PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

SCHOOL OF ENGINEERING

STUDY OF AN INTEGRATED PROCESS OF EXTRACTION AND PURIFICATION OF POLYPHENOLIC EXTRACTS FROM CARMÉNÈRE POMACE

NILS LEANDER HUAMAN CASTILLA

Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences

Advisor:
JOSÉ RICARDO PÉREZ CORREA

Santiago de Chile, January, 2020

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Dedicado a mi hijo Eduardo,
a mi esposa Gladys y a mis
queridos Padres.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF FIGURES vii

LIST OF TABLES viii

LIST OF PAPERS ix

PROCEEDINGS x

RESUMEN xi

ABSTRACT xiv

1. INTRODUCTION 1

1.1. Are agroindustrial discards a potential source of polyphenols? 2

1.2. Polyphenols of grape pomace 3

1.3 Hot Pressurized Liquid Extraction (HPLE) an alternative technology to obtain polyphenolic extracts 5

1.4 Relevant role of solvent characteristics on the selectivity of specific polyphenols. 7

1.5 Purification as a green alternative to obtain selective polyphenolic extracts 8

1.6 Integration of clean technologies to obtain selective polyphenolic extracts 9

1.7 Integrated HPLE - RP process: an attractive economic activity for industrial 9

1.8 Polyphenols as bioactive compounds 13

1.9 Scope and objectives of the thesis 14

2. POLYPHENOLS OF *CARMÉNÈRE* GRAPES 22

2.1. Introduction 22

2.2. Polyphenols of *Carménère* 25
2.2.1 Flavonols 25
2.2.2 Anthocyanins 29
2.2.3 Flavanols 34
2.3. Bioactive polyphenols found in *Carménère* 38
2.4. *Carménère* grape pomace as a source of valuable polyphenol extracts 40
2.5. Conclusion 42

3. IMPACT OF AN INTEGRATED PROCESS OF HOT PRESSURIZED LIQUID EXTRACTION-MACROPOROUS RESIN PURIFICATION OVER THE POLYPHENOLS, HYDROXYMETHYLFURFURAL AND REDUCING SUGARS CONTENT OF *CARMÉNÈRE* POMACE EXTRACTS. 51
3.1. Introduction 51
3.2. Materials and methods 53
3.2.1. Chemicals and analytic reagents 53
3.2.2. Grape pomace 53
3.2.3. Liquid extraction of Carménère pomace polyphenols 53
3.2.4. Macroporous resin purification process of Carménère pomace extracts 54
3.2.5. Total polyphenols content (TPC) 54
3.2.7. Quantification of HMF 54
3.2.8. Quantification of fructose and glucose 55
3.2.9. Proanthocyanidins analysis 55
3.2.10. Statistical Analyses 56
3.3. Results and discussion 56
3.3.1. Changes occurred in the chemical composition of Carménère pomace extracts during HPLE 56
3.2.2. Impact of an integrated process of HPLE-RP over chemical composition of Carménère pomace extracts 58
3.4. Conclusions 62

4. THE IMPACT OF TEMPERATURE AND ETHANOL CONCENTRATION ON THE GLOBAL RECOVERY OF SPECIFIC POLYPHENOLS IN AN INTEGRATED HPLE/RP PROCESS. 65
4.1. Introduction 65
4.2. Materials and Methods 69
4.2.1. Chemicals and analytic reagents 70
4.2.2. Wine pomace 70
4.2.3. Hot pressurized liquid extraction (HPLE) of Carménère pomace 71
4.2.4. Purification process (RP) of Carménère pomace raw extracts 71
4.2.5. Total polyphenols content (TPC) 71
4.2.6. Antioxidant capacity 72
4.2.7. Quantification of 5- hydroxymethylfurfural (HMF) concentration 72
4.2.8. Quantification of fructose and glucose concentration 73
4.2.9. Quantification of target polyphenols 73
4.2.10. Computational Chemistry calculations 74
4.2.11. Statistical analysis 75
4.3. Results and discussion 75
4.3.1. Effect of ethanol as co-solvent in HPLE 75
a) Phenolic acids 78
b) Flavanols 79
c) Flavonols 80
d) Stilbenes (resveratrol) 81
e) Interfering compounds 81
4.4. Global antioxidant properties 82
4.5. Purification with macroporous resin (RP) 82
a) Phenolic acids 82
b) Flavanols 84
c) Flavonols 84
d) Stilbenes (resveratrol) 85

e) Interfering compounds 85
4.6. Conclusions 87

5. GENERAL CONCLUSIONS AND FUTURE PROSPECTS 94
5.3. General conclusions 94
5.4. Future prospects 96

LIST OF FIGURES

Figure 1.1. Agroindustrial discards. 3
Figure 1.2. Polyphenols present in the grape pomace 4
Figure 1.3. Recovery of specific polyphenols using pure water as solvent in HPLE 6
Figure 1.4. Effect of water-ethanol mixtures on recovery of specific polyphenols 7
Figure 1.5. Overview on the potential of grape pomace as source of polyphenols 14
Figure 2.1. Chemical structures of flavonols 26
Figure 2.2. Flavonols content in *Carménère* grape skin 27
Figure 2.3. Flavonols content in grape skins of *Carménère, C. Sauvignon* and *Merlot* ..28
Figure 2.4. Chemical structures of anthocyanins 31
Figure 2.5. Anthocyanins content in *Carménère* grape skin. 32
Figure 2.6. Malvidin-3-*O*-glucoside content in grape skins of *Carménère, Cabernet Sauvignon* and *Merlot*. 33
Figure 2.7. Chemical structure of flavanols. 35
Figure 2.8. Flavanols content in *Carménère* grape seed 36
Figure 2.9. Flavanols content in *Carménère* grape skin 37
Figure 2.10. Epigallocatechin content in grape *Carménère, Cabernet Sauvignon* and *Merlot* 38
Figure 2.11. Content of phenolic compounds present in *Carménère* grape pomace 41
Figure 2.12. Total polyphenol content in agroindustrial biomass 42
Figure 3.1. Impact of different integrated processes on the chemical composition of *Carménère* pomace extracts 60
Figure 3.2. Changes produced by the integrated process of HPLE-RP on the oligomeric distribution (DPm) of the total proanthocyanidins content 61
Figure 4.1. Effect of the temperature and ethanol concentration on the recovery of specific polyphenols 76
Figure 4.2. Effect of co-solvent addition on the recovery of specific polyphenols in an integrated HPLE-RP process 83

