Universidade do Minho
Escola de Engenharia

Meirielly Santos de Jesus

A biorefinery approach for valorization of vine pruning residue

Julho de 2019

UMinho | 2019 Meirielly Santos de Jesus **A biorefinery approach for valorization of vine pruning residue**

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Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho elaborado sob a orientação do
Doutor José António Couto Teixeira

e da
Doutora Lucília Maria Alves Ribeiro Domingues

Julho de 2019

ACKNOWLEDGMENTS

Na realização da presente tese, contei com o apoio direto ou indireto de múltiplas pessoas e instituições às quais estou profundamente grata. Sem esses contributos esta investigação não teria sido possível. Correndo o risco de injustamente não mencionar algum dos contributos quero deixar expresso os meus agradecimentos:

- Ao meu orientador o Doutor Professor José António Couto Teixeira, pela orientação prestada, pelo seu incentivo, disponibilidade e apoio que sempre demonstrou. Aqui lhe exprimo a minha gratidão.

- A orientadora Doutora Professora Lucília Domingues, pelo seu incentivo, pela sua disponibilidade, pela sua compreensão nos momentos desafiadores e igualmente pelo seu apoio na elaboração deste trabalho.

- Agradeço à Doutora Professora Cristina Pereira Wilson, por aceitar o desafio de me orientar e realizar um trabalho tão complexo num curto espaço de tempo. Por permitir realizar os ensaios de atividade anticancerígeno no Laboratório de Biologia Animal, que me foram extremamente úteis. Muito obrigada.

- Aos colegas de trabalho Lina, Ricardo e Cristina Carvalho, que colaboraram em algumas etapas dos trabalhos desenvolvidos no CEB e no Departamento de Biologia. Obrigada pela parceria, auxilio no trabalho laboratorial, incentivo e revisões dos artigos.

- À Diana, pelo auxílio na preparação das soluções e compras de reagentes para os trabalhos. Muito obrigada!

- À Alóia, pela ajuda preciosa dada no ínício da tese com auxílio no desenvolvimento do meu trabalho laboratorial. O meu muito obrigada.

- À Tina, quero de uma forma especial, expressar o meu muito obrigada...por tudo. Pela parceria no trabalho ao longo destes anos, pela forma amiga e generosa com que sempre me incentivou e ajudou, e pelo estímulo sentido após cada conversa, que me faziam “carregar baterias”.

- Aos amigos que ganhei durante esta jornada aqui em Portugal, bem como aqueles que deixei no Brasil que, mesmo com a distância, permanecem presentes.... Agradeço a amizade e o carinho que sempre me disponibilizaram.

- Aos colegas dos Departamentos de Engenharia Biológica e de Biologia, pela amizade e companheirismo.

- Sou muito grata a todos os meus familiares pelo incentivo recebido ao longo destes anos.

- Ao meu irmão Denilson...por ser meu irmão. Obrigada por cuidares e fazeres companhia à nossa mãe nos meus momentos de ausência.

- À minha mãe Maria Fernandes e à minha avó Judite, que sempre acreditaram no meu potencial e que mesmo distantes se fizeram presentes a me incentivar. Sem vocês, não teria tido esta oportunidade de lutar pelos meus sonhos e objectivos.

- Aos meus sogros Rui Fernando e Adelaide pelas palavras de incentivo e encorajamento valorizando o meu potencial, nos momentos mais difíceis. Obrigada pelo amor, alegria e atenção sem reservas...

- Ao Rui Filipe, meu marido, agradeço por todo o amor, carinho, admiração, dedicação e pela presença incansável com que me apoiou ao longo do período de elaboração desta tese. Agradeço ainda a parceria, paciência demonstrados nos meus momentos menos bons, por sempre acreditar nas minhas capacidades.

- O meu profundo e sentido agradecimento a todas as pessoas que contribuíram para a concretização desta tese, estimulando-me intelectual e emocionalmente. A todos os meus sinceros agradecimentos.

Este trabalho foi realizado durante uma bolsa de estudos apoiada pela Cooperação Internacional Programa Ciência sem fronteiras na Universidade do Minho, financiado pelo CNPq- Conselho Nacional de Desenvolvimento Cientifico e Tecnológico do CNPq, agência do Ministério da Ciência, Tecnologia e Inovação do Brasil.

This work was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2019, the MultiBiorefinery project (POCI-01–0145-FEDER-016403) and the BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

UMA ABORDAGEM DE BIOREFINERIA PARA A VALORIZAÇÃO DE RESÍDUOS DE PODAS DE VIDEIRA

O continente europeu é considerado o maior produtor mundial de vinho e apresenta uma grande biodiversidade de castas e alto potencial de mercado. No entanto, ao longo dos diferentes estágios do processo de produção de vinho, muitos resíduos são gerados (resíduo de poda de videira-VPR, engaço e bagaço) e geralmente não são utilizados, sendo descartados. Portugal gera anualmente cerca de 178 mil toneladas de resíduos. O interesse em redirecionar o uso desses materiais lignocelulósicos tem aumentado devido à superprodução, grande disponibilidade e baixo custo. A utilização da biomassa lignocelulósica derivada da indústria vinícola poderá beneficiar economicamente os produtores, causando impacto benéfico ao meio ambiente. Esses subprodutos podem ser submetidos a pré-tratamentos (físicos, químicos e biológicos), para fracionamento e conversão a diferentes compostos com alto interesse industrial, reduzindo o desperdício do agronegócio e aumentando o lucro industrial. Os VPR, além de fonte de açúcar, possuiem alto valor nutritivo, servem como fonte compostos fenólicos, capazes de ser fracionados e aproveitados na bioconversão e na obtenção de produtos com alto valor agregado por fermentação seguindo um conceito de biorrefinarias. Poucos trabalhos foram feitos em relação a este conceito direcionado às podas de videira, portanto, diferentes fatores ainda precisam ser estudados e otimizados. A necessidade de desenvolver um pré-tratamento adequado para o fracionamento de VPR foi recentemente identificada, com o objetivo de aumentar a suscetibilidade durante a sacarificação enzimática ou reduzir a produção de inibidores para o processo de fermentação. Neste contexto, este projeto otimizou o fracionamento de VPR através de pré-tratamentos hidrotérmicos que é um tratamento suave e consiste no uso de água e altas temperaturas para fracionamento de biomassa lignocelulósica e para a produção de diferentes produtos com interesse industrial seguindo o conceito de biorrefinaria. Portanto, neste trabalho foi aplicada a auto-hidrólise em dois estágios sequenciais (severidade de 4,60) para melhorar a suscetibilidade do material liginocelulósico à hidrólise enzimática, onde se obtiveram 99 % de conversão de glicose, atingindo 96 % de bioetanol após 48 h de sacarificação e 8h de fermentação separada e 83 % em sacarificação e fermentação simultânea, recuperando 13,1kg de etanol/100 kg de VPR nas melhores condições. Através dos pré-tratamentos de auto-hidrólise foi possível obter 13,6 kg/100 kg de VPR de xiloligossacarídeos, 3,1 kg/100 kg de compostos fenólicos, 13,1kg/100 kg de etanol e 27,0 kg/100 kg de lignina em quatro correntes separadas. Além disso, nesta tese estudou-se o potencial bioativo das podas de videira, otimizando a extração de compostos fenólicos através de soluções hidroalcoólicas em diferentes métodos (aquecimento convencional-CHE, micro-ondas assistido - MAE e aquecimento ómico-OH em duas intensidades distintas- campo elétrico baixo - LEF e campo elétrico intermédio - IEF). Bem como, a caracterização dos extratos obtidos *in vitro*, capacidade antioxidante, atividade antimicrobiana e efeito antiproliferativo em linhas celulares cancerígenas. No tratamento CHE foi possível observar que as melhores condições de extração foram 46 % de etanol/água, 80 °C durante 120 minutos. Entretanto, o tratamento MAE mostrou ser mais eficiente, devido à redução do tempo de extração comparando com o CHE. Os extratos obtidos através da extração MAE apresentaram também melhores resultados em comparação com a autohidrólise, embora este tratamento mostre maiores concentrações de compostos fenólicos totais. Quanto aos tratamentos por OH, foi possível observar maiores concentrações de apigenina, acido elágico e quercetina comparando com os outros tratamentos, além de identificar a presença da hisperedina que não foi identificada nos extratos obtidos pelos tratamentos por auto-hidrólise, CHE e MAE. Devido ao seu potencial bioativo, os extratos obtidos por OH foram testados, avaliando a capacidade de inibir o crescimento microbiano em fungos e a citotoxicidade dos extratos em células tumorais humanas de mama, hepatocelular e colo retal, bem como em células não-carcinogénicas de cólon retal mostrando resultados positivos quanto às duas atividades estudadas. Estes resutados mostraram que VPR apresenta grande potencial de valorização, e que através de pretratamentos, se pode direcionar as diferentes frações extraídas, para produção de diferentes produtos que abrangem diversos setores industriais. Desta forma, a valorização deste resíduo pode contribuir para alcançar modelos econômicos mais sustentáveis e circulares, dando origem a modelos de produção altamente competitivos que impactariam positivamente o cenário econômico.

Palavras-chave: Resíduo de poda de videira, conceito de biorrefinaria, biomassa lignocelulósica, bioetanol, compostos bioativos.

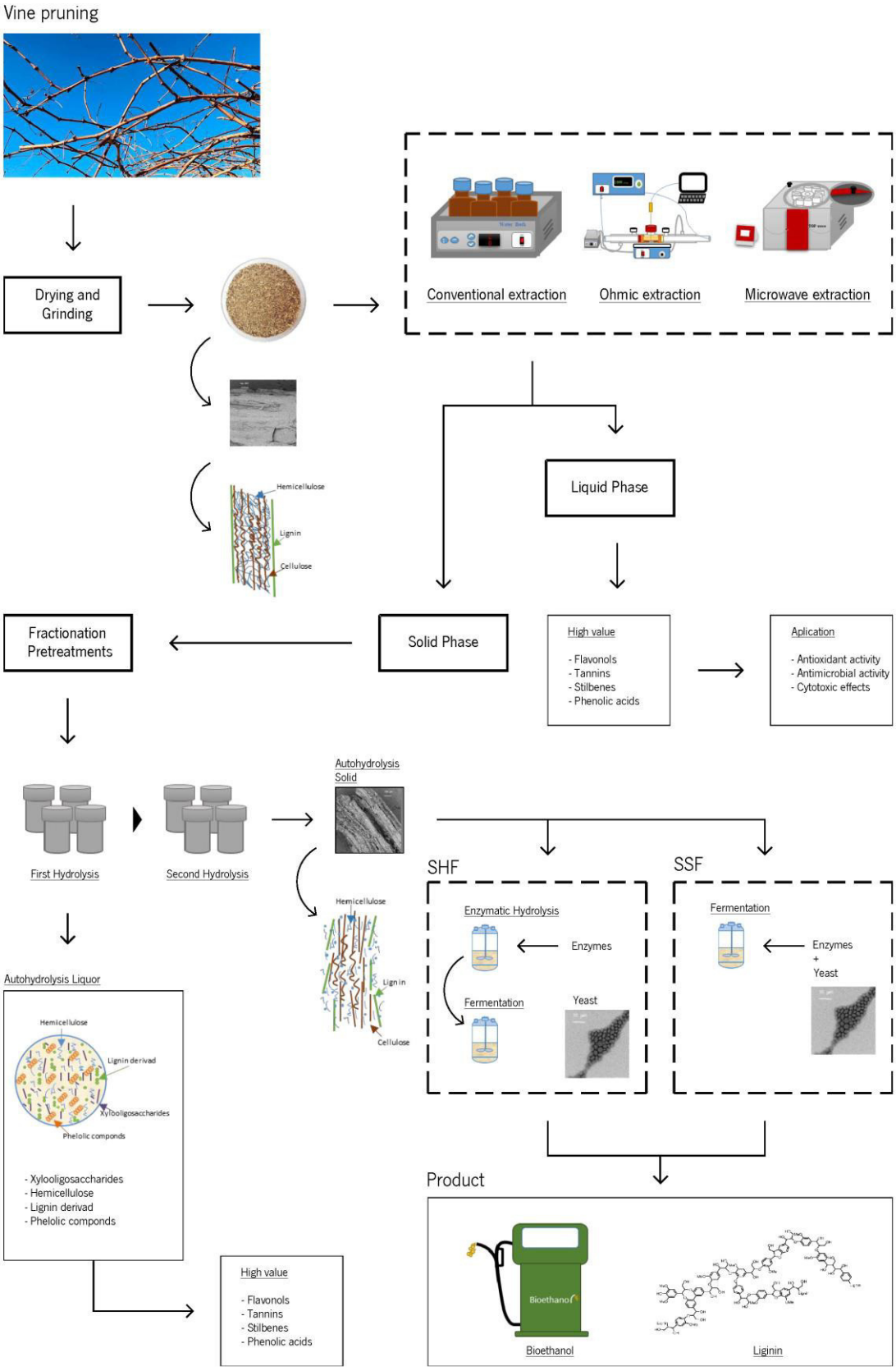
ABSTRACT

A BIOREFINERY APPROACH FOR VALORIZATION OF VINE PRUNING RESIDUE

The European continent is considered the world's largest producer of wine and features a large biodiversity of grape varieties and high market potential. However, throughout different stages of wine production process many wastes are generated (Vine Pruning residue, Stalks and Grape Marc) and usually are not used, being discarded. Portugal annually generates approximately 178 thousand tons of waste. In this context, the interest in redirecting the use of these lignocellulosic materials has increased due to overproduction, great availability and low cost. The utilization of lignocellulosic biomass derived from the wine industry will benefit economically the producers, while causing beneficial impact to the environment. These byproducts can be submitted to pretreatments (physical, chemical and biological), for its fractionation and conversion to different compounds with high industrial interest, reducing the waste of agribusiness and increasing industrial profitability. The Vine Pruning Residue (VPR) besides being a source of sugar, has high nutritional value, serves as a source phenolic compounds, is capable of being fractionated and used in bioconversion and in obtaining products with high added value per conversion following the concept of biorefinery. Few works have been done in relation to this concept directed to vine pruning, therefore different factors still need to be studied and optimized. The need to develop adequate pretreatment for VPR fractionation has recently been identified, with the aim of increasing susceptibility during enzymatic saccharification or reducing the production of inhibitors for the fermentation process. In this context, this project optimized VPR fractionation through hydrothermal pretreatments, which is a mild treatment and consists of the use of water and high temperatures for lignocellulosic biomass fractionation and for the production of different products with industrial interest following the concept of biorefinery. Therefore, in this work, two sequential stages autohydrolysis (severity of 4.60) were applied to improve the susceptibility of lignocellulosic material to enzymatic hydrolysis, where 99% glucose conversion was obtained, reaching 96% bioethanol after 48h saccharification, and 8h of separate fermentation and 83 % in saccharification and simultaneous fermentation, recovering 13.1kg of ethanol 100kg of VPR under the best conditions. Through the autohydrolysis pretreatments it was possible to obtain 13.6 kg/100kg of xyloligosaccharides VPR, 3.1 kg/100kg of phenolic compounds, 13.1 kg/100kg of ethanol and 27.0 kg/100 kg of lignin in four separate streams.In addition, this thesis studied the bioactive potential of vine pruning, optimizing the extraction of phenolic compounds through hydroalcoholic solutions in different methods (conventional heating - CHE, assisted microwave - MAE and ohmic - OH heating at two different intensities- low electric field extraction - LEF and intermediate electric field extraction - IEF). Moreover, the characterization of the obtained extracts was done *in vitro* in terms of antioxidant capacity, antimicrobial activity and antiproliferative effect on cancer cells. In the CHE treatment it was possible to observe that the best extraction conditions were 46 % ethanol/water, 80°C for 120 minutes. However, MAE treatment proved to be more efficient due to the reduction of extraction time compared to CHE. Extracts obtained by MAE also showed better results compared to auto-hydrolysis, although this treatment shows higher concentrations of total phenolic compounds. Regarding the treatments by OH, it was possible to observe higher concentrations of apigenin, ellagic acid and quercetin compared to the other treatments, besides identifying the presence of hesperidin that was not identified in the extracts obtained by the treatments by autohydrolysis, CHE and MAE. Due to their bioactive potential, the extracts obtained by OH were tested for their ability to inhibit microbial growth in fungi and the cytotoxicity of the extracts in human tumor breast, hepatocellular and colon rectal and non-carcinogenic colon rectal cells was evaluated, showing positive results for both activities studied.The results obtained in this study showed that vine pruning is a waste with great valorization potential which, through pretreatment, can direct the different fractions extracted to the production of different products that cover different industrial sectors. Thus, VPR valorization may contribute to achieving more sustainable and circular economic models, giving rise to highly competitive production models that would positively impact the economic scenario.

Keywords: Vine Pruning residue, concept of biorefinery, lignocellulosic biomass, bioethanol, bioactive compound.

GRAPHICAL ABSTRACT



OUTPUTS

PAPERS IN PEER-REVIEWED INTERNATIONAL JOURNALS

Jesus, Meirielly S., Romaní, A., Genisheva, Z., Teixeira, J. A., Domingues, L. Integral valorization of vine pruning residue by sequential autohydrolysis stages. *Journal of Cleaner Production*, 168, 74-86, 2017.

Jesus, Meirielly S., Genisheva, Z., Romaní, A., Pereira, R. N., Teixeira, J. A, Domingues, L. Bioactive compounds recovery optimization from vine pruning residues using conventional heating and microwave-assisted extraction methods. *Industrial Crops and Products*, 132, 99-110, 2019.

PAPERS UNDER PREPARATION

Jesus, Meirielly S., Ballesteros, C., Genisheva, Z., L. F., Pereira, R. N., Carvalho, A. C., Pereira-Wilson, Teixeira, J. A, Domingues, L. Ohmic heating polyphenolic extracts from vine pruning residue with enhanced biological activity (submitted).

Jesus, Meirielly S., Romaní, A., Genisheva, Z., Ballesteros, L. F., Teixeira, J. A, Domingues, L. *Evaluation of vine pruning residue for the production of biofuels, biopolymers, antioxidants and biocomposites following the concept of biorefinery (manuscript in preparation).*

ABSTRACTS AND PROCEEDINGS IN INTERNATIONAL CONFERENCES

Romaní, A., **Jesus, Meirielly S.**, Teixeira, J. A., Domingues, L. (06/2016). Bioethanol production from vine pruning residue by sequential steps of autohydrolysis. BIOIBEROAMÉRICA 2016 - Salamanca, Spain.

Jesus, Meirielly S., Romaní, A., Domingues, L., Teixeira, J. A. (09/2016) Biorefinery approach for the valorization of vine pruning residue. XXI Congresso Brasileiro de Engenharia Química (COBEQ 2016). Fortaleza, Brazil.

Jesus, Meirielly S.; Genisheva, Z., Romaní, A., Teixeira, J. A., Domingues, L. (12/2017) Extraction of polyphenols from vine pruning residues optimization. Congress of Microbiology and Biotechnology 2017 (Microbiotec'17). No. P-027, Porto, Portugal.

Jesus, Meirielly S.; Genisheva, Z., Romaní, A., Pereira, R. N., Teixeira, J. A., Domingues, L. (09/2018) Hydroalcoholic extraction of polyphenol compounds assisted by a microwave digester from residues of vine pruning. 12th European Symposium on Biochemical Engineering Sciences (ESBES 2018). Lisbon, Portugal.

Jesus, Meirielly S., Ballesteros, L. F., Pereira, R. N., Genisheva, Z., Teixeira, J. A, Domingues, L. (09/2019) Extraction of bioactive compounds from vine pruning residues by using ohmic heating and conventional heating techniques. 2nd Food Chemistry Conference. Seville, Spain.

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SYMBOLS

Ω Empirical parameter (14.75 K)

A_0 Absorbance of control (DPPH or ABTS)

A_i Absorbance of extracts or liquor

B Biomass concentration (g/L)

C Cellobiose concentration (g/L)

CV cell viability (%)

E_a Activation energy (J/mol)

ESR Enzyme to solid ratio (FPU/g)

$EtOH_0$ Ethanol concentration at the beginning of the fermentation (g/L)

$EtOH_f$ Ethanol concentration at the end of the fermentation (g/L)

F Glucan fraction in dry biomass (g/g)

G Glucose concentration (g/L)

GGC Glucan to glucose conversion (%)

Ia Inhibition activity (%)

IC_{50} Concentration of sample or standard that can inhibit 50% of DPPH, ABTS (mg/mL) or 50 % of cell viability (µg/mL)

LSR Liquid to solid ratio (g/g)

R Ideal gas constant (J/mol K)

R_0 Reaction ordinate (min)

S_0 Severity (dimensionless)

SY Solid yield (g autohydrolyzed VPR/100 g of VPR, oven dry basis)

$T_{(t)}$ Represent the temperature profile in the heating stage (K)

$T'_{(t)}$ Represent the temperature profile in the cooling stage (K)

t_F Time needed for the whole heating–cooling period (min)

T_{MAX} Target temperature (K)

t_{MAX} Time needed to achieve the target temperature (min)

T_{REF} Reference temperature (373.15 K)

Y_{Et} Ethanol yield (%)

V_1 cell viability of control sample

V_2 cell viability of extracts

ABBREVIATIONS

ABTS 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid

ATCC American Type Culture Collection

ATR Attenuated total reflectance

CHE Conventional heating extraction

DMEM Dulbecco's Modified Eagle's Medium

DMSO dimetil sulfóxido

DPPH 2,2-diphenyl-1-picryl-hydrazyl-hydrate

DTG Derivative Thermogravimetric

ECCA European Collection of Authenticated Cell Cultures

FBS Fetal Serum Bovine

FE (II) ferrous equivalents

FPU Filter Paper Units

FT-IR Fourier-transform infrared

GAE Gallic Acid Equivalent

H hours

HMF Hydroxymethylfurfural

HPLC High-performance Liquid Chromatography

HVED High-voltage electrical discharge

IEF Intermediate electric field

LEF Low Electric Field

MEM Minimum Essential Medium

Min Minute

MTT Methylthiazolyldiphenyl-tetrazolium bromide

OH Ohmic heating

PEFE Pulsed electric field extraction

PFE Supercritical fluid extraction

PLE Superheated liquid extraction

PSE pressurize solvent extraction

PTFE polytetrafluoroethylene

RPMI-1640 Medium Roswell Park Memorial Institute-1640

RT Room temperature

SD Standard deviation

SEM Standard error of the mean

SEM Scanning Electron Microscopy

SFE supercritical fluid extraction

SHF Separate Hydrolysis and Fermentation

SHLE superheated liquid extraction

SSF Simultaneous Saccharification and Fermentation

SWE subcritical water extraction

TAPPI Technical Association of the Pulp and Paper Industry

TGA Thermogravimetric Analysis

UAE ultrasound assisted extraction

UHPLC Ultra-high-performance liquid chromatography

VPR Vine Pruning Residue

CHAPTER I

AIM AND OUTLINE OF THE THESIS

This chapter introduces the main topics of the thesis and its main goals. The outline of the thesis is also presented.

1.1 RESEARCH AIMS

Nowadays, environmental concerns and reliance on fossil resources encourage the scientific community to devote great efforts to find renewable sources for energy and platform chemicals production for a circular bioeconomy. Agri-food lignocellulosic residues represent the main sources of biomass; moreover, vine pruning residues (VPR) are low cost biomass that can be a sustainable alternative with great biotechnological potential. However, to have access to lignocellulosic compounds it is necessary to apply pretreatment or combination of different pretreatments to allow this fractionation and the use of all lignocellulosic fractions. Nevertheless, there is not a sole strategy to attain this proposal since this will depend on the composition and structure of the source material. Thus, it is necessary to search for environmentally friendly pretreatments to effectively fractionate biomass, improving performance and productivity in the fermentation process. The lignocellulosic biomass derived from Vine Pruning Residue (VPR) due to its chemical composition, is a promising material for the production of different products of great industrial interest.

Therefore, this thesis proposes to use the different fractions obtained from VPR processing for the production of different products with industrial interest, following the concept of biorefinery, promoting a circular economy through the valorization of residues from agrifood sectors.

The main aims of this thesis were:

1. Chemical characterization of VPR;

2. Study of VPR fractionation by hydrothermal pretreatment;

3. Study of enzymatic susceptibility and ethanol production from treated VPR;

4. Comparison of different phenolic and post-fractionation extraction techniques;

5. Characterization of phenolic compounds tanned after fractionation;

6. Comparison of different phenolic compounds extraction techniques;

7. Evaluation of antioxidant, an antimicrobial and antitumor activity of VPR extracts.

1.2 OUTLINE OF THE THESIS

To address the above-mentioned objectives, this thesis has been structured in 6 chapters as follows:

Chapter I – In the current chapter, a general overview of the current demand for more sustainable processes, and particularly, the need for valorization processes of residues generated by the wine industry, which are generated in high amounts and have high biotechnological potential, is addressed. The main motivations for this work are highlighted, while the aims and structure are further referenced.

Chapter II - It presents a comprehensive review of previous studies developed using VPR as raw material for the production of products of industrial interest. In particular, a critical analysis of recent advances in the development of different treatments' strategies, processes and applications of VPR within the concept of biorefinery is addressed.

Chapter III – It presents a study of the integral valorization of VPR, where the characterization and fractionation of VPR using sequential treatment of autohydrolysis is performed. The study of the enzymatic susceptibility and optimization of ethanol production from VPR is included, as well as the recovery of compounds of high industrial interest such as xyloligosaccharides, phenolic compounds and lignin.

Chapter IV – Optimization of extraction of bioactive compounds from VPR using conventional CHE and microwave-assisted heating. The extracts characterization is addressed regarding its phenolic content and antioxidant activity.

Chapter V – It presents the study of the extraction of bioactive compounds using the extraction by ohmic heating in two different electric field intensities, comparing with the CHE extractions at room temperature. In addition, the phenolic profile characterization and evaluation of antioxidant, antimicrobial activity in fungi and antitumor activity in diverse human cell lines is discussed.

Chapter VI – It reports the final conclusions of this work, as well as some perspectives on future research based on the critical assessment of the results obtained throughout this work.

CHAPTER II

GENERAL INTRODUCTION

ABSTRACT

The European continent is considered the largest producer of wine worldwide and features high market potential. However, from the different stages of wine production process, many wastes are generated (Vine Pruning, Stalks and Grape Marc) and usually these residues are not used, being discarded. Portugal annually generates approximately 178 thousand tons of wine production wastes. In this context, the interest in redirecting the use of these lignocellulosic materials has increased due to overproduction, great availability and low cost. The utilization of lignocellulosic biomass derived from the wine industry would benefit economically the producers, while causing beneficial impact to the environment. These byproducts can be submitted to pretreatments (physical, chemical and biological), for separation of different compounds with high industrial interest, reducing the waste of agro-industrial activities and increasing industrial profitability. The Vine Pruning Residue (VPR) besides being a source of sugar, has high nutritional value, serves as a source of proteins and phenolic compounds. These compounds can be obtained by bioconversion following a concept of biorefinery. In this context, the current options for the valorization of VPR will be hereby presented and evaluated.

Keywords: Vine Pruning Residue, biorefinery concept.

The information presented in this chapter will be part of a review article that is under preparation.

Jesus, Meirielly S., Genisheva, Z., Ballesteros, L. F., Teixeira, J. A, Domingues, L. Evaluation of vine pruning residue for the production of biofuels, biopolymers, antioxidants and biocomposites following the concept of biorefinery (*manuscript in preparation*).

CONTENTS

2.1 INTRODUCTION

Nowadays, environmental concerns and dependence of fossil resources encourage the scientific community to devote great efforts in the pursuit of renewable sources for the manufacturing of energy and products. Strategies and policies for the development of sustainable growth based in the efficient use of natural resources are one of the main targets to achieve a Bioeconomy (European Commission, 2018). In fact, it is expected an increase of biomass demand for bioenergy and biomaterials requirements as the result of growing population by 2050 (Black et al., 2016). One of the main sources of renewable biomass remains in lignocellulosic materials due to its high regeneration capacity, as the most widespread and abundant carbon source in the world (Fatma et al., 2018).

Lignocellulosic materials are composed by 0 - 10 % of non-structural (including ashes, extracts, other components in minor amounts) and structural compounds (40 to 50 % of cellulose, 15 to 25 % of hemicellulose and 15 – 25 % of lignin) (Gullón et al., 2011; IOV, 2013). The source of lignocellulosic materials can include residues of forestry or crop production as well as energy crops using whole plant biomass (Romero-García et al., 2014). Residues from agro-food activities represent great part of lignocellulosic biomass generated in the world. The waste management associated to these activities is normally related with an environmental impact since some of these residues are burned releasing unhealthy compounds (Devesa-Rey et al., 2011). In addition, these residues are enriched with bio-based compounds with a potential interest in the industry. Therefore, the valorization of these residues could contribute to attain a sustainable industrial sector by means of an alternative scheme of food processing (Lin et al., 2014).

Among food industries, the wine making sector represents one of the most important in the world. In this context, 7.4 million hectares of vineyard were dedicated to grape crop reaching a production of 77.8 million tons in 2018. European Union has stood out in world production of grapes (31.3 %) and wine (177.5 million hectoliters), among the European countries, Italy (18.7 %), France (16.6 %) and Spain (15.2 %) had the highest production in the year 2018 where the wine industry is an important part of the economy and culture (IOV, 2013). Nevertheless, Portugal is the second largest producer in the world as a percentage of vineyard area in relation to the total area of the country achieving 194 thous and of hectares (kha) and 2.10 %, losing only to Italy that exhibits 695kha (2.31 %). The third country in the ranking presenting 967kha (1.91 %), is France that has an area of 786 kha (1.22 %). According to the International Organization of Vine and Wine, currently Portugal appears as the 11 th wine producing country and the 9 th exporter in the world, these statistics are

promising for a country with 9221 kha (Fraga et al., 2017; OIV, 2018). Generally, 40 % of the wine production wastes are burned in soil, resulting in greenhouse gas emission such as CH_4, N_2O, CO and NO_2, and heat to the atmosphere without recovery of resources, often creating environmental problems (APA, 2014; Raposo et al., 2016). According to Ruiz-Moreno and coworkers (Ruiz-Moreno et al., 2015) there is a great possibility to increase the added value of waste generated by wineries around the world, promoting studies on this matter.

The residues of the winemaking industry could be an interesting lignocellulosic biomass to be used in a biorefinery scheme due to their enriched composition in antioxidant compounds (phenolic compounds derived from lignin processing) (Peixoto et al., 2018), xylooligosaccharides with potential use as prebiotic (Dávila et al., 2019) and biofuels from hydrolysis and fermentation of cellulose to ethanol (Corbin et al., 2015).

In this chapter, identification and composition of lignocellulosic residues derived from winemaking industry, as well as the pretreatments used for the valorization of its main fractions were approached. In addition, different strategies for the integral use of this biomass were highlighted.

2.2 BIOMASS RESIDUES FROM GRAPE AND WINE

The harvest of vine and the process of wine elaboration (known as vinification) involves seasonal stages in which large amount of residues (including product enriched with a biodegradable content) are generated (Devesa-Rey et al., 2011). Among these wastes, it is important to highlight: biomass derived from pruning of vine, grape stalk from desteming stage, grape marc or grape pomace obtained from the pressing stage. Briefly, grapes are harvested mechanically or by hand. Stems are removed before fermentation in the process called desteming. Fermentation is carried out by yeast already present on the grapes, or inoculated. After days of maceration (contact between grape skins and must), the grape must is pressed to separate wine from grape skins, resulting in grape marc (Torchio et al., 2016). These by-products are the main waste generated in the wine industry, presenting themselves as a raw material of lignocellulosic source of great potential for the industry. Figure 2.1 summarizes the main stages of vinification where lignocellulosic residues are generated.

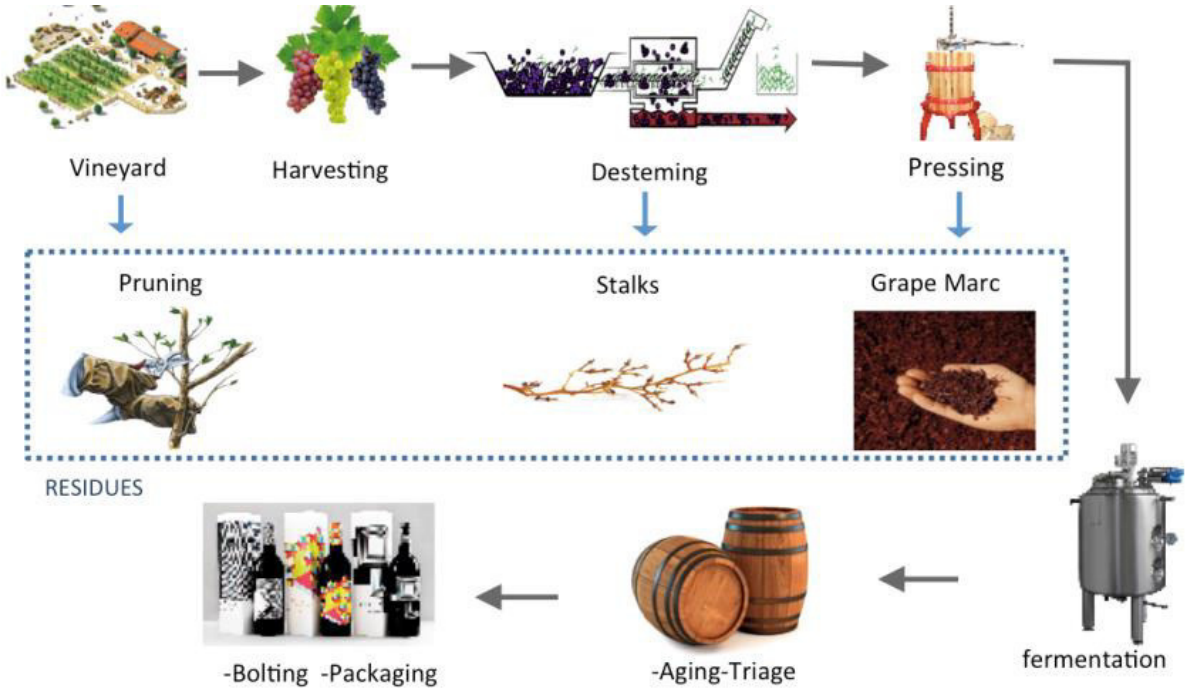

Figure 2.1 Main stages of vinification where lignocellulosic residues are generated.

