternary combinations. For each experiment the foam parameters HM, HS and TS were measured.

Sugar analysis. Monosaccharides were released from cell wall polysaccharides by a pre-hydrolysis in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC. Neutral sugars were analyzed after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (*23, 24*). A Perkin Elmer Clarus 400 GC apparatus with split injector and a FID detector was used, equipped with a 30 m column DB-225 (J&W) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC and then 220 ºC for 7 min, followed by an increase until 230 ºC at rate of 20 ºC/min, being this temperature maintained for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1 mL/min.

Uronic acids (UA) were quantified by a modification (*23*) of the 3-phenylphenol colorimetric method (*25*). Samples were prepared by hydrolysis in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature followed by 1 h in 1 M H_2SO_4 at 100 ºC. A calibration curve based on D-galacturonic acid as standard was used to calculate UA concentration.

Glycosidic-linkage composition of polysaccharide fractions. Glycosidic-linkage composition was determined by GC-qMS of the partially methylated alditol acetates (*26*). The sample (1–2 mg) was weighed into glass tubes and placed in a vacuum oven, at 40ºC,

overnight in the presence of P_2O_5 (s). Afterwards, it was dispersed in 1 mL of anhydrous DMSO and stirred over night for total solubilization. NaOH pellets (30 mg) were powdered under argon, added to the solution and kept stirring during 30 min. The polysaccharides were methylated with 80 µL of methyl iodide, added with a syringe into the closed tube with a cap with a silicone septum. The mixture was allowed to react for 20 min under stirring. Two mL of water were added, and the solution was neutralized with HCl 1 M. The methylated material was then extracted with 3 mL of CH_2Cl_2 and the aqueous phase was removed after centrifugation. The dichloromethane phase was then washed three times with 2 mL of water until the dichloromethane phase became limpid. The organic phase was transferred to a clean tube and dried by centrifugal evaporation (Univapo 100 ECH, UniEquip, Germany). This methylation procedure was repeated. The permethylated polysaccharides were hydrolyzed with 0.5 mL of 2 M TFA (1 h at 121ºC) (*27*) and dried by centrifugal evaporation. The reduction of monosaccharides was performed during 1 h at 30ºC with 20 mg of sodium borodeuteride (Isotec^{TM}, Switzerland) in 300 µL of 2 M NH_3. The reaction was terminated by the addition of 0.1 mL of glacial acetic acid. The acetylation was performed with 3 mL of acetic anhydride using 450 µL 1-methylimidazole as catalyst, during 30 min at 30ºC. Then, 3 mL of distilled water were added to decompose the acetic anhydride, and the acetylated sugars were extracted with 5 mL of CH_2Cl_2. The organic phase was washed three times with water and then dried by centrifugal evaporation. The partially methylated alditol acetates were dissolved in 70 µL of acetone and 0.2 µL was injected and analyzed by GC-qMS on Agilent Technologies 6890N Network gas chromatograph, equipped with a 30 m × 0.25 mm (i.d.), 0.1 µm film thickness DB-1 fused silica capillary column (J&W Scientific Inc., CA, USA), connected to an Agilent 5973 quadrupole mass selective detector. The oven temperature was programmed from 45 ºC (5 min) to 140 ºC (5 min) at 10 ºC/min, to 170 ºC (1 min) at 0.5 ºC/min and then to 280 ºC (5 min) at 15 ºC/min. Helium carrier gas had a flow of 1.7 mL/min and a column head pressure of 2.8 psi. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 40-500 *m/z*, in a full scan acquisition mode. Identification was achieved comparing the standard mass spectra and other spectra with a laboratory made database. Methylation analysis was assayed for MP1, AG0, AG1, and AG2.

For the permethylated fractions of AG0, AG1, and AG2, prior to acid hydrolysis, the dichloromethane solutions were split in two portions and a carboxyl reduction was performed. The permethylated polysaccharides were dried, dissolved in 1 mL anhydrous tetrahydrofuran and 20 mg lithium aluminium deuteride (Aldrich, WI, USA) was added under argon. The suspension was kept at 65ºC during 4 h under stirring (*28*). The reagent in excess was eliminated by adding 2–3 drops of ethanol and 2–3 drops of distilled water. The solution was neutralized by addition of 1 M HCl. Two mL of $CHCl_3$/methanol 2:1 (v/v) mixture were then added. The reduced polymers were removed from the white precipitate by centrifugation and washed thoroughly with the chloroform/methanol solution. The supernatant was collected, evaporated, and the carboxyl-reduced material was submitted to hydrolysis with TFA, reduction, and acetylation, as described above.

Protein analysis. Protein quantification was based on the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as standard, using the Bicinchoninic Acid Protein Assay Kit from Sigma (Aldrich-Chemie, Steinheim, Germany). The samples were incubated in a water bath at 60 ºC during 15 min. The absorbance was measured at 562 nm with a 6405 Jenway UV–vis spectrophotometer (UK) against a blank in the reference cell. The data were correlated with the calibration curve of BSA standard (concentration range of 0.05–0.40 mg/mL), also analyzed in the same conditions of the samples. At least three replicates of each concentration were carried out for all experiments. Protein analysis was assayed for all samples except AGs.

Determination of total phenolic compounds. Total phenolic composition was determined by the Folin-Ciocalteu colorimetric method (*29*). The samples were dissolved in hydroalcoholic solution (10% v/v of ethanol) and 0.125 mL of this solution was mixed with 0.5 mL of water and 0.125 mL of Folin-Ciocalteu reagent. After homogenization with a vortex, the sample was allowed to react during 5 min, and 1.250 mL of Na_2CO_3 (75 g/L) and 1.0 mL of water were added. The mixture was homogenized in a vortex and reaction occurs during 90 min at room temperature. The absorbance was measured at 760 nm (6405 Jenway UV–vis spectrophotometer, UK). The calibration curve was built using gallic acid as standard in the concentration range 12.7–101.8 mg/L. At least three replicates of each concentration were carried out for all experiments. The analysis of total phenolic

compounds was performed for the following samples: ppHMW, AqHWM, MeHMW, AqIMW, MeIMW, and MeLMW.