LIST OF TABLES

Table 1.1. Analysis of the manufacturing costs (MC) to obtain polyphenol extracts, through different extraction methods using water-ethanol mixtures 10
Table 1.2. Price of polyphenol extracts from agroindustrial by-products.....11
Table 1.3. Price of purified polyphenols from grape (skin and seed).....12
Table 1.4. Comparative prices between mining and natural products 13
Table 2.1. Distribution of polyphenols in *Carménère* grapes.....24
Table 2.2. Polyphenols content comparing *Carménère* with *Cabernet Sauvignon* 25
Table 2.3. Potential health benefits of *Carménère* polyphenols. Main biological effects in vitro and in vivo 39
Table 3.1. Chemical characterization of Carménère pomace extracts obtained under different operating conditions 58
Table 3.2. Chemical characterization of Carménère pomace extracts obtained using an integrated process of HPLE-RP. 59
Table 4.1. Analytical features of Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) method for polyphenol quantification in grape 74
Table 4.2. Polyphenols profile of extracts obtained by HPLE process.....77
Table 4.3. Changes of the Gibbs free energies of solvation of chlorogenic and gallic acids in ethanol-water systems with increasing ethanol contents 79
Table 4.4. Polyphenols profile of purified extracts obtained by integrated HPLE-RP process.....86

LIST OF PAPERS

This thesis is based on the following papers, referred in the text by their respective chapters:

CHAPTER 2: Huamán Castilla N.L.; Mariotti M.S.; Pérez-Correa, J.R. (2017). **Polyphenols of *Carménère* grapes.** *Mini-Reviews in Organic Chemistry,* 14: 176–186.

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PROCEEDINGS

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Huamán Castilla N.L.; Mariotti M.S.; Pérez, J. R. Obtaining safe polyphenols extracts from *Carménère* pomace using an integrated extraction-purification process. In: Proceedings of the X Food Science and Technology Congress, December 10-15, Puno, Perú (2017).

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PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
ESCUELA DE INGENIERÍA

ESTUDIO DE UN PROCESO INTEGRADO DE EXTRACCIÓN Y PURIFICACIÓN DE EXTRACTOS POLIFENÓLICOS DE ORUJO DE UVA *CARMÉNÈRE*

Tesis enviada a la Dirección de Investigación y Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

Nils Leander Huamán Castilla

RESUMEN

El orujo de uva *Carménère*, un subproducto agroindustrial del proceso de vinificación presenta altas concentraciones de polifenoles. Este residuo ha despertado el interés de la industria alimentaria y farmacéutica debido a sus propiedades bioactivas y nutraceúticas. Una buena opción para obtener extractos de subproductos agroindustriales es la extracción líquida caliente presurizada (ELCP). Sin embargo, la extracción de polifenoles a altas temperaturas es responsable no solo de su degradación térmica, sino también de la formación de compuestos tóxicos como el hidroximetilfurfural (HMF) y la recuperación de azúcares. Las mezclas agua-etanol han permitido reducir la temperatura de extracción, pero el efecto sobre la reducción de azúcares y compuestos tóxicos aún no ha sido ampliamente estudiado. Por lo tanto, para obtener extractos enriquecidos en polifenoles libres de compuestos no deseados (HMF y azúcares), los extractos pueden ser purificados utilizando resinas macroporosas. Sin embargo, la presencia de etanol en el extracto puede afectar la adsorción de los polifenoles disminuyendo sus rendimientos. En este sentido, la integración de dos tecnologías limpias como ELCP y purificación con

resinas (PR), utilizando diferentes concentraciones de etanol, puede desempeñar un papel relevante en la recuperación y selectividad de polifenoles específicos.

Por ello, nosotros determinamos los efectos de la temperatura de extracción y características del solvente sobre la extracción y selectividad de polifenoles en un proceso integrado de ELCP-PR a partir del orujo de uva *Carménère*. En ese sentido, la información disponible sobre el orujo de *Carménère* fue exhaustivamente analizada con la finalidad de conocer sus características químicas y posibles futuras aplicaciones en la industria alimentaria y farmacéutica. Los efectos de las bajas concentraciones de etanol (0 - 15%) y moderadas temperaturas (60 – 90°C) sobre el contenido de polifenoles totales, recuperación de azúcares y formación de compuestos tóxicos en un proceso integrado ELCP-PR fueron analizados. Finalmente, nosotros evaluamos el efecto de las características del solvente (agua-etanol: 15 – 50%) a altas temperaturas (90 - 150°C) sobre la recuperación de polifenoles específicos en un proceso integrado ELCP-PR.

Según nuestros resultados, el orujo de *Carménère* presenta una fracción importante de polifenoles retenidos tales como malvidina (antocianina), quercetina (flavonol) y epigalocatequina (flavanol), así como polímeros de alto peso molecular (procianidinas). Las mezclas agua-etanol (15%) a 90°C permitieron recuperar ~0,8 veces más polifenoles totales en comparación con el agua pura bajo las mismas condiciones. La efectividad de las mezclas agua-etanol puede ser atribuida al efecto sinérgico de ambas moléculas. Las moléculas de agua permiten la ruptura de las paredes celulares liberando los polifenoles, mientras que el etanol reduciría la polaridad del solvente de extracción mejorando la solubilidad de estos compuestos. El uso de bajas concentraciones de etanol (15%) no solo permitió reducir la temperatura de extracción de 130°C a 90°C sin disminuir los rendimientos de polifenoles, sino también redujo la recuperación de azúcares debido a la baja polaridad del etanol que disminuye la solubilidad de estos compuestos. La PR permitió recuperar ~60% del contenido total de polifenoles, mientras el contenido de azúcares y HMF fueron reducidos significativamente (~95%). Sobre la base de estos resultados, un incremento en la concentración de etanol hasta un 50% a 150°C permitió una extracción selectiva de polifenoles durante la ELCP. Las concentraciones de etanol

más apropiadas fueron 15% para flavonoles, 32,5% para flavanoles y estilbenos, y 50% para ácidos fenólicos. Finalmente, el uso de etanol al 15% en ELCP permitió una mayor recuperación global de ácidos fenólicos (65%), flavanoles (57%) y estilbenos (64%) durante la PR. Contrariamente, la recuperación más alta de flavonoles (61%) durante la purificación se logró con un 50% de etanol en ELCP.

Desde una perspectiva científica, se concluye que el uso de etanol como cosolvente es una alternativa promisoria que permite mejorar los rendimientos y una extracción selectiva de polifenoles específicos durante la ELCP. La temperatura y concentración de etanol en la extracción, así como la estructura química de los polifenoles, determinan la selectividad y eficiencia de extracción de estos compuestos en el proceso integrado ELCP-PR. Los resultados obtenidos en esta tesis podrán ser usados para valorizar el orujo de uva *Carménère*, un residuo producido en grandes cantidades en Chile. Además, la nueva metodología desarrollada en esta tesis podrá utilizarse para mejorar la recuperación de compuestos bioactivos de diferentes subproductos agroindustriales.