In Portugal, the cultivation of vineyards produces about 2.500 kg of waste per hectares per year, from those around 50 % of production is residue and only a small volume is used. About 15% of the grapes produced in Portugal is directed to the wine industry, which generates up to 1.5 million tons of solid waste per year, consisting of different by-products being more prominent grape marc, stems and pruning (APA, 2014). Table 2.1 shows the chemical composition of lignocellulosic materials of the wine industry. As it can be seen, a variability in composition is an important aspect of these residues as well as their seasonality.

Table 2.1 Composition of lignocellulosic materials (in %) present in vine waste (Casazza et al., 2016; Gloria et al., 2019; Mendes et al., 2013; Ping et al., 2011; Prozil et al., 2012; Buratti et al., 2015; Dávila et al., 2017, 2016).

Residues	Cellulose	Hemicellulose	Lignin	Ashs	Proteins	Extractives
Grape marc	20.8	2.5	29.8	4.2 - 7.8	12.1 – 18.8	39.1
Stalks	20 - 36	21 - 24.5	17.4 - 34	3.9 - 7	6.1	1.7 - 2.3
Vine pruning	33 - 39.9	5.8 - 27	26.7 - 46.8	2.6 - 3.0	2.0 - 2.7	3.1 - 12.2

2.2.1 Grape marc

The Grape marc is the main waste product of winemaking and accounts for 20 to 30 % of the total weight of the grapes (Brenes et al., 2016). Every 6 liters of wine generates approximately 1kg of grape marc, and it is estimated that 10 million tons per year are produced annually (Zhang et al., 2017). Grape marc consists of seeds (38 - 52 %), skin (38 - 52 %), residual pulp and stems (2 - 10 %) (Brenes et al., 2016; Corbin et al., 2015). The chemical composition and amount of grape marc depends on several factors such as grape cultivation, pressing process and fermentation steps (Beres et al., 2017). In the production of red wine, the grapes are pressed in the fermentation process and the juice and the bagasse are fermented together. However, in white wine production, the bagasse is removed after the pressing and does not take part of the fermentation process (Beres et al., 2017). This differential process leads to dissimilarities in the white and red bagasses, with the bagasse from white vinification presenting higher content of pulp and residual sugars (12 - 25 %) than the one resulting from red vinification (11 – 22 %) (Mendes et al., 2013).

The grape marc contains 20.8 % of cellulose, 12.5 % of hemicellulose, 7.2 to 18.8 % of proteins, 29.8 % of lignin and 4.2 to 7.8 % of ash (w/w) (Table 2.1) (Casazza et al., 2016; Gloria et al., 2019; Mendes et al., 2013). According to Corbin and co-workers (Corbin et al., 2015), the grape marc composition depends on the grape variety, processing method, environmental conditions and the proportion of the skin, seeds and cuttings. The compounds extracted from waste sources are of great importance in several sectors, such as food, cosmetics, pharmaceuticals, biofertilizer and energy (Beres et al., 2017; Campanella et al., 2017; Garrido et al., 2019; Ibn Ferjani et al., 2019).

2.2.2 Grape stalk

Grape stalks (grape cluster skeleton) are an important by-product of the wine industry that is present in the bagasse, which represents about 3 to 6 % (w/w) of processed material (Garcia-Perez et al., 2010). The bagasse consists of 30.3 to 36 % cellulose, 21 to 24.5 % hemicellulose and 17.4 to 34 % lignin, 3.9 to 7 % ash, 6.1 % protein, 1.7 to 2.3 % of extractives, (Prozil et al., 2012; Pujol et al., 2013). According to Pujol and co-workers (Pujol et al., 2013) grape stems are a promising source of valuable natural products. In this sense, some authors have developed studies to obtain polyphenolic compounds, as adsorbent for the removal of caffeine from aqueous solution, activated carbon, among others (Portinho et al., 2017; Teixeira et al., 2018).

2.2.3 Vine pruning residue

The vine pruning residue is an abundant lignocellulosic material, consisting of a significant percentage of cellulose 33 to 39.9 % followed by lignin Klason 26.7 to 46.8 % and hemicelluloses 5.8 to 27 %, 2.6 of 3.0 % ashes, 2.0 to 2.7 % of proteins and 3.1 to 12.2 % extractives (Table 2.1). Due to its chemical composition, availability and low cost, several works have been developed in the sense of fractionating and valorizing this residue to serve as a biologic basis for different processes and products following the biorefinery concept (Buratti et al., 2015; Dávila et al., 2017, 2016).

2.3 FRACTIONATION OF LIGNOCELLULOSIC BIOMASS FOR ITS REVALORIZATION: A BIOREFINERY APPROACH

2.3.1 Pretreatment as first step of a biorefinery for valorization of vine pruning residue

The bioconversion of lignocellulosic biomass into biofuels, biochemical and biomaterials by a biorefinery scheme is one of the potential approaches for sustainable growth based on a suitable use of renewable sources. The integral use of lignocellulosic materials can be divided into two groups: global use (integrated use without previous separation) and fractionated use (selective separation of lignocellulosic components prior to its use). Figure 2.2 graphically shows the valorization of lignocellulosic materials following a biorefinery concept. The global use includes methods of combustion, gasification, pyrolysis or liquefaction. Combustion is a chemical reaction between two or more reactants (fuels and oxidants) with release of energy in the form of heat. Gasification is based on the chemical transformation of solid or liquid fuels into a synthesis gas, which is capable of being ignited immediately for energy production, or serves as raw material for industrial products. The pyrolysis is based on the thermal decomposition of the lignocellulosic material in the controlled presence of gases synthesizing products in different stages as gas (H, CO, CO_2 and hydrocarbons of low molar mass), liquid (gaseous condensation product) and solid (biochar). The liquefaction is based on the conversion of a gas into a liquid. On the other hand, the fractionated approach of lignocellulosic biomass is present as alternative to global use by selective fractionation in the main compounds (cellulose, hemicellulose and lignin). Nevertheless, its main challenge is the difficulty of components separation without chemical degradation of some of them. These processes can be classified considering the main fraction obtained: delignification methods (solubilization of lignin), hydrolysis of polysaccharides (solubilization of polysaccharides) and combination of both.

The first step in a biorefinery is a process to alter the recalcitrant structure of lignocellulosic biomass also known as pretreatment. A physical, chemical, physico-chemical or biological pretreatment plays a significant role in the fractionation of lignocellulosic materials to obtain suitable fractions to produce the desired compounds or value added products. To follow the concept of biorefinery, we must first carry out a physical treatment. It consists of reducing the size by milling or milling dry biomass, improving processing efficiency.

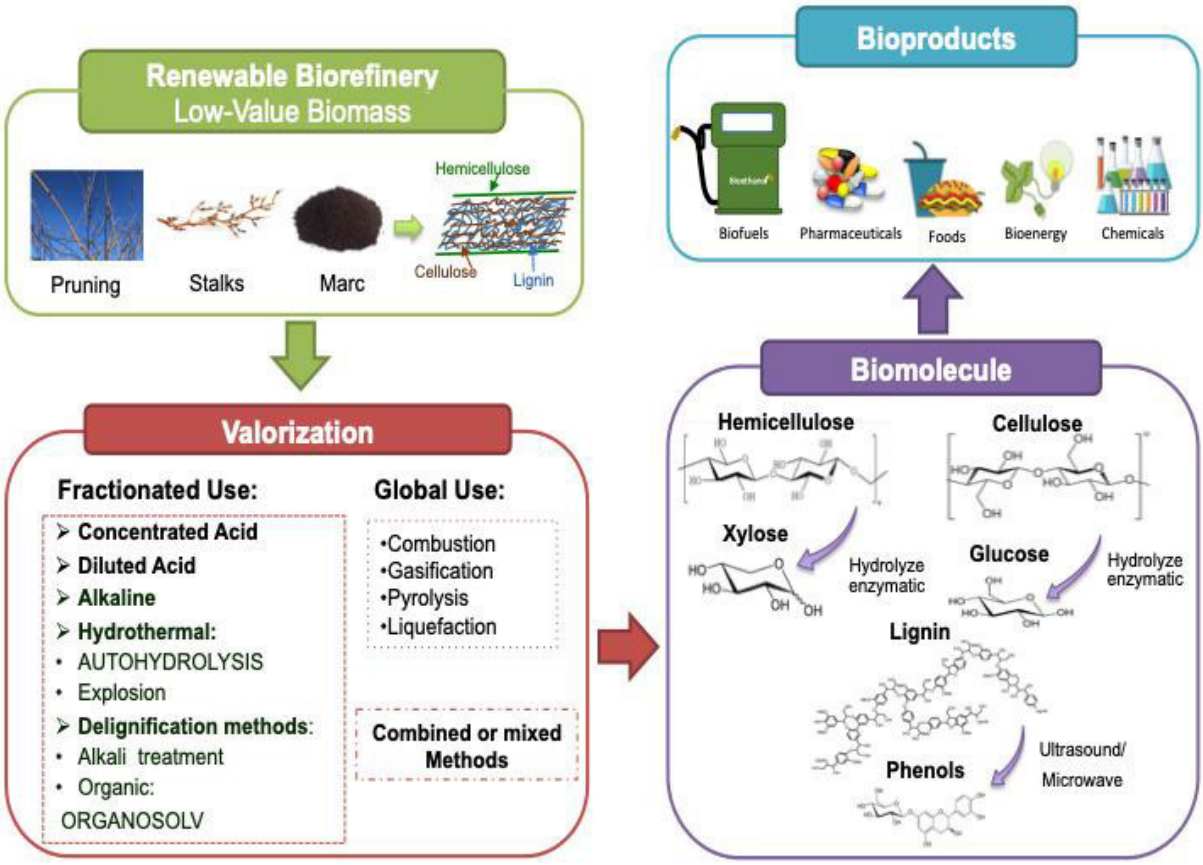

Figure 2.2 Valorization of lignocellulosic biomass generated in wine processing following the biorefinery concept.

Other physical treatments such as ultrasound, vaporization and extrusion, autoclaving, voltage electrical discharges, among others, are also widely exported to alter the structure of lignocellulosic materials, increasing the surface area of the biomass. Chemical pre-treatment consists of the use of different catalysts, such as acids, alkali and oxidizing agents. The most commonly used acid for the fractionation of lignocellulosic materials is H_2SO_4. It converts hemicelluloses into reducing sugars. Alkaline pretreatment involves the use of bases such as NaOH that has been extensively studied, which is responsible for breaking down the lignin structure of the biomass, leading to cellulose swelling, partial decrystallization of cellulose resulting in improved accessibility of the enzymes to cellulose and

hemicellulose. The physico-chemical pretreatment consists of thermal treatments such as liquid hot water, autohydrolysis, steam explosion, among others. This process solubilizes the components of the lignocellulosic material according to pH, moisture content and temperature. Biological treatments are based on the use of biological agents, such as fungi, which act as biocatalysts with the function of selectively degrading lignin while preserving cellulose. The enzymatic hydrolysis also has the function of catalyzing fermentable sugars from insoluble cellulose or hemicellulose releasing it into the medium (Rajha et al., 2018; Vecino et al., 2017). The objectives are to increase the surface area and porosity of the substrate, reducing the crystallinity of cellulose and disrupt the heterogeneous structure of cellulosic materials. So far, no single method of pretreatment was found to meet all these requirements; instead a combination of different methods may be applied (El Achaby et al., 2018; Pérez-Rodríguez et al., 2016; Rajha et al., 2018). An "ideal" pretreatment should satisfy these requirements:

I. Simple and economical operation
II. Limited requirements of energy, process water and chemicals
III. Limited corrosion
IV. Ability to alter the structure of lignocellulosic material
V. Selectivity towards polysaccharides losses
VI. High recovery of valuable hemicellulose-derived products
VII. Limited production of undesired degradation products
VIII. Production of substrates with high cellulose content and susceptibility towards enzymatic hydrolysis
IX. Generation of high quality lignin or lignin-derived products
X. Limited generation of wastes

In basis of this approach, residues of wine making have been used as raw material for the production of value added products such as xylitol, ethanol, antioxidant compounds, etc. Vine pruning (VPR) is a promising lignocellulosic biomass, due to its high sugar content, low cost and abundance in Europe. Most free phenolic compounds are present in cell vacuoles. However, it is in the cell wall that lignin, flavonoids and insoluble polyphenols are conjugated to sugars, carbohydrates, organic acids, proteins and polysaccharides. However, to access to these compounds one or more pre-treatments are required generally followed by enzymatic hydrolysis to release the fermentable sugars. Table 2.2 reports published work on the processing of vineyard waste and resulting products.

Table 2.2 Treatments of VPR for the production of different products following the concept of biorefinery.

Treatments	Products	References
Acid and alkaline hydrolysis	Phenolic compounds	(Max et al., 2010)
Autohydrolysis	Phenolic compounds	(Gullón et al., 2017)
CHE and β-cyclodextrin	Phenolic compounds	(Rajha et al., 2015)
Alkaline hydrolysis and HVED	Phenolic compounds	(Rajha et al., 2014)
SHLE, MAE and UAE	Phenolic compounds	(Delgado-Torre et al., 2012)
CHE and MAE	Phenolic compounds	(Moreira et al., 2018)
MAE	Phenolic compounds	(Piñeiro et al., 2017)
Enzymatic hydrolysis, HVED and alkaline hydrolysis	Phenolic compounds and proteins	(Rajha et al., 2018)
PSE and PFE	Phenolic compounds	(Zachová et al., 2018)
CHE	Phenolic compounds	(Sáez et al., 2018)
-	Enological additives	(Cebrián-Tarancón et al., 2019)
CHE, SLDE, PSE and MAE	Viticultural biostimulant	(Sánchez-Gómez et al., 2017b)
CHE	Foliar fertilizer	(Sánchez-Gómez et al., 2016a)
Autohydrolysis	Prebiotic oligosaccharides	(Dávila et al., 2019)
Hydrolysis acid	Biosurfactants	(Vecino et al., 2017)
Dilute acid hydrolysis, delignification and enzymatic hydrolysis	Biosurfactants	(Cortés-Camargo et al., 2016)
Steam explosion and enzymatic hydrolysis	Bioethanol	(Buratti et al., 2015)
Alkaline hydrolysis and enzymatic hydrolysis	Bioethanol	(Cotana et al., 2015)
Alkaline hydrolysis	Biocomposite	(Vecino et al., 2015)
UAE and enzymatic hydrolysis	Biogas	(Pérez-Rodríguez et al., 2016)
Alkaline hydrolysis, bleaching and acid hydrolysis	Cellulose nanocrystals for nanocomposite materials	(El Achaby et al., 2018)
-	Wood chips and ashes	(Giorio et al., 2019)
Pyrolysis	Ultra-microporous adsorbents	(Manyà et al., 2018)
Pyrolysis	Biochar	(Azuara et al., 2017)

2.3.2 Phenolic extraction and composition of vine pruning

Although it represents a small fraction, the extractives have high commercial value, since they consist mainly of phenolic and volatile compounds, among them stilbenes (ε-viniferine, trans-resveratrol, trans-piceid), flavonoids (catechin, epicatechin, vanillin, luteolin) and phenolic acids (caffeic acid, gallic acid, p-coumaric acid, ferulic acid) (Moreira et al., 2018; Piñeiro et al., 2017; Raposo et al., 2016). According to Sánchez-Gómez and co-workers (Sánchez-Gómez et al., 2016b), lignin can release low molecular weight phenolic compounds, alcohols, aldehydes, ketones or acids. In this sense, several studies employ different methods to obtain bioactive compounds such as conventional heating (CHE), microwave-assisted extraction methods (MAE), high temperature hydrothermal treatment, ultrasonic-assisted extraction (UAE), alkaline hydrolysis treatments, pulsed electric field extraction (PEFE), solid-liquid dynamic extraction (SLDE), pressurized solvent extraction (PSE), supercritical fluid extraction

(SFE), superheated liquid extraction (SHLE), subcritical extraction of water (SWE), pressurized liquid extraction (PLE), and combined treatments such as SFE and PSE, alkaline hydrolysis and enzymatic hydrolysis assisted by pretreatment with physical by high-voltage electric discharges (HVED), among others. In addition, the use of vine lignin for the extraction of phenolic compounds (ferulic acid, p-coumaric acid and other phenolic compounds) was also evaluated using a combination of two pre-treatments (acid and alkaline hydrolysis). The acidic prehydrolysis (3% H_2SO_4 for 15 min at 130 °C, liquid solid ratio 7.64: 1 g/g) allowed conversion of the major polysaccharides into monosaccharides. The resulting solid from the acid prehydrolysis was subjected to an alkaline hydrolysis (4-12% NaOH, for 30-120 min, 50-130 °C) to promote delignification and the liquid phase was subjected to phenolic profile analysis. The main hydroxycinnamate acids were ferulic and *p*-camaric presenting concentrations of 141.0 and 31.5 mg/L, respectively. The hydroxybenzoic acid that presented the highest concentration was gallic acid with a concentration of 164.4 mg/L (Max et al., 2010). Some authors have used physical treatments such as PEF, HVED shocks and UAE to extract polyphenol and proteins. There was a significant increase in the phenolic compounds extraction in both treatments, although at different energy intensities. The treatment that obtained better results with lower energy expenditure was by HVED with energy input of 254 kJ/kg, where it caused greater damage in the cellular structure of the treated VPR. The highest yields of total phenolic content (TPC) were 34.5 mg of gallic acid equivalent (GAE) per g of VPR with 89 % purity and 4.4 mg/g proteins (Rajha et al., 2014). The physical treatment by HVED was combined in another work with alkaline (delignification) and biological treatments (enzymatic hydrolysis) in VPR to obtain different products, such as polyphenols, reducing sugars and soluble lignin. The TPC (3.7 mg GAE/g VPR), reducing sugars (110 mg/g VPR) and soluble lignin (1.5 %) presented higher yields when high voltage electric shocks were applied before of the enzymatic hydrolysis also improving the subsequent delignification process where it reduced the lignin by 10 %. This combination of treatments also positively influenced concentration of individual phenolic compounds such as ferulic acid (186 μg/g VPR), resveratrol (26 μg/g VPR) and *p*-coumaric acid (140 μg/g VPR). These results show the efficiency of the use of HVED shocks combined with enzymatic hydrolysis, which favors the extraction of phenolic compounds in the delignification process (Rajha et al., 2018).

In addition, some studies suggest the application of high pressure technologies in VPR as a good alternative for the extraction of polyphenolic compounds. Some authors have evaluated the potential of two varieties of VPR (Touriga Nacional and Tinta Roriz) for extraction of bioactive compounds by comparing three different methods, MAE, SWE and CHE. The Tinta Roriz variety showed better results

for flavonoids (18.7 mg epicatechin equivalents (EE)/g VPR) in the treatment by subcritical extraction of water, and TPC (32.1 mg GAE/g VPR) for microwave extraction.

These methods produced extracts with higher concentrations of bioactive compounds, where the major phenolic compounds identified were gallic acid (175 mg/100 g), catechin (592 mg/100 g), myricetin (281 mg/100 g) and kaempferol-3-O-rutinoside (125 mg/100 g) (Moreira et al., 2018; Zachová et al., 2018). Other high pressure techniques were also applied to the extraction of VPR stilbenes, such as SFE and PLE. These extraction methods were compared with the ethanolic extraction in Soxhlet which reached 231 mg/100 g of *trans*-resveratrol, 156 mg/100 g of *trans*-ε-viniferin and 69 mg/100 g of r2-viniferin. The stilbene yields obtained by PFE (85 - 210 mg/100 g trans-resveratrol, 20 - 92 mg/100 g *trans*-ε-viniferin and 1 - 18 mg/100 g r2-viniferine) were lower than the yields obtained by PLE and Soxhlet, these differences are attributed to the molecular dimensions where the solubility is reduced according to the dimensions of the solute molecules. The extracts obtained by PLE yielded 240 - 258 mg/100 g of *trans*-resveratrol, which were higher when ethanol was added at 10 MPA and 80 - 100 °C, with extraction times and solvent consumption lower than those used in extraction with Soxhlet. However, r2-viniferine 10 - 70 mg/100 g was greater than or equal to the yield obtained in Soxhlet when using temperatures of 100 °C or less. Nevertheless, a decline was observed when the extraction was performed above 100 °C probably due to thermal degradation. However, when combined with PSE and PFE treatments it was possible to obtain a yield of 10 – 172 mg/100 g *trans*-resveratrol, 7 – 99 mg/100 g *trans*-ε-viniferin and 0 - 40 mg/100 g r2-viniferine these values were lower than those obtained by Soxhlet and PLE. Therefore, although high pressure extraction techniques are more economical and efficient for the extraction of phenolic compounds of VPR, further studies are necessary to optimize parameters combination (Zachová et al., 2018).

It is described in the literature that the hydrothermal treatments can be an environmentally friendly alternative to obtain extracts with high content of high value-added phenolic compounds. From extracts obtained by hydrothermal treatments and ethyl acetate extraction, extract yield was obtained between 0.9 and 3.8 g extract/100 g VPR, with maximum TFC yield of 0.9 g rutine equivalents (RE)/100 g of VPR and TPC of 1.6 g of GAE/100 g of VPR at the temperature of 215 °C. In this study, the compounds identified were derived from sugar and lignin, probably due to the severity of the treatments. The major compounds identified were vanillin (0 - 3.5 mg/100 g extract), acetovanilone (0 - 8 mg/100 g extract), guacylacetone (0 - 3.1 mg/100 g extract), syringaldehyde (3.7 - 3.5 mg/100 g extract) extract) and acetosyringone (2.7 - 12.9 mg/100 g extract) (Gullón et al., 2017). However,

further studies are needed to optimize the parameters used for the extraction of bioactive VPR compounds using environmentally sound methodologies using non-toxic solvents such as hydroalcolic mixtures.

2.3.3 Applications of VPR extracts in agriculture and pharmacological properties

Due to their great potential, the bioactive compounds of the VPR were explored in search of different and innovative applications. Some authors have proposed the aqueous extracts by CHE of VPR (Airén variety) as a wine biostimulant, which are efficient as foliar fertilizers, which increase the amino acid content of the wine (Sánchez-Gómez et al., 2017b), other studies have evaluated the efficiency of aqueous extracts of VPR (Muscat variety) previously treated by toasting as leaf biostimulants in the Arién variety, where it was observed that the production of grapes presented higher yield, produced wines with lower alcolic content and with higher contents of aroma compounds like norisoprenoids (β-damascenona), vanillin derivatives (vanillin, acetovanilone) and volatile phenols (guaiacol, syringol) (Sánchez-Gómez et al., 2016a).

Cebrián-Tarancón and co-workers (Xavier et al., 2013), evaluated the release of phenolic compounds of VPR (Airén and Cencibel) cultivars, roasted and unroasted, in two concentrations (4 and 12 g/L) during hot maceration times in wine models composed of ethanol/water (12.5/87.5 v/v) and 5 g/L of tartaric acid, adjusted to pH 3.5 with 1M NaOH. Where the concentrations of vanillin or guaiacol were higher than the wine odor limits, depending on the variables applied, as well as high concentrations of trans-resveratrol and ellagic acid. Due to the high transfer rate of compounds, it can be affirmed that the VPR has oenological capacity that can increase the sensorial and functional quality of wines. Another study evaluated the effect of VPR addition of the cultivars Airén and Cencibel as oenological additives. VPR were previously crushed in pellets and granules, in the concentration of 12 g/L, put in contact with wines during different vinification stages. In this study, the (-)-epicatechin and (+)-catechin compounds were elevated in comparison to the control wines. For white wines Airén guaiacol had higher concentrations and *trans*-resveratrol increased its concentration to 4 mg/L. As for the red wines Cencibel, the compound that presented the highest concentration was the vanillin with values four times higher than its odor threshold in all grapevine cultivars at all times of addition and independent of the grape variety, shape and moment of addition. The β-ionol was detected only in wines in which VPR was added after the fermentation process. In this sense, the use of VPR as an

inovating alternative to modulate the chemical composition of wines, elaborating distinct wines, thus connecting viticulture and oenology in a new concept of circular viticulture, is preferred (Cebrián-Tarancón et al., 2019).

The antifeedant activity (against insects: *Spodoptera littoralis* Boisd (Lepidoptera: Noctuidae), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (polyphagous/olyphagous insects chewing) and the aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), antioxidant and phytotoxic (*Lactuca sativa* cv. and *Lolium perenne* seeds), of the aqueous extract of VPR were also investigated. This study proved that the extracts presented significant antifeedant activity against *L. decemlineata*, phytotoxic activity against *L. perenne* and stimulated the root lengthening of *L. sativa* (Sánchez-Gómez et al., 2017a).

Over the years the emergence of drug resistant microorganisms in different environments has been increasing, this fact has reduced the efficiency of antibiotics. Due to the high presence of high value compounds of VPR extracts, some authors evaluated extracts obtained by hydrothermal treatments and extraction with ethyl acetate for its antioxidant potential and antimicrobial activity against six strains of gram positive and negative bacteria (*Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Salmonella sp.*), from extracts obtained by hydrothermal treatments and extraction with ethyl acetate with extract yield between 0.95 and 3.80 g of extract/100 g of VPR. In this study, the identified compounds were vanillin, acetovanilone, guacylacetone, syringaldehyde and acetosyringone. The antimicrobial activity of the extracts presented minimal inhibitory concentration and minimal bactericidal concentration between 5 - 20 mg/mL. Thus, the results obtained showed that VPR extracts obtained by autohydrolysis and extraction by ethyl acetate showed great potential of bioactive compounds with antimicrobial and antioxidant activity (Gullón et al., 2017). Other authors evaluated the inhibition of α-amylase, antioxidant activity and antimicrobial activity of extracts of VPR (Cultures Tinto Ruiz and Touriga Nacional) by MAE, SWE and CHE against gram negative bacteria (*E.* coli and *E. coli* ESA37 resistant to cephalosporins), gram positive bacteria (*Streptococcus mitis* and *S. mitis* ESA65 from Lactam Resistance) and against yeasts (*Candida albicans* ATCC 10231 and *C. albicans* from amphotericin resistance). The Touriga Nacional cultivar was associated with higher antioxidant activities with FRAP values of 24.3 mg of ascorbic acid equivalent (AAE)/g of VPR and of DPPH of 35.3 mg of trolox (TE)/g VPR, both extracts obtained by SWE. However, for the protective effect on hemolysis induced by AAPH2,2'-azobis-2-amidinopropane and the antimicrobial activity the extracts of the cultivar Touriga Nacional obtained by MAE showed better results ranging from 0.6 - 4.0 mg of extract/mL for minimal inhibitory concentration, 1.5 - 6.0

mg extract/mL for minimal lethal concentration, and IC_{50} of 9.59μg/mL for inhibition of oxidative hemolysis (Moreira et al., 2018). Thus, the results showed that the VPR extracts presented great potential of bioactive compounds with antimicrobial, antioxidant and inhibitory activity against the α-amylase and acetylcholinesterase enzymes, and could be used as adjuvants in the treatments of diseases such as Alzheimer's and diabetes. In addition, the extracts obtained through MAE extraction presented better results in comparison with autohydrolysis, although the showed higher concentrations of total phenolic compounds. This difference is probably due to the phenolic composition present in each extract, according to the severity of the treatment. The extracts obtained by autohydrolysis presented higher concentrations of lignin-derived compounds whereas in the extracts obtained by MAE the majority compounds are derived from the cell wall, another factor that may have influenced is the synergy of the complex mixture of the bioactive compounds. Recent studies have tested the antioxidant and antiproliferative activities of stilbenes isolated from extracts: *(E)*-ε-viniferine, *(E)*-resveratrol, *(E)*-piceatanol, ampelopsin A, vitisin B, palidol, *(E)*-δ-*(E)*-ω-viniferine, *(E)-trans-cis*-benzole C, isorhapontigenin, scirpusina A, and a new isomer called isoscirpusine A, the results obtained in this study showed that the oligostilbenoids isolated from VPR have potential benefits to health (Sáez et al., 2018).

2.3.4 Application of polysaccharides from vine pruning in the production of bioproducts

There are reports in the literature proposing the use of oligosaccharides obtained by the acid hydrolysis (H_2SO_4) of grapevine pruning in the production of adsorbent compounds for the treatment of agroindustrial effluent. It was verified that the non-encapsulated VPR hydrolyzate removed 27.8 %, while the encapsulated VPR hydrolyzate in calcium alginate spheres removed 77.3 % of the dyes. In view of these results, it can be stated that VPR are potential biocomposites in the treatment of industrial effluents (Vecino et al., 2015). Other authors studied the production of glycolipopeptide biosurfactants by *Lactobacillus paracasei* from the hemicellulosic fraction obtained by acid pretreatment (H_2SO_4) followed by alkaline delignification (NaOH), where they obtained about 70.7 % cellulose, 1.7 % hemicelluloses and 25.5 % lignin. After the enzymatic hydrolysis the glucose yield was 47 %, the ability to reduce the surface tension of 25.1 mN/m and the minimum concentration to maintain the lower surface tension of an aqueous solution of 1.35 mg/mL of biosurfactant, these values represent an advantage from an industrial point of view, due to their greater efficiency (Vecino et al., 2017). The fraction of VPR cellulose previously fractionated by alkali treatments (NaOH 4%), bleaching (from

acetate buffer and aqueous sodium chlorite v:v), followed by an acid hydrolysis (NaOH) process was also exploited for the production of cellulose nanocrystals for the development of nanocomposite materials. The obtained cellulose nanocrystals presented a needle shape with an average length of 456 nm and an average diameter of 14 nm, cellulose with high crystallinity index (82 %), thermal stability comparing with nanocomposites obtained from other raw materials and the same process of extraction and greater capacity of reinforcement raising the mechanical resistance of nanocomposites based on biopolymer (El Achaby et al., 2018).

Recent studies have investigated the probiotic activity of VPR oligosaccharides obtained by the autohydrolysis treatment (severity of 4.69), concentrated by ultrafiltration (1kDa to the concentration ratio by volume of 5.1) and purified by ion exchange resin (IRA-96). The purified oligosaccharides (99 %) were submitted to gastrointestinal digestion *in vitro*, simulating the three stages of digestion (mouth, gastric digestion and small bowel conditions) and prebiotic effect (fermented in vitro with human feces). The results showed that the oligosaccharides obtained were resistant to gastrointestinal digestion. However, when the undigested mixture was subjected to fermentation in human feces for 48 h, it was verified that 80 % of the oligosaccharides were consumed, in addition to increasing the *Bifidobacterium* population by 14 %. These results have demonstrated efficiencies of the use of VPR oligosaccharides treated as a prebiotic constituent with functional properties important to human health (Dávila et al., 2019).

2.3.5 Evaluation of vine pruning for the production of biofuels

Previous studies have shown that VPR was also exploited as an energy source for boilers in the wine industry, replacing pine pellets with VPR chips. Where combustion tests were carried out in a biomass boiler and evaluated the viability of stable steady state combustion and the environmental impact. The gases emitted during the combustion presented values lower than the limits required by the European legislation, however the particles were higher than the established limit, but the large and medium sized boilers have systems of traces that prevent the release of these particulates to the environment, a technology that does not exist in the household equipment. The results showed that VPR pellets can be used for energy conversion in medium and large biomass boilers and are not feasible for domestic use. Ashes produced during the coarsening process showed high levels of copper, with the limits for the use as agricultural fertilizer in some European countries. Thus, to make VPR a viable

alternative in economic and energy terms, it is necessary to develop environmentally friendly technologies for the valorization process (Giorio et al., 2019).

Methodologies such as pyrolysis lead to a significant production of combustible gases and a good source of graphite carbon with high calorific value, due to its high lignin content, grape pruning is an excellent raw material for the production of biochar. Some authors have studied the effects of pure CO_2 and N_2 use at two absolute pressure levels (0.1 and 1.1 MPa) at 600 °C on pyrolysis of VPR. Where it was found that pyrolysis in CO_2 and N_2 atmosphere yielded similar fixed carbon yields and biochar mass. In CO_2 atmosphere, CO_2 yield was reduced however, CO yield increased. Therefore, these results indicate that the recycling of CO_2 obtained by the combustion of VPR as the pyrolysis atmosphere is a promising substitute for N_2 which is a relatively expensive gas (Azuara et al., 2017). Other authors evaluated the production of VPR biochar obtained by physical and chemical activation, using CO_2 and KOH, by the CO_2 absorption capacity at different temperatures (0, 25 and 75 °C), apparent CO_2 selectivity on N_2, and isosteric adsorption heat. The charcoal activated chemically at 25 °C presented higher adsorption capacity of CO_2 overcoming results obtained in similar works with other lignocellulosic materials. However, the coals prepared with physical activation at 800 °C during 1h of immersion presented higher adsorbent capacity of CO_2 with high adsorption rates at higher temperature (Manyà et al., 2018). Based on the results described above, it can be stated that VPR biochar is a promising material that can also be used as an efficient adsorbent with several applications such as CO_2 after combustion, biogas upgrading and H_2 purification.

For the production of biofuels through lignocellulosic biomass, it is necessary to perform different pre-treatments (hydrolysis) to break down the recalcitrant lignin structure, aiding the enzymatic and microbial accessibility (Buratti et al., 2015; Cotana et al., 2015; Pérez-Rodríguez et al., 2016). The combination of methodologies such as UAE and enzymatic hydrolysis were suggested as pre-treatments for biogas production through anaerobic digestion. However, methane and biogas yields were higher for VPR treated only with enzymatic hydrolysis (Pérez-Rodríguez et al., 2016). Some authors used pre-treatments by alkaline hydrolysis and enzymatic hydrolysis of VPR for the production of glucose as carbon source for the production of bioethanol. The results indicated that the reaction time and the NaOH concentration influenced the response, where the optimum glucose yield (202 g glucose/kg of VPR) was reached at 2.5 % NaOH for 40 minutes at 120 °C (Cotana et al., 2015). In the search for environmentally friendly methodologies, some studies have been developed using innovative technologies that apply thermal and water treatments followed by enzymatic hydrolysis to obtain

bioethanol, some authors propose the use of pretreatments such as steam explosion (Buratti et al., 2015) to improve the enzymatic saccharification of VPR for the production of bioethanol. Some authors used pre-treatments by VPR vapor blast followed by enzymatic hydrolysis and fermentation for the production of glucose as a carbon source. According to Buratti and coauthors, the treatment of the vapor extraction in the severity of 4.56 was efficient for the production of bioethanol obtaining maximum of 8.9 kg of ethanol/100 kg of VPR, referring to a yield of 81.09 % of ethanol (Buratti et al., 2015). Based on the work previously described using VPR as a sugar source for bioethanol production, it has been found that there is no effective and environmentally friendly pretreatment technique to favor enzymatic saccharification of VPR, so further studies are needed to increase susceptibility to enzymatic hydrolysis of VPR by achieving higher levels of glucan to glucose conversion.

2.4 Conclusions

The composition of lignocellulosic biomass may vary depending on geographic location, climate, process and variety of the studied grape. There are several lignocellulosic biomass conversion studies of wine residues and these are reviewed here. In addition, fractionation studies of wine residues, related to the extraction and production processes of phenolic extracts and antioxidants were also highlighted. Still, a biorefinery approach could be addressed to obtain simultaneously added-value products from the different fractions of residues generated in the winemaking. The main limitation on the effective use of lignocellulosic materials obtained from winemaking is to know the chemical composition and to use a suitable pretreatment for the proposed objective.

This review addressed the most recent studies that use the valorization of VPR as a raw material for the production of products of industrial interest, as well as their perspectives, their potential and future challenges. The main limitations encountered were the diversity of processes and products found and the lack of standardization of the units of measurement which increased the complexity of the comparisons. Based on the analyzes, it was possible to observe that there is a need to find an effective, clean and economically viable methodology for the effective fractionation of VPR, which is a high value-added byproduct, making possible the full use of all the existing fractions within a biorefinery concept, directing the products to various industrial sectors and thus contributing to a circular economy.

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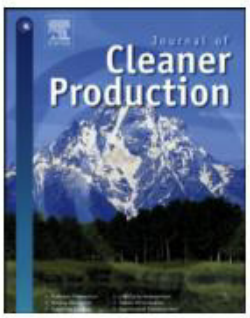
CHAPTER III

INTEGRAL VALORIZATION OF VINE PRUNING RESIDUE BY SEQUENTIAL AUTOHYDROLYSIS STAGES

ABSTRACT

Wine processing generates a large amount of residue, in particular pruning residue of vine. In this work, autohydrolysis in two sequential stages was proposed for the integral valorization of this residue. In a first stage, vine pruning residue was submitted to autohydrolysis treatment at 180 °C for 60 min (severity of 4.13) and liquid to solid ratio of 6 g water per g vine pruning residue. In these conditions, 63.7 % of xylan was recovered in the liquid phase as xylooligosaccharides (17 g/L) and 2.35 g/L of phenolic compounds with antioxidant activity were also extracted. Autohydrolyzed vine pruning residue was subjected to a second autohydrolysis at temperature in the range 180 - 200 °C and time 30 - 40 min. After sequential treatments, enzymatic hydrolysis of cellulose was significantly improved from 73 % to 99 % of conversion. At selected conditions (severity of 4.60), ethanol production was successfully obtained from two strategies of, separately and simultaneously, saccharification and fermentation, thus achieving ethanol yield of 96 and 83 %, respectively. Overall, two sequential stages of the process allowed the recovery of 13.7 kg of xylooligosaccharides, 3.1 kg of phenolic compounds, 13.1 kg of ethanol and 27 kg of lignin per 100 kg of vine pruning residue. Sequential autohydrolysis stages were shown as a suitable strategy for the integral valorization of vine pruning residue.

Keywords: Vine pruning residue, Hydrothermal treatment, Oligosaccharide Bioethanol, Phenolic compound.



The information presented in this Chapter was published in *Journal of Cleaner Production.*

Meirielly S. Jesus, Aloia Romaní, Zlatina Genisheva, José A. Teixeira, Lucília Domingues (2017). Integral valorization of vine pruning residue by sequential autohydrolysis stages. *Journal of Cleaner Production 168,* 74-86.

CONTENTS

3.1 INTRODUCTION

The bioeconomy is based on the optimal use of renewable resources (such as lignocellulosic biomass) through the development of biorefineries for the co-production of fuels and high-value bio-based products as chemicals and materials (Hennig et al., 2016; Karlsson et al., 2014). In particular, high-value bio-based products are obtained by a sustainable process based on principles of green chemistry (Peleteiro et al., 2014).

Lignocellulosic biomass includes wastes from agriculture, forestry and related industries (Ferreira et al., 2009). In Portugal, the wine industry generates during wine processing a lot of residues (such as vine pruning residue, VPR) with an estimated annual production of 1.2-3.5 t/ha (Brito et al., 2014). These residues are typically left in the agricultural field, used as domestic fuel due to calorific power or burnt in the field which causes environmental pollution (Devesa-Rey et al., 2010). The potential energy that these residues could provide has been estimated in 190GW per year or alcohol equivalent of 357 million liters (Ferreira et al., 2009). The Portuguese experience is limited to combustion of biomass. Nevertheless, VPR is a lignocellulose biomass enriched with bio-based compounds with potential to be transformed into commercial products by selective fractionation of the main components (cellulose, hemicellulose and lignin) (Dávila et al., 2016). Therefore, the valorization of these residues under a biorefinery approach could contribute attaining an energy and rural sustainable development (Lin et al., 2014).

Several pretreatments have been proposed for the processing of vine pruning residue, such as alkali pretreatment for glucose production (Cotana et al., 2015), enzymatic hydrolysis and ultrasounds pretreatments to obtain methane and biogas (Pérez-Rodríguez et al., 2016), or ethylene glycol pulping for paper sheets manufacturing (Jimenez et al., 2008). Recently, hydrothermal treatment (known as autohydrolysis) has been used for xylooligosaccharides (Dávila et al., 2016) and ethanol production (Buratti et al., 2015). Moreover, this residue is enriched in polyphenolic compounds (Max et al., 2010; Pérez-Rodríguez et al., 2016) with potential antioxidant features. Autohydrolysis has also been proposed for the extraction of phenolic compounds from lignocellulosic biomass (Egües et al., 2012). Therefore, autohydrolysis is considered a green and sustainable technology suitable to be used in lignocellulosic biorefineries (Gullón et al., 2012). Yet, an integrated approach for the valorization of whole biomass fractions is mandatory to achieve a cost-competitive process (Singhvi et al., 2014). In this sense, one of the autohydrolysis limitations is the difficulty to attain an optimal condition for oligosaccharides manufacturing and suitable saccharification of cellulose into glucose for fuel

production (Romaní et al., 2010). Typically, cellulose is more prone to saccharification after harsh conditions of pretreatment while in these severe conditions the xylose and xylooligosaccharides are degraded into sugars and/or inhibitors compounds such as furfural (Guilliams et al., 2016). Thus, the strategy employed for this two-target goal usually includes the use of two treatments (such as an autohydrolysis combined with a delignification process) (Romaní et al., 2011; Romaní et al., 2016). Delignification processes solubilize the lignin and, consequently, the solid fraction is enriched in glucan being more susceptible to enzymatic hydrolysis (Romaní et al., 2011). Nevertheless, delignification treatments use alkalis and organic solvents, demanding additional steps of washing and neutralization. Moreover, the handling of these chemicals is less safe (Chaturvedi and Verma, 2013). As alternative to delignification process, autohydrolysis in two sequential stages at milder conditions (with water as only catalyst) is shown as an attractive and cleaner solution to disrupt lignocellulosic matrix attaining a suitable recovery of all its components or derivatives (Lee et al., 2010; Min et al., 2015).

In this study, a process using autohydrolysis in two sequential stages was used for the fractionation and integral valorization of VPR. The conditions of operation (temperature and time, severity in the range of 3.36 - 4.90) were evaluated in order to maximize the hemicellulose-derived compounds recovery (as oligosaccharides) and for quantification and identification of phenolic compounds in autohydrolysis liquor. Moreover, an alternative second autohydrolysis of pretreated solid from the first autohydrolysis was carried out (severity in the range of 4.36 - 4.69) to improve the enzymatic accessibility of cellulose for glucose production and further fermentation into ethanol by simultaneous and separate saccharification and fermentation (SSF and SHF, respectively).

3.2 Materials and Methods

3.2.1 Raw material and analysis of chemical composition Vine

Vine pruning residue was provided from a local producer from Minho region, Northern Portugal. VPR was air-dried, milled to pass an 8mmscreen, homogenized in a single lot to avoid differences in the composition, and stored at room temperature in a dark and dry place until use. The raw material was analyzed by TAPPI (Technical Association of the Pulp and Paper Industry) standards for extractives, moisture, ashes and quantitative acid hydrolysis with 72 % (w/w) sulphuric acid (T-264-cm-07; T-211-cm-93; T-249-em-85). VPR was Soxhlet extracted with water and ethanol as described in Romaní and

co-workers (2016). Liquors from quantitative acid hydrolysis and aqueous extract were analyzed by High-Performance Liquid Chromatography (HPLC) for sugars (glucose, xylose, arabinose) and acetic acid, using a Refractive Index detector and an 87H (300, 7.8 mm) Aminex (BioRad) column eluted with 0.005M H_2SO_4, flow rate of 0.6 mL/min at 60 °C. The content of poly- saccharides (glucan, xylan, arabinan) and acetyl groups was calculated from HPLC data. The Klason lignin content of VPR was gravimetrically measured from the insoluble solid residue obtained after the quantitative acid hydrolysis step.

3.2.2 Autohydrolysis of vine pruning residue in two sequential stages: solid and liquid characterization

3.2.2.1 First autohydrolysis stage

The VPR was submitted to autohydrolysis treatments under conditions listed in Table 3.1. For this, water was mixed with VPR at liquid to solid ratio (LSR) of 8g per g, placed in stainless steel reactor and submerged in silicone oil bath in a 160 mL total volume batch cylinder reactor fabricated from 316 stainless steel at distinct temperatures (180 - 200 °C) and different reaction times (10 - 90min) (heating time of 5min). After autohydrolysis treatment, the reactor was removed from the oil bath and cooled down in an ice-water bath for 5min. The hardness of autohydrolysis treatments can be expressed in terms of "severity" (S_0) (Lavoie et al., 2010), defined as the logarithm of the reaction ordinate (R_0) (Overend and Chornet, 1987; Abatzoglou et al., 1992), which was calculated using the following equation:

$$S_0 = \log R_0 = \log(R_0\text{HEATING} + R_0\text{COOLING}) = \log\left[\int_0^{t_{MAX}} \exp\left(\frac{\mathrm{T(t)}-T_{REF}}{\omega}\right)\mathrm{dt}\right] + \left[\int_{t_{MAX}}^{t_F} \exp\left(\frac{\mathrm{T'(t)}-T_{REF}}{\omega}\right)\mathrm{dt}\right] \quad (1)$$

According to this expression, R_0 is the reaction ordinate (min), t_{MAX} (min) is the time needed to achieve the target temperature T_{MAX} (K), t_F (min) is the time needed for the whole heating–cooling period, $T(t)$ and $T'(t)$ represent the temperature profiles in the heating and cooling stages (K), respectively, T_{REF} is the reference temperature (T_{REF} = 373.15 K) and ω is an empirical parameter related with the activation energy of hemicellulose solubilization reaction, which can be expressed as (Garrote et al., 2002):

$$\omega = \frac{R\,{T_{REF}}^2}{E_a} \quad (2)$$

where, R is the ideal gas constant (R = 8.314 J/mol K) and E_a is the activation energy of hemicellulose solubilization reaction in J/mol. Although ω can be evaluated and optimized (Garrote et al., 2002), it is common to select a value of ω = 14.75 K (González-Muñoz et al., 2011).

After autohydrolysis treatment, solid and liquid phases were separated by filtration for determination of chemical composition. An aliquot of autohydrolysis liquors (liquid phase) was filtered through 0.45 μm membranes and used for direct HPLC determination of glucose, xylose, arabinose, acetic acid, hydroxymethylfurfural (HMF) and furfural, using the same method specified above.

A second aliquot was subjected to quantitative posthydrolysis with 4 % (w/w) sulphuric acid at 121 °C for 30 min, filtered through 0.45 μm membranes, and analyzed by HPLC for oligosaccharides concentration. Solid phase from autohydrolysis was washed with distilled water, air-dried and employed for solid yield (*SY*) determination (expressed as g autohydrolyzed VPR per 100 g VPR, oven dry basis). Pretreated VPR was analyzed for glucan, xylan and Klason lignin using the analytical procedure described in section 3.2.1.

Table 3.1 Operational conditions of first autohydrolysis treatment at Liquid to Solid Ratio = 8 g per g and main results obtained: composition of solid and liquid phases

Temperature (T_{aw}, °C)	180	180	180	180	180	180	180	180	200	200	200	200
time (min)	10	20	30	40	50	60	70	80	30	40	60	90
S_0 (-)	3.36	3.66	3.83	3.96	4.05	4.13	4.20	4.26	4.42	4.55	4.72	4.90
Solid Yield (g per 100g of raw material)	86.4	83.7	74.8	68.7	68.6	65.7	64.5	63.5	63.4	60.1	60.9	62.4
Solid Phase Composition (g of component per 100 g of autohydrolyzed VPR oven-dry basis)												
Glucan	36.3 ± 0.45	37.3 ± 0.72	39.3 ± 0.65	38.3 ± 1.82	40.3 ± 1.58	42.6 ± 2.30	45.6 ± 3.74	44.5 ± 2.71	43.5 ± 1.60	43.6 ± 2.10	43.0 ± 1.96	40.7 ± 0.68
Xylan	14.07 ± 0.21	17.48 ± 0.01	11.1 ± 0.45	9.14 ± 1.09	8.90 ± 0.23	7.38 ± 0.01	5.48 ± 0.23	5.53 ± 0.03	3.96 ± 0.43	2.95 ± 0.0	2.37 ± 0.01	1.53 ± 0.12
Klason lignin	30.6 ± 0.54	31.4± 0.03	36.6 ± 1.35	36.6 ± 2.54	40.6 ± 0.32	42.5 ± 1.40	43.8 ± 0.93	42.9 ± 1.80	44.1 ± 0.80	45.7 ± 0.14	45.2 ± 1.59	44.6 ± 0.46
Acetyl groups	3.40 ± 0.50	3.18 ± 0.02	1.76 ± 0.58	1.68 ± 0.38	1.52 ± 2.06	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Liquid Phase Composition (g/L)												
Glucose	0.9 ± 0.01	1.9± 0.56	1.9 ± 0.58	1.6 ± 0.39	1.6 ± 0.26	1.3 ± 0.02	1.3 ± 0.15	1.2 ± 0.08	0.4 ± 0.17	0.7 ± 0.02	0.5 ± 0.04	0.2 ± 0.23
Xylose	2.5 ± 0.53	2.1 ± 0.16	2.0 ± 0.18	1.8 ± 1.71	1.8 ± 0.13	2.0 ± 0.02	1.8 ± 0.08	2.0 ± 0.21	2.3 ± 0.37	2.8 ± 0.29	2.0 ± 0.06	1.1 ± 0.10
Arabinose	2.6 ± 0.24	0.3 ± 0.14	0.7 ± 0.34	0.6 ± 0.81	0.6 ± 0.10	0.4 ± 0.02	0.5 ± 0.13	0.4 ± 0.18	0.0 ± 0.32	0.0 ± 0.19	0.0 ± 0.11	0.0 ± 0.11
Acetic Acid	0.16 ± 0.05	0.31 ± 0.14	0.83 ± 0.31	1.13 ± 0.02	1.9 ± 0.02	2.13 ± 0.02	2.66 ± 0.08	2.33 ± 0.23	3.02 ± 0.36	3.28 ± 0.46	3.51 ± 0.52	3.86 ± 0.70
HMF	0.1 ± 0.30	0.1± 0.03	0.1 ± 0.02	0.1 ± 0.96	0.1 ± 0.17	0.1 ± 0.02	0.1 ± 0.79	0.0 ± 0.0	0.4 ± 0.70	0.4 ± 0.44	1.0 ± 0.17	1.0 ± 0.81
Furfural	0.0 ± 0.43	0.1 ± 0.03	0.2 ± 0.05	0.3 ± 0.66	0.3 ± 0.90	0.4 ± 0.02	0.3 ± 0.98	0.4 ± 0.95	1.2 ± 0.05	2.0 ± 0.64	3.0 ± 0.44	2.1 ± 0.27
Glucooligosaccharides	5.9 ± 0.52	5.5 ± 4.09	5.0 ± 1.20	4.8 ± 0.28	5.4 ± 0.84	5.0 ± 0.42	5.0 ± 1.01	4.5 ± 0.13	3.2 ± 0.31	2.5 ± 0.14	1.0 ± 0.04	0.3 ± 0.04
Xylooligosaccharides	3.4 ± 0.12	3.5 ± 2.97	10.3 ± 1.56	11.3 ± 0.24	12.6 ± 0.59	13.2 ± 0.42	13.0 ± 0.35	13.0 ± 0.72	12.0 ± 1.01	7.0 ± 0.35	1.7 ± 0.01	0.9 ± 0.03
Arabinooligosaccharides	2.0 ± 0.18	2.1 ± 0.45	1.4 ± 0.02	0.6 ± 0.57	0.9 ± 0.05	1.1 ± 0.29	0.5 ± 0.19	0.7 ± 0.20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	0.56 ± 0.39	1.18 ± 3.50	2.73 ± 0.65	2.4 ± 2.57	2.13 ± 0.37	1.97 ± 0.51	1.69 ± 0.11	1.87 ± 0.15	2.61 ± 0.08	2.7 ± 0.56	2.7 ± 0.31	2.08 ± 0.41
Total phenolic compounds	1.04 ± 0.08	0.98 ± 0.02	1.01 ± 1.61	1.33 ± 0.03	1.47 ± 0.91	2.09 ± 0.64	1.70 ± 0.65	1.66 ± 0.77	1.64 ± 0.93	1.91 ± 0.91	1.86 ± 0.31	1.99 ± 0.44

3.2.2.2 Second autohydrolysis stage

Second autohydrolysis treatment was performed mixing water and pretreated VPR from first autohydrolysis (Figure 3.1) at LSR of 6 g per g. Conditions of the second stage of autohydrolysis were listed in Table 3.2 and carried out at LSR of 6 g per g.

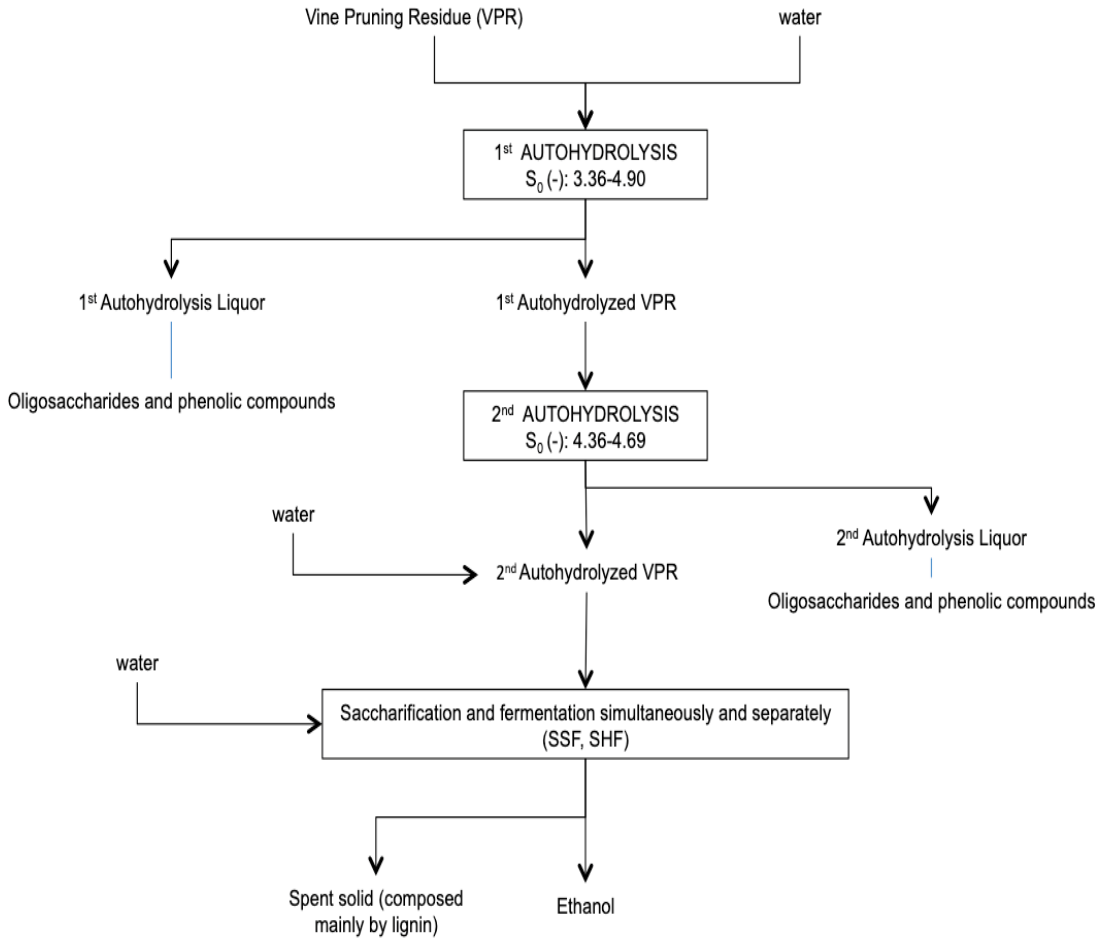


Figure 3.1 Scheme proposed in this work for integral valorization of vine pruning residue

Table 3.2 Conditions of second autohydrolysis treatment of VPR from first autohydrolysis (S0 = 4.13) at Liquid to Solid Ratio = 6 g per g and chemical characterization of solid and liquid phases (first and second autohydrolysis stages).

	1st Stage Autohydrolysis	2nd Stage Autohydrolysis			
Temperature (T_{MAX}, °C)	180	180	180	200	200
time (min)	60	40	60	30	40
S_0(-)	4.13	3.96	4.13	4.42	4.55
Total S_0[a] (-)	-	4.36	4.43	4.60	4.69
Solid Yield	66.02	66.60	62.01	62.61	63.60
Solid phase composition	(g per 100 g of 1st autohydrolyzed VPR)	(g per 100 g of 2nd autohydrolyzed VPR)			
Glucan	41.31 ± 3.58	40.40 ± 0.46	41.35 ± 1.70	41.72 ± 0.98	40.35 ± 0.59
Xylan	9.96 ± 2.44	6.98 ± 0.16	6.58 ± 0.20	6.29 ± 0.12	4.28 ± 0.08
Klason Lignin	42.20 ± 0.69	40.05 ± 3.83	40.11 ± 0.53	40.55 ± 2.45	39.58 ± 4.17
Liquid phase composition	(g/L)	(g/L)			
Glucose	1.50 ± 0.79	0.11 ± 0.02	0.14 ± 0.15	0.14 ± 0.03	0.17 ± 0.04
Xylose	1.99 ± 0.93	0.53 ± 0.01	0.81 ± 0.34	1.18 ± 0.03	1.47 ± 0.60
Arabinose	0.72 ± 0.21	0.11 ± 0.01	0.10 ± 0.62	0.12 ± 0.02	0.09 ± 0.09
Acetic Acid	0.96 ± 1.06	0.59 ± 0.09	0.84 ± 0.77	0.98 ± 0.04	1.45 ± 0.24
Furfural	0.36 ± 0.27	0.42 ± 0.02	1.07 ± 0.67	0.99 ± 0.54	1.24 ± 0.52
HMF	0.66 ± 0.42	0.82 ± 0.01	2.47 ± 1.64	3.08 ± 0.88	4.50 ± 0.12
Glucooligosaccharides	6.29 ± 0.63	0.61 ± 0.01	0.55 ± 0.01	0.68 ± 0.02	0.57 ± 0.04
Xylooligosaccharides	17.22 ± 0.73	5.20 ± 0.01	5.50 ± 0.02	6.88 ± 0.12	4.89 ± 0.09
Arabinooligosaccharides	1.60 ± 0.56	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	6.10 ± 0.30	1.74 ± 0.03	1.64 ± 0.02	2.17 ± 0.09	1.82 ± 0.02

[a] Total S_0 was calculated taking into account the severity of first stage and second

3.2.3 Phenolic compounds analysis and antioxidant activity of autohydrolysis liquors

Autohydrolysis liquors were analyzed for total phenolic compounds (expressed as gallic acid equivalents, GAE) by absorbance following Folin-Ciocalteu method (as described in Conde et al., 2011). In addition, some phenolic compounds were also identified by Ultra-high-performance liquid chromatography (UHPLC) using a Shimatzu Nexpera X2 UHPLC chromatograph equipped with Diode Array Detector (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Acquity UHPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; from Waters) at 40 °C. The flow rate was

0.4 mL/min. The HPLC grade solvents used were water/formic acid (0.1 %) as solvent A and acetonitrile as solvent B. The elution gradient for solvent B was as follows: from 0.0 to 5.5 min at 5 %, from 5.5 to 17 min a linear increase to 60 %, from 17.0 to 18.5 min a linear increase to 100 %, then column equilibration from 18.5 to 30.0 min at 5 %.

Antioxidant activity of autohydrolysis liquors was also determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay and the radical cation decolorization of 2,2´-azino-bis (3-ethylbenzothiazoline-6—sulphonic acid) (ABTS) assay following the methods described in Karacabey et al. (2012) and Ballesteros and co-workers (2015). The percentage of inhibition was calculated as a function of the concentration of autohydrolysis liquor and Trolox. IC_{50} (concentration of sample required to reduce 50% of DPPH or ABTS) was calculated by interpolation of the inhibition activity (*Ia*, %), calculated by the following equation:

$$Ia = \frac{A_0 - A_1}{A_1} 100 \quad (3)$$

where A_0 is the absorbance of the control (DPPH or ABTS), and the A_1 is the absorbance of the autohydrolysis liquor.

3.2.4 Enzymatic hydrolysis of vine pruning residue from autohydrolysis treatment

In order to evaluate the enzymatic susceptibility of leftover glucan in pretreated vine pruning residue, enzymatic hydrolysis assays were carried out using commercial enzymes Cellic Ctec2 (cellulase) and Htec2 (hemicellulase), kindly provided by Novozymes (Denmark). Enzymatic activities for cellulases and hemicellulases were determined (Ghose, 1987; Bailey et al., 1992) corresponding to 120 Filter Paper Unit (FPU)/mL and 1690 International Unit (IU)/mL, respectively. The enzymatic hydrolysis was carried out in an orbital shaker at 50 °C and 150 rpm using 4 % and 10 % of solids and enzyme loading of 25 FPU per g of substrate in a 0.05N sodium citrate buffer (pH 4.85). Samples were withdrawn between 0 – 72 h and analyzed by HPLC for glucose and xylose concentration. All determinations were performed in duplicate. The results obtained from enzymatic hydrolysis can also be expressed as glucan to glucose conversion (GGC, %) using the following equation:

$$GGC = \frac{G + 1.053\,C}{1.111\,f\,B} 100 \quad (4)$$

where, *G* is glucose concentration (g/L), *C* is cellobiose concentration (g/L), *B* is dry biomass concentration (g/L), *f* is glucan fraction in dry biomass (g per g), the multiplication factor, 1.053, converts cellobiose to equivalent glucose and 1.111 is the stoichiometric factor that converts glucan to equivalent glucose. In all experiments, cellobiose was not detected.

3.2.4.1 Inoculum preparation

Yeast used in this work was Saccharomyces cerevisiae PE-2 strain (isolated from Brazilian Bioethanol Distillery) (Pereira et al., 2014). Stock culture was maintained on yeast peptone dextrose medium (2% of glucose, 2% of peptone and 1 % of yeast extract) agar plates at 4 °C. Yeast was grown in Erlenmeyer flasks containing 20 g/L of glucose, 20 g/L of peptone and 10 g/L of yeast extract for 15 h at 30 °C and 200 rpm. Cells were separated from culture media by centrifugation (10 min at 4 °C and 7500 g) and resuspended in 0.9 % NaCl. Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) experiments were inoculated with 5mg of fresh yeast per mL (final concentration).

3.2.4.2 Saccharification and fermentation

For ethanol production, separate and simultaneous saccharification and fermentation assays (SHF and SSF) were carried out using as substrate the pretreated VPR obtained from autohydrolysis in two sequential stages (Figure 3.1). Enzymes used for saccharification were Cellic Ctec2 and Cellic Htec2. Nutrients (peptone and yeast extract) were sterilized in autoclave separately from pretreated VPR at 121 °C for 15 min. Inoculum and enzymes were added to SSF assays. Experiments were carried out at 35 °C and 150 rpm. For SHF assays, enzymatic hydrolysis (at 50 °C) was carried out for 4h. After this, temperature was decreased to 30 °C and yeast cells were added. Samples from SHF and SSF were withdrawn at desired times and analyzed by HPLC for ethanol concentration. Ethanol yield (Y_{Et}) was calculated by the following equation:

$$Y_{Et} = \frac{\mathrm{EtOH_f} - \mathrm{EtOH_0}}{0.5\mathrm{l}\ f\ B\ 1.111}\ 10 \qquad (5)$$

where, $EtOH_f$ is the ethanol concentration produced during the fermentation (g/L), $EtOH_o$ is the ethanol concentration at the beginning of the fermentation (g/L) which was zero, *B* is dry biomass concentration at the beginning of the fermentation (g/L), *f* is glucan fraction of dry biomass (g per g),

0.51 is conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast. The stoichiometric factor that converts glucan to equivalent glucose is 1.111.

3.2.5 Structural analysis of raw material and pretreated samples

3.2.5.1 Scanning electron microscopy (SEM) analysis

Micrographs of raw material, autohydrolyzed vine pruning residue from the first autohydrolysis (180 °C for 60 min, S_0=4.13) and autohydrolyzed vine pruning residue from the second autohydrolysis (200 °C for 30 min, S_0=4.60) were obtained using a desktop scanning electron microscope (Phenom-World BV, Netherlands). The images were obtained using a voltage of 5kV at 270-fold magnification.

3.2.5.2 Thermal analysis of spent residue recovered after SHF assay

Thermogravimetric analysis (TGA) of spent residue recovered after saccharification and fermentation assay was carried out in Thermogravimetric (TGA 4000 and DSC 6000) Analyzer. The analysis was carried out in the range of 25-600 °C with a linear increase of 10 °C per min.

3.2.5.3 Fourier-transform infrared (FT-IR)

The chemical groups and bonding arrangement of constituents of the spent residue obtained after saccharification and fermentation assay were determined by Fourier transform infrared spectroscopy (FT-IR) using a Jasco infrared spectrometer (FT-IR-4100) equipped with a diamond-composite attenuated total reflectance (ATR) cell. The FT-IR spectrum was obtained operating with a resolution of 4cm^{-1}, 16 scans, and frequency range of 4000-600 cm^{-1}. The FI-TR bands were identified for comparison with those reported in the literature (Santos et al., 2015; Dávila et al., 2017).

3.3 Results and Discussion

3.3.1 Raw material

Chemical composition of vine pruning residue (expressed in g per 100 g VPR on oven-dry basis ± standard deviation based in three replicate determinations) was as follows: 32.9 ± 0.66 of cellulose (as glucan); 14.87 ± 0.17 of xylan; 0.40 ± 0.06 of arabinan; 3.95 ± 0.52 of acetyl groups; 29.5 ± 1.21 of Klason lignin; 13.7 ± 1.02 of extractives in water; 2.94 ± 0.89 of extractives in ethanol and 3.32 ± 0.56 of ashes. Structural components of VPR (cellulose, Klason lignin and hemicellulose) represented 81.6% of the raw material. Extractives in water were analyzed by HPLC for determination of glucose concentration which represented 1.20 % of raw material oven-dry basis. VPR was composed mainly of cellulose followed by lignin. Hemicellulose fraction was mainly composed of xylan which represented 77.4 % of total identified hemicellulose compounds. Chemical composition was similar to the one reported by other authors for vine shoots (Rivas et al., 2007; Dávila et al., 2016).

3.3.2 Process configuration

Process configuration of this work was shown in Figure 3.1. First, autohydrolysis stage was proposed in order to solubilize hemicellulose fraction into added value compounds such as oligosaccharides, recognized prebiotic functional food (Patel and Prajapati, 2015), and phenolic compounds, valued by their bioactive properties and antioxidant activity (Rivas et al., 2013). Autohydrolyzed VPR from the first stage was assayed for enzymatic saccharification. After this evaluation, second autohydrolysis stage was proposed in order to improve the enzymatic susceptibility of cellulose. Finally, pretreated VPR from sequential stages of autohydrolysis was subjected to saccharification and fermentation for ethanol production. A systematic evaluation of operational conditions on fractionation of VPR was carried out as shown below.

3.3.3 First stage of autohydrolysis pretreatment of vine pruning residue

3.3.3.1 Fractionation of vine pruning residue

After the first stage of autohydrolyisis treatment, pretreated biomass and autohydrolysis liquor were analyzed to evaluate the degree of fractionation achieved. Table 3.1 showed the operational conditions of autohydrolysis treatment (temperature and time and its corresponding severity, S_0) and

the results obtained. As observed in Table 3.1, solid yield (S_Y) decreased with the increase of severity. Glucan content varied in the range of 36.3 - 45.6 g of glucan per 100 g of autohydrolyzed VPR. The glucan recovery was 77.2 - 95.2 % with respect to glucan content in raw material at S_0=4.90 and 3.36, respectively (boundary conditions evaluated in this work). The lowest glucan recovery at S_0=4.90 revealed high cellulose loss, that is an undesirable condition for vine pruning processing from a biorefinery approach. Buratti and coworkers (2015) reported a 69.1 % of cellulose recovery from steam exploded residue from vineyard at S_0=4.24. The wide range of severities studied in this work displayed the behavior of the raw material to selective fractionation of autohydrolysis treatment. As observed from data listed in Table 3.1, xylan solubilization increased with the severity of treatment achieving up to 93 % of xylan solubilization at S_0=4.90. Lignin content ranged from 30.60 to 45.66 g of lignin per 100 g of autohydrolyzed VPR which corresponded to 90 and 100 % of recovery, respectively. Similar range of severities were evaluated for fractionation of agricultural residues as barley straw (Vargas et al., 2015), rice straw (Moniz et al., 2015) or olive tree pruning (Silva-Fernandes et al., 2015) in which cellulose recovery of 90% were obtained at values of S_0 in the range of 3.15 - 4.36.

Table 3.1 also provides the chemical composition of liquors from first autohydrolysis step. As expected, xylooligosaccharides were the main component at milder conditions of treatment (S_0=3.83-4.26). As severity increases, the hydrolysis of oligosaccharides into monosaccharides is more effective but after a critical value leads to the subsequent dehydration of sugars in furfural and HMF. This behavior is typical of the severity rise effect on autohydrolysis treatment of lignocellulosic materials (Garrote et al., 1999; Ruiz et al., 2013). A 16 - 50 % of xylan solubilization into xylooligosaccharides was obtained at S_0 3.36-3.80, achieving a maximal extraction of xylan as xylooligomers (65.1 %) at S_0=4.13. The lowest recovery of xylan as xylooligosaccharides (4.4 %) was obtained at the highest severity factor evaluated (S_0=4.90). The maximal concentration (measured as sum) of xylooligosaccharides and xylose (13.19 and 2.01 g/L, respectively) were also obtained at S_0=4.13 which corresponded to 75.9 % recovery of xylan in the raw material. Similar concentration of xylooligosaccharides (12.2 g/L) or xylose (14.8g/L) were obtained by autohydrolysis treatment using trimming vine shoots at S_0=4.01 (Dávila et al., 2016) and by sequential stages of autohydrolysis at S_0=4.08 and acid hydrolysis with 1% H_2SO_4 (w/w) (Moldes et al., 2007), respectively. The concentration of glucooligosaccharides and acetyl groups linked to oligosaccharides achieved an average value of 5.2 g/L ± 0.39 and 2.05 g/L ± 0.67, respectively. Regarding degradation compounds, furfural and HMF concentrations were higher than 1 g/L at S_0=4.42 and 4.72, respectively. On the other hand, acetic acid from deacetylation of hemicellulose had a significant increase up to 3.86 g/L at S_0=4.90.

Taking into account that by-products from wine-making are enriched in phenolic compounds with valuable antioxidant properties to be used in food, pharmaceutical and cosmetic industries (Delgado-Torre et al., 2012; Teixeira et al., 2014), total phenolic compounds in autohydrolysis liquor were also quantified and included in Table 3.1. Phenolic compounds obtained by autohydrolysis achieved an average value of 1.5 g/L ± 0.39, achieving a maximal concentration of 2.09 of GAE g/L (or 1.88 g GAE per 100 g of VPR) at S_0=4.13. Autohydrolysis treatment has been employed for extraction of phenolic compounds, obtaining approximately 2 GAE g per 100g from lignocellulosic wastes such as corncobs, eucalyptus wood and grape pomace at high temperatures (240 °C) in non-isothermal regime (Conde et al., 2011). Traditionally, extraction methods for phenolic compounds recovery from vine by-products use toxic solvents such as methanol (Delgado-Torre et al., 2012). Water extraction at high temperatures is an environmentally-friendly alternative to traditional extraction methods.

3.3.3.2 Enzymatic susceptibility of vine pruning residue

For a global evaluation of pretreatment, the enzymatic susceptibility of pretreated biomass was also studied. Effect of autohydrolysis pretreatment on time course of enzymatic saccharification was represented in Figure 3.2 (assays were carried out in duplicate with a calculated relative error ≤ 10 %). At S_0=4.05 - 4.42, glucan to glucose conversion (GGC) at 72 h of enzymatic hydrolysis achieved values of 73 - 78 %. Under these conditions of pretreatment, more than 60 % of xylan was solubilized into xylooligosaccharides, disclosing a suitable range of conditions to attain a biorefinery scheme. The highest glucan conversion (91%) was achieved at S_0=4.90 in which oligosaccharides and sugars were completely degraded in the liquid phase. Glucose yield higher than 90 % was also obtained from alkaline pretreated residue from vineyard using 2.5 % NaOH at 100 °C for 30 min (Cotana et al., 2015). Enzymatic saccharification yield of steam exploded residue from vineyard attained 86 % at S_0=4.56 (Buratti et al., 2015). Xylose was also generated in enzymatic hydrolysis assays at a concentration of 0.4 - 2.3 g/L, corresponding to a xylan conversion of 12 - 100 %, respectively. In this work, the increment in the severity factor significantly enhanced the enzymatic susceptibility of cellulose with 5.4-fold higher conversion comparing to the lowest severity (S_0=3.36).

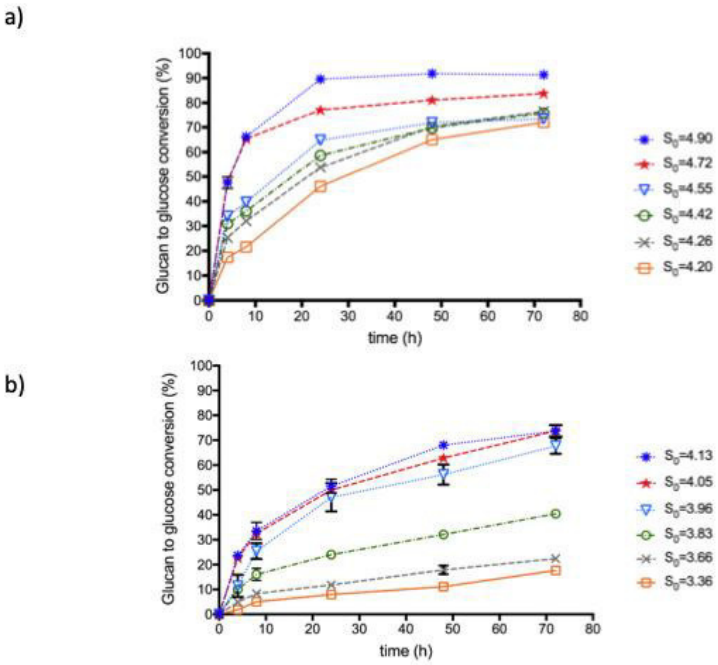


Figure 3.2 Time course of cellulose to glucose conversion at S_0 in the range of 3.36 - 4.90.

Considering the autohydrolysis liquor composition in xylooligosaccharides (13.19 g/L) and enzymatic susceptibility of solid phase (glucan to glucose conversion of 73.7 %), operational condition of S_0=4.13 was selected to advance on further fractionation of VPR. For that, an additional treatment was carried out reducing the LSR up to 6 g per g with the objective of reducing water consumption in the treatment and increase the concentration of hemicellulose-derived compounds in autohydrolysis liquor. The solid and liquid phase composition was shown in Table 3.2. As seen, oligosaccharides concentration was considerably increased up to 31.2 g/L (17.2 g/L as xylooligosaccharides).

Some of the phenolic compounds present in the autohydrolysis liquor were identified by UHPLC. Phenolic acids from wine by-product include benzoic and cinnamic acid derivatives, being hydroxycinnamic acid the most abundant in these residues (Teixeira et al., 2014). In accordance, hydroxycinnamic acids were the ones found in the highest percentage in the autohydrolysis liquor (caffeic acid, p-coumaric acid, chlorogenic acid, rosmarinic acid and ferulic acid) (Table 3.3). Moreover, hydroxybenzoic acids such as vanillic, gallic and syringic acid were also identified. Grape stems constitute an enriched source of flavonoids and stilbenes (containing resveratrol in high concentration) (Anastasiadi et al., 2012). More recently, shoots, leaves and tendrils from six grapevine varieties were analyzed for their content in resveratrol in which 9.25-12.5 mg of trans-resveratrol per kg of shoots from *St. Laurent vine* variety was reported using an extraction method with methanol: ethyl acetate (Lachman

et al., 2016). In this study, the extraction of resveratrol from VPR by autohydrolysis achieved a concentration of 12.46 mg/L (or 79mg per kg of VPR).

Antioxidant activity of autohydrolysis liquor was also reported in Table 3.3 showing antioxidant capacity expressed as Trolox equivalent. Antioxidant activity of phenolic compounds obtained by autohydrolysis treatments, liquid hot water and steam explosion, of olive pruning was reported to be 7.33 and 6.31 mmol DPPH per 100 g of extract, respectively (Conde et al., 2009). Recently, antioxidant, antifeedant and phytotoxic activities of aqueous extracts from vine-shoots have been reported showing the potential of this residue to be applied in cosmetics, nutraceuticals or pharmaceuticals (Sánchez-Gómez et al., 2017). Values IC_{50} for extract samples from vine shoots of 32 µg/mL and 38 µg/mL were reported using conventional solid-liquid extraction and microwave extraction, respectively (Sánchez-Gómez et al., 2017).

Table 3.3. Antioxidant activity and phenolic compounds composition in liquors from first stage (S_0=4.13) and second stage (S_0=4.60) of autohydrolysis.

Severity Factor (S_0)	4.13	4.60
Antioxidant activity		
ABTS (mg per L Trolox equivalent)	247.05 ± 0.34	35.56 ± 0.03
DPPH (mg per L Trolox equivalent)	173.45 ± 0.03	45.2 ± 0.21
ABTS IC_{50} (mg of autohydrolysis liquor per mL)	10.97 ± 0.32	50.2 ± 0.15
DPPH IC_{50} (mg of autohydrolysis liquor per mL)	9.89 ± 0.58	57.25 ± 0.25
Total phenol (GAE g/L)	2.35 ± 0.02	1.43 ± 0.06
Phenolic Compound	(mg/L)	(mg/L)
catechin	47.54	14.86
syringic acid	18.88	12.65
chlorogenic acid	34.70	0.0
caffeic acid	33.60	0.0
vanillic acid	21.96	16.57
ferulic acid	5.0	0.0
gallic acid	8.39	5.06
p-coumaric acid epicatechin	5.54	4.60
o-coumaric acid	10.32	4.72
hesperidin	21.84	0.0
cinnamic acid	7.26	6.91
resveratrol	12.46	11.78
rutin	20.62	10.40
rosmarinic acid	12.24	11.72

3.3.4 Second stage of autohydrolysis pretreatment of vine pruning residue

3.3.4.1 Effect on solid and liquid composition

Sequential second stage of autohydrolysis was studied for the improvement of enzymatic cellulose susceptibility. VPR from autohydrolysis treatment at S_0=4.13 and LSR of 6 g per g was employed as feedstock in a second autohydrolysis step in the range of temperatures from 180 to 200 °C for 30 - 60 min, corresponding to total S_0=4.36 - 4.69 (including the severity from the first stage of autohydrolysis). Table 3.2 collected solid and liquid composition from autohydrolysis in two sequential stages. Under the evaluated conditions of treatment, high glucan recovery was obtained (94.0 - 96.6 g of glucan per 100 g of glucan in first autohydrolyzed VPR). Glucan content increased with the severity factor achieving 41.7 g of glucan per 100 g of second autohydrolyzed VPR at S_0=4.60. These results were comparable with glucan obtained from first autohydrolysis stage for S_0 of 4.55 and 4.72 (Table 3.1). Xylan recovery was in the range of 67.5 - 41.4 g of xylan per 100 of xylan in first autohydrolyzed VPR for the boundary conditions (S_0: 4.36 and 4.69, respectively). On the other hand, lignin recovery remained almost constant with an average value of 90.56 ± 0.96 g of lignin per 100 g of lignin in the first autohydrolyzed solid. Consequently, the main compound in the liquid phase (or liquor from second autohydrolysis) were xylooligosaccharides which achieved a maximum concentration (6.88 g/L) at S_0=4.60. Considering the xylooligosaccharides extraction in the first autohydrolysis stage, 81.2 % of xylan was recovered as xylooligosaccharides from first and second autohydrolysis stages (73.4 and 18.4 % at S_0=4.13 and S_0=4.60, respectively). Moreover, phenolic compounds present in the liquor from second autohydrolysis were also analyzed by UHPLC and included in Table 3.3. The concentration of identified phenolic compounds in the second step of autohydrolysis was reduced. Nevertheless, the antioxidant activity was similar probably caused by the higher concentration of HMF (3.8 g/L) formed due to the harder conditions of the second autohydrolysis.

3.3.4.2 Effect on enzymatic susceptibility of vine pruning residue

Regarding enzymatic hydrolysis from the second stage of autohydrolysis, Table 3.4 showed operational conditions evaluated and the main results obtained at 72 h of hydrolysis. The percentage of solids was increased from 4 % up to 10 % in order to increase glucose concentration and consequently ethanol in subsequent fermentation assays. Glucan to glucose conversion was significantly increased, achieving 99 % of conversion at the highest severity (S_0 = 4.69). The maximal concentration of glucose (45.4 g/L) was obtained at S_0 = 4.69 and 10 % of solids. The increase of solid loading up to 10 %

reduced the glucan to glucose conversion between 1.6 - 4.6 % achieving a glucose concentration 2.2-fold higher than the concentration obtained when operating at 4 % of solids. Glucan conversion from S_0 = 4.60 was higher than 80 %. In comparison with the saccharification of first autohydrolysis stage (S_0 = 4.13), an increase of 35.6 % of enzymatic hydrolysis conversion was obtained at S_0 = 4.69 and 4 % of solids. On the other hand, 43.7 % augment in glucose conversion was achieved using 10 % of solid loading and severity of S_0= 4.69.

The improvement of enzymatic hydrolysis after autohydrolysis in two sequential stages can be due to structural changes. Scanning Electronic Micrographs (SEM) of raw material and pretreated samples were taken (see Figure 3.3). These treated samples correspond to treatment at 180 °C for 60 min (first autohydrolysis, S0=4.13) and 200 °C for 30 min (second autohydrolysis, Total S0=4.60). Clear differences between native and pretreated biomasses were shown. The raw material displayed an ordered structure of fibers. On the other hand, pretreated sample from the first autohydrolysis showed a more porous structure and the sample from autohydrolysis in two sequential stages presented a more defragmented and open structure.

Table 3.4 Enzymatic hydrolysis conditions and main results obtained from saccharification of second autohydrolysis stage at 72 h.

run	Operational conditions				Main Results				
	Temperature (°C)	Time (min)	Severity (S_0) or Total Severity (Total S_0)	Solid loading (%)	Enzyme to Substrate Ratio (ESR, FPU per g)	Glucose at 72 h (g/L)	Xylose at 72h (g/L)	Glucan to glucose Conversion (%)	Xylan to glucose Conversion (%)
1	180	60	4.13	4	25	13.20 ± 0.01	3.51 ± 0.30	73.21± 0.06	77.5 ± 0.74
2				10	25	29.85 ± 0.34	8.21 ± 0.12	68.45 ± 0.78	72.5 ± 2.99
3	180	40	4.36[a]	4	25	12.80 ± 0.28	3.05 ± 0.10	72.63 ± 1.60	96.1 ± 2.56
4				10	25	27.55 ± 0.07	7.80 ± 0.05	70.84 ± 3.18	98.3 ± 1.28
5	180	60	4.46[a]	4	25	13.47 ± 0.13	2.88 ± 0.25	74.69 ± 0.75	96.3 ± 6.40
6				10	25	34.95 ± 2.89	7.20 ± 0.20	71.25 ± 6.65	96.3 ± 0.43
7	200	30	4.60[a]	4	25	15.50 ± 0.14	2.80 ± 0.10	85.16 ± 0.91	97.9 ± 2.56
8				10	25	40.50 ± 1.55	7.05 ± 0.34	83.21 ± 3.53	98.6 ± 0.78
9	200	40	4.69[a]	4	25	17.48 ± 0.74	1.91 ± 0.30	99.30 ± 4.21	98.2 ± 7.69
10				10	25	45.43 ± 0.03	4.80 ± 2.86	97.70 ± 0.08	98.7 ± 5.98

[a] Total S_0 was calculated taking into account the severity of first stage and second

Few works have reported the effect of autohydrolysis in two stages on lignocellulosic biomass being the results obtained in this work favorably compared with the literature (Park et al., 2016; Guilliams et al., 2016). Recently, this strategy has been employed to reduce the inhibitor loading in wheat straw and hardwoods hydrolysates (Min et al., 2015; Park et al., 2016). It was also employed for the improvement of enzymatic cellulose saccharification, an increase from 67 to 75 % of glucose yield with 30 FPU per g was obtained by two steps of autohydrolysis using coastal Bermuda grass (Lee et al., 2010). On the other hand, combined autohydrolysis treatment at S_0=4.66 achieved a 66 % of sugar recovery from wheat straw (Min et al., 2015).

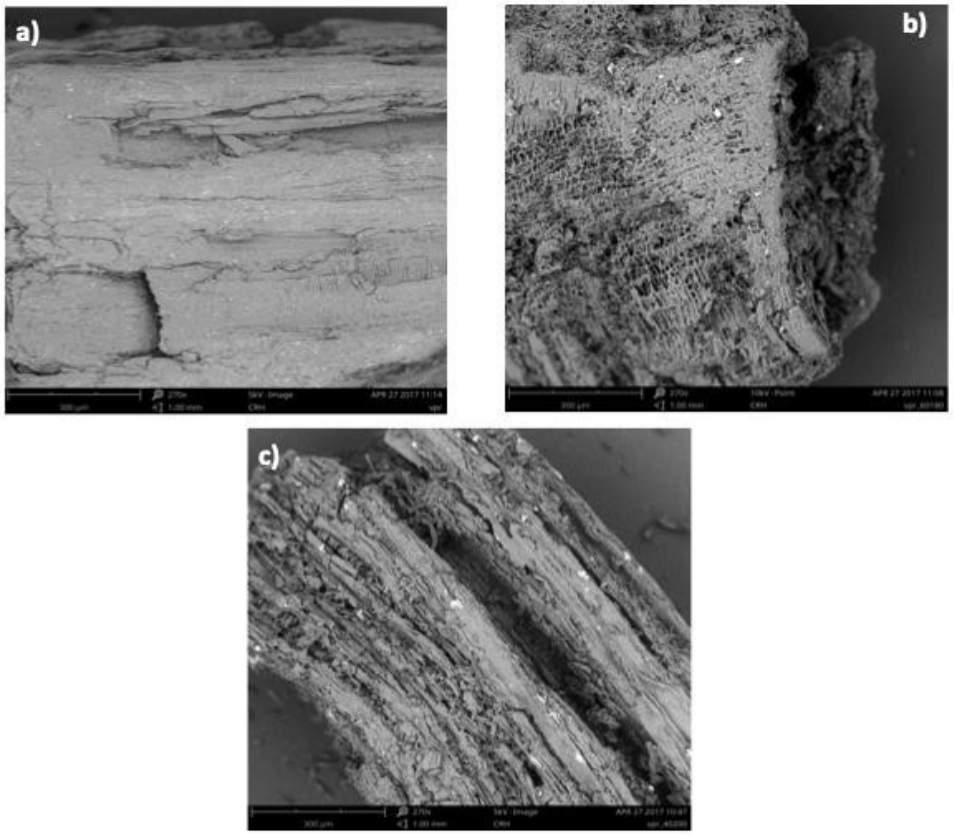


Figure 3.3 Scanning Electron Microscopy images: **a)** vine pruning residue; **b)** autohydrolyzed vine pruning residue from first autohydrolysis treatment (180°C for 60 min, S_0 of 4.13); **c)** autohydrolyzed vine pruning residue from sequential stages of autohydrolysis (200 °C for 40 min, Total S_0 of 4.60).

3.3.5 Bioethanol production by separate and simultaneous saccharification and fermentation

Taking into account the results discussed in the above section, VPR treated by autohydrolysis in two sequential stages (Total S_0=4.60) was chosen for ethanol production. At this condition, 96 % of glucan was recovered after treatment, xylooligosaccharides in autohydrolysis liquor achieved the

highest concentration (6.9 g/L) and enzymatic saccharification conversion was higher than 80 %. For ethanol production, two strategies were evaluated: separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Operational conditions for SHF and SSF were included in Table 3.5. SHF experiments were shown in Figure 3.4a (assays were carried out in duplicate with a calculated relative error ≤ 10 %). Glucose concentration achieved the maximal concentration of 47.2 and 16.5 g/L for 10 and 4 % of solids, respectively. After 48 h of enzymatic hydrolysis, yeast cells were added achieving maximal ethanol concentration within 4 and 8 h of fermentation with an ethanol yield > 90 %.

Table 3.5 Operational conditions of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) and main results obtained from vine pruning residue from sequential stages of autohydrolysis (Total S_0=4.60)

Run	Operational conditions		Main results	
	Solid loading (%)	Enzyme Substrate Ratio (FPU per g)	Ethanol max (g/L)	Ethanol yield, Y_{Et} (%)
SHF1	4	25	8.84 ± 0.08	95.26 ± 0 86
SHF2	10	25	20.97 ± 0.21	93.50 ± 1.07
SSF1	16.7	25	30.08 ± 0.85	83.41 ± 2.36
SSF2	16.7	15	19.09 ± 0.33	52.94 ± 0.92

SSF has been proposed as a strategy for improvement of ethanol yield since glucose is simultaneously produced and consumed avoiding end-product inhibition of cellulose enzymes (López-Linares et al., 2014). In this sense, solid loading was increased up to 16.7 % and enzyme loading was decreased (15 FPU per g) to evaluate the SSF strategy under demanding conditions. Ethanol profiles of SSF experiments were shown in Figure 3.4b (assays were carried out in duplicate with a calculated relative error ≤ 10 %). Ethanol production was affected by a reduction of enzyme loading achieving low ethanol yields (Y_{Et}=52.9 %) (Table 3.5). Ethanol concentration of 30 g/L corresponding to an Y_{Et} of 83 % was obtained with enzyme loading of 25 FPU per g. Ethanol production was strongly affected by the enzyme loading. Nevertheless, recent studies on enzyme recycling suggested the possibility of enzyme recovery being used in sequential batches reducing the overall enzyme loading (Gomes et al., 2016).

Ethanol results obtained in this work can be compared with reported data using residues from wine-making process. SHF strategy was also used for ethanol production from residue derived from vineyard pruning treated by steam explosion (S_0=4.56), obtaining 8.8 g/L of ethanol with 81.09 % of

ethanol yield (Buratti et al., 2015). By-products from wine-making processing such as grape skins (containing water-soluble carbohydrates) have been used as raw material for ethanol production obtaining a maximal concentration of ethanol (22 g/L) with ethanol yield of 0.51 g per g using sequential treatments of hexane and water extraction and acid hydrolysis (Mendes et al., 2013). Pretreated grape stalks by autohydrolysis followed by acid hydrolysis were also used for ethanol production obtaining 20.84 g/L (0.35 g ethanol per g sugars) (Egüés et al., 2013).

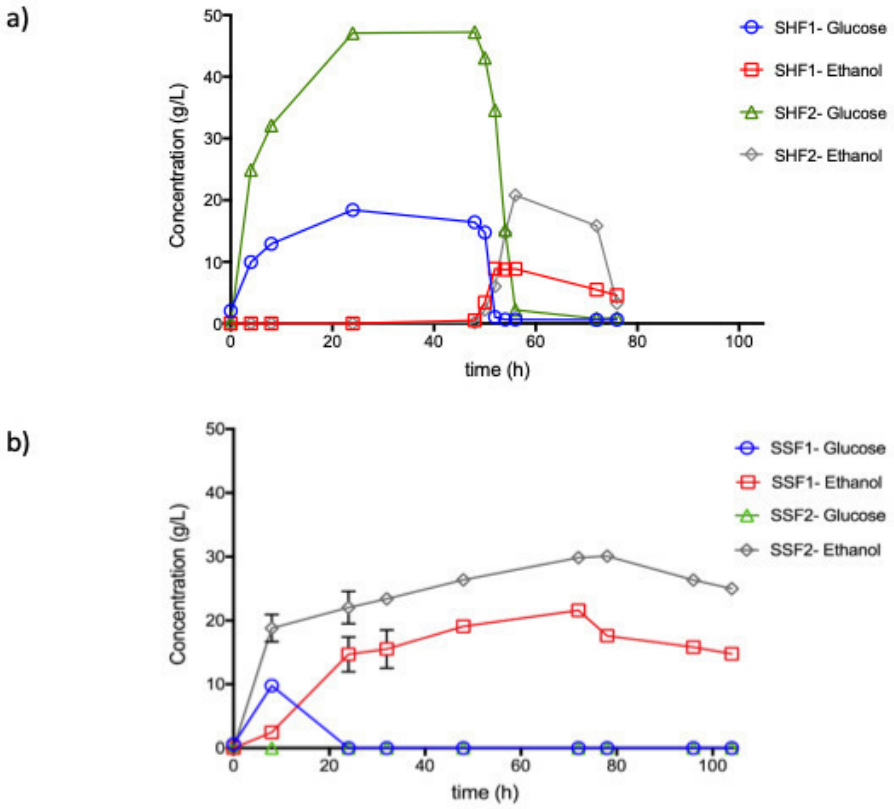


Figure 3.4 a) Separate hydrolysis and fermentation experiments using 4 % (SHF-1) and 10 % (SHF-2) of solids and 25 FPU per g and **b)** Simultaneous saccharification and fermentation (SSF) using 16.7 % of solids and 25 (SSF-1) and 25 FPU per g (SSF-2).

3.3.6 Recovered spent residue after saccharification and fermentation process

Finally, spent solid residue was recovered after SHF (using 4 % solids) and analyzed for chemical composition. Results showed a solid residue mainly composed by lignin (80.7 g of Klason lignin per 100 g of spent solid from SHF). This result can be compared to Klason lignin from isolated lignins (81-86 %) obtained by autohydrolysis followed by organosolv, acetosolv and alkali processes (Dávila et al.,

2017). The chemical structure of the recovered residue was analyzed by FT-IR (Figure 3.5a). The FT-IR analysis is used to determine the presence of functional groups, lignin purity and lignin units composition (Santos et al., 2015). The spectrum showed the characteristic bands for lignin functional groups: hydroxyl group and phenol compounds between 3500 and 3000 cm^{-1}, aromatic ring in the region of 1500 cm^{-1}, carbonyl and carboxyl groups in the band of 1700 cm^{-1}. The FT-IR spectrum of spent residue from SHF also showed an intensity of monolignols present in the structure of lignin: signal assigned to syringyl units (the bands at 1330 and 833 cm^{-1}) and guaiacyl units (the bands observed at 1511, 1421 and 913 cm^{-1}). This spectrum can be compared with FT-IR spectra from isolated lignins obtained by sequential stages of autohydrolysis and organosolv, acetosolv and 8 % NaOH of vine shoot (Dávila et al., 2017).

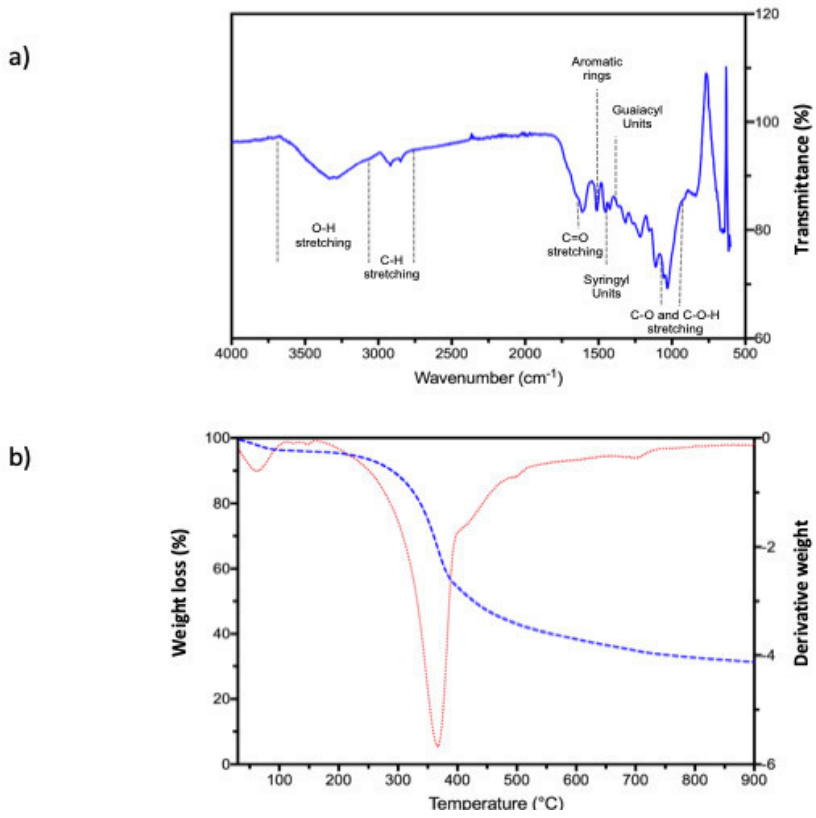


Figure 3.5 Analysis of spent residue after enzymatic saccharification and fermentation: **a)** FT-IR spectrum; **b)** Thermogravimetric analysis (TGA) and its derivative thermogravimetric (DTG) curve.

On the other hand, thermogravimetric analysis (TGA) of this sample was also carried out (Figure 3.5b), weight loss (4.7 %) at 100 °C was due to moisture removal. In the range of 100 - 280 °C, the

weight loss was almost constant (corresponding to 6 %). The highest weight loss (30 %) was achieved at 368 °C, which was similar to those reported by previous workers using lignin extracted from enzymatic hydrolysis process (Tana et al., 2016). In the range of 420 - 900 °C, the weight loss (20 %) was less pronounced. Final residue yield for spent solid recovered after SHF was 30 %, corresponding to ashes and protein contents (Tana et al., 2016). Moreover, residual yeast from fermentation process is also present in this kind of residues (Yunus et al., 2015). Therefore, the use of spent residue from saccharification and fermentation process as new protein and antioxidants source could be an interesting alternative leading to widened valorization of VPR.

3.4 Conclusions

In this work, autohydrolysis in two sequential stages was proposed for the integral valorization of VPR. High concentration of oligosaccharides with antioxidant activity was obtained from the first step of autohydrolysis. The second autohydrolysis improved the enzymatic saccharification of VPR. Selected conditions led to the following products yield (per 100 kg of VPR): 13.6 kg of xylooligosaccharides, 3.1 kg of phenolic compounds, 13.1 kg of ethanol and 27.0 kg of lignin in four separate streams. As a whole, 69 kg of value added compounds were obtained from processing of 100 kg VPR being suitable for manufacture in energy, pharmaceutical and food industries.

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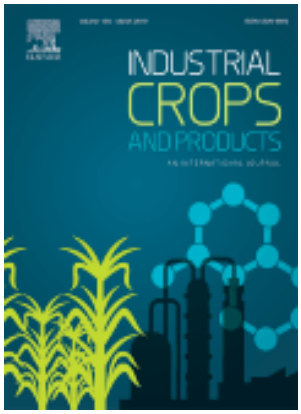
CHAPTER IV

BIOACTIVE COMPOUNDS RECOVERY OPTIMIZATION FROM VINE PRUNING RESIDUE USING CONVENTIONAL HEATING AND MICROWAVE-ASSISTED EXTRACTION METHODS

ABSTRACT

Polyphenol compounds from vine pruning residue (VPR) were extracted by conventional heating and microwave-assisted treatments. For each treatment, total phenolic compounds and their antioxidant activity were optimized by experimental design. Maximal extraction of polyphenolic compounds (2.17 g/100 g VPR) was obtained at 80 °C, 120 min and 45 % of ethanol by conventional heating, and 2.37 g/100 g of VPR were extracted by microwave-assisted process at 120 °C, 5 min and 60% of ethanol. Ellagic acid and apigenin were the predominant polyphenolic compounds in the extracts, achieving concentration of 68.65 and 208.23 mg/100 g VPR, respectively for conventional heating and 185.15 and 118.84 mg/100 g of VPR for microwave-assisted treatment. The results showed reduction of extraction time and energy consumption for microwave-assisted treatment leading to cost-effective technology for the extraction of polyphenol compounds. Furthermore, the results hereby compiled allow for the tailor-made extraction of specific high-value compounds from a renewable biomass as vine pruning residue.

Keywords: Vine pruning reidue, Extraction, Polyphenols, Antioxidant.



The information presented in this Chapter was published in *Industrial Crops and Products*.

Meirielly S. Jesusa, Zlatina Genishevaa, Aloia Romaní, Ricardo Pereira, José A. Teixeira, Lucília Domingues (2019). Bioactive compounds recovery optimization from vine pruning residue using conventional heating and microwave-assisted extraction methods. *Industrial Crops and Products 132, 99-110.*

CONTENTS

4.1 INTRODUCTION

High volumes of agricultural wastes and byproducts are produced from agro-food industry every year. In Mediterranean regions, the wine industry generates a significant amount of these residues (namely stems, peels, seeds, vine leaves, vine pruning or shoots) with no economic value, which are directly discarded causing environmentally problems (Delgado-Torre et al., 2012; Teixeira et al., 2014). Recycling and reusing these wastes to obtain added-value compounds is mandatory to promote the circular Economy, and thus achieve a sustainable growth of our society. In this context, these residues are recognized sources of polyphenolic compounds with interesting biological activities which help for the prevention of inflammatory processes, cardiovascular problems and prevention of oxidative reactions (Teixeira et al., 2014). Therefore, the research on the extraction of these natural bioactive compounds from winemaking byproducts has recently increased (Domínguez-Perles et al., 2014; Lachman et al., 2016; Piñeiro et al., 2017; Pintać et al., 2018).

Among these residues, vine pruning residue is a lignocellulosic material composed by non-structural components (extractives, ashes, proteins) and structural components (cellulose, hemicellulose and lignin) which can be processed for the production of oligosaccharides and cellulosic ethanol (Jesus et al., 2017). In addition, extractives can be also considered as target fraction for an integral valorization of these lignocellulosic materials (Gullón et al., 2017). The content of the extractives in vine pruning residue can vary from 3.1 to 16.6 % (Dávila et al., 2016; Buratti et al., 2015; Jesus et al., 2017), which could depend on the cultivar, different growth regions (Delgado-Torre et al., 2012) and/or conditions of post-pruning and time of storage (Cebrián et al., 2017). In spite of representing a low percentage fraction, the recovery and separation from extractives of valuable products with high market price (such as terpenes, esters, stilbenes, flavonoids or other antioxidant phenols) could improve the economic profitability of the process (Cebrián et al., 2017).

Traditional methods used for the phenolic compounds extraction employ toxic solvents such as methanol-water mixtures (Delgado-Torre et al., 2012) and long times of heating, which results in high energy requirements and also in the degradation of thermal-labile molecules (Galanakis et al., 2013). The application of the extracted bioactive compounds for a human use makes mandatory the optimization of processes based on nontoxic solvents such as water or ethanol-water mixtures. Alternative techniques of extraction have been used for the recovery of phenolic compounds from vine pruning residue, including hydrothermal treatment at high temperatures (Jesus et al., 2017; Gullón et al., 2017), ultrasonic-assisted extraction (Jing et al., 2015; Kazibwe et al., 2017), alkaline hydrolysis

treatments (Max et al., 2010), pulsed electric field extraction (Rajha et al., 2015) and microwave-assisted extraction (MAE) (Calinescu et al., 2017; Ranic et al., 2014; Spigno and De Faveri, 2009). Among these alternatives, the microwave-assisted treatment is considered an efficient method of extraction since, it requires low energy and short times of extraction (Delgado-Torre et al., 2012; Luque-Rodríguez et al., 2006; Piñeiro et al., 2017; Proestos and Komaitis, 2008). The electromagnetic dipole rotation between the molecules and the heating generated in the MAE solvent are responsible for the higher recovery of extracts with high content of the target compound. Polar molecules, such as water inside the plant cell, are heated instantaneously by microwave, where evaporation generates high pressure on the walls of cells due to the swelling of the plant cell, pushing and stretching the cell wall causing them to be destroyed. This process facilitates the release of the bioactive compounds from the cells, increasing the extraction yield. This efficiency can vary accordingly to the parameters adopted for the extraction such as time, temperature and solid-liquid ratio, concentration and type of solvent used (Mandal et al., 2007; Proestos and Komaitis, 2008).

Recently, vine pruning residue was subjected to two sequential stages of hydrothermal treatment to produce cellulosic ethanol and hemicellulosic liquors enriched in xylooligosaccharides and phenolic compounds with antioxidant activity (Jesus et al., 2017). On the basis of the previous work, a study of phenolic compounds extraction, as first stage for the biorefinery of vine pruning residue, is proposed. Thus, the aim of this work was the optimization of phenolic compound extraction by conventional heating and microwave assisted methods through an experimental design in which temperature, time and percentage of ethanol-water were evaluated. The antioxidant activity (based on FRAP, DPPH and ABTS methods) were determined and phenolic compounds were identified and quantified.

4.2 MATERIALS AND METHODS

4.2.1 Raw material and analysis of chemical composition

Vine pruning residue (VPR) from *V. vinifera* variety Loureiro were collected and gently given away by a producer from the Minho region (North of Portugal), in January of 2015. The material was dried at room temperature, milled (Retsch SM100) in to pass an 8 mm mesh and homogenized in a single batch. The moisture content was determined using a Moisture Analyser (Radwag MAC 50/1/NH) and

was 17.6 ± 0.12 and stored at room temperature in a dark and dry place until its use. The chemical composition of the VPR was previously analyzed by Jesus et al. (2017). VPR composition was as follows (expressed in g per 100 g VPR on oven-dry basis ± standard deviation based in three replicate determinations): 32.9 ± 0.66 of cellulose (as glucan); 14.87 ± 0.17 of xylan; 0.40 ± 0.06 of arabinan; 3.95 ± 0.52 of acetyl groups; 29.5 ± 1.21 of Klason lignin; 13.7 ± 1.02 of extractives in water; 2.94 ± 0.89 of extractives in ethanol and 3.32 ± 0.56 of ashes.

4.2.2 Extraction of antioxidant compounds

4.2.2.1 Conventional heating extraction (CHE)

Conventional heating extraction (CHE) treatment was carried out in silicone oil bath with circulating heater and temperature control using 160 mL cylindrical reactors fabricated from 316 stainless steel. Previous study was performed to evaluate the range of variables to be evaluated (Table 4.1). For CHE assays, 0.5 g of VPR was mixed with 20 mL of ethanol-water (according to Moreira et al., 2018) ranging from 30 to 60 %, varying the temperature between room temperature (25 °C) and 100 °C and time between 60 and 180min. Then, the extracted solution was recovered by filtration and the VPR extracts were stored at 4 °C until further use.

Table 4.1 Experimental runs of the preliminary analyzes using different time (min) temperature (°C) and ethanol (%) and total polyphenolic compound responses obtained.

Runs	Ethanol (% v/v)	Temperature (°C)	Time (min)	TPC (g GAE/100 g VPR)
1	60	80	120	1.9 ± 0.05^{cd}
2	30	80	120	2.0 ± 0.09^{cd}
3	45	80	60	1.7 ± 0.01^{bc}
4	45	80	180	2.1 ± 0.03^{d}
5	45	60	120	1.5 ± 0.01^{a}
6	45	100	120	2.0 ± 0.01^{cd}
7	45	25	120	1.2 ± 0.02^{a}

* The averages followed by the same letters do not differ by the *Tukey* test ($p < 0.05$). TPC: total phenolic compounds; GAE: gallic acid equivalents.

After evaluation of preliminary results, an experimental design (2^3) was carried out for the optimization of phenolic compounds extraction, where the variables extraction time (19 – 221 min), ethanol concentration (20 – 70 % v/v) and extraction temperature (46 – 114 °C) were evaluated. A

liquid to solid ratio of 40:1 mL/g of VPR was selected for CHE experiments. Table 4.2 shows the conditions of experiments carried out. After conventional extraction, VPR extracts were recovered by filtration, as described above. All the experiments were conducted in triplicate.

The independent variables: extraction time (x_1, min), extraction temperature (x_2, °C), and concentration of ethanol (x_3, %) were correlated with the dependent variables (phenolic compounds and antioxidant activity) by the follow equation:

$$y_i = \beta_{0i} + \beta_{1i} x_1 + \beta_{2i} x_2 + \beta_{3i} x_3 + \beta_{11i} x_1^2 + \beta_{22i} x_2^2 + \beta_{33i} x_3^2 + \beta_{12i} x_1 x_2 + \beta_{13i} x_1 x_3 + \beta_{23i} x_2 x_3 \quad (1)$$

Where, y_i (i = 1 to 6) are the dependent variables corresponding to concentration of total phenolics extracted, antioxidant activity (g GAE/100 g VPR, g FE (II)/100 g VPR and g TE/100 g VPR) or phenolic compounds (apigenin mg/100 g VPR and ellagic acid mg/100 g VPR); x_1, x_2 and x_3 value of independent variables; β_{0i}, β_{1i}, β_{2i}, β_{3i}, β_{11i}, β_{22i}, β_{33i}, β_{12i}, β_{13i} and β_{23i} are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed model using commercial software (Statistica). The goodness of model fitting was evaluated by the coefficient determination R^2 and the statistical significance by the *Fisher´s F-test* for analysis of variable with a 95 % confidence level. Significant differences among the results were evaluated by the one-way analysis of variance (*ANOVA*) followed by the *Tukey's* HSD.

Table 4.2 Experimental runs using coded levels of time (min. x_1), temperature (°C x_2) and ethanol (% x_3) according to the 2^3 full factorial central composite design and extraction of polyphenolic compounds and antioxidant activity obtained under those conditions.

Independent variables	Symbol	Range and levels				
		-1.68	-1	0	1	1.68
Extraction time (min)	x1	19	60	120	180	221
Extraction Temperature (°C)	x2	46	60	80	100	114
Concentration of ethanol (%)	x3	20	30	45	60	70

Runs	Coded variables levels							
	x_1 (min)	x_2 (°C)	x_3 (% v/v)	TPC (g GAE /100 g VPR)	FRAP (g FE/100 g VPR)	DPPH (g TE/100 g VPR)	IC_{50} (g/L)	ABTS (g TE/100 g VPR)	IC_{50} (g/L)
1	60	60	30	1.5 ± 0.04^{ab}	1.8 ± 0.06^{a}	3.4 ± 0.22^{abc}	12.3 ± 0.02	1.0 ± 0.01^{a}	8.1 ± 0.41
2	60	60	60	1.6 ± 0.06^{bcd}	1.7 ± 0.03^{a}	3.1 ± 0.08^{ab}	12.8 ± 0.05	1.3 ± 0.02^{b}	6.7 ± 0.15
3	180	60	30	1.2 ± 0.013^{a}	1.9 ± 0.05^{a}	4.2 ± 0.14^{cde}	6.2 ± 0.11	1.8 ± 0.02^{c}	3.7 ± 0.10
4	180	60	60	1.5 ± 0.05^{abc}	3.0 ± 0.16^{c}	3.9 ± 0.12^{1abcd}	7.6 ± 0.26	2.0 ± 0.02^{cd}	3.3 ± 0.04
5	60	100	30	1.5 ± 0.03^{abc}	2.3 ± 0.08^{ab}	4.0 ± 0.12^{bcd}	12.8 ± 0.22	2.1 ± 0.02^{def}	5.2 ± 0.06
6	60	100	60	1.8 ± 0.06^{def}	1.9 ± 0.05^{a}	2.9 ± 0.18^{ab}	12.6 ± 0.01	2.2 ± 0.05^{fghi}	2.9 ± 0.11
7	180	100	30	1.7 ± 0.08^{cdf}	2.9 ± 0.07^{bc}	3.8 ± 0.14^{abcd}	7.7 ± 0.07	2.3 ± 0.03^{fghi}	2.9 ± 0.14
8	180	100	60	2.1 ± 0.05^{fg}	3.0 ± 0.04^{c}	4.4 ± 0.12^{bcd}	6.6 ± 0.02	2.4 ± 0.01^{hi}	2.6 ± 0.03
9	19	80	45	2.0 ± 0.15^{efg}	2.0 ± 0.13^{ab}	3.7 ± 0.07^{abcd}	8.4 ± 0.05	2.2 ± 0.01^{efgh}	3.0 ± 0.09
10	221	80	45	2.2 ± 0.05^{g}	1.9 ± 0.05^{a}	5.3 ± 0.02^{ef}	5.6 ± 0.08	2.5 ± 0.06^{a}	2.5 ± 0.15
11	120	46	45	1.5 ± 0.03^{bcd}	2.3 ± 0.07^{ab}	3.1 ± 0.03^{ab}	13.0 ± 0.01	2.4 ± 0.06^{ghi}	3.2 ± 0.05
12	120	114	45	2.2 ± 0.02^{g}	2.3 ± 0.17^{a}	5.7 ± 0.04^{f}	4.9 ± 0.12	2.3 ± 0.01^{efgh}	2.3 ± 0.09
13	120	80	20	1.3 ± 0.03^{ab}	2.2 ± 0.2^{a}	2.8 ± 0.08^{a}	7.3 ± 0.15	2.0 ± 0.02^{de}	3.1 ± 0.02
14	120	80	70	1.5 ± 0.16^{2bcd}	2.1 ± 0.13^{a}	4.5 ± 0.06^{de}	6.9 ± 0.31	2.0 ± 0.02^{cd}	2.2 ± 0.01
15	120	80	45	2.2 ± 0.08^{g}	4.2 ± 0.04^{d}	4.7 ± 0.18^{def}	6.5 ± 0.10	2.2 ± 0.09^{efgh}	2.3 ± 0.21
16	120	80	45	2.2 ± 0.06^{g}	4.3 ± 0.18^{d}	4.3 ± 0.11^{cde}	6.5 ± 0.08	2.2 ± 0.01^{efgh}	2.1 ± 0.19
17	120	80	45	2.1 ± 0.06^{g}	4.5 ± 0.17^{d}	4.7 ± 0.18^{cde}	6.6 ± 0.04	2.3 ± 0.01^{fgh}	2.3 ± 0.14
18	120	80	45	2.2 ± 0.03^{g}	4.7 ± 0.03^{d}	4.7 ± 0.23^{cde}	6.2 ± 0.17	2.3 ± 0.02^{fgh}	2.7 ± 0.02

* The averages followed by the same letters do not differ by the *Tukey* test ($p < 0.05$). TPC: total phenolic compounds; GAE: gallic acid equivalents; FE ferrous equivalents; TE: trolox equivalents. Antiradical activity is expressed as a mean (n = 3) of IC_{50} values (g of extract/L of solution).

4.2.2.2 Microwave-assisted extraction (MAE)

Alternative microwave-assisted extraction of phenolic compounds from VPR, were carried out in a Speedwave 4 microwave digester. Conditions of microwave extraction were selected based on conventional heating extraction treatment. For the extraction of phenolic compounds, the liquid to solid ratio was 40 mL of ethanol-water per 1 g of VPR. The percentage of ethanol-water varied from 0 to 60 % (v/v). Microwave extraction assays were carried out at temperature ranging from 60 to 120 °C for 5 or 40 min (Table 4.3). Extracts were recovered by filtration. The experiments were conducted in triplicate.

Table 4.3 Total phenolic content and antioxidant activity from extracts obtained by microwave-assisted extraction. The results were expressed as g per 100 g dry material (g/100 g VPR).

Runs	Ethanol concentration (% v/v)	Temperature (°C)	Time (min)	TPC (g GAE/100 g)	FRAP (g FE/100 g)	DPPH (g TE/100g)	IC_{50} (g/L)	ABTS (g TE/100 g)	IC_{50} (g/L)
1	60	120	40	2.3 ± 0.10^{c}	9.5 ± 0.45^{e}	3.2 ± 0.02^{ab}	4.9 ± 0.40	3.3 ± 0.03^{b}	1.3 ± 0.17
2	60	120	25	2.2 ± 0.10^{bc}	8.5 ± 0.02^{ef}	3.2 ± 0.02^{ab}	5.4 ± 0.43	3.2 ± 0.10^{ab}	1.4 ± 0.27
3	60	120	10	2.3 ± 0.02^{c}	6.5 ± 0.03^{ab}	3.1 ± 0.05^{ab}	5.3 ± 0.21	3.3 ± 0.04^{b}	2.4 ± 0.25
4	60	120	5	2.4 ± 0.01^{c}	7.5 ± 0.07^{bcd}	4.3 ± 0.06^{c}	6.4 ± 0.12	3.4 ± 0.20^{b}	3.0 ± 0.38
5	60	90	10	2.15 ± 0.02^{bc}	7.2 ± 0.01^{abc}	2.3 ± 0.06^{a}	6.5 ± 0.50	3.0 ± 0.10^{ab}	2.0 ± 0.04
6	60	90	5	2.1 ± 0.02^{bc}	6.2 ± 0.03^{ab}	3.8 ± 0.01^{bc}	6.8 ± 0.64	2.7 ± 0.04^{ab}	4.0 ± 0.26
7	60	60	10	1.6 ± 0.02^{a}	6.3 ± 0.03^{ab}	3.2 ± 0.01^{ab}	-	2.9 ± 0.04^{ab}	-
8	60	60	5	1.8 ± 0.05^{ab}	5.8 ± 0.09^{a}	3.2 ± 0.02^{ab}	-	2.8 ± 0.02^{ab}	-
9	30	120	10	2.3 ± 0.01^{c}	8.4 ± 0.03^{cde}	4.0 ± 0.10^{bc}	5.5 ± 0.13	2.7 ± 0.03^{ab}	2.2 ± 0.19
10	30	120	5	2.4 ± 0.01^{c}	7.5 ± 0.10^{bcd}	4.0 ± 0.01^{bc}	3.6 ± 0.14	3.3 ± 0.10^{b}	1.1 ± 0.13
11	30	90	10	2.3 ± 0.02^{c}	6.8 ± 0.03^{abc}	3.9 ± 0.01^{bc}	3.7 ± 0.39	2.8 ± 0.03^{ab}	2.6 ± 0.67
12	30	90	5	2.3 ± 0.02^{c}	4.1 ± 0.02^{ab}	4.0 ± 0.04^{c}	6.7 ± 0.07	2.8 ± 0.07^{ab}	2.4 ± 0.05
13	30	60	10	1.6 ± 0.01^{a}	6.2 ± 0.09^{ab}	3.3 ± 0.03^{bc}	-	2.5 ± 0.04^{ab}	-
14	30	60	5	1.8 ± 0.03^{ab}	5.9 ± 0.06^{ab}	3.4 ± 0.06^{ab}	-	2.2 ± 0.02^{c}	-
15	0	120	10	1.5 ± 0.01^{a}	5.6 ± 0.06^{a}	3.5 ± 0.02^{bc}	-	2.7 ± 0.01^{ab}	-
16	0	120	40	2.4 ± 0.01^{c}	6.3 ± 0.05^{c}	3.7 ± 0.20^{ab}	6.9 ± 0.67	3.0± 0.03^{ab}	2.6 ± 0.25

* The averages followed by the same letters do not differ by the *Tukey* test ($p < 0.05$). TPC: total phenolic compounds; GAE: gallic acid equivalents; FE (II) ferrous equivalents; TE: trolox equivalents. Antiradical activity is expressed as a mean (n = 3) of IC_{50} values (g of extract/L of solution).

4.2.3 Total phenolic compounds (TPC) analysis

Total phenol compounds in VPR extracts were analyzed based on the spectrophotometric method described by Meneses et al. (2013). Briefly, 5 μL of the extract, 60 μL of 7.5 % (w/v) aqueous sodium carbonate solution, 15 μL of Folin-Ciocalteu reconstitution agent and 200 μL of distilled water were mixed in 96-well microplate. The mixture was incubated for 6 minutes at 60 °C and the absorbance was measured at 700 nm against a blank sample using a Microplate reader for

UV-Vis absorbance (Synergy HT-BIOTEK). A calibration curve was prepared using standard solution of gallic acid (125, 250, 500, 750, 1000, 2000, and 3000 mg/L). The results were expressed as g of gallic acid equivalents per 100 g dry material (g GAE/100 g VPR).

4.2.4 UHPLC analysis

Phenolic compounds present in the VPR extracts from conventional heating and microwave treatments were analyzed and quantified by Shimatzu Nexpera X2 UHPLC chromatograph equipped with Diode Array Detector (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; from Waters) and a precolun of the same material at 40 °C. The flow rate was 0.4 mL/min. The HPLC grade solvents used were water/formic acid (0.1 %) as solvent A and acetonitrile as solvent B. The elution gradient for solvent B was as follows: from 0.0 to 5.5min eluent B at 5 %, from 5.5 to 17 min a linear increase to 60 %, from 17.0 to 18.5 min a linear increase to 100 %, then column equilibration from 18.5 to 30.0 min at 5 %. Phenolic compounds were identified by comparing their UV/Vis spectra and retention times with that of corresponding standards. Compounds were quantified and identified at different wave-lengths: ellagic acid at 250 nm; vanillic acid at 254 nm; catechin, narigenin, *p*-cumaric acid, epicatechin, gallic acid, syringic acid, *o*-cumaric acid and cinnamic acid at 280nm, *trans*-resveratrol at 308 nm; apigenin and rosmarinic acid at 329 nm; rutin at 350nm and quercetin at 370 nm. All calibration curves had R^2 between 0.997 and 0.999. Concentration of the individual compounds in the calibration curves were between 250 mg/L and 1.25 mg/L.

4.2.5 Determination of antioxidant capacity

4.2.5.1 Ferric reducing antioxidant power (FRAP)

The antioxidant activity by the ferric reducing antioxidant power (FRAP) was made according to the methodology previously described (Ballesteros et al., 2015; Meneses et al., 2013). FRAP reagent was freshly prepared by mixing a 10mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40mM HCl with a 20mM, $FeCl_3$ solution and 0.3M acetate buffer (pH 3.6) in a proportion 1:1:10 (v/v/v). A calibration curve was prepared with aqueous solution of $FeSO_4$ (10, 30, 50, 60, 70, 80, 110, 140, 200,

and 275 mg/L). In a 96-well microplate was added 10μL of the filtered and duly diluted extract with 290 μL of FRAP reagent. Then, the reaction mixture was incubated at 37 °C for 15 min. After that, the absorbance was determined at 593 nm against a blank prepared with distilled water. FRAP values were expressed as grams of ferrous equivalent per 100 g of VPR extract (g FE/100 g VPR).

4.2.5.2 DPPH radical scavenging activity

The activity DPPH was determined by measuring free radical scavenging the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (Meneses et al., 2013; Sánchez-Gómez et al., 2017a). The method consists in the reduction of the DPPH radical in the presence of hydrogen-donating antioxidant and in the formation of the non-radical DPPH-H form at the end of the reaction. The percentage of remaining DPPH was inversely proportional to the antioxidant concentration. The reaction was carried out in a 96-well microplate, samples (10 μL) with extract were mixed with 290 μL of DPPH in methanol (6 x 10-5M dissolved in methanol absolute to an absorbance value of 0.700 at 515 nm), incubated for 1 h at room temperature and the absorbance was measured at 515 nm. The negative and positive controls were made with methanol and the Trolox standard, respectively. The inhibition activity (%) of the DPPH radicals was calculated using the following equation:

$$\%\ \text{inhibition activity} = \frac{A_0 - A_1}{A_1}\ x\ 100 \qquad (2)$$

Where A_0 was an absorption of the negative control and A_1 for an absorbance of the extracts.

The sample concentration for the 50 % reduction of DPPH radicals (IC_{50}) was calculated by interpolation. The IC_{50} values were expressed as g of Trolox equivalent per 100 g of dry weight material (g TE/100 g VPR).

4.2.5.3 Radical ABTS elimination capability

The ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) cation decolorization assay was according to the methodology described by Ahmad Mir et al., (2017) adapted for 96-well microplates to

evaluate the ability of the sample to eliminate free ABTS + radicals. ABTS (7.4 mM) diluted in ethanol and potassium persulfate (2.6 mM) were mixed in 1:1 (v/v) ratio and incubated for 16 h at room temperature in the dark. To a 200 μL of ABTS radical solution were added 10 μL sample solution or ethanol and the mixture was allowed to stand for 1h at 30 °C to obtain a stable absorbance. Absorbance was measured at 734 nm against a blank. The results were expressed as percent inhibition using the Eq. 2 as described in the item 4.4.2.

4.2.6 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean value ± standard deviation. The statistical analysis of model was carried out using StatSoft Statistica software (version 10).

4.3 RESULTS AND DISCUSSION

4.3.1 Conventional heating extraction (CHE)

4.3.1.1 Effect of temperature, time and ethanol percentage on phenolic compounds extraction: preliminary experiments

The temperature, solvent concentration and time of extraction are important factors that influence the efficiency of the extraction process of phenolic compounds. Preliminary experiments were carried out to evaluate the range of variables for the extraction of phenolic compounds. The conditions of these experiments and the results obtained are listed in Table 4.1.

As seen in Table 4.1, the lowest extraction of total phenolic compounds was achieved at the room temperature. Improvement in the extraction was observed, when the temperature was increased up to 80 and 100 °C. On the other hand, the percentage of ethanol, varying between 30 – 60 %, showed similar results. For the time of extraction, the highest concentration of total phenolic compounds (TPC) was obtained at higher extraction periods. However, there were no significant differences between the experiments carried out at 120 and 180 min. The highest total phenolic compounds extraction (2.0, 2.1 and 1.9 g GAE/100 g VPR) was obtained under conditions of

experiment 2 (30 %, 80 °C and 120 min), experiment 4 (45 %, 80 °C and 180 min) and experiment 6 (45 %, 100 °C and 120 min), respectively. The lowest phenolic compounds extraction (1.2 and 1.5 g GAE/100 g VPR) was achieved in experiments 7 and 5 (Table 4.1), respectively. In this preliminary study, it is observed that the temperature is the most important factor for the extraction of polyphenols compounds from vine pruning residue. On the other hand, the time of extraction has a lower influence on the TPC extraction than the ethanol concentration and temperature.

4.3.1.2 Total phenolic compounds and antioxidant activity CHE method

Based on previous results (Table 4.1), an experimental design (2^3) was proposed to optimize the phenolic compounds extraction and to evaluate their antioxidant capacity. The range of operational conditions (temperature, time and percentage of ethanol) and dependent variables studied in this work were listed in Table 4.2.

The total phenolic compounds obtained by CHE ranged from 1.2 to 2.2 g GAE/100 g of VPR. Maximum concentration of total phenolic compounds (2.2 g GAE/100 g) was obtained under conditions of the central points (120 min, 80 °C and 45 % ethanol concentration) and minimum concentration of total phenolic compounds (1.2 g GAE/100 g) was obtained in run 3 (180 min, 60 °C and 30 % ethanol concentration). These results are in agreement with the values found by other authors (Çetin et al., 2011; Karacabey and Mazza, 2008; Luque-Rodríguez et al., 2006; Moreira et al., 2018) for CHE using different ethanol concentrations. Studies in the literature have shown that the total content of phenols in VPR can vary significantly according to the grape variety (Çetin et al., 2011; Delgado-Torre et al., 2012; Farhadi et al., 2016). Previous work confirmed the influence of solvent concentration on the extraction process of phenolic compounds (Alexandru et al., 2014; Moreira et al., 2018). Mixtures of alcohols/water were found to be more efficient in extraction of phenolic constituents than the corresponding mono-component solvent system due to the increase in permeability in the membrane of the lignocellulosic material (Alara et al., 2018; Bouras et al., 2015). However, despite increasing the extraction of total phenolic compounds, it does not have much influence on the antioxidant activity (Pinelo et al., 2006; Spigno et al., 2007; Yilmaz et al., 2006).

The antioxidant activities of the extracts varied, according to the analysis method, between 1.7 and 4.7 g FE/100 g for the FRAP, 2.9 to 5.7 and 1.0 to 2.4 g GAE/100 g and IC_{50} 0.6 to 1.3 and 0.2 to 0.8, for DPPH and ABTS, respectively. Maximum antioxidant activity for FRAP (4.7 g FE/100 g) were

obtained under conditions of the central points (120 min, 80 °C and 45 % ethanol concentration). For the ABTS and DPPH methods, the maximum antioxidant activity was achieved in one of the axial points; runs 10 and 12, respectively, both made using ethanol 45 %. The highest antioxidant activity measured by DPPH was 2.4 g TE/100 g VPR (run 12) and the highest antioxidant activity quantified by ABTS was of 5.7 g TE/100 g VPR (run 10). The lowest concentration of total phenolic compounds was obtained in the run 3 (180 min, 60 °C and 30 %). The antioxidant activity of vine pruning extracts was superior to the results obtained by Moreira et al., (2018), for FRAP (0.72 g AAE/100 g VPR) and DPPH (0.83 g TE/100 g VPR).

The experimental variables were correlated following the polynomial eq. 1, of the second order, presented in point 4.2.2.2. The proposed mathematical models describing the extraction time (x_1), temperature time (x_2) and ethanol concentration (x_3) as function and using normalized values are described by Eqs. 3, 4, 5 and 6 for TPC (y1), FRAP (y_2), DPPH (y_3) and ABTS (y_4), respectively.

$$y_1 = 2.75 + 0.11\,x_1 + 0.09\,x_1^2 + 0.08\,x_2 - 0.25\,x_2^2 - 0.35\,x_3 - 0.48\,x_3^2 + 0.09\,x_1x_2 + 0.04x_1x_3 + 0.03\,x_2x_3 \quad (3)$$

$$y_2 = 4.64 + 0.11\,x_1 - 0.97\,x_1^2 + 0.04\,x_2 - 0.91\,x_2^2 - 0.61\,x_3 - 0.84\,x_3^2 + 0.03\,x_1x_2 - 0.16\,x_1x_3 + 0.20\,x_2x_3 \quad (4)$$

$$y_3 = 2.53 + 2.25\,x_1 + 0.12\,x_1^2 + 0.03\,x_2 - 0.13\,x_2^2 - 0.12\,x_3 - 0.24\,x_3^2 - 0.13\,x_1x_2 - 0.03\,x_1x_3 + 0.01\,x_2x_3 \quad (5)$$

$$y_4 = 4.66 + 0.24\,x_1 + 0.53\,x_1^2 + 0.12\,x_2 - 0.14\,x_2^2 - 0.17\,x_3 + 0.43\,x_3^2 - 0.06\,x_1x_2 + 0.01\,x_1x_3 + 0.20\,x_2x_3 \quad (6)$$

The R^2 of the models presented in Eqs. 3-6 were: 0.94, 0.90, 0.69 and 0.73, respectively. The calculated *F-value* ($F_{5,3}$) were: 38.35 (Eq. 3); 67.21 (Eq. 4); 16.95 (Eq. 5) and 78.16 (Eq. 6). The models were validated by ANOVA analysis and *F*-test at 95% of confidence level before building the response surface graphs presented in Figure 4.1a-d. All models were statistically significant since the calculated *F-values* were higher than the listed *F-value* ($F_{5,3}$= 9.01) at 95 % of confidence level.

The statistical significance of the model (based on *Fischer´s F* parameter) showed the good fitting of the evaluated variables total phenolics compounds and FRAP ($R^2 \geq 0.9$).

Table 4.4 shows the regression coefficients and variance analysis of linear, quadratic and interactions between variables. The total phenolic compounds (TPC) and antioxidant activity of VPR extracts presented significant effects (p <0.05, p <0.01 or p <0.0001), whereas the interactions between the variables presented different meanings according to the applied analysis method (p <0.05, p <0.01 or p <0.0001). Therefore, the linear and quadratic effects of independent variables were the main determinant terms for antioxidant activities.

Figure 4.1a-d show the effect of temperature and extraction time, with a fixed concentration of 45 % ethanol, on the extraction of TPC and antioxidant activity measured by FRAP, DPPH and ABTS. As shown in Figure 4.1a, concentration of total phenolic compounds above 2 g GAE/100 g of VPR was obtained when the temperature was >80 °C, regardless of the extraction time. The temperature had a notable positive effect on the concentration of total phenolic compounds. Figure 4.1b shows the response surface and the Pareto plot of the effects of the two variables, namely extraction time, extraction temperature (ethanol concentration set at 45 %), for FRAP assays. Quadratic and linear effects of the variables were all significant. Control of model parameters, R^2, *F-exp*, confirmed the model adequacies for total phenolics compounds and FRAP (Table 4.4).

Table 4.4 Analysis of variance of the regression parameters of the predicted second order polynomial models for total phenol compounds and antioxidant activities (FRAP, DPPH and ABTS).

Coefficientes	y_1	y_2	y_3	y_4	y_5	y_6
β_0	2.75	4.64	2.53	4.66	0.071	0.218
β_{11}	0.23 [a]	0.11 [a]	2.25 [a]	0.24 [a]	-0.002 [a]	0.001 [d]
β_{22}	0.18 [b]	0.97 [a]	0.12 [a]	0.53 [b]	0.011 [a]	0.031 [a]
β_{33}	0.17 [b]	0.04 [b]	0.03 [a]	0.12 [a]	0.006 [a]	0.056 [a]
β_{111}	-0.50 [a]	-0.91 [a]	-0.13 [a]	-0.14 [a]	-0.007 [a]	-0.025 [a]
β_{221}	-0.70 [a]	-0.61 [a]	-0.12 [a]	-0.17 [a]	-0.009 [a]	-0.040 [a]
β_{331}	-0.96 [a]	-0.84 [a]	-0.24 [a]	0.43 [a]	-0.012 [a]	-0.045 [a]
β_{121}	0.19 [b]	0.03 [c]	-0.13 [a]	-0.06 [a]	-0.005 [a]	0.020 [a]
β_{131}	0.09 [c]	-0.16 [a]	0.03 [d]	0.01 [b]	0.006 [a]	-0.021 [a]
β_{231}	0.06 [d]	0.20 [a]	0.01 [a]	0.20 [d]	0.001 [a]	0.012 [a]
R^2	0.94	0.90	0.69	0.73	0.74	0.90
F-exp	38.35	67.21	16.95	78.16	517.19	592.64
lack of fit	51.36 [a]	434.36 [a]	377.60 [a]	803.74 [a]	8217.06 [a]	135.79 [a]

[a] Significant coefficients at the p < 0.001 confidence level; [b] Significant coefficients at the p < 0.01 confidence level; [c] Significant coefficients at the p < 0.05 confidence level; [d] not significant p > 0.05

The antioxidant activity measured by DPPH, showed significant effects for all variables and their interactions, except for the interaction between the extraction time and the ethanol concentration, but increasing the temperature and extraction time at low ethanol concentrations reduced the antioxidant capacity of the extracts (Figure 4.1c). With the increase of the extraction temperature, DPPH values increased significantly. However, no significant differences were found in DPPH when ethanol concentration increased above 45 %. The interaction of temperature and ethanol concentration was significant for DPPH method.

Figure 4.1d shows the effect of time and temperature on the antioxidant activity measured by ABTS. The extraction time presented a linear effect, while the extraction temperature and the ethanol concentration had quadratic effects on the response. Under these conditions, the maximum antioxidant activity measured by ABTS can be obtained with an extraction temperature of 80 °C and 45 % ethanol. However, the linear effects of the extraction time demonstrate that an increase on the extraction time, lead to higher antioxidant activity, according to the method ABTS. Previous studies with VPR showed a significant linear increase of the antioxidant activity (ABTS), with increase temperature and low ethanol concentrations (50 %). However, the effect of temperature diminished in higher ethanol concentrations (60 %) (Karacabey and Mazza, 2010). The ethanol concentration, time and temperature on total phenolics compound and antioxidant activities of VPR, has been also reported by Luque-Rodríguez et al., (2006), and Karacabey and Mazza, (2010). The authors showed that the only significant variable was the temperature (16.4 - 240 °C); the percentage of ethanol (20 - 100 %) and extraction time (20 - 90 min) had negative and positive effects, respectively. The lack of adjustment test at the 0.05 level does not indicate evidence of lack of fit for both the total phenolic compound models and antioxidant activity measured by FRAP, DPPH and ABTS.

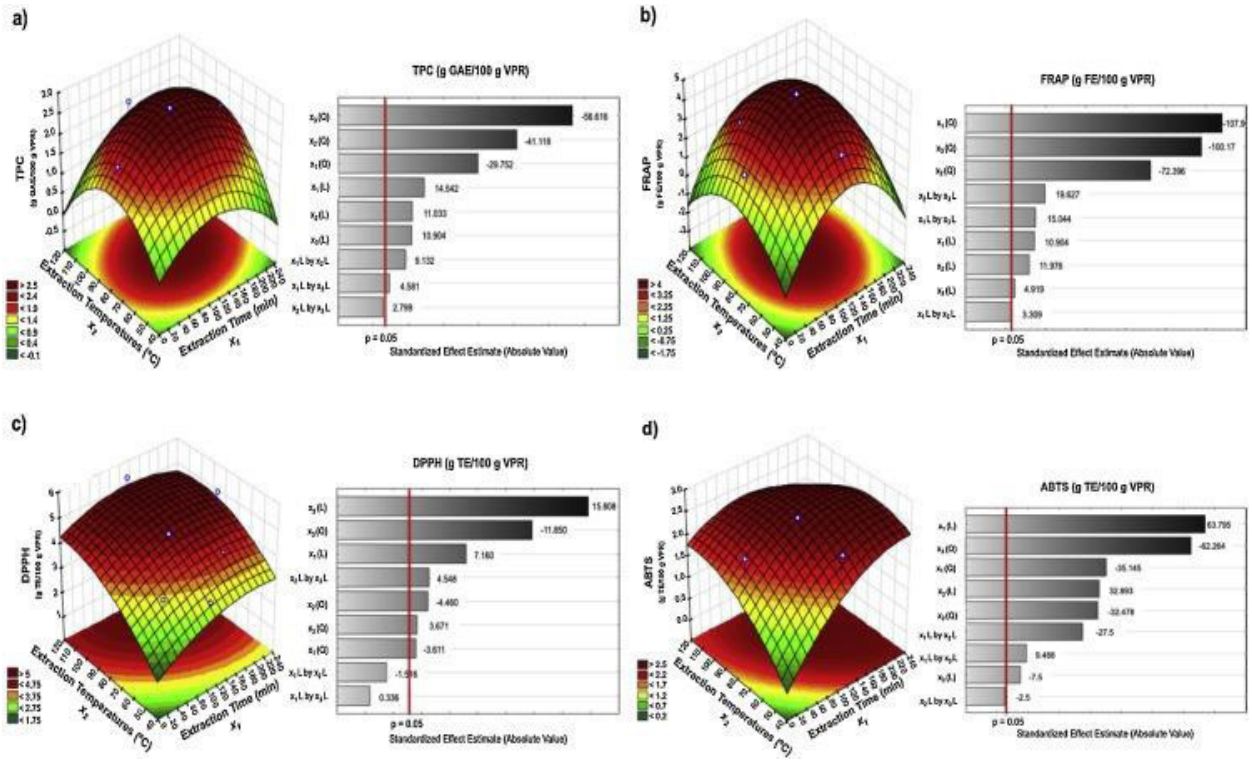


Figure 4.1 Pareto diagram for standardized effects and response surface graphs as function of time of extraction and temperature of extraction (fixed enthanol concentration at 45 % of: a) total phenolicas compounds in GAE g/100g. and antioxidant activity. b) FRAP in g FE/100 g. c) DPPH in g TE/100g and d) ABTS in g TE/100g ($p > 0.05$).

4.3.1.3 Process optimization and validation

In the search to find the highest antioxidant activity (using FRAP, DPPH and ABTS methods), and the total polyphenolic content, maintaining the minimum concentration of ethanol, time and temperature, it was possible to optimize the extraction process. The optimal extraction conditions for the present study were different according to the analysis performed. Optimized parameters for antioxidant activities were included in Table 4.5. As observed, operational conditions to maximize the antioxidant activity for different methods varied in the range of 80 - 115 °C, 137-175 min and 45 - 53 % of ethanol concentration. The antioxidant activities, measured by FRAP, ABTS and total phenolic compounds showed similar optimal conditions between them, temperature of 80 to 83 °C and ethanol concentration of 45 to 46 %, however the extraction time ranged from 124 to 175 min, where the potential values were 4.4 g FE/100 g VPR, 2.5 g TE/100 g VPR and 2.3 g GAE/g, respectively. For DPPH the optimum conditions were: extraction time of 147 min, temperature of 115 °C and ethanol concentration of 53 %, where the antioxidant potential value was 5.1 g TE/100 g VPR. The validation

experiments carried out under the predicted conditions derived from the experimental design analysis demonstrated that the experimental values were close to the predicted values, confirming the validity and adequacy of the predicted models. In addition, the verification experiments also proved that the predicted values of total polyphenolic compounds and antioxidant activity (using FRAP method) for the VPR model can be satisfactorily achieved within the 95% confidence interval. Therefore, the regression equations obtained in this study can be used to obtain extracts with optimal antioxidant activity and total polyphenols.

Table 4.5 Comparison between predicted and experimentally obtained values for investigated optimum conditions based on individual response, between the experimental and predicted values responses.

Responses	Process variables			Predicted value	Experimental value
	X_1 (min)	X_2 (°C)	X_3 (% v/v)		
TPC (g GAE/100g)	137	83.45	46.68	2.26	2.27 ± 0.15
FRAP (g FE/100g)	124	81.72	45.42	4.38	4.45 ± 0.24
DPPH (g TE/100g)	147	115.33	53.39	5.22	5.12 ± 0.24
ABTS (g TE/100g)	175	80.01	45.06	2.37	2.54 ± 0.13

4.3.1.4 UHPLC analysis of phenolic compounds of CHE

Qualitative and quantitative analysis of phenolic compounds of the extract at optimized condition were analyzed by ultra high performance liquid chromatography (UHPLC). Table 4.6 lists the concentrations of the identified compounds (results expressed in mg/100 g of VPR). The highest concentration of phenolic compounds was obtained under conditions of the central point of experimental design (120 min, 80 °C and 45 %). Fifteen phenolic compounds of different classes were detected: flavonoids, phenolic acids and stilbenes, the concentrations of the compounds varied according to the extraction conditions applied in each experiment. The flavonoids present in the extracts obtained were catechin (12.7 - 21 mg/100 g), narigenin (4.9 - 6.2 mg/100 g), apigenin (6.4 - 218.0 mg/100 g), quercetin (23.5 -27.7 mg/100 g) and rutin (9-16.5 mg/100 g). The phenolics acids obtained were rosmarinic acid (13.5 - 15.3 mg/100 g), cinnamic acid (12.6-16.1mg/100 g), syringic acid (13.5 – 20 mg/100 g), vanillic acid (19.7 - 23.1 mg/100 g), ferulic acid (5.8 - 6.9 mg/100 g), gallic acid (6.3 - 12.4 mg/100 g), *o*-cumaric acid (7.4 - 16 mg/100 g) and ellagic acid (5.0 - 75.5 mg/100 g). The retention time of the epicatechin and the *p*-coumaric acid were the same and it was

not possible to separate these compounds, so the concentrations are presented together (5.3 - 6.7 mg/100 g). The stilbene resveratrol was found in concentrations between 13.3 mg and 14.9 mg/100 g VPR. The most abundant flavonoids in VPR are (+) - catechin and (-) - epicatechin, however, in this work these compounds were not the most abundant which is not in accordance with previous studies (Çetin et al., 2011; Delgado-Torre et al., 2012a; Luque-Rodríguez et al., 2006; Sánchez-Gómez et al., 2017b; Sánchez-Gómez et al., 2014). The highest concentration of (+) - catechin was 21.5 mg/100 g of VPR (run 6, 60 min, 100 °C and 60 % of ethanol). The highest content of *p*-coumaric acid + epicatechin 6.7 mg/100 g of VPR, was found at run 10 (221min, 80 °C and 45 % of ethanol). The highest concentration of resveratrol obtained in this study was 14.9 mg/100 g of VPR (221min, 80 °C and 45%). These values were higher than those found by Sánchez-Gómez et al., (2014) in extracts of VPR (2.8 mg/100 g). But, the values of resveratrol concentration were lower than those reported for VPR hydroethanolic extracts (Cebrián et al., 2017; Soural et al., 2015; Vergara et al., 2012). However, in this work the most abundant flavonoid was apigenin with 207.9 mg/100g of VPR (120min, 80 °C and 45 %), although it has not been reported in previous studies using VPR. However, apigenin was identified in extracts of grape marc (Pintać et al., 2018).

Table 4.6 Polyphenolic compounds identified in vine pruning residue extracts (expressed as mg per 100 g VPR).

Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Time (min)	60	60	180	180	60	60	180	180	19	221	120	120	120	120	120	120	120	120
Temperature (°C)	60	60	60	60	100	100	100	100	80	80	46	114	80	80	80	80	80	80
Ethanol concentration (%)	30	60	30	60	30	60	30	60	45	45	45	45	20	70	45	45	45	45
Phenolics Compounds (mg/100 g VPR)																		
catechin	13.2^{ab}	14.6	14.9^{bc}	14.9^{abc}	16.3^{cd}	21.0^{e}	15.8^{cd}	14.4^{abc}	16.4^{bc}	15.7^{bcd}	17.4^{d}	15.0^{bcd}	14.4^{bc}	12.7^{a}	17.6^{cd}	13.3^{abc}	15.1^{bc}	15.8^{de}
narigenin	ND	ND	ND	6.2^{d}	ND	ND	ND	5.5^{bc}	ND	5.9^{bc}	ND	5.4^{bc}	ND	ND	5.7^{bc}	4.9^{b}	5.3^{ab}	5.4^{ab}
apigenin	ND	164.7^{d}	6.4^{a}	193.7^{ef}	7.8^{a}	60.4^{bc}	66.9^{c}	198.1^{ef}	160.4^{d}	187.4^{e}	76.1^{c}	191.5^{ef}	46.5^{b}	190.1^{fg}	215.1^{g}	194.9^{fg}	203.6^{fg}	218.0^{g}
quercetin	ND	25.4^{cde}	23.5^{b}	24.3^{cde}	ND	25.3^{cde}	ND	27.7^{e}	23.5^{b}	25.9^{def}	24.6^{bcd}	24.1^{bcd}	ND	23.9^{bc}	25.2^{cde}	23.8^{bcd}	24.3^{bcd}	26.1^{ef}
rutin	ND	11.9^{c}	13.5^{d}	14.9^{def}	ND	9.7^{b}	11.6^{c}	15.6^{efg}	14.2^{de}	15.3^{efg}	10.6^{bc}	15.0^{def}	9.0^{b}	14.9^{def}	16.2^{fg}	14.7^{efg}	15.5^{efg}	16.5^{g}
p-cumaric acid + epicatechin	5.7 ^{ab}	6.1^{bcd}	5.3^{a}	5.8^{abc}	6.3^{bcd}	6.6^{cd}	6.4^{bcd}	6.6^{cd}	6.0^{bcd}	6.7^{d}	6.4^{bcd}	6.2^{bcd}	6.1^{bcd}	6.2^{bcd}	6.1^{bcd}	6.0^{bcd}	6.0^{bcd}	6.5^{bcd}
gallic acid	ND	11.1^{efg}	ND	10.2^{cde}	6.3^{b}	11.7^{gh}	9.4^{c}	11.3^{fgh}	10.0^{cd}	11.8^{fg}	9.8^{cd}	12.4^{h}	6.8^{b}	10.5^{def}	11.1^{fgh}	10.8^{fgh}	10.9^{fgh}	11.9^{gh}
syringic acid	20.0^{e}	16.1^{d}	16.0^{d}	15.3^{c}	15.1^{c}	ND	13.5^{b}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
vanillic acid	19.7^{a}	21.1^{abc}	19.9^{ab}	21.4^{bcd}	21.1^{abc}	23.0^{fg}	22.6^{def}	23.0^{fg}	21.0^{abc}	23.1^{g}	22.3^{def}	21.9^{cde}	21.2^{bcd}	21.4^{efg}	21.6^{efg}	20.6^{def}	20.8^{def}	22.7^{efg}
ferulic acid	6.1^{abcd}	6.5^{bcd}	6.0^{abc}	6.2^{bcd}	6.3^{bcd}	6.2^{abc}	6.4^{bcd}	6.6^{bcde}	6.0^{abc}	6.7^{bcd}	6.4^{bcd}	5.8^{ab}	6.5^{e}	5.8^{a}	6.5^{cde}	6.3^{bcd}	6.3^{bcd}	6.9^{ef}
o- cumaric acid	ND	14.6^{fgh}	16.0^{hi}	16.3^{i}	ND	7.4^{b}	9.0^{b}	14.4^{fg}	11.4^{d}	14.1^{fg}	12.5^{de}	13.5^{ef}	9.4^{c}	13.4^{56}	15.5^{ghi}	13.6^{fg}	14.4^{fg}	15.4^{ghi}
ellagic acid	23.9^{b}	5.0^{a}	59.0^{g}	66.5^{ghi}	5.1^{a}	32.7^{cd}	37.2^{d}	50.3^{ef}	59.7^{g}	58.4^{fg}	49.6^{e}	59.8^{g}	27.4^{bc}	62.4^{gh}	75.5^{i}	60.9^{ghi}	67.6^{gh}	70.4^{hi}
cinnamic acid	13.2^{ab}	14.6^{def}	14.2^{bcd}	15.3^{cde}	12.6^{a}	14.0^{bc}	14.7^{def}	16.8^{g}	14.2^{bcd}	16.1^{efg}	13.3^{ab}	15.3^{def}	14.0	14.9^{def}	15.8^{efg}	14.7^{def}	15.1^{def}	16.1^{fg}
rosmarinic acid	13.6^{b}	15.0^{cd}	13.9^{ab}	14.2^{bcd}	ND	15.2^{d}	14.7^{bcd}	15.2^{d}	13.9^{bc}	15.3^{d}	14.8^{bcd}	14.2^{bcd}	13.7^{bcd}	14.1^{bcd}	14.0^{bcd}	13.6^{bcd}	13.7^{bc}	15.0^{cd}
trans- resveratrol	ND	14.6^{bc}	13.5^{b}	13.8^{bc}	ND	14.8^{c}	14.3^{bc}	14.8^{c}	13.5^{b}	14.9^{c}	14.4^{bc}	13.9^{bc}	13.7^{bc}	13.8^{bc}	13.7^{bc}	13.3^{bc}	13.4^{b}	14.6^{bc}

* The averages followed by the same number do not differ by the *Tukey* test (p <0.05).

ND: not detected.

The concentrations of phenolic acids: gallic acid, vanillic acid, syringic acid and ferulic acid were within the range (0.5 - 2.1, 0.3 - 0.8, 0.6 and 0.2-14.3 mg/100 g, respectively) reported in literature (Cebrián et al., 2017; R. Sánchez-Gómez et al., 2017b; Sánchez-Gómez et al., 2014). Among the identified phenolic acids, the most abundant in VPR extracts was ellagic acid, with concentration of 68.6 mg/100 g VPR at the central point (120 min, 80 °C and 45 %), these values were higher than those found in the literature Cebrián et al., (2017) (1.36 - 2.1 mg/100 g VPR), Luque-Rodríguez et al., (2006) (17.9 - 57 mg/100 g VPR), Sánchez-Gómez et al., (2017b) (0.8 - 5.4 mg/100 g VPR), Sánchez-Gómez et al., (2014) (0.4 - 1.4 mg/100 g VPR). As a general trend, the total concentration of phenolic compounds in the extract, identified by UHPLC, increased with temperature. According to Luque-Rodríguez et al., (2006), the temperature was the most influential variable on the composition of the extracts of VPR, whereas the percentage of ethanol and extraction time had only an influence on the amount of each compound.

Ellagic acid is a polyphenol of great interest due to its high prebiotic, antioxidant and anti-inflammatory activity (García-Niño and Zazueta, 2015; Landete, 2011; Yu-Qing et al., 2017). In addition, ellagic acid was one of the most abundant compounds found in VPR extracts from this study. The ellagic acid content in extracts of VPR obtained using CHE ranged from 5.0 to 75.5 mg/100 g VPR. The results obtained in this work were superior to those described in previous works for the ellagic acid content in extracts of VPR obtained using the CHE technique (Cebrián et al., 2017; Luque-Rodríguez et al., 2006; Sánchez-Gómez et al., 2017b; Sánchez-Gómez et al., 2014). According to our results, the highest ellagic acid content was achieved using 45 % ethanol, temperature of 80 °C and extraction time of 120 min. On the other hand, the lowest content was obtained in the extract prepared using 60 % ethanol, extraction temperature of 60 °C and 60 minutes of extraction time. Figure 4.2a shows the response surface for the temperature-time relationship with the ethanol concentration set at 45 % for the extraction of ellagic acid. All extraction parameters investigated showed a significant positive impact on the final ellagic acid content (Figure 4.2 b). The concentration of ethanol showed the greatest positive impact on ellagic acid content in VPR extracts. The levels of ethanol, extraction temperature and extraction time significantly affected the ellagic acid content (p <0.001) (Table 4.4). The interactions between time/ethanol concentration, time/temperature and temperature/ethanol concentration, (p < 0.001) also significantly affected the ellagic acid content.

The final predictive equation to describe the extraction efficiency to reach the maximum ellagic acid content (y_5) of VPR is as follows:

$$y_5 = 0.07 - 0.002\,x_1 + 0.011\,x_1^2 + 0.006\,x_2 - 0.007\,x_2^2 - 0.009\,x_3 - 0.012\,x_3^2 - 0.005\,x_1x_2 - 0.006\,x_1\,x_3 + 0.001\,x_2x_3 \quad (7)$$

According to the *p*-value, it can be concluded that the model showed a good approximation for the investigated responses (p <0.001). The R^2 for ellagic acid was 0.74, the non-significant value of lack of fit (F = 135.79) showed the model is fitted to the spatial influence of the variables to the response with good prediction, with a *F-value* of 517.19, higher than the *F-value* table ($F_{5,3}$= 9.01).

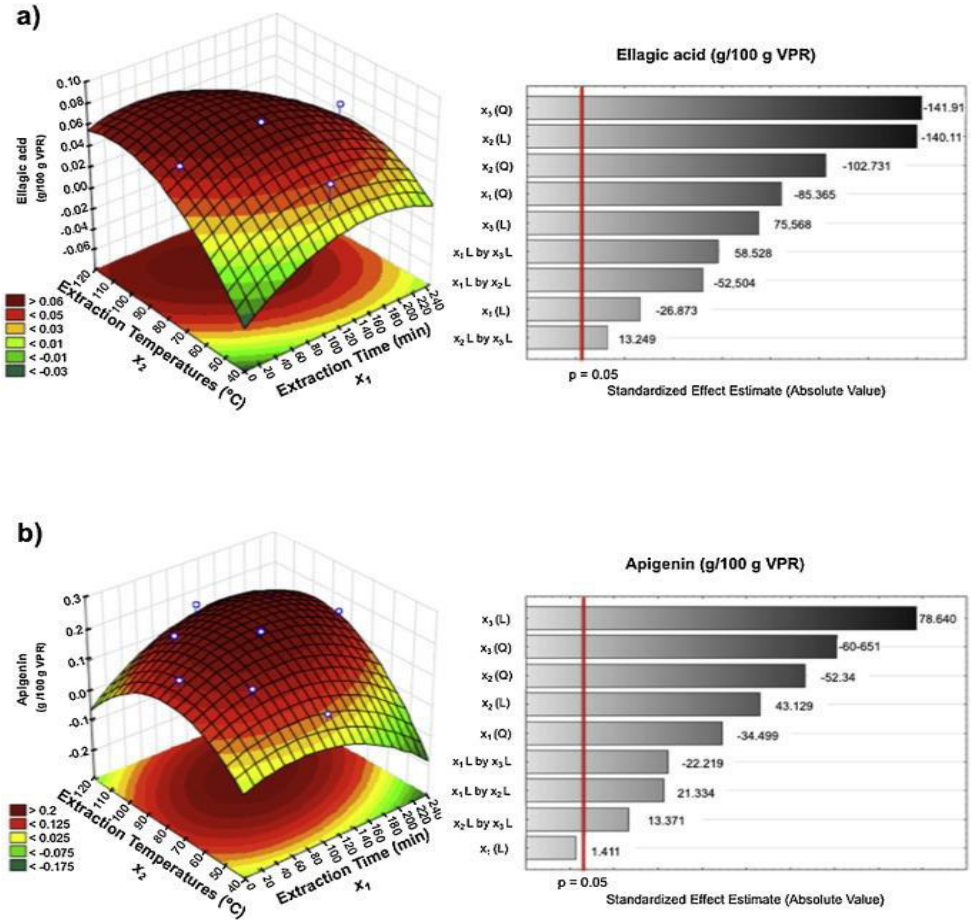


Figure 4.2 Response surface and Pareto diagram for standardized effects showing the effect of temperature and time of extraction (fixed enthanol concentration at 45% of: **a)** Ellagic acid mg/100g. **b)** Apigenin mg/100g. (p > 0.05).

The content of apigenin in VPR extracts obtained using CHE ranged from 6.4 to 2.2 mg/100 g VPR. In our study, the extract with the highest apigenin content was obtained using the following parameters: 45 % ethanol, temperature of 80 °C and extraction time of 120 min. However, it was not

identified using 60min of extraction time, extraction temperature of 60 °C and 30 % of ethanol. The lowest content was obtained in the extract prepared using 180 min, 60 °C and 30 % ethanol. The results show that the concentration of apigenin increased up to 80 °C and 120 minutes of extraction, at higher temperatures and times there was a decrease of the concentration, probably due to its degradation. The response surface shows the interaction of the extraction time and temperature with the ethanol concentration set at 45% (Figure 4.2a). The Pareto graph (Figure 4.2d) showed that the most dominant factor influencing the extraction of apigenin was the concentration of ethanol, and this factor positively affected its extraction ($p < 0.001$). The quadratic and linear levels of extraction temperature, quadratic extraction time and the interactions between time/temperature, time/ethanol concentration and temperature/ethanol concentration also significantly altered the apigenin content, while the linear parameter of extraction time was insignificant ($p > 0.5$) (Table 4.4). The predictive equation to describe the extraction efficiency to reach the maximum apigenin content (y_6) of VPR is as follows:

$$y_6 = 0.21 + 0.001x_1 + 0.0001\,x_1^2 + 0.031\,x_2 + 0.056\,x_2^2 - 0.025\,x_3 - 0.039\,x_3^2 - 0.045\,x_1x_2 + 0.020\,x_1\,x_3 + 0.021\,x_2x_3 \quad (8)$$

The variance analysis (ANOVA) was able to confirm that the model was significant ($p < 0.001$), and with R^2 values of 0.90 and calculated *F-value* 592.64 higher than the table $F_{5,3} = 9.01$ to 95 % of confidence level (Table 4.4). Negligible *lack of fit* ($p > 0.05$) was also evidence of good fit of the model.

4.3.2 Microwave-assisted extraction (MAE)

Based on the optimization of the conventional extraction method, study of the extraction of the polyphenolic compounds using microwave-assisted method was initiated in order to find the minimum conditions of extraction (concentration of ethanol (%), temperature (°C) and time)) to obtain maximum extraction yield. The experiments were conducted at different concentrations of ethanol (0 - 60 %), temperatures (60 - 120 °C) and time (5 - 40 min).

4.3.2.1 Total phenolic compounds and antioxidant activity MAE method

According to Rombaut et al., (2014) and Vinatoru et al., (2017) from an industrial point of view, the conventional extraction technique has some disadvantages, such as insufficient recovery of extracts, extensive extraction time and intensive heating and/or mixing, resulting in high energy consumption. In contrast MAE is a more advantageous methodology with its selective heating of the vegetal material versus solvent that improves the extraction mechanism by increasing the extraction yield and decreasing the extraction time. The predicted optimum conditions for TPC extraction were calculated as extraction time of 137 min, extraction temperature of 83.45 °C, ethanol concentration 46.7 %, and the model predicted a maximum TPC concentration of 2.3 g GAE/100 g VPR. The experimental concentration achieved under these conditions was similar CHE 2.3 g GAE/100 g VPR. For MAE the total phenolic content concentrations ranged between 1.5 and 2.4 g GAE/100 g VPR. The total phenolic content (TPC) as function of the extraction conditions are shown on Table 4.3. The results showed that the highest concentration of TPC was reached using 30 % ethanol, 120 °C and 5 min for MAE. According to these results, MAE can be considered a better method for polyphenols extraction (2.4 ± 0.01 g GAE/100 g VPR) than the conventional heating extraction (CHE) (2.2 ± 0.1 g GAE/100 g VPR). In addition, the antioxidant capacity of extracts obtained by MAE was significantly higher than the obtained by CHE. These values are in agreement with the results obtained by Çetin and coworkers (2011) (2.5 to 3.6 g GAE/100 g VPR) and were superior to those found by Cebrián and coworkers, and Karacabey and Mazza, (2017; 2008) (0.1 to 0.2 and 0.3 to 0.8 g GAE/100 g VPR, respectively). The superheated extraction of ethanol and water from VPR polyphenols yielded 1.7 to 4.1 g of GAE/100 g, depending on the experimental parameters. Experiments 15 and 16 (0 % ethanol, 120 °C, 10 min. and 0 % ethanol, 120 °C, 40 min.) showed lower quantities of phenolic compounds in comparison to the other extractions using the same times and temperatures. This is due to the higher dielectric constant (ε = 80) that water has in comparison to other solvents. This feature causes significantly lower dissipation than other solvents (δ = 1500 × 104). This phenomenon causes overheating of the water leading to degradation of the bioactive compounds (Proestos and Komaitis, 2008).

The FRAP, DPPH and ABTS assays are often used to determine the total antioxidant activities of natural compounds (Oroian and Escriche, 2015). The antioxidant activities FRAP, DPPH and ABTS ranged from 4.1 to 9.5 g FE/100 g VPR, 2.3 to 4.3 g TE/100 g VPR and 2.2 to 3.4 g TE/100 g VPR, respectively (Table 4.3). The extracts that presented lower TPC concentrations were not submitted to IC_{50} inhibition analysis for DPPH and ABTS antioxidant activities. The highest antioxidant capacity

measured by FRAP using MAE method was 9.5 g TE/100 g VPR using 60 % ethanol at 120° C for 40 min, these results were superior to those found in this work using the CHE method and that is in contrast to that described by (Gullón et al., 2017). These authors tested different severities in autohydrolysis for the extraction of VPR phenolic compounds. They described FRAP values between 0.7 and 2.7 g TE/100 g VPR. The results of ABTS radical censor sequestering activity were consistent with the DPPH tests, and showed moderate oxidant activity relative to ABTS. The highest antioxidant activity was measured by DPPH and ABTS methods (4.3 TE/100 g of VPR and 3.4 g TE/100 g of VPR, respectively), using 60 % ethanol, at 120 °C, during 5 minutes. The ABTS results were in accordance with those obtained by Karacabey and Mazza (2010) in conventional extractions using a temperature of 83.6 °C, solvent to solid ratio of 70 (mL/g) and 58 % ethanol. The antioxidant activities (IC_{50}) of the VPR extracts obtained by MAE varied from (DPPH) IC_{50}=3.6 to 6.9 g/L and (ABTS) IC_{50}=1.1 to 4.0 g/L, respectively. These values show high efficiency as DPPH and ABTS reducers compared to those obtained by the CHE method (IC_{50} =4.9 to 13 g/L, IC_{50}=2.1 to 8.1 g/L, respectively). The greater ability to reduce DPPH and ABTS obtained in MAE was when 30 % ethanol was used at a temperature of 120 °C for 5 min (IC_{50}=3.6 g/L and 1.1 g/L, respectively). According to Ruiz-Moreno et al., (2015) the direct comparison of the antioxidant activities of wine derivatives with the literature is difficult due to the grape variety discrepancy and the variety of extractive techniques and quantification methods.

4.3.2.2 UHPLC analysis of phenolic compounds of MAE

Among the 15 polyphenolics compounds identified in the extraction by the CHE method, only 12 compounds were identified in the extracts obtained by the MAE method. The major phenolic compounds identified by UHPLC for MAE were catechin, narigenin, apigenin, rutin, *p*-cumaric acid + epicatechin, ferulic acid, gallic acid, syringic acid, vanillic acid, *o*-coumaric acid, ellagic acid, cinnamic acid, rosmarinic acid, *trans*-resveratrol (Table 4.7). The presence of these compounds is in agreement with other authors (Cebrián et al., 2017; Çetin et al., 2011; Delgado-Torre et al., 2012; Jesus et al., 2017; Sánchez-Gómez et al., 2017b; Sánchez-Gómez et al., 2014).

Table 4.7 Phenolic compounds in the vine pruning residue samples extracted using the microwave-assisted extraction method (mg/100 g VPR).

Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Time (min)	40	25	10	5	10	5	10	5	10	5	10	5	10	5	10	40
Temperature (°C)	120	120	120	120	90	90	60	60	120	120	90	90	60	60	120	120
Ethanol concentration (%)	60	60	60	60	60	60	60	60	30	30	30	30	30	30	0	0
Phenolics Compounds (mg/100 g VPR)																
Catechin	27.1^{g}	27.5^{gh}	26.6^{678}	25.5^{fgh}	23.4^{defg}	23.4^{defg}	24.4^{fgh}	19.2^{abi}	16.6^{a}	24.9^{fg}	23.3^{cde}	22.6^{cdi}	20.8^{abc}	20.4^{bcd}	21.4^{cde}	17.3^{ab}
Narigenin	3.1^{a}	1.5^{a}	1.1^{a}	0.3^{a}	0.7^{a}	0.2^{a}	0.6^{a}	0.2^{a}	0.5^{a}	ND	0.3^{a}	ND	ND	ND	0.4^{a}	0.2^{a}
Apigenin	104.8^{def}	93.9^{de}	150.1^{fj}	118.8^{efg}	94.4^{def}	184.7^{i}	126.4^{fg}	174.0^{hi}	59.0^{bi}	16.0^{a}	70.0^{cd}	34.6^{ab}	30.8^{ab}	44.8^{bc}	12.4^{a}	9.4^{a}
Quercetin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rutin	13.3^{d}	13.2^{d}	10.6^{bcd}	11.2^{bcd}	12.3^{cd}	9.7^{bc}	10.9^{bcx}	9.6^{abc}	10.3^{bc}	9.3^{abc}	9.1^{ab}	9.6^{bc}	9.7^{bcd}	11.1^{234}	4.3^{a}	9.4^{abc}
p-cumaric acid + epicatechin	6.9^{cd}	7.4^{d}	7.0^{cd}	7.3^{d}	6.9^{cd}	6.9^{bc}	7.2^{d}	6.8^{ab}	6.7^{bc}	6.8^{bc}	6.8^{bc}	6.6^{ab}	6.4^{a}	6.9^{34}	6.5^{ab}	6.8^{bc}
rosmarinic acid	32.8^{e}	29.7^{de}	35.5^{ef}	36.2^{f}	31.7^{ef}	32.5^{e}	34.0^{ef}	27.3^{d}	22.9^{c}	21.5^{3}	25.8^{d}	22.6^{c}	22.4^{c}	22.4^{c}	15.9^{b}	ND
cinnamic acid	9.3^{b}	10.0^{bc}	10.8^{cd}	11.0^{cd}	9.2^{b}	11.1^{cd}	10.2	10.9^{cd}	9.9^{bc}	ND	9.7^{bc}	11.3^{cd}	10.9^{d}	10.2^{bc}	ND	ND
syringic acid	16.8^{d}	17.1^{d}	17.3^{d}	17.7^{e}	16.5^{bc}	16.6^{cd}	24.4^{e}	16.2^{abi}	16.6^{cc}	16.3^{abx}	16.3^{abi}	16.2^{abi}	15.6^{a}	16.6^{34}	15.7^{ab}	16.3^{bc}
vanillic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ferulic acid	6.9^{abcd}	7.1^{efg}	7.0^{bcd}	7.3^{g}	6.7^{ab}	7.1^{efg}	7.0^{bcd}	6.8^{abcd}	7.1^{efg}	7.2^{efg}	6.8^{abcd}	6.8^{abcd}	6.6^{a}	7.1^{def}	6.7^{abcd}	6.8^{abcd}
gallic acid	14.1^{de}	13.9^{de}	13.9^{de}	15.1^{e}	11.7^{cd}	10.8^{abc}	10.9^{bcd}	10.3^{abc}	10.5^{abx}	14.4^{de}	9.7^{abc}	9.2^{abc}	6.8^{a}	7.1^{ab}	7.3^{abc}	11.8^{cd}
o-cumaric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ellagic acid	75.3^{c}	64.8^{b}	172.5^{h}	185.1^{i}	73.5^{cd}	131.2^{g}	119.6^{fg}	29.5^{a}	68.3b	88.5^{cde}	109.2^{ef}	97.7^{de}	74.4^{c}	86.0^{de}	ND	ND
Trans-resveratrol	29.5^{fg}	32.3^{gh}	34.9^{gh}	36.0^{h}	31.3^{gh}	32.3^{g}	33.6^{gh}	27.3^{cd}	22.9^{cc}	19.6^{ab}	25.6^{bcc}	22.4^{cd}	22.4^{cd}	22.4^{34}	16.3^{b}	0.3^{a}

* The averages followed by the same number do not differ by the Tukey test ($p < 0.05$) ND: not detected

Among the quantified phenolic compounds, the most abundant flavonoids were apigenin (9.4 to 184 mg/100 g of VPR) and catechin (16.6 to 27.1 mg/100 g of VPR), their concentration varied according to the extraction conditions. The concentration of apigenin significantly exceeded the other flavonoids, with the highest concentration of 184 mg/100 g of VPR with 60 % ethanol at 90 °C for 5 minutes. Meanwhile, this value was lower than the optimum obtained in CHE, 207.9 mg/100 g of VPR (120 min, 80 °C and 45%). Pintać et al., (2018) was able to extract between 0.30 and 9.1 mg/100 g of grape pomace apigenin, using CHE and 80 % ethanol and acetone as extraction solvents, respectively. The highest concentration of catechin was 27.1 mg/100 g of VPR. The results obtained by MAE for catechin are in agreement with previous studies on extracts of grapevine shoots (Sánchez-Gómez et al., 2017b).

Stilbenes are the most studied group of compounds in VPR due to their important properties for the pharmaceutical industry (Guerrero et al., 2016; Piñeiro et al., 2017, 2016; Shen et al., 2009; Soural et al., 2015). Resveratrol is the most studied stilbene in VPR, in this study the resveratrol concentration ranged from 0.3 to 36.0 mg/100 g of VPR in the MAE method. The highest content of resveratrol was 36.0 mg/100 g of VPR when 60 % ethanol was used at 120 °C for 5 minutes. These results are superior to those found by Çetin et al., (2011) and Sánchez-Gómez et al., (2014), in extracts of VPR, but still inferior to the results obtained by Delgado-Torre et al., (2012) using 1 g of solid and 20 mL of ethanol of 80 % (v/v), Soural et al., (2015) 1.5 g of grape canes and 100 mL of methanol and Vergara et al., (2012) using 2 g of solid and 16 mL of ethanol of 80 % (v/v), both used higher amounts of solid and organic solvent in a higher concentration than the one used in this work.

The most abundant phenolic acids were ellagic acids (6.67 to 185.1 mg/100 g of VPR), syringic (15.6 to 24.4 mg/100 g of VPR) and rosmarinic (15.87 to 36.25 mg/100 g of VPR). The highest concentration of syringic acid (24.4 mg/100 g of VPR) was obtained when using 60 % ethanol at 60 °C for 10 minutes and 20.02 mg/100g VPR, this concentration was higher than that obtained by CHE when submitted 30 % ethanol at 60 °C for 60 minutes. These results are superior to those described in the literature (Cebrián et al., 2017; Sánchez-Gómez et al., 2014). Extracts submitted to 60 % ethanol, at 120 °C for 5 minutes, achieved the highest ellagic acid extraction (185.1 mg/10 0g of VPR) and rosmarinic acid (36.2 mg/100 g of VPR). These results were superior to those obtained by CHE (8.6 and 14.1 mg/100 g of VPR, respectively) using 45 % ethanol at 80 °C for 120 minutes. The ellagic acid content varies remarkably according to the solvent and/or the extraction procedure used (Cebrián et al., 2017; Luque-Rodríguez et al., 2006; Sánchez-Gómez et al., 2014).

The experiments that presented the highest concentration of phenolic compounds were 3 and 4 (60 % ethanol, 120 °C for 10 min and 60 % ethanol, 120 °C for 5 min, respectively). The mixture of organic solvent and water is more efficient in the extraction of phenolic compounds than pure solvents Bouras et al., (2015). According to the results of Liazid et al., (2007), most phenolic compounds can be extracted without degradation at temperatures up to 125 °C for an extraction time of 20 min. The results obtained for the polyphenolic profile indicate that the concentration of each compound determined after the extraction with the different concentrations of ethanol, time and temperature increased significantly using microwaves in comparison with the conventional extraction, except for some compounds (apigenin, cinnamic acid, resveratrol, rutin, rosmarinic acid, ellagic acid, quercetin, vanillic acid and *o*-cumaric acid) and especially for the water extraction, possibly due to the higher dielectric constant. The obtained results are in accordance with Chemat et al., (2017), concluding that, compared to CHE, the MAE is a greener method, as it uses less energy, shorter extraction time and less percentage of ethanol.

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Even though, MAE has been successfully used in the extraction of valuable components in laboratory scale, the number of industrial-scale applications remain very small. However, MAE is considered a key process for the application of the biorefinary concept to any industrial production, like is the extraction of natural products (Vinatoru et al., 2017).

4.4 Conclusions

The extraction of polyphenolic compounds (TPC) was optimized for conventional heating and microwave-assisted extraction using environmentally friendly solvents *i.e.* ethanol and water. The obtained TPC and antioxidant activity were according to the predicted by the linear and quadratic models developed from the optimization study. Extraction of TPC from VPR had greater efficiency when using MAE than CHE methodology. The use of MAE significantly reduced the extraction time and improved the extraction efficiency of TPC of high industrial interest such as ellagic acid and apigenin. The extraction of valuable TPC from VPR contributes to increase the integral valorization of this residue. For first time was reported the presence of the compound apigenin in the extracts of VPR. MAE generally increased extraction of ellagic acid, *trans*-resveratrol, rosmarinic acid, cinnamic acid and catechin when compared to CHE; similar values were obtained for *p*-coumaric acid + epicathechin, apigenin, rutin, syringic, ferulic and gallic acid. Lower amounts of naringin, quercetin, vanillic and *o*-cumaric acids were detected in MAE than in CHE.

4.5 References

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CHAPTER V

OHMIC HEATING POLYPHENOLIC EXTRACTS FROM VINE PRUNING RESIDUE WITH ENHANCED BIOLOGICAL ACTIVITY

Vine Pruning residue was submitted to conventional heating (CHE) and ohmic heating (OH) for the extraction of bioactive compounds and these extracts were analyzed for total phenolic content (TPC), polyphenolic profile, antioxidant activity, antimicrobial activity and anticancer activity in human cancer cell lines. The OH extracts were obtained using Low electric field - LEF (496.0 V/cm) or Intermediate electric field - IEF (840.0 V/cm). The tests were performed using 45 % (v/v) ethanol-water extraction solution at 80 °C at different extraction times (20 to 90 min). The extract that stood out among the others concerning anticancer potential was the one obtained by OH when used, IEF, where the TPC was significantly higher than in the other extracts which correlated with higher antioxidant, antimicrobial and anti-proliferative activity on different tumor cell lines (HepG2, MDA-MB-231, MCF-7 and Caco2). Vine pruning OH extracts obtained using green solvents by an eco-friendly procedure were revealed as a source of compounds with relevant antioxidant and anticancer activity.

Keywords: Vine pruning residue; Ohmic extraction; Phenolic compounds; Anticancer activity; Antimicrobial activity; antioxidant activity

The information presented in this chapter is part of an article being submitted.

Jesus, Meirielly S., N., Ballesteros, L., Genisheva, Z., Pereira, R., Carvalho, A. C., Pereira-Wilson, C., Teixeira, J. A, Domingues, L. Ohmic heating polyphenolic extracts from vine pruning residue with enhanced biological activity (*submitted*).

CONTENTS

5.1 INTRODUCTION

Vine Pruning residue (VPR) is a lignocellulosic material widely generated in the Mediterranean region by the wine industries, as a consequence of the great wine market. Conversely, this waste represents a pollution hazard if discharged into the environment. On the other hand, VPR is economically underutilized, however, it has attracted enormous attention due to its interesting properties and its possible use as a raw material to obtain added-value compounds. In fact, this by-product can be considered a rich source of energy and bioactive compounds, and the recovery of these compounds from VPR could avoid the possible economic and environmental concerns. In this context, some authors have proposed different alternative applications for the valorization of this residue. Thus, VPR can be used to produce biochar (Azuara, Sáiz, Manso, García-Ramos, & Manyà, 2017), enological additives (Cebrián-Tarancón et al., 2019), bioethanol (Jesus, Romaní, Genisheva, Teixeira, & Domingues, 2017) bioactive compounds (Jesus et al., 2019; Moreira et al., 2018), foliar fertilizers (R. Sánchez-Gómez, Garde-Cerdán, et al., 2016), prebiotic oligosaccharides (Dávila, Gullón, Alonso, Labidi, & Gullón, 2019), or biosurfactants (Vecino et al., 2017) as well as others. In spite of these applications, VPR is still underutilized as a valuable material for industrial processes.

Nowadays, studies for reusing wine production residues as potential sources of phenolic compounds have gained great interest since these compounds present multiple biological effects, including antioxidant (Jesus et al., 2019), anti-mutagenic, anti-inflammatory, antimicrobial and anti-carcinogenic properties (Tartaglione et al., 2018), which provide enormous benefits for human health. According to some authors, extracts of different residues from the wine industry, such as leaves, seeds and, vine pruning may be toxic to different human cancer cells. As a result, leaf extracts were tested for human colon cancer cells and A549 lung cancer cells (Abed, Harb, Khasib, & Saad, 2015; Ramadan, Abou-Taleb, Galal, & Abdel-Hamid, 2017). The extracts from the grape seeds, for example, were purified for the isolation of resveratrol, being tested against prostate cancer cells (El-Elimat, Jarwan, Zayed, Alhusban, & Syouf, 2018), and vine pruning residue was subjected to the extraction, purification and isolation of oligostilbenoids in order to be tested against normal MRC-5 lung fibroblasts, AGS gastric adenocarcinoma cells, SK-MES-1 lung stem cells and bladder carcinoma cells J82 (Vânia Sáez 2018).

Factors such as the origin and composition of the raw material, the type of solvent used and its concentration, the solid/solvent ratio, time of contact, temperature, and extraction methodology, significantly influence the efficiency of the process extraction of bioactive compounds (Jesus et al., 2019). Previous works regarding the extraction of phenolic compounds using VPR as a raw material

have tested different solvents including methanol (Ju et al., 2016), butanone and acetone (Alexandru et al., 2014). However, these solvents are mostly toxic to humans. Taking into account the potential application of these extracts in human health sector, researchers are looking for technologies able to use green solvents such as water (Gullón et al., 2017; Jesus et al., 2017; Moreira et al., 2018; R. Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2016), ethanol (Alexandru et al., 2014) or ethanol-water mixtures (Alexandru et al., 2014; Jesus et al., 2019; Moreira et al., 2018) in order to ensure the attainment of phenolic compounds suitable for the food, pharmaceutical and cosmetic industries

Thus, alternative eco-friendly methodologies including alkaline hydrolysis treatments (Max, Salgado, Cortés, & Domínguez, 2010; Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2015b), ultrasound-assisted extraction (Alexandru et al., 2014; Delgado-Torre, Ferreiro-Vera, Priego-Capote, Pérez-Juan, & Luque De Castro, 2012; Farhadi, Esmaeilzadeh, Hatami, Forough, & Molaie, 2016), hydrothermal treatment at high temperatures (Gullón et al., 2017; Jesus et al., 2017), solid-liquid dynamic extraction (Delgado-Torre et al., 2012; Luque-Rodríguez et al., 2006; Rosario Sánchez-Gómez, Zalacain, Alonso, & Salinas, 2014), subcritical water extraction (Gabaston et al., 2018; Moreira et al., 2018), pressurize solvent extraction (Karacabey et al., 2012), as well as high-voltage electrical discharge (Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2015a), have been applied. However, the current methods used for the extraction of bioactive compounds present limitations due to the long extraction times and high process temperatures, which can cause oxidation and denaturation of the phenolic compounds since they are relatively unstable, leading to low extraction percentages. Therefore, it is necessary to study novel extraction methodologies capable of extracting high concentrations of bioactive compounds using less aggressive conditions and environmentally friendly and sustainable solvents. One of the eco-friendly trends in the techniques of extracting polyphenolic compounds is the use of emerging technologies such as ohmic heating (OH) to reduce the deleterious effects of conventional thermal processes

OH is a less aggressive heat treatment because of its ability to heat materials evenly and rapidly, where the passage of alternating electric current is used to generate heat internally, thereby preventing thermosensitive substances such as pigments, polyphenolic compounds and, vitamins from being denatured (Khan et al., 2018; Ramírez-Jiménez, Rangel-Hernández, Morales-Sánchez, Loarca-Piña, & Gaytán-Martínez, 2019). Some authors have reported that the use of OH causes destruction of the cell membrane making it permeable and thus, raising the release of phenolic compounds from plant tissues (Kulshrestha and Sastry, 2010, Lebovka et al., 2007). The permeabilization increases with the rise of

the intensity and temperature of the electric field (El Darra, Grimi, Vorobiev, Louka, & Maroun, 2013). Previous studies showed that the use of ethanol/water blends in pulsed ohmic heating accelerates the extraction kinetics of this type of compounds from grape marc (El Darra et al., 2013).Currently, studies of bioactive compound extractions performed from VPR have been concentrated on the qualitative/quantitative evaluation of polyphenols and on their antioxidant activity and antimicrobial capacity onto bacteria and yeasts. However, to the best of our knowledge, there are no studies that use the sustainable and environmentally friendly ohmic heating to recovery bioactive compounds from VPR. In addition, there is no study in the literature that reports the antimicrobial inhibition of VPR extracts onto filamentous fungi, as well as the anti-carcinogenic activity when used a VPR integral extract in order to evaluate the synergistic effects of the set of bioactive compounds present in that extract. Therefore, the aim of this study was to investigate the levels of polyphenolic compounds and their profile, as well as the functional properties, including antioxidant and antimicrobial capacities, and cytotoxic activity in human tumor cell lines, in produced extracts from VPR by using the emerging OH extraction technique at different intensities: Intermediate electric field (IEF) and Low electric field (LEF).

5.2 MATERIALS AND METHODS

5.2.1 Raw material and analysis of the chemical composition

The VPR was randomly sampled from the Portuguese region of Minho (Amares Braga-PT). Samples were dried at room temperature, milled to pass an 8 mm mesh, homogenized in a single batch and stored at room temperature into a dark and dry place until use. The VPR used in this work was previously studied and characterized (Jesus et al., 2019; Jesus et al. 2017) and presents the following chemical composition: 32.9 ± 0.6 of cellulose (as glucan); 14.9 ± 0.2 of xylan; 0.4 ± 0.01 of arabinan; 3.9 ± 0.5 of acetyl groups; 29.5 ± 1.2 of Klason lignin; 13.7 ± 1.0 of extractives in water; 2.9 ± 0.9 of extractives in ethanol and 3.3 ± 0.5 of ashes, expressed in g per 100 g VPR on oven-dry basis ± standard deviation based in three replicate determinations.

5.2.2 Extraction process

The extraction of phenolic compounds from VPR was performed by CH and OH techniques. The extraction parameters defined in this study were based on previous results (Jesus, et al., 2019). Briefly, the extractions were carried out in a glass cylindrical reactor of 30 cm total length and 2.3 cm of internal diameter containing two electrodes of stainless steel insulated with polytetrafluoroethylene, which kept constant at 7 cm of distance. Voltages were controlled using a function generator (Agilent 33.220 A, Bayan Lepas, Malaysia, 1 Hz-25 MHz, and 1-10 V) with a sinusoidal wave at a 25 kHz frequency, connected to an amplifier (Peavey CS3000, Meridian, MS, USA, 0.3-170 V). The electric fields ranged from 400 to 1600 V/cm, and the temperature was monitored by a k-type thermocouple (precision temperature ± 1 °C, Omega, 709, USA) placed in the geometric center of the sample volume and connected to a data logger software (National Instruments, USB-9161, USA), according to previous reports (Pereira et al., 2010; Rodrigues, Vicente, Petersen, & Pereira, 2019).

For the extractions, 1 g of VPR was suspended in 40 mL of a hydroalcoholic solution at 45 % (v/v), put inside the reactor and maintained to a constant agitation with a magnetic stirrer. NaCl was used to adjust the electrical conductivity of the solution (2.3 mS/cm) in order to ensure a homogeneous current flow. The power was controlled to reach a temperature of 80 °C, and the extraction time ranged from 20 to 90 min. Extraction of polyphenolic compounds was performed for samples subjected at four different treatments, all of them carried out into the glass cylindrical reactor. Thus, an IEF (840.0 V/cm) and LEF (496.0 V/cm) treatment (using an extraction time of 60 min) were applied. For the IEF only the electrodes were used to achieve 80 °C, while for LEF, an additional thermo-stabilized water bath circulating in the reactor jacket was used to reach the temperature. On the other hand, CHE extraction treatment was made using a circulating thermo-stabilized water bath in the reactor jacket, and finally, an extraction treatment at room temperature, used as a control, was performed (RT). All experiments were made in triplicate.

At the end of each treatment, the obtained extracts were immediately cooled down in an ice bath in order to stop the reaction, and then, filtered with a paper filter of cellulose. The ethanol was evaporated on an orbital shaker at 40 °C and 150 rpm, and the extracts lyophilized and stored for further analysis.

5.2.3 Analytical methodology

5.2.3.1 Total phenolic compounds analysis

The total phenolic compounds (TPC) of the VPR extracts were determined by using the Folin-Ciocalteu reagent, adapted to 96-well microplate, as described by Singleton, Orthofer, & Lamuela-Raventós, (1999). Briefly, 10 μL of sample was mixed with 60 μL of sodium carbonate solution (7.5 %, w/v) and 15 μL of Folin-Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO, USA). Subsequently, 200 μL of distilled water was added and then, the mixtures were incubated at 60 °C for 6 minutes, and the absorbance was measured at 700 nm against a blank sample using UV-*Vis* (Synergy HT-BIOTEK). The total phenolic content was calculated as equivalents of gallic acid from a standard curve (Y = 0.0007x + 0.0671, r = 0.99433). The results were expressed in grams of equivalent gallic acid per 100 g dry matter (g GAE/100 g VPR).

5.2.3.2 UHPLC analyses

In addition, phenolic compounds were also identified and quantified by Shimatzu Nexpera X2 UHPLC chromatograph equipped with Diode Array Detector (Shimadzu, SPD-M20A). The separation was performed on a reversed-phase Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; from Waters) at 40 °C. The flow rate was 0.4 mL/min. The HPLC grade solvents used were water/formic acid (0.1 %) as solvent A and acetonitrile as solvent B. The elution gradient for solvent B was as follows: from 0.0 to 5.5 min at 5 %, from 5.5 to 17 min a linear increase to 60 %, from 17.0 to 18.5 min a linear increase to 100 %, then column equilibration from 18.5 to 30.0 min at 5 %. The phenolic compounds were identified by comparing their UV/Vis spectra and retention times, at the most suitable wave-length for each compound, with that of corresponding standards (Jesus et al., 2019).

5.2.3.3 Antioxidant activity analysis

The effect of OH and CH techniques on the antioxidant activity of the extracted bioactive compounds was evaluated by using different antioxidant assays as described below.

Ferric reducing antioxidant power (FRAP) assay, was determined according to the methodology described by (Ballesteros, Cerqueira, Teixeira, & Mussatto, 2015; Meneses, Martins, Teixeira, & Mussatto, 2013). Thus, FRAP reagent was prepared from a 0.3 M acetate buffer solution, 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) and a 40 mM aqueous ferric chloride solution in a ratio of 10:1:1 (v/v/v). For the analysis, 10 μL of the filtered extract was added to 290 μL of the FRAP reagent into a 96-well microplate and then, the samples were incubated at 37 °C for 15 minutes. The absorbance was recorded at 593 nm (Synergy HT-BIOTEK). The $FeSO_4$ (10 to 275 mg/L) was used as a standard and water used as a blank. The results were expressed in grams of ferrous equivalent per 100 g of VPR extract (g FE/100 g VPR).

DPPH radical scavenging assay was carried out preparing a solution of 2,2-diphenyl-1-picrylhydrazine (DPPH) radical in methanol (6 x 10^{-5} M to an absorbance of 0.700 at 515 nm) as described by (Meneses et al., 2013; R. Sánchez-Gómez, Zalacain, Pardo, Alonso, & Salinas, 2017). Briefly, aliquots of 10 μL of each sample were added to 290 μL of DPPH solution into a 96-well microplate, and the samples were kept in the absence of light for 1 h at room temperature, and then, measured at 515 nm using detector UV-Vis (Synergy HT-BIOTEK). Negative and positive controls were made with methanol, and the standard curve was linear between 48 and 719 μM Trolox.

On the other hand, radical cation decolorization (ABTS) assay was determined according to Ahmad Mir et al. (2017). ABTS radical cation was prepared by mixing 7.4 mM 2.2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) dissolved in ethanol with potassium persulfate (2.6 mM) in a ratio of 1:1 (v/v). The mixture was incubated for 16 h at room temperature in the dark. After 16 h, 1 mL of ABTS radical cation solution was added to approximately 50 mL of solution absolute ethanol and then adjusted to an absorbance of 0.700 at 734 nm using detector UV-*Vis* (Synergy HT-BIOTEK). In a 96-well microplate, aliquots of 10 μL of each sample were added to 200 μL of the ABTS radical cation solution and the samples were kept in the absence of light for 6 min at 30 °C.

$$\%\ Inhibition\ activity = \frac{A_0 - A_1}{A_1}\ \mathrm{x}\ 100 \qquad (1)$$

Where A0 is the absorbance of the negative control and A1 is the absorbance of the extracts. The sample concentration for the inhibition concentration at 50 % (IC_{50}) for both methodologies was calculated by interpolation. The antioxidant activity was expressed as the amount of antioxidant able to reduce the initial concentration of DPPH and ABTS by 50 %. The IC_{50} values were expressed as g of Trolox equivalent per 100 g of dry weight material (g TE/100 g VPR).

5.2.3.4 Antimicrobial activity analysis

5.2.3.4.1 Microbial strains

Antimicrobial evaluation was performed against five food pathogenic fungi including *Alternaria sp*. (MUM 02.42), *Cladosporium cladosporioides* (MUM 97.06), *Phoma violacea* (MUM 97.08), *Penicillium italicu*m (MUM 02.25) and *Penicillium expansum* (MUM 02.14), being obtained from the collection of the Mycology Laboratory of the Minho University (MUM), Portugal. All strains were cultured into potato dextrose agar (PDA) and incubated at 25 ± 2 °C during 15 days before the antimicrobial test.

5.2.3.4.2 Micro-dilution methodology for filamentous fungi

The determination of the optimal inhibitory concentration of the obtained extracts from VPR against microbial strains was performed using the micro-dilution methodology for filamentous fungi described by the Clinical and Laboratory Standards Institute (CLSI, 2002). Thus, the fungi cell number was adjusted to approximately 106 CFU (colony forming unit)/mL (0.5 on the McFarland scale). Additionally, a lyophilized fraction (2 mg/mL) of the VPR extracts obtained from each treatment were serially two-fold diluted in the synthetic culture medium RPMI 1640 (with glutamine and without sodium bicarbonate buffered with bicarbonate 3-(N-morpholino) propanesulfonic acid - MOPS), in order to obtain final concentrations from 1000 to 1.95 µg/mL. Experiments were carried out in a sterile 96-well microplate, in which 100 µL of inoculum suspension was added to 100 µL sample. The microplate was incubated at 25 ± 2 °C for 96 h and the absorbance was measured at 530 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). The behavior of the samples was evaluated against growth and sterility controls, which consisted in using 100 µL of medium RPMI 1640 plus 100 µL of inoculum suspension as a microbial growth control (negative control) and 200 µL of medium RPMI 1640 as a sterility control (positive control). Moreover, fluconazole solutions (concentrations from 0.19 to 100 µg/mL) were used as standard controls. All the treatments were analyzed against the 5 fungi, and the concentrations of each treatment capable of inhibiting growth when compared with the negative control were considered as the optimal conditions.

5.2.3.5 Anticancer activity analysis

5.2.3.5.1 Cell culture and experimental conditions

Caco2 human colon carcinoma cells, MDA-MB-231 and MCF-7 human breast cancer cells were kindly provided by Dr. Raquel Seruca (Ipatimup, University of Porto, Portugal). HepG2 human hepatocellular carcinoma cells (HB-8065) and CCD841 CoN normal human colonic cells (CRL-1790) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained at 37 °C in a humidified 5 % CO_2/95 % air (v/v) atmosphere in the appropriate culture medium. Caco-2 cells were maintained in DMEM supplemented with 10 mM HEPES, 10 % FBS, 1 % antibiotic/antimycotic solution, and 1 % non-essential amino acids solution. On the other hand, MDA-MB-231cells were cultivated in DMEM with 10 mM HEPES, 1.5 g/L sodium bicarbonate, 10% FBS, and 1 % antibiotic/antimycotic solution, while MCF-7 in RPMI-1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10 % FBS, and 1 % antibiotic/antimycotic solution. Finally, HepG2 and CCD841 CoN cells were maintained in MEM supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10% FBS, and 1 % antibiotic/antimycotic solution.

For cell experiments, Caco2 cells were seeded at 50000 cells/mL, MDA-MB-231 and MCF-7 at 75000 cells/mL, HepG2 at 200000 cells/mL, and CCD841 CoN at 75000 cells/mL, 24 h before incubation with the different extracts. Stock solutions of lyophilized extracts were prepared in dimethyl sulfoxide (DMSO) and kept in aliquots at -20 °C. Extracts were then added to the culture medium just before incubation, keeping the DMSO concentration not higher than 0.5 % (v/v). Controls contained only DMSO.

5.2.3.5.2 Assessment of anticancer effect on cancer cell lines

The effect of varying concentrations of the tested extracts obtained from the distinct extraction procedures (in 24 and 48 h incubations) on cell proliferation was evaluated by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay as previously described (Lima, Pereira-Wilson, & Rattan, 2011). Briefly, 2 h before the end of the treatment period, the cells were incubated with MTT to a final concentration of 0.5 mg/mL. After removing the medium, the formazan crystals formed by the cell's capacity to reduce MTT were dissolved with a DMSO: ethanol solution 50:50 (v/v), and measured at an absorbance of 570 nm. The results were expressed as a percentage relative to the control (cells without any test extract). The extract concentration that inhibited cell growth

by 50 % (IC_{50}) relative to control was calculated using GraphPad Prism 7.0 software (San Diego, CA, USA).

5.2.4 Statistical analysis

Data were expressed as mean ± SD of at least three independent experiments. Statistical significances were assessed by two-way ANOVA and Tukey post hoc test (95 % confidence interval), using Statistica software (version 10) and GraphPad Prism 7.0. Differences between groups were considered to be significant when $p \leq 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Determination of ohmic heating extraction conditions: Preliminary assays

The extraction time can significantly influence the concentration of TPC, impacting on the antioxidant activity of the extracts (Jesus et al., 2019), besides reducing the energy consumption and cost of the extraction process. Therefore, it is important to evaluate the extraction time to optimize the recovery of the bioactive compounds. For defining the extraction process conditions, preliminary tests were performed in order to evaluate the optimum extraction time (20 to 90 min) for OH treatments in comparison to the tests carried out by the CHE and RT methods, being the last one used as a control. The temperature (80 °C) and the liquid/solid ratio (40:1 mL/g of VPR) were defined according to results obtained in previous CH extractions (Jesus et al., 2019). The extraction time efficiency was evaluated in terms of the recovered TPC and the antioxidant activity evaluated by different methods, including FRAP, DPPH and ABTS (Figure 5.1). For evaluating the treatments, the TPC concentration and the antioxidant activity of the samples obtained by OH and CH were compared with the control extraction (RT) method, inferred by two-way ANOVA.

The results showed no significant differences ($p > 0.05$) for the polyphenol content in the extracts obtained by CH and OH (3.2 ± 0.1 and 3.1 ± 0.2 g GAE/100 g, respectively, at 60 min) for all evaluated times, correlating with the values previously reported by other authors (Çetin, Emine Sema, Altinöz, Duygu, Tarçan, Ecehan, & Baydar, 2011; Jesus et al., 2019; Moreira et al., 2018). However, there were significant between the different treatments used with respect to the antioxidant activity

where OH treatment presented the best FRAP value (3.1 ± 0.3 g FE/100 g), and also the highest values when DPPH (3.2 ± 0.4 g TE/100 g) and ABTS (1.50 ± 0.2 g TE/100 g) assays were determined. In general, it was possible to observe significant differences ($p < 0.05$) between the extraction treatments and the time of extraction in the OH treatment, with the better result obtained with 60 min of extraction. Therefore, 60 min may be considered an adequate time to be used in all the extraction processes, which is an advantage, since the use of the extraction times shorter result in important economic viewpoints.

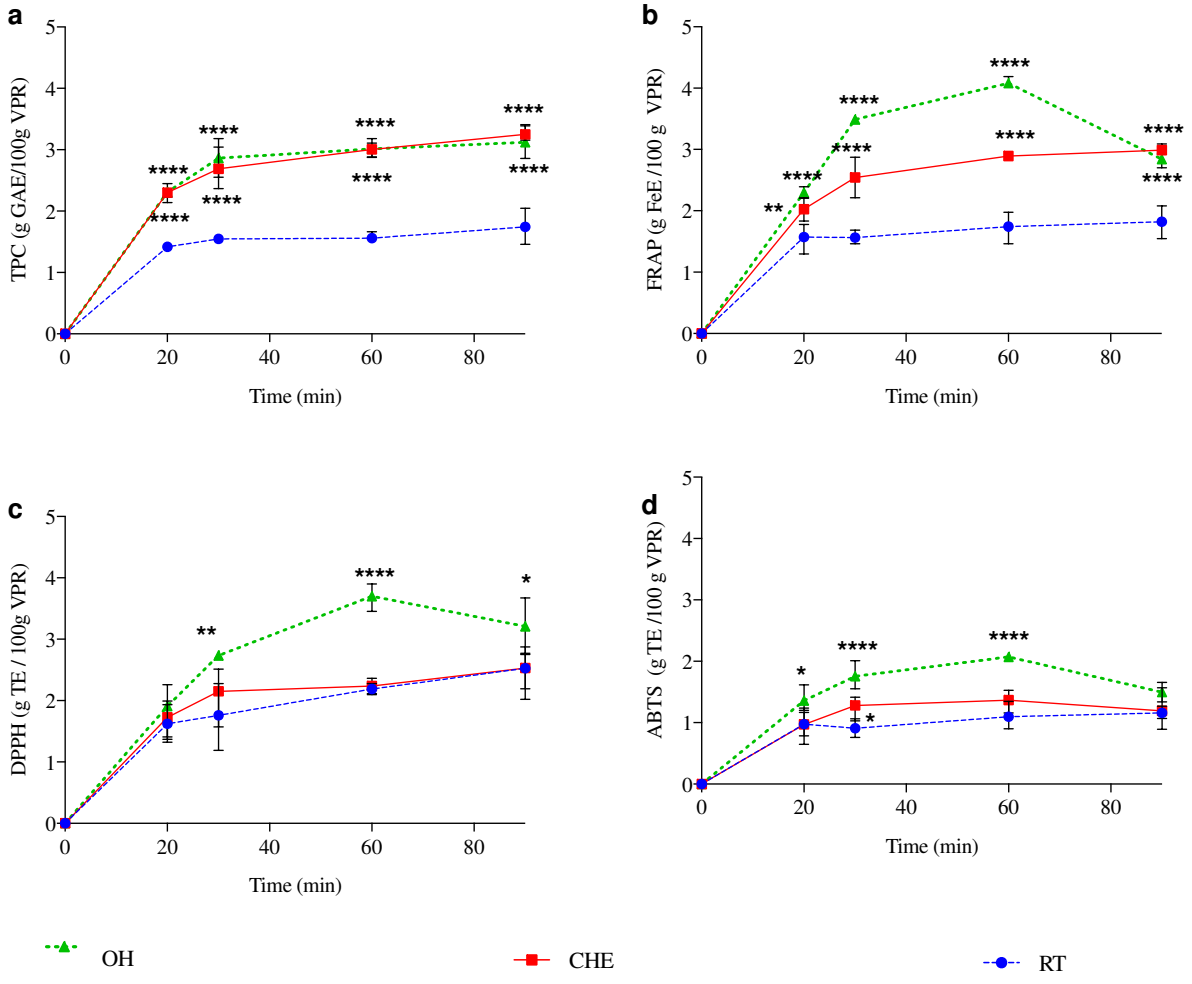

Fig. 5.1. Extraction of phenolic compounds from vine pruning residue using different extraction times (20, 30, 60 and 90 min) and methods of heating: room temperature (RT), conventional heating (CHE) and ohmic heating (OH). (a) Total phenolic compounds; (b) FRAP; (c) DPPH; (e) ABTS. Fixed conditions: solid liquid ratio (1:40 w/v), ethanol concentration (45 %) and temperature (80 °C). All experiments were done in triplicate and the results expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.2 Experimental TPC and antioxidant extraction

Bioactive compounds from VPR, were extracted with a 40 % hydroalcohol solution, at 80 °C, for 60 min. The VPR were subjected to three methods of extracting, including CHE, OH with two different electric field intensities (LEF- Low Electric Field and IEF-Intermediate Electric Field), and a treatment at RT, which was used as a controls Table 5.1. The total content of phenolic compounds extracted by OH using IEF was 23 % higher than that obtained in previous works from high electrical voltage (2.0 g GAE/100 g VPR) (Rajha et al., 2018). In addition, significant ($p < 0.05$) variations of TPC in the extracts obtained through the different extraction methods were observed, being IEF and LEF the extract with the highest TPC values (3.4 ± 0.1 and 3.1 ± 0.2 g GAE/100 g VPR, respectively). The extract obtained through RT treatment (1.2 ± 0.1g GAE/100 g VPR), as expected, exhibited the lowest TPC values, which is in agreement with other authors, who observed a marked reduction of TPC in extracts obtained at low temperatures (Jesus et al., 2019; R. Sánchez-Gómez, Sánchez-Vioque, et al., 2017). The temperature directly influences the TPC concentration, since mild treatments are not able to solubilize the phenolic compounds present in the lignicellulosic materials. The extracts produced by the IEF treatments had statistically higher concentrations ($p > 0.05$) compared to the RT, CHE and LEF treatments indicating that the electric field strength could positively affect the final TPC concentration (Rajha et al., 2015b).

Table 5.1 Total phenolic compounds (TPC) and antioxidant activity FRAP, DPPH and ABTS of the extracts produced from vine pruning residue by using different methods of heating: room temperature (RT), conventional heating (CHE) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field).

Runs	TPC	FRAP	DPPH		ABTS	
	g GAE/100 g VPR	g FE/100g VPR	g TE/100 g VPR	IC_{50}	g TE/100 g VPR	IC_{50}
IEF	3.4 ± 0.1^{c}	4.6 ± 0.2^{c}	4.1 ± 0.1^{d}	0.76^{a}	3.1 ± 0.1^{c}	0.34^{a}
LEF	3.1 ± 0.2^{b}	4.1 ± 0.3^{b}	3.2 ± 0.1^{c}	0.90^{b}	1.9 ± 0.2^{b}	0.44^{b}
CHE	3.0 ± 0.2^{b}	3.7 ± 0.1^{d}	2.7 ± 0.2^{b}	0.95^{b}	1.9 ± 0.1^{b}	0.40^{b}
RT	1.2 ± 0.1^{a}	1.7 ± 0.2^{a}	2.2 ± 0.1^{a}	1.25^{c}	1.01 ± 0.1^{a}	0.94^{c}

* The averages followed by the same letters within a column do not differ by the Tukey test ($p < 0.05$). GAE: gallic acid equivalents; FE ferrous equivalents; TE (II): Trolox equivalents. Antiradical activity is expressed as a mean ($n = 3$) of IC_{50} values (g of extract/L of solution).

The experimental results showed that the highest antioxidant activities evaluated by FRAP (4.6 ± 0.2 g FE/100 g), DPPH (4.0 ± 0.1 g TE/100 g) and ABTS (3.1 ± 0.1 g TE/100 g) assays were achieved when the ohmic IEF heating was used. The LEF treatment presented higher antioxidant activities for FRAP (4.1 ± 0.3 g FE/100 g) and DPPH (3.2 ± 0.1 g TE/100 g) assays compared with the obtained values when CH and RT methods were carried out. However, the ABTS values presented no significant differences ($p > 0.05$) (1.9 ± 0.1 g TE/100 g) between both, LEF and CHE treatments. Previous works have shown that there is a great influence of the applied electric field on the antioxidant activity values when FRAP and DPPH assay were determined, showing better results with respect to other extraction methodologies (Cappato et al., 2018; Loypimai, Moonggarm, & Chottanom, 2009). When the comparison based on the extraction method is conducted, (Table 5.1), it becomes evident that RT and CHE were the worst methods for obtaining extracts with relevant antioxidant activity. The increase of the intensity of the electric discharges in the IEF treatment raises the TPC and the antioxidant activity in the VPR extracts, probably due to the microscopic damages induced by shock waves of pressure that lead to the deterioration of the structural components of the residue, facilitating the rupture of the tissues by cavitation bubbles (Boussetta et al., 2012). These results highlight the significant effects of using IEF to extract antioxidant phenolic compounds in relation to other extraction methods.

Additionally, half of the maximal inhibitory concentration (IC_{50}) was also calculated by the DPPH and ABTS assays. The highest value was obtained in the extracts obtained by the CHE and RT treatments, which means that the electric fields have influenced positively the extraction of bioactive compounds in VPR.

5.3.3 Chemical composition of VPR extracts

The samples extracted using OH at two different intensities (LEF and IEF), CHE and RT techniques were analyzed by UHPLC (Ultra high performance liquid chromatography) and the identification and quantification of the phenolic compounds present in the extracts were carried out. The compounds identified in VPR extracts (results expressed in mg/100 g of VPR) are listed in Table 5.2.

Twelve different types of polyphenolic compounds including phenolic acid, flavonoids, phenylethanoids and stilbenes, were identified and quantified using their corresponding standards. The LEF and IEF extracts showed the highest concentrations of flavonoids. However, apigenin and

hesperidin flavonoids, were more abundant in IEF treatment (287.2 and 180.3 mg/100 g VPR, respectively) than LEF, and were not identified in the extracts obtained by CH and RT treatments. The concentration of apigenin in LEF was higher than that previously described (Jesus et al., 2019), which was 207.9 mg of apigenin per 100 g of VPR when a conventional liquid-solid method using a 45 % hydroalcoholic solution and 120 min of extraction was applied. Other authors employed a conventional heating method to extract the same compound from grape pomace, using an 80 % hydroalcoholic solution and acetone as extraction solvents, obtaining extracts with 0.3 and 9.1 mg apigenin /100 g of grape pomace (Pintać et al., 2018; Dutra et al. 2018; respectively).

Table 5.2 Polyphenolic composition of the VPR extracts (Expressed as mg/100 g VPR) obtained by different methods of heating: room temperature (RT), conventional heating (CHE) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field).

Polyphenols (mg/100 g VPR)	IEF	LEF	CHE	RT
Phenolics acid				
Gallic acid	2.9^{b}	3.5^{a}	ND	ND
o-Cumaric acid	15.8^{b}	26.5^{c}	14.2^{b}	6.6^{a}
Ferulic acid	46.6^{a}	46.1^{a}	ND	ND
Ellagic acid	222.9^{b}	77.7^{a}	ND	ND
Vanillic acid	68.4^{bc}	70.3^{c}	67.2^{b}	31.2^{a}
Flavonoids				
Hesperidin	180.3^{b}	149.0^{a}	ND	ND
Apigenin	384.2^{b}	157.5^{a}	ND	ND
Quercetin	287.2^{bc}	286.8^{bc}	281.6^{b}	132.8^{a}
Taxifolin	23.7^{c}	21.8^{bc}	19.8^{a}	ND
Simple phenols				
HidroxiTyrosol	152.4^{c}	151.6^{bc}	149.6^{bc}	ND
Tyrosol	142.3^{c}	139.8^{b}	137.1^{b}	64.2^{a}
Stilbenes				
trans-resveratrol	65.4^{a}	137.3^{b}	ND	ND

*Where The averages followed by the same letters within a file do not differ by the Tukey test (p <0.05). ND: not detected.

Hesperidin was also found analyzed the extraction of phenolic compounds from conventional grape juice (4.04 mg/L^{-1}), conventional wines (4.9 mg/L^{-1}) and organic wines (4.3 mg/L^{-1}). On the other hand, quercetin was present in all extracts, IEF, LEF and CH (287.2, 286.8 and 281.6 mg/100 g VPR, respectively) with significantly higher concentrations ($p < 0.05$) than in RT (132.8 mg/100 g VPR). These values were higher than those obtained in previous works when used CH extraction (27.7 mg/100 g VPR) (Jesus et al., 2019) and microwave extraction (82.1 mg/100 g VPR) (Moreira et al., 2018). The identification of this compound in the VPR extracts is relevant, since quercetin is the main flavonol present in vine leaves of white and red grape varieties, representing more than 70 % of total flavonols (Flamini, Mattivi, De Rosso, Arapitsas, & Bavaresco, 2013).

Single phenols were identified as hydroxytyrosol and tyrosol. Hydroxytyrosol was not identified in the RT extracts and did not present statistically significant ($p > 0.05$) differences in the concentrations found in the extracts obtained by CH, LEF and IEF (149.6, 151.6, and 152.4 mg/100 g VPR, respectively). IEF extract presented the highest tyrosol concentration (142.3 mg/100 g VPR), followed by LEF and CH (139.8 and 137.1 mg/100 g VPR, respectively), which showed no statistically significant differences ($p > 0.05$) and RT extract (64.2 mg/100 g VPR). Hydroxytyrosol and tyrosol were also identified in samples of commercial white Greek wines of Vitis vinifera L. cv. Malagusia (Tourtoglou, Nenadis, & Paraskevopoulou, 2014). According to Vauzour, Corona, & Spencer, (2010), hydroxycinnamates, phenolic acids and phenolic alcohols are capable of inducing significant neuroprotective effects.

Although stilbenes are extensively explored in VPR studies, in this study trans-resveratrol was the unique compound identified in the extracts. The highest trans-resveratrol concentration was obtained in the LEF treatment (137.3 mg/100 g VPR), where it was twice as high as the IEF extract value (65.4 mg/100 g VPR). These values are in agreement with those obtained by other authors (Moreira et al., 2018), who evaluated the extraction of trans-resveratrol (136 mg/100 g VPR) from two varieties of Portuguese grapes by using microwave extraction.

As it is well known, residues of the wine industry have been extensively studied for the extraction of bioactive compounds. However, the different cultivars, parts of the plant, geographical location, climatic conditions and storage time can influence on the properties and chemical composition of the recovered compounds (Cebrián-Tarancón et al., 2018; Gorena et al., 2014; Houillé et al., 2015). The main phenolic compounds found in this work were apigenin, quercetin, ellagic acid and hesperidin. According to Delgado-Torre and co-workers, catechin was the main phenolic compound isolated in VPR

extract (Delgado-Torre, Ferreiro-Vera, Priego-Capote, Pérez-Juan, & Luque De Castro, 2012;), while in other studies, (E) -resveratrol followed by (E)-ε-viniferine were also identifieds as the main phenolic compounds in VPR extracts (Gabaston et al., 2017; Lambert et al., 2013; Vergara et al., 2012). On the other hand, studies based on the polyphenolic composition extracted from VPR by using heating by electric fields are scarce, and most of them use pulsed electro technologies and water as the extraction solvent followed by other pre-treatments. In addition, these studies have described the presence of kaempferol, epicatechin, resveratrol, hydrodobenzoic acid, p-coumaric acid and ferulic acid (Rajha, Boussetta, Louka, Maroun, & Vorobiev, 2014; Rajha et al., 2015a, 2018).

5.3.4 Antimicrobial activity of VPR extracts

The VPR extracts obtained in this study through LEF, IEF, CHE and RT treatments were screened for antimicrobial activity against five fungi using a micro-dilution methodology. All fungi were evaluated as a function of the incubation time (assessing the growth rate at 24, 48, 72 and 96h) at 25 ± 2 °C. Thus, different concentrations of each extract (1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95 μg/mL) were tested against *Alternaria sp., Cladosporium cladosporioides, Phoma violacea, Penicillium italicum* and *Penicillium expansum* in order to observe the fungal growth and determine the optimal inhibitory extract concentrations for each treatment. The main results are summarized in Table 5.3, where the two better concentration of LEF, IEF, CHE and RT extracts against each fungus are shown. All the extracts exhibited antifungal activity against the studied fungi, showing greater activity after 96 h of exposure at the different extract concentrations. Antifungal activity varied according to the concentrations and extracts, although none of the concentrations used presented 100 % inhibition. Some authors suggest plant extracts as potential natural antifungal agents because of the need for low dosages to achieve antimicrobial bioactivity and reducing negative sensory impact on food (Kumar, Kujur, Singh, & Prakash, 2019).

Table 5.3 Percent inhibition of the VPR extracts by using different methods of heating: room temperature (RT), conventional heating (CH) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field) against the growth of different fungi calculated after 96 h of exposure.

Extraction technique	**IEF**		**LEF**		**CH**		**RT**	
Fungi	Extracts concentration (µg/mL)	Inhibition (%)	Extracts concentration (µg/mL)	Inhibition (%)	Extracts concentration (µg/mL)	Inhibition (%)	Extracts concentration (µg/mL)	Inhibition (%)
Penicillium italicum	7.8	30.5 ± 1.9	7.8	38.8 ± 3.4	15.6	38.5 ± 8.1	15.6	37.3 ± 2.5
	1000	68.2 ± 1.2^{a}	1000	66.5 ± 1.4^{a}	1000	67.8 ± 4.5^{a}	1000	64.3 ± 2.9^{a}
Penicillium expansum	7.8	21.3 ± 9.6	3.9	13.1 ± 5.9	15.6	24.6 ± 3.4	15.6	18.1 ± 6.1
	1000	53.3 ± 10.3^{a}	1000	54.5 ± 13.7^{a}	1000	34.7 ± 3.4^{b}	1000	25.0 ± 8.3^{c}
Alternaria sp.	15.6	20.9 ± 2.1	15.6	25.8 ± 5.4	15.6	24.9 ± 11.7	15.6	27.8 ± 6.0
	1000	40 ± 5.2^{d}	1000	45.4 ± 7.5^{c}	1000	32.1 ± 1.6^{a}	1000	35.1 ± 1.2^{b}
Phoma violacea	3.9	24.5 ± 2.1	7.8	14.2 ± 3.1	15.6	12.3 ± 6.4	15.6	21.4 ± 11.5
	1000	41 ± 5.9^{c}	1000	33.9 ± 4.5^{a}	1000	31.6 ± 2.1^{a}	1000	28.5 ± 5.8^{b}
Cladosporium Cladosporioides	3.9	38.4 ± 3.3	15.6	27.1 ± 2.7	15.6	30.3 ± 17.9	15.6	26.0 ± 10.4
	1000	62.4 ± 7.9^{a}	1000	59.9 ± 2.7^{a}	1000	43.8 ± 5.4^{b}	1000	18.1 ± 9.2^{c}

*The statistic was carried out only for the concentration of 1000 µg/mL. The averages followed by the same letters within a line do not differ by the Tukey test (p <0.05).

The highest antimicrobial inhibition was obtained when the maximum extract concentration (1000 μg/mL) was tested after 96 h of incubation. In addition, IEF, LEF and CHE extracts expressed much more significant inhibitory effects against P. italicum, P. expansum and C. cladosporioides when the same concentration (1000 μg/mL) was used, in contrast to RT extract that only presented a high percent inhibition against *P. italicum*. In general, VPR extracts showed 18.1 to 68.2 % growth inhibition (Table 5.3) under the best concentration used. On the other hand, the concentration 15.6 μg/mL showed for CHE (12.3 - 43.8 %) and RT (18.1 – 37 %) extracts was being, greater than or equal to all extracts, for the five fungi tested.

The highest antimicrobial inhibition was obtained when the maximum extract concentration (1000 μg/mL) was tested after 96 h of incubation. In addition, IEF, LEF and CHE extracts expressed much more significant inhibitory effects against *P. italicum, P. expansum* and *C. cladosporioides* when the same concentration (1000 μg/mL) was used, in contrast to RT extract that only presented a high percent inhibition against *P. italicum*. In general, VPR extracts showed a growth inhibition from 18.1 to 68.2 % (Table 5.3) when the maximum concentration was applied. On the other hand, the minimum inhibitory concentration for CHE (12.3 - 43.8 %) and RT (18.1 – 37 %) extracts was 15.6 μg/mL, being greater than or equal to all extracts, for the five fungi tested. The results showed that the IEF extract presented the highest inhibitory power against *C. cladosporioides* (38.4 %) and *Phoma violacea* (24.5 %) with the lowest extract concentration (3.9 μg/mL extract) reported in Table 5.3, against *C. cladosporioides* followed by *P. italicum and P. expansum*, which were inhibited in a 30.5 % and 21.3 %, respectively, when 7.8 μg/mL extract was tested.), and also, a lower inhibitory intensity for A*lternaria sp*. (inhibition of 20.9 % to 15.6 μg/mL extract) As regards the LEF extract, the minimum inhibitory concentration was 3.9 μg/mL for *P. expansum*, which was able to inhibit 13.1 %, whereas for P. italicum and *Phoma violacea* the concentration 7.8 μg/mL was able to inhibit 38.8 % and 14.2 % of the fungal growth, respectively. Finally, *Alternaria sp*. and *C. cladosporioides* showed a percent growth inhibition of 25.8 % and 27.1 %, respectively, to 15.6 μg/mL of extract. The LEF and IEF extracts showed minimal inhibitory concentrations that expressed stronger antifungal activities for *Alternaria sp.*, *C. cladosporioides, P. violacea, P. italicum* and *P. expansum*. These results are related to the phenolic profile and antioxidant activity previously provided.

Some authors claim that the antifungal effects of plant extracts and essential oils rich in bioactive compounds such as flavonoids are related to the disruption of the fungal cell endomembrane system (plasma membrane and mitochondria) and mitochondrial dysfunction, inducing metabolic stagnation

(Hu, Zhang, Kong, Zhao, & Yang, 2017; Yang et al., 2017). Inhibition of fungal growth and germination can be explained by the presence of flavonoids such as quercetin, hesperidin and apigenin, and phenolic acid such as ellagic acid, which have the capacity to reduce microbial growth, making a preferential alternative to synthetic antimicrobial agents, as these are environment friendly and easy to use (Kumar et al., 2019; Yang et al., 2017).

5.3.5 Anticancer activity of VPR extracts

The anticancer potential of VPR extracts was evaluated by their capacity to decrease the cell proliferation of four different cancer cell lines (MDA-MB-231, MCF-7, HepG2, and Caco2) and a non-carcinogenic cell line (CCD 841 CoN) being assessed by the MTT assay. As shown in Table 5.4, all VPR extracts inhibited cell growth in a concentration- and time-dependent manner in all cell lines tested. In recent years, several studies have attributed to flavonoids the selective reduction of cell viability of cancer cells (Mahmoudi et al., 2019; Tavsan & Kayali, 2019; Zhu, Wang, Jia, & Xie, 2019). These results can be attributed, among other compounds, to the abundant presence of quercetin in all extracts, which is considered to have anticancer potential due to its ability to induce apoptosis and inhibit the progression of numerous human cancer cell lines (Rauf et al., 2018). The IEF extract showed the most potent activity against MDA-MB-231, MCF-7, HepG2, Caco2, and CCD 841 CoN cells with IC_{50} values after 48 h of exposure of 62.8, 54.7, 89.7, 49.7, 71.0 μg/mL, respectively (Table 5.4). The distinct polyphenolic composition of the VPR extracts may explain the different growth inhibitory capacity between the VPR extracts. Taking into account the chemical characterization of the VPR extracts, apigenin ellagic acid hesperidin and quercetin are more abundant in the IEF extract than in the other extracts. Some authors recognize apigenin as a potential inhibitor of viability of human lung carcinoma cells (A549), while not showing significant side effects in healthy human umbilical vein endothelial cells (HUVEC) (Mahmoudi et al., 2019). Apigenin is considered an efficient inhibitor of the expression of nuclear factor E2-related factor 2 important contributor to chemoresistance in cancer therapy (Gao, Ke, Shi, Sun, & Chen, 2013; Paredes-Gonzalez et al., 2015). Over the years, studies with hesperidin have shown potent ant-inflammatory effects, anticancer and chemopreventive antioxidants (Kamaraj et al., 2019; Roohbakhsh, Parhiz, Soltani, Rezaee, & Iranshahi, 2015). In earlier studies hesperidin significantly inhibited the cell viability of HT-144 melanoma cells (Mashhadi Akbar Boojar, Mashhadi Akbar Boojar, Golmohammad, & Yazdi, 2018), human lung cancer A549 cells (Kamaraj et al., 2019), A-494 renal cell carcinoma (Boojar, Boojar, Golmohammad, & Bahrehbar, 2018), Caco2

cells (El-Readi, Hamdan, Farrag, El-Shazly, & Wink, 2010) and MCF-7 breast cancer cells (Febriansah et al., 2014), these works suggest hesperidin as a new agent in carcinoma therapy. Other authors show that keratin enhances anticancer and proapoptotic effects in colon CO115 and HCT1 cell lines and has also been shown to act on KRAS and PI3K. (Xavier et al., 2009; Xavier, Lima, Rohde, & Pereira-Wilson, 2011). Previous publications showed that ellagic acid isolated from *Phyllanthus emblica* L. exert a dose-dependent anticancer activity against MCF-7 cells in a concentration range between 5 and 50 μg/mL (Luo et al., 2011).

The cancer cell lines MDA-MB-231, MCF-7, HepG2, and Caco2 were more sensitive to VPR extracts than CCD 841 CoN non-carcinogenic cells under the same treatment conditions. An ideal chemopreventive agent should have a minimal effect on healthy cells, but a strong inhibitory effect on cell proliferation and carcinogenic pathways of cancer cells, therefore natural therapies for the treatment of cancer by plant extracts may reduce the side effects and toxic effects of therapies (Liu, Wang, Tang, Bowater, & Bao, 2019; Ramadan et al., 2017). However, HepG2 cancer cells were less sensitive to VPR extracts than CCD 841 CoN cells. This may be explained by the fact that HepG2 cells retain many of the specialized function of normal hepatocytes (Liu et al., 2019).

These results demonstrated that the IEF extract inhibits cell growth and its effect may be related to its chemical composition. Since, the inhibition of cell growth evaluated by the MTT assay could be attributed to decreased cell proliferation, induction of cell death or both, it would be interesting in the future to evaluate the effects of the VPR extracts on cell cycle and death. The results of this study represent preliminary results that will be beneficial for the evaluation of the bioactive potential of extracts of VPR and the development of novel chemotherapeutic agents.

Table 5.4 IC_{50} values (μg/mL) of VPR extracts obtained by different methods of heating: room temperature (RT), conventional heating (CHE) and ohmic heating at different electric fields (LEF: Low Electric field; IEF: Intermediate Electric field) against different cancer cell lines (MDA-MB-231- human breast, MCF-7- human breast, HepG2- human hepatocellular, and Caco2- human colon) and a non-carcinogenic cell line (CCD 841 CoN- human colon), calculated after 24 h and 48 h of exposure.

Cell lines	IC_{50} (μg / mL)							
	IEF		LEF		CHE		RT	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDA-MB-231	91.7 ± 12.7^{ab}	62.8 ± 6.5^{a}	116.1 ± 6.5^{a}	74.2 ± 5.6^{a}	119.9 ± 14.8^{a}	79.1 ± 8.2^{a}	137 ± 4.8^{ab}	86.97 ± 14.2^{a}
MCF-7	154.6 ± 21.7^{bc}	54.7 ± 5.3^{a}	167.2 ± 76.5^{bc}	62.7 ± 4.9^{a}	186 ± 14.6^{b}	65.7 ± 6.1^{a}	222.9 ± 64.6^{cd}	70.8 ± 10.1^{a}
HepG2	134.3 ± 27.7^{ab}	89.7 ± 13.7^{ab}	193.4 ± 51.1^{a}	117.7 ± 15.3^{a}	198.8 ± 29.9^{a}	117.2 ± 16.1^{a}	216.2 ± 28.7^{bc}	122.7 ± 25.0^{a}
Caco2	76.2 ± 4.0^{ab}	49.7 ± 6.0^{a}	104.3 ± 12.6^{a}	62.7 ± 3.7^{a}	105.2 ± 8.5^{b}	65.9 ± 6.3^{a}	115.2 ± 9.5^{b}	83.8 ± 7.4^{ab}
CCD 841 CoN	283.4 ± 5.6^{c}	71.0 ± 0.3^{a}	337.9 ± 5.4^{b}	78.1 ± 2.1^{a}	435.6 ± 8.1^{b}	81.6± 1.1^{a}	430.9 ± 8.8^{b}	99.0 ± 2.1^{a}

*Where Values are expressed as mean± SEM of three independent experiments. The averages followed by the same letters within a line do not differ by the Tukey test (p <0.05).

5.4 CONCLUSIONS

In this study, the extraction of polyphenolic compounds from Vine Pruning Residue (VPR) was evaluated through different electric field intensities (LEF electric field and intermediate electric field - IEF) using the environmentally friendly OH extraction procedure and compared to two conventional extraction (Room Temperature- RT and Conventional Heating-CHE). Overall, VPR was validated as a relevant source of phenolic compounds that can be exploited as ingredients for application on the food, pharmaceutical or cosmetic industries. The extracts obtained by the environmentally friendly OH technique presented better results in comparison to the other treatments most probably due to the rupture of the cells by the electric current passage. These results suggest that OH in IEF is an effective technique for extracting phenolic compounds from VPR. In addition, the IEF extract, which used higher electrical intensity, showed greater enrichment in the phenolic compounds, antioxidant activity, antimicrobial activity, and cancer cell growth inhibitory activity. Besides its enhanced biological activity, these extracts have additional advantages for application in food, pharmaceutical or cosmetic industries in comparison with other extraction techniques as no toxic solvents have to be removed.

5.5 References

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CHAPTER VI

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The final remarks of this work are about the general conclusions that were obtained from the developed research. Moreover, some suggestions for future activities are proposed.

6.1 General Conclusions

The main objective of the present thesis was to develop efficient and environmentally friendly methodologies involving low cost and nontoxic solvents for VPR fractionation and to use the different fractions obtained by processing to produce different products with industrial interest, following the concept of biorefinery. For this, the VPR was submitted to two sequential autohydrolysis treatments using different severities to select the ideal condition for attaining the different products. In addition, the extraction of phenolic compounds from VPR was optimized using different CHE, MAE methodologies and their phenolic content characterization and antioxidant potential was studied. The optimal condition obtained by CHE treatment was selected for studies with OH at two different electric field intensities. The extracts obtained in this study were characterized for their phenolic profile and the antioxidant, antimicrobial potential in fungi and antitumor potential in human cell lines were evaluated.

Therefore, the main conclusions drawn from this thesis are summarized below:

- Based on its chemical characterization, it can be stated that VPR is a lignocellulosic residue rich in biobased compounds with potential to be transformed into commercial products through its selective fractionation separating cellulose, hemicellulose, lignin and extractives.

- The severity of hydrothermal treatment significantly increased the cellulose enzymatic susceptibility with 5.4 times higher conversion compared to the lower severity. However, by performing an additional fractionation (severity of 4.60) of the solid obtained in the best condition, it was possible to achieve a better conversion of glucan to glucose (99 % comparing to 73.7 %).

- The solid obtained by auto-hydrolysis in two sequential stages (4.60 severity) was submitted to enzymatic hydrolysis reaching 96 % bioethanol after 48 h saccharification and 8 h separate fermentation and 83 % after simultaneous saccharification and fermentation, recovering 13.1 kg ethanol/100 kg VPR under the best condition. Based on previous work using VPR as a sugar source for bioethanol production, the most effective and environmentally friendly pretreatment technique to date is the two sequential autohydrolysis, as it favored subsequent saccharification, increasing the susceptibility of VPR to saccharification by enzymatic hydrolysis achieving 99 % conversion of glucan to glucose using only water as extraction solvent.

- Liqueurs obtained by autohydrolysis with severity of 4.13 showed significant concentrations of TPC (2.3 GAE g/L) with a yield of 3.1 GAE g/100 g of VPR, these values were higher than those obtained by other authors using 4.69 and ethyl acetate purification. The main compounds present in the liquors were: hydroxycinnamic acids, hydroxybenzoic acids, flavonoids and stilbenes. The compounds with the highest concentrations were catechin, chlorogenic acid, caffeic acid, vanillic acid, rutin, syringic acid, resveratrol, rosmarinic acid, p-coumaric acid, gallic acid, cinnamic acid, and ferulic acid.

- Through sequential autohydrolysis and enzymatic hydrolysis treatments, it was possible to obtain the fractionation of different products of great industrial interest such as xylooligosaccharides, phenolic compounds, ethanol and lignin (13.6, 3.1, 13.1, 27.0 per 100 kg of VPR, respectively) in four separate streams. Conferring a total of 69Kg of compounds per 100Kg of treated VPR, making this raw material suitable for use in various industrial sectors.

- By extracting bioactive VPR compounds by CHE, it was possible to reach peak concentrations using 45% ethanol for 120 min at 80 °C, where TPC of 2.17 GAE g/100 g VPR was extracted. However, for MAE extraction it was possible to reach the maximum TPC concentration (2.3 g/100 g VPR) using 60% ethanol for 5 min at 120 °C.

- The main compounds found in CHE and MAE extraction were ellagic acid (68.6 and 185.1 mg/100 g VPR, respectively) and apigenin (208.23 and 118.8 mg/100 g VPR). These results show that MAE treatments can reduce extraction time and consequently energy consumption, proving to be an efficient technology for the extraction of polyphenolic compounds, being able to selectively extract specific compounds according to the conditions applied in the extraction process. Therefore, it can be stated that the extracts obtained by microwave treatments in hydroalcoholic solutions have high ellagic acid and apigenin content and interesting antioxidant activity.

- Evaluation of the extraction of VPR polyphenolic compounds through different electric field intensities (LEF and IEF) by OH proved to be a more efficient and environmentally friendly technique compared to conventional extraction techniques due to cell disruption caused by passage of the electric current.

- The extracts obtained by IEF, where it was applied the highest electrical intensity, presented higher concentration of phenolic compounds compared to the treatment by CHE and MAE. The main compounds identified in this condition were apigenin (384.2 mg/100 g VPR), quercetin (287.2 mg/100 g VPR), ellagic acid (222.9 mg/100 g VPR) and hesperidin (180.3 mg/100 g VPR). Quercetin and

hesperidin were not detected in the extracts obtained by MAE. Extraction by IEF also showed great antioxidant, antimicrobial and growth inhibitory potential of diverse cancer cell lines with better results than extracts obtained by CHE. These extracts showed to be a promising alternative for the development of new and more efficient cancer therapies.

- Therefore, due to their high concentration of bioactive compounds and use of non-toxic solvents, VPR extracts obtained by MAE and OH in IEF have great potential for application in the food, pharmaceutical or cosmetic industries compared to other extraction techniques.

In conclusion, the results of this work contribute significantly to the full valorization of VPR, where its extracts can be obtained by ecologically correct and efficient treatments such as hydroalcoholic extraction by MAE and OH. These extracts have polyphenolic compounds with high biological potential making them an appealing alternative to synthetic bioactive compounds. In addition, the solids resulting from this extraction can be subjected to hydrothermal treatments and their obtained fractions can be directed to various industrial sectors, increasing the added value of this raw material according to the biorefinery concept, thus contributing to a circular economy.

6.2 Future perspectives

This thesis has shed some light on the full appreciation of VPR and its great potential, but further studies are still needed in order to effectively apply VPR liqueurs obtained by auto-hydrolysis, as well as the enhancement of lignin obtained after the fermentation process and effective applications of VPR extracts obtained by OH.

The liquors obtained by auto-hydrolysis can be applied as prebiotic and antioxidant functional ingredients, referring to the presence of xylooligosaccharides and bioactive compounds in their composition. Therefore, further studies devoted to the evaluation of the probiotic potential of integral VPR liqueurs for the purpose of its application as food ingredients are required. Due to the high lignin content present after the fermentative processes, further works focused on the evaluation of depolymerization methods for its effective valorization and industrial application are envisaged. It is

possible to produce derivative materials such as solvents, resins, dispersants and composites. Lignins can also be applied as a bacterial growth substrate for triglyceride lipid synthesis.

Given the great bioactive potential of extracts obtained by OH treatments, further studies are needed to explore their applicability in different industrial sectors. As shown in this work, VPR extracts have microbial growth inhibitory activity and due to the use of clean solvents to obtain them, can be incorporated into food products such as packaging films and thus increasing product shelf life. Recent studies show a great interest for new effective anticancer agents derived from natural products. Based on the selective growth inhibitory activity in human cancer cell lines from the VPR extracts obtained in this work, further studies are suggested to evaluate the effects of the extracts on cell functions, evaluating apoptosis induction and cell cycle distribution in human tumor cells.