RESULTS AND DISCUSSION

Characterization of wine isolated fractions. The wine was fractionated into twelve different fractions according to their molecular weight, polarity, and solubility (**Scheme 1**). The fractions, nine polymeric: MP1, snMP2, ppMP2, AG0, AG1, AG2, MeHMW, ppHMW, AqHMW, and three fractions of intermediate and low molecular weight: AqIMW, MeIMW, MeLMW, were characterized concerning their abundance in wine, sugar composition, and content in protein and phenolic compounds (**Table 1**). Also, the snHMW material, that gave origin to the mannoprotein and arabinogalactan fractions, and AG, originating the three arabinogalactan fractions, were analyzed.

Concerning the three mannoprotein fractions, the most abundant was MP1 (48.8 mg/L of wine), contrasting with snMP2 and ppMP2, presenting only 1.3 and 2.2 mg/L, respectively. These fractions had different protein contents, 5%, 38% and 64% for MP1, snMP2, and ppMP2, respectively. All these fractions contained mannose as the main sugar. Glycosidic-linkage analysis of MP1 showed that 2,6-Man*p* (31.9 mol%), terminally-linked Man*p* (29.8 mol%), 2-Man*p* (20.2 mol%), and 3-Man*p* (10.9 mol%) were the most abundant linkages (Table 2), confirming that they are mannoproteins from yeast origin (*11*). The material not retained by the Concanavalin A medium, accounting for 85.2 mg/L of wine, was very rich in sugars (93%), mainly galactose (45 mol%) and arabinose (28 mol%), containing only 5 mol% of mannose. This shows that this fractionation allowed separating the mannoprotein components from those arising from arabinogalactans (AG). The material recovered after fractionation through the Concanavalin A medium accounted only for 57% of the material eluted (snHWM). However, the recovery of mannose was 77% and arabinose and galactose were almost totally recovered (**Table 1**), showing that the main material lost through this purification step was protein. According to the protein content of the different fractions, it can be estimated a loss of approximately 50% of the protein eluted.

Table 1- Yield of the fractions isolated from wine, sugar composition, total sugar, total protein and total phenolic content.

Fraction	Yield (mg/L)	*Mol %*								Total Sugars (%, w/w)	Protein (%, w/w)	Phenolics (%, w/w)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur. Ac.			
snHMW	255.2	2	0	25	0	26	32	7	7	75	8	-
MP1	48.8	1	0	1	0	90	3	2	3	74	5	-
snMP2	1.3	0	0	0	0	90	1	2	6	33	38	-
ppMP2	2.2	1	0	0	0	83	4	3	9	29	64	-
AG	85.2	2	0	28	0	5	45	12	8	93	-	-
AG0	38.1	1	0	24	0	5	38	28	4	62	-	-
AG1	32.0	3	0	28	0	7	47	3	11	77	-	-
AG2	4.4	2	0	28	1	5	51	3	10	77	-	-
ppHMW	38.6	2	0	8	1	17	10	9	53	3	0.2	0.1
AqHMW	4.8	3	2	23	4	21	16	14	17	12	80	8
MeHMW	64.0	7	1	38	1	8	16	11	18	65	40	10
AqIMW	17.7	7	1	6	3	30	11	10	32	53	19	18
MeIMW	23.9	3	1	15	6	10	4	45	18	9	57	73
MeLMW	356.3	2	1	10	5	20	4	49	10	7	39	6

Three fractions of arabinogalactans were recovered from wine: AG0, AG1, and AG2, accounting for 38.1 mg/L of wine, 32.0 mg/L, and 4.4 mg/L, respectively. These fractions were composed mainly by arabinose (24-28 mol%) and galactose (38-51%) (**Table 1**). Glycosidic-linkage analysis showed that the major linkages (**Table 2**) are 3,6-Gal*p* (25-35 mol%), 6-Gal*p* (9-13 mol%), 3-Gal*p* (5-9 mol%), terminally-linked Gal*p* (5-8 mol%), terminally-linked Ara*f* (8-18 mol%), and 5-Ara*f* (2-5 mol%), together with the occurrence of terminally-linked glucuronic acid. This composition is consistent with the presence of arabinogalactans (*16*). The neutral fraction (AG0), in addition to the arabinogalactan, contained a glucan, identified by the presence of glucose (28 mol%) (**Table 1**) and 4-Glc*p* and 6-Glc*p* (**Table 2**). Comparing the amount of AG material eluted through the anion-exchange chromatography and the amount of material recovered in the three fractions, it was possible to observe a recovery of 88% of the material, with no significant difference for the recovery of polysaccharides (**Table 1**).

Table 2 - Glycosyl linkage composition of MP1, AG0, AG1 and AG2 fractions isolated from wine.

Glycosyl linkage[a]	MP1	AG0	AG1	AG2
T-Fuc*p*	tr	tr	0.1	tr
2-Fuc*p*	-	-	0.2	tr
Total	***0.0***	***0.0***	***0.3***	***0.1***
T-Rha*p*	0.1	0.1	0.3	0.3
4-Rha*p*	-	tr	0.4	-
3-Rha*p*	-	-	0.1	-
2,4-Rha*p*	-	-	0.3	0.1
Total	***0.1***	***0.2***	***1.1***	***0.3***
T-Ara*f*	0.1	8.3	14.4	17.8
T-Ara*p*	tr	0.2	0.5	0.4
2-Ara*f*	-	0.2	0.4	0.6
3-Ara*f*	-	0.4	0.5	0.5
5-Ara*f*	0.3	4.5	4.4	2.2
3,5-Ara*f*=3,4-Ara*p*	-	2.0	1.5	0.4
3-Ara*p*	0.2	0.2	0.3	0.1
Total	***0.6***	***15.7***	***21.9***	***22.0***
4-Xyl*p*	0.24	-	0.1	-
Total	***0.2***		***0.1***	
T-Man*p*	29.8	0.9	0.7	0.3
2-Man*p*	20.2	-	-	-
3-Man*p*	10.9	-	-	-
6-Man*p*	2.9	-	-	tr
2,3-Man*p*	0.3	0.1	0.2	tr
2,4-Man*p*	0.1	-	-	-
4,6-Man*p*	tr	-	-	-
2,6-Man*p*	31.9	0.1	-	-
3,6-Man*p*	1.3	-	-	-
2,3,6-Man*p*	0.4	-	-	-
2,3,4,6-man*p*	0.1	-	-	-
Total	***96.0***	***1.0***	***0.9***	***0.4***
T-Gal*p*	0.1	4.8	7.9	8.3
2-Gal*p*	-	0.5	0.8	0.2
4-Gal*p*	0.3	0.3	0.6	0.4
3-Gal*p*	0.1	5.3	6.7	8.7
6-Gal*p*	0.1	9.4	12.8	11.7
4,6-Gal*p*	-	1.4	2.2	1.7
3,6-Gal*p*	0.2	25.2	31.4	34.7
3,4,6-Gal*p*	-	4.5	7.3	6.9
2,3,6-Gal*p*	-	0.1	0.2	0.3
2,3,4,6-Gal*p*	-	-	tr	0.1
Total	***0.8***	***51.5***	***70.0***	***72.9***
T-Glc*p*	0.4	0.5	0.2	0.1
3-Glc*p*	-	0.2	0.1	0.1
4-Glc*p*	-	19.4	1.4	1.3
6-Glc*p*	0.2	10.2	0.3	0.2
3,4-glc*p*	tr	0.8	1.9	2.0
3,6-Glc*p*	-	-	0.1	-
4,6-glc*p*	-	0.3	-	-
2,3,6-Glc*p*	-	tr	0.1	tr
2,3,4,6-Glc*p*	-	0.1	tr	tr
Total	***0.6***	***31.5***	***4.1***	***3.8***
T-GlcA*p*	-	0.6	0.8	0.4
4-GlcA*p*	-	0.1	0.2	-
4-GalA*p*	-	tr	0.4	-
Total	***-***	***0.7***	***1.3***	***0.4***

[a] The molar ratios are the means of two repetitions.
tr- Trace amounts.

The fraction containing the hydrophobic high molecular weight material (MeHMW), accounting for 64.0 mg/L of wine, was rich in sugars (65%), mainly arabinose (38 mo%), uronic acids (18 mol%), and galactose (16 mol%), a sugar composition characteristic of a

highly branched pectic polysaccharide (*30*). It is possible that the retention of this fraction in the C_{18} resin may be due to the linkage of the polysaccharides to hydrophobic material, namely phenolic compounds and/or protein. According to **Table 1**, this fraction is also rich in protein and phenolic compounds. However, because the methods used to determine these compounds interfere one with the other, the values achieved may be overestimated. The fraction of the high molecular weight material not retained by the C_{18} stationary phase and that precipitate upon concentration (ppHMW) accounted for 38.6 mg/L wine. This white powder recovered showed only to contain 3% of sugars, no phenolic compounds, and no protein (**Table 1**). The high molecular weight material fraction that was slightly sorbed in C_{18} stationary phase (AqHMW), accounted for only 4.8 mg/L, and was mainly composed by protein (80%); sugars account for 12% and phenolic compounds 8% (**Table 1**).

The fraction of low molecular weight sorbed in C_{18} resin (MeLMW) was the largest fraction recovered, accounting for 356.3 mg/L of sparkling wine. It was composed by proteins (39%), sugars (7%), and phenolic compounds (6%) (**Table 1**). The fraction of intermediate molecular weight retained in the C_{18} resin (MeIMW) accounted for 23.9 mg/L. It showed a high amount of phenolic compounds and protein, preventing their accurate estimation with the methodology used. Sugars accounted for only 9%, whereas glucose was the major sugar (45 mol%), probably resultant from the glycosylation of phenolic compounds. The fraction of intermediate molecular weight not sorbed in the C_{18} resin (AqIMW), accounting for 17.7 mg/L, was composed mainly by sugars (53%), protein (19%), and phenolic compounds (18%). The main sugar residues were uronic acids (32 mol%) and mannose (30 mol%) (**Table 1**), indicating that this fraction should be a mixture of degraded pectic polysaccharides and mannoproteins.

Evaluation of foam aptitude of the fractions isolated from wine. The twelve different fractions isolated from wine were individually used to prepare wine model solutions containing 10% ethanol and 0.5% of tartaric acid at pH 3.5. The amount of material used was that recovered for each fraction, as shown in the yield column in **Table 1**. A model solution containing the snHMW material, that gave origin to the mannoprotein and arabinogalactan fractions, was also prepared. All these solutions were tested to evaluate their foam aptitude, namely, the maximum height reached by foam after CO_2 injection through the glass frit, expressed in cm (HM), the foam stability height during CO_2 injection, expressed in

cm (HS), and the foam stability time, expressed in s (TS), as shown in **Figures 1** and **2** and **Table 3**.

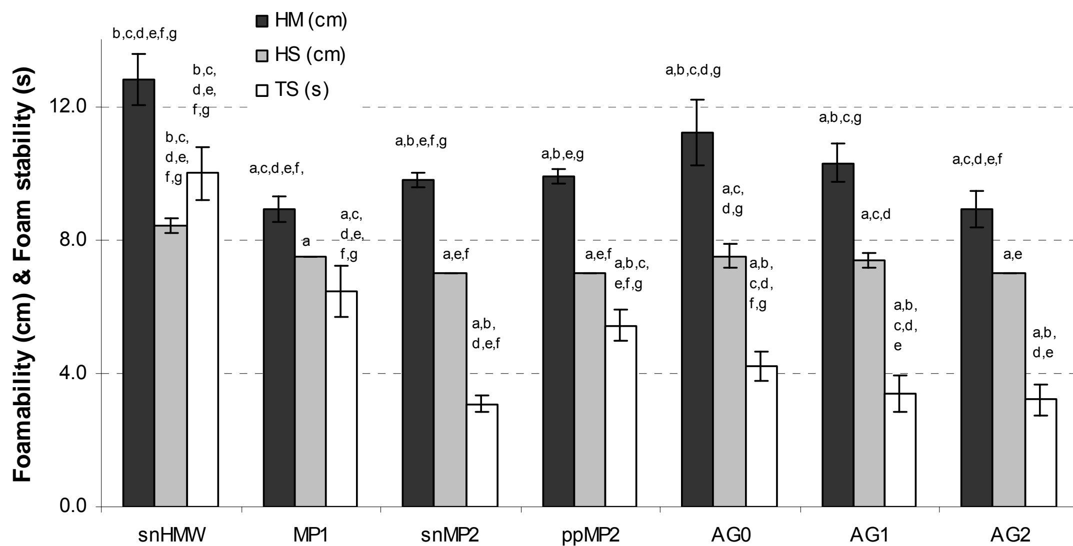


Figure 1 - Foamability, HM and HS, and foam stability, TS, measured for snHMW (supernatant of high molecular weight material) and all fractions obtain from that (3 mannoprotein fractions with different protein contents – MP1, snMP2, and ppMP2; and 3 arabinogalactans fractions one neutral and two acidic fractions – AG0, AG1, and AG). All fractions were in wine concentration in the model solution.

a- significantly different ($p > 0.05$) from snHMW, **b**- significantly different ($p > 0.05$) from MP1, **c**- significantly different ($p > 0.05$) from snMP2, **d**- significantly different ($p > 0.05$) from ppMP2, **e**- significantly different ($p > 0.05$) from AG0, **f**- significantly different ($p > 0.05$) from AG1, **g**- significantly different ($p > 0.05$) from AG2.

The HM ranged between 8.4 and 12.8 cm, being the maximum HM observed for snHMW material, followed by MeHMW, MeLMW, and AG0, and the minimum was observed for AqHMW and AG2 fractions. As the analysis of the blank wine model solution, composed only by ethanol and tartaric acid, at pH 3.5, showed an HM of 8.2 cm, with a standard deviation of 0.4 cm (5 replicates), it can be inferred that all fractions, with the exception of AqHMW and AG2, are contributing to the HM properties of these solutions. The higher values observed for snHMW than those observed for all six fractions obtained from it allows to conclude that when the different mannoprotein or arabinogalactan-rich material are assayed individually, they have lower HM values than when they are assayed together in a mixture. This can be explained by the higher amount of polymeric material used in the

solutions of snHMW than in the others (**Table 1**). Anyway, although MP1 was the fraction with higher amount of material from these six, it was not the fraction with higher HM, showing that other parameters should be also involved in this property. At wine concentration, the high and low molecular weight fractions retained in C_{18} and recovered with acidic methanol are important for HM.

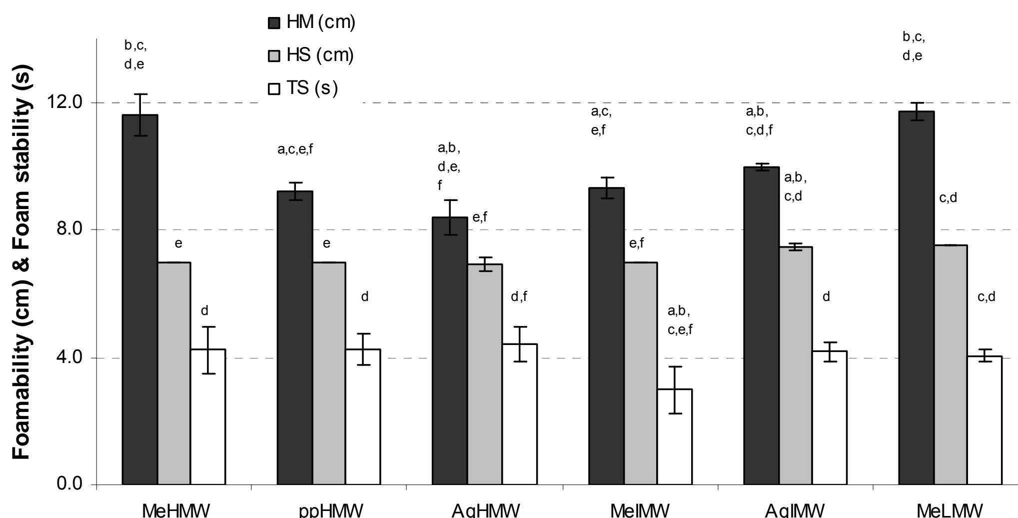


Figure 2 - Foamability, HM and HS, and foam stability, TS, measured for six fractions 3 polymeric (MeHMW, ppHMW, AqHMW), 2 of intermediate molecular weight compounds (MeIMW and AqIMW), and one of low molecular weight compounds MeLMW). All fractions were in wine concentration in the model solution.

a- significantly different ($p > 0.05$) from MeHMW, **b**- significantly different ($p > 0.05$) from ppHMW, **c**- significantly different ($p > 0.05$) from AqHMW, **d**- significantly different ($p > 0.05$) from MeIMW, **e**- significantly different ($p > 0.05$) from AqIMW, **f**- significantly different ($p > 0.05$) from MeLMW.

The HS of the fractions analyzed at wine concentration varied only between 6.9 and 7.5 cm. Although these values are close to those measured for the blank wine model solutions (7.0 cm), it was found significant differences for snHMW, MP1, AG0, AG1, AqIMW, and MeLMW. Concerning TS, it varied between 3.0 and 10.0 s, whereas the blank solution was 3.2 s. The higher TS was observed for snHMW, which was very much higher than that observed for the fractions derived from it (6.5 to 3.1 s). Nevertheless, fractions MP1 and ppMP2 were those that, among the 12 fractions understudy, presented higher TS, showing that the mannoproteins are relevant molecules to explain the foam stability characteristics of wine. Other fractions that contribute to the TS were AG0, MeHMW, ppHMW, AqHMW, AqIMW, and MeLMW, showing the acidic AG fractions are not relevant to explain the wine foam stability.

The foam properties observed for snHMW were higher in all three parameters measured (HM, HS, and TS) than those of the fractions derived from it. As the concentration of the solution of snHMW was 4-195 times more concentrated than the other ones, it shows that, although not explaining all the foam properties, the concentration of the compounds in each model solution cannot be neglected. Also, considering that the concentration used for reconstitution of the fractions may be underestimated due to the manipulation and natural loss of material during the fractionation process, resulting in more diluted solutions than those present in real wines, it is a requirement to study the effect of the concentration of each fraction on foam properties.

Effect of concentration of wine isolated fractions in foam aptitude. In order to evaluate the concentration effect, the foam aptitude of model solutions up to 10 folds the wine concentration was measured for all 12 fractions. However, for MeLMW, the maximum concentration possible to dissolve in the wine model solution was 2 fold its concentration in wine.

Table 3 - HM, HS and TS of all fractions isolated measured in 1, 2, 5 and 10 fold their average wine concentration.

	n[wine]	MP1	snMP2	ppMP2	AG0	AG1	AG2	MeHMW	ppHMW	AqHMW	MeIMW	AqIMW	MeLMW
HM (cm)	1	8.9±0.4*	9.8±0.2*	9.9±0.2*	11.2±1.0*	10.3±0.6*	8.9±0.5	11.6±0.7*	9.2±0.3*	8.4±0.5	9.3±0.3*	10.0±0.1*	11.7±0.3*
	2	11.0±0.5*	-	-	-	-	-	-	-	-	-	-	13.8±0.4*
	5	13.0±1.6*	-	-	-	-	-	-	-	-	-	-	-
	10	19.3±0.5*	10.1±0.2	9.5±0.4	10.9±0.7	9.8±0.3	11.8±0.6*	13.4±1.2*	10.8±0.7*	11.0±0.6*	13.0±0.4*	9.0±0.0*	-
HS (cm)	1	7.5±0.0*	7.0±0.0	7.0±0.0	7.5±0.4*	7.4±0.2*	7.0±0.0	7.0±0.0	7.0±0.0	6.9±0.2	7.0±0.0	7.4±0.1*	7.5±0.0*
	2	8.1±0.1*	-	-	-	-	-	-	-	-	-	-	12.9±0.4*
	5	8.7±0.3*	-	-	-	-	-	-	-	-	-	-	-
	10	8.1±0.3*	7.3±0.1*	7.9±0.1*	7.5±0.0	7.4±0.1	7.7±0.4*	7.6±0.2*	7.5±0.1*	7.4±0.2*	7.1±0.1	7.0±0.0*	-
TS (s)	1	6.5±0.8*	3.1±0.2	5.4±0.5*	4.2±0.4*	3.4±0.5	3.2±0.4	4.2±0.7*	4.2±0.5*	4.4±0.5*	3.0±0.7	4.2±0.3*	4.1±0.2*
	2	5.2±0.3*	-	-	-	-	-	-	-	-	-	-	11.5±0.4*
	5	10.3±1.6*	-	-	-	-	-	-	-	-	-	-	-
	10	54.3±19.1*	4.8±0.1*	7.0±0.4*	5.9±0.7*	5.4±0.6*	5.0±0.4*	8.0±0.9*	4.3±0.5	4.8±0.3*	3.1±0.6	3.6±0.3*	-

* Each fraction at wine concentration is significantly different ($p > 0.05$) from wine model solution (data in the text); for the concentrations where $n > 1$[wine], it is significantly different ($p > 0.05$) from the fraction at immediately lower concentration.

Concerning the HM, only 6 of the 11 fractions studied showed an increase of this foam parameter when 10 times concentrated solutions of each individual fraction were used (**Table 3**). MP1 increased more than twice, from 8.9 to 19.3 cm, whereas AG2, MeHMW, ppHMW, AqHMW, and MeIMW increased 16-40% for a concentration increase of 1000%. Testing the HM for fraction MP1 using the intermediate concentrations of 2 and 5 folds its wine concentration, it was observed a linear increase with concentration (HM (cm) = 1.10 n[wine] + 8.17, with an R^2 = 0.96). A comparable increase was observed for fraction

MeLMW at 2 fold wine concentration (from 11.7 to 13.8 cm), reaching a HM value higher than MP1 for 2 fold, and also higher than those observed for all other fractions assayed at 10 fold their wine concentrations. The fractions that promoted a significant increase in HS were MP1, snMP2, ppMP2, AG2, MeHMW, ppHMW, AqHMW, and MeLMW. Although the later was only tested for the twofold wine concentration, it achieved a HS higher than the other fractions at 10 fold wine concentration (**Table 3**).

The foam parameter TS showed to increase in 9 of the 11 fractions tested with concentrations 10 times higher. A 7.4 times increase was observed for MP1, whereas snMP2, ppMP2, AG0, AG1, AG2, MeHMW, AqHMW, and AqIMW increased 10-90%. When tested for the intermediate concentrations of 2 and 5 folds wine concentration, fraction MP1 showed an exponential increase with concentration (TS (s) = $3.77e^{0.257(n[\text{wine}])}$, with an R^2 = 0.95), allowing to conclude that the higher the concentration of the mannoproteins with 5% of proteic material the higher is its influence in foam stability of the wine model solution. The increase of foamability and foam stability with the increase of the concentration of mannoproteins was also observed by other authors, where sparkling wines were supplemented with increasing concentrations of yeast extracts (*6*). Concerning fraction MeLMW, the two-fold increase in concentration allowed an increase from 4.1 s to 11.5 s, a value higher than those observed for all fractions, with the exception of MP1 at 10 fold (**Table 3**), showing that the low molecular weight hydrophobic material is also relevant to explain the foam stability of the solutions.

These results allowed concluding that not all fractions presented the same contribution to wine model solutions foam properties. Nevertheless, the foam aptitude is influenced by the concentration of some wine constituents. From all fractions isolated, MP1 (mannoproteins), MeLMW (low molecular weight hydrophobic material), and AG0 (neutral arabinogalactans) seems to be the most relevant ones. All these fractions were recovered in high yield from wine and presented a significant impact on foam properties of the wine model solutions. These three fractions were selected to build wine model solutions where binary and ternary combinations were performed in order to evaluate any possible synergistic effect at the average concentration they occur in wine.

Evaluation of foam aptitude of binary and ternary combinations of MP1, AG0, and MeLMW fractions. Fig. 3 shows the foam aptitude of the wine model solutions containing the different combinations of MP1, AG0, and MeLMW fractions.

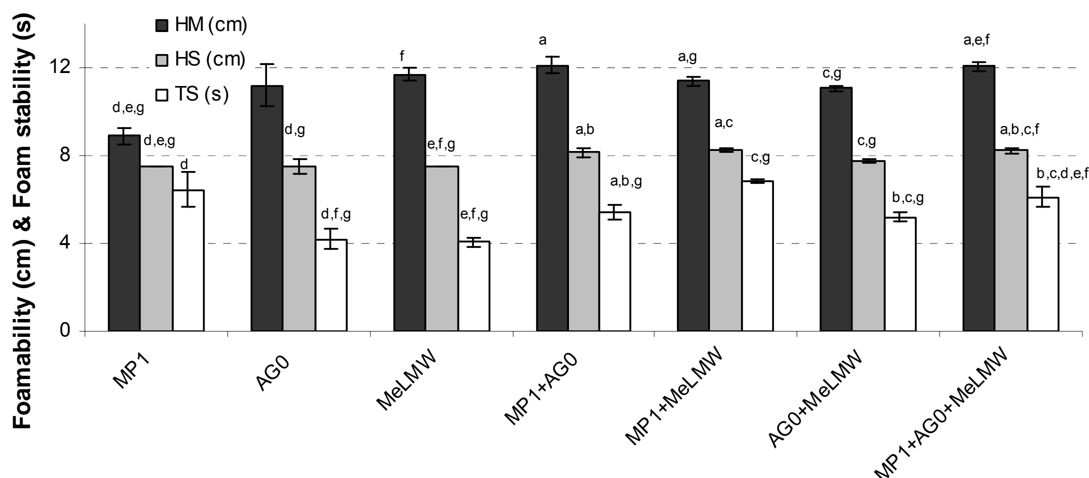


Figure 3 - Foamability, HM and HS, and foam stability, TS, measured for the three fractions that contribute more to the foamability and stability individually and in binary combination and the tertiary combinations of these fractions. All fractions were in wine concentration in the model solution.

a- significantly different ($p > 0.05$) from MP1, **b**- significantly different ($p > 0.05$) from AG0, **c**- significantly different ($p > 0.05$) from MeLMW, **d**- significantly different ($p > 0.05$) from MP1 + AG0, **e**- significantly different ($p > 0.05$) from MP1 + MeLMW, **f**- significantly different ($p > 0.05$) from AG0 + MeLMW, **g**- significantly different ($p > 0.05$) from MP1 + AG0 + MeLMW.

Concerning MP1, its combinations, both binary and ternary, all showed significant increases in HM (28-36%) and HS (8-10%) when compared with the fraction alone. This shows a synergistic effect of the mannoproteins with other wine components concerning foamability. However, for TS, no significant differences were observed for MP1+MeLMW neither for the ternary mixture, when compared to the MP1 fraction alone. However, for MP1+AG0, a significant decrease of 16% was observed, allowing to conclude that the presence of both mannoprotein and arabinogalactan prevents a higher value for foam stability. The observation that there is a balance in wine between constituents that act negatively and positively on foam as already been stated by Viaux *et al.* , namely the particles or macromolecular complexes retained by filter with 0.45 μm cut-off are able to destroy foam stability (*31*). This is consistent with the fact that arabinogalactans, due to

their highly branched structure that confers them high solubility, are molecules with the highest molecular weight when compared with all other soluble polysaccharides present in wines (*15, 16*).

Concerning AG0 fraction, its addition to the other fractions promotes a significant increase in the TS (24-46%) of the mixtures when compared with the single fraction. HM parameter is not significantly different, and HS showed a slight increase for the combination MP1 + AG0 (8%) and for the ternary combination (10%). Regarding the fraction MeLMW, its combination with the other fractions increases significantly the HS and TS parameters (3-10% and 28-68%, respectively). The HM decreased (6%) when combined with AG0 in comparison with the value obtained for MeLMW fraction alone. For the other combinations, HM was not significantly different.

In most cases, the ternary combination showed better foam parameters than the fractions individually. These results show that the foam stability of sparkling wines seems to be mainly influenced by mannoproteins with low content of protein (5%) and the foamability by arabinogalactans and a hydrophobic low molecular weight fraction (<1kDa). The binary combination of MP1 and MeLMW presents a synergistic effect where all foam parameters were improved. This MeLMW fraction was shown in previous study to be composed by tensioactive molecules that seem to be involved in foam stabilization (*20*).

Other major wine components are also relevant to explain sparkling wine foam properties. For example, the presence of glycerol and glycerol plus ethyl octanoate also influence the foam parameters, namely HM and TS (**Fig. 4**). Glycerol represents almost 5% of wine composition (*18*) and is known to contribute to the viscosity of the solution. Also, ethyl esters of fatty acids have been were positively correlated with foamability (*32*). From these, ethyl octanoate was the major ester present in these sparkling wines (*22*). In almost experiments shown in **Fig. 4**, the supplementation of the wine model with ethyl octanoate did not increment HS and TS when compared with the incorporation of glycerol, but the addition of ethyl octanoate decreased the relative standard deviation of the foam parameters of these solutions.

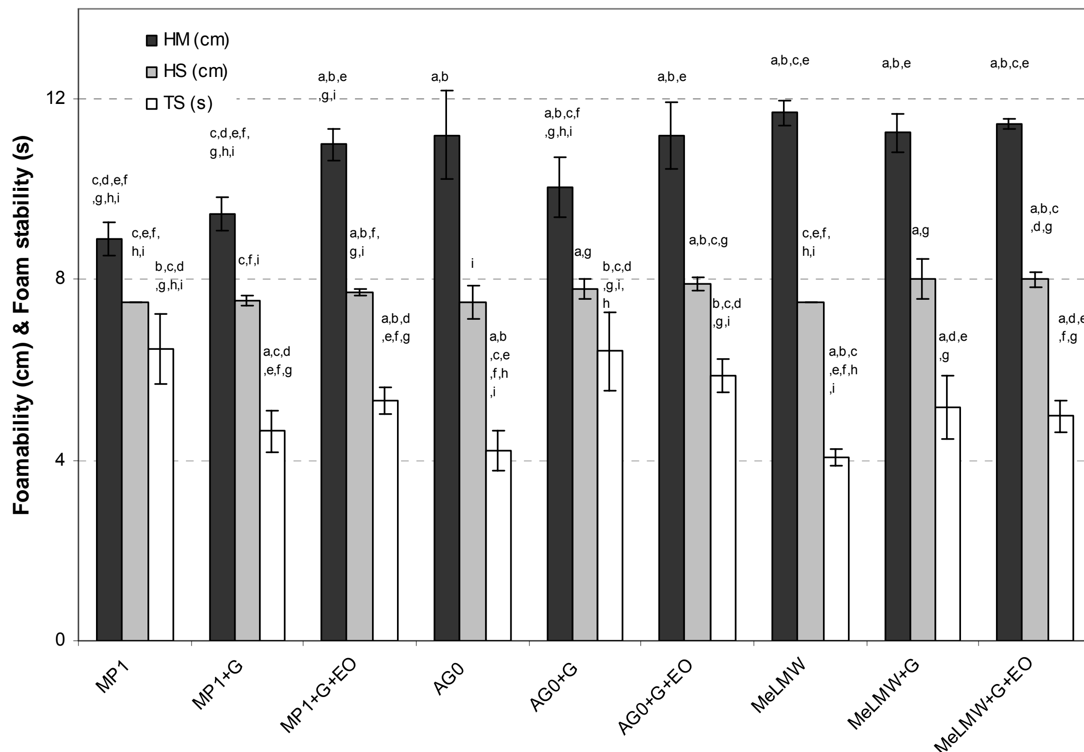


Figure 4 - Foamability, HM and HS, and foam stability, TS, measured for the three fractions that contribute more to the foamability and stability in three different model solutions. All fractions were in wine concentration in the model solution.

a- significantly different ($p > 0.05$) from MP1, **b**- significantly different ($p > 0.05$) from MP1+glycerol, **c**- significantly different ($p > 0.05$) from MP1+glycerol+ethyl octanoate, **d**- significantly different ($p > 0.05$) from AG0, **e**- significantly different ($p > 0.05$) from AG0+glycerol, **f**- significantly different ($p > 0.05$) from AG0+glycerol+ethyl octanoate, **g**- significantly different ($p > 0.05$) from MeLMW, **h**- significantly different ($p > 0.05$) from MeLMW+glycerol, **i**- significantly different ($p > 0.05$) from MeLMW+glycerol+ethyl octanoate.

In conclusion, this work shows that the foam properties of sparkling wines are ruled by a large number of molecules that act in a synergistic way. Nevertheless, some compounds are more relevant than others to explain their foam properties. The synergistic effect of mannoproteins with low content of protein (5%) and the components present in the low molecular weight fraction (<1kDa), shown to contain surfactant compounds (*20*), play a key role in the foamability and foam stability properties of sparkling wines.

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CAPÍTULO IV – CONCLUSÕES

A espuma e o aroma são os parâmetros de qualidade mais importantes na apreciação geral de um vinho espumante. A vindima para os vinhos espumantes normalmente é realizada prematuramente, de forma a ser obtido um produto final acídulo. No entanto, se as uvas não forem colhidas na expressão máxima do seu aroma, poderá verificar-se uma perda significativa do seu potencial varietal volátil. No caso da Bairrada ainda não era conhecida a evolução dos aromas das castas mais abundante e usadas para a produção de espumante. Para responder a esta questão foi estudada a composição volátil das uvas tintas Baga (BG) e brancas Fernão-Pires (FP) durante a maturação, em duas vinhas, com vista a avaliar este efeito na expressão máxima de aroma. Para o efeito foi usada a metodologia de micro-extracção em fase sólida em espaço de cabeça seguida de análise por cromatografia de gás acoplada à espectrometria de massa com quadrupolo (HS-SPME-GC–qMS). No caso das uvas BG observou-se um aumento acentuado na expressão máxima de compostos voláteis próximo da maturidade da uva, mantendo-se constante durante a pós-maturidade. Para a determinação deste perfil volátil foram identificados 66 compostos varietais nas uvas provenientes da vinha de Pedralvites e 45 na vinha do Colégio. Estes compostos dividem-se nas seguintes classes: monoterpenóides (13), sesquiterpenóides (40 dos quais 23 são comuns), norisoprenóides em C_{13} (10 com 6 em comum), álcoois aromáticos (2) e um diterpenóide. Na maturidade, os sesquiterpenóides representaram 56% e 80% dos compostos varietais na vinha do Colégio e de Pedralvites, respectivamente. Os sesquiterpenóides, devido à sua abundância em número e em área cromatográfica, podem ser considerados marcadores da casta BG.

A evolução da composição volátil ao longo da maturação das uvas FP foi diferente do das uvas BG, sendo a expressão máxima de compostos voláteis expressa durante um curto período de tempo (1 semana), que coincide com a maturidade da uva. Depois de atingido este pico, observa-se uma diminuição drástica logo na semana seguinte. Foram identificados e semi-quantificados 25 compostos voláteis varietais e pré-fermentativos. Os compostos varietais das uvas da casta FP foram detectados em igual número em ambas as vinhas e são pertencentes às seguintes classes de compostos: monoterpenóides (16), norisoprenóides em C_{13} (2), álcoois aromáticos (2) e compostos em C_6 (5). Estes resultados mostram que o potencial volátil quer das uvas FP quer das uvas BG quando são colhidas precocemente (uma semana antes da maturidade) para a produção de espumante é observada uma redução significativa do que estas variedades expressam na maturidade.

Para a análise da composição volátil dos vinhos espumantes foi optimizada uma metodologia de microextracção que permite usar uma maior quantidade de fase estacionária, a extracção sorptiva em barra de agitação (SBSE). A barra de agitação usada contém 26 μL de fase estacionária enquanto que a fibra de SPME contém 0,5 μL. Tendo sido usada a metodologia de SBSE seguida de uma desorção líquida dos compostos extraídos pela fase estacionária, vários parâmetros tiveram de ser optimizados, permitindo propor para a extracção o uso de 60 min, e para a desorção líquida o uso de 200 μL de pentano durante 15 min. Em virtude de ter sido usada a desorção líquida foram também optimizados parâmetros relativos à instrumentação, propondo-se o fluxo de hélio no injector de 10 mL/min e temperatura do injector 10 ºC. O método foi optimizado usando 10 padrões de compostos voláteis representativos das principais famílias químicas presentes no vinho, nomeadamente ésteres, monoterpenóides, sesquiterpenóides, norisoprenóides em C_{13} e álcoois. O método proposto apresenta uma boa linearidade para as concentrações testadas, os coeficientes de correlação são superiores a 0,982 e a reprodutibilidade varia entre 8,9 e 17,8%. Os limites de detecção para a maioria dos compostos é bastante baixo, entre 0,05 e 9,09 μg L^{-1}, só o 2-feniletanol é que apresenta um limite de detecção superior, pois este tem uma baixa recuperação pelo SBSE. A aplicação da metodologia de extracção sorptiva em barra de agitação seguida de análise por cromatografia de gás com injecção de grandes volumes com detecção por espectrometria de massa com quadrupolo (SBSE-LD/LVI-GC-qMS) permitiu a quantificação de 71 compostos voláteis em concentrações inferiores aos seus respectivos limites de detecção sensorial.

A metodologia de SBSE-LD/LVI-GC-qMS foi aplicada ao estudo da composição volátil dos vinhos espumantes. Os vinhos espumantes da casta FP apresentaram uma maior concentração de compostos voláteis, podendo dar origem a vinhos espumantes com maior potencial de aroma do que a casta BG. Relativamente à avaliação dos diferentes estados de maturação, verificou-se que as uvas maduras e as da colheita tardia (uma semana depois) deram origem a vinhos com maior quantidade de compostos voláteis, incluindo os compostos varietais. Para os três tipos de solo estudados (arenoso, argiloso e argilo-calcário), o vinho obtido a partir de uvas colhidas no solo argilo-calcário mostrou a maior concentração de compostos voláteis varietais. Assim, para estes vinhos verificou-se que o parâmetro que apresentou maior influência na composição volátil foi a casta, seguido do

tipo de solo e do estado de maturação das uvas.

A espuma destes vinhos foi também avaliada quanto à sua quantidade e estabilidade. O vinho espumante que apresenta uma maior estabilidade da espuma é o vinho produzido a partir da casta FP proveniente de uma colheita tardia e solo argiloso (FP_{EC}). Os vinhos provenientes dos solos arenosos e argilo-calcários são os que apresentam valores mais baixos de estabilidade da espuma.

Com vista a avaliar quais os conjuntos de moléculas do vinho que estão relacionados com as propriedades da espuma e possíveis sinergismos entre eles, para cada vinho espumante foi separada a fracção hidrofóbica de baixo peso molecular (MeLMW) como também a fracção de elevado peso molecular (HMW) e duas fracções de peso molecular intermédio (AqIMW e MeIMW). Os vinhos que apresentam valores de estabilidade da espuma (TS) menores são aqueles em que se obteve um rendimento menor na fracção MeLMW. Os valores de TS observados para as soluções modelo reconstituídas com estas fracções individualmente foram muito superiores para a fracção HMW comparativamente com as restantes, mas todas com valores de espumabilidade e estabilidade da espuma muito inferiores aos valores observados no vinho espumante. A combinação da fracção HMW com a MeLMW produz um efeito sinergético, sendo o TS 2,7 vezes maior do que o observado para a fracção HMW isoladamente. Este aumento do TS ainda é maior quando se combina a fracção HMW com as subfracções obtidas da fracção MeLMW. Dentro destas, observa-se um aumento do TS com o aumento da polaridade das fracções. A fracção hidrofóbica mais polar (F3) foi caracterizada por espectrometria de massa de por ionização por *electrospray* (ESI-MS) e ESI-MS/MS, tendo sido identificada um série de oligómeros de polietileno glicol e o 8-hidroxi-tridecanoato de dietilenoglicolgliceril acetato, um potencial tensioactivo.

Para confirmar se os compostos anteriormente identificados no vinho espumante como sendo importantes para a estabilidade da espuma se encontram realmente presentes na espuma do vinho espumante, os compostos hidrofóbicos de baixo peso molecular presentes na espuma foram caracterizados por ESI-MS/MS. Foram identificados vários potenciais tensioactivos, dois monoacilgliceróis (C16:0 e C18:0) e quatro derivados de ácidos gordos com gliceriletilenoglicol (dodecanoato de monoetileno glicol gliceril formato, nonanoato de dietileno glicol gliceril acetato, 6-hidroxi-undecanoato de dietileno glicol gliceril acetato e 8-hidroxi-tridecanoato de dietileno glicol gliceril acetato).

Para se melhor poder relacionar os parâmetros da espuma com os constituintes do vinho, o vinho foi fraccionado em doze grupos de moléculas: três fracções de manoproteínas, três de arabinogalactanas, três fracções de mistura de polissacarídeos, proteínas e compostos fenólicos, três fracções de peso molecular intermediário e baixo, compostas por uma mistura de hidratos de carbono, péptidos e compostos fenólicos. Posteriormente, foram preparados simulantes de vinho partindo de uma matriz hidroalcoólica à qual foram adicionados as diferentes fracções anteriormente isoladas, com vista à reconstituição combinatória do vinho para avaliar o efeito de cada uma das diferentes fracções nas características e propriedades da espuma. As propriedades da espuma foram avaliadas para cada fracção individualmente na concentração presente no vinho. As fracções apresentaram valores de espumabilidade máxima (HM) de 8-12 cm, espumabilidade na estabilidade (HS) de 7-8 cm e TS de 3.0-6.5 s. O aumento da concentração para dez vezes a concentração do vinho faz com que a fracção rica em manoproteínas, com 5% de proteína, (MP1) aumente para mais do dobro a HM e o TS é 7,4 vezes maior. O aumento da concentração de MP1 apresenta um aumento linear e exponencial da HM e do TS, respectivamente. O aumento para duas vezes a concentração do vinho na fracção MeLMW permite observar para os três parâmetros de espuma avaliados aumentos significativos, no caso do TS para mais do dobro. A combinação entre a fracção MP1 e MeLMW mostrou aumentos significativos dos valores de HM, HS e TS.

Em conclusão podemos afirmar que para se incrementar a estabilidade e a quantidade da espuma do vinho espumante, os compostos chave do vinho são o resultado da combinação dos compostos de elevado peso molecular e os compostos hidrofóbicos de baixo peso molecular, dado que a sua combinação produz um efeito sinergético. Dentro dos compostos de elevado peso molecular, os que mais influenciam os parâmetros da espuma são as manoproteínas com baixo teor em proteína (5%). Para os compostos hidrofóbicos de baixo peso molecular, a presença de tensioactivos não iónicos derivados de ácidos gordos com glicerriletilenoglicol, são os que influenciam os parâmetros da espuma do vinho espumante.