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PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
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STUDY OF AN INTEGRATED PROCESS OF EXTRACTION AND PURIFICATION OF POLYPHENOLIC EXTRACTS FROM *CARMÉNÈRE* POMACE

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences by

Nils Leander Huamán Castilla

ABSTRACT

Carménère pomace, an agroindustrial by-product from the winemaking process presents high concentrations of polyphenols. This residue has awoken the interest of the food and pharmaceutical industry due to its bioactive and nutraceutical properties. A good option to obtain polyphenol extracts from agroindustrial by-products is Hot Pressurized Liquid Extraction (HPLE). However, extraction of polyphenols at high temperatures is responsible not only of their thermal degradation, but also of the formation of toxic compounds such as hydroxymethylfurfural (HMF) and sugar recovery. Water-ethanol mixtures have allowed to reduce the extraction temperature, but the effect on reduction of sugars and toxic compounds have not been widely studied yet. Thus, to obtain extracts enriched in polyphenols free of undesirable compounds (HMF and sugars), crude extracts can be purified using macroporous resins. However, the presence of ethanol in the extract, may affect the adsorption of the polyphenols decreasing their yields. In this sense, integrating two clean technologies like HPLE and resin purification (RP) combining

different ethanol concentrations can play a relevant role on the recovery and selectivity of specific polyphenols.

Thus, we determined the effects of extraction temperature and solvent characteristics on the extraction and selectivity of specific polyphenols in an integrated HPLE-RP process from the *Carménère* pomace. In this sense, the available information about *Carménère* pomace was exhaustively analyzed in order to know their chemical characteristics and possible future applications in the food and pharmaceutical industries. The effects of low ethanol concentrations (0 - 15%) and moderate temperatures (60 - 90°C) on total polyphenols content, sugar recovery and formation of toxic compounds in an integrated HPLE-RP process were analyzed. Finally, we evaluated the effect of solvent characteristics (water-ethanol: 15 - 50%) at high temperatures (90 - 150°C) on the recovery of specific polyphenols in an integrated HPLE-RP process.

According to our results, *Carménère* pomace presents an important fraction of polyphenols retained such as malvidin (anthocyanin), quercetin (flavonol) and epigallocatechin (flavanol), as well as polymers of high molecular weight (procyanidins). Water-ethanol mixtures (15%) at 90°C allowed to recover ~0.8 times more total polyphenols compared to pure water under the same conditions. The effectiveness of water-ethanol mixtures can be attributed to a synergistic effect of both molecules. Water molecules allows the rupture of cell walls releasing the polyphenols, while ethanol would reduce the polarity of the extraction solvent improving the solubility of these compounds. The use of low ethanol concentrations (15%) not only allowed to reduce the extraction temperature from 130°C to 90°C without decreasing the polyphenol yields, but also reduced the sugars recovery due to the low ethanol polarity that decreases the solubility of these compounds. Integrated HPLE-RP process allowed to recover ~60% of total polyphenols, while the sugar and HMF content were significantly reduced (~95%). Based on these results, an increased in the ethanol concentration up to 50% at 150 °C allowed a selective extraction of polyphenols during HPLE. The most adequate ethanol concentrations were 15% for flavonols, 32.5% for flavanols and stilbenes, and 50% for phenolic acids. Finally, the presence of ethanol at 15% in HPLE extracts allowed a greater

overall recovery of phenolic acids (65%), flavanols (57%) and stilbenes (64%) after RP. In contrast, the highest recovery of flavonols (61%) during RP was achieved when 50% of ethanol was used in HPLE.

From a scientific perspective, it is concluded that the use of ethanol as a co-solvent is a promising alternative to improve yields and selectivity of specific polyphenols during HPLE. Extraction temperature, ethanol concentration and polyphenol´s chemical structure, determine their extractability and selectivity in an integrated HPLE-RP process. The results obtained in this thesis can be used to valorize *Carménère* grape pomace, a residue produced in large amounts in Chile. In addition, the methodology developed in this thesis can be used to improve the recovery of bioactive compounds from different agroindustrial by-products.

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1. INTRODUCTION

A broad range of agroindustrial by-products (skin, seed, steam and leaves) derived from the processing of fruits and vegetables are generated each year. These by-products present considerable levels of bioactive compounds like polyphenols, which have awoken the interest of the pharmaceutical and food industries due to their biological and technological properties (Kumar et al., 2017; Sagar et al., 2018).

Conventional extraction (solid – liquid) at atmospheric conditions is one of the most used techniques to obtain extracts rich in polyphenols from agroindustrial by-products (Ameer, Shahbaz and Kwon, 2017). However, this traditional method presents some disadvantages such as large solvent volumes, long extraction times, low yields and use of toxic solvents (acetone, methanol and hexane) with the subsequent environmental and human risks (Fontana, Antoniolli and Bottini, 2013; Wang and Weller, 2006a). Thus, the development of clean technologies with low production costs in the extraction of polyphenols should be considered.

Hot Pressurized Liquid Extraction (HPLE) is recognized as a clean technology, where the temperature of the extraction solvent is increased from 100 to 250°C and pressure is applied to maintain the solvent in liquid state (Petersson et al., 2010). This alternative technology allows to extract between 2 and 10 times more polyphenols than atmospheric extraction (Ameer et al., 2017; Mauromoustakos et al., 2009). Pure water is the solvent most used in HPLE to obtain polyphenols from vegetable matrices (Ko, Cheigh, Cho and Chung, 2011; Vergara-Salinas et al., 2013). However, the use of high temperatures (≥120°C) presents some limitations related to the degradation of specific polyphenols. Additionally, high temperatures promote the recovery and the formation of undesirable compounds such as sugars and hydroxymethylfurfural (HMF), respectively (Plaza, Abrahamsson and Turner, 2013; Vergara-Salinas et al., 2013).

In HPLE, water-ethanol mixtures have been successfully used to obtain polyphenol extracts (Wijngaard and Brunton, 2009). These mixtures allow to reduce the extraction temperature without decreasing the recovery of polyphenols (Jablonsky, 2015;

Karacabey et al., 2012; Mauromoustakos et al., 2009; Olech and Nowak, 2012). Ethanol presents a polar and non-polar fraction in its chemical structure, it increases the intermolecular interactions with polyphenols improving their solubility (Galanakis et al., 2013). In this sense, the use of low polarity co-solvents such as ethanol in HPLE could allow a selective extraction of specific polyphenols without sacrificing the extraction yields.

Crude extracts contain undesirable compounds after the extraction process, therefore they should be purified in a subsequent adsorption/desorption process using macroporous resins (Sandhu and Gu, 2013; Soto, Moure, Domínguez, and Parajó, 2011). Normally, the adsorption is carried out using extracts obtained with pure water allowing a strong resin/polyphenols interaction. Afterwards, desorption with an ethanol rich solution as eluent allows recovering a high proportion (65 – 85%) of the polyphenols retained in the resin (Li et al., 2017; Lin, Zhao, Dong, Yang and Zhao, 2012; Sandhu & Gu, 2013). Therefore, using extraction solvents different from water could reduce resin/polyphenols interaction during the adsorption stage, affecting significantly the recovery of polyphenols in the desorption stage.

1.1. Are agroindustrial discards a potential source of polyphenols?

Large amount of agroindustrial by-products (~1.3 billion tons/year) are generated during food processing (Ravindran, Hassan, Williams and Jaiswal, 2018). Fruits and vegetables present the highest proportions of discards (~50%) compared to other natural sources such as cereals (~20%), fish (~35%) and meat (~15%) (FAO, 2019; Ravindran et al., 2018). These discards are commonly used in low value animal foods and organic fertilizers, causing economic losses (~US$ 680 billion per year) and environmental pollution (Vilariño, Franco and Quarrington, 2017).

Fruits such as mango, pineapple and grapes present the higher proportion of agroindustrial by-products compared to other agricultural products such as onion, potato

and tomato (Figure 1.1a) (Sagar et al., 2018). Among these discards, grape pomace (skin and seed), a solid organic wastes from the wine industry, presents the highest total polyphenols content (TPC) (Figure 1.1b) (Kumar et al., 2017; Sagar et al., 2018). Grape pomace is an attractive source of polyphenols, that can be exploited to increase wineries income and reduce their environmental impact (Vijayalaxmi, Jayalakshmi and Sreeramulu, 2015).

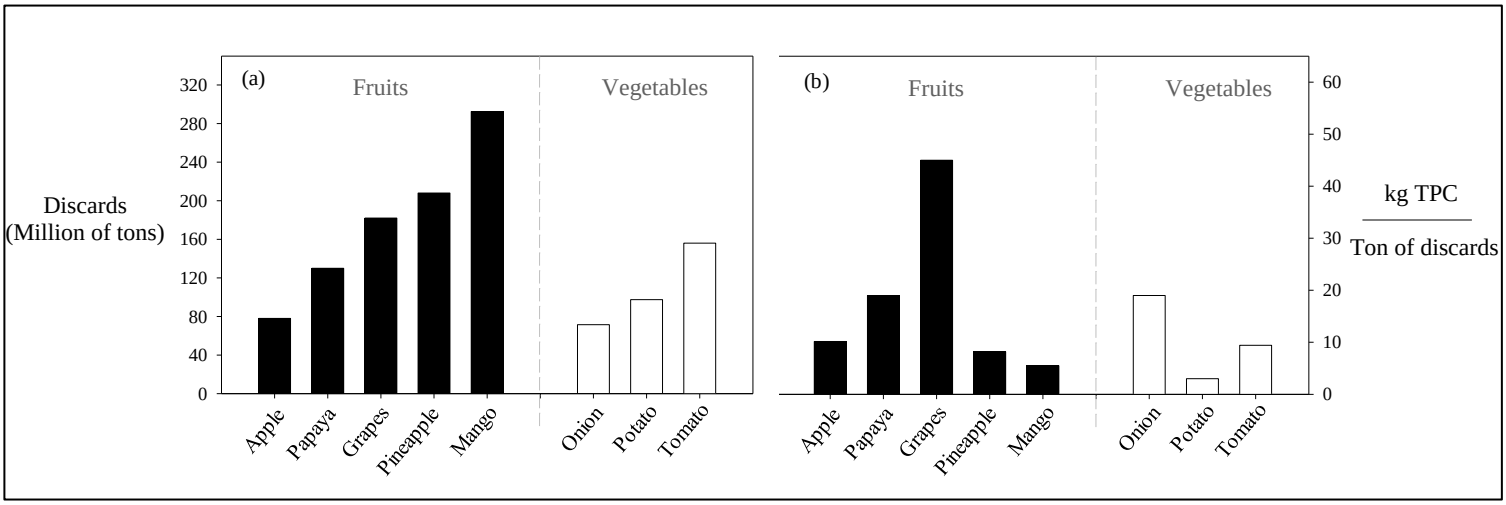

Figure 1.1. Agroindustrial discards, a) production in general adapted from Sagar et al. (2018); b) Total polyphenols content adapted from Sagar et al. (2018); Ang et al. (2012); Kabir, Sultana and Kurnianta (2015); Makris and Kefalas (2015); Masibo and Qian (2008); Sudha, Baskaran and Leelavathi (2007).

1.2. Polyphenols of grape pomace

Grape pomace presents a broad range of polyphenols, which can be classified as non-flavonoids (phenolic acids and stilbenes) and flavonoids (flavonols, flavanols and anthocyanins) (Figure 1.2)(Peixoto et al., 2018).

The distribution of polyphenols in grape pomace may present differences, depending on some factors such as the varietal differences and the winemaking procedures (Fontana et al., 2013; Ruberto et al., 2007).

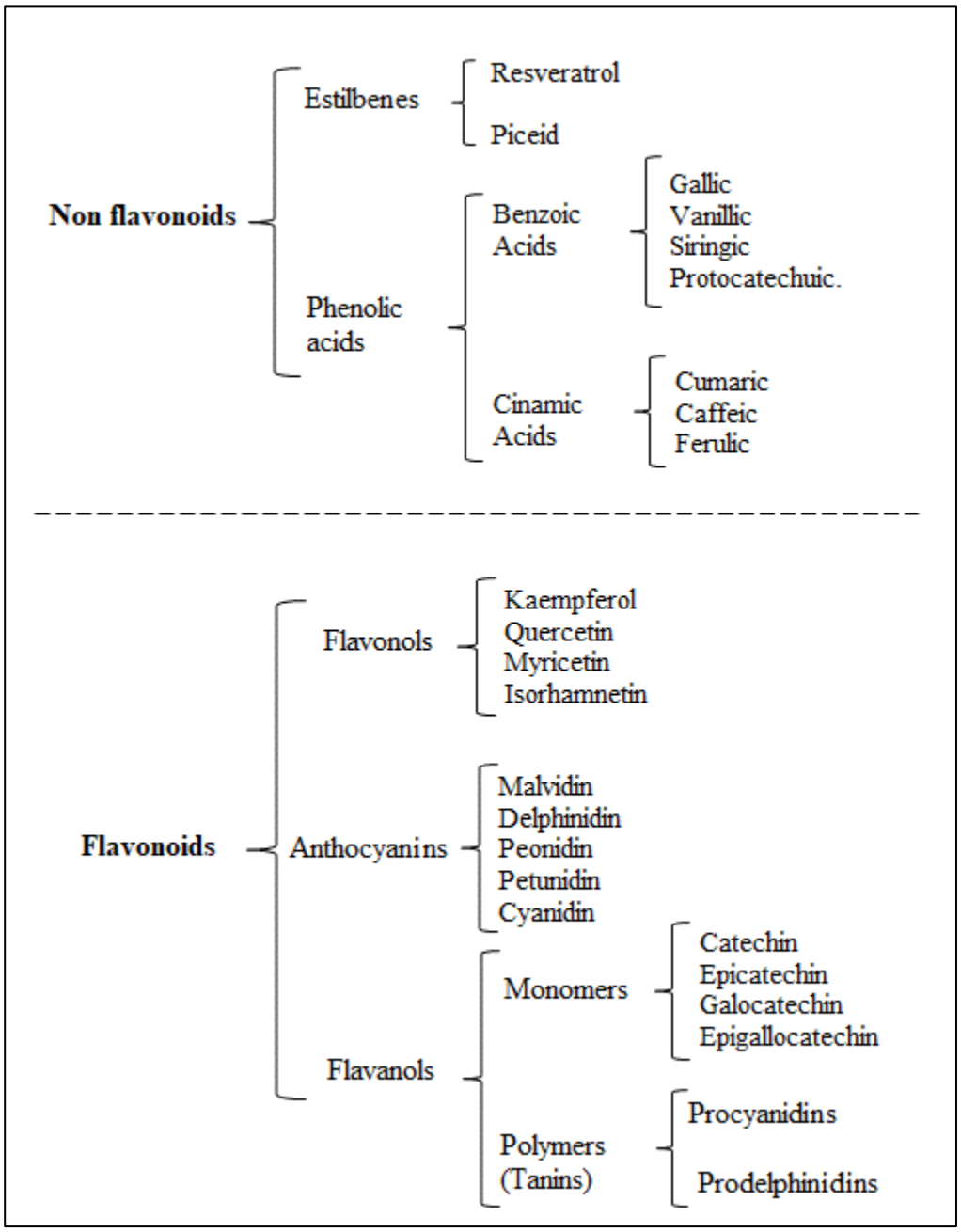

Figure 1.2. Polyphenols present in the grape pomace adapted from Peixoto et al. (2018)

Chilean industry produces several wine varieties such as Cabernet Sauvignon (~28%), Sauvignon Blanc (~16%), Merlot (~13%), *Carménère* (~8%) and Syrah (~5%) (ODEPA, 2019). *Carménère*, the emblematic wine of Chile, presents a distinctive deep red color and low astringency due to its particular polyphenols profile (240 – 350 mg GAE/L) (Fernández, Kennedy and Agosin, 2007). This wine generates ~80,000 tons of grape pomace/year (SAG, 2018), representing an important natural source of polyphenols with potential applications in functional foods and nutraceuticals.

1.3. Hot Pressurized Liquid Extraction (HPLE) an alternative technology to obtain polyphenolic extracts

HPLE is a clean technology for the recovery of polar and non-polar substances (Plaza & Turner, 2015). The high temperatures (50 – 200°C) and elevated pressures (0.1 – 22 MPa) applied during the process allow to reduce the polarity and maintain the liquid state of the solvent, improving the solubility and mass transfer of analytes (Ameer et al., 2017; Plaza and Turner, 2015). However, high temperatures (T ≥120°C) promote the degradation of some bioactive compounds and increase the formation of HMF as well as the extraction of sugars (Plaza et al., 2013; J. R. Vergara-Salinas et al., 2015).

Pure water is the most used solvent in HPLE, which combined with high temperatures have allowed to obtain extracts rich in polyphenols from grape pomace (Suárez et al., 2014; Vergara-Salinas et al., 2015; Vergara-Salinas et al., 2013). However, temperatures higher than 100°C and 150°C decrease significantly the recovery of anthocyanins (Figure 1.3a) and promote the hydrolysis of tannins releasing monomers and oligomers (Figure 1.3b) (Vergara-Salinas et al., 2013). In contrast, some monomers such as myricetin (flavonol) and epicatechin (flavanol) are highly stable at high temperatures (Figure 1.3a) (Vergara-Salinas et al., 2015).

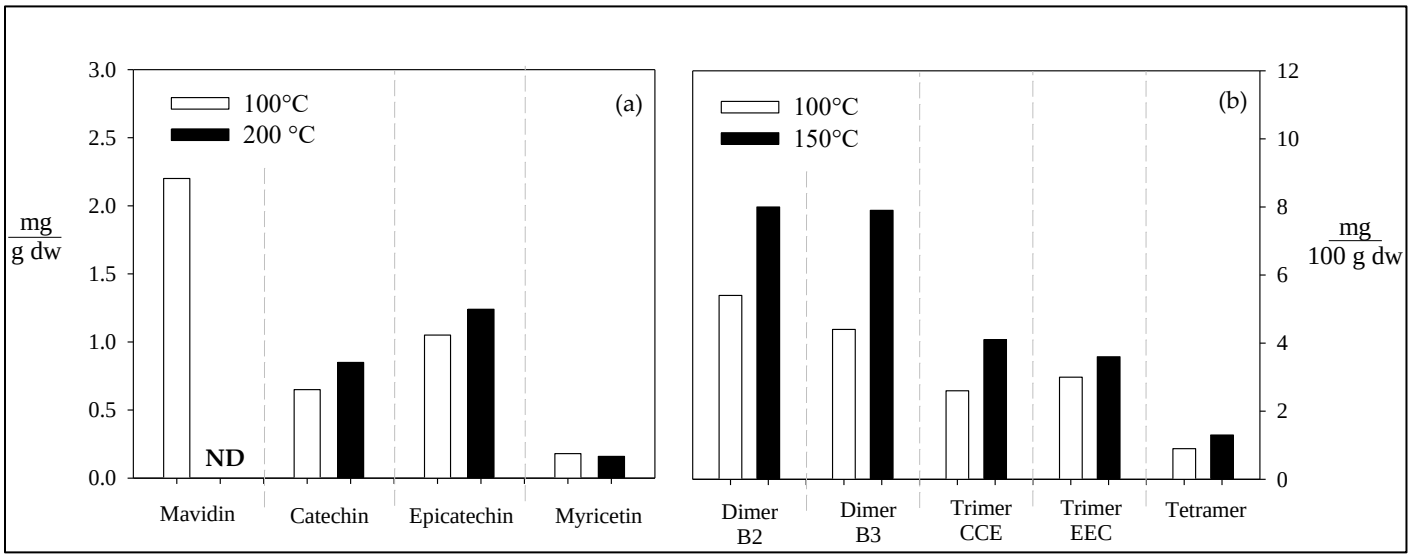

Figure 1.3. Recovery of specific polyphenols using pure water as solvent in HPLE from grape pomace adapted from Suárez et al. (2014); Vergara-Salinas et al. (2015). Malvidin is expressed as malvidin-3-glucoside. Dimer B2: Epicatechin-Catechin, Dimer B3: Catechin-Catechin, Trimer CCE: Catechin-Catechin-Epicatechin, Trimer EEC: Epicatechin-Epicatechin-Catechin. ND: no detected.

In this context, the use of water-ethanol mixtures have allowed to reduce the extraction temperature without decreasing the polyphenols recovery from grape pomace (Mauromoustakos et al., 2009; Monrad et al., 2010). For example, water-ethanol mixtures (10%-30%) recovered more anthocyanins than pure water and also improved the stability of these compounds at high temperatures (≥ 120°C) (Figure 1.4a) (Monrad et al., 2010). Mauromoustakos et al. (2009) reported that water-ethanol mixtures (10% - 30%) were more effective to extract procyanidins than pure water under the same conditions (Figure 1.4b). In contrast, Wijngaard and Brunton (2009) found that an increment in the ethanol content from 15% to 50% at 100°C decreased the flavonols recovery in ~40%. Since previous studies do not show consistent trends, finding the right conditions to improve the recovery of specific families of polyphenols is challenging; hence, a holistic approach should be applied, considering experimental, empirical and computational analyses.

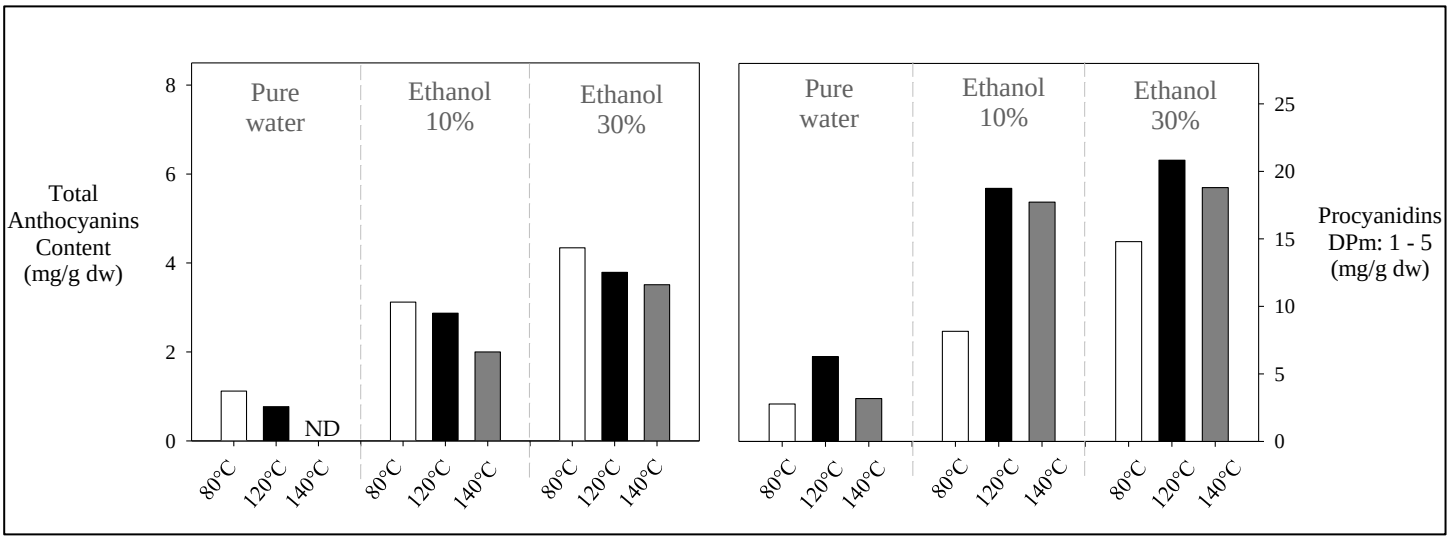

Figure 1.4. Effect of water-ethanol mixtures on recovery of specific polyphenols from grape pomace, adapted from Mauromoustakos et al. (2009) and Monrad et al. (2010). DPm: polymerization grade. ND: no detected.

1.4. Relevant role of solvent characteristics on the selectivity of specific polyphenols.

Polyphenols present aromatic rings (non-polar fraction) and one or more hydroxyl groups (polar fraction) in their chemical structure (Vijayalaxmi et al., 2015). These chemical features and the characteristics of the solvent explain the solubility of polyphenols during the extraction process (Cheigh et al., 2015). Although, solvent polarity is usually characterized in terms of the dielectric constant (Ɛ), this parameter does not consider the intermolecular interactions between solute and solvent. Thus, a correct definition of the solvent polarity should include all possible intermolecular interactions between solute molecules and solvent molecules (Katritzky et al., 2004; Reichardt, 1994).

Kamlet–Taft solvatochromic parameters allow to understand the solvent affinity for an specific compound and its ability to form hydrogen-bonds with it, which are expressed as polarity/polarizability (π*) and acidity (α) respectively (Jessop, 2011). Additionally, pure water and ethanol are recognized as protic solvents due to the presence

of hydroxyl groups in their chemical structure (Jessop et al., 2012). Although, under atmospheric conditions at 30°C water present a higher polarity (π*: 1.14) than ethanol (π*: 0.51), the ability of both solvents to form hydrogen bonds (α) is relatively similar, being 0.98 for ethanol and 1.07 for water (Jessop et al., 2012). Thus, polyphenols are highly soluble in water–ethanol mixtures due to the capacity of ethanol to modulate the solvent polarity and form a high number of intermolecular interactions with the polyphenols functional groups (Galanakis et al., 2013).

1.5. Resin purification as a green alternative to obtain selective polyphenolic extracts

Crude extracts obtained with HPLE are rich not only in polyphenols but also in other undesired compounds that are difficult to handle in food and nutraceutical applications (e.g., atomization) (Muzaffar, Nayik and Kumar, 2015; Plaza et al., 2013). Consequently, a subsequent purification stage is necessary. Adsorption/desorption with macroporous resins (RP) is one of the most frequently used techniques to obtain purified polyphenol extracts. This process is low cost and environmentally friendly, as well as simple to scale up, operate and regenerate (Yang, Zhao and Lin, 2016).

RP processing includes four stages: adsorption, washing, desorption and regeneration. For example, during the adsorption step, the polyphenols are retained in the packed column according to their affinity with the macroporous resins. Then, the resin is washed with distilled water to remove polar compounds (sugars and HMF). Finally, during desorption, between 65% and 85% of the polyphenols retained in the resin are recovered using hydro-alcoholic solutions (Buran et al., 2014; Silva et al., 2013; Lin et al., 2012).

Normally, the presence of pure water in the extracts allows that polyphenols can be retained in the resin through π- π stacking interactions during the adsorption step (Li et al., 2017; Lin et al., 2012; Silva et al., 2013). However, the presence of ethanol in the extract could affect the adsorption of the interest compounds in the resin due to a higher

affinity between polyphenol and solvent. In this sense, process optimization will require specific studies that consider the relevant role of the extraction solvent during purification.

1.6. Integration of clean technologies to obtain selective polyphenolic extracts

Sustainable technologies such as HPLE and RP have been proposed as alternatives for an efficient extraction and purification to obtain polyphenolic extracts for future applications in the industry (Ameer et al., 2017; Li and Chase, 2010). Therefore, it seems reasonable to integrate both technologies, HPLE and RP, and assess the role of the extraction solvent in the whole integrated process.

The evaluation of water-ethanol mixtures in the integrated HPLE-RP process can improve the understanding about the effect of solvent characteristics and chemical structure of polyphenols on their selective recovery. For example, during the extraction step, the hydroxyl groups in the polyphenols would allow a higher dipole-dipole interaction with the hydroxyl group of ethanol. Additionally, the aromatic rings of the polyphenols can interact with the non-polar fraction of ethanol through London dispersion forces. In addition, during the purification step, the aromatic rings of the polyphenols will interact with the aromatic rings of the resin through π-π stacking interactions. However, the presence of ethanol in the crude extracts could favored solvent-polyphenol interactions instead of polyphenol-resin interactions, affecting significantly the global polyphenols recovery.

1.7. Integrated HPLE - RP process: an attractive economic activity for industry

Sustainable separation of polyphenols through an integrated HPLE-RP process to obtain safe polyphenols extracts. This new approach also seems to be a useful tool to reduce the manufacturing cost (MC), considering a future technology transfer to the industry.

Santos, Veggi and Meireles (2010, 2012) estimated the manufacturing costs (MC) of obtaining polyphenol extracts using different extraction techniques (Table 1.1). MC represent the total capital investment cost and operating cost. This analysis showed that an increased on the production volume allows to reduce the MC in all techniques. Contrary, long process times present in Soxhlet and conventional extraction increase the MC between 2.58 and 1.58 US$ per gram of polyphenol, respectively (Table 1.1). In contrast, the MC for HPLE was up to 8 times lower compared to other technologies, due to its short extraction time (0.15 h), low solvent consumption and high polyphenols yield.

Table 1.1. Analysis of the manufacturing costs (MC) to obtain polyphenol extracts, through different extraction methods using water-ethanol mixtures (30%).

Technique	**Condition Process**	**Extractor capacity (m^3)**	**MC for phenolic compounds (US$/g)**
Conventional	30°C – 2h	0.05	3.80
		0.10	2.50
		0.30	1.58
Soxhlet	30°C – 8h	0.05	9.23
		0.10	5.50
		0.30	2.38
Ultrasound	30°C – 2h	0.05	3.70
		0.10	2.46
		0.30	1.86
HPLE	120°C – 0.15h	0.05	0.32
		0.10	0.29
		0.30	0.26

Adapted from Santos, Veggi and Meireles (2012) and Santos, Veggi, & Meireles (2010)

The global polyphenols market has increased significantly from 757 million US$ (2015) to 1,950 million US$ (2018) (GVR, 2019). Moreover, the development of more efficient process technologies and the higher availability of low cost agroindustrial discards, may boost this market even more rapidly (Di Donato et al., 2018; Ravindran, Hassan, Williams and Jaiswal, 2018). For example, the Chinese industry has developed alternative technologies to obtain powder extracts rich in polyphenols from agroindustrial by-products such as grape seed, grape skin, apple skin, pineapple skin and onion (AG, 2019; GVR, 2019). The prices of their polyphenol extracts are relatively low compared with other competing producers; between 16 and 190 US$ per kilogram, depending on their purity (Table 1.2).

Table 1.2. Price of polyphenol extracts from agroindustrial by-products.

Agroindustrial by-products	**Polyphenolic extracts (20 – 50% purity) (US$/kg)**
Grape skin	35.00 – 100.00
Grape seed	32.00 – 140.00
Apple	50.00 – 100.00
Pineapple	16.00 – 100.00
Onion	50.00 – 190.00
Potato	40.00 – 60.00

Adapted from Chinese trading companies in Alibaba Group (2019).

RP, is a relatively low cost technology that selectively retain those polyphenols present in crude extracts (Silva, Castellanos and Ottens, 2018). A subsequent purification of polyphenol extracts with this technology, would allow to obtain extracts with higher amounts of specific polyphenols, that reach much higher sale prices. For example, the

price of gallic acid (≥99%), catechin (≥99%), resveratrol (≥99%), quercetin (≥99%) and procyanidin B1 (≥99%) is 910 US$, 9,500 US$, 16,000 US$, 24,000 US$ and 38,000 US$ per kilogram (Table 1.3). Therefore, the integration of two green technologies like HPLE and RP to obtain enriched polyphenol extracts, is technically feasible an extremely attractive from and economic point of view.

Table 1.3. Price of purified polyphenols from grape (skin and seed).

	Purified extracts			
Specific polyphenols	20 - 40% purity (US$/kg)	60% purity (US$/kg)	80% purity (US$/kg)	≥99% purity (US$/kg)
Gallic acid	25.00	60.00	150.00	910.00
Catechin	80.00	120.00	980.00	9,500.00
Resveratrol	50.00	250.00	1,600.00	16,000.00
Quercetin	70.00	280.00	1,900.00	24,000.00
Procyanidin B1	85.00	310.00	2,500.00	38,000.00

Adapted from Chinese trading companies in Alibaba Group (2019).

We believe that Latin American (LA) countries should strongly support the development of the bioactive compounds industry, due to its evident economic and competitive advantages. However, governments and private companies still tout to the mining as the most important economic activity in many LA countries, without considering its high environmental and health risks, and the extremely high investments required (Ríos, 2018).

Chile is considered as one of the most important mining countries in the world and its activity represents ~20% of national GDP and 60% of total exports (Ghorbani & Kuan,

2017). While food industry only represents ~2.4% of national GDP and 3.5% of total exports (ODEPA, 2019; OEC, 2019). Although, production of polyphenol extracts through sustainable processes may be much more profitable and environmentally friendly than copper mining (Table 1.4).

Table 1.4. Comparing mining and natural products production.

Description	**Refined copper**	**Crude polyphenols extracts**
Manufacturing costs (per kg)	4.7 US$	15.53 US$
Sale price (kg)	6.2 US$	35.00 US$

Adapted from OEC (2019), Alibaba Group (2019) and Santos, Veggi, & Meireles (2010)

1.8. Polyphenols as bioactive compounds

Both crude and purified extracts obtained from grape pomace present different families of polyphenols such as flavanols, flavonols, anthocyanins, stilbenes and phenolic acids, which can be effectively applied in the treatment and prevention of various chronic diseases (Vergara et al., 2013; Huamán et al, 2017; Mariotti et al., 2018). Particularly, anthocyanins can modulate not only the oxidation of LDL-lipoprotein but also several inflammatory responses associated with type 2 diabetes mellitus (Bell et al., 2017). Stilbenes have shown to possess antioxidant and anti-inflammatory features both *in vitro* assays and *in vivo* assays (Lancon et al., 2016; Zhang et al., 2015). Flavanols have shown an antiproliferative ability against breast cancer cells by inducing apoptosis and due to the presence of several hydroxyl groups (OH) also show a strong ability to reduce free radicals (superoxide anion and singlet oxygen) (Chi et al., 2004; Koteswari et al., 2018). Flavonols

inhibit the release of histamine; therefore, they show anti-inflammatory and anti-allergic bioactivities (Shi et al., 2004; Mlcek et al., 2016). Phenolic acids inhibit cytochrome P-450 bioactivation of various carcinogens (Hour et al., 2009; Negi et al., 2006). Given the wide spectrum of applications of polyphenols, the development and optimization of sustainable extraction/purification technologies to recover them from agroindustrial wastes is a highly active research area (Figure 1.5).

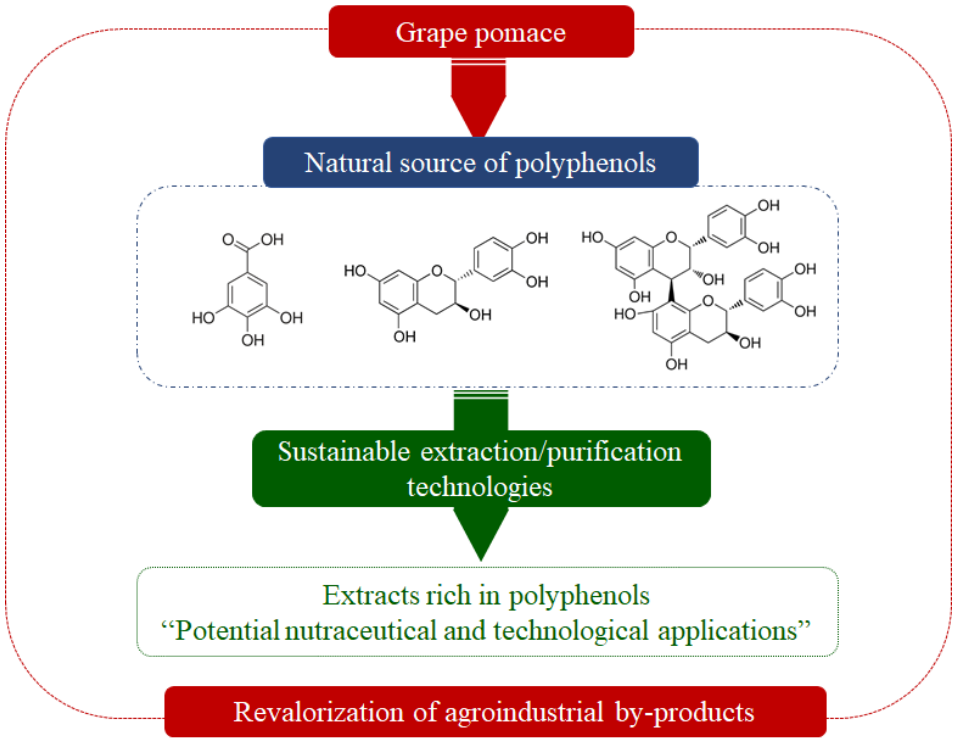

Figure 1.5. Overview on the potential of grape pomace as natural source of polyphenols

1.9. Scope and objectives of the thesis

The hypothesis that support this thesis, is that it is possible to significantly improve the global yield, safety, purity and selectivity of specific polyphenol extracts in an

integrated HPLE-RP process, by changing extraction conditions such as temperature and solvent composition.

The overall objective that delimits the scope of this thesis was to determinate the effects of temperature and solvent characteristics on the recovery, safety, purity and selectivity of polyphenol families and specific compounds in an integrated HPLE-RP process from *Carménère* grape pomace.

To achieve this goal, the thesis was divided into the following specific objectives:

- Review the main polyphenols present in skin and seed of *Carménère* pomace (considering chemical structure and amounts) and analyze their possible applications in the food and pharmaceutical industry. This objective was accomplished by the publication of a review in the journal Mini-Reviews in Organic Chemistry, which is presented in Chapter 2 of this thesis.

- Evaluation of the effect of low ethanol concentrations and moderate temperatures on the recovery from *Carménère* pomace of total polyphenols, oligomers profile and undesirable compounds (HMF and sugars) in an integrated HPLE-RP process. This objective was accomplished by the publication of a research paper in the International Journal of Food Science and Technology, which is presented in Chapter 3 of this thesis.

- Evaluation of the effect of solvent composition on the recovery of specific families of polyphenols such as flavanols, flavonols, stilbenes and phenolic acids in an integrated HPLE–RP process from *Carménère* pomace. This objective resulted in the publication of an article in the journal Molecules, which is presented in Chapter 4 of this thesis.

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2. POLYPHENOLS OF *CARMÉNÈRE* GRAPES

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2.1. Introduction

How people eat has drastically changed over recent years; nutritional foods associated with wellness and health are growing in importance (Goldberg, 1994). These foods contain bioactive compounds which help in the prevention and treatment of various chronic diseases (Kitts, 1994). For example, it has been shown that a diet rich in polyphenols helps in preventing various oxidative stress related diseases like cancer, and several cardiovascular and neurodegenerative diseases (Scalbert and Williamson, 2000).

Polyphenols are bioactive compounds naturally present in fruits and vegetables, which are characterized by their huge antioxidant capacity. