Universidade de Aveiro Departamento de Química
2013

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

Apoio financeiro do POCTI 2010 no âmbito do III Quadro Comunitário de Apoio.

Ciência.Inovação 2010 Programa Operacional Ciência e Inovação 2010
MINISTÉRIO DA CIÊNCIA, INOVAÇÃO E ENSINO SUPERIOR

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

À minha família

“Ninguém caminha sem aprender a caminhar, sem aprender a fazer o caminho caminhando, refazendo e retocando o sonho pelo qual se pôs a caminhar.”

Paulo Freire

o júri

Presidente

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Doutora Sílvia Maria da Rocha Simões Carriço

professora auxiliar da Universidade de Aveiro (orientador)

agradecimentos

Gostaria de expressar o meu agradecimento àqueles que contribuíram directa ou indirectamente para o meu trabalho ao longo destes anos.

Aos meus orientadores Professora Sílvia Maria Rocha e Professor Manuel António Coimbra, pelo apoio e orientação ao longo deste trabalho. Por tudo o que me foi ensinado, por vossa paciência e disponibilidade sempre que eu precisava, fica aqui a minha sincera gratidão.

À Professora Fátima Martel pela sua colaboração neste trabalho, por me receber no seu laboratório e disponibilizar os meios para a realização do mesmo, bem como pela discussão dos resultados e troca de ideias. Ao Pedro Gonçalves, João Araújo, Elisa Keating e a Ana Faria, por toda vossa ajuda durante o tempo que passei no laboratório do Serviço de Bioquímica da Faculdade de Medicina do Porto.

À Estação Vitivinícola da Bairrada, em especial ao Eng. Dias Cardoso, pela cedência de vinhos que foram objecto de estudo nesta tese.

Ao Departamento de Química da Universidade de Aveiro pelas condições proporcionadas para a realização deste trabalho de diferentes formas.

À Universidade de Aveiro e Fundação para a Ciência e Tecnologia pelo financiamento através das bolsas de Doutoramento, e à Unidade de Investigação 62/94, QOPNA.

À Alzira Yamazaki, minha Professora e amiga que foi a ponte para a minha vinda para Portugal. Obrigada pelo apoio, pela amizade, sem a tua ajuda nada disso teria acontecido.

À todos os amigos do laboratório de Bioquímica e Química Alimentar e do Departamento de Química, o meu agradecimento pelo vosso companheirismo e amizade ao longo de todos esses anos. Em especial à Alisa Rudnitskaya, Ana Reis, Cláudia Passos, Cláudia Nunes, Elisabete Coelho e Isabel Silva obrigada pelo companheirismo, amizade e incentivo. À Alisa obrigada pelo desenvolvimento do modelo estatístico.

À Professora Paula Andrade por me ter dado a oportunidade de crescer profissionalmente no seu laboratório. Aos amigos que fiz na Gnosia.

Ao Ronaldo, por acreditar em mim e me incentivar, sempre com muito carinho e paciência, obrigada.

Aos meus pais João Gilberto e Maria Elisabete, aos meus irmãos Eduardo e Alexandra, a minha cunhada Nidiara e aos meus sobrinhos Tomás e Caetana, as minhas avós Zita e Dalva e todos os meus familiares, obrigada por todo suporte e amor, pelo incentivo e pela confiança, sem vocês nada disso faria sentido.

Palavras-chave

Vitis vinifera L., compostos voláteis varietais, metodologias rápidas, SPME, cromatografia em fase gasosa, compostos sesquiterpénicos, propriedades antioxidantes, efeito antiproliferativo, hepatoproteção, quantificação da relação estrutura actividade

Resumo

O ponto de partida para o desenvolvimento da presente tese de doutoramento foi a *Vitis vinifera* L., que é o fruto mais cultivado a nível mundial. Este tema foi explorado tendo em consideração duas tendências atuais: i) o desenvolvimento de métodos rápidos, simples e com elevada sensibilidade, minimizando a manipulação da amostra e ii) a valorização de produtos naturais como fonte de compostos com potencial efeito benéfico para a saúde.

Os compostos voláteis varietais, nomeadamente os mono e sesquiterpenóides e os norisoprenóides em C_{13}, representam as famílias-alvo em estudo, uma vez que estes apresentam propriedades biológicas interessantes do ponto de vista sensorial, podendo contribuir para o aroma do vinho, e com potenciais benefícios para a saúde humana.

Foram desenvolvidas duas novas metodologias para quantificação de compostos norisoprenóides em C_{13} em vinhos. A primeira metodologia, denominada de método rápido, baseou-se na combinação da microextracção em fase sólida em modo espaço de cabeça com cromatografia em fase gasosa-quadrupolo espectrometria de massa, em modo de monitorização de ião selecionado (HS-SPME/GC–qMS-SIM), utilizando condições cromatográficas que permitiram a obtenção do perfil volátil em norisoprenóides em C_{13}.Este método não requer nenhum pré-tratamento da amostra e a composição do vinho em norisoprenóides em C_{13} é avaliada com base no perfil cromatográfico e fragmentos *m/z* específicos, sem a completa separação cromatográfica dos compostos. A segunda metodologia, usada como método de referência, foi muito similar à anterior. Neste caso, foram definidas as condições cromatográficas adequadas para que ocorresse separação dos vários componentes voláteis do vinho. Foram preparadas curvas de calibração externas usando a β-ionona como padrão, tendo-se obtido coeficientes de regressão (r^2) de 0,9968 (RSD 12,51 %) e 0,9940 (RSD 1,08 %) para o método rápido e método de referência, respetivamente, com baixos limites de deteção (1,57e 1,10 μg L^{-1}, respetivamente). Foram analisados 17 vinhos brancos e tintos, nos quais os isómeros do vitispirano e o TDN (1,1,6-trimetil-1,2-dihidronaftaleno) apresentaram concentrações entre 158 e1529 μg L^{-1} e entre 6,42 e 39,45 μg L^{-1}, respetivamente. Os dados obtidos pelos dois métodos desenvolvidos para as concentrações dos isómeros do vitispirano e do TDN estão altamente correlacionados (r^2 de 0,9756 e 0,9630, respetivamente).

O perfil volátil varietal dos vinhos brancos da casta Portuguesa *Vitis vinifera* L. cv. Fernão-Pires (FP) foi estabelecido por um método rápido baseado na HS-SPME seguida de cromatografia em fase gasosa abrangente bidimensional (HS-SPME/GC×GC-TOFMS). Foram analisados vinhos monovarietais de diferentes colheitas, Regiões Demarcadas e produtores. O estudo focou-se em compostos voláteis que podem contribuir para o caráter varietal dos vinhos, nomeadamente os compostos mono e sesquiterpenóides e norisoprenóides em C_{13}. Foram estabelecidos espaços cromatográficos bidimensionais específicos para as 3 famílias químicas em estudo, utilizando os fragmentos *m/z* 93, 121, 161, 175 e 204: para os monoterpenóides (^1t_R = 255-575 s, ^2t_R = 0,424-1,840 s), para os norisoprenóides em C_{13} (^1t_R = 555-685 s, ^2t_R = 0,528-0,856 s) e para os sesquiterpenóides (^1t_R = 695-950 s, ^2t_R = 0,520-0,960 s).

Através da aplicação desta metodologia foram identificados um total de 170 compostos varietais em vinhos FP. Quarenta e cinco dos 170 compostos identificados são comuns a todos os vinhos da casta FP, permitindo assim estabelecer o perfil varietal para a casta. Destes, 15 compostos foram descritos pela primeira vez para esta casta. A combinação da metodologia de HS-SPME/GC×GC-TOFMS com o processamento de dados por utilização de uma amostra de classificação-referência permitiu a identificação rápida do perfil varietal de vinhos. Esta abordagem foi muito útil para eliminar a maioria dos compostos não-terpénicos e não norisoprenóides em C_{13} uma vez que: 1) permite definir um espaço cromatográfico bidimensional que contém estes compostos; 2) simplifica a complexidade e o número de dados obtidos comparativamente com os dados originais; e 3) reduz o tempo de análise.
A presença de compostos sesquiterpénicos em produtos de *Vitis vinifera* L., aos quais são atribuídos vários efeitos benéficos para a saúde foi um fator motivador para o estudo de vários efeitos biológicos associados a estes compostos, nomeadamente das atividades antioxidante, antiproliferativa e hepatoprotetora. Inicialmente, foi avaliada a capacidade antioxidante do *trans,trans*-farnesol, *cis*-nerolidol, α-humuleno e o guaiazuleno usando modelos químicos ($DPPH^{\bullet}$, radical hidroxilo) e biológicos (células do tipo Caco-2). A avaliação da atividade anti-radicalar mostrou que o guaiazuleno inibe eficientemente o $DPPH^{\bullet}$ (IC_{50} 0,73), enquanto o *trans,trans*-farnesol (IC_{50}= 1,81 mM) e o *cis*-nerolidol (IC_{50}=1,48 mM) foram mais ativos em relação ao radical hidroxilo. A avaliação da atividade antioxidante em condições não citotóxicas (≤ 1 mM) revelou que todos os compostos, com exceção do α-humulene, foram capazes de proteger as células do tipo Caco-2 contra o stresse oxidativo induzido pelo hidroperóxido de *tert*-butilo. Os compostos foram também avaliados quanto à sua ação antiproliferativa. O guaiazuleno e o *cis*-nerolidol foram capazes de interromper o ciclo celular na fase da síntese do ADN mais efetivamente que o *trans,trans*-farnesol e o α-humuleno, sendo o último praticamente inativo.
Foi ainda avaliada a capacidade hepatoprotetora relativa de 15 compostos sesquiterpenénicos com estruturas químicas diversas e comuns em plantas, alimentos e bebidas derivadas de plantas. A peroxidação lipídica endógena e a peroxidação lipídica induzida pelo hidroperóxido de *tert*-butilo foram avaliadas em homogeneizado de fígado de rato. Todos os compostos em estudo (1 mM), com exceção do α-humuleno, foram eficientes na redução dos níveis de malonaldeido na peroxidação lipídica endógena (35%) e na peroxidação induzida (70%). Foram desenvolvidos modelos 3D-QSAR, que relacionam a actividade hepatoprotetora com descritores moleculares, os quais apresentaram valores de calibração de R^2_{LOO} > 0,81) e poder de previsão de Q^2_{LOO} >0,97 e SDEP < 2%. Verifica-se que há uma rede de efeitos associados com caraterísticas estruturais e químicas dos compostos, tais como a configuração da molécula, maior ou menor grau de ramificação, simetria e a presença de fragmentos eletronegativos, que podem modular a atividade hepatoprotetora observada para estes compostos.
Em conclusão, este estudo permitiu o desenvolvimento de métodos rápidos e que também fornecem informação detalhada sobre os compostos voláteis varietais, com potencial impacto positivo nas caraterísticas sensoriais e benéficas para a saúde relacionadas com a *Vitis vinifera* L. A aplicação deste tipo de metodologia pode ser alargada a outro tipo de matrizes, tais como uvas, mostos e muitos outros tipos de matrizes vegetais. Além disso, é ainda de realçar que os resultados bastante promissores dos ensaios *in vitro* abrem novas perspetivas ao uso dos compostos sesquiterpénicos, com estruturas químicas similares aos estudados no âmbito deste trabalho, como agentes antioxidantes, hepatoprotetores e antiproliferativos, o que está em linha com os desafios atuais relacionados com as doenças civilizacionais atuais.

Keywords

Vitis vinifera L., varietal volatile compounds, rapid methodologies, SPME, gas chromatography, sesquiterpenic compounds, antioxidant properties, antiproliferative effect, hepatoprotection, quantification of structure activity relationship.

Abstract

Vitis vinifera L., the most widely cultivated fruit crop in the world, was the starting point for the development of this PhD thesis. This subject was exploited following on two actual trends: i) the development of rapid, simple, and high sensitive methodologies with minimal sample handling; and ii) the valuation of natural products as a source of compounds with potential health benefits.

The target group of compounds under study were the volatile terpenoids (mono and sesquiterpenoids) and C_{13} norisoprenoids, since they may present biological impact, either from the sensorial point of view, as regards to the wine aroma, or by the beneficial properties for the human health.

Two novel methodologies for quantification of C_{13} norisoprenoids in wines were developed. The first methodology, a rapid method, was based on the headspace solid-phase microextraction combined with gas chromatography-quadrupole mass spectrometry operating at selected ion monitoring mode (HS-SPME/GC–qMS–SIM), using GC conditions that allowed obtaining a C_{13} norisoprenoid volatile signature. It does not require any pre-treatment of the sample, and the C_{13} norisoprenoid composition of the wine was evaluated based on the chromatographic profile and specific *m/z* fragments, without complete chromatographic separation of its components. The second methodology, used as reference method, was based on the HS-SPME/GC–qMS–SIM, allowing the GC conditions for an adequate chromatographic resolution of wine components. For quantification purposes, external calibration curves were constructed with β-ionone, with regression coefficient (r^2) of 0.9968 (RSD 12.51 %) and 0.9940 (RSD of 1.08 %) for the rapid method and for the reference method, respectively. Low detection limits (1.57 and 1.10 μg L^{-1}) were observed. These methodologies were applied to seventeen white and red table wines. Two vitispirane isomers (158-1529 μg L-1) and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (6.42-39.45 μg L-1) were quantified. The data obtained for vitispirane isomers and TDN using the two methods were highly correlated (r^2 of 0.9756 and 0.9630, respectively).

A rapid methodology for the establishment of the varietal volatile profile of *Vitis vinifera* L. cv. 'Fernão-Pires' (FP) white wines by headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (HS-SPME/GC×GC-TOFMS) was developed. Monovarietal wines from different harvests, Appellations, and producers were analysed. The study was focused on the volatiles that seem to be significant to the varietal character, such as mono and sesquiterpenic compounds, and C_{13} norisoprenoids. Two-dimensional chromatographic spaces containing the varietal compounds using the *m/z* fragments 93, 121, 161, 175 and 204 were established as follows: ^1t_R = 255-575 s, ^2t_R = 0,424-1,840 s, for monoterpenoids, ^1t_R = 555-685 s, ^2t_R = 0,528-0,856 s, for C_{13} norisoprenoids, and ^1t_R = 695-950 s, ^2t_R = 0,520-0,960 s, for sesquiterpenic compounds.

For the three chemical groups under study, from a total of 170 compounds, 45 were determined in all wines, allowing defining the “varietal volatile profile” of FP wine. Among these compounds, 15 were detected for the first time in FP wines. This study proposes a HS-SPME/GC×GC-TOFMS based methodology combined with classification-reference sample to be used for rapid assessment of varietal volatile profile of wines. This approach is very useful to eliminate the majority of the non-terpenic and non-C_{13} norisoprenic compounds, allowing the definition of a two-dimensional chromatographic space containing these compounds, simplifying the data compared to the original data, and reducing the time of analysis.

The presence of sesquiterpenic compounds in *Vitis vinifera* L. related products, to which are assigned several biological properties, prompted us to investigate the antioxidant, antiproliferative and hepatoprotective activities of some sesquiterpenic compounds. Firstly, the antiradical capacity of *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene was evaluated using chemical ($DPPH^{\bullet}$ and hydroxyl radicals) and biological (Caco-2 cells) models. Guaiazulene (IC_{50}= 0.73 mM) was the sesquiterpene with higher scavenger capacity against DPPH•, while *trans,trans*-farnesol (IC_{50}= 1.81 mM) and *cis*-nerolidol (IC_{50}= 1.48 mM) were more active towards hydroxyl radicals. All compounds, with the exception of α-humulene, at non-cytotoxic levels (≤ 1 mM), were able to protect Caco-2 cells from oxidative stress induced by *tert*-butyl hydroperoxide. The activity of the compounds under study was also evaluated as antiproliferative agents. Guaiazulene and *cis*-nerolidol were able to more effectively arrest the cell cycle in the S-phase than *trans,trans*-farnesol and α-humulene, being the last almost inactive.

The relative hepatoprotection effect of fifteen sesquiterpenic compounds, presenting different chemical structures and commonly found in plants and plant-derived foods and beverages, was assessed. Endogenous lipid peroxidation and induced lipid peroxidation with *tert*-butyl hydroperoxide were evaluated in liver homogenates from Wistar rats. With the exception of α-humulene, all the sesquiterpenic compounds under study (1 mM) were effective in reducing the malonaldehyde levels in both endogenous and induced lipid peroxidation up to 35% and 70%, respectively. The developed 3D-QSAR models, relating the hepatoprotection activity with molecular properties, showed good fit ($R^2_{LOO} > 0.819$) with good prediction power ($Q^2 >$ 0.950 and SDEP < 2%) for both models. A network of effects associated with structural and chemical features of sesquiterpenic compounds such as shape, branching, symmetry, and presence of electronegative fragments, can modulate the hepatoprotective activity observed for these compounds.

In conclusion, this study allowed the development of rapid and in-depth methods for the assessment of varietal volatile compounds that might have a positive impact on sensorial and health attributes related to *Vitis vinifera* L. These approaches can be extended to the analysis of other related food matrices, including grapes and musts, among others. In addition, the results of *in vitro* assays open a perspective for the promising use of the sesquiterpenic compounds, with similar chemical structures such as those studied in the present work, as antioxidants, hepatoprotective and antiproliferative agents, which meets the current challenges related to diseases of modern civilization.

Abbreviations and symbols

^{1}D: First chromatographic dimension

$^{1}t_R$: Retention time on the first column

^{2}D: Second chromatographic dimension

$^{2}t_R$: Retention time on the second column

3D: Three Dimensional

3D-MoRSE: Three dimensional-Molecule Representation of Structures based on Electron diffraction

CAT: Catalase

CW-DVB: CarboWax-DiVinylBenzene

DMAPP: Dimethylalyl pirophosphate

DNA: DeoxyriboNucleic Acid

DPPH$^{\bullet}$: 2,2-DiPhenyl-1-PicrylHydrazyl radical

EDTA: EthyleneDiamineTetraacetic Acid

e-nose: electronic nose

eV: electron Volt

FPP: Farnesyl PiroPhosphate

GA: Genetic Algorithm

GA3P: GlycerAldehyde-3-Phosphate

GC: Gas Chromatography

GC×GC: Comprehensive two-dimensional Gas Chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

GETAWAY: Geometry, Topology, and Atom-Weights AssemblY

GPP: Geranyl PiroPhosphate

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Reduced Glutathione

GST: Glutathione-S-Transferase

HMG-CoA: 3-Hydroxy-3-MethylGlutaryl-CoA

HMG-CoAR: 3-Hydroxy-3-MethylGlutaryl-CoA Reductase

HPLC: High Performance Liquid Chromatography

HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry

HS: HeadSpace

IC_{50}: half maximal Inhibitory Concentration

IPP: Isopentenyl pirophosphate

LOD: Limit of Detection

LOF: Lack of Fit

LOQ: Limit of Quantification

MDA: Malonaldehyde

MLR: MultiLinear Regression

MS: Mass Spectrometry

MTT: 3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylTetrazolium bromide

OT: Odour Threshold

PCA: Principal Component Analysis

PCR: Principal Component Regression

PLS: Partial Least Square regression

QR: Quinone Reductase

QSAR: Quantitative Structure Activity Relationship

RI: Retention Index

RLH: Rat Liver Homogenate

RMSECV: Root Mean Square Error in Cross-Validation

RSD: Relative Standard Deviation

SDEC: Standard Deviation Errors in Calculation

SDEP: Standard Deviation Errors in Prediction

SEM: Standard Error Mean

SIM: Selected Ion Monitoring

SPME: Solid Phase MicroExtraction

SPME–MS-MVA: Solid Phase Microextraction-Mass Spectrometry-MultiVariate Analysis

SOD: SuperOxide Dismutase

SRB: SulfoRhodamine B

TCA: TriChloroacetic acid

TBA: ThioBarbituric Acid

TBAR's: ThioBarbituric Acid Reactive substances

TDN: 1,1,6-Trimethyl-1,2-DihydroNaphtalene

TIC: Total Ion Current

TOF: Time Of Flight

TPS: TerPene Synthases

WHIN: Weighted Holistic INvariant molecular

Publications related to this thesis

Publications in international journals with referee:

Vinholes, J., Coimbra, M. A. and Rocha, S. M. (2009). Rapid tool for assessment of C_{13} norisoprenoids in wines. Journal of Chromatography A. 1216, (47), 8398-8403.

Vinholes, J., Rudnitskaya, A., Gonçalves, P., Martel, F., Coimbra, M. A. and Rocha, S. M. (2014). Hepatoprotection of sesquiterpenoids: a quantitative structure-activity relationship (QSAR) approach. Food Chemistry. 146, 78-84.

Vinholes, J., Gonçalves, P., Martel, F. Coimbra, M. A. and Rocha, S. M. Antioxidant and antiproliferative effects of sesquiterpenoids in Caco-2 cells. (Submitted, October 2013)

Vinholes, J., Coimbra, M. A. and Rocha, S. M. Rapid varietal profile assessment: Fernão-Pires monovarietal wines a case study. Under preparation.

Vinholes, J., Coimbra, M. A. and Rocha, S. M. Biological role of sesquiterpenoids present in wines: aroma and health benefits. Review manuscript. Under preparation.

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Vinholes, J., Rocha, S. M., Delgadillo, I. and Coimbra, M. A.. Estimation of sesquiterpenoids concentration in white and red wines by HS-SPME/GC-MS. XXIXth World Congress of Vine and Wine 4th General Assembly of the O.I.V. CD-ROM OENOLOGY 2.3 Vinholes, Juliana, Oral Presentation. Logroño, Espanha, 2006.

Posters communications in international scientific meetings:

1. Vinholes, J., Coimbra, M. A., Rocha, S. M. Rapid assessment of wine varietal volatile profile classification by comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry, 34nd International Symposium on Capillary Chromatography e

7th GC×GC Symposium, Riva del Garda, Itália, 2010, K33, pg. 298.

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Posters communications in national scientific meetings:

1. Vinholes, J., Coimbra, M. A. and Rocha, S. M. Rapid tool for assessment of C_{13} norisoprenoids in wines, 6º Encontro Nacional de Cromatografia, Funchal, Madeira, Portugal, 2009.

2. Vinholes J., Gonçalves, P., Barros, A. S., Martel, F, Rocha, S. M. and Coimbra, M. A. Sesquiterpenoids protection against lipid peroxidation in hepatocytes, XVI Congresso Nacional de Bioquímica, Ponta Delgada, Açores, Portugal, 2008.

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4. Rocha, S. M., Coelho, E., Vinholes, J., Coimbra, M. A. *Vitis vinifera* L. as a potential source of sesquiterpenoids to be exploited for different applications. Workshop em Produtos Naturais, Universidade da Madeira, Funchal, Portugal, 2007.

5. Vinholes, J. R., Coelho, E., Rocha, S. M., Delgadillo, I. and Coimbra, M. A.: HS-SPME using a carbowax/divinylbenzene coating fibre as a powerful tool for characterization of sesquiterpenoids from *Vitis vinífera* L. grapes and wines. 4º Encontro Nacional de Cromatografia, Évora, Portugal, 2005.

Contents

Chapter 1- Introduction

Introduction

Summary

This chapter comprises the framework and main goals of this thesis and the state of the art of the most relevant topics related to field under study. The main volatile compounds responsible for the wine aroma are described with special attention to the varietal composition of wines, covering the mono and sesquiterpenoids, and C_{13} norisoprenoids, their aroma descriptors, odour threshold and relevance for the wine aroma properties. A special focus was done on the varietal composition of the Portuguese varieties under study: Alfrocheiro, Baga, Fernão-Pires and Touriga Nacional. The microextraction and gas chromatography based methodologies used to characterize the wine volatile compounds are reviewed, highlighting the methodologies/techniques used in this work. The potential health benefits of sesquiterpenic compounds identified in *Vitis vinifera* L. and other plants and plant-derived foods and beverages are also reviewed with focus on their antioxidant and antiproliferative effects. Moreover, comprehensive effect evaluation of these biological activities, using QSAR models, is also presented.

1.1. Thesis framework and Aims

Vitis vinifera L., the most widely cultivated fruit crop in the world, was the starting point for the development of this PhD thesis. This subject was exploited regarding two actual trends: i) the development of rapid, simple, and high sensitive methodologies with minimal sample handling; and ii) the valuation of natural products as a source of compounds with potential health benefits.

Wine is the most important *Vitis vinifera* L. product, and its quality and typicity are highly correlated with its aroma (1, 2). The wine aroma results from a complex mixture of compounds belonging to different chemical families, however only a small part of these compounds can contribute to its complexity (3). Among several chemical families, the varietal compounds play a key role on the peculiar characteristics of the wines. Thus, the target varietal volatile compounds chosen for this study were the volatile terpenoids, comprising mono and sesquiterpenic compounds, and the C_{13} norisoprenoids, since they present specific sensorial attributes. The development of rapid and reliable methods to access the characterization of varietal volatile compounds is a very stimulating challenge. Thus, the following specific aims were established:

1. The development of a rapid methodology for the quantification of C_{13} norisoprenoids compounds in wines by HS-SPME/GC–MS-SIM.

2. The development of a rapid characterization method for the establishment of the varietal volatile profile of wines by HS-SPME/GC×GC-TOFMS.

The methods of extraction and analysis chosen to accomplish the first and second aims were the solid phase-microextraction followed by gas chromatography mass spectrometry (uni and bidimensional). This combination has proved to be a very important tool in wine characterization, allowing increasing the knowledge about different varieties and their potentialities providing qualitative and quantitative information that can be used for wine quality control. Moreover, the analysis can be performed in different stages of winemaking, and cover molecules from different chemical pathways constituting a way to control the winemaking process. Monovarietal wines from Alfrocheiro, Baga, Fernão-Pires

and Touriga Nacional were used for the methods development.

Besides the particular sensorial properties related with the target volatile compounds, some of them, specially the sesquiterpenoids present in *Vitis vinifera* L. grapes and wines, have been related with interesting biological properties (4). These properties may be associated with the capability of inhibiting the oxidation process, involved in the initial development steps of chronic degenerative diseases such as cardiovascular, cancer and neurodegenerative diseases, that are described for wines (4-8). This fact had prompted us to evaluate the biological potential of some sesquiterpenic compounds described in *Vitis vinifera* L. (grapes and wines) and other natural matrices, mainly focused on their *in vitro* antioxidant and antiproliferative effects, using chemical and biological models. The specific aims regarding the sesquiterpenic compounds biological potential were:

3. The assessment of antioxidant and antiproliferative effects of sesquiterpenoids in *in vitro* Caco-2 cells model.

4. The evaluation of the hepatoprotection of sesquiterpenoids and establishment of a quantitative structure-activity relationship approach.

Four sesquiterpenic compounds identified in wines were used to evaluate their antioxidant and antiproliferative effects on Caco-2 cells: *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene. $DPPH^{\bullet}$ and hydroxyl radicals were chosen to cover different antioxidant properties. Caco-2 cells was chosen since it is a well-established model for *in vitro* investigations of the antioxidant effect of bioactive compounds and present several biochemistry aspects that resembles the small intestine cells.

The sesquiterpenoids hepatoprotection was evaluated in hepatocytes from Wistar rats. The compounds *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, guaiazulene, (-)-α-bisabolol, *trans*-β-farnesene, (+)-aromadendrene, (-)-α-cedrene (9), (-)-α-copaene (10), germacrene D, β-caryophyllene, (+)-valencene, and (+)-cyclosativene (11-13), identified in *Vitis vinifera* L., grapes and wines and isocaryophyllene and (-)-α-neoclovene (14, 15) identified in other plants and plant-derived foods and beverages were studied. The hepatocytes were chosen since it is connected with detoxification process, thus being subject to high levels of oxidative stress.

1.2. Volatile compounds of wines

The aroma of the wine is considered an important factor since it defines its quality and typicity. These characteristics are responsible for wine acceptance, satisfaction and distinction, especially for white wines.

Among the compounds that are involved in the complex mixture that is the wine, only the volatile and semi-volatile compounds are able to stimulate the olfactory cells. The volatile compounds represent in average 0.5 % of the total composition of wines (Figure 1.1) (16-18).

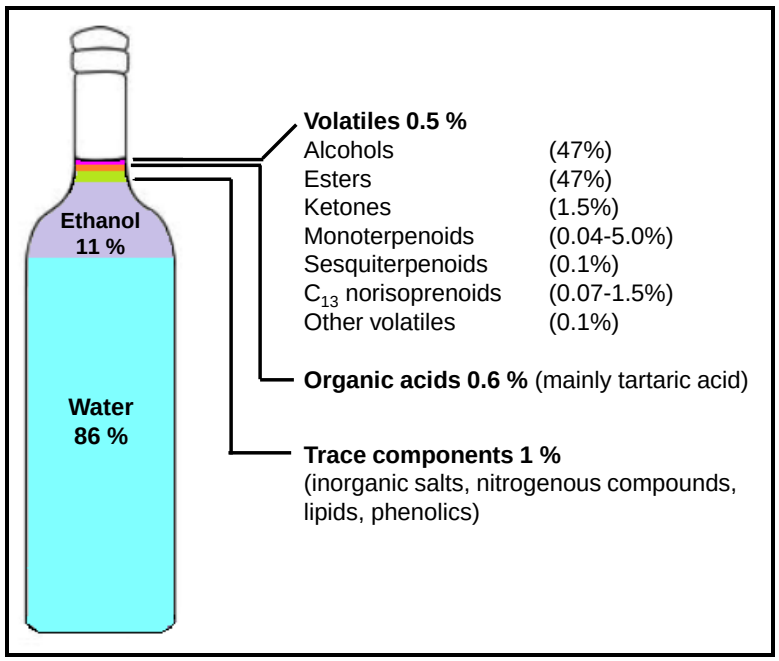


Figure 1.1. Average composition of wines based on data collected from (16-18).

Up to date hundreds of volatile compounds have been found in wine composition. These compounds share some physico-chemical characteristics such as low molecular weight, high vapour pressure and low solubility in water, which promote their release into the vapour phase. These characteristics allow their contact with the olfactory system and thus their perception in the brain is further translated in the aroma. Two pathways are known for the nasal aroma perception, the direct, where the compounds are in direct contact with the olfactory system, and via retro nasal, when the compounds are released from the chewing and enter in contact with the through (19). Therefore, the intensity of an

olfactory sensation depends not only on the compound concentration in the vapour and liquid phase but also on its volatility, vapour pressure and odour threshold (OT) (20).

The compounds responsible for the aroma may be directly recognizable in grapes as volatile odorants as they are present in their free form, or they can be present as non-volatile and non-odorous form, where the molecules are linked to a non-volatile compound, usually a sugar residue. This glycosidically linked form is constituted by a compound (aglycone) which may be a terpenoid, a C_{13} norisoprenoid, aliphatic alcohols, or aromatic alcohols, linked to sugars residue frequently D-β-glucopyranose or disaccharides such as 6-O-α-L-rhamnopyran-β-D-glucopyranose, 6-O-α-L-arabinofuran-β-glucopyranose and 6-O-α-L-apiofuran-β-D-glucopyranose (Figure 1.2) (21).

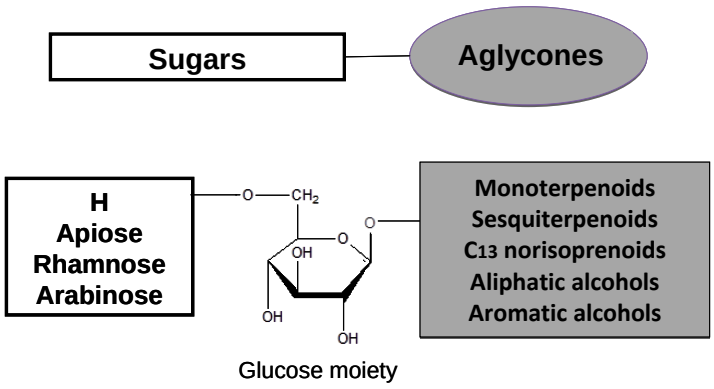


Figure 1.2. General structure of the mono- and disaccharides linked to the aglycones present in wines (adapted from (22)).

Among the volatile compounds present in wine, several chemical classes are described such as alcohols, terpenic compounds (mono and sesquiterpenoids) C_{13} norisoprenoids, esters, aldehydes, ketones, acids, ethers and lactones (23). Esters and higher alcohols are the compounds detected at higher concentration, in the order of mg L^{-1} (24, 25). By the other hand, varietal compounds such as linalool, α-terpineol, geraniol, α- and β-ionone that are related with typicity of wines are present at lower concentrations, even in the order μg L^{-1} (26, 27).

1.2.1. Classification according to the winemaking process steps

The wine bouquet is the product of several biochemical and technological steps that promote the formation of several volatile and semi-volatile compounds (23). These compounds can be classified according to their origin along the winemaking process:

- Varietal compounds- These compounds constitute the primary aroma. They arise from grapes skins and pulp, and are characteristic of each variety, presenting fruit and flower aromas. The compounds include mono and sesquiterpenoids, norisoprenoids, phenylpropanoids, methoxypyrazines, and volatile sulphur compounds. Their abundance depends on factors such as soil type, climate, health and status of grapes ripening, viticulture conditions used and winemaking procedure (28-34).

- Pre-fermentative compounds- These compounds are formed before fermentation due to mechanical operations (transportation, crushing and grape maceration) and the technological manipulations (clarification) (28, 35). Aldehydes and alcohols with 6 atoms of carbon are examples of pre-fermentative compounds. These compounds are formed from the degradation of polyunsaturated fatty acids, primary metabolites of grapes lipid membrane, after reduction by alcohol dehydrogenase enzyme (36).

- Fermentative compounds- These compounds are produced during fermentation and their occurrence is influenced by many factors, namely by temperature and the type of yeast used. In this stage, the higher alcohols (containing more than two atoms of carbon), ketones, aldehydes, fatty acids and esters are formed (28, 37, 38).

- Post-fermentative compounds- These compounds are formed during the storage and aging phases. Depending on the wine ageing conditions, some physico-chemical phenomena may occur such as oxidation, reduction or rearrangements resulting in alterations of the volatile composition (28, 39-42). For instance, lactones and furfurals profiles may be changed with the ageing processes (39, 41)

1.2.2. Varietal compounds

The varietal compounds are those that can be directly related with the grape variety. From the sensorial point of view two important characteristics differentiate the varietal volatile compounds from the others volatile compounds present in wines: i) present pleasant aroma descriptors, frequently associated with flower and fruit, among others; and ii) present very low odour thresholds, allowing their perception at low concentrations (43). This combination of factors has a great impact on wine acceptability, since consumers' prefers wines with flower and fruit attributes (2). Thus, the presence of these compounds in wines can be responsible for the unique sensory characteristics reported for some of them (3) as indicated by different sensorial studies. For example, the concentration of certain terpenic compounds (i.e. linalool, geraniol, nerol and *cis*-rose oxide) in wines is positively related with floral, sweet, citrus, Muscat and lychee aromas in Muscat and Gewürztraminer wines (44-46). The kerosene aroma of Riesling wines, associated with 1,1,6-trimethyl-1,2-dihydronaphthalene (47), the bell pepper aroma of 3-isobutyl-2-methoxypyrazine in Red Bordeaux and Loire wines (48) and the pepper aroma of rotundone in Shiraz wines (49, 50) are other examples. In addition, β-damascenone (violet aroma descriptor) and thiols (4-mercapto-4-methylpentan-2-one and 3-mercaptohexan-1-ol, blackcurrant and grapefruit aroma descriptors, respectively) were also reported as contributors of varietal character of Maccabeo and Sauvignon Blanc (51-53).

The varietal volatile compounds profile can be exploited for several purposes, namely to i) establish wine quality; ii) trace varieties identity, and iii) determine wine origin.

i) Quality- wine classification according to the quality characteristic is mainly attributed to the sensorial attributes related with the varietal profile. For instance, table wines with flower and fruit characteristics (characteristic of some varietal volatile compounds) are preferred by consumers (2);

ii) Varieties- wine classification according with different varieties is explained by studies recognizing that the varietal composition and their concentration are distinct among varieties, which influences the aroma of wines allowing

their distinction, that is frequently done based on the terpenic compounds (23, 54-56). This fact can be related with the high number of *Vitis vinifera* (Vv) terpene synthases (TPS) genes (39 *Vv*TPS gene products), being capable of synthesize 21 monoterpenes and 47 sesquiterpenes (57). Since the synthesis of these compounds are encoded by specific genes (57-59) their presence in grapes, and thus in wines, is variety dependent;

iii) Origin - wine classification based on their origin is evidenced by studies describing that subtle differences in the varietal profiles of wines can also occur within the same variety from different Appellations (60-62). This property enables to trace the wines varietal origin.

Besides the terpenic compounds other varietal compounds are also present in wines such as the methoxypyrazines, originated from the metabolism of amino acids and volatile thiols formed from the action of carbon-S lyase enzymes on cysteine-S-conjugated compounds during alcoholic fermentation (43, 63). Some pleasant aromas are associated with these groups of compounds such as green pepper and earth aromas (43), citrus zest, grapefruit and passion fruit (52).

1.2.2.1. Monoterpenoids

The monoterpenoids contribution for the average composition of the wine can vary from 0.04 to 5% of the volatile fraction, representing concentrations in the ng L^{-1} to mg L^{-1} levels (Figure 1.1).

Monoterpenoids are synthesized from isopentenyl pirophosphate (IPP) and dimethylallyl pirophosphate (DMAPP) units which may be formed by two independent metabolic pathways (Figure 1.3): i) The mevalonate pathway, that occurs in the cytosol due to the sequential condensation of three molecules of acetyl-CoA resulting in 3-hydroxy-3-methylglutaryl-CoA (64), and ii) the 2-C-methylerythritol-4-phosphate plastidial pathway that involves pyruvate and glyceraldehyde-3-phosphate (65). The C_5 units formed are head-to-tail condensate via prenyl-transferases, and the monoterpenic compounds are formed

from geranyl pirophosphate (GPP, C_{10}) through the action of TPS.

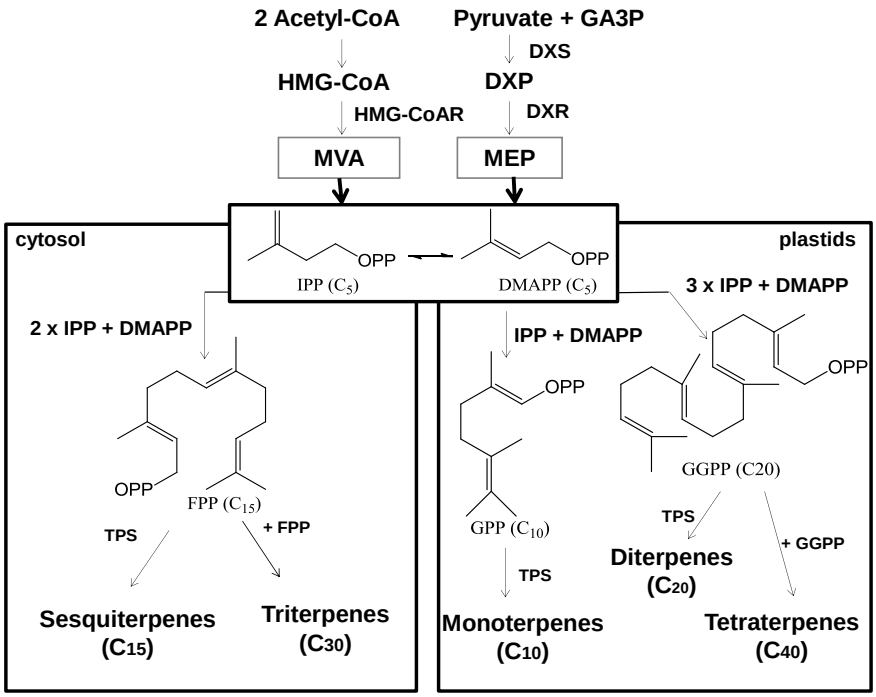


Figure 1.3. Terpenoids biosynthesis by mevalonate (MVA) and 2-C-methylerythritol-4-phosphate (MEP) pathways. Acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IPP, isopentenyl pirophosphate; DMAPP, dimethylalyl pirophosphate; FPP, farnesyl pirophosphate; TPS, terpene synthases; GA3P, glyceraldehyde-3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-xylulose-5-phosphate reductoisomerase; GPP, geranyl pirophosphate; GGPP, geranyl-geranyl pirophosphate (66, 67).

Different monoterpenic compounds are found in wines, such as hydrocarbons (i. e. limonene, cymene), alcohols (linalool, nerol), aldehydes (i. e. citronellal, neral), oxides (i. e. linalool and nerol oxides), acids (i.e. geranic acid) and esters (linalyl acetate) and polyols (compounds with more than 1 hydroxyl group, 2,6-dimethyl-7-octene-2,6-diol). The most common monoterpenoids of wines are displayed in Figure 1.4.

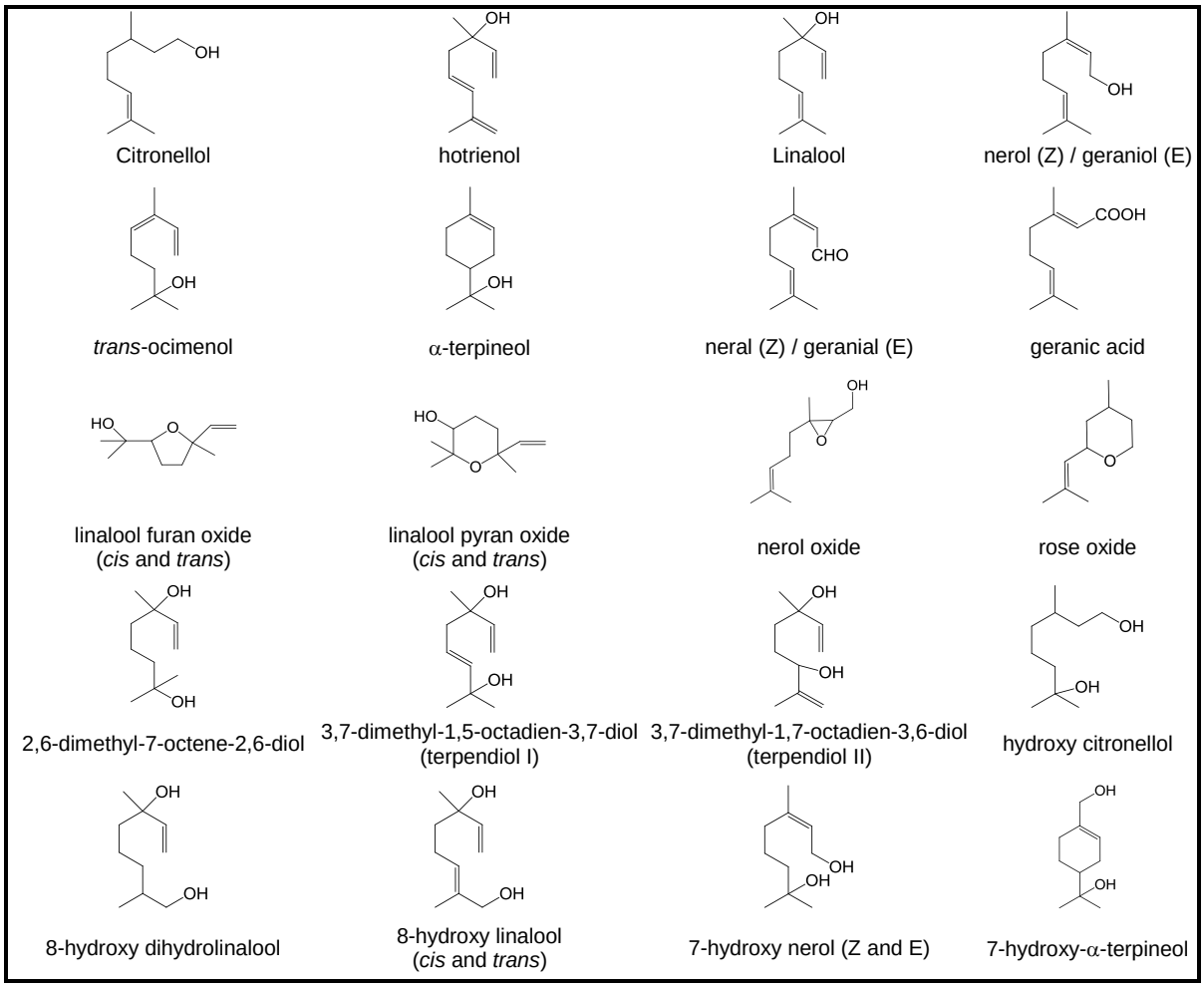


Figure 1.4. Main monoterpenoids described in wines (68, 69).

Monoterpenoids can be found in free and/or their glycoconjugated forms (70). The release of the aglycons from the glycoconjugated form may be performed by the action of the endogenous β-glucosidases or by the addition of commercial preparations. Besides the release of the aglycones, the pH of wine is also responsible for other reactions, for instance, the linalool hydroxylation, cyclization and isomerization that can lead to the formation of hydroxylinalool, α-terpineol and geraniol, and nerol, respectively. Other example is the production of hotrienol by dehydratation of the odourless terpendiol I (3,7-dimethyl-1,5-octadien-3,7-diol) (40, 71). Considering these chemical phenomena the contents of these compounds can change during wine bottle storage and wine maturation stages (72).

Table 1.1- Monoterpenoids reported in wines from the varieties studied in the present thesis, and the respective aroma descriptors and odour threshold (OT).

Compounds	Wine	Aroma descriptor	OT μg L^{-1}
Alloocimene	FP	citrus (73)*	
α-Pinene	FP	pine, turpentine (74) piney (75)	62 (76)*
Limonene	FP	lemon, orange (74)	10 (76)
α-Terpinene	FP	lemon (74)	
α-Terpinolene	FP	sweet, piney (74)	200 (76)
Citronellol	FP	rose (74)	18 (43)
Geraniol	TN	citrus (74), sweet (77), floral (75)	130 (43)
Hotrienol	FP	hyacinth (74)	110 (43)
Linalool	FP, TN	floral, fruity, sweet (77), lavender (74)	80 (78), 50 (43)
Nerol	TN	sweet (74)	400 (43)
α-Terpineol	FP, TN	fruity, fragrant, oily (77)	100 (79)
Linalyl acetate	FP	sweet, fruity (74)	
Geranyl acetate	FP	floral (75)	
Linalool *Z*-furan oxide	FP	flower (74)	>6000 (54)
Linalool *E*-furan oxide	FP	flower (74)	>6000 (54)
Nerol oxide	FP	green fruit, citrus (79)	100 (79)
Rose oxide	FP	green, floral (46)	0.2 (45)

FP-Fernão-Pires (31, 80-83); TN- Touriga Nacional (84). *References.

Limonene, citronellol, geraniol, hotrienol, linalool, α-terpineol, nerol oxide and rose oxide are the key compounds for wine aroma, since they have lower odour thresholds and pleasant aroma descriptors (Table 1.1, Figure 1.4). High correlations between floral sensory attributes and high levels of linalool and α-terpineol are well documented (69, 85-88).

In Portuguese wines such as those produced with FP (Fernão-Pires) grapes, linalool and α-terpineol represented the major monoterpenoids (Table 1.1) (81, 82). This profile is in agreement with their presence in grapes and musts indicating their potential floral aroma contribution (89-91). Nevertheless, the presence of some monoterpene oxides in FP wines, even in small quantities, can be also relevant to aroma properties due to their floral characteristics since these compounds have low odour thresholds (Table 1.1) (68). Besides the compounds presented in Table 1.1, FP wines are also characterized by the presence of geranic acid and oxide, herboxides (Z and E) and terpendiols (I, II; 2,6-dimetilocta-2,7-dieno-1,6-diol (Z and E); and 3,7-dimethylocta-1-en-3,7-diol) (31, 81). Nevertheless, there is no information available about the aroma descriptors and odour threshold for these compounds, and the terpendiols are odourless. Concerning the monoterpenoids in TN

(Touriga Nacional) wines, linalool seems to be the most important one, since it was found in concentrations above their odour threshold limit, indicating a positive contribution for the floral aroma of these wines (84). There are no references available in the literature regarding the monoterpenic composition of Alfrocheiro and Baga wines.

1.2.2.2. Sesquiterpenoids

The contribution of sesquiterpenoids for the volatile fraction of wines is on average 0.1% representing concentrations in the ng L^{-1} to μg L^{-1} levels. Sesquiterpenoids are synthesized from isopentenyl pirophosphate (IPP) and dimethylallyl pirophosphate (DMAPP) (Figure 1.3) (65). As described for the monoterpenoids the C_5 units are head-to-tail condensate by prenyl-transferases, and the sesquiterpenic compounds are formed from farnesyl pirophosphate (FPP, C_{15}) through the action of TPS.

Throughout the last decade, an increasing in the study of the sesquiterpenic compounds in wines was observed, which may be explained by the use of more sensitive techniques of extraction and detection. The sesquiterpenoids structures present in wines represent hydrocarbons (i. e. cadalene, α-calacorene), linear and cyclic alcohols (i. e. farnesol, α-cadinol) and ketones (i. e. α-gurjunene). Figure 1.5 displays the most frequently sesquiterpenoids reported as wine components.

Studies concerning the role of sesquiterpenoids in grape-derived aroma are scarce comparatively to those addressed to monoterpenoids. To date, few studies reported the presence of sesquiterpenoids as aglycones (92) as it is common for monoterpenoids and C_{13} norisoprenoids. In addition, few sesquiterpenoids have been described with impact to the wine aroma (49, 50). Although their contribution remains to be unraveled, some of the sesquiterpenoids present in wines have interesting aroma descriptors and low odour thresholds. Table 1.2 summarizes the sesquiterpenic compounds already reported in wines from the grape varieties under study, their aroma descriptors and odour thresholds.

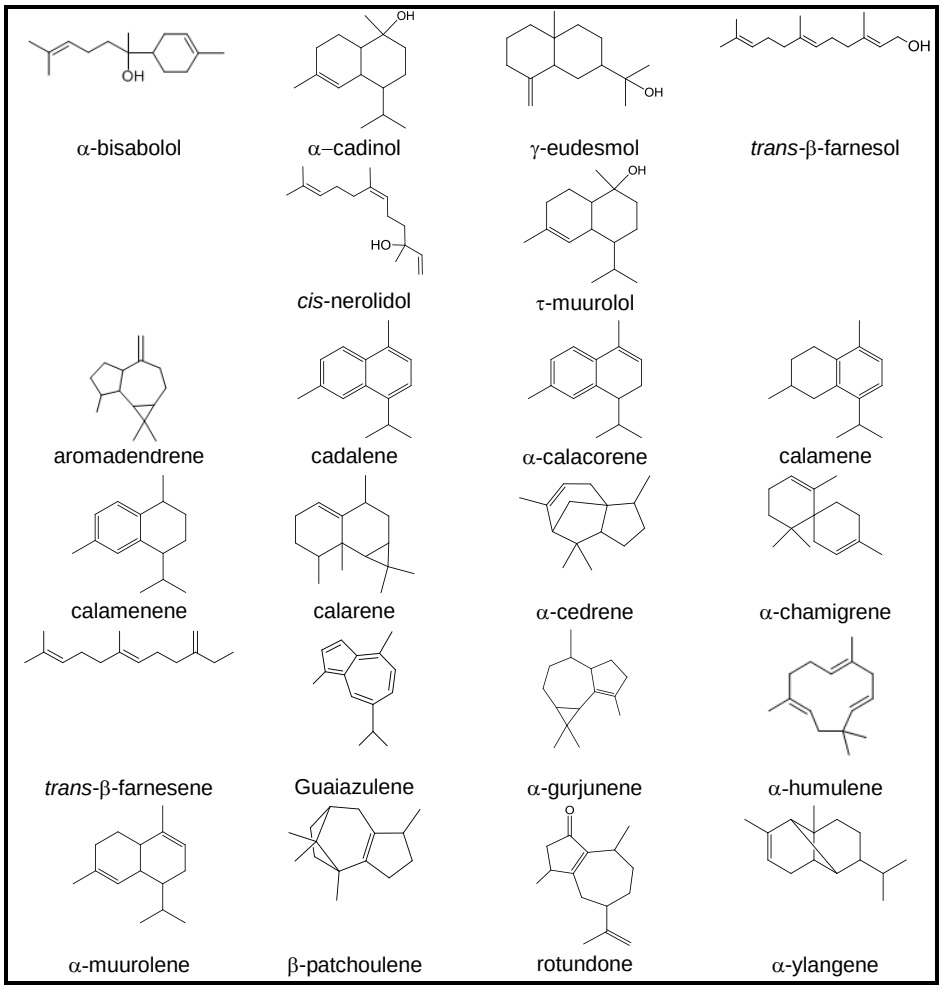


Figure 1.5. The most frequently sesquiterpenoids found in wines (4, 9, 11, 18, 31, 49, 50, 55, 93-96) .

Besides the compounds listed on Table 1.2, other compounds were identified in BG (Baga) and FP wines, such as 1(5),3-aromadenedradiene and α-chamigrene (BG), α-cedrene (BG and FP), α-cadalene, β-chamigrene, cycloisolongifolene and guaiazulene (FP) (4, 31), but any aroma descriptor have been yet associated to these compounds.

From the six sesquiterpenic compounds detected in BG wines (Table 1.2), only α-calacorene, cadelene, and α-muurolene are common with the respective grapes (4). Some of the compounds present in wines and not detected in grapes can be rearrangement products of the major constituent of grapes germacrene D, a well known precursor compound of many sesquiterpene hydrocarbons (4, 97).

Table 1.2- Sesquiterpenic compounds reported in wines from the grape varieties under study and the respective aroma descriptors and odour thresholds (OT).

Compound	Wine	Aroma descriptor	OT μg L^{-1}
Aromadendrene	ALF	sweet, dry (98)*	0.63[a] (98)*
α-Bisabolol	FP	sweet, citrus (99)	3.92[b] (99)
α–Cadinol	BG	herb, wood, floral (74, 100)	-
α–Calacorene	BG	wood (74)	-
Calamenene	BG	spicy (101)	-
γ-Eudesmol	BG	sweet, woody (74)	-
cis-β-Farnesene	FP	green, citrus (77)	-
trans-β-Farnesene	FP	oily, fruit, citrus (77)	87 (102)
Farnesol	FP	flower, oil (74)	>100 (103)
α-Gurjunene	FP	woody, balsam, fatty (74, 104)	-
α-Humulene	FP, ALF	Herbal, woody (98)	0.12 (105)
α-Muurolene	BG	fruity, grassy, citrusy, floral (104, 106)	-
Nerolidol	BG, FP	sweet, woody , floral (107)	>100 (103)
β-Patchoulene	FP	moss, moldy, earthy, woody (108)	-

[a] Estimated from (98); [b] Estimated from (99); ALF-Alfrocheiro (unpublished data) ; BG-Baga (4); FP-Fernão-Pires (31, 82, 109). *References.

Some of the sesquiterpenic compounds present in FP wines can be related with the floral characteristics described for its wines, namely *cis* and *trans* β-farnesene, farnesol and nerolidol.

1.2.2.3. C_{13} norisoprenoids

C_{13} norisoprenoids compounds can represent 0.07 to 1.50 % of the total volatile composition of wines in concentrations ranging ng L^{-1} to mg L^{-1} (110-112). The C_{13} norisoprenoids are products of secondary metabolism of carotenoids (C_{40} tetraterpenes) that can be generated by the direct oxidative degradation of grape carotenoids such as β-carotene, lutein, neoxanthin, and violaxanthin (111, 113). From the carotenoids degradation process other products are also formed such as C_9, C_{10}, C_{11} (43), C_{12}, C_{14}, and C_{15} (114) norisoprenoids. Figure 1.6 exemplifies the formation of C_9, C_{10}, C_{11} and C_{13} from the carotenoid degradation. Among these products, the C_{13} norisoprenoids are the most

important compounds for the wine aroma due to their low odour thresholds and pleasant aroma descriptors which are associated with tea, violet, exotic flowers, stewed apples, eucalyptus, and camphor (43, 74, 110).

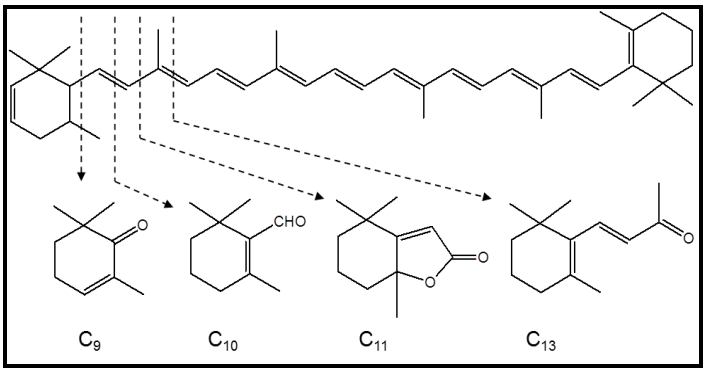


Figure 1.6. Origin of C_9, C_{10}, C_{11}, and C_{13} norisoprenoids from the carotenoids oxidative degradation.

These compounds are present in grapes in their glycoconjugated forms that are accumulated during the grape maturation. Therefore, their presence in wines in their free form is due to chemical or enzymatic hydrolysis that takes places during winemaking or along the wine maturation (111, 112, 115-117).

The C_{13} norisoprenoids compounds can be classified in two main groups, megastigmanes and non-megastigmanes. The megastigmane group has a sub-classification that differs according to the position of the oxygen functional group: i) Damascones - oxygen at carbon 7, like β-damascone (Figure 1.7) and, ii) Ionones - oxygen at carbon 9 as observed for β-ionone (Figure 1.7) (118). The non-megastigmane forms are all the other C_{13} norisoprenoids derivatives.

In Figure 1.7 are displayed the most frequent structures of the C_{13} norisoprenoids present in wines. Table 1.3 reports the main C_{13} norisoprenoids found in the wines from the grape varieties under study.

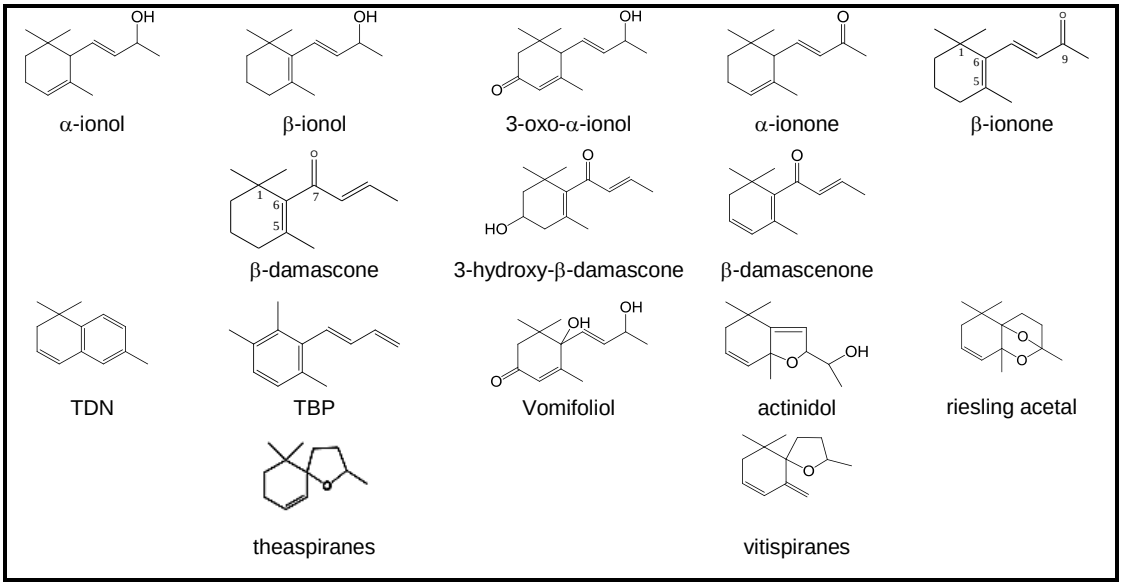


Figure 1.7. C_{13} norisoprenoids derivatives commonly found in wines (80, 81, 84, 119). TDN- 1,1,6-trimethyl-1,2-dehydronafhthalene; TBP: 4-(2,3,6-trimethylphenyl)buta-1,3-diene.

From the sensorial point of view β-damascenone and β-ionone are the most important compounds since they have the lowest odour threshold and interesting aroma descriptors (Figure 1.7, Table 1.3) (120).

Table 1.3- Main C_{13} norisoprenoids found in wines from the grape varieties under study, and the respective aroma descriptors and odour thresholds (OT).

Compound	Wine variety	Aroma descriptor	OT (μg L^{-1})
β-Ionone	FP, TN	violet, floral, raspberry (74)*	0.80 (43)*
β-Damascenone	FP, TN	apple, rose, honey (74)	0.045 (43)
TDN	FP, TN	kerosene, truffle, and flowery (110, 121)	2 (47), 20 (110)
Theaspiranes	FP	honey, acid drops (122)	-
Vitispiranes	FP, TN	camphor (110)	800 (110)

FP- Fernão-Pires (80, 81); TN- Touriga Nacional (84). *References.

Among the C_{13} norisoprenoids frequently described in FP wines, β-damascenone and β-ionone can explain the floral characteristic aroma of these wines. Concerning the TN composition, β-ionone was described as the most important compound and it is present in TN wines above the OT indicating the contribution of this compound to the floral characteristic described for these wines (84). No references are available in the literature about the presence of C_{13} norisoprenoids in wines from ALF and BG varieties.

1.3. Methods for the characterization of wine volatile compounds

One of the main objectives of wine volatile characterization is to obtain qualitative and quantitative data about compounds that can play important role in its aroma. The first step on the wine volatile analysis is the compounds extraction from the wine matrix, followed by separation and identification using chromatographic methods.

Up to date, different techniques of extraction of volatile compounds have been applied to the characterization of wines, such as liquid-liquid extraction (117, 123) and microextraction (124), simultaneous distillation-extraction with solvents (125), solid phase extraction (37, 126, 127), supercritical fluid extraction (128), microwave extraction (129), ultrasonic extraction (130), stir bar sorptive extraction (31, 131, 132) and solid phase microextraction (SPME) (4, 55, 133-137). Among the methods previously reported, the use of SPME has been increasing in the last two decades due to its facility to use, may be applied in a wide range of analytes sorption at low concentration ranges (ng L^{-1} and μg L^{-1}), and is solvent free. These characteristics are in the base of the selection of this technique for further analysis included in the present thesis.

The analysis of volatile fraction of wines is very difficult owing to its complex composition, since more than 1000 volatile molecules can be present. Consequently, qualitative and quantitative analyses are a challenge due to the high number of peaks acquired during a gas chromatographic analysis. Gas chromatography coupled to a mass detector seems to be the most suitable technique, since it enables the detection and identification of compounds present in very different amounts. However, due to the complexity of the matrix different strategies can be employed, which will be dependent on the objective of the study:

- Target analysis – If the aim is to detect and accurately quantify a specific pre-determined analyte or a set of compounds, making use of authentic standards (138, 139).

- Non-target analysis – If the aim is to determine the samples chemical profile, taking into account the information of analytes identified by authentic standards and also unknown compounds. Is a more comprehensive analysis that can be used to

understand the compounds that play a key role in specific biochemical processes, flavour characteristics, or any other aspect of specific interest, irrespective of whether they have already been identified or not (138, 139).

Both strategies have been applied in the varietal volatile analysis of wines especially those from the grapes varieties under study (4, 80, 82, 83).

1.3.1. Solid Phase MicroExtraction

SPME is a sample preparation technique based on sorption (absorption and/or adsorption, depending on the fiber coating), which is very useful for the simultaneously extraction and concentration either by submersion in a liquid phase or by exposure to a gas phase. After the fiber coating contact with the samples, the sorbed analytes can be thermically desorbed on the gas chromatograph injector.

SPME was developed by Janusz Pawliszyn and co-workers and was first used in water analysis (140). However, it spread rapidly to the analysis of various solid, liquid and gas matrices (141). This methodology has been extensively applied for wine volatile characterization, including the varietal components, and distinction of different varieties (9, 82, 123, 134, 135) despite its use in studies from a large number of products (142). SPME present many advantages over conventional techniques, since it is solvent free, allows sampling, extraction, concentration and clean-up in a single step of compounds with different physico-chemical characteristics (i. e. hydrophobic and hydrophilic compounds), reducing the total number of sample handling steps (83). Moreover, SPME can be easily automatized for gas chromatography and liquid chromatography couplings.

The principle of the SPME applied for liquid matrices is based on the partition of an analyte between two or three phases (140). In the first case, the liquid sample occupies the total volume of the vial, involving two phases; the liquid matrix and the fiber coating. In a three-phase system, the most common used in wine analysis and used in this thesis, the liquid matrix occupies part of the vial. Therefore, the system is composed by the liquid

matrix (L), the headspace (HS) of the sample (gaseous phase) and the fiber coating (F) as represented in Figure 1.8 (140). The same partition process is considered for gaseous and solid matrices, being the first case a two-phase system and the last a three-phase system.

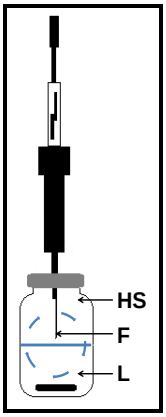


Figure 1.8. Three-phase system mode of extraction in SPME. HS-headspace; F-fiber coating and L-liquid.

In this system two types of equilibrium are established: $K_{liquid\text{-}headspace}$ and $K_{headspace\text{-}fiber\ coating}$. The amount of analyte extracted by the fiber coating can be determined by the following mathematical Equation 1:

$$n = \frac{C_0 \times V_F \times V_L \times K_{HS\text{-}F} \times K_{L\text{-}HS}}{K_{HS\text{-}F} \times K_{L\text{-}HS} \times V_F + K_{L\text{-}HS} \times V_{HS} + V_L} \quad (1)$$

where n is the mass of analyte extracted by the fiber coating, C_0 is the initial concentration of the analyte in the sample, V_1, V_2 and V_3 are the volumes of fiber coating, liquid and headspace, respectively, $K_{HS\text{-}F}$ is the analytes distribution constant between the headspace and the fiber coating and $K_{L\text{-}HS}$ is the distribution constant between the liquid and the headspace.

SPME is available in different geometric configurations, such as suspended particles, disk/membrane, stirrer, tube and vessel walls (143). However, syringe configuration continuous to be the most widely used apparatus (Figure 1.8). There are different stationary phases commercially available for SPME, thus, it is necessary to adapt the choice of coating to the sample and compounds to be analysed (144). The analytes

interaction with the SPME fiber coatings can be through absorption or adsorption phenomena (Figure 1.9) or by a combination of both (mixed fiber coatings). In absorptive interactions, the analytes are solvated in the interior of the fiber coating and the extraction is mainly dependent on the film thickness and the size of the analyte (141).

Concerning adsorptive interactions, the analytes are retained on the surface of a porous crystalline structure that can have micro (2-20 Å), meso (20-500 Å), and macro porous (>500 Å). Adsorptive fiber coatings have a limited number of sorption sites, in comparison with the absorptive ones, leading to competitive phenomena between the analytes (141).

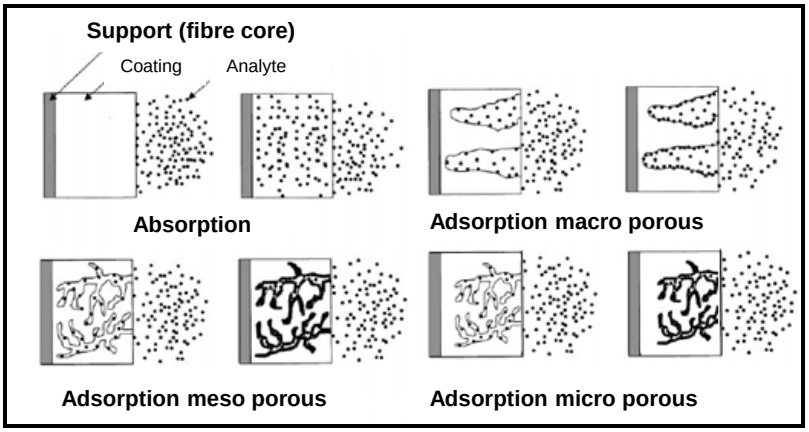


Figure 1.9. Representation of absorptive and adsorptive extractions, and adsorption for macro, meso and micro porous (143).

Mixed coatings join the best characteristics of both absorption and adsorption phases, revealing a high capacity to retain the analyte, which is possible due to a synergistic effect between two or more phases. For instance, fiber coatings such as Carbowax-Divinylbenzene (CW-DVB) are appropriate for the analysis of varietal volatile compounds in wines, as they can extract compounds with different physico-chemical characteristics within a wide range of molecular weights (40-275 MW) (144, 145). Moreover, its high capacity to retain the analyte, characteristic of mixed coatings, renders them suitable for the analysis of trace compounds (144).

SPME experimental parameters are dependent on the matrix composition. Therefore, to assure reproducibility by SPME analysis it is essential that the experimental parameters, such as extraction time, extraction temperature, addition of salt, stirring and

samples volume, must be optimized and maintained along the analysis (83, 140).

Extraction time. The time of exposure of the fiber coating during sampling increases the analyte concentration on the fiber coating, until the equilibrium is reached (140). From this point, its concentration should be constant, and small variations in the extraction time do not affect the amount of analyte extract by the fiber coating. On the contrary, under equilibrium, the amount of analytes extracted by the fiber coating varies with small variations of time (146). Therefore, the extraction time must be optimized and if the analysis were performed under the equilibrium state this time must be controlled.

Temperature. The temperature affects the equilibrium during extraction: an increase in temperature change the equilibrium distribution of analytes in the sample and the headspace, that consequently alters the amounts of analytes extracted by the fiber coating (143). An increase on temperature increases the analytes diffusion from the liquid matrix to the headspace, allowing an increase on the amount of analytes extracted, by reducing the equilibrium time (147). Otherwise, the extraction temperature may also affect the analyte solubility. The temperature effect on the amount of volatiles sorbed is the result of a compromise between the solubility and volatility (83).

Salting out. The addition of an electrolyte in a solution is frequently used to decrease the solubility of analytes in the aqueous phase and change the properties of the phase boundary (147). Since the water molecules form spheres around the ionic salts, a reducing in the concentration of water available to dissolve the analytes of the sample is observed, increasing therefore their availability on the headspace (83, 144). This phenomena is called the "salting out" effect and is widely used to increase sensitivity of analytical methods (148). However, depending on the physicochemical properties of the molecule a high interaction with the electrolyte can be established diminishing its release to the headspace (147).

Stirring. The system agitation facilitates the release of volatile analytes by increasing the surface of the liquid-vapour interface allowing a faster equilibration and a more uniform extraction (144). Sample agitation increases the extraction efficiency and reduces the time of extraction, especially for compounds with high molecular weight and diffusion coefficient.

Sample volume. In headspace analysis, the ratio headspace volume/sample volume (β ratio) has a great impact on the amounts of analytes extracted by the fiber coating (149). This parameter is dependent on the compounds' coefficient of partition in the headspace (149). It is described in the literature that in general the analytes extraction increases with the increase of the headspace volume (149, 150).

1.3.2. Gas chromatographic methods

Gas chromatography (GC) is an analytical technique that separates the components present in a mixture (151). For GC analysis, two essential conditions are required regarding the analyte: i) it must be volatile at working temperature; and ii) stable at this temperature in order to avoid its own degradation.

1.3.2.1. One-dimensional GC-qMS

In GC analysis a mixture of volatile components are separated in a chromatographic column as a result of their distribution between a mobile and a stationary phase (152). GC when coupled to a mass spectrometry detector becomes a powerful tool for identification and quantification of complex matrices such as wines. A scheme representing a GC-qMS system is shown in Figure 1.10.

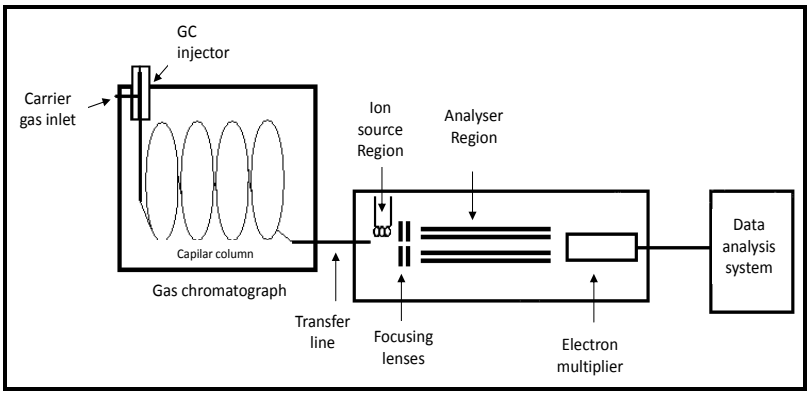


Figure 1.10. Representative scheme of a gas chromatography-mass spectrometry system (153).

After chromatographic separation the analytes are transferred to the ion source where they are bombarded with high-energy electrons emitted by filaments, thus leading to structure breakdown forming ions that are separated according to a ratio mass/charge (*m/z*) (Figure 1.11). In the sequence, fragments are rapidly formed, generating a charged fragment and a radical which can be further decomposed into smaller fragments. The positive ions are then repelled from the ionization chamber by applying a positive charge through the "repeller" (Figure 1.11).

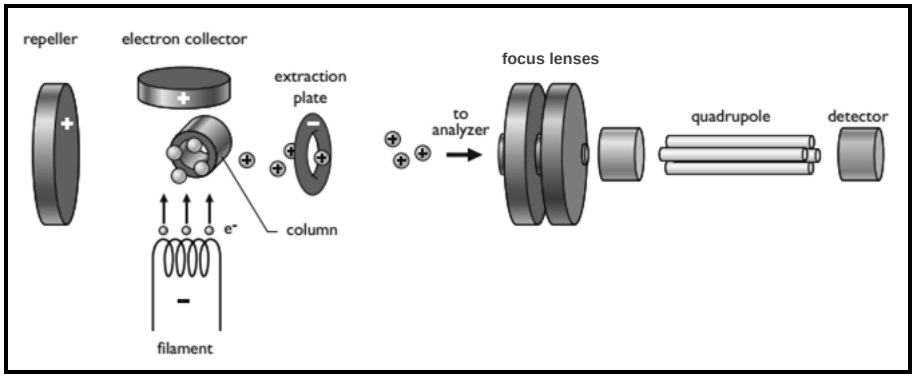


Figure 1.11. Ionization source scheme (154).

Generally, electron beans of 70 eV are used, allowing the breaking of high-energy bonds and removing an electron to form the molecular ion (M^+). The application of small voltages on the focusing lens (Figure 1.11) is responsible to the ions focus towards the quadrupole analyser (Figure 1.11). In the quadrupole instrument a radio frequency and direct current potential are then applied in order to establish a stable trajectory for the detection of the *m/z* fragment (153).

Some MS instrumental parameters can greatly influence the sensitivity and reproducibility of a quantitative analysis. These parameters can be optimized in order to increase the sensitivity for specific fragments. For instance, soft ionization energies can be used, which are still able to fragment most of the compounds. Figure 1.12 exemplifies the mass spectra of a compound obtained by two ionization energies 70 and 12 eV. As can be observed, the mass spectra obtained with 70 eV (Figure 1.12a) is more complex than that of 12 eV (Figure 1.12b), being the last dominated by few largest characteristics fragments, carrying important structural information (155).

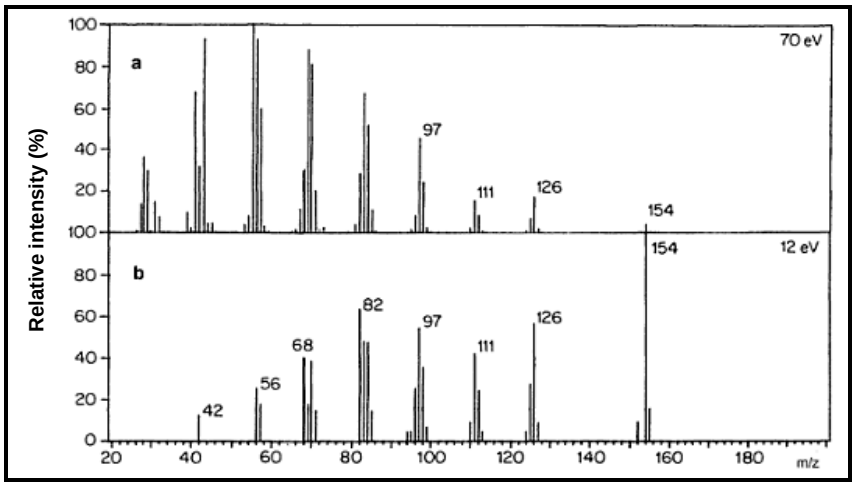


Figure 1.12. Undecan-1-ol mass spectra obtained at different Electron Impact (EI) energies: a) 70 eV and b) 12 eV (155).

Other parameters can be optimized in order to modify the intensity of specific fragments, such as the repeller and the ion focus lens voltages (Figure 1.11) (156). In Figure 1.13A it is exemplified the effect of the repeller voltage on the peak intensity of the selected fragment.

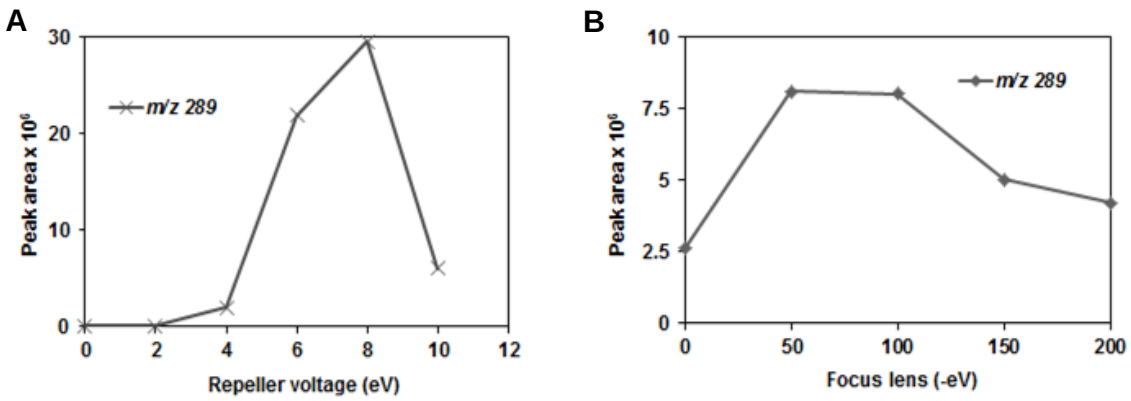


Figure 1.13. Influence of the repeller (A) and ion focus (B) voltages on specific fragments (adapted from (157).

It can be observed from that example, that an increase on the repeller voltage increases the peak intensity for the fragment *m/z* 289, where the optimum intensity is obtained for setting the repeller at 8 eV (Figure 1.13A). An opposite behaviour is observed for the ion focus lens that increases the response for the fragment *m/z* 289 with the lower voltages (Figure 1.13B)

The sequence of fragmentation of the analyte is acquired with the assistance of a computing system and data processing and is displayed as a chart bar, the mass spectrum. The abundance of the generated fragments is reported as percentage of the most abundant fragment (base peak 100%). Two modes of mass spectra acquisition can be used:

i) full-scan mode that allows the detection of all ions formed. A graphical representation of all information obtained is given by plotting the total ion current (TIC). The TIC is a signal representing the sum of the ion current for the detected peaks in each mass spectra scan (153).

ii) selected ion monitoring (SIM) mode that allows the detection of specific ions. The main advantage of the SIM mode is an increase in the order of magnitude of detection, with a considerable increase in sensitivity compared to full-scan mode (158). This technique is used to analyse target components, where selection of representative *m/z* fragment of the analyte, normally the base peak, is used for monitorization. Once SIM enables the selection of a single ion, specific separation of co-eluted analytes may also be another application of this mode.

1.3.2.2. Two-dimensional GC×GC-TOFMS

One-dimensional chromatographic processes are widely used for food matrix analyses. Although this method often provides rewarding analytical results, the complexity of many natural matrices exceeds the capacity of any single separation system. In recent years considerable research has been devoted to the combination of independent techniques aiming the strength of resolving power such as two-dimensional comprehensive chromatography (GC×GC) (59, 159, 160).

GC×GC employs two orthogonal mechanisms to separate the constituents of the sample within a single analysis. The technique is based on the application of two GC columns coated with different stationary phases, such as one non-polar and one polar (NP/P), connected in series through a special interface (modulator). The interface cuts the first dimension (^{1}D) eluate by cryofocusing in small (few seconds) portions and re-injects

them onto the second column (^{2}D). Each ^{1}D peak is modulated several times, which allows the preservation of the ^{1}D separation. The second column is very short and narrow and consequently each modulated portion is "flash" separated before the next modulation starts. Using this instrumental approach, compounds co-eluting from the first column undergo additional separation on the second one (59, 161). Therefore, the separation potential is greatly enhanced when compared to the one-dimensional GC. Besides chromatographic separation, sensitivity and limits of detection are also improved due to the peak focusing in the modulator and the analytes separation from chemical background (162) which is fundamental to study trace compounds. Figure 1.14 illustrates a scheme of GC×GC equipment.

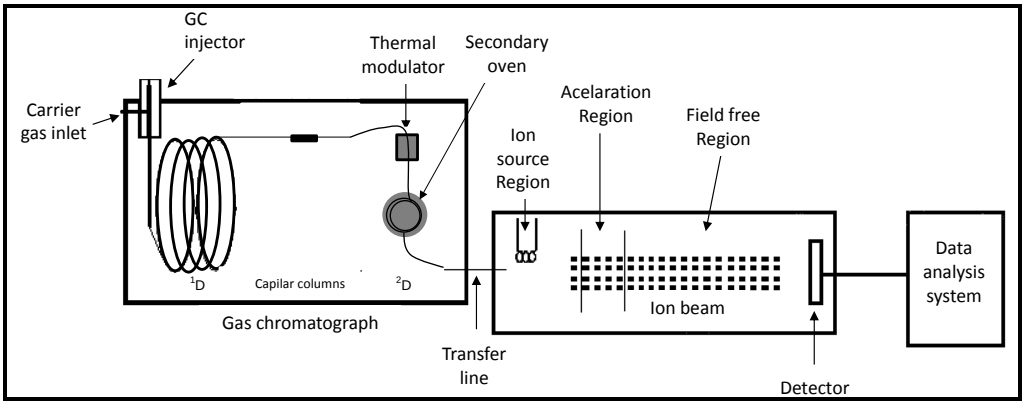


Figure 1.14. Representative scheme of a comprehensive two-dimensional gas chromatography time of flight mass spectrometry (163).

The ions separation in the time of flight analyser (TOF) is based on the migration time, after acceleration in the region of the open field. Since the produced ions have equivalent kinetic energies, those with lower *m/z* arrive first at the detector, followed by those with heavier *m/z* (153). TOF analysers present many advantages over quadrupole analysers as: i) no limit to the upper mass range of a TOF instrument; ii) high ion transmission efficiency that leads to very high sensitivity, indicating that ions produced in a short time span will be temporal separated allowing all of them to reach the detector; iii) very high analysis speed; and iv) spectrum over a broad mass range can be obtained in micro-seconds (164).

The recording of the detector signal in the system of GC×GC versus time is a continuous sequence of chromatograms obtained for each fraction eluted from the ^{2}D. The graphs obtained are three-dimensional and represent the signal from the detector, retention time of the 1st column ($^{1}t_R$) and retention time in the second column ($^{2}t_R$).

Figure 1.15 illustrates an example of three compounds co-eluted in a broad band in ^{1}D (a), fractionation and elution of this portion in the ^{2}D represented by the chromatogram without deconvolution (b). The individual chromatograms (c) are treated for generating three-dimensional graphics representing the signal in $^{1}t_R \times {}^{2}t_R$ (d), or a series of monochromatic curves levels called contours diagram (e), or by chromatic scales called colours diagrams (f). Another important aspect to stress is the increase in the analytes detection since the modulation allows their elution as a series of narrow and intense peaks, with increased signal-to-noise relation facilitating their detection (163).

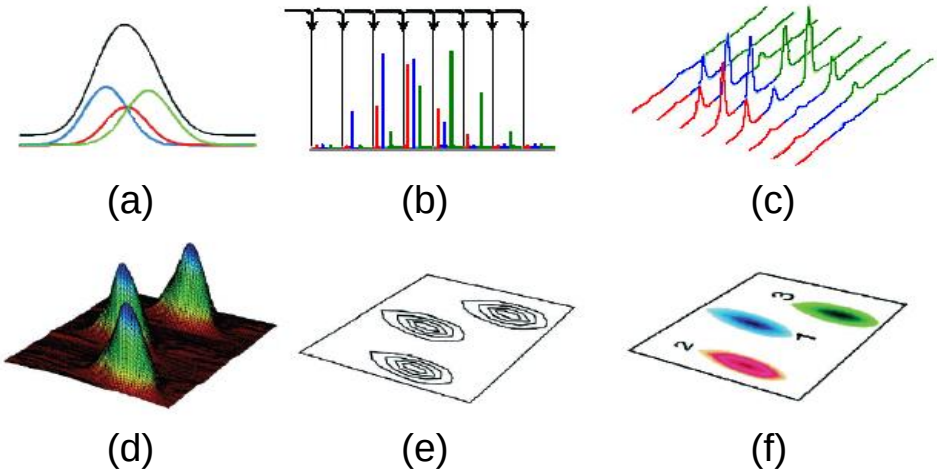


Figure 1.15. Steps for the construction of tri-dimensional diagrams signs in GC×GC (163).

GC×GC also offers new opportunities to develop relationships between molecular structure and retention times in the two dimensional space defined by the GC×GC retention times in the combined dimensions (165). Compound identification based on the organized structure of the peaks of structurally related compounds in the GC×GC contour plot (Figure 1.16) represents a valuable approach, as the ordered-structure principle can considerably help the establishment of the composition of samples (59). This is particularly important not only as a rapid screening tool but also for the characterization of natural matrices, which in many cases chemical standards are not commercially available.

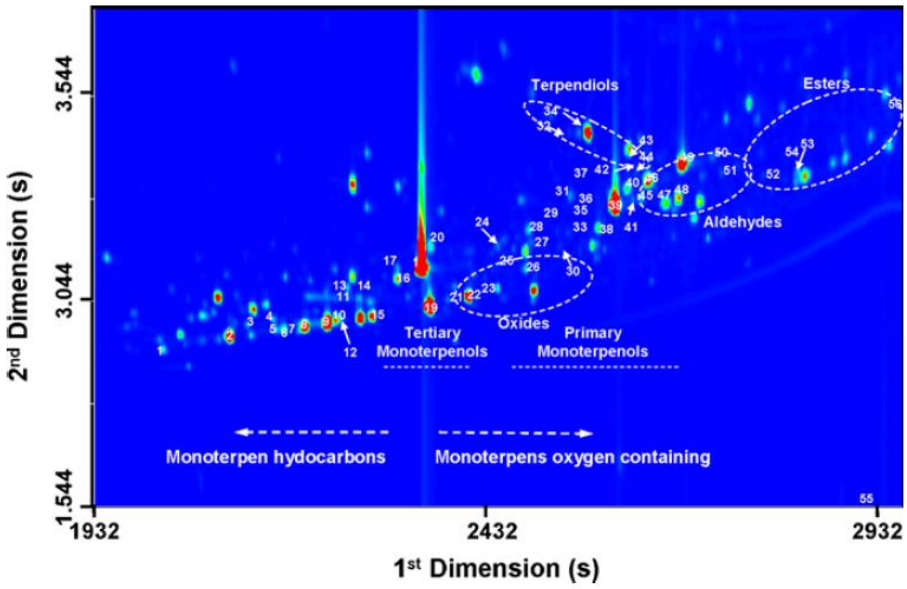


Figure 1.16. GC×GC chromatogram contour plot exemplifying the ordered-structure principle (59).

GC×GC analysis has been applied for target analysis of methoxypyrazines in Sauvignon Blanc wines (166), 2-aminoacetophenone in different white wines (167), ethyl carbamate and markers of aging in Madeira wines (168, 169), and the full characterization of Cabernet Sauvignon (170), Pinotage (94, 95, 171), Brazilian Merlot (96), Pinot noir and Dornfelder (170) wines.

1.3.2.3. Rapid methods based on GC-MS

Reduction of the time used in analytical procedures has become a priority in several laboratories. Faster analyzes are attractive not only to reduce the response time of delivery, but also the costs of operation in various fields. A comprehensive approach to reducing time of analysis is to reduce both the sample preparation and analytical steps. In the analysis of the volatile fraction of wine samples, SPME seems to offer a rapid way of sample extraction and pre-concentration, as previously mentioned and because of its high efficiency, selective, and sensitive GC remains a separation method frequently selected. Moreover, it is easy to coupling GC with mass spectrometry that offers the analytes identity. In this context, a methodology based on solid-phase microextraction–mass

spectrometry-multivariate analysis (SPME–MS-MVA) was developed to rapidly assess off-flavours in milk as an alternative approach to commercial e-nose instruments (electronic noses) (172). The principle of this methodology was the extraction of the volatiles present in the headspace of the sample by SPME, following by direct injection in the ionization chamber of the mass spectrometer, without chromatographic separation.

In SPME–MS-MVA methods, the GC is used as a transfer liner responsible for coupling the injection module to the mass spectrometer (Figure 1.17). Different strategies can be employed in order to obtain the sample spectral fingerprint such as by using shorten chromatographic columns (0.5-5m) depending on the internal diameter (0.05-0.20 mm), increasing the gas flow, and setting the column oven temperature high enough that no separation takes place allowing obtaining the unresolved profile (173, 174).

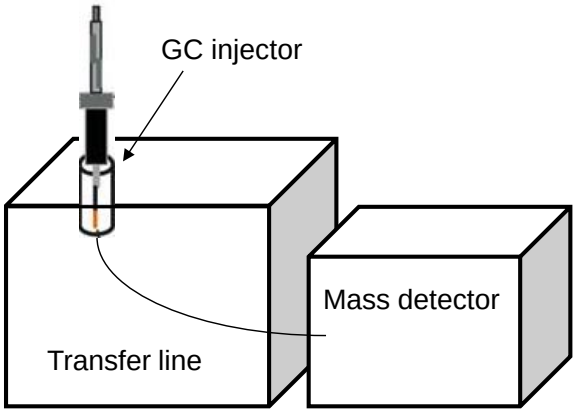


Figure 1.17. SPME-GS-MS scheme (adapted from (174))

The sample volatile signature or spectral fingerprint is obtained in few minutes, as the example given in Figure 1.18 (174). The fingerprint is characteristic to the product being analysed, since the obtained spectrum is the result of the simultaneous ionization and fragmentation of the mixture of molecules injected. The analysis of this spectral information allows determining the composition of the sample (175). These volatile spectral fingerprints have been used for different purposes such as samples classification, sensory properties prediction, estimation of technological parameters, and detection of compounds responsible for the odours of different foods (172, 174, 176, 177). An important advantage of the spectral fingerprint obtained with this methodology is the chemical information that is not possible with current e-nose instruments.

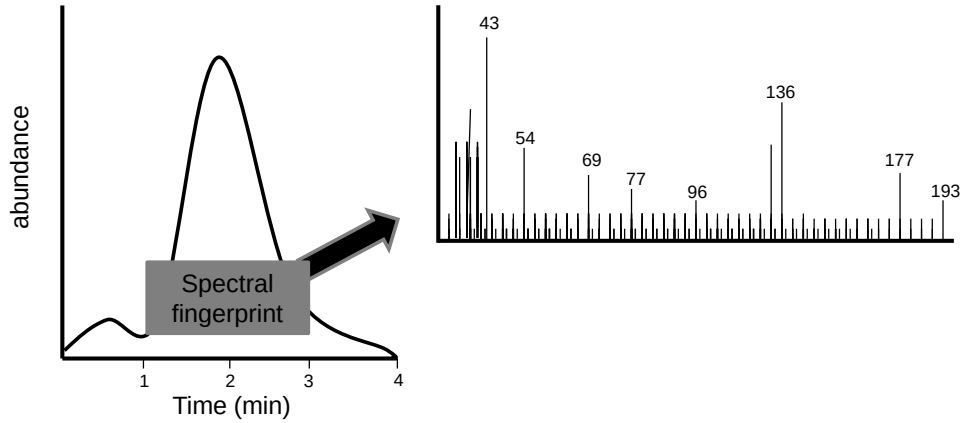


Figure 1.18. Mass spectral fingerprint obtained by HS-MS (adapted from (174))

This methodology was first applied in wines by Rocha et al. (80), allowing their rapid distinction by principal component analysis (PCA). The global volatile signature of the wine headspace (chromatographic profile and *m/z* pattern of fragmentation in each scan) was evaluated without complete chromatographic separation of its components. It was possible to extract chemical information based on the *m/z* fragments (markers) which enables the characterization and distinction of the wines varieties (80). The results obtained with this methodology indicate that it is possible to focus the analysis only in some zones of the chromatographic profiles. Hence, defining varietal markers may allow the distinction and possible classification of wines.

1.4. Potential health benefits of sesquiterpenic compounds

Several studies show a beneficial association between the consumption of fruit, vegetable, herbs and food and drink based on these matrices (178-181) and the development of certain pathologies (i. e. cardiovascular disease, cancer and neurodegenerative diseases such as Alzheimer and Parkinson diseases). This association suggests that the lower incidence of diseases in populations with high consumption of plant food may be attributed in part to the cumulative or synergistic impact of the compounds present in the matrix.

Sesquiterpenoids are commonly found in fruits and vegetables and also in different commercial products exhibiting different health applications (4, 182, 183). During the last 27 years Fraga (184-209), provided annual reviews about sesquiterpenic compounds from natural sources, their biosynthesis and biological properties. More than three hundred compounds were identified each year and their biological potential explored revealing a broad-spectrum of activities. Antibacterial and enhancers of bacterial susceptibility to antibiotics (210-216), antimalarial (213), anti-inflammatory (217-220), anti-carcinogenic (221-226), anti-mutagenic (227, 228) and antioxidant effects (214, 229-231) are examples of the effects exerted by sesquiterpenic compounds among many others.

Considering the description of sesquiterpenoids in *Vitis vinifera* L. grapes and wines and the potential health benefits that can be expected for these compounds, this thesis aims to evaluate the antioxidant and antiproliferative effect for some of the compounds identified in this matrices and in plants and plant-derived foods and beverages. In this sense, the following sections present the theoretical basis behind the antioxidant and antiproliferative effects, the methods used to evaluate these effects, and the literature review for the compounds under study.

1.4.1. Antioxidants effects

1.4.1.1. Reactive species and their role in the organism

A free radical is defined as a neutral species containing an odd number of electrons, thus having an unpaired electron in their valence orbital. The stability of the radical is reached when the valence orbital is filled with an electron abstracted from another molecule, which in turn becomes a new radical (232). The radicals species (RS) formed can be radicals derived from oxygen and nitrogen species, ROS and RNS respectively. By the other hand, the antioxidant is a molecule able to donate an electron and stabilize itself. Great emphasis has been given to antioxidants in recent years, since these compounds can have an essential role in the control of reactive oxygen species produced endogenously in the body such as superoxide ($O_2^{\bullet-}$) in the mitochondrial respiration (233), or produced exogenously as the singlet oxygen and the superoxide radical ($^1O_2$ and $O_2^{\bullet-}$) by ultra-violet radiation (234). Normally, the organism is able to control the concentration of these species, mainly by the production of enzymes that catalyses the reactions of inactivation of radicals. However, an increase on the generation of intracellular radicals, or a insufficiency in the antioxidant mechanisms of defense can deregulate this control system producing the denominate oxidative stress (232). When RS exceeds cellular antioxidant capacity, this may result in damage of proteins, DNA and lipids. The alterations caused by RS on cell membrane lipids lead to changes in its permeability altering the flux control of ionic and non-ionic substances and selectivity control for the way in and out of toxic substances. Consequently, DNA alterations and low-density lipoprotein oxidation can be observed. This process can result in cell death and neoplasia which are probably involved in the incidence of several diseases, among which are cancer, atherosclerosis, rheumatism, arthritis, osteoarthritis and degenerative diseases such as Parkinson's and Alzheimer's (235).

1.4.1.2. Lipid peroxidation process

RS can alter the cell membrane function by a process known as lipid peroxidation that involves three different pathways: (i) enzymatic pathway involving cyclooxygenases and lipoxygenases; (ii) non-enzymatic pathway where the reactive oxygen and nitrogen species, transition metals and others free radicals are implicated; and (iii) non-enzymatic and non-radical (i. e. photo-oxidation) (236). After lipid peroxidation cycle initiation, the propagation process can only be broken due the intervention of antioxidants. The mechanism of antioxidant action involves membrane stabilisation and neutralisation of free radicals (237). Therefore, the antioxidants are considered effective inhibitors of carcinogenesis and pathogenically associated with oxidative mechanisms (238).

The mammalian cell membrane lipid fraction is composed by phosphoglicerolipids, sphyngolipids and sterols. Phosphoglicerolipids, the major lipid group, are composed, in its simpler form, of a molecule of glycerol bonded to a phosphate group (polar head group) and to two fatty acids containing one or more unsaturations. Sphyngolipids are composed of one molecule of the long-chain amino alcohol sphingosine or one of its derivatives, one molecule of a long-chain fatty acid, and a polar head group (phosphate or a sugar group). Cholesterol, the sterol present in mammalian cell membrane, contains a polar head group (the hydroxyl group) and a nonpolar hydrocarbon body (the steroid nucleus and the hydrocarbon side chain), about as long as a 16-carbon fatty acid in its extended form (239, 240). These lipids (L) are vulnerable to reactive oxygen species attack, due to the presence of unsaturations, being the first step on the lipid peroxidation process. This phenomenon can be divided in three phases: initiation, propagation and termination (Scheme 1.1).

(1) Initiation

$LH + OH^{\bullet}$ (or $LO^{\bullet}$) $\rightarrow L^{\bullet} + H_2O$ (or LOH)

(2) Propagation

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

(2) Propagation

$LH + LOO^{\bullet} \rightarrow L^{\bullet} + LOOH$

(3) Termination

$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$

(3) Termination

$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$

Scheme 1.1- Lipid peroxidation phases, initiation, propagation and termination.

In the initiation phase the allylic hydrogen of the polyunsaturated fatty acid is abstracted forming the carbon radical which is then stabilized by a molecular re-arrangement forming a conjugated diene (241). In aerobic environment, the alkyl radical is combined with an oxygen given the peroxyl radical that can abstract other allylic hydrogen from other fatty acid, promoting the propagation phase since other carbon radical is formed (Scheme 1.1) (241). The extinction of free radicals by the formation of non-radicals products occurs in the termination phase (Scheme 1.1, (3)) (241). Peroxyl and alkoxyl radicals can also give rise to aldehydes by dismutation or β-cleavage, or form a covalent bond with amino acid residues, or re-arrangement forming secondary products of the lipid peroxidation.

The peroxidation products can be assessed by different methodologies, such as: i) the conjugated dienes by spectrophotometry; ii) conjugated hydroperoxides by iodometry and high performance liquid chromatography (HPLC) with different detectors (ultraviolet, MS and chemioluminescence); iii) aldehydes, mainly malonaldehyde (MDA), by thiobarbituric acid reactive substances (TBARs), HPLC-MS and GC-MS; iv) ethane and pentane by GC-MS; v) mono and dihydroxyl polyunsaturated fatty acids by GC-MS and HPLC; and vi) isoprostanes by GC-MS and immunoassays (242). Among these methods the TBAR's is the assay most frequently used to evaluate the extension of lipid peroxidation. The principle of the method is the reaction of the secondary product of polyunsaturated fatty acids oxidation (MDA), with the thiobarbituric acid (TBA) in acidic medium and under heat, resulting in a coloured compound detectable at 532 nm.

1.4.1.3. Sesquiterpenic compounds and their antioxidants properties

Different studies have been carried out concerning the antioxidant activity of sesquiterpenic compounds. In Table 1.4 is presented a summary of the *in vitro* and *in vivo* antioxidant activity already reported for some standard compounds, the concentration range tested, the concentration needed to inhibit 50 % of the radical (IC_{50}), the type of assay used and the compounds mode of action.

Table 1.4- Antioxidant activity of sesquiterpenic compounds, concentration range tested, type of assay used and mode of action.

Compounds	Tested Concentration mM	IC_{50} mM	Type of assay	Mode of action	Ref
(+)-Aromadendrene α-Bisabolol α-Cedrene β-Caryophyllene *trans,trans*-Farnesol α-Humulene (+)-Valencene	0.5-5.0 0.45-4.5 0.5-5.0 0.5-5.0 0.45-4.5 0.5-5.0 0.5-5.0		egg yolk homogenates (*in vitro*)	Inhibition of lipid peroxidation	(243)
	0.017-0.14		human neutrophil bursts and cell-free systems (*in vitro*)	Improvement of the antioxidant network and restore of the redox balance by antagonising oxidative stress	(244)
α-Bisabolol	0.45 and 0.90		rats (*in vivo*)	Gastroprotection effect against ethanol injury probably associated with an increase of gastric sulfhydryl groups	(245)
	0.45 and 0.90		rats (*in vivo*)	Gastroprotection effect against ethanol injury by lipid peroxidation reduction and increase of SOD	(246)
	0.23 and 0.45		rats (*in vivo*)	Colon protection effect against 1,2-dimethylhydrazine by lipid peroxidation reduction, increase in SOD, CAT, GPx, GR, GST, QR and GSH	(247)
trans,trans-Farnesol	0.23 and 0.45		rats (*in vivo*)	Protection against cigarette smoke toxicants by reducing the lipid peroxidation, increase in GSH content and decrease in H_2O_2	(248)
	0.11-1.1		Swiss albino mice (*in vivo*)	Skin protection effect against 12-O-tetradecanoylphorbol-13-acetate by lipid peroxidation reduction and increase of CAT, GPx, GR, GST and GSH	(249)
Guaiazulene	0.1-0.4 5-20 0.005-0.02 1.26	0.4 > 20 0.0098	*in vitro* *in vitro* hepatic microsomal fraction (*in vitro*) rats (*in vivo*)	Inhibition of $DPPH^{\bullet}$ Inhibition of hydroxyl radicals Reduction of lipid peroxidation Hepatoprotection against paracetamol by restoring the levels of GSH	(250)
	1.26		rats (*in vivo*)	Hepatoprotection effect against paracetamol by increasing its metabolic activation preventing the cytochrome P450 degradation and the NAPQI-induced toxicity, and the decreasing of GSH	(251)
α-Humulene β-Caryophyllene	0.007 0.01		Astrocytic cells (*in vitro*)	Protection against hydrogen peroxide injury	(252)
	0.05-0.5	0.5 and 0.5	*in vitro*	Inhibition of $DPPH^{\bullet}$ and hydroxyl radicals	(253)
β-Caryophyllene	0.0001-1 0.000001-1	0.65, 0.001 and 0.0005 0.00007 0.005	*in vitro* *in vitro* hepatic microsomal fraction (*in vitro*) CFS-2G hepatic stellate cells (*in vitro*) rats (*in vivo*)	Inhibition of $DPPH^{\bullet}$, hydroxyl radicals and $O_2^{\bullet-}$ Inhibition of xanthine oxidase and 5-lipoxygenase Reduction of lipid peroxidation Reduction of lipid peroxidation caused by Fe^{2+}/ascorbate system Hepatoprotection effect against carbon tetrachloride by reducing the lipid peroxidation	(254)
	0.001-0.1	0.01	human neoroblastoma cells (*in vitro*)	Neuroprotection effect against the oxidative damage caused by ischemia/reperfusion	(255)

SOD-superoxide dismutase; CAT-catalase; GPx-glutathione peroxidase; GR-glutathione reductase; GST-glutathione-S-transferase; QR-quinone reductase; GSH-reduced glutathione.

As can be observed in Table 1.4, the antioxidant activity of sesquiterpenic compounds have been tested in a large range of concentrations, between 0.000001 and 20 mM by different *in vitro* and *in vivo* studies.

The antioxidant activity of the sesquiterpenic compounds in *in vitro* systems is related with the inhibition of radicals such as the stable radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), hydroxyl and peroxyl radicals and protective effects using cells. Concernig $DPPH^{\bullet}$ inhibition similar values of IC_{50} can be observed for guaiazulene and β-caryophyllene. However, β-caryophyllene is twice more effective than guaiazulene in the inhibition of lipid peroxidation in hepatic microsomal fraction and against hydroxyl radicals (Table 1.4). According with the results obtained by Ruberto and Baratta (243), for the sesquiterpenic compounds with different chemical structures, oxygenated compounds were more effective in inhibit peroxyl radicals than the hydrocarbons.

In general, the concentrations of sesquiterpenic compounds in studies *in vitro* models (0.000001-1 mM) are lower than in *in vivo* tests (0.11-1.26 mM). Normally, in biological assays an external injury with a chemical agent (i. e. ethanol, 1,2-dimethylhydrazine, 12-O-tetradecanoylphorbol-13-acetate and paracetamol) is provoked in order to initiate the oxidative stress which in turn initiates the lipid peroxidation process. Thus, the cells antioxidant system is activated in order to overcome the injury and the levels of these compounds drops. As can be observed in Table 1.4, the cells pre-treatment with sesquiterpenic compounds increases the enzymatic, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and quinone reductase (QR) and non-enzymatic, reduced glutathione (GSH) antioxidant status levels when cells are further exposed to the oxidant agent. This protective effect has direct consequence on the decrease of the lipid peroxidation since the oxidation process is diminished or stopped.

1.4.2. Antiproliferative effects

1.4.2.1. Isoprenoids antiproliferative mode of action

As previously stated, antioxidants compounds can have a pivotal role on the organism protection against oxidative damages, thus preventing the development of different pathologies such as cancer.

Different hypothesis have been already postulated to the mode of action of antiproliferative compounds. The most frequently associated with isoprenoids, including sesquiterpenic compounds, is the suppression of the mevalonate pathway which supports pools of farnesyl pirophosphate, geranylgeranyl pirophosphate, and dolichol phosphate, products essential for cell survival and proliferation. The mevalonate pathway chemotherapeutic agents inhibit either the activities providing mevalonate-derived intermediates (statins, phenylacetate, and phenylbutyrate) or the activity transferring the farnesyl moiety to small G proteins (farnesyl protein transferase inhibitors) (221, 256-259). The inhibitory actions attributed to the cyclic isoprenoids, include inhibition of farnesyl protein transferase, activation of allyl pirophosphate pirophosphatase with a concomitant increase in the signaling molecule, farnesol, causing HMG-CoAR degradation and inhibiting translation of HMG-CoAR mRNA (Figure 1.19). An anomaly associated with tumour growth is their resistance to the sterol feedback, and overexpression of the HMG-CoAR activity. This characteristic renders the mevalonate pathway sensitive to isoprenoid-mediated post-transcriptional downregulation.

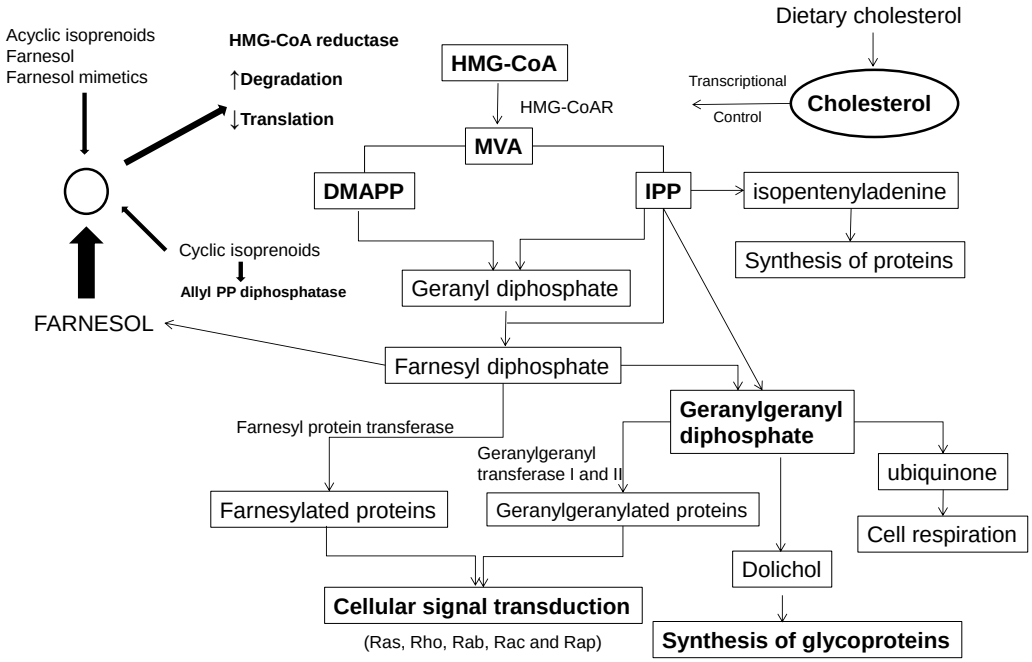


Figure 1.19. Schematic diagram of the mevalonate pathway (260).

As referred above, antiproliferative effect aims at assessing the ability of compounds in preventing cell cycle progression. For this purpose, cells must be between G0-G2 phases, thereby preventing that mitosis takes place (Figure 1.20). Cell cycle arresting properties associated with the isoprenoids was confirmed as for instance farnesol and nerolidol were reported to arrest cell cycle at the G_0-G_1/S interface (Figure 1.20) (222, 261).

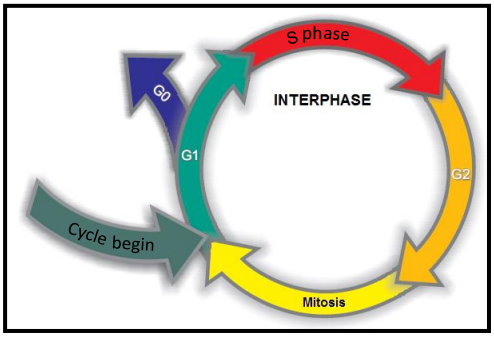


Figure 1.20. Cell cycle scheme. G0- resting; G1- cell duplication with increase in size and number of organelles, enzymes and other molecules; S phase- duplication of DNA and associated proteins; G2- preparation for division, chromosome condensation starts; Mitosis-cell division.

1.4.2.2. Sesquiterpenic compounds and their antiproliferative effects

In recent years, several studies have been conducted on the ability of sesquiterpenic compounds to act as antiproliferative agents. Table 1.5 summarizes the antiproliferative effect already reported for a set of four sesquiterpenic compounds commonly detected in wines and under study in this thesis, the concentration needed to inhibit 50 % of the cell proliferation (IC_{50}), and the model used.

Table 1.5- The antiproliferative activity of sesquiterpenic compounds commonly detected in wines, the cytotoxic concentration and the biological model used.

Compounds	IC_{50} (μM)	Method	Model	Ref
Nerolidol	65	trypan blue	Mouse melanoma cells (B16F10)[a]	(222)
	26		Promyelocytic leukemia cells (HL-60)[a]	
	10	sulforhodamine B and MTT	Human hepatocellular liver carcinoma cells (HepG2)[a]	(221)
	20	cell proliferation reagent WST-1	Human cervical carcinoma cells (HeLa)[a]	(262)
Farnesol	28	trypan blue	Mouse melanoma cells (B16F10)[a]	(222)
	30		Promyelocytic leukemia cells (HL-60)[a]	
	20	Coulter counter	Human pancreatic ductal adenocarcinoma cells (MIA PaCa-2)[a]	(261)
	60		Human pancreatic ductal adenocarcinoma cells (BxPC-3)[a]	
	230	[^{3}H]thymidine incorporation	Skin tumorogenenisis Swiss albino mice	(249)
Guaiazulene	400	MTT	Human gingival fibroblast（HGF)[b]	(263)
	190		Human pulp cell (HPC)[b]	
	190		Human periodontal ligament fibroblast (HPLF)[b]	
	110		Human oral tumour cell line (HSG)[a]	
	160		Human oral tumour cell line (HSC-2)[a]	
	120		Human oral tumour cell line (HSC-3)[a]	
α-humulene	129	MTT	Monkey kidney (Vero)[b]	(264)
	159		Macrophages[b]	
	18		Human lung carcinoma cells (A-549)[a]	
	35		Human cervical carcinoma cells (HeLa)[a]	
	24		Human colon adenocarcinoma cells (HT-29)[a]	

[a] Cancer cells
[b] Normal cells

As observed from Table 1.5, all compounds showed antiproliferative effect in a concentration ranging from 10-400 μM. This effective concentration is dependent of the compound and the biological model under study. Different cell parameters were used in order to assess the antiproliferative effects, such as the cell death assayed by trypan blue stain, the mitochondrial respiration that can be measured by reducing tetrazolium salts such

as bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), measuring the total protein by the method of sulforhodamine B (SRB), and by the incorporation of tritiated thymidine ([^{3}H]thymidine) during DNA synthesis (S phase) as described by (265).

Considering the compounds listed in Table 1.5 some key observations can be made: i) farnesol was twice more effective than *cis*-nerolidol on B16F10 cells, however, they are not statistically different on HL-60 cells; ii) farnesol was three times more effective in MIAPaCa-2 cells when compared with BxPC-3 cells, the two human pancreatic ductal adenocarcinoma cells studied by Wiseman *et al.* (261); iii) farnesol effective concentration in *in vivo* antiproliferative test is almost 4 to 12 times higher than the *in vitro* assays (249); iv) low concentrations of nerolidol were needed to inhibit HepG2 and HeLa cells proliferation (221, 262); and v) higher antiproliferative effect was observed for guaiazulene and α-humulene against tumour cells than to normal cells (263, 264).

1.4.3. Comprehensive effect evaluation: QSAR models

1.4.3.1. Role of chemical structure on biological activity

The biological effect of bioactive compounds results from interactions with biological systems and is dependent on factors related to their chemical structure and, consequently, their physicochemical properties. These factors, i.e. hydrophobic, electronic, or steric character, influence the interaction of the compound with the biophase (the place that a drug must reach to exert their therapeutic action), and their distribution in compartments that comprises the biological system. Consequently, it is expected that compounds structurally similar, differing only by one atom or its position in the molecule, may have different physicochemical properties and, thus, different biological activities, both quantitative and qualitatively (266). For instance, from Figure 1.21 it can be observed that the neuroprotection activity is dependent upon the compound tested. However, the activity is not directly correlated with the molecular properties MW (constitutional

descriptor) and/or LogP (molecular descriptor) of the different compounds, suggesting that other structural parameters, or the combination of different molecular descriptors, can also influence the activity under evaluation.

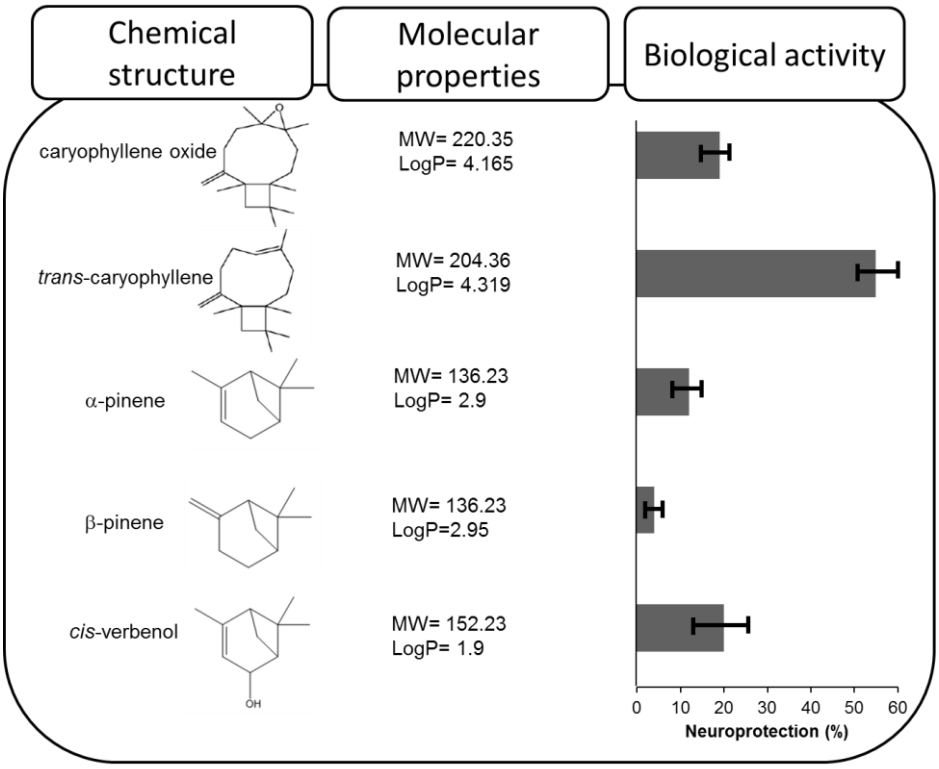


Figure 1.21. Chemical structure, molecular properties and biological activity for different isoprenoids (adapted from (255). MW-molecular weight, LogP partition coefficient of octanol/water.

Compounds biological activity (BA) is dependent on their chemical characteristics C, and also to their molecular descriptors (numerical values encoding features of the structure) (M) that can be written as the Equation 2.

$$BA = f(C) = f(M) \quad (2)$$

This equation is considered the first general application of the quantitative structure activity relationship (QSAR) (267). QSAR approaches can be defined as mathematical models that correlate the physicochemical parameters or structural descriptors of a series of analogues compounds with their observed biological activity (268). If the correlation

between the molecular descriptor and the activity is direct a linear regression equation can be obtained. Nevertheless, if more than one descriptor is necessary to explain the activity, a multilinear regression will be more suitable. These models should be able to explain, with a certain degree of confidence, the complex relationships between the independent (molecular descriptors that codifies the structural and physico-chemical aspects of interest) and dependent (biological activity of a series of compounds with similar structure) variables under study. These linear, multilinear or non-linear relationships may reveal an important role in the mechanism of action of each compound. Another feature of QSAR models is their predictive power, once the molecular properties that influence the biological activity are established, the foreknowledge of this property for each compound can be used to predict its activity (268). In addition, QSAR models can also be used in the design of molecules based on the descriptors with high influence on the activity (269).

1.4.3.2. 3D descriptors in quantitative structure activity relationship

QSAR using three dimensional (3D) descriptors is a recent method that consider the 3D characteristics of a molecule as a whole instead of considering individual substituents or moieties. The most important molecular features in 3D QSAR are its overall size and shape, and its electronic properties (270). After obtaining the biological activity for a series of compounds, the QSAR model can be built following the steps showed in Figure 1.22.

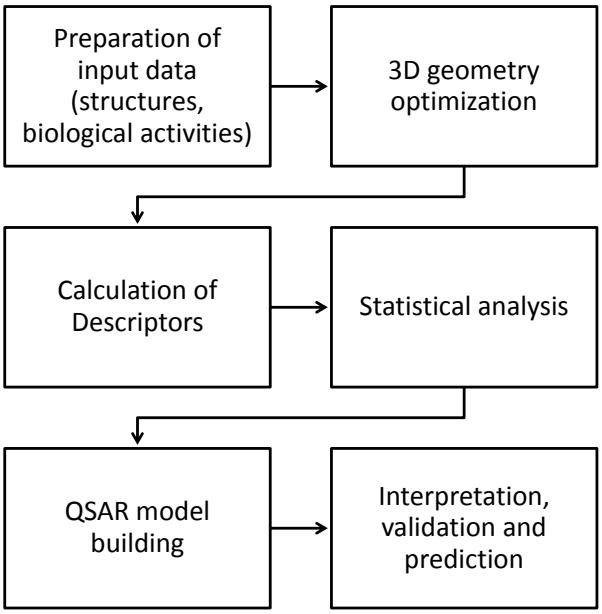


Figure 1.22. Steps involved in the development of a QSAR model.

The structure drawing and molecular modeling can be obtained using different programs, however prior descriptors generation the 3D structure must be optimized to their lowest energy. To calculate the molecule physicochemical and structural descriptors, 3D structures are inserted into specific programs such as ADAPT, ADMET Predictor, ADRIANA.Cod, ALMOND, CODESSA, DRAGON, GRID, JOELib, MOE, MOLCONN -Z, MOLGEN-QSPR, PowerMV, PreADMET, Sarchitect, Tsar, ChemSketch, HyperChem, among others. Different molecular descriptors can be calculated, which may be constitutional, molecular, physicochemical, topological, geometric, and others, which can result in over 1600 descriptors.

A high number of descriptors is usually generated, thus it is necessary to select the variables in order to identify highly correlated descriptors that can reduce the quality of the QSAR predicting model (271). In this sense, descriptors with constant or almost constant values are eliminated, followed by high correlated descriptors among the same group ($r \geq 0.7$). Descriptors that show low correlation with the activity are also eliminated (271). The elimination criteria used for the descriptors selection can vary, since the analyst can determine the correlation limits that will be used among the descriptors and descriptors-activity as well (255, 272-274). When the systematic search, or a combination of the different descriptors for choosing the best model is not feasible, one option is to use the

strategy of selecting descriptors by using Genetic Algorithms (GA) (275).

Different mathematical approaches can be used in order to obtain the models such as multilinear regression, projections methods as principal component regression (PCR) and partial least square (PLS), and non-linear methods based on artificial neural network. Nevertheless, the final model should contain a limit number of descriptors (271). In the case of QSAR models created by partial least squares regression (PLS), the descriptors pre-treatment is not required since this type of regression is not sensitive to variables with a high degree of inter-correlation (276). However, according to literature, the same criteria for selecting the descriptors discussed above may be adopted (277).

The final step is the model validation that can be performed by internal and external methods. Internal validation can be carried out in two different ways: i) model capacity to adjust the data, explained by the variance, using unilinear regression (Pierson correlation, r) or the coefficient of multiple determination (R^2) with the studied biological activity, the standard deviation of adjustment or calibration and the significance expressed by the Fisher test; and ii) the quality of the internal prediction, or the predicted variance, determined by cross-validation, in this case one of the compounds is removed from the "data set" and all other compounds serve as "training set" for building the model, which is then used to predict the activity of the compound removed. This procedure is repeated for each compound in the data set, until obtaining the individual prediction of all compounds. The predictive power of the model is evaluated by the correlation coefficient (r^2) and the coefficient of prediction (q^2) (275). External validation is used to predict the activity for a set of known compounds that were not used in the model construction from which the activity of interest were also evaluated using the same protocol.

Different QSAR studies involving sesquiterpenic compounds and biological properties have already been reported (255, 277-283). Nevertheless, biological data concerning the hepatoprotection activity of sesquiterpenic compounds is scarce. Only, Paukku et al. (278) reported the hepatoprotection activity of 22 sesquiterpene lactones using a 2D model. No data was available regarding 3D models. Results obtained by these authors (278) indicating that steric effects caused by functional groups, the higher lipophilic and higher electronic distribution modulates the hepatoprotection effect.

Chapter 2 - Rapid tool for assessment of C_{13} norisoprenoids in wines

Rapid tool for assessment of C_{13} norisoprenoids in wines

Summary

In this Chapter, two novel methodologies for quantification of C_{13} norisoprenoids in wines were developed. The first methodology, method A (reference method) was based on the headspace solid-phase microextraction combined with gas chromatography–quadrupole mass spectrometry operating in selected ion monitoring mode (HS-SPME/GC–qMS–SIM). This methodology allowed selecting the GC conditions for an adequate chromatographic resolution of wine components. The second methodology, method B (rapid method) was based on the HS-SPME/GC–qMS–SIM, using GC conditions that allowed obtaining a C_{13} norisoprenoid volatile signature. In the later, the GC capillary column of 30 m at 220 ºC was used acting as a transfer line of the components sorbed by the SPME fiber coating to the mass spectrometer, which acts as a sensor for *m/z* fragments 142 and 192. It does not require any pre-treatment of the sample, and the C_{13} norisoprenoid composition of the wine was evaluated based on the chromatographic profile and specific *m/z* fragments, without complete chromatographic separation of its components. For quantification purposes, external calibration curves were constructed with β-ionone chemical standard. Calibration curves with regression coefficient (r^2) of 0.9940 and 0.9968, RSD of 1.08 % and 12.51 %, and detection limits of 1.10 and 1.57 μg L^{-1} were obtained for methods A and B, respectively. These methodologies were applied to seventeen white and red table wines. Two vitispirane isomers (158-1529 μg L^{-1}) and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (6.42-39.45 μg L^{-1}) were quantified. The data obtained for vitispirane isomers and TDN using the two methods were highly correlated (r^2 of 0.9756 and 0.9630, respectively). Considering the fast and robust character of the proposed method B, its application can be extended for new analytes if specific *m/z* fragments would be established.

2.1. Aim of the study

The present Chapter aims to develop a novel rapid tool to characterize the C_{13} norisoprenoids of *Vitis vinifera* L. by HS-SPME–GC–qMS. In order to increase selectivity and sensitivity, the MS is operated in the selected ion monitoring mode (SIM) using the *m/z* fragments characteristics of C_{13} norisoprenoids. To test the applicability of this methodology, seventeen white and red table wines were studied. Further, the results obtained were compared to those obtained by a conventional GC–qMS methodology.

2.2. Experimental

2.2.1. Wine samples

A total of seventeen table wines were used. Four monovarietal white wines from Fernão-Pires variety produced in two Appellations (Bairrada and Península de Setúbal), from two harvests (2002 and 2003): Fernão-Pires 2002 (FP1) and Fernão-Pires 2003 (FP2) from Bairrada Appellation (from the same producer), and Fernão-Pires 2003 (FP3 and FP4) from Península de Setúbal Appellation (from different producers). Thirteen monovarietal red wines from three varieties were analysed: Touriga Nacional, Alfrocheiro, and Baga. Touriga Nacional (TN1) and Alfrocheiro (ALF1 and ALF2) produced in Dão Appellation from 2005 harvest. Several Baga wines produced in Bairrada Appellation from 2004 harvest were used: BG1-BG10 (from different producers and winemaking processes). The morphological aspect of grapes branch and leafs of the four varieties under study is presented in Figure 2.1.

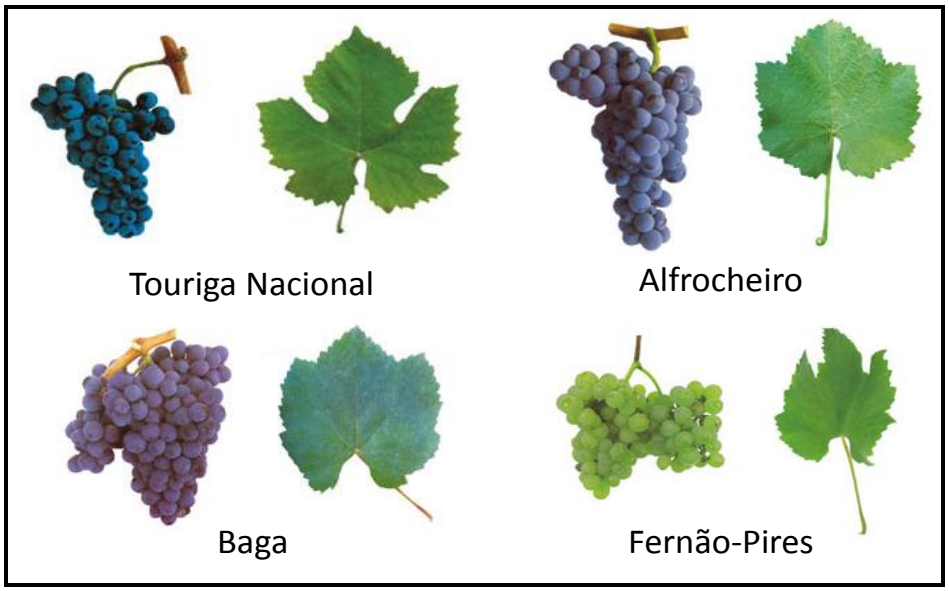


Figure 2.1. Morphological aspect of grapes branch and leafs of Touriga Nacional, Alfrocheiro, Baga and Fernão-Pires varieties (284).

2.2.2. Reagents and standards

In the present study β-ionone (ca. 97% GC, supplied from Sigma–Aldrich Química S.A. (Madrid, Spain) was prepared in ethanol (purity ≥99.8%, Riedel-de Haën, Seelze, Germany). The SPME holder for manual sampling and fiber coating used in the analyses were purchased from Supelco (Aldrich, Bellefonte, PA). The SPME device included a fused silica fiber partially cross-linked with 65 μm carbowax-divinylbenzene (CW-DVB). According to the producer's recommendations, CW-DVB is an adsorbent fiber coating, containing macro (>500 Å), meso (20-500 Å), and microporous (2-20 Å), and seems to be adequate for the analysis of complex matrices such as wine. Furthermore, adsorbent fiber coatings are good for trace level extractions. The mutually synergistic effect of adsorption and absorption of the stationary phase promotes a high retention capacity and, consequently, a higher sensitivity than fiber coatings based on absorption only. The SPME fiber coating was conditioned at 250 ºC for 30 min in the GC injector, according to the manufacturer's recommendations.

2.2.3. General conditions for C_{13} norisoprenoids extraction by SPME

The SPME experimental parameters previously developed by Rocha *et al.* (80) were used. Aliquots of 20 mL of wine were placed in a 60 mL glass vial containing 2.0 g of NaCl and a stirring bar (2.0 cm × 0.5 cm) at 200 rpm. A PTFE septum and an aluminium cap (Chromacol Ltd., Herts, UK) sealed hermetically the vial, which was placed in a thermostated bath adjusted to 40.0 ± 0.1 ºC. The fiber coating was manually inserted into the headspace of the vial immediately after sealing and remained inside the vial for a period of 10 min. Since headspace volume can be a critical factor determining the precision of the results in three-phase systems (liquid, headspace and fiber coating), vials from the same producer and lot were used. Blanks, corresponding to the analysis of the fiber coating not submitted to any extraction procedure, were run between sets of three analyses. All measurements were made with, at least, three replicates, being each replicate the analysis of one different aliquot of standard solution or wine.

2.2.4. GC–MS analysis

2.2.4.1. General conditions

An Agilent Technologies 6890N Network gas chromatograph, equipped with a 30 m × 0.32 mm I.D., 0.25 μm film thickness DBFFAP fused silica capillary column (J&W Scientific, Folsom, CA, USA), connected to an Agilent 5973 quadrupole mass selective detector was used. After the extraction/concentration step, the SPME fiber coating was manually introduced into the GC injection port at 250 ºC and kept for 1 min for desorption. Splitless injections were used (1 min). The transfer line was heated at 250 ºC. The carrier gas (He) had a flow of 1.7 mL min^{-1} and the column head pressure was 12 psi. Data recording and instrument control were performed by the MSD ChemStation software (G1701DA; version D.00.00.38; Hewlet-Packard, Agilent Technologies, Santa Clara, CA, USA).

2.2.4.2. Conditions for screening of C_{13} norisoprenoids in wines using method A

In order to identify and quantify C_{13} norisoprenoids, all the wines were analysed according to the general SPME and GC–MS conditions described in Sections 2.2.3 and 2.2.4.1. GC oven was programmed from 35 (3 min) to 65 ºC at 2 ºC min^{-1}, followed by 65-90 ºC at 1 ºC min^{-1}, 90 (3 min) to 150 ºC at 2 ºC min^{-1}, and finally 150-220 ºC at 10 ºC min^{-1}.

For identification purposes, MS was operated in full-scan acquisition mode, with electron impact at 70 eV and scanning the range *m/z* 30–300 in a 3 s cycle. The mass spectra were compared with the Wiley (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA) and Nist (Nist Mass Spectral Search Program; version 2.0f, Nist Data Center, Gaithersburg, MD, USA) libraries reference spectral bank, and other published data (285, 286).

For quantification purposes, MS was operated in SIM mode with 3 s cycle, using the mass fragments *m/z* 142 and 192. Previous full-scan analysis of wines has shown that no significant signals associated to these fragments were detected for other compounds beyond the C_{13} norisoprenoids. In order to improve signal sensitivity, three MS parameters were optimised: electron impact energy (20, 50, or 70 eV), ion focus (60 or 90 eV), and repeller (22 or 30 eV).

2.2.4.3. C_{13} norisoprenoid volatile signature (method B)

The GC oven temperature was set at 220 ºC for a period of 3 min. The mass spectrometer was operated in SIM mode, using the parameters described in Section 2.4.2 for quantification purposes.

2.2.5. Analytical plots

For quantification purposes, calibration curves were performed for β-ionone, over the concentration range 0.87-6000 μgL^{-1} according to methods A and B previously described (Sections 2.2.4.2 and 2.2.4.3, including electron impact energy of 20 eV, ion focus 60 eV, and repeller of 30 eV). The analytical plots were made in a wine model solution (20 mL) with 10 % ethanol, 0.5 % tartaric acid adjusted to pH 3.0 with NaOH, fortified with a β-ionone standard. A minimum of eight concentration levels was used to build the analytical curves (see Table 2.1 for concentration range). All the experiments were performed at least in triplicate. The calibration curves were constructed with the β-ionone standard using the *m/z* fragments 142 and 192. The vitispirane isomers were quantified directly from the calibration curves; β-ionone and vitispirane isomers present the same base peak. As this was not observed between β-ionone and TDN, correction was done for the data of TDN to maintain the same abundance ratio. The base peak of TDN is the *m/z* fragment 157. This fragment cannot be used since it is in common with the majority of superior aldehydes, thus interference effects may be expected when the rapid method B is used.

2.2.6. C_{13} norisoprenoids determination in wines

Seventeen white and red table wines were analysed according to both methodologies previously described, using electron impact energy of 20 eV, ion focus 60 eV, and repeller of 30 eV. All experiments were performed at least in triplicate. The reproducibility was expressed as relative standard deviation (RSD) in the tables.

2.3. Results and discussion

2.3.1. Screening analysis of C_{13} norisoprenoids in wines

The C_{13} norisoprenoids present in the headspace of white and red wines were identified using full-scan mode. Three compounds, two vitispirane isomers and TDN (Figure 2.2) were identified in the wines under analysis as shown in the chromatogram obtained for FP white wine (Figure 2.3). To increase specificity and sensitivity, several strategies may be improved, such as ion extracted chromatography (31) or SIM (93), which allow the analysis of volatile components by combining the spectral evidence with the selected ion and retention time, thus minimizing the co-elution and increasing peak area.

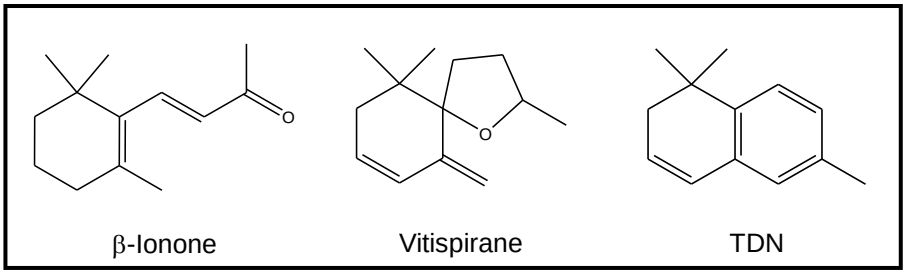


Figure 2.2. Chemical structure of C_{13} norisoprenoids. β-ionone was used as standard, and vitispirane and TDN were detected in wines.

By using the SIM mode, the lack of chromatographic resolution can be definitely overcome by the remarkable spectrometric selectivity (93). SIM mode is always performed by selecting characteristic target ions from the analytes under study. In Figure 2.3 the SIM chromatogram obtained for FP wine headspace using the *m/z* 142 and 192 confirmed the successful applicability of this approach.

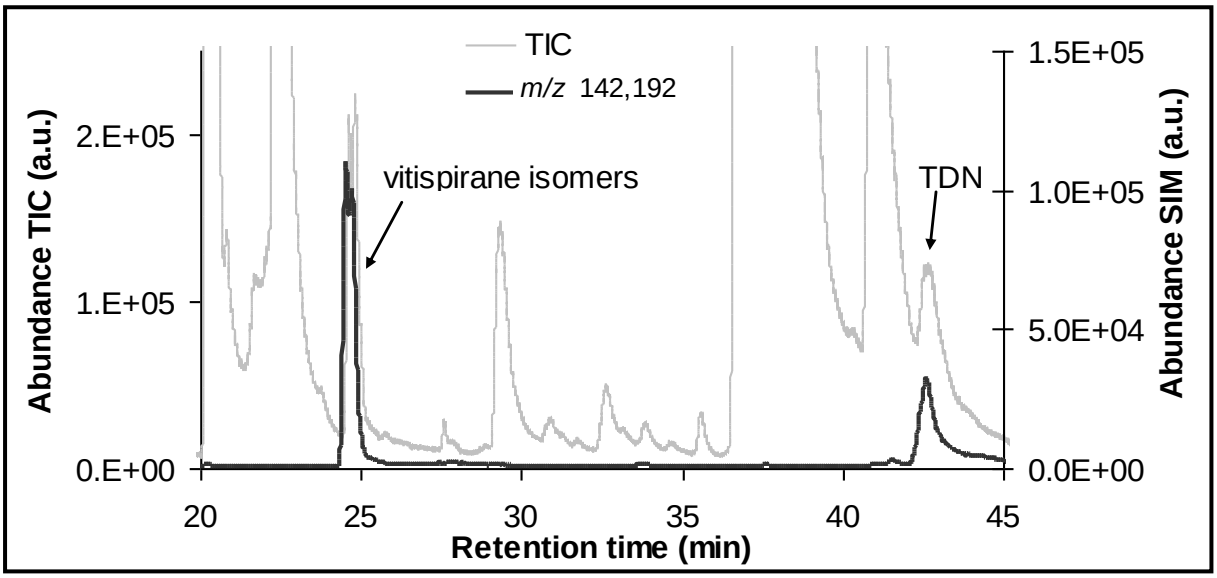


Figure 2.3. Total ion current (TIC) and selected ion monitoring (SIM, obtained at *m/z* 142 and 192) chromatograms of FP wine. (a.u. arbitrary units).

However, no separation was achieved for the two vitispirane isomers as their retention time is very close, as previously reported for these isomers (286, 287). As a consequence, the quantification of vitispirane isomers was established as the total concentration of vitispiranes in wines.

2.3.1.1. Optimisation of mass spectrometry parameters

In order to increase signal (area) for the C_{13} norisoprenoids detected during wine screening, the β-ionone standard was analysed under different electron impact energy as well as conditions of analyses on the ionization chamber (ion focus and repeller). SIM chromatograms of β-ionone were acquired at different mass spectrometer parameters: electronic impact (20, 50, and 70 eV), ion focus (60 and 90 eV), and repeller (22 and 30 eV) (Figure 2.4). According to the manual of the MS equipment, decreasing the ion focus voltage an improvement in sensitivity at higher masses can be noted. The similar effect is expected when using higher repeller voltage values. The lower energy produces larger fragments. Thus an increase in the signal is expected if an adequate set of values of these three parameters is obtained.

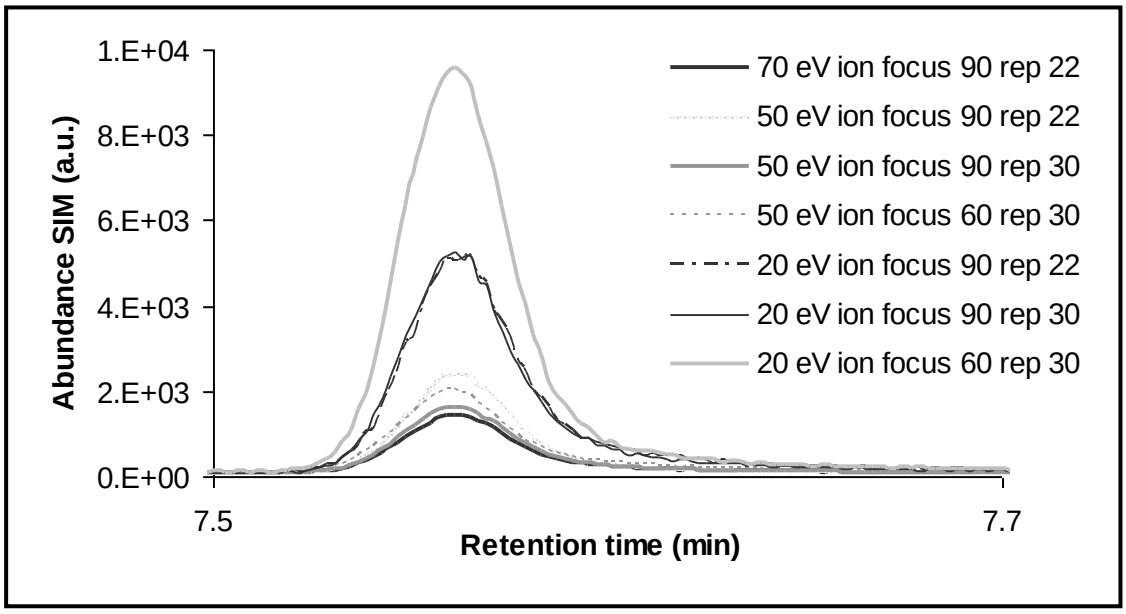


Figure 2.4. Selected ion monitoring (SIM) chromatograms (*m/z* 142 and 192) of β-ionone acquired at different mass spectrometer parameters: electronic impact (20, 50, and 70 eV), ion focus (60 and 90 eV), and repeller (22 and 30 eV). (a.u. arbitrary units)

Results of Figure 2.4 show that an electron impact energy of 20 eV, with an ion focus of 60 eV and repeller of 30 eV increased the signal 7 times when comparing with the standard conditions usually used for the analysis of volatiles (electron impact energy 70 eV, ion focus 90 eV and repeller 22 eV). Thus, these conditions showing the highest chromatographic area were chosen.

2.3.2. C_{13} norisoprenoid volatile signature

The C_{13} norisoprenoids volatile signature for the headspace of white and red wines was obtained using the SIM mode (*m/z* 142 and 192) and optimised MS parameters (see discussion of Section 2.3.1). Figure 2.5 shows C_{13} norisoprenoids chromatographic profiles for white (FP) and red wines (BG, TN, and ALF). Two resolved peaks were observed at 0.89 min for the vitispirane isomers and at 1.0 min for the TDN, indicating that it is possible to quantify separately these compounds (total of vitispirane isomers and TDN). Furthermore, Figure 2.5 exhibits four different volatile signatures (chromatographic profiles), corresponding to four different monovarietal wines, which indicate that these

wines present different C_{13} composition.

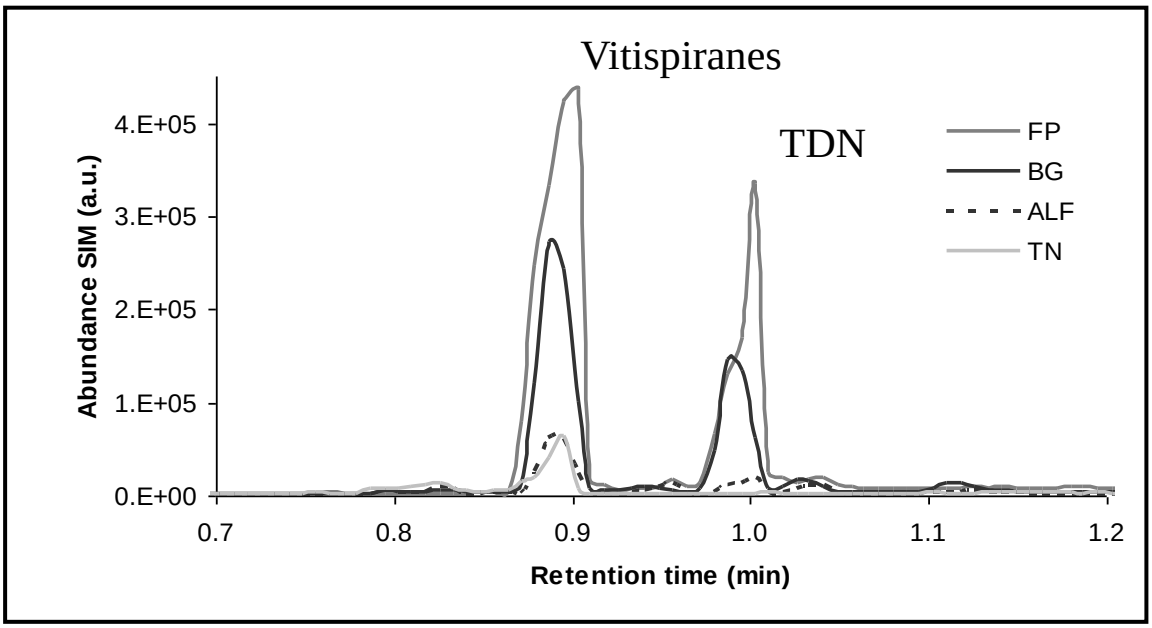


Figure 2.5. C_{13} norisoprenoid chromatographic profile (volatile signature) of wines headspace, FP, BG, TN and ALF, obtained by HS-SPME–GC–qMS-SIM (*m/z* 142 and 192), for 10 min of extraction. (a.u. arbitrary units).

Due to the use of a manual injection mode, small deviations can be detected in the retention time (up to 0.03 min) of the chromatographic fingerprint peaks from different analyses. This deviation is even accepted for the regular resolved chromatographic methods, such as the method A. Deviation in retention times higher than 0.03 min was not observed.

2.3.3. Calibration curves

For quantification purposes, external calibration curves were constructed with the β-ionone standard using the *m/z* fragments 142 and 192 (*m/z* 192 corresponds to the base peak and *m/z* 142 represents 3 % of it). These specific fragments were used for quantification of vitispirane isomers and TDN. The SIM-mass spectrum of vitispirane isomers only exhibited the *m/z* fragment 192, also corresponding to the base peak for β-ionone. However, this *m/z* is not present in the TDN SIM-mass spectrum (is higher than

TDN molecular weight). In this case, the *m/z* fragment 142 was used as a qualifier ion common between TND and β-ionone, and a correction was done to maintain the same abundance ratio (31). It is important to point out that excellent signal-to-noise ratio was observed for the individual ions selected. β-Ionone was tested in eight levels of concentration and in triplicate, calculating the regressions lines for both methods, as shown in Table 2.1.

Table 2.1 - Calibration parameters for β-ionone in headspace of wine model solutions analysed by two methods (A- HS-SPME followed by a GC-qMS-SIM analysis, using GC conditions that allowed an adequate chromatographic resolution of wine components, and B- HS-SPME–GC–qMS-SIM, using GC conditions that allowed to obtain a C_{13} norisoprenoid volatile signature - rapid tool): selected ions at *m/z* 142 and 192, linear concentration range 0.87-6000 μg L^{-1}, slope, interception, *P*-value of lack-of-fit test (LOF), correlation coefficients (r^2), limit of detection (LOD), limit of quantification (LOQ), and relative standard deviation (RSD).

Method	Slope	Interception	LOF *P*-value	Correlation coefficient (r^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	R.S.D.(%)
A	2.84E+04	-7.83E+03	0.881	0.9940	1.10	3.67	1.08
B	2.99E+03	9.37E+03	0.849	0.9968	1.46	4.87	12.51

This two analytical methods were developed for quantitative analysis of vitispirane isomers and TDN in wines: method A – HS-SPME followed by a GC–qMS-SIM analysis, using GC conditions that allowed an adequate chromatographic resolution of wine components, and method B (rapid method) – HS-SPME–GC–qMS-SIM, using GC conditions that allowed to obtain a C_{13} norisoprenoid volatile signature – rapid tool. Both methods were validated with respect to linearity, sensitivity (limits of detection and quantification), and reproducibility (Table 2.1). Good regression coefficients (r^2) were obtained in both cases 0.9940 for method A and 0.9968 for method B. The limits of detection or the minimum amount of each compound that can be reliably distinguished were achieved as the amount of each compound to provide a signal-to-noise ratio of 3 above the variability; the limits of quantification (LOQ) were achieved as the amount of each compound to provide a signal-to noise ratio of 10 above the variability. The method A had LOD of 1.10 μg L^{-1} and LOQ of 3.67 μg L^{-1} and the method B had 1.46 and

4.87 μg L^{-1}, respectively, as shown in Table 2.1. The lack-of-fit test performed for the analytical plots showed for both curves no lack of linear fit for $\alpha = 0.05$, as all *P*-values, shown in Table 2.1, were higher than 0.05. The reproducibility (RSD) was calculated by performing three consecutive extractions to the lower concentration of each volatile compound, giving the results of 1.08 % and 12.51 %, respectively. Although, a higher RSD value was observed for method B, this result was considered very good because an acquisition time of 3 min (plus 10 min of HS-SPME as in the method A) instead of 80 min allowed the identification and quantification of vitispirane isomers and TDN in wines.

2.3.4. C_{13} norisoprenoids determination in wines

Both methods were applied to seventeen white and red table wines from several varieties, harvests, Appellations, and producers. The results obtained are shown in Table 2.2. As can be seen, the values obtained for vitispirane isomers and TDN by the method A were closely related to those obtained for the method B. The high correlation values observed between the two methods were confirmed with an r^2 of 0.9756 for vitispirane isomers and r^2 of 0.9630 for TDN, as can be seen in Figure 2.6.

A slope near 1 is also observed for both compounds, although for vitispirane isomers, possibly due to the higher concentrations obtained, it was close to the unity. The statistics of the lack-of-fit also confirmed that both method regressions fits well as *P*-value observed for vitispiranes and TDN were higher than 0.05 (0.185 for vitispirane isomers and 0.135 for TDN). Considering methods A and B, the concentrations of vitispirane isomers obtained for white and red wines ranged from 158.43 to 1529 μg L^{-1} and for TDN from 12.13 to 39.45 μg L^{-1} (when quantified, some samples exhibited values <LOD). The FP wines analysed by method A, exhibit concentrations of vitispirane isomers varying from 826 to 1268 μg L^{-1} and TDN concentrations from 18.56 to 39.45 μg L^{-1}.

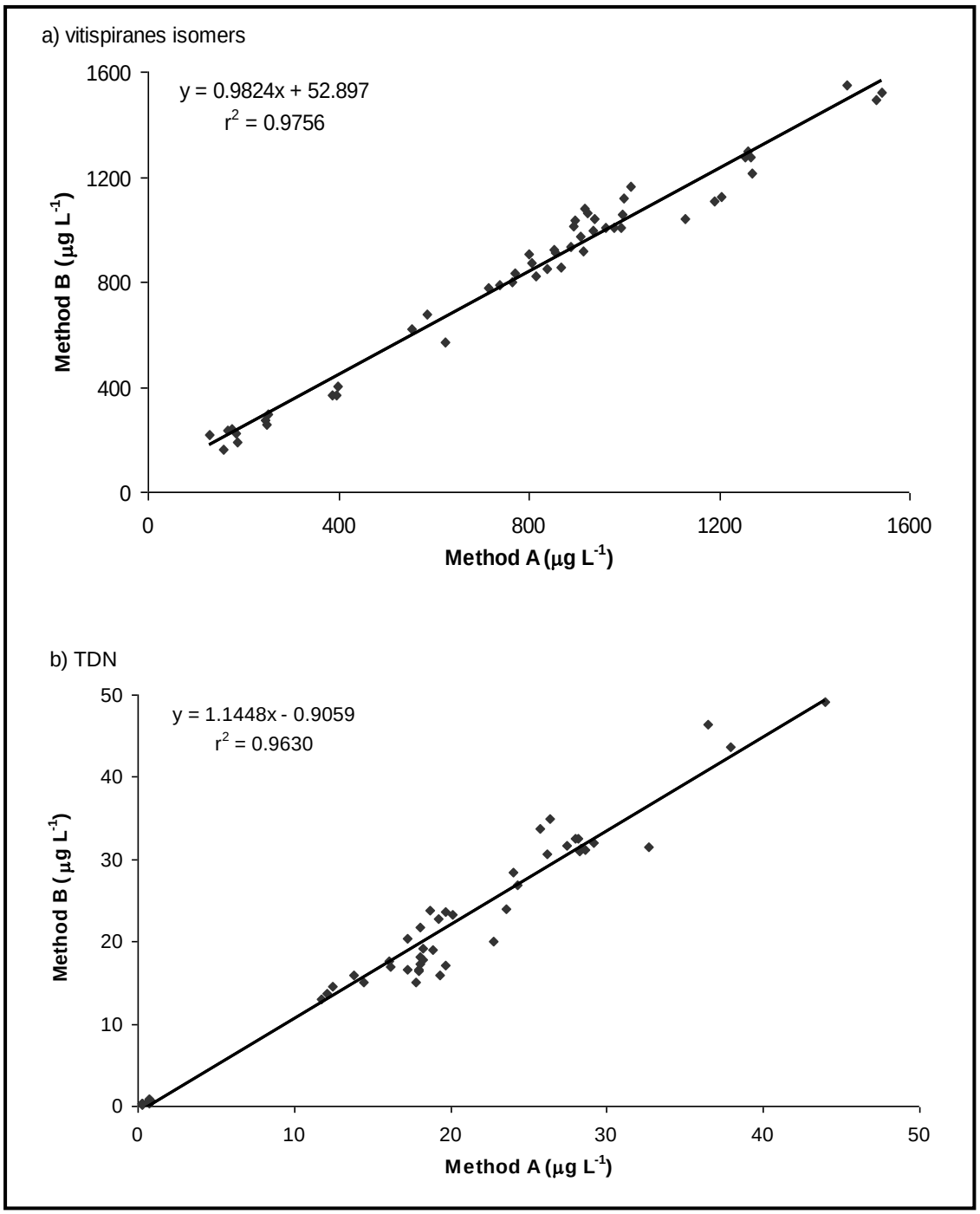


Figure 2.6. Quantification correlation for vitispirane isomers (a) and TDN (b) obtained for white and red wines headspace by the Method A and Method B (rapid tool).

Table 2.2- Concentration of C_{13} norisoprenoids in headspace of white and red wines obtained by two methods: **A**- HS-SPME followed by a GC-qMS-SIM analysis, using GC conditions that allowed an adequate chromatographic resolution of wine components, and **B**- HS-SPME–GC–qMS-SIM, using GC conditions that allowed to obtain a C_{13} norisoprenoid volatile signature - rapid tool.

Wine samples	Concentration μg L^{-1} (n=3)			
	Vitispirane isomers		TDN	
	Method A	Method B	Method A	Method B
White wines				
FP1	859.18 (3)[a]	902 (5)	26.95 (4)	32.31 (7)
FP2	1191.15 (5)	1142 (10)	26.47 (8)	30.89 (7)
FP3	1264.82 (4)	1273 (4)	39.45 (10)	46.40 (6)
FP4	935.75 (3)	992 (17)	18.56 (7)	22.62 (8)
Red wines				
TN1	175.73 (5)	232.61 (4)	<LOD	<LOQ
ALF1	158.43 (18)	190.53 (15)	<LOD	<LOD
ALF2	249.18 (1)	276.00 (8)	<LOD	<LOD
BG1	394.18 (2)	381.83 (10)	12.13 (6)	13.71 (7)
BG2	924.38 (3)	1065.30 (6)	19.13 (15)	22.67 (7)
BG3	1529.12 (4)	1521.46 (2)	30.01 (7)	31.46 (2)
BG4	856.83 (7)	911.40 (0)	17.99 (3)	16.46 (8)
BG5	929.39 (6)	1036.20 (2)	23.52 (6)	23.67 (21)
BG6	765.00 (7)	801.01 (3)	17.56 (21)	16.47 (3)
BG7	772.11 (4)	831.62 (5)	16.03 (2)	17.57 (9)
BG8	1028.71 (16)	1048.07 (16)	17.65 (2)	17.07 (12)
BG9	951.83 (2)	1080.28 (4)	27.53 (16)	32.50 (4)
BG10	587.33 (6)	623.03 (9)	18.03 (1)	17.31 (4)

[a] Relative standard deviation

Comparing with the amounts obtained by method B, similar results were observed. FP2, for instance, showed vitispirane isomers concentrations of 1191 μg L^{-1}, quantified by method A and 1142 μg L^{-1} by method B. Moreover, concentrations of TDN found for this wine was 26.47 μg L^{-1} by method A and 30.89 μg L^{-1} by method B. The same behaviour was observed for red wines when comparing both methods. For ALF1, vitispirane isomers concentration accounted for 158 μg L^{-1} using method A and 191 μg L^{-1} with method B. For ALF2, vitispirane isomers concentrations accounted for 249 and 276 μg L^{-1} using methods

A and B, respectively. The amount of TDN was under the LOD and LOQ for both methods.

The BG wines, produced with different treatments, showed higher vitispiranes and TDN concentrations than the other red varieties. BG3 showed the highest concentration of vitispiranes. Amounts of vitispirane isomers obtained for this wine were 1529 and 1521 μg L^{-1} using methods A and B, respectively. TDN accounted for 30.01 μg L^{-1} by method A and 31.46 μg L^{-1} by method B.

Vitispirane isomers possess odour descriptors of camphor, eucalyptus (112), woody, and spicy (286), and an odour threshold (OT) of 800 μg L^{-1} (79, 112). Due to the difficulty to separate the two isomers, the OT value is reported for the mixture. The total of vitispirane isomers was above its OT in wines FP1, FP2, FP3, FP4, BG2, BG3, BG4, BG5, BG8, and BG9, which suggests its individual contribution to the wine aroma. TDN was described as presenting kerosene-like (23, 112), truffle, and flowery odour notes (121), with an OT of 20 μg L^{-1} (23). The TDN was found in FP1, FP2, FP3, FP4, BG2, BG3, BG5, and BG9 wines in concentrations above its OT which suggests its individual contribution to the wine aroma.

2.4. Concluding remarks

Two approaches were developed to study the C_{13} norisoprenoids in wines: method A, used as reference, and method B, which represents a rapid tool. The validation were done using β-ionone chemical standard. On the basis of this study, it is concluded that both methodologies showed good linearity over the concentration range tested, with correlations coefficients of 0.9940 and 0.9968, respectively. A good reproducibility was also attained (1.08-12.51 %) and, additionally, method A had LOD of 1.10 μg $L^{-1}$and LOQ of 3.67 μg L^{-1} and method B had 1.46 and 4.87 μg L^{-1}, respectively. The proposed methodologies were tested in seventeen white and red table wines, where two vitispirane isomers and TDN were quantified. As the detection limits are above their odour threshold, it is possible to evaluate the expected contributions of these compounds to the wine aroma properties.

Concentrations ranging from 158 to 1529 μg L^{-1} were found for vitispirane isomers and from 12.13 to 39.45 μg L^{-1} for TDN (when quantified, some samples exhibited values <LOD). The data obtained for vitispirane isomers and TDN in wines using the methods A and B were highly correlated (r^2 of 0.9756 and 0.9630, respectively). It is important to point out that method B allows to determine in 13 min (10 min SPME + 3 min instrumental analysis) the vitispirane isomers and TDN in the headspace of white and red wines, instead of using the time consuming methods. Furthermore, the proposed methodology does not require any pre-treatment of the sample, and allows extracting specific information about chemical composition, which was done without complete chromatographic separation of wine components. This was done by selecting adequate MS operating parameters and *m/z* fragments.

The properties of method B, in terms of simplicity of use, sensitivity, and precision, make it suitable for quantitative analysis of the C_{13} norisoprenoids in wines. This study was focused on the C_{13} norisoprenoids detected in wines under study, but this methodology can be extended to other C_{13} norisoprenoids if specific *m/z* fragments are established for the new analytes. All these characteristics are in accordance with the actual demands of the laboratories of the industries as well as of the research centres, where the development and/or implementation of rapid and robust methodologies represent an area in extensive growth.

Chapter 3- Rapid varietal profile assessment: Fernão-Pires monovarietal wines a case study

Rapid varietal profile assessment: Fernão-Pires monovarietal wines a case study

Summary

This Chapter aims the development of a rapid methodology for the establishment of the varietal volatile profile of *Vitis vinifera* L. cv. 'Fernão-Pires' (FP) white wines by headspace solid-phase microextraction combined with comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (HS-SPME/GC×GC-TOFMS). The study was focused on the volatiles that seem to be significant to the varietal character, such as mono and sesquiterpenic compounds, and C_{13} norisoprenoids. Monovarietal wines from different harvests, Appellations, and producers were analysed. Two-dimensional chromatographic spaces containing the varietal compounds using the *m/z* fragments 93, 121, 161, 175 and 204 were established as follows: ^1t_R = 255-575 s, ^2t_R = 0.424-1.840 s, for monoterpenoids, ^1t_R = 555-685 s, ^2t_R = 0.528-0.856 s, for C_{13} norisoprenoids, and ^1t_R = 695-950 s, ^2t_R = 0.520-0.960 s, for sesquiterpenic compounds. For the three chemical groups under study, from a total of 170 compounds, 45 compounds were determined in all wines, allowing defining the "varietal volatile profile" of FP wine. Among these compounds, 30 have been already reported for FP variety, and 15 were detected for the first time. This study proposes a HS-SPME/GC×GC-TOFMS based methodology combined with classification-reference sample to be used for rapid assessment of varietal volatile profile of wines. This approach is very useful to eliminate the majority of the non-terpenic and non-C_{13} norisoprenic compounds, i) allowing the definition of a two-dimensional chromatographic space containing these compounds, ii) simplifying the data obtained, and iii) reducing the time of analysis. This approach can be extended to the analysis of other related food matrices, including grapes and musts, and can be applied as a powerful tool to trace their varietal origin.

3.1. Aim of the study

Considering the potentiality of GC×GC in wine analysis, the main objective of this study is the rapid establishment of the varietal volatile profile of wines based on mono and sesquiterpenic compounds, and C_{13} norisoprenoid components in *Vitis vinifera* L.. Monovarietal wines from FP variety (Figure 2.1, Chapter 2) were chosen due to its importance for the Portuguese wine industry. In order to obtain the varietal profile wines from different harvests, Appellations, and producers were analysed by combining the great HS-SPME extractability potential with the powerful GC×GC-TOFMS separation system and a specific data treatment available in ChromaTOF software.

3.2. Experimental

Nine monovarietal wines from 'Fernão-Pires' *Vitis vinifera* L. variety from different harvests, Appellations, and producers were used for this study: FP from Bairrada Appellation (Maria Gomes 2003 (B-03) and Quinta de Pedralvites 2002 (B-02)), from Ribatejo Appellation (Solar de Caniços 2001 (R-01), Quinta de São João Baptista 2002 (R-02-1), Prova Real 2002 (R-02-2), Prova Real 2003 (R-03-1) and Quinta de São João Baptista (R-03-2)), and from Península de Setúbal Appellation (Padre Pedro 2003 (PS-03-1 and Terras do Pó (PS-03-2)).

3.2.2. HS-SPME methodology

The SPME fiber coating and the experimental parameters were established according to a methodology previously developed in our laboratory for wine analysis (83). The SPME holder for manual sampling and the fiber coating used were purchased from Supelco (Aldrich, Bellefonte, PA, USA). The SPME device included a fused silica fiber coating partially cross-linked with 65 μm Carbowax-divinylbenzene (CW-DVB). The SPME fiber coating was conditioned at 250 ºC for 30 min in the GC injector, according to

the manufacturer's recommendations. For headspace sampling, 40 mL of wine was transferred to a 120 mL glass vial, which corresponds to a ratio of the volume of the liquid phase to the headspace volume (1/β) of 0.5. The vial was capped with a PTFE septum and an aluminium cap (Chromacol, Welwyn Garden City, UK). After the addition of 8 g of NaCl and stirring bar (25 × 5 mm) at 1000 rpm, the vial was placed in a thermostated bath adjusted to 40.0 ± 0.1 ºC and the SPME fiber coating was manually inserted into the sample vial headspace for 45 min. Each sample was analysed, at least, in triplicate. Fiber coating reproducibility, corresponding to the analysis of 50 μL of 1-octanol standard solution (8 mg mL^{-1}) submitted to a rapid extraction procedure (10 min, 40 °C), were run each day. Fiber coating was discarded if differences of 1-octanol peak area were higher than 10 %. Blanks, corresponding to the analysis of the fiber coating not submitted to any extraction procedure, were run between sets of three analyses.

3.2.3. GC×GC-TOFMS analysis

The SPME fiber coating containing the headspace volatile compounds was manually inserted into the GC×GC-TOFMS injection port at 250 ºC and kept for 3 min for desorption. The injection port was lined with a 0.75 mm I.D. splitless glass liner. Splitless injections were used (30 s). LECO Pegasus 4D (LECO, St. Joseph, MI, USA) GC×GC-TOFMS system consisted of an Agilent GC 7890 gas chromatograph with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven. The detector was a high-speed TOF mass spectrometer. An HP-5 30 m × 0.32 mm I.D., 0.25 μm film thickness (Supelco) was used as the first dimension column and a DB-FFAP 1 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco) was used as a second-dimension column. The carrier gas was helium at a constant flow rate of 2.0 mL min^{-1}. The primary oven temperature was programmed from 40 (1 min) to 230 ºC (2 min) at 10 ºC min^{-1}. The secondary oven temperature was programmed from 70 (1 min) to 250 ºC (1 min) at 10 ºC min^{-1}. The MS transfer line temperature was 250 ºC and the MS source temperature was 220 ºC. The modulation time was 5 s; the modulator temperature was kept at 30 ºC offset (above primary oven). The TOFMS system was operated at a spectrum storage rate of 125 spectra s^{-1}. The mass spectrometer was operated in the EI mode at 70 eV using a range of

m/z 33-350 and the detector voltage was 1630 V. Total ion chromatograms (TIC) were processed using the automated data processing software ChromaTOF (LECO) at S/N threshold 100. Contour plots were used to evaluate the general quality of the separation and for manual peak identification. Two commercial databases (Wiley 275 and US National Institute of Science and Technology (NIST) V. 2.0- Mainlib and Replib) and a laboratory-made database of terpenoids comprising 40 compounds were used. Mass spectral match factor, similarity > 850, was used to decide whether a peak was correctly identified or not. Furthermore, more careful attention was provided by the manual inspection of the mass spectra and/or by the use of additional data, such as the experimentally determined retention index (RI) values and the values reported in the literature for comprehensive GC×GC system with Equity-5, DB-5 and VF-5ms for the 1D column (9, 59, 94, 288, 289) and, for one dimensional GC with 5%-Phenyl-methylpolysiloxane GC column or equivalent (31, 131, 290-297) (Table 3.1). For the determination of the RI, a C_8-C_{20} n-alkanes series was used (the solvent *n*-hexane was used as C_6 standard) and calculated according to the Van den Dool and Kratz equation (298). The DTIC (Deconvoluted Total Ion Current) GC×GC area data were used as an approach to estimate the relative content of each volatile component.

3.3. Results and discussion

3.3.1. HS-SPME/GC×GC-TOFMS data analysis

In a first step of GC×GC-TOFMS analysis, automated data processing was used to find all peaks in the GC×GC chromatograms with a signal-to-noise at minimum of 100. Within the automated data processing, the software finds peaks at individual single ion traces over the whole mass range measured. Therefore, not only major sample compounds were found, but also the trace compounds hidden under TIC baseline can be detected. After peak detection, modulated peaks are automatically combined by mass spectral deconvolution, i. e. mathematical separation of spectra of co-eluted peaks is performed. The contour plot of total ion chromatogram obtained by GC×GC (data not shown)

exhibited several hundreds of peaks. In the present work, the peak table generated automatically by ChromaTOF software has been further checked and compounds identification has been confirmed or changed based on the criteria described in Section 3.2.3. Thus, for varietal peaks establishment (mono and sesquiterpenic compounds, and C_{13} norisoprenoids) a careful analysis of compounds mass spectra was done, and diagnostic ions were used to define the two-dimensional chromatographic spaces containing these varietal compounds (93, 121, 161, 175, and 204 *m/z* fragments). The following chromatographic spaces were established: ${}^{1}t_R$ = 255-575 s, ${}^{2}t_R$ = 0.424-1.840 s, for monoterpenoids, ${}^{1}t_R$ = 555-685 s, ${}^{2}t_R$ = 0.528-0.856 s, for C_{13} norisoprenoids, and ${}^{1}t_R$ = 695-950 s, ${}^{2}t_R$ = 0.520-0.960 s, for sesquiterpenic compounds.

Subsequently, a classification method was created which consists in drawing the limits of the two-dimensional chromatographic spaces for each chemical group (mono and sesquiterpenic compounds, and C_{13} norisoprenoids) in a chromatogram contour plot obtained for FP wine B-03. The FP wine B-03 was chosen because it presented the highest number of varietal compounds (113), highest number of common compounds between all samples under study, as well as the highest total chromatographic areas (Figure 3.1).

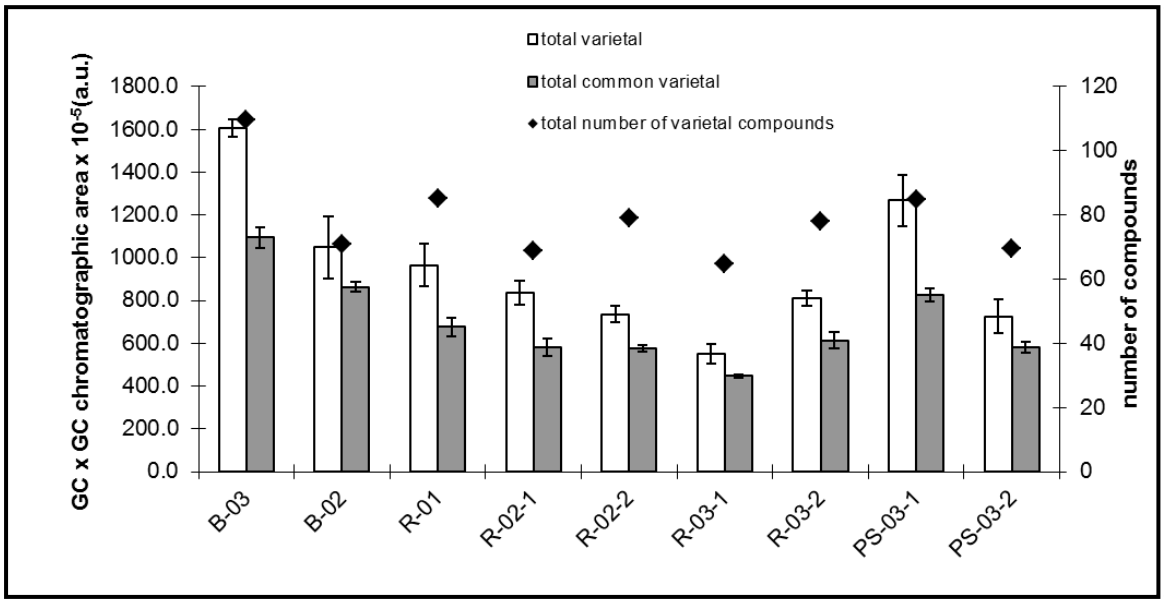


Figure 3.1. Number of varietal volatile compounds (mono and sesquiterpenic compounds, and C_{13} norisoprenoids) common to all FP wines analysed by HS-SPME/GC×GC-TOFMS, their area, and area of total varietal volatile compounds.

As a consequence, a reference peak table was built with the varietal compounds found in FP B-03 wine sample, which was then applied for the establishment of the FP varietal profile. The occurrence of non-varietal compounds in their specific two-dimensional chromatographic space was observed. To overcome this problem, the reference peak table was carefully checked and the non-terpenic and non-C_{13} norisoprenic compounds were eliminated. Therefore, comparison between the reference and the other samples were processed with the classification-reference approach.

In Figure 3.2A is displayed the total ion chromatogram (TIC) classification, containing all varietal volatile compounds classified by homologous series of monoterpenoids (orange bubbles), C_{13} norisoprenoids (green bubbles), and sesquiterpenic compounds (yellow bubbles).

The bubble plots were used as they easily enable the distinguishing of compounds classification: each colour represents one chemical group, and bubble diameter is directly related to peak area. By using this plot, the compounds from the chemical groups under study could be observed in the TIC chromatogram without the interference of other compounds. Thus, this approach was very helpful to eliminate the majority of the non-terpenic and non-C_{13} norisoprenic compounds, allowing the definition of a two-dimensional chromatographic space containing only these compounds. The establishment of two-dimensional spaces for the varietal compounds by the classification method and the use of a reference sample in the sample-to-sample comparison simplify the data obtained and reduce the time of analysis.

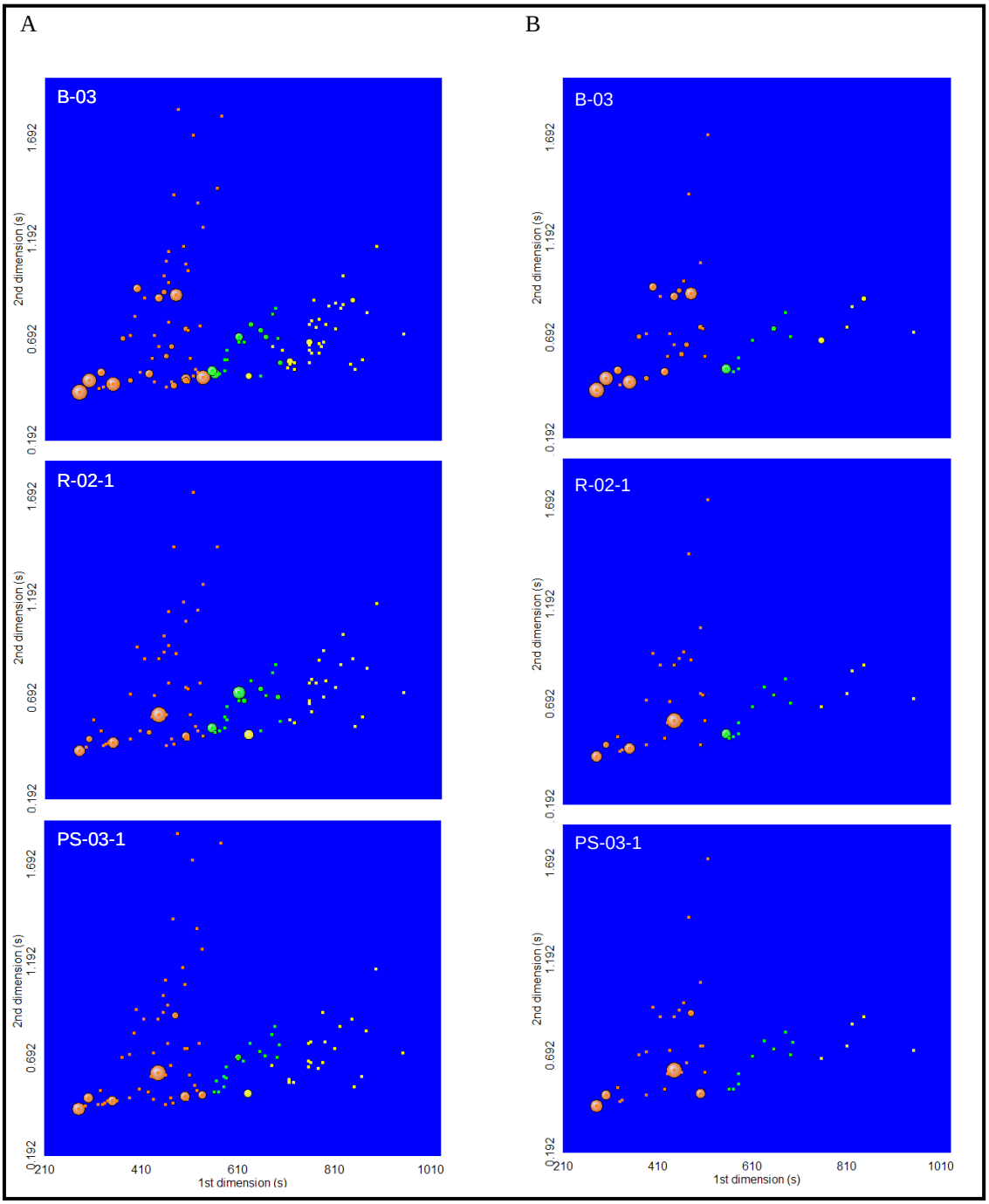


Figure 3.2. GC×GC total ion chromatogram bubble plots of sample-to-sample classification using mono (orange bubbles) and sesquiterpenic compounds (yellow bubbles), and C_{13} norisoprenoids (green bubbles) of FP wines from different harvests, Appellations and producers: B-03 (Bairrada), R-02-1 (Ribatejo) and PS-03-2 (Península de Setúbal). (A) Total varietal compounds, (B) Common varietal compounds.

3.2.2. Varietal volatile profile of FP wines

Over the years, different studies have described the aroma (volatile compounds) of the wine as one of the main factors that determine its nature and excellence. It is well established that the volatile compounds of wine can be influenced by different factors such as: environment (soil and climate); grape variety; ripeness; fermentation conditions; biological factors (yeast and other components of the oenological microflora); as well as the wine production and aging (299). However, among the diversity of volatile compounds present in wines a particular group, the varietal volatile ones namely the terpenoids, have a greater impact in wines since they have low odour thresholds and pleasant aroma descriptors. Besides, they are considered specific to a variety and are marginally influenced by the growing area, in qualitative terms (23).

In the present study FP white wines showed a total of 170 different varietal volatile compounds, being 45 of them common to all wines analysed. Among these, fifteen new compounds were identified for FP wines accounting with seven monoterpenoids: *o*-cymene, 2-bornene, ocimene quintoxide, 3,9-epoxy-δ-*p*-menthene, myrcenol, cyclogeraniol and camphor; five C_{13} norisoprenoids: 1,1,6-trimethyl-1,2,3,4-tetrahydronaphtalene (α-ionene), edulan I, hydroxydihydroedulan, geranyl acetone, 3,4-dehydro-β-ionone; and three sesquiterpenic compounds: α-calacorene, 4,5,9,10-dehydro-isolongifolene and γ-eudesmol. When comparing the results obtained in this study with the varietal compounds present in Pinotage (9, 94, 171), Cabernet Sauvignon (94, 95, 171), Brazilian Merlot (96), Pinot noir and Dornfelder wines (170) FP wines are richer in this subject.

As the 45 compounds were present in FP wines from different harvests, Appellations and producers analysed by HS-SPME/GC×GC-TOFMS, they seem to be characteristic of FP variety, therefore, we can infer that they represents the "varietal volatile profile". Figure 3.2B displays the TIC classification chromatogram, represented by bubble plots, for the common varietal volatile compounds detected in FP wines.

The FP varietal volatile profile obtained by GC×GC is shown in Table 3.1. From the 45 compounds identified 10 were confirmed with pure standards being the others

tentatively identified considering the match with NIST database, by using fit and retrofit scores of 850 and 800 respectively. In addition, the RIs, calculated according to the van den Dool and Kratz equation (298), were compared with those already reported in the literature for GC×GC systems with similar GC columns (9, 59, 94, 288, 289) or with the RIs values reported for 5 % phenyl polysilphenylene-siloxane GC column or equivalents for one-dimensional separation (31, 131, 290-297) (Table 3.1). In the first case, the maximum difference for the absolute RI ($|RI_{cal} - RI_{lit}|$) was 5-30, with the exception of *E*-2,3-epoxycarane (absolute RI of 52). Comparing the RI_{cal} and the values obtained from the literature for the one-dimensional system, the maximum difference for the absolute RI were 0-39. The exception was geranic oxide and β-damascenone (absolute RI 42 and 68, respectively), however in the last case the compound was confirmed with pure standard.

According to Table 3.1, the monoterpenic compounds were the group with higher number of compounds (29), followed by C_{13} norisoprenoids (11), and sesquiterpenic compounds (5). In FP wines the monoterpenic compounds were the major chemical group found with highest number of compounds (29) and also showed the highest total chromatographic area (Table 3.1). Different types of monoterpenic compounds were found in FP wines: 4 monoterpene hydrocarbons, 12 oxides, 8 monoterpenols, 1 monoterpendiol, 3 aldehydes, and 1 monoterpene ketone. Among monoterpenic compounds, monoterpene oxides exhibited also the highest chromatographic areas. The compounds detected were geranic oxide, herboxides isomers, 1,8-cineole, citroxide, linalool-furanic oxide isomers, rose oxide, E-2,3-epoxycarane, nerol oxides isomers and 3,9-epoxy-δ-p-menthene. Monoterpenols were the second predominant group, represented by hotrienol, myrcenol, three ocimenol isomers, cyclogeraniol, p-cymen-8-ol and α-terpineol. In addition, four monoterpenes (o-cymene, limonene, α-terpinolene, and 2-bornene), one monoterpene ketone (camphor), and three monoterpene aldehydes (p-menth-1-en-9-al isomers and β-cyclocitral) were also detected. From these 29 common monoterpenoids, 7 compounds have not been previously described in FP grapes, musts and wines (31, 59, 81, 89-91, 300, 301), namely, *o*-cymene, 2-bornene, ocimene quintoxide, 3,9-epoxy-δ-*p*-menthene, myrcenol, cyclogeraniol and camphor. All these compounds have been already found in wines (9, 94, 302), and cyclogeraniol was reported in grapes (303).

Two classes of C_{13} norisoprenoids were detected in FP wines and the oxygen-

containing compounds presented higher number of compounds and chromatographic area when compared with the C_{13} norisoprenoid hydrocarbons. Eight C_{13} norisoprenoids oxygen-containing compounds were detected in the volatile varietal profile of FP wines, while the C_{13} norisoprenoids hydrocarbons were represented by three compounds.

The vitispirane was the predominant compound representing the C_{13} norisoprenoids oxygen-containing compounds, followed by β-damascenone, geranyl acetone, hydroxyedulan, 3,4-Dehydro-β-ionone, theaspiranes A and B, and edulan I. From these compounds, theaspiranes, vitispirane and β-damascenone were previously reported in FP grapes, musts and wines (31, 80, 89-91, 300, 301). Edulan I and hydroxydihydroedulan have not been described for FP variety. These compounds were reported as product of hydrolysis of megastigma-3,6,9-triols (304) both compounds have been already described in the volatile composition of wine (9, 305) and, edulan was reported in rum (292).

Among the three C_{13} norisoprenoid hydrocarbons, TDN and its isomer were the predominant compounds while α-ionene has shown lower chromatographic area. TDN have been already described in the volatile composition of FP wines (219) while 1,1,6-trimethyl-1,2,3,4-tetrahydro-naphthalene (α-ionene) was present in Pinotage wines (95). Vitispirane and TDN were the main C_{13} norisoprenoids compounds found in FP wines, both compounds were previously quantified in FP monovarietal wines by HS-SPME/GC-qMS in selected ion monitoring mode (SIM) (219).

Sesquiterpenoids are widespread found in several plants and fruits, including the *Vitis vinifera* L., where compounds such as α-ylangene, β-bourbonene, germacrene D, γ-cadinene, and farnesol were detected in grapes (4). Five sesquiterpenic compounds were found in FP wines varietal volatile profile, three sesquiterpenes namely α-calacorene, 4,5,9,10-dehydro-isolongifolene and cadalene, and two sesquiterpenols, γ-eudesmol, and *trans*,*trans*-farnesol. All these compounds have been already reported as wine components (4, 9). However, we were able to identify α-calacorene, 4,5,9,10-dehydro-isolongifolene and γ-eudesmol as new components of FP variety since cadalene and *trans*,*trans*-farnesol have been already reported in FP sparkling wines (31). Interesting aroma descriptors are related to this group of compounds, however their impact in the wine aroma has not been explored yet.

Table 3.1- Common volatile varietal compounds (mono and sesquiterpenoids, and C_{13} norisoprenoids) identified by GC×GC-TOFMS in *Vitis vinifera* L. cv. 'Fernão-Pires' white wines from different harvests, Appellations and producers.

[1]tR (s), [2] tR (s)	RI_{calc} [a]	RI_{lit} [b]	RI_{lit} [c]	Compound	Identification [d]	Lit. [e]	GC x GC chromatographic area (10^5) B-03 n=3		B-02 n=3		R-01 n=6		R-02-1 n=6		R-02-2 n=3		R-03-1 n=3		R-03-2 n=6		PS-03-1 n=3		PS-03-2 n=3	
Monoterpenic compounds																								
Monoterpenes																								
325 , 0.536	1022	1022	1022	*o*-Cymene	B, C	-	22.53	(6)[f]	17.34	(6)	9.48	(18)	6.84	(8)	20.54	(7)	8.60	(4)	10.94	(15)	10.84	(11)	7.30	(20)
330 , 0.472	1028	1030	1031	Limonene	A, B, C	X	4.81	(17)	17.74	(9)	1.69	(20)	1.32	(9)	1.39	(3)	2.39	(7)	6.80	(3)	2.01	(9)	1.36	(9)
385 , 0.496	1089	1113	1087	α-Terpinolene	B, C	X	12.77	(6)	2.65	(8)	1.33	(14)	5.26	(15)	3.19	(5)	4.62	(5)	1.58	(12)	3.15	(10)	2.40	(13)
510 , 0.608	1215	-	-	2-Bornene	B, C	-	5.56	(5)	3.62	(4)	4.35	(7)	3.99	(5)	4.28	(4)	3.74	(2)	5.92	(3)	4.96	(3)	4.64	(5)
Sub-total							**45.67**	**(3)**	**42.75**	**(4)**	**16.23**	**(6)**	**18.73**	**(7)**	**30.09**	**(1)**	**19.09**	**(10)**	**27.22**	**(7)**	**22.26**	**(3)**	**14.23**	**(16)**
Monoterpene oxygen-containing compounds																								
Monoterpene epoxides																								
280 , 0.440	929	971	968	Geranic oxide (dehydroxylinalool oxide A)	B, C	X	69.11	(5)	54.42	(3)	45.90	(4)	35.32	(10)	23.13	(11)	17.05	(20)	45.36	(8)	61.90	(5)	42.47	(18)
300 , 0.488	985	1006	1002	*Z*-Herboxide (dehydroxylinalool oxide)	B, C	X	69.72	(6)	54.45	(2)	32.32	(4)	18.57	(6)	29.74	(3)	18.19	(3)	21.04	(5)	57.53	(7)	36.88	(4)
315 , 0.512	1011	1021	1008	*E*-Herboxide (dehydroxylinalool oxide)	B, C	X	64.54	(16)	32.24	(10)	23.70	(19)	10.44	(16)	20.47	(3)	18.81	(23)	7.97	(17)	31.65	(23)	24.74	(13)
335 , 0.472	1033	1033	1032	1,8-Cineole	A, B, C	X	16.79	(17)	10.94	(10)	7.36	(14)	5.72	(18)	8.20	(16)	2.83	(8)	7.77	(5)	14.07	(10)	8.39	(19)
350 , 0.480	1050	1046	1049	Ocimene quintoxide	B, C	-	69.21	(2)	29.57	(22)	41.52	(3)	41.16	(6)	28.48	(3)	14.45	(3)	23.42	(4)	68.36	(1)	33.60	(5)
370 , 0.704	1072	1067	1074	Linalool *Z*-furanic oxide	B, C	X	25.51	(8)	16.94	(12)	11.45	(15)	7.63	(23)	9.80	(18)	4.90	(17)	12.12	(7)	23.99	(4)	11.54	(6)
385 , 0.720	1089	1081	1088	Linalool *E*-furanic oxide	B, C	X	9.56	(14)	5.49	(4)	2.78	(13)	2.90	(12)	3.45	(7)	1.96	(21)	3.82	(7)	10.17	(1)	4.73	(6)
425 , 0.528	1133	1128	1121	*Z*-Rose oxide	B, C	X	31.56	(5)	19.73	(12)	12.46	(9)	8.65	(7)	5.85	(2)	3.82	(2)	3.66	(10)	18.62	(3)	10.86	(13)
430 , 0.608	1139	1191	-	*E*-2,3-Epoxycarane	B, C	X	10.05	(2)	7.15	(7)	5.02	(34)	2.23	(17)	3.25	(7)	2.39	(9)	3.71	(16)	5.66	(16)	3.81	(4)
445 , 0.664	1156	1156	-	Nerol oxide	B, C	X	86.72	(13)	95.23	(1)	71.54	(13)	54.76	(5)	63.77	(2)	43.92	(1)	57.30	(7)	71.17	(1)	57.34	(2)
460 , 0.616	1172	-	-	Nerol oxide isomer	B, C	X	14.25	(3)	6.70	(1)	4.21	(9)	2.40	(3)	1.73	(17)	1.34	(5)	1.02	(20)	5.43	(16)	2.85	(12)
470 , 0.664	1183	-	1235	3,9-Epoxy-δ-*p*-menthene	B, C	-	24.96	(9)	17.60	(5)	7.38	(2)	2.10	(19)	2.06	(5)	2.75	(5)	2.33	(12)	5.52	(4)	6.33	(2)
Sub Total							**491.92**	**(2)**	**349.21**	**(4)**	**265.87**	**(4)**	**189.31**	**(4)**	**203.53**	**(2)**	**132.35**	**(4)**	**189.45**	**(3)**	**376.49**	**(3)**	**251.25**	**(2)**
Monoterpenols																								
400 , 0.952	1106	1114	1108	Hotrienol	A, B, C	X	41.89	(11)	40.10	(3)	23.75	(13)	4.67	(7)	16.46	(7)	12.22	(8)	5.02	(13)	33.42	(3)	9.68	(5)
415 , 0.904	1122	-	1118	Myrcenol	B, C	-	9.15	(9)	3.42	(8)	2.95	(17)	2.95	(19)	2.35	(8)	1.31	(9)	1.61	(13)	8.18	(4)	2.97	(18)
445 , 0.904	1156	-	-	*cis*-Ocimenol isomer	B,C	X	37.98	(12)	15.75	(0)	14.49	(21)	12.82	(6)	10.98	(5)	4.76	(4)	4.60	(13)	34.37	(1)	8.85	(2)
455 , 0.936	1167	1175	-	*cis*-Ocimenol isomer	B, C	X	22.55	(6)	9.38	(1)	7.17	(20)	6.39	(8)	6.59	(7)	3.51	(10)	4.81	(4)	21.88	(4)	7.56	(4)
465 , 0.984	1178	-	1213	Cyclogeraniol	B, C	-	24.12	(5)	11.70	(2)	8.89	(6)	5.32	(14)	4.03	(8)	4.76	(7)	6.11	(10)	17.39	(9)	9.24	(9)
475, 1.416	1189	1213	1183	*p*-Cymen-8-ol	B, C	X	4.24	(10)	2.39	(9)	1.81	(24)	1.53	(7)	1.10	(11)	8.84	(2)	2.19	(17)	2.33	(21)	1.57	(10)
480 , 0.920	1194	1195	1189	α-Terpineol	A, B, C	X	72.69	(10)	32.07	(4)	19.65	(21)	4.08	(13)	24.34	(2)	40.34	(18)	24.19	(20)	40.99	(13)	28.36	(2)
500 , 0.496	1209	-	-	Ocimenol isomer	B, C	X	24.41	(2)	10.39	(9)	27.94	(41)	21.15	(24)	2.14	(5)	15.82	(6)	27.42	(24)	31.80	(19)	34.79	(12)
Sub Total							**237.01**	**(7)**	**135.67**	**(3)**	**113.55**	**(11)**	**62.81**	**(9)**	**91.89**	**(3)**	**88.39**	**(9)**	**81.01**	**(5)**	**203.17**	**(1)**	**109.31**	**(3)**
Monoterpenediol																								
515 , 1.712	1218	1237	-	3,7-Dimethyl-1-octen-3,7-diol	B, C	X	6.66	(13)	3.46	(16)	1.91	(4)	1.91	(3)	1.62	(5)	1.79	(21)	2.07	(21)	7.79	(10)	3.29	(10)
Sub Total							**6.66**	**(13)**	**3.46**	**(16)**	**1.91**	**(4)**	**1.91**	**(3)**	**1.62**	**(5)**	**1.79**	**(21)**	**2.07**	**(21)**	**7.79**	**(10)**	**3.29**	**(10)**
Monoterpene ketone																								
435 , 0.728	1144	1139	1143	Camphor	B, C	-	1.70	(13)	3.20	(8)	4.06	(28)	1.87	(10)	6.19	(7)	0.68	(8)	5.30	(6)	3.11	(4)	2.03	(6)
Sub Total							**1.70**	**(13)**	**3.20**	**(8)**	**4.06**	**(28)**	**1.87**	**(10)**	**6.19**	**(7)**	**0.68**	**(8)**	**5.30**	**(6)**	**3.11**	**(4)**	**2.03**	**(6)**
Monoterpene aldehydes																								
500 , 0.752	1209	1228	-	*p*-Menth-1-en-9-al isomer	B, C	X	12.33	(5)	5.37	(2)	3.31	(9)	1.06	(16)	1.81	(10)	2.51	(1)	2.04	(11)	2.83	(5)	3.69	(6)
500 , 1.072	1209	-	-	*p*-Menth-1-en-9-al isomer	B, C	X	5.35	(11)	3.60	(1)	2.19	(12)	8.64	(11)	0.53	(5)	3.60	(13)	8.48	(4)	1.75	(12)	1.89	(7)
505 , 0.744	1212	1229	1220	β-Cyclocitral	B, C	X	8.15	(11)	4.71	(11)	3.59	(12)	4.58	(6)	3.49	(10)	3.60	(13)	3.17	(4)	5.46	(8)	5.32	(15)
Sub Total							**25.82**	**(8)**	**13.68**	**(4)**	**9.08**	**(7)**	**6.51**	**(5)**	**5.84**	**(5)**	**9.69**	**(3)**	**6.06**	**(3)**	**10.04**	**(6)**	**10.89**	**(9)**
Total							**802.18**	**(1)**	**548.77**	**(2)**	**411.2**	**(4)**	**283.4**	**(3)**	**334.10**	**(1)**	**250.6**	**(0)**	**310.83**	**(3)**	**615.43**	**(1)**	**384.49**	**(5)**

C_{13} norisoprenoid																
C_{13} norisoprenoid hydrocarbons																
495 , 0.552	1206	-	1208	1,1,6-Trimethyl-1,2,3,4-tetrahydro-naphthalene (α-ionene)	B, C	-	3.56 (13)	2.87 (11)	2.07 (12)	1.78 (15)	0.97 (22)	1.37 (5)	1.90 (26)	1.36 (10)	1.06 (14)
610 , 0.688	1276	-	1278	1,1,6-Trimethyl-1,2-dihydro-naphthalene isomer	B, C	X	54.27 (24)	85.75 (21)	44.54 (24)	89.89 (20)	22.59 (1)	48.62 (24)	61.31 (35)	45.48 (17)	71.40 (4)
655 , 0.744	1311	1358	1339	1,1,6-Trimethyl-1,2-dihydro-naphthalene (TDN)	B, C	X	23.30 (8)	13.52 (6)	10.09 (21)	12.05 (8)	9.94 (8)	9.18 (10)	9.39 (12)	14.48 (7)	8.36 (7)
Sub Total							**82.23 (14)**	**102.32 (15)**	**58.74 (21)**	**104.23 (17)**	**33.47 (15)**	**60.23 (10)**	**72.21 (25)**	**61.23 (14)**	**81.06 (9)**
C_{13} norisoprenoid oxygen-containing compounds																
555 , 0.544	1242	1287	1242	Vitispirane	B, C	X	72.48 (21)	133.33 (4)	146.63 (11)	146.78 (26)	153.43 (6)	121.37 (1)	137.11 (18)	135.82 (23)	93.54 (19)
570 , 0.528	1252	1322	1289	Theaspirane B	A, B, C	X	2.20 (9)	2.56 (10)	0.50 (18)	1.02 (18)	0.43 (7)	0.75 (18)	0.46 (18)	0.57 (12)	0.58 (5)
580 , 0.544	1258	1305	1263	Theaspirane A	A, B, C	X	2.17 (18)	1.43 (9)	0.54 (24)	0.75 (18)	4.85 (16)	0.63 (7)	0.61 (9)	0.73 (2)	0.60 (2)
580 , 0.600	1258	1317	1257	Edulan I	B, C	-	1.76 (10)	2.34 (10	0.74 (11)	0.73 (3)	0.74 (6)	1.43 (4)	0.76 (33)	1.10 (4)	0.95 (0)
635 , 0.776	1291	1364	1359	β-Damascenone	A, B, C	X	31.81 (10)	18.30 (8)	12.69 (21)	12.90 (15)	6.27 (7)	7.34 (9)	15.74 (31)	14.68 (2)	8.01 (1)
680 , 0.824	1366	-	-	Hydroxydihydroedulan	B, C	-	10.94 (9)	4.85 (2)	1.96 (13)	2.43 (17)	3.44 (7)	7.06 (3)	2.50 (18)	4.79 (6)	5.33 (7)
690 , 0.704	1389	-	1417	Geranyl acetone	A, B, C	-	16.49 (18)	31.16 (3)	42.61 (23)	17.44 (16)	11.53 (24)	1.47 (7)	14.51 (24)	61.29 (16)	14.61 (17)
710 , 0.880	1460	-	1485	3,4-Dehydro-β-ionone	B, C	-	5.68 (8)	3.62 (2)	1.47 (8)	2.24 (22)	2.08 (30)	2.06 (4)	1.94 (19)	3.80 (18)	1.58 (10)
Sub Total							**143.73 (12)**	**197.40 (7)**	**207.43 (10)**	**184.93 (14)**	**182.66 (4)**	**190.09 (6)**	**142.48 (4)**	**222.89 (8)**	**125.01 (10)**
Total							**223.42 (8)**	**301.85 (8)**	**268.33 (10)**	**285.78 (14)**	**212.72 (5)**	**201.91 (7)**	**246.82 (17)**	**285.30 (8)**	**206.65 (10)**
Sesquiterpenic compounds																
Sesquiterpene																
755 , 0.688	1547	1550	1542	α-Calacorene	A, B, C	-	22.10 (6)	10.57 (12)	7.27 (20)	8.06 (5)	6.01 (4)	7.96 (20)	8.56 (24)	5.00 (11)	4.66 (19)
810 , 0.752	1616	1535	-	4,5,9,10-Dehydro-isolongifolene	B, C	-	10.01 (9)	4.57 (2)	3.05 (14)	3.22 (12)	4.65 (28)	5.63 (17)	3.74 (14)	2.50 (9)	2.30 (16)
845 , 0.896	1650	1684	1673	Cadalene	B, C	X	23.26 (14)	14.52 (5)	8.53 (28)	10.01 (14)	8.29 (9)	10.66 (13)	10.03 (15)	6.82 (12)	6.89 (11)
Sub Total							**55.37 (8)**	**29.66 (6)**	**18.85 (18)**	**21.42 (7)**	**18.94 (9)**	**24.24 (15)**	**22.39 (17)**	**14.32 (6)**	**13.85 (7)**
Sequisterpenols																
820 , 0.856	1625	1651	1629	γ-Eudesmol	B, C	-	18.52 (17)	71.07 (9)	9.22 (29)	9.65 (8)	11.09 (29)	12.26 (22)	17.37 (18)	11.21 (23)	6.40 (4)
950 , 0.728	1800	-	1829	*trans,trans*-Farnesol	A, B, C	X	4.77 (24)	9.31 (21)	6.74 (30)	4.87 (26)	5.05 (17)	4.95 (23)	3.46 (9)	9.25 (17)	2.35 (11)
Sub Total							**23.3 (11)**	**80.38 (10)**	**15.96 (23)**	**14.53 (7)**	**16.14 (25)**	**17.22 (22)**	**20.83 (14)**	**20.50 (14)**	**8.75 (6)**
Total							**78.67 (6)**	**110.76 (7)**	**34.81 (19)**	**35.95 (7)**	**35.09 (12)**	**41.46 (17)**	**43.16 (15)**	**34.82 (10)**	**22.26 (6)**

[a] RI_{calc}: retention index obtained through the modulated chromatogram.
[b] RI_{lit}: retention index reported in the literature for comprehensive GC×GC system with Equity-5, DB-5 and VF-5ms for the first dimension ((9, 59, 94, 288, 289).
[c] RI_{lit}: retention index reported in the literature for one dimensional GC with 5%-Phenyl-methylpolysiloxane GC column or equivalent (31, 131, 290-297).
[d] reliability of the identification or structural proposal is indicated by 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.
[e] Compounds previously detected in grapes, musts and wines of FP variety (31, 59, 80, 81, 83, 89-91, 131, 301).
[f] Relative standard deviation in percentage.

FP wines have been described by the Interprofessional Association of Bairrada Appellation (Comissão Vitivinícola da Região da Bairrada) as exhibiting mature citrus and floral notes, such as orange, orange-tree, and mimosa. The results previously reported showed that hotrienol, α-terpineol, linalool *E*-pyranic oxide and geraniol were always reported as FP components, even using different extractions and detection methodologies (31, 59, 81, 89-91, 300, 301). Among these, linalool, hotrienol, geraniol, and α-terpineol have been described as contributors for the aroma of FP monovarietal wines (81). These compounds have specific aroma descriptors: linalool has characteristic citrus-like, sweet and flowery notes, hotrienol, α-terpineol, and geraniol exhibit flowery and sweet aromas (54, 306). In the present study, hotrienol and α-terpineol were found in all wines under study, however the absence of linalool in some samples can be explained by its ability, under acidic conditions, to originate other terpenes, via the hydration of double bonds, and dehydration reactions (307). The same behaviour can be observed for geraniol, which under acidic conditions can originate cyclogeraniol, present in all FP wines analysed. Besides those compounds already described as contributors for the FP aroma additional information can be added with the compounds *p*-cymen-8-ol and limonene with their citrus aroma, and the linalool-furanic oxides isomers, rose oxide, nerol oxides isomers, β-damascenone, geranyl acetone, TDN and *trans,trans*-farnesol with their floral notes, reinforcing the characteristics described for FP wines specialists.

3.4. Concluding remarks

The GC×GC-TOFMS combined with SPME showed to be a powerful methodology for the establishment of the varietal composition of FP wines, considering both mono and sesquiterpenic compounds, and C_{13} norisoprenoids. The structured chromatograms obtained for the varietal compounds were very helpful since the compounds organization according to their different chemical groups allows the definition of specific two-dimensional spaces, making possible a more accurate tentative identification of FP components. The analysis of the mass spectra data, together with the analytes retention times and their positioning in the 2D plot, allowed the identification of 170 compounds. It is also important to point out that remarkable data were obtained in

terms of retention index and comprehensive mass spectral databases, which responds to a current limitation of the spectral libraries and databases for comprehensive systems.

The development of a varietal classification-reference approach used for sample-to-sample analysis resulted in a reliable and efficient approach for the establishment of wines varietal identification. Thus, simplification of the data obtained and reduction of the time of analysis were achieved. The developed methodological approach was applied to FP monovarietal wines obtained from different harvests, Appellations and producers. From the total 170 mono and sesquiterpenic compounds, and C_{13} norisoprenoids, 45 components were detected in all wines under study, as allowing obtaining a "varietal volatile profile". Among these, comparing with literature, the GC×GC-TOFMS allowed to confirm the identity of 30 previously detected compounds in FP variety, together with 15 detected for the first time. The major varietal group was the monoterpenoids followed by C_{13} norisoprenoids and sesquiterpenic compounds. Among these compounds, hotrienol and α-terpineol, described as FP wine aroma contributors, have been detected in all wines under analysis. This approach can be extended to the analysis of other related food matrices, such as grapes, and musts, and can be applied as a powerful tool to trace their varietal origin.

Chapter 4- Assessment of antioxidant and antiproliferative effects of sesquiterpenoids in *in vitro* Caco-2 cells model

Assessment of antioxidant and antiproliferative effects of sesquiterpenoids in *in vitro* Caco-2 cells model

Summary

In this chapter, the antiradical capacity of *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene was evaluated. Chemical (DPPH$^{\bullet}$ and hydroxyl radicals) and biological (Caco-2 cells) models were used. Guaiazulene (IC_{50}= 0.73 mM) was the sesquiterpene with higher scavenger capacity against DPPH$^{\bullet}$, while *trans,trans*-farnesol (IC_{50}= 1.81 mM) and *cis*-nerolidol (IC_{50}= 1.48 mM) were more active towards hydroxyl radicals. All compounds, with the exception of α-humulene, at non-cytotoxic levels (≤ 1 mM), were able to protect Caco-2 cells from oxidative stress induced by *tert*-butyl hydroperoxide. The activity of the compounds under study was also evaluated as antiproliferative agents. Guaiazulene and *cis*-nerolidol were able to arrest the cells cycle in the S-phase more effectively than *trans,trans*-farnesol and α-humulene, being the last almost inactive. The results obtained for the sesquiterpenic compounds by these *in vitro* assays opens a perspective for their promising use as antioxidants and antiproliferative agents. However, *in vivo* tests (animals and humans) should be carried out in the future to confirm their safety and effectiveness.

4.1. Aim of the study

The aim of the present study is to screening the antiradical activity ($DPPH^{\bullet}$ and hydroxyl radicals) of four sesquiterpenic compounds, and test them as cytoprotectors, at non cytotoxic levels, against *tert*-butyl hydroperoxide (*tert*-BuOOH) toxicity in Caco-2 cells. In addition, they will be evaluated as antiproliferative agents. Moreover, the mechanisms by which they exert their activities will be further discussed. The four sesquiterpenic compounds studied comprises linear alcohols (*trans,trans*-farnesol and *cis*-nerolidol), cyclic and bicyclic hydrocarbons (α-humulene and guaiazulene, respectively) (Figure 4.1) which are found in *Vitis vinifera* L. (grapes and wines) and in different matrices from natural origin.

4.2. Materials and methods

4.2.1. Chemicals

In the present study four sesquiterpenic compounds with different chemical structures and functions were evaluated (Figure 4.1). *Trans,trans*-farnesol (≥ 95 %) and α-humulene (≥ 95 %) were purchased from Fluka (Buchs, Switzerland), *cis*-nerolidol (≥ 96 %) was from Aldrich Chemical Co (Milwaukee, WI, USA), and guaiazulene (> 98 %) was from TCI Europe N.V. (Zwijndrecht, Belgium). Minimum essential medium (MEM), glucose, ethanol, methanol, fetal calf serum, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), penicillin, streptomycin, amphotericin B, trypsin-EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Trizma® hydrochloride (Tris-HCl), Trizma® base (Tris-base), acetic acid, sodium hydroxide, hydrochloric acid, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), ethylenediaminetetraacetic acid disodium salt (EDTA-Na), iron sulphate heptahydrate, salicylic acid, hydrogen peroxide solution (30 % w/w), sulforhodamine B (SRB) and trypan blue were all from Sigma-Aldrich (St. Louis, USA). *tert*-Butyl hydroperoxide (*tert*-BuOOH) were purchased from Aldrich Chemical Co (Milwaukee, WI, USA), trichloroacetic acid (TCA) was from Merck® and methyl-[^{3}H]-thymidine (88.0 Ci/mmol) from Amershan (Arlington Heights, IL). Stock solutions of each sesquiterpenic compound (10 mM) were prepared in ethanol.

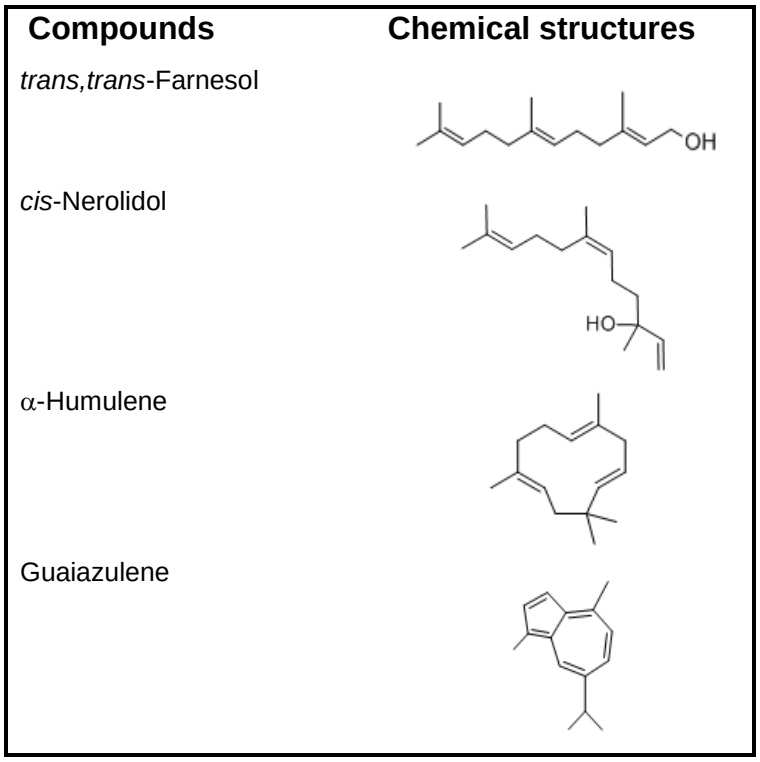


Figure 4.1. Chemical structure of the four sesquiterpenic compounds under study: *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, and guaiazulene.

4.2.2. Antiradical activities

4.2.2.1. DPPH$^{\bullet}$

The hydrogen atoms or electrons donation ability of the four sesquiterpenic compounds was measured from the bleaching of purple coloured methanol solution of DPPH$^{\bullet}$ (308). This compound is a stable free radical capable of accept an electron or a hydrogen atom becoming a non-radical species very hardly oxidizable (231, 309). Because of the unpaired electron, the DPPH has a strong absorbance at 515 nm; if this electron is paired the absorbance disappears, yielding a compound of yellow color. This assay is frequently used for the screening of the antiradical activity of different matrix and isolated compounds, due to its reproducibility and fastness.

Briefly, 100 μL of each sesquiterpenoid solution, *trans*,*trans*-farnesol, *cis*-nerolidol, α-humulene (0.65-5 mM final concentration) and guaiazulene (0.10-1.85 mM final concentration) and ascorbic acid (0.003-0.03 mM final concentration) were added to 3.9 mL of a 0.6 mM $DPPH^{\bullet}$ methanol solution. The reaction kinetic was read against blank at 0, 1 and after periods of 15 minutes until the steady-state was reached (180 min), at room temperature. Percentage of inhibition of free radical $DPPH^{\bullet}$ (I %) was calculated in the following way:

$$I\,\% = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the tested compound in the reaction mixture. Inhibition values were achieved from three independent assays performed in triplicate and results are expressed as the arithmetic mean ± standard error mean (SEM).

4.2.2.2. Hydroxyl radical

Hydroxyl radical scavenging activity was measured according to Smirnoff and Cumbes (310) with slight modification. This radical, conversely to $DPPH^{\bullet}$, is present in the physiological process, and is the most reactive oxygen species, which can cause enormous biological damage, being therefore closely related with the origin of different human diseases (311). The method is based on compounds capacity of competition with salicylic acid in the hydroxyl radical generation/detection system (312). The hydroxyl radicals ($OH^{\bullet}$) were generated by the Fenton reaction as follows:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

The hydroxyl radicals formed reacts with salicylic acid originating different products, being the 2,3-dihydroxy benzoic acid and 2,5-dihydroxy benzoic acid the major ones. The subsequent complexation of dihydroxy benzoic acids with Fe^{3+} gives rise to a red complex with maximum absorption at 515 nm data can be determined spectrophotometrically. If the antiradical/antioxidant is capable of inhibiting the hydroxyl

radicals the complex will not be formed.

The individuals reaction mixture containing 0.25 mL of each sesquiterpenoid solution (0.1-2 mM final concentration) and ascorbic acid (0.5-5 mM final concentration), plus 1.1 mL of iron sulphate heptahydrate solution (8 mM, prepared in EDTA-Na 20 μM) and 0.5 mL of H_2O_2 solution (7 mM), was initiated by the addition of 0.75 mL of salicylic acid (3 mM). After vortexing, the reaction was incubated at 37 ºC for 30 minutes. Thus, 1 mL of supernatant was transferred to reduced volume plastic cuvettes and the absorbance was read at 515 nm. The percentage of inhibition of hydroxyl radicals (I %) was calculated as follows:

$$I\,\% = [A_{control} - (A_{sample} - A_{blank})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the reaction with the tested compounds (containing all reagents), and A_{blank} is the absorbance of the compounds in the mixture containing all reagents excepting salicylic acid. Values are expressed as arithmetic mean ± SEM calculated from three independent assays performed in triplicate.

4.2.3. Sesquiterpenoids protection against oxidative damage

4.2.3.1. Cell culture

The Caco-2 cell line was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany) and was used between passage numbers 47 and 61. The cells were maintained in a humidified atmosphere of 5% CO_2, 95% air and were grown in MEM containing 5.55 mM glucose and supplemented with 15 % fetal calf serum, 25 mM HEPES, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25 % trypsin-EDTA, 5 min, 37 ºC), split 1:3, and sub-cultured in plastic

culture dishes (21 cm^2; Ø 60 mm; Corning Costar, Corning, NY, USA). For studies, Caco-2 cells were seeded on 12, 24 or 96 well plastic cell culture clusters (Corning Costar), and the experiments were performed 7-15 days after the initial seeding. Cells were free of fetal calf medium 24 h before experiments.

4.2.3.2. Cytotoxicity assay

The reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a colorimetric assay used to evaluate the mitochondrial metabolism and the activity of the cell respiratory chain in response to different factors such as their exposure to chemicals and environmental factors (313). The method is based in the following sequence (314): i) MTT is accumulated in cells by endocytosis; ii) MTT is metabolized by the enzyme succinate desidrogenase mitochondrial; iii) the reduction of the tetrazolium ring results in the formation of insoluble crystal of formazan (blue colour); iv) the crystals are accumulated in endossomals and/or lipossomals enclosures, and are subsequently released into the extracellular space by exocytosis; v) the formation of formazan is quantified spectrophotometrically at 540-660 nm and is directly proportional to the number of viable cells.

For analysis of cytotoxic compounds effect, viable cells were quantified by incubating Caco-2 cells with MTT (314). Confluent cells, cultured in 96 wells plastic culture dishes, were treated with *trans*,*trans*-farnesol, *cis*-nerolidol, α-humulene, guaiazulene and ascorbic acid in a final concentration of 1 mM prepared in medium containing 1 % of ethanol for all sesquiterpenic compounds and water for ascorbic acid. Experiments were carried out in four different exposition times: 0.5, 6, 24 and 48 hours. In the 48 h experiment, the medium was changed once (24 h) by adding fresh medium with the compounds at same concentration. Negative controls were run for all treatments (ethanol for all sesquiterpenic compounds and water for ascorbic acid). Three hours before the end MTT (20 μL) 10 μM was added. At the end of the experiment the medium was removed and the purple insoluble formazan, produced by viable cells, was dissolved in 200 μL of dimethylsulfoxide and quantified by measuring the absorbance at 540-660 nm. Cell cytotoxicity was calculated as follows:

$$Cell\ viability\ (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ was measure of the formazan formed in negative control cells and A_{sample} was the measure of the formazan formed after compounds exposure. Assays were performed in triplicate and cytotoxic effects were expressed as arithmetic mean ±SEM.

4.2.3.3. Sesquiterpenoids cytoprotection against *tert*-BuOOH

Cytoprotection was evaluated by means of the trypan blue assay (315). This is a dye exclusion method based on the principle that live cells possess intact cell membranes that exclude certain dyes. Thus, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Cells are then transferred to a Neubauer chamber and visually examined to determine the number of viable and nonviable cells.

Caco-2 cell monolayers were cultured in 12-well plastic culture dishes and tests were performed under quiescent conditions and when cells were subjected to *tert*-BuOOH-induced oxidative stress (316). Cells were exposed to each sesquiterpenic compound or ascorbic acid (1 mM final concentration prepared in medium) for 24 h. Negative controls were kept in fresh culture medium or in medium containing ethanol 1 %. After 24 h the cells were washed, trypsinized, stained by trypan blue (0.4%) and cells viability was calculated as follows:

$$\%\ Viability = [(viable\ cells_{control} - viable\ cells_{treatment})/viable\ cells_{control}] \times 100$$

where $viable\ cells_{control}$ was the number of intact cells in negative controls (only medium or medium with 1 % ethanol) and $viable\ cells_{treatment}$ was the number of viable cells after compounds pre-treatment.
The effectiveness of the treatment was achieved by the percentage of cytoprotection that was calculated as:

$$\%\ Cytoprotection = \%\ viable\ cells_{treatment\ tert\text{-}BuOOH} - \%\ viable\ cells_{control\ tert\text{-}BuOOH}$$

where *% viable cells*$_{treatment\ tert\text{-}BuOOH}$ was the viability of cells pre-treated with sesquiterpenic compounds and then exposed to *tert*-BuOOH (1 mM, 1 h), and *% viable cells*$_{control\ tert\text{-}BuOOH}$ was the percentage viability of cells on negative controls treated with *tert*-BuOOH. The obtained results were expressed as arithmetic mean ± SEM calculated from three independent assays performed in triplicate.

4.2.4. Sesquiterpenoids antiproliferative activity

In order to achieve the antiproliferative effect of sesquiterpenic compounds two methods were used: the SRB and the [^{3}H]-Thymidine incorporation assays.

4.2.4.1. Sulphorhodamine B (SRB) assay

The SRB method is based on the measurement of the whole-culture protein content as an index of tumour cell proliferation. The sulphorhodamine B (SRB) is an anionic pink dye that is combined electrostatically to the cells basic amino acids. This combination has a direct relationship, indicating that the more intense the colour, the higher the absorbance, and hence the number of cells. Thus, the fixed dye, measured spectrophotometrically after solubilisation, is correlated with the total rate of protein synthesis, and then with cellular proliferation (317).

The sesquiterpenic compounds ability to inhibit Caco-2 cells growth was assessed according with Faria *et al.* (318). Briefly, cell cultures were plated on 96-well plates, and exposed to 200 μL of culture medium containing the sesquiterpenic compounds (with a final concentration of ethanol equal to 1 %) or ascorbic acid in concentration levels of 0.01, 0.1 and 1 mM. Negative controls were made in the presence of 1 % ethanol and water for the sesquiterpenic compounds and ascorbic acid, respectively. After 24 h of exposure, cells were fixed by adding TCA (50 %) for 1 h at 4 ºC in the dark. Plates were then washed with tap water to remove the TCA, air-dried, and stained for 15 min with 0.4 % (w/v) SRB (in 1 % acetic acid). The culture was then rinsed with 1 % acetic acid (four times) to remove residual dye, plates were air-dried again and the bounded dye was solubilized in

200 μL of Tris base solution (10 mM, pH 10.5). The absorbance was determined at 492 nm in a microplate reader. Results were expressed as arithmetic mean ± SEM calculated from three independent assays performed in triplicate and reported as percentage of the control.

4.2.4.2. [^{3}H]-Thymidine incorporation assay

This method is based on the ability of proliferating cells to incorporate [^{3}H]-thymidine into replicating DNA (S phase of cell cycle) (265). In turn, the quantification of the radiation of compound incorporated into DNA during cell division is performed by a liquid scintillation beta counter. The thymidine is characterized by the nitrogenous base (thymine) linked to deoxyribose, via 3-N-glycoside bound. The incorporation of thymidine to DNA is accomplished in multiple steps: i) initial stage- phosphorylation of deoxythymidine to deoxythymidine-5-monophosphate (dTMP) in a reaction catalysed by the enzyme thymidine kinase 1. The nucleoside is then converted into nucleotides, which does not cross the cell membrane and remains trapped in the cell, ii) the next step is the conversion of dTMP to 5-deoxythymidine diphosphate and then to 5-deoxythymidine triphosphate to incorporation into DNA (319, 320).

The ability of sesquiterpenic compounds to arrest Caco-2 cells into S-phase was assessed according with Faria *et al.* (318). Briefly, Caco-2 cells were seeded into 24-well plates in a final volume of 0.5 mL culture medium containing fetal calf serum (10 %). After 24 h in culture, cells were treated with sesquiterpenic compounds or ascorbic acid at 0.01, 0.1 and 1 mM concentrations (negative controls were made in medium with 1 % ethanol and water). Cells were exposed to the compounds during 24 h and, after this period, a volume of 0.2 mL of methyl-[^{3}H]-thymidine (0.5 μCi/well) was added and incubated for 4 h. The medium was then removed and cells were fixed with TCA (10 %) for 1 h at 4 ºC. Cells were washed twice with TCA 10 % to remove unbounded radioactive compounds. The plates were air-dried and cells were lysed with NaOH 1 M (0.28 mL/well). An aliquot of 0.25 mL of lysate was neutralized with a diluted HCl solution before the addition of scintillation fluid. Samples radioactivity was quantified by a liquid scintillation counter. Results were expressed as the mean of the counts (disintegrations per min) against the controls.

4.2.5. Statistical analysis

Values are expressed as the arithmetic mean ±SEM of analyses done at least in triplicate and values of IC_{50} were obtained from the linear regression equation. Statistical significance of the difference between different compounds was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni test. Differences were considered to be significant when $p < 0.05$.

4.3. Results and discussion

4.3.1. Antiradical activity

As observed in Figure 4.2A, *trans,trans*-farnesol, *cis*-nerolidol and guaiazulene were able to inhibit $DPPH^{\bullet}$ in a dose-dependent manner. Nevertheless, only for guaiazulene it was possible to compute the IC_{50} value (Table 4.1). At the highest concentration tested (5 mM) mild scavenging activities were observed for *trans,trans*-farnesol (23 %), *cis*-nerolidol (21 %), and α-humulene (4 %) (the less effective one). Ascorbic acid, a hydrophilic antioxidant used as comparison, showed the lowest IC_{50} value (Table 4.1).

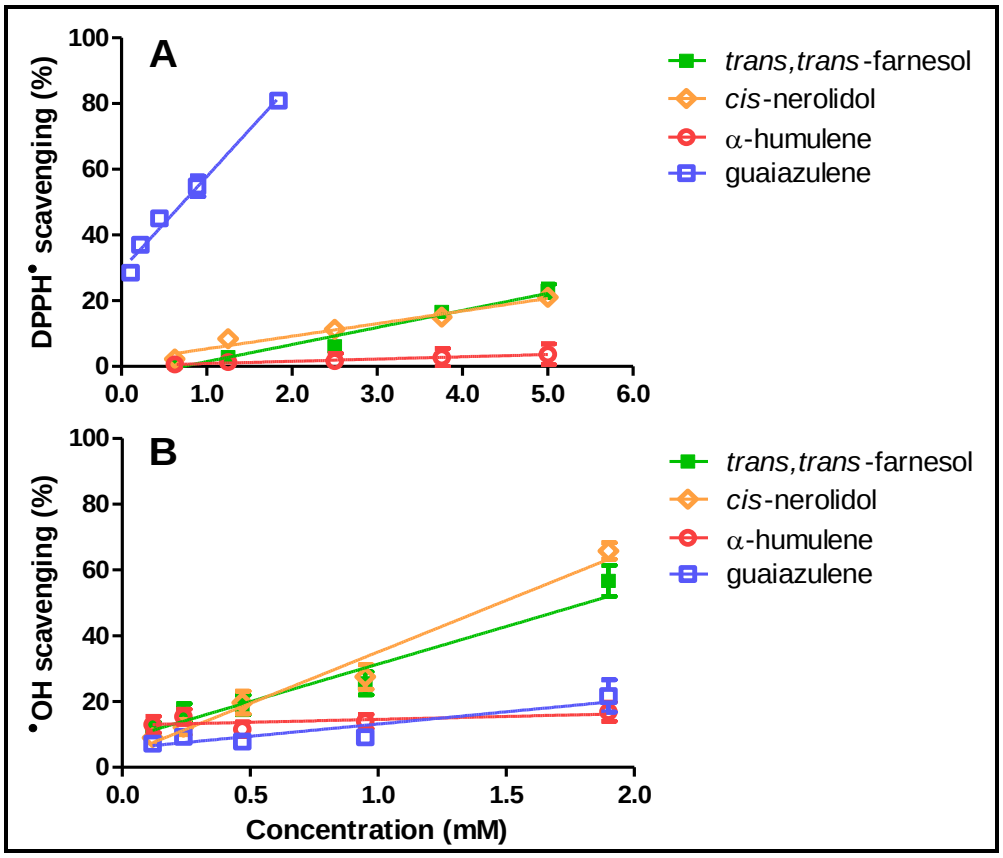


Figure 4.2. Antiradical activity of *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, and guaiazulene against DPPH• (A) and hydroxyl (B). Results show mean ± SEM of three experiments performed in triplicate.

Concerning the antiradical activity of the sesquiterpenic compounds towards hydroxyl radicals, in this assay, *cis*-nerolidol and *trans,trans*-farnesol were those able to scavenger it in a dose-dependent manner (Figure 4.2B). According to their IC_{50}, *cis*-nerolidol was more effective than *trans,trans*-farnesol and both compounds were more effective than ascorbic acid (Table 4.1). α-Humulene and guaiazulene showed poorer hydroxyl radicals scavenger capacity, which at the highest tested concentration was 17 % and 22 %, respectively.

Table 4.1- IC_{50} values (mM) determined for DPPH$^{\bullet}$ and hydroxyl radicals for the ascorbic acid (reference compound), *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene.

Compounds	IC_{50}	
	DPPH$^{\bullet}$	$^{\bullet}OH$
Ascorbic acid	0.02 ±0.001[a]	2.67 ±0.15
trans,trans-Farnesol	-	1.81 ±0.13
cis-Nerolidol	-	1.48 ±0.17
α-Humulene	-	-
Guaiazulene	0.73 ±0.06	-

[a] Values are mean ± standard deviation of three independent assays done in triplicate

4.3.2. Cytotoxicity

The cytotoxicity effect of sesquiterpenic compounds on Caco-2 cells was assessed with the MTT assay. The sesquiterpenic compounds at 1 mM were tested concerning their cytotoxicity by measuring the cells viability at different times of exposure: 0.5, 6, 24 and 48 hours. Cell viability was not affected by the sesquiterpenic compounds up to 24 h of exposure (Figure 4.3). However, after 48 h of exposure (chronic effect) all sesquiterpenic compounds drastically reduced cell viability, *trans,trans*-farnesol, *cis*-nerolidol and guaiazulene being the most toxic ones. In fact these compounds showed reductions on cell viability around 70 % for *trans,trans*-farnesol and *cis*-nerolidol and 80 % for the guaiazulene (Figure 4.3). Ascorbic acid did not affect cell viability at any exposure concentration tested.

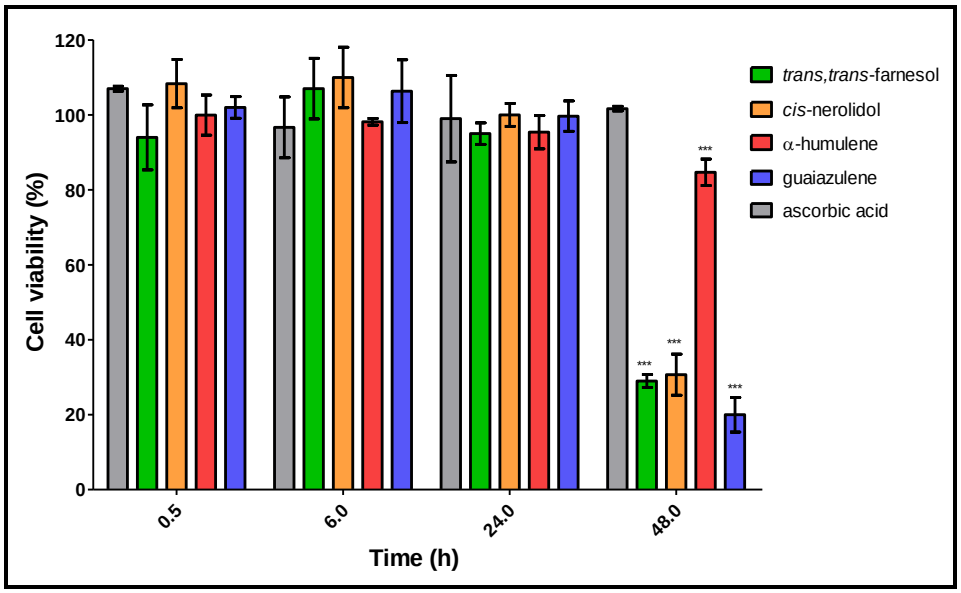


Figure 4.3. Individual compounds cytotoxicity on Caco-2 cells monolayers evaluated by the percentage of viable cells (MTT assay) after treatment with *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, guaiazulene, and ascorbic acid (0.5, 6, 24 and 48 h) at 1 mM concentration. Results are expressed by mean value ±SEM of three experiments done in triplicate. Significance differences were compared with the respective control (*** $p < 0.0001$).

4.3.3. Sesquiterpenoids cytoprotection against (*tert*-BuOOH)

The ability of the sesquiterpenic compounds to protect Caco-2 cells against the oxidative stress caused by *tert*-BuOOH was assessed in quiescent conditions by means of the trypan blue assay. In order to evaluate the potential protective effects of sesquiterpenic compounds, Caco-2 cells were pre-treated with the sesquiterpenic compounds under non-toxic conditions (≤ 1 mM, ≤ 24 h of incubation). After this period, the cells were exposed to 1 mM *tert*-BuOOH for 1 h, because under these conditions cellular viability was reduced by ca. 60 % (Figure 4.4). The results showed that *trans,trans*-farnesol, *cis*-nerolidol and guaiazulene were able to significantly protect Caco-2 cells against *tert*-BuOOH toxicity as these compounds were able to reduce de damage by around 30 % (Figure 4.4). Although a slight protection was observed for α-humulene (18 %) this result was not statistically significant. Ascorbic acid was not effective against the oxidant (Figure 4.4).

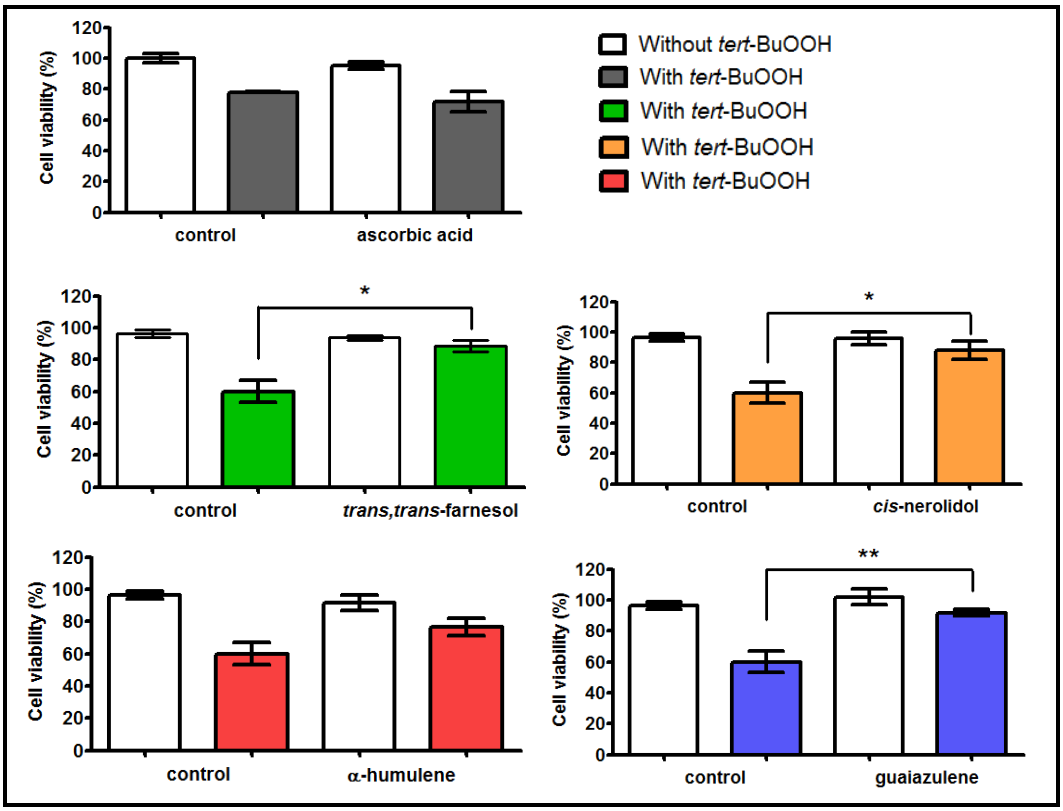


Figure 4.4. Effect of ascorbic acid, *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, and guaiazulene (1 mM, 24 h) on Caco-2 cells viability, with and without *tert*-BuOOH-induced oxidative stress (1 mM, 1 h). Values show mean ± SEM (n = 3). Mean values were significantly different compared with the respective control (* $p < 0.05$ and ** $p < 0.01$).

4.3.4. Sesquiterpenoids antiproliferative activities

Two distinct methods were used to access the antiproliferative activities of *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene (0.01, 0.1 and 1 mM) after 24 h of incubation: SRB method that measures the cellular protein, and methyl-[^{3}H]-thymidine that measures the DNA synthesis. As can be seen in Figure 4.5A, all sesquiterpenic compounds were able to reduce Caco-2 cell proliferation (SRB method) but only at 1 mM. More prominent results were observed for *trans,trans*-farnesol and *cis*-nerolidol with inhibitions of almost 90 %, followed by guaiazulene (80 %), and α-humulene (60 %). The results obtained with the incorporation of methyl-[^{3}H]-thymidine were more pronounced (Figure 4.5B). By this method, guaiazulene was able to inhibit cell

proliferation at all concentrations tested, with inhibitions varying from 30 % to 90 %. *cis*-Nerolidol was responsible for a inhibition of 60 % to 90 % at the two higher concentrations. The cell proliferation activities for *trans,trans*-farnesol and α-humulene were observed only at the highest concentration tested with inhibitions of 80 % and 75 %, respectively. Ascorbic acid did not show antiproliferative effect at any concentration tested.

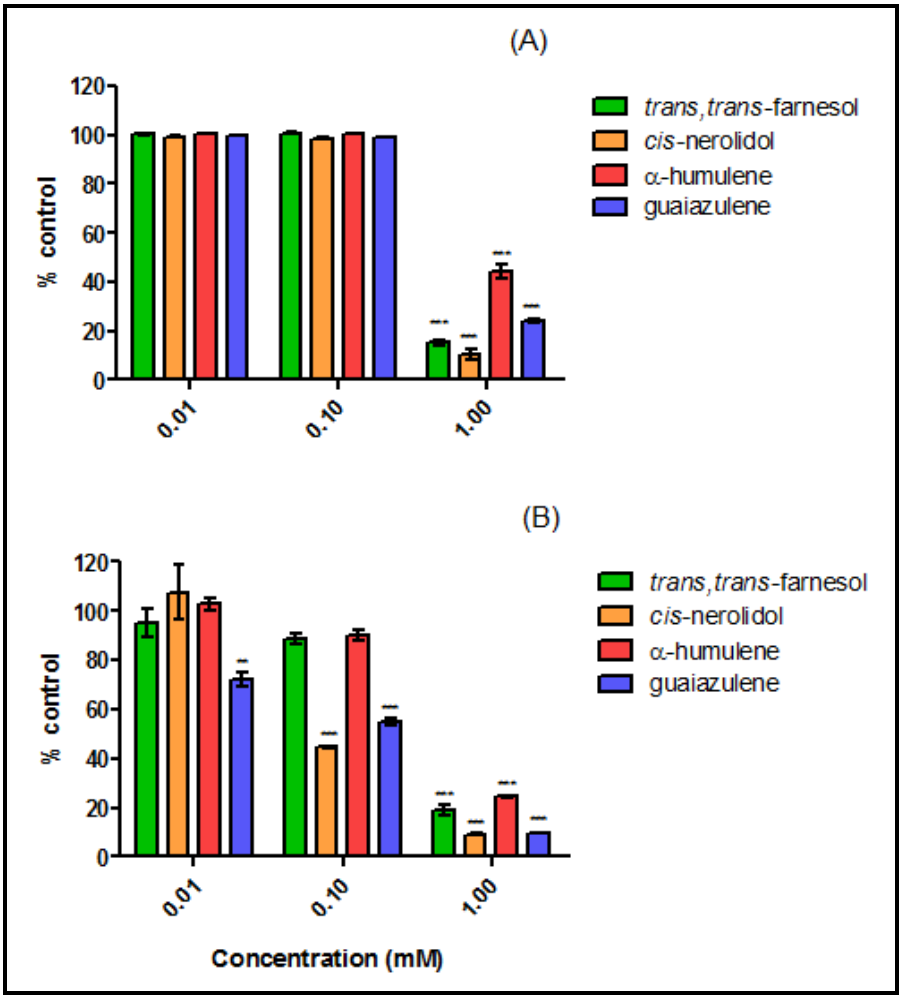


Figure 4.5. Antiproliferative effect of ascorbic acid, *trans,trans*-farnesol, *cis*-nerolidol, α-humulene, and guaiazulene on Caco-2 cells, after 24 h pre-treatment, evaluated by SRB (A) and methyl-[^{3}H]-thymidine (B). Values show mean ± SEM (n = 3). Mean values were significantly different compared with the respective control (** $p < 0.01$ and *** $p < 0.0001$).

4.3.5. Sesquiterpenoids effects on chemical and biological models

Different *in vitro* methods can be used to measure the efficiency of natural antioxidant compounds either as pure or as plant mixtures. Owing to the complex nature of sesquiterpenoids and their mechanisms of action, a single method is not capable to provide a comprehensive view of their antioxidant profile. In addition, the comparison of results with those found in the literature is very difficult since the assays conditions cannot be exactly the same and the relative effectiveness of antiradical compound is highly dependent on their concentration, test system, time and selected assay. Thus, we tested the sesquiterpenoids radical-scavenging activity by means of two assays: $DPPH^{\bullet}$ and hydroxyl radicals. The results (Figure 4.2 and Table 4.1) showed that, under the assays conditions, guaiazulene was able to scavenger $DPPH^{\bullet}$ radical efficiently, while mild effects were observed for the remaining compounds. Its capacity to scavenger $DPPH^{\bullet}$ may be explained by the formation of a tertiary radical that is stabilized by the presence of double bounds in conjugated position giving rise to resonance structures (Figure 4.6) (250). The antiradical activity of *trans,trans*-farnesol, *cis*-nerolidol, and α-humulene against the $DPPH^{\bullet}$ was poor. Nevertheless, this is the first report about their ability to scavenger this radical, as far as we know. All compounds were less effective than ascorbic acid, which IC_{50} (Table 4.1) is in accordance to that reported in the literature (321).

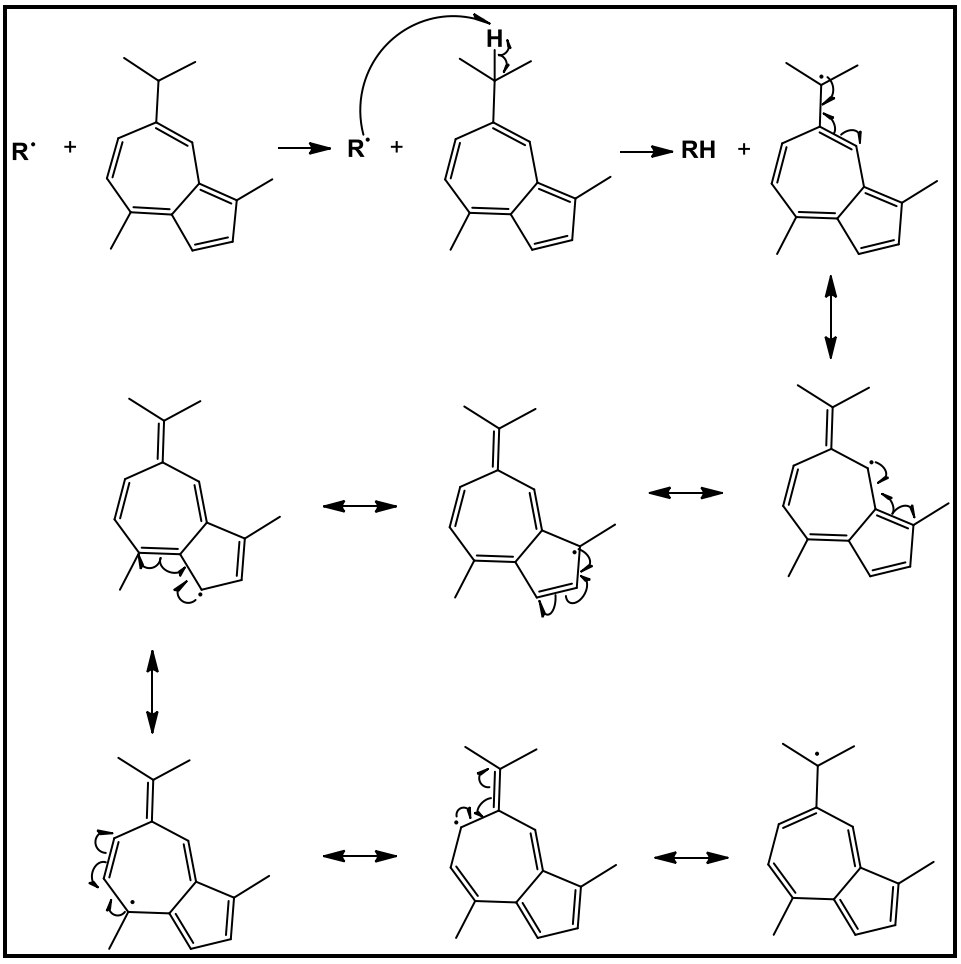


Figure 4.6. Mechanism of free radical (R•) stabilization by the sesquiterpene guaiazulene.

Conversely to the results we obtained for the DPPH•, the compounds with highest scavenging capacity against hydroxyl radicals were *trans,trans*-farnesol and *cis*-nerolidol. The observed activity of the sesquiterpenols against this free radical can be due their ability to donate the hydrogen present in the hydroxyl group and/or by the abstraction of their hydrogen's in allylic position (Figure 4.1). To the best of our knowledge, only guaiazulene have been already studied as hydroxyl radical scavenger (250). Ascorbic acid was less active than *trans,trans*-farnesol and *cis*-nerolidol but more active than guaiazulene and α-humulene. The IC_{50} value of ascorbic acid is in accordance with that reported in the literature (322).

Considering the antiradical effects obtained for the sesquiterpenic compounds we decided to test their ability to protect Caco-2 cells against the oxidative injure caused by

tert-BuOOH. Thus, the concentration of 1 mM was chosen for the subsequent tests, based on an intermediary value of IC_{50} inhibitory activity between the most potent antiradical compounds for $DPPH^{\bullet}$ and hydroxyl radical (Table 4.1). Therefore, the sesquiterpenic compounds cytotoxicity was the preliminary assay to assess a possible cellular damage caused by the compounds and it was used to determine the ideal time of exposure. Caco-2 cells are a well-established model for *in vitro* investigations of the antioxidant effect of bioactive compounds (323). These cells when confluent and differentiated have properties similar to the enterocytes, forming confluent monolayers and expressing several morphological, biochemical and biological characteristics of small intestine, mainly related to the absorption, transport, and metabolism of compounds such as lipids, lipoproteins, anthocyanins, phenolic compounds among others (324-327). Thus, confluent Caco-2 cells were exposed to the compounds at 1 mM, in different times. All sesquiterpenic compounds studied were non-cytotoxic up to 24 h of exposure but were cytotoxic at 48 h (Figure 4.3). Consequently, the protective effect of the sesquiterpenic compounds against *tert*-BuOOH on Caco-2 cells was carried out at non-cytotoxic time of exposure ($\leq$ 24 h).

The main cause of toxicity of *tert*-BuOOH on Caco-2 cells model is related with the lipid peroxidation of the cells membrane (328). *tert*-BuOOH, when metabolized originates *tert*-butoxyl, peroxyl and methyl radicals (329), promoting a significant decrease in the levels of the molecules involved in the cell defense mechanism and, consequently, increase lipid peroxidation (330).

The results obtained showed that all sesquiterpenic compounds were able to protect the cells against *tert*-BuOOH cytotoxicity, with the exception of α-humulene that showed around 20 % of protection but was not statistically different from the control. The protective effect of sesquiterpenic compounds against hydrophobic oxidants may be modulated by their lipophilicity. This idea may be also supported as ascorbic acid showed no protective effect.

Since *trans,trans*-farnesol, *cis*-nerolidol and guaiazulene showed higher protective behaviour, it can be assumed that their mechanism of action might be due to their ability to scavenger free radicals. These results are in agreement with the results obtained for $DPPH^{\bullet}$ and hydroxyl radical assays (Table 4.1, Figure 4.2A and 4.2B). In addition, pre-treatment of biological models with *trans,trans*-farnesol increased the levels of glutathione and

induced the enzymatic detoxification systems, reducing the injury promoted by different agents (247, 248). Guaiazulene also showed protection against the *in vivo* hepatotoxicity of paracetamol by decreasing its metabolic activation by preventing both cytochrome P450 activity and NAPQI-induced glutathione depletion, which was attributed to its ability to act as a chain-breaking antioxidant (251).

Undifferentiated Caco-2 cells are a cancer cell line that grow exponentially after seeding, enabling its use to assess the antiproliferative effect of different compounds. In addition, these cells are reported to be very sensitive to the suppression of growth mediated by isoprenoids (256, 258). Therefore, compounds showing no cytotoxicity on confluent cells and that are able to supress the cells when they are in exponential growth are good anticancer candidates. In this sense, the sesquiterpenic compounds were evaluated as antiproliferative agents, by means of the two assays, by SRB that determines the cells whole protein content and by the methyl-[^{3}H]-thymidine incorporation assay that measures DNA synthesis.

All sesquiterpenic compounds under study were able to inhibit cell proliferation in Caco-2 cells only at the highest concentration tested (1 mM) (Figure 4.5A). In addition, they also induced an S-phase cell cycle arrest in the concentration range from 0.01 to 1 mM, being guaiazulene and *cis*-nerolidol the most potent compounds (Figure 4.5B). Ascorbic acid showed no antiproliferative effect at any concentration tested, which is in accordance with the literature for another colon carcinoma cell line (LS174T) (331). Guaiazulene, *trans,trans*-farnesol, *cis*-nerolidol and α-humulene have been already mentioned to have antiproliferative effects in other *in vitro* assays at similar concentration levels. For instance, guaiazulene showed antiproliferative effect against different human oral tumor cell lines, being more effective against cancer cells (IC_{50}=0.11-0.16 mM) than normal cells (IC_{50}=0.19-0.40 mM) (263). *trans,trans*-Farnesol and *cis*-nerolidol were reported to suppress cell proliferation in two cell models, B16F10 (mouse melanoma cell line) and HL-60 (acute promyelocytic leukemia cells), with IC_{50} values lower than 0.065 mM (222). In addition, α-humulene has been reported to have cytotoxic effect against A-549 (human lung carcinoma), HeLa (human cervical carcinoma) and HT-29 (human colon adenocarcinoma) at low concentrations (IC_{50} in the range of 0.10 to 0.17 mM), while higher concentrations were needed for normal Vero cells (monkey kidney) and

macrophages (IC_{50} > 0.60 mM for both) (264).

Different hypothesis have been already postulated to the mode of action of different bioactive compounds against cancer cells: i) they can directly affect the expression of genes involved in cell signaling that regulates cell proliferation, cycle, apoptosis and differentiation; or ii) they can suppress enzymatic pathways that are responsible for the provision of essential products for the post-translational process and biological activity of proteins essential in cell proliferation (257-259). This last hypothesis has been frequently associated with the antiproliferative action of different isoprenoids. In fact, Tatman and Mo (222) found that the antiproliferative effect of *trans,trans*-farnesol and *cis*-nerolidol is related with their ability to mimic the endogenous modulator (*trans,trans*-farnesol) of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity. The inhibition of this enzyme slows down the mevalonate pathway, which is an essential process for the cell cycle progression and achievement of mitosis (260). More recently, Ferreira et *al.* (221) reported that nerolidol suppressed 50 % of the HepG2 (human hepatocellular liver carcinoma cells) proliferation at a concentration of approximately 10 µM. These authors have found that nerolidol decreases the mitochondrial membrane potential and the phosphorylation activity by inhibiting the ATPase. Both parameters are essential to assure the cell cycle progression, thus the dysfunction caused by nerolidol at this level should be probably in the origin of its antiproliferative effect.

4.4. Concluding remarks

In summary, the sesquiterpenic compounds scavenger capacity studied is dependent upon the assay used, being guaiazulene the most active against $DPPH^{\bullet}$, while *trans,trans*-farnesol and *cis*-nerolidol the more efficient against the hydroxyl radical. These antiradical capacities were also proved when guaiazulene, *trans,trans*-farnesol and *cis*-nerolidol, at non cytotoxic conditions, were able to protect Caco-2 cells against the toxicity caused by *tert*-BuOOH. Moreover, all sesquiterpenic compounds showed antiproliferative effect, being guaiazulene and *cis*-nerolidol the most promising ones. This effect is probably related to their antioxidant properties or to their ability to interfere with cellular enzymes

responsible for the cell cycle process. Since these compounds are present in different natural products, which in some cases take part of our diet, they could have a positive effect on human health due to their ability to overcome the oxidative damage and interfere with cancer cells proliferation. Furthermore, the presence of different isoprenoids in human daily diet may improve their potential benefits due to cumulative (259), potentiation (332), and additive (256, 261) effects.

Although these *in vitro* models provided very useful information and promising results on the possible beneficial effect of sesquiterpenoids to human health, it's *in vivo* assessment (animals and humans) is also important and essential to confirm their real safety and effect

Chapter 5- Hepatoprotection of sesquiterpenoids: a quantitative structure-activity relationship (QSAR) approach

Hepatoprotection of sesquiterpenoids: a quantitative structure-activity relationship (QSAR) approach

Summary

In this Chapter, the relative hepatoprotection effect of fifteen sesquiterpenic compounds, commonly found in plants and plant-derived foods and beverages was assessed. Endogenous lipid peroxidation (assay A) and induced lipid peroxidation (assay B) were evaluated in liver homogenates from Wistar rats by the thiobarbituric acid reactive species test. Sesquiterpenic compounds with different chemical structures were tested: *trans,trans*-farnesol, *cis*-nerolidol, (–)-α-bisabolol, *trans*-β-farnesene, germacrene D, α-humulene, β-caryophyllene, isocaryophyllene, (+)-valencene, guaiazulene, (–)-α-cedrene, (+)-aromadendrene, (–)-α-neoclovene, (-)-α-copaene, and (+)-cyclosativene. Ascorbic acid was used as a positive antioxidant control. With the exception of α-humulene, all the sesquiterpenoids under study (1 mM) were effective in reducing the malonaldehyde levels in both endogenous and induced lipid peroxidation up to 35% and 70%, respectively. The 3D-QSAR models developed, relating the hepatoprotection activity with molecular properties, showed good fit (R^2 0.819 and 0.972 for the assays A and B, respectively) with good prediction power (Q^2 > 0.950 and SDEP < 2%, for both models A and B). A network of effects associated with structural and chemical features of sesquiterpenoids such as shape, branching, symmetry, and presence of electronegative fragments, can modulate the hepatoprotective activity observed for these compounds.

5.1. Aim of the study

The present study aimed to investigate the potential hepatoprotection activity of sesquiterpenoids, commonly found in *Vitis vinifera* L. (grapes and wines) and plant-derived foods and beverages against lipid peroxidation using liver homogenate from Wistar rats in an *in vitro* study. In order to establish the structure-activity relationships for future hepatoprotection prediction, fifteen sesquiterpenic compounds representing different chemical classes were used: linear alcohols, a cyclic alcohol, a linear hydrocarbon, cyclic hydrocarbons, bicyclic hydrocarbons, tricyclic hydrocarbons and tetracyclic hydrocarbons (Figure 5.1).

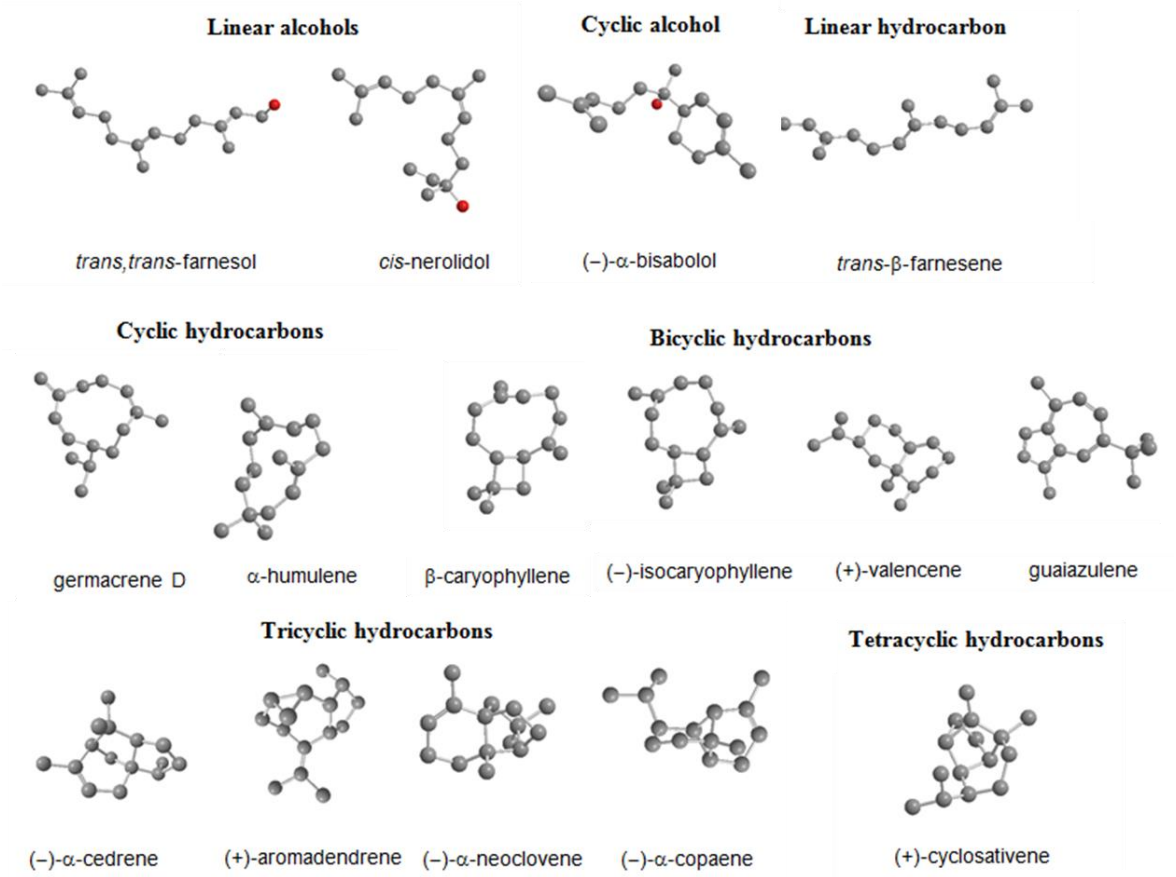


Figure 5.1. 3D Chemical structures of the fifteen sesquiterpenic compounds tested: *trans,trans*-farnesol, *cis*-nerolidol, (−)-α-bisabolol, *trans*-β-farnesene, germacrene D, α-humulene, β-caryophyllene, isocaryophyllene, (+)-valencene, guaiazulene, (−)-α-cedrene, (+)-aromadendrene, (−)-α-neoclovene, (−)-α-copaene, and (+)-cyclosativene.

5.2. Materials and methods

5.2.1. Materials

Ethanol (purity ≥ 99.8 %) from Riedel-de Haën (Seelze, Germany), 3-amino-1,2,4-triazole (95 %), mercaptosuccinic acid (97 %), and *tert*-butyl hydroperoxide (*tert*-BuOOH) were purchased from Aldrich Chemical Co (Milwaukee, WI, USA). Pentobarbital sodium salt, potassium phosphate monobasic (> 99 %), and sodium phosphate dibasic (> 99 %) were purchased from Sigma (St. Louis, MO, USA). Triton X-100 was purchased from Merck (Darmstadt, Germany). The ascorbic acid was purchased from AnalaR BDH Chemical Ltd. (London, United Kingdom). 2-Thiobarbituric acid (TBA) (≥ 98 %) was purchased from Fluka (Buchs, Switzerland). Fifteen sesquiterpenic compounds were used (Figure 5.1): β-caryophyllene (≥ 98.5 % GC) , (−)-α-cedrene (≥ 99 %), (+)-aromadendrene (> 97 % GC), α-humulene (>98% GC), (−)-α-copaene (≥ 90 %), (+)-cyclosativene (≥ 99 %), isocaryophyllene (> 98 %), (−)-α-neoclovene (≥ 95 %), (+)-valencene (≥ 70 %), *trans*-β-farnesene (90 %), *trans,trans*-farnesol (≥ 95 %), and (−)-α-bisabolol (≥ 95 %) were purchased from Fluka (Buchs, Switzerland). *cis*-Nerolidol (≥ 96 %) was purchased from Sigma-Aldrich (St. Louis, Mo.), guaiazulene (> 98 %) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium), and germacrene D (40%, natural extract enriched in germacrene D, this extract containing also other sesquiterpenoids, where β-farnesene ca. 10 % is the second most abundant) was kindly offered by Tecnufar Ibérica, (Madrid, Spain). Stock solutions of each sesquiterpenic compound (10 mM) were prepared in ethanol.

5.1.2. Animals and hepatocytes isolation

Female Wistar rats weighting *ca.* 400-600 g were used in the experiments. Animals were kept one per cage under controlled environmental conditions: 12.00 h light - dark cycle, room temperature (24 ºC), and food and tap water were allowed *ad libitum*. Animals were anesthetized with pentobarbital (50 mg/kg). The livers were removed and placed (0.5 g/mL) in a glass tube with homogenization buffer (KH_2PO_4 62.5 mM, Na_2HPO_4 50.0 mM and Triton X-100 0.1 %). For the experiments, the liver tissue were homogenized in a glass-teflon homogenizer and kept continuously on ice. Rat liver homogenate (RLH) inhibition of catalase and glutathione peroxidase activities were done by the addition of 3-amino-1,2,4-triazol and mercaptosuccinic acid (both at 10 mM), respectively.

5.1.3. Evaluation of sesquiterpenoids hepatoprotection potential

The individual relative hepatoprotection of the fifteen sesquiterpenic compounds was evaluated for endogenous (assay A) and induced (assay B) lipid peroxidation effects, as shown in Figure 5.2, according to (333). Relative hepatoprotection of ascorbic acid was evaluated and used as positive antioxidant control. In order to assess molecular structure - activity relationships, sesquiterpenoids were tested at the same molar concentration. The procedure consisted in the individual addition of 40 μL of each sesquiterpenoid solution (1 mM, final concentration) or ascorbic acid (1 mM, final concentration) to aliquots of 400 μL of RLH and incubation at 37 ºC for 1 hour. Compounds were tested at 1 mM, based on the results obtained on Chapter 4 and the protective effects reported in the literature for some of them (246, 251). In the initial step a concentration 10 times lower (0.1 mM) was tested, but none of the compounds under study were able to reduce the MDA levels (data not shown). Thus, the hepatoprotection evaluation effect was performed using standards at 1 mM. Subsequently, *tert*-BuOOH (1 mM) was added to the assay B to induce lipid peroxidation, and the assay was kept at the same temperature for one more hour. Negative controls were run for all treatments (ethanol for all sesquiterpenoids and water for ascorbic acid assay).

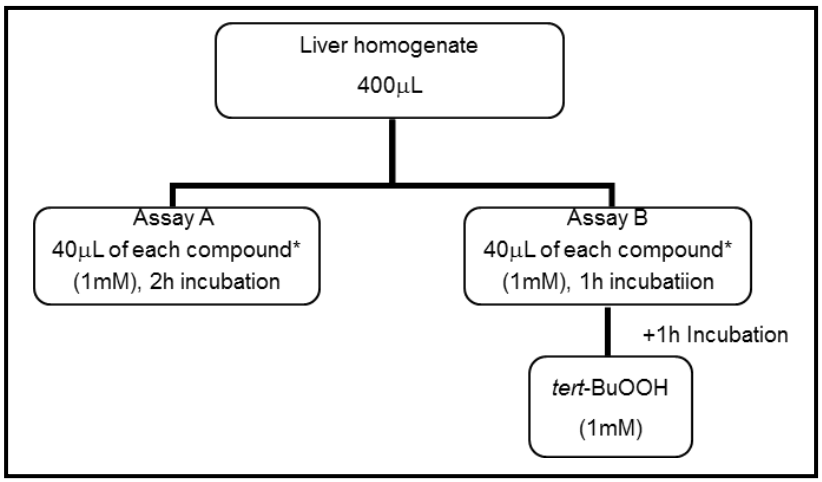


Figure 5.2. Experimental procedure for hepatoprotection evaluation in rat liver homogenate. * Ascorbic acid, *trans,trans*-farnesol, *cis*-nerolidol, (−)-α-bisabolol, *trans*-β-farnesene, germacrene D, α-humulene, β-caryophyllene, isocaryophyllene, (+)-valencene, guaiazulene, (−)-α-cedrene, (+)-aromadendrene, (−)-α-neoclovene, (−)-α-copaene and (+)-cyclosativene.

After incubation, the level of malonaldehyde (MDA) in the assays A and B was measured using the thiobarbituric acid reactive substances (TBAR's) assay adapted from Fernandes *et al.* (333). Briefly, the homogenates were centrifuged at 13000 rpm for 10 min at 4 ºC, and the supernatant (100 μL) was collected and precipitated with 200 μL of TCA 10 %. Then, centrifugation was carried out at 13000 rpm for 2 min at 4 ºC and 100 μL of supernatant was added to 100 μL of TBA (1 %). Samples were kept in water bath at 96 ºC for 10 min and, after cooling to room temperature, the absorbance at 535 nm was read. The concentration of MDA was determined using the extinction coefficient of 1.56×10^5 1/M cm, and results were expressed as percentage of reduction of nmol MDA/mg protein in relation to negative controls. Four independent experiments were performed in triplicates at least on 4 different days.

5.1.4. Protein determination

The concentration of MDA formed during lipid peroxidation was normalized by the protein tissue homogenates content, thus a protein determination was carried out as described by Bradford (1976) (334) using human serum albumin as standard.

5.1.5 Molecular descriptors

The 3D-structures of all the compounds were drawn and their minimum energy conformations were obtained by the mechanic method of Allinger (MM2) using HyperChem software evaluation version 7.0 (Hypercube, Inc. Gainesville, Florida, USA 2002). Then, they were transferred into the Dragon program evaluation version 5.4 (Talette SRL, Milan, Italy) and the molecular descriptors were calculated. The molecular descriptors are organized in twelve groups: (a) constitutional; (b) molecular properties; (c) atom-centered fragments; (d) topological; (e) connectivity indices; (f) information indices; (g) functional group counts; (h) geometrical; (i) radial Distribution Functions (RDF); (j) Molecule Representation of Structures based on Electron diffraction (3D-MoRSE); (k) Weighted Holistic Invariant Molecular descriptors (WHIM) and (l) Geometry,Topology, and Atom-Weights AssemblY (GETAWAY). The meaning of these molecular descriptors and the calculation procedures are summarized by (335).

5.1.6. QSAR model development

A total of 803 molecular descriptors were calculated by the Dragon software. Correlation coefficients between the activity (dependent variable) and the descriptor or descriptors were determined by correlation analysis. Reduced descriptors were obtained by discarding highly inter-correlated ($r > 0.9$) descriptors and molecular descriptors with constant or near constant values, resulting in 80 molecular descriptors. Further, selection of the best subset of descriptors for the prediction of sesquiterpenoids hepatoprotection activity was done using genetic algorithm (GA) and Principle Component Regression (PCR). GA is an optimization technique based on the principles of evolutionary selection. GA is effective for finding global minimum (or maximum) for the complex tasks in particular ones that involve large number of independent variables (336, 337). GA has been widely applied to the selection of the best subset of the molecular descriptors in QSAR and quantitative structure-property relationships analysis in different fields (337-340).

The purpose of the optimization using GA was minimization of the prediction error for the QSAR model. Thus, Root Mean Square Error in Cross-Validation (RMSECV) calculated using PCR was used as objective function. PCR was employed for calculating calibration models during the optimization step due to the large number of descriptors compared to the number of samples. Optimization was done using activity data from both assays A and B.

Calibration models with respect to the hepatoprotection activities were build using the best subsets of molecular descriptors and Multilinear Regression (MLR). MLR calibration models were validated using leave-one-out cross-validation and evaluated using the following statistics: adjusted R^2 *and* Q^2, Standard Deviations Errors in Calculation (SDEC) and Prediction (SDEP). As the number of samples is relatively small compared to the number of the descriptors necessary to achieve the best model, statistics adjusted for the number of the variables was used as recommended by Todeschini (341). All calculations were done in MATLAB 7.3.0. GA was implemented using GA toolbox v. 1.2 available from the Department of Automatic Control and Systems Engineering of The University of Sheffield, UK (342).

5.2. Results and discussion

5.2.1. Hepatoprotection of sesquiterpenoids.

Relative hepatoprotection of fifteen sesquiterpenoids with different molecular structures (Figure 5.1, Table 5.1) was evaluated by the estimation of MDA levels in rat liver homogenates for endogenous (assay A) and induced (assay B) lipid peroxidation assays. The amounts of MDA formed on assay A for water and ethanol controls were 4.47 ±0.27 and 4.23 ±0.38 nmol MDA/mg protein, respectively. These values were three and seven times higher on controls exposed to *tert*-BuOOH (assay B), being 15.51 ±2.02 nmol MDA/mg protein for water control, and 29.50 ±0.59 nmol MDA/mg protein for ethanol control. The percentage of MDA reduction, displayed on Figure 5.3 and Table 5.1, for ascorbic acid and sesquiterpenic compounds was calculated against their respective controls.

For endogenous hepatoprotection (assay A), (–)-α-neoclovene showed the strongest reduction (35.46 ±1.25 %), followed by *cis*-nerolidol (25.60 ±0.98 %), *trans,trans*-farnesol (23.82 ±1.03 %), (–)-α-copaene (23.66 ±0.71 %), and *trans*-β-farnesene (21.82 ±1.94 %) (Figure 5.3). Moreover, lower activities were observed for guaiazulene (17.20 ±0.61 %), (+)-valencene (14.77 ±0.49 %), β-caryophyllene (14.61 ±1.43 %), germacrene D (13.55 ±1.50 %), (–)-α-cedrene (8.80 ±0.76 %), (+)-cyclosativene (7.60 ±0.37 %), and (+)-aromadendrene (4.46 ±0.45 %). Isocaryophyllene (1.50 ±0.19 %) and (–)-α-bisabolol (0.32 ±0.06 %) had very low activity and for α-humulene no protection activity was observed.

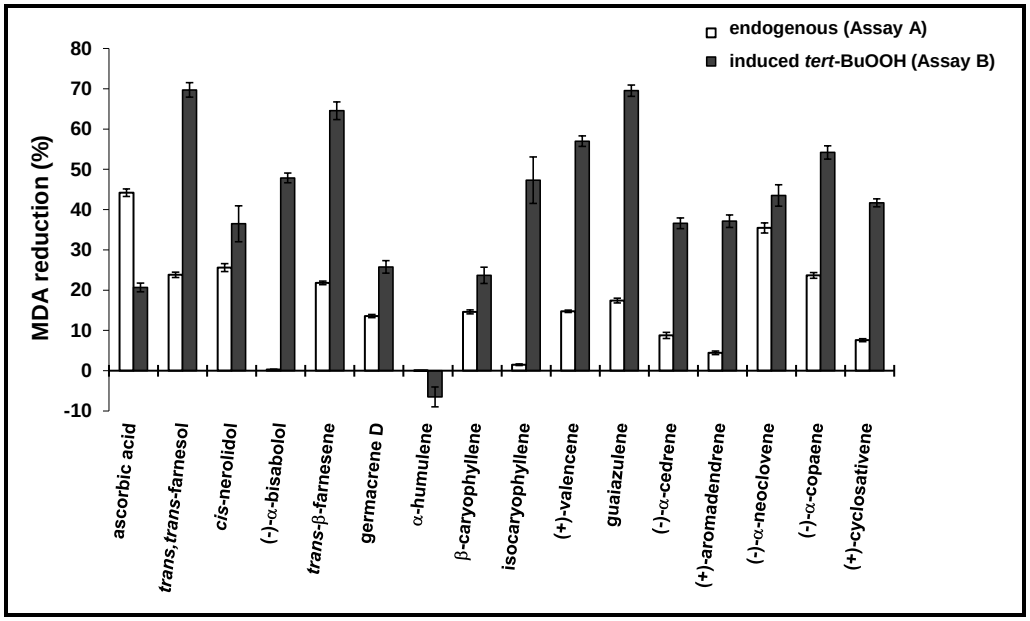
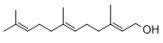
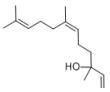
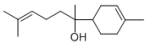
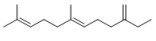


Figure 5.3. Relative hepatoprotection activity of ascorbic acid and sesquiterpenoids (1mM) in endogenous and induced *tert*-butyl hydroperoxide (1mM) assays. Mean activity values with standard errors of mean (SEM) from four independent experiments performed in triplicate in different days are plotted.

The sesquiterpenoids hepatoprotection for assay B ranged from 23.70 % to 69.72 % (Figure 5.3, Table 5.1). *trans,trans*-Farnesol, guaiazulene, and *trans*-β-farnesene showed the highest hepatoprotector effects with 69.72 ±1.82 %, 69.53 ±1.42 % and 64.55 ±2.20 % of MDA reduction, respectively. Lower reduction effects were observed for (+)-valencene (56.97 ±1.31 %), (–)-α-copaene (54.19 ±1.66 %), (–)-α-bisabolol (47.86 ±1.21 %), isocaryophyllene (47.31 ±4.77 %), (–)-α-neoclovene (43.50 ±2.65 %), (+)-cyclosativene (41.68 ±1.01 %), (+)-aromadendrene (37.13 ±1.56%), (–)-α-cedrene (36.60 ±1.33 %), *cis*-

nerolidol (36.50 ±4.47 %), germacrene D (25.77 ±1.55 %), β-caryophyllene (23.70 ±2.02 %) and α-humulene (-6.48 ±2.49%).

Table 5.1- Sesquiterpenic compounds 2D structure, formula and molecular weight. Relative hepatoprotection of ascorbic acid and sesquiterpenoids (1mM) on endogenous (assay A) and *tert*-butyl hydroperoxide (1mM) induced (assay B).

Compound	Structure	Formula	Molecular Weight	Relative hepatoprotection	
				assay A	assay B
Positive control					
Ascorbic acid				44.19 ±0.92	20.67 ±1.08
trans,trans-Farnesol		$C_{15}H_{26}O$	222.37	23.82 ±1.03	69.72 ±1.82
cis-Nerolidol		$C_{15}H_{26}O$	222.37	25.60 ±0.98	36.50 ±4.47
(–)-α-Bisabolol		$C_{15}H_{26}O$	222.37	0.32 ±0.06	47.86 ±1.21
trans-β-Farnesene		$C_{15}H_{24}$	204.36	21.82 ±1.94	64.55 ±2.20
Germacrene D		$C_{15}H_{24}$	204.36	13.55 ±1.50	25.77 ±1.55
α-Humulene		$C_{15}H_{24}$	204.36	0.01 ±0.00	-6.48 ±2.49
β-Caryophyllene		$C_{15}H_{24}$	204.36	14.61 ±1.43	23.70 ±2.02
(–)-Isocaryophyllene		$C_{15}H_{24}$	204.36	1.49 ±0.19	47.31 ±5.77
(+)-Valencene		$C_{15}H_{24}$	204.36	14.77 ±0.49	56.97 ±1.31
Guaiazulene		$C_{15}H_{18}$	198.30	17.42 ±0.61	69.53 ±1.42
(–)-α-Cedrene		$C_{15}H_{24}$	204.36	8.78 ±0.76	36.60 ±1.33
(+)-Aromadendrene		$C_{15}H_{24}$	204.36	4.46 ±0.45	37.13 ±1.56
(–)-α-Neoclovene		$C_{15}H_{24}$	204.36	35.46 ±1.25	43.50 ±2.65
(–)-α-Copaene		$C_{15}H_{24}$	204.36	23.66 ±0.71	54.19 ±1.66
(+)-Cyclosativene		$C_{15}H_{24}$	204.36	7.59 ±0.37	41.68 ±1.01

* Values expressed as a mean ± standard deviation of at least four experiments done in triplicate.

The comparison between results obtained for the assays A and B showed that under oxidative stress conditions the sesquiterpenoids hepatoprotection activity was higher. The decrease of MDA formation in the assay B was 150 times higher compared to the assay A for α-bisabolol (47.86 % and 0.32 %, respectively), while for isocaryophyllene was 30 times higher (47.31 % and 1.49 %, respectively). Other sesquiterpenic compounds showed smaller differences (1 to 8 times higher) though the same tendency was maintained. The results also show that the sesquiterpenoids are able to protect the hepatocytes from hydrophobic oxidants more efficiently than ascorbic acid (which produced a 20.6 ±1.08 % reduction), possibly due to their lipophilicity. However, for endogenous hepatoprotection none of the sesquiterpenic compounds was as efficient as ascorbic acid, as this compound acts mainly in the aqueous phase by increasing the serum oxygen-radical absorbance and inhibiting peroxyl radicals (343).

Most of the hepatoprotective drugs belong to the group of free radical scavengers or antioxidants, and their action involves membrane stabilisation and neutralisation of free radicals (237). To achieve membrane stabilisation the compound should be able to penetrate it, which is a physico-chemical process highly dependent on its solubility and diffusion across the lipid bilayer. Compound size, lipophilicity, and shape are parameters that highly influence that process (344). Since the largest portion of the cell membrane is lipophilic, simple diffusion for lipophilic molecules can be attained with few restrictions. The lipophilicity of the molecule depends on various physical and chemical characteristics, such as molecular surface area, molecular volume and polarity (345). The sesquiterpenoids under study present a lipophilic character with a ALogP (Ghose-Crippen octanol-water partition coeff. (logP)) ranging from 3.6 to 5.7. This fact was determinant when the oxidative stress was induced by *tert*-BuOOH on assay B, since both sesquiterpenic compounds and the membrane-permeant oxidant *tert*-BuOOH (328) have high affinity to the cell membrane lipid fraction.

The results obtained herein are in agreement with the previous study (Chapter 4), since *trans,trans*-farnesol, *cis*-nerolidol and guaiazulene were able to protect the Caco-2 cells when they were submitted to oxidative stress conditions, with the exception of α-humulene that was not active and ascorbic acid that was less active under oxidative stress caused by the hydrophobic oxidant. Moreover, these results are also in agreement with the

data of lipid peroxidation reduction and antioxidant effects determined for some of the sesquiterpenic compounds under study using different methodological approaches. Ruberto & Baratta (243) found that the inhibition of lipid peroxidation follows this order: farnesol (mixture of isomers) > *trans,trans*-farnesol > (±)-α-bisabolol > (+)-valencene > α-cedrene > (+)-aromadendrene > α-humulene.

Guaiazulene exhibits the highest activity in assay B. This compound was reported as protecting against the hepatotoxicity of paracetamol *in vivo* by decreasing its metabolic activation by preventing both cytochrome P450 activity and NAPQI-induced glutathione depletion (251). This property was attributed to the ability of guaiazulene to act as a chain-breaking antioxidant. Additionally, very efficient inhibition by guaiazulene of membrane lipid peroxidation *in vitro* has been reported, which is in agreement with the high activity observed for this compound in assay B (250). Also, (–)-α-bisabolol was shown to protect the gastric mucous membrane of male Swiss mice against the injuries caused by ethanol by reducing lipid peroxidation and increasing the superoxide dismutase activity, which is in agreement with the activity of this compound in the assay B (246).

trans,trans-Farnesol, possessing also high hepatoprotection activity in assays A and B, has been shown to be able to reduce the lipid peroxidation on egg yolk homogenates (243). Moreover, *in vivo* protection against oxidative damage caused by 1,2-dimethylhydrazine in the colon of Wistar rats (247), and against cigarette smoke toxicants in the trachea of Wistar rats (248) has been reported for *trans,trans*-farnesol.

β-Caryophyllene, with mild MDA reduction in both A and B assays, has been reported to have antioxidant activity, which explain its role as neuroprotector (255), has also scavenger ability in relation to hydroxyl and superoxide anion radicals, and also inhibits the enzymes xanthine oxidase and 5-lipoxygenase, which are involved in the initiation of the lipid peroxidation (254). Neuroprotective activity was related to the lipophilicity, shape and electrostatic parameters (255).

Another mechanism of hepatoprotection may involve the interaction of these compounds with the cell defense system. The protection against lipid peroxidation in biological models involves different biochemical molecules such as the endogenous antioxidants (glutathione, ascorbic acid, tocopheryl) and the detoxification enzyme system

(i. e. catalase, glutathione peroxidase, superoxide dismutase, glutathione-S-transferase redox system, quinone reductase) (242). The treatment of cells with an oxidant agent such as *tert*-BuOOH, that is metabolized originating *tert*-butoxyl, peroxyl and methyl radicals, originates a significant decrease in the levels of the biochemical molecules involved in the cell defense mechanism in order to overcome the injury, and consequently, an increase in the lipid peroxidation is observed (330). Different studies reported the increase of glutathione levels and induction of the enzymatic detoxification system by *trans,trans*-farnesol and β-caryophyllene when biological models are pre-treated with this compound before injury by different agents (247, 248, 254).

5.2.2. QSAR model for sesquiterpenoids hepatoprotection

A QSAR model based on three-dimensional molecular descriptors for the fifteen sesquiterpenoids (Tables 5.2 and 5.3) for the prediction of the relative MDA reduction (Figure 5.3 and Table 5.1) was developed.

Table 5.2- Subsets of 3D molecular descriptors, selected by genetic algorithm, of the QSAR regression models reported in this study

Descriptor*	Meaning	Model
GETAWAY		
HATS6v	leverage-weighted autocorrelation of lag 6 / weighted by atomic van der Waals volumes	A
HATS2v	leverage-weighted autocorrelation of lag 2 / weighted by atomic van der Waals volumes	A and B
R1u+	R maximal autocorrelation of lag 1 / unweighted	B
R3u+	R maximal autocorrelation of lag 3 / unweighted	B
R4u+	R maximal autocorrelation of lag 4 / unweighted	A
R6u+	R maximal autocorrelation of lag 6 / unweighted	A
R7u+	R maximal autocorrelation of lag 7 / unweighted	B
R8u+	R maximal autocorrelation of lag 8 / unweighted	B
R1m+	R maximal autocorrelation of lag 1 / weighted by atomic masses	B
R3m+	R maximal autocorrelation of lag 3 / weighted by atomic masses	A
R6m+	R maximal autocorrelation of lag 6 / weighted by atomic masses	B
R4e+	R maximal autocorrelation of lag 4 / weighted by atomic Sanderson electronegativities	A and B
R5e+	R maximal autocorrelation of lag 5 / weighted by atomic Sanderson electronegativities	B
R4p+	R maximal autocorrelation of lag 4 / weighted by atomic polarizabilities	A
WHIN		
G2e	2st component symmetry directional WHIM index / weighted by atomic Sanderson electronegativities	B
Gm	total symmetry index / weighted by atomic masses	A
3D-MORSE		
Mor02m	3D-MoRSE - signal 02 / weighted by atomic masses	B
Mor24p	3D-MoRSE - signal 24 / weighted by atomic polarizabilities	B

*Calculated using Dragon program evaluation version 5.4.

First, the number of descriptors was reduced using GA, resulting in two optimal subsets of molecular descriptors for the assays A and B. Optimal descriptors subsets are shown in the Table 5.2. The MLR models were validated by leave-one-out validation, resulting in the following equations and respective statistical data:

Model A= 66 + 1.1e^{+03}R6u+ - 631HATS2v - 431R4p+ + 288R4e+ - 287Gm - 8HATS6v + 166R4u+ + 219R3m+

SDEC= 0.82,
SDEP= 2.09,
R^2_{LOO}= 0.819,
Q^2_{LOO}= 0.961

Model B= -83 + 1.8e^{+03}R5e+ - 760HATS2v - 531R1m+ + 460R3u+ + 415R1u+ + 332R8u+ - 322R4e+ + 312G2e + 109R7u+ - 66R6m+ + 3Mor24p + 2Mor02m

SDEC= 0.09,
SDEP= 1.45,
R^2_{LOO}=0.972,
Q^2_{LOO}=0.994

The resulting models showed high correlations coefficients, both to calibration (R^2_{LOO}= 0.819 and R^2_{LOO}=0.972, for assays A and B, respectively) and prediction (Q^2_{LOO}= 0.961 and Q^2_{LOO}=0.994), as well as low errors (SDEC and SDEP < 2.09 %).

Table 5.3- Values of 3D descriptors selected by GA as the best subsets for prediction of activity in the assays A and B. Descriptors from three different groups (GETAWAY, WHIN, and 3D-MoRSE) were selected.

	GETAWAY												WHIN		3D-MORSE	
Compounds	HATS2v	HATS6v	R1u+	R3u+	R6u+	R8u+	R1m+	R3m+	R6m+	R4e+	R5e+	R4p+	Gm	G2e	Mor02m	Mor24p
trans-*trans*-Farnesol	0.058	0.055	0.111	0.055	0.028	0.028	0.062	0.018	0.009	0.039	0.042	0.010	0.186	0.204	13.053	0.064
cis-Nerolidol	0.083	0.089	0.104	0.060	0.027	0.021	0.057	0.040	0.017	0.047	0.035	0.013	0.170	0.180	13.879	-0.500
(−)-α-Bisabolol	0.084	0.083	0.102	0.047	0.024	0.027	0.039	0.022	0.019	0.044	0.038	0.015	0.180	0.169	14.520	0.003
trans-β-Farnesene	0.075	0.067	0.130	0.051	0.036	0.030	0.061	0.026	0.010	0.041	0.047	0.017	0.186	0.173	11.110	-0.133
Germacrene D	0.097	0.146	0.102	0.049	0.040	0.035	0.053	0.024	0.018	0.045	0.036	0.017	0.172	0.198	8.211	-0.215
α-Humulene	0.097	0.177	0.106	0.040	0.027	0.023	0.042	0.020	0.014	0.048	0.033	0.016	0.191	0.159	7.395	-0.111
β-Caryophyllene	0.091	0.156	0.099	0.048	0.027	0.022	0.039	0.023	0.012	0.046	0.041	0.014	0.168	0.173	9.108	-0.159
Isocaryophyllene	0.090	0.157	0.104	0.047	0.028	0.023	0.040	0.023	0.013	0.046	0.040	0.014	0.184	0.198	9.134	-0.123
(+)-Valencene	0.081	0.135	0.101	0.047	0.030	0.025	0.041	0.026	0.013	0.049	0.049	0.020	0.189	0.159	11.366	0.159
Guaiazulene	0.089	0.145	0.124	0.078	0.030	0.046	0.038	0.022	0.015	0.068	0.042	0.017	0.179	0.205	11.323	0.446
(−)-α-Cedrene	0.092	0.116	0.098	0.040	0.031	0.040	0.038	0.022	0.022	0.045	0.040	0.018	0.183	0.159	14.125	0.168
(+)-Aromadendrene	0.089	0.175	0.110	0.042	0.030	0.026	0.040	0.022	0.015	0.050	0.043	0.018	0.189	0.198	9.679	-0.077
(−)-α-Neoclovene	0.081	0.140	0.108	0.044	0.051	0.000	0.037	0.023	0.002	0.042	0.044	0.022	0.171	0.191	15.191	0.044
(+)-Cyclosativene	0.101	0.116	0.097	0.060	0.028	0.028	0.045	0.026	0.014	0.048	0.041	0.014	0.176	0.211	14.097	-0.142
(−)-α-Copaene	0.087	0.141	0.101	0.045	0.039	0.029	0.039	0.024	0.016	0.048	0.048	0.018	0.163	0.173	12.640	-0.143

The scatter plots of the experimental data (Relative hepatoprotection obs.) versus the predicted one (Relative hepatoprotection calc.) using the obtained equations are shown in Figure 5.4 A and B for the models A and B, respectively. The agreement observed between the predicted and experimental values confirmed the efficiency of these QSAR methods.

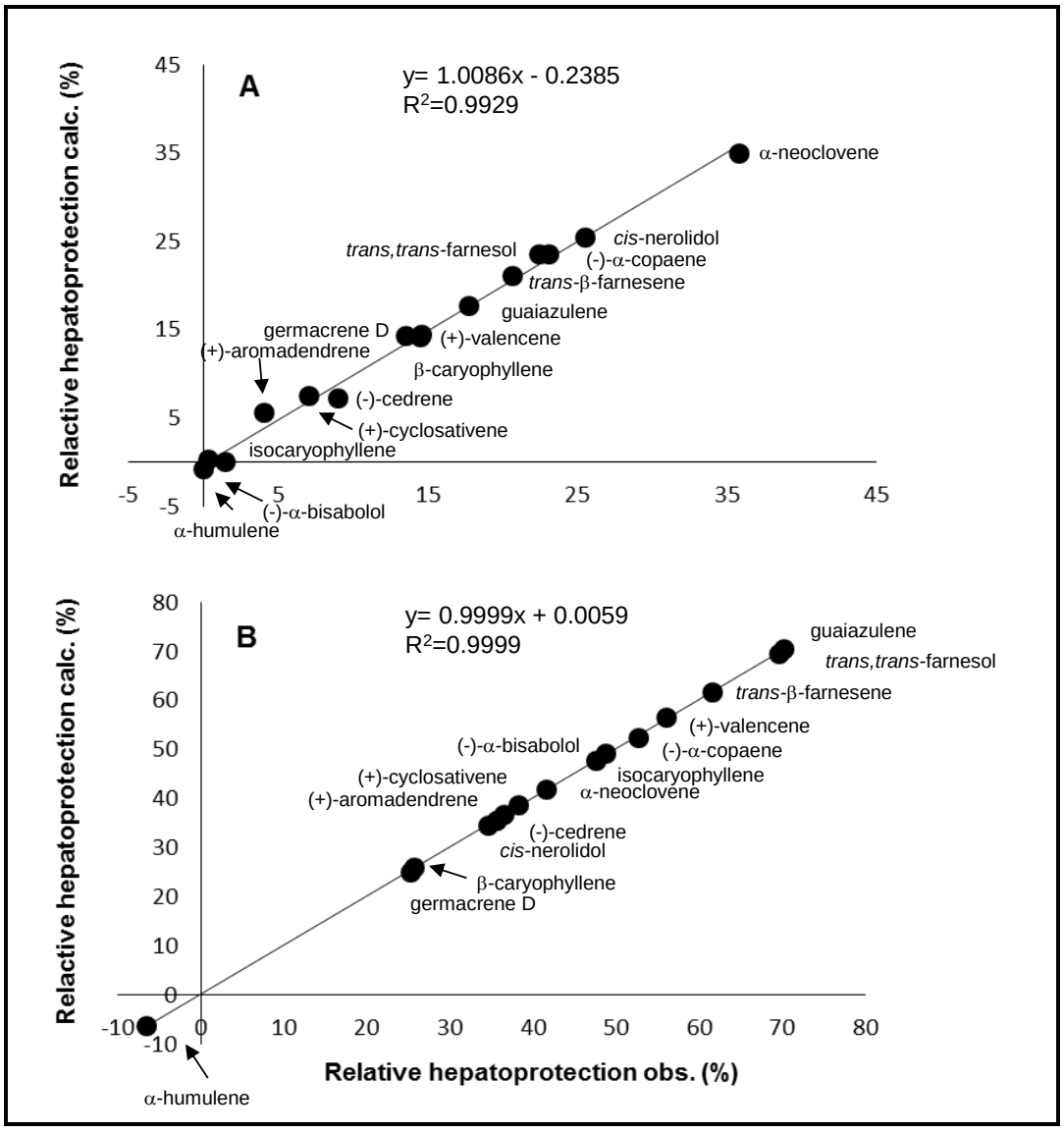


Figure 5.4. Scatter plots of observed *vs.* calculated hepatoprotection for assay A (A) and assay B (B). Results are shown for the cross-validation data. MLR models A and B were calculated using optimized descriptor subsets (Table 5.2).

5.3.3. QSAR model interpretation

According to the obtained models, the most relevant molecular descriptors related to the hepatoprotection properties belong to the three groups: the 3D GEometry, Topology, and Atom-Weights AssemblY (GETAWAY), Weighted Holistic Invariant Molecular (WHIM) and Molecule Representation of Structure based on Electron diffraction (MoRSE) descriptors, and (Tables 5.2 and 5.3). Due to the complexity of 3D descriptors interpretation is usually done on the series of structurally similar compounds (346-348).

GETAWAY descriptors encode both geometrical and topological information. While geometrical information is given by the influence molecular matrix (H-GETAWAY descriptors), the topological one is given by the influence/distance matrix R. This matrix R combines the molecular matrix influence with the molecular inter-atomic geometrical distance weighted by physicochemical properties such as atomic mass, polarizability, van der Waals volume and electronegativity (R-GETAWAY descriptors) (349).

The models obtained for the assays A and B included several R-GETAWAY descriptors, among which descriptors R6u+ and R5e+ had the highest positive regression coefficients, for each model, respectively. Larger values of the autocorrelation descriptors at topological distance (lag) correspond to the most external atoms that are simultaneously next to each other in the molecular space, i.e. terminal atoms located next to other terminal atoms. Moreover, they can be related to the molecular shape, where higher values are associated with compact molecules and lower values are associated with more linear conformations (349). A tendency in the value of the R6u+ descriptor can be observed among the compounds with higher activity in the assay A, indicating that an increase in molecular compactness may be related with the increase of the activity in the assay A. Thus, molecules possessing higher R6u+ values as for example α-neoclovene (R6u+=0.051), (–)-α-copaene (R6u+=0.039) and *trans*-β-farnesene (R6u+=0.036) were more efficient than α-humulene (R6u+=0.027) and (–)-α-bisabolol (R6u+=0.024) that have low values of this descriptor.

The most important descriptor in the model B is R5e+, which is a R index weighted by atomic Sanderson electronegativity. The weighting of the R indexes encode information about substituents differently from unweighted indexes. In this case, largest values of this

descriptor can be expected when high electronegative atoms are situated far from the centre of the molecule at a topological distance of 5 bonds. Since this descriptor had a positive contribution to the model is expected that the hepatoprotective activity increases with the increase of its values. Therefore, compounds with MDA reduction above 50% such as guaiazulene, *trans,trans*-farnesol, *trans*-β-farnesene, (+)-valencene and (–)-α-copaene had higher values of R5e+ descriptor (with values between 0.042 and 0.049) compared to molecules with lower hepatoprotective activity such as α-humulene and germacrene D (R5e+= 0.033 and 0.036). *trans,trans*-Farnesol has a terminal hydroxyl group while *trans*-β-farnesene and (+)-valencene have a terminal alkene, which can explain the higher values of electronegativity observed for R5e+ descriptor. Recently, the antioxidant activity of di(hetero)arylamines derivatives of benzo[b]thiophenes has been related to the descriptors encoding atomic electronegativities, and the effect was evidenced to be due to the presence of electron-donating substituents (350).

The second most important descriptor in both models is HATS2v, which belongs to the H-GETAWAY group. H-GETAWAY descriptors are calculated using diagonal elements of the molecular influence matrix accounting for the relative position of each atom in the 3D molecular space weighted by different atomic properties, in this case atomic van der Waals volume (349). High values of this descriptor indicate higher ramification of the molecules, while low values of this descriptor are typical for more linear molecules. The negative influence of HATS2v on both hepatoprotection models indicates that molecules possessing lower values of this descriptor are more active. The most active compounds in models A and B were *trans,trans*-farnesol, *trans*-β-farnesene, (–)-α-neoclovene, *cis*-nerolidol, (–)-α-copaene, and guaiazulene (HATS2v ranging from 0.058 to 0.089), while α-humulene, the less active compound for both assays, has an HATS2v value of 0.097.

The two WHIM descriptors involved in models A and B, Gm and G2e, respectively, represent different sources of chemical information for the whole 3D molecular structure in terms of size, shape, symmetry, and atom distribution. They are calculated by performing a PCA on a weighted covariance matrix of the centered Cartesian coordinates of a molecule, obtained from different weighting schemes for the atoms (351). G2e descriptor encodes the symmetry of the molecules along the second component

weighted by electronegativity, while Gm is the total symmetry that tends to 1 as the molecule shows a central symmetry along each axis and to 0 when there is a decrease in the symmetry along at least one axis (335). For model A, Gm showed a negative effect, indicating that the reduction of MDA increases with the decrease of symmetry. For instance, the less active compound, α-humulene, has the highest value (Gm=0.191), While compounds with activity higher than 20% showed lower values of this descriptor namely, *trans*-β-farnesene, *trans,trans*-farnesol, (–)-α-copaene, *cis*-nerolidol and (–)-α-neoclovene (with Gm ranging from 0.163 to 0.186).

In model B the hepatoprotection activity increases for more symmetric molecules, considering the second component and an electronegative fragment. Thus, the compounds with more than 50% MDA reduction such as guaiazulene, *trans,trans*-farnesol, *trans*-β-farnesene and (–)-α-copaene, all with high values of the G2e descriptor (values ranging from 0.173 to 0.205), were more active than α-humulene (G2e=0.159).

From the structural features described by the QSAR models the following key observations can be made: 1. (–)-α-neoclovene and (–)-α-copaene have one allylic hydrogen atom that can be abstract giving rise to a more stable radical; 2. (–)-α-copaene can form an extremely stable radical due to the presence of an allylic hydrogen in a tertiary carbon; 3. *cis*-nerolidol, *trans,trans*-farnesol and *trans*-β-farnesene also have additional allylic hydrogens and the first two also have a hydroxyl group from which the hydrogen can be abstracted. Besides, they are polyunsaturated compounds with lipophilic backbone similar to the lipid core of the cell membrane, being particularly susceptible to oxidation damage.

The high activity observed for guaiazulene in assay B can be associated with its rigid and therefore less ramified structure, which is reflected in a relatively low value of HATS2v descriptor, and its symmetry in the second component, reflected by the high value of G2e descriptor. Another important characteristic for the antioxidant activity of guaiazulene is the number of double bounds and their conjugated position, which allows easier free radical stabilization.

5.3. Concluding remarks

This is the first attempt to develop 3D-QSAR models of the hepatoprotective activity of sesquiterpenic compounds with quite different backbone structures using an *in vitro* model system. The developed models allowed extracting relevant information suggesting that sesquiterpenoids possessing more compact molecular structures ((–)-α-neoclovene and (–)-α-copaene), low ramification (*trans*,β-farnesene, *trans,trans*-farnesol and *cis*-nerolidol) and less symmetric (according to Gm- total symmetry of the molecule) (*trans*-β-farnesene, *trans,trans*-farnesol, (–)-α-copaene, *cis*-nerolidol and (–)-α-neoclovene) will be more effective for endogenous hepatoprotection. Otherwise, compounds with electronegative substituents (guaiazulene, *trans,trans*-farnesol, *trans*-β-farnesene, (+)-valencene and (–)-α-copaene), less ramified (*trans,trans*-farnesol, *trans*-β-farnesene, (–)-α-copaene and guaiazulene) and with more symmetry (according to G2e-symmetry considering the second component) with an electronegative terminal fragment (guaiazulene, *trans*-β-farnesene and *trans,trans*-farnesol) seem to be more effective for the induced hepatoprotection. This knowledge can be useful for future valorisation of plants or related materials containing such structures. Finally, it is important to point out that the concentration tested (1 mM) to evaluate the hepatoprotective effects is higher than that usually observed for these compounds in plant materials. Thus, this study supports the current tendencies of valorisation of natural products as a source of bioactive compounds for the formulation of foods and/or nutraceuticals enriched extracts.

Chapter 6- Conclusions and Future Perspectives

Conclusions and Future Perspectives

In this PhD thesis, *Vitis vinifera* L. has been exploited as a potential source of compounds with biological impact, either from the sensorial point of view, as regards to the wine aroma, or with beneficial properties for the human health. The wine is the major product of *Vitis vinifera* L., and it is well known that its properties result from the presence of several biomolecules, being the volatile ones the responsible for the first olfactory impact sensation having a crucial role in the product acceptance and quality. Among the compounds responsible for the wine aroma, the varietal volatile compounds, namely the mono and sesquiterpenic compounds and C_{13} norisoprenoids, can be highlighted due to their pleasant aroma descriptors and low odour thresholds. Thus, considering the particular attributes related to the target varietal volatile compounds, this research was focused on the development of rapid and in-depth methodologies for their assessment in wines, aiming to reduce the time of analysis and associated costs. Experimental parameters related to the sample preparation, chromatography and mass spectrometry conditions, and data processing were considered.

A novel method based on HS-SPME/GC–qMS-SIM enabled the rapid assessment (*ca*. 13 min.) of C_{13} norisoprenoid volatile signature. The GC capillary column of 30 m at 220 ºC was used acting as a transfer line of the components sorbed by the SPME fiber coating to the mass spectrometer, which acts as a sensor for *m/z* fragments 142 and 192. This method was compared to a reference method using the same SIM conditions, but with adequate chromatographic resolution of wine components. After optimization, external calibrations curves for β-ionone were acquired using the *m/z* fragments 142 and 192 and the MS operated with electron impact energy of 20 eV, ion focus of 60 eV and repeller of 30 eV for both reference and rapid methods. Good analytical parameters were obtained for both methods with $r^2 > 0.99$ and quantification limits < 1.6 μg L^{-1}. The quantification of

vitispiranes and TDN above their odour thresholds may indicate their potential contribution for the aroma. Moreover, the C_{13} norisoprenoids quantification in wines by the rapid method is almost 8 times faster than the reference method without losing sensitivity and limit of detection.

The GC×GC-TOFMS combined with SPME showed to be a suitable methodology for the rapid establishment of the target volatile varietal composition of wines. Taking the advantage of the GC×GC structured chromatograms, i. e., organization of the analytes according to their physicochemical properties corresponding to a specific 2D chromatographic spaces, a more accurate tentative identification of FP components could be obtained. The combination of this concept with the use of specific *m/z* fragmens was very useful to eliminate the majority of the non-terpenic and non-C_{13} norisoprenic compounds, i) allowing the definition of a two-dimensional chromatographic space containing these compounds, ii) simplifying the data obtained, and iii) reducing the time of analysis. A varietal classification-reference approach allowed the identification of 170 compounds. From these, 45 compounds were detected in all wines under study (wines from different harvests, Appellations and producers), allowing defining the "FP varietal volatile profile". Fifteen compounds were described for FP variety for the first time, herein accounting with 7 monoterpenoids, 5 C_{13} norisoprenoids, and 3 sesquiterpenoids. Among the identified compounds, hotrienol and α-terpineol, described as FP wine aroma contributors, have been detected in all wines under analysis.

Besides the importance of the target varietal volatile compounds (mono and sesquiterpenoids and C_{13} norisoprenoids) for the wine aroma, the presence of sesquiterpenoids in *Vitis vinifera L.* have been associated with beneficial properties for the human health. Therefore, in this study the antioxidant, anti-proliferative and hepatoprotective activities of sesquiterpenic compounds commonly found in wines and other food matrices were evaluated. Firstly, the antiradical capacity of *trans,trans*-farnesol, *cis*-nerolidol, α-humulene and guaiazulene was evaluated using chemical ($DPPH^{\bullet}$ and hydroxyl radicals) and biological (Caco-2 cells) models. From these, with the exception of α-humulene, all compounds showed antiradical activity in the different assays. Guaiazulene was the most active against $DPPH^{\bullet}$, while *trans,trans*-farnesol and *cis*-nerolidol were more active against the hydroxyl, with IC_{50} values in the range of 0.73 and

1.81 mM. Moreover, at non-cytotoxic conditions (1 mM, 24 ≤ h exposure) these three compounds were able to protect the Caco-2 cells against the oxidative damage caused by *tert*-BuOO$^{\bullet}$. Moreover, all sesquiterpenic compounds showed antiproliferative effect, being guaiazulene and cis-nerolidol the most promising ones. This effect is probably related to their antioxidant properties or to their ability to interfere with cellular enzymes responsible for the cell cycle process.

The hepatoprotection ability of fifteen sesquiterpenic compounds was also assessed, using liver homogenates from Wistar rats. Endogenous lipid peroxidation and induced lipid peroxidation (caused by *tert*-BuOO$^{\bullet}$) were evaluated by measuring the malonaldehyde (MDA) content, as a lipid peroxidation product, using the thiobarbituric acid reactive species test. The pre-treatment of rat liver homogenate with the sesquiterpenic compounds at 1 mM concentration showed that they are capable of reducing the endogenous and induced lipid peroxidation. The compound with the highest efficiency in reducing the MDA for the endogenous assay was (-)-α-neoclovene (35.46%), and *trans,trans*-farnesol, guaiazulene and *trans*-β-farnesene were the most efficient compounds for the induced assay (~70%). The hepatoprotection activity for each compound was related with their molecular descriptors, affording models that allowed quantifying this relationship for both endogenous and induced lipid peroxidation. Models, obtained by genetic algorithm followed by multiple linear regression, presented good statistical parameters with $R^2_{LOO} > 0.82$ and prediction errors lower than 2. A network of effects associated with structural and chemical features of sesquiterpenic compounds such as shape, branching, symmetry, and presence of electronegative fragments, can modulate the hepatoprotective activity observed for these compounds.

In conclusion, the developed methodologies for the quantification of C_{13} norisoprenoids in wines (HS-SPME/GC-qMS-SIM) and the methodology for the varietal volatile characterization of FP wines by GC×GC-TOFMS offer rapid assessment and in-depth information about varietal components from *Vitis vinifera* L., including those with relevant contribution for aroma properties and/or with potential health benefits. Moreover, the results of all the biological assays can be useful for future valorisation of plants or related materials, namely the *Vitis vinifera* L. that present sesquiterpenic compounds with similar chemical structures such as those studied in this thesis. It is important to point out

that the concentration tested (1 mM) to evaluate the antioxidant and hepatoprotective effects is higher than that usually observed for these compounds in *Vitis vinifera* L. and other plant materials. However, due to the lipophilic character of the sesquiterpenic compounds, they can exert cumulative effects, suggesting that the consumption of a diversified diet, rich in this type of compounds, should provide satisfactory concentrations to achieve health benefits.

From the results obtained herein future studies can be proposed:

i) application of the rapid methodologies for wine and or/and other related food matrices characterization such as grapes and musts, for quality control analysis, as a monitoring tool in the winemaking process and to trace their varietal origin.

ii) *in vitro* evaluation of the potential cumulative, potentiation, and additive effects associated to the presence of two or more sesquiterpenic compounds on the antiproliferative activity;

iii) *in vitro* evaluation of the ability of suppress the mevalonate pathway by sesquiterpenes such as guaiazulene and α-humulene;

iv) application of the developed 3D-QSAR models to valorize vegetable-related matrices by prediction of the hepatoprotection activity of their sesquiterpenic components;

v) implementation of *in vivo* tests (animals) to confirm the safety and effectiveness regarding the antiproliferative effect of the sesquiterpenic compounds studied.

Chapter 7- References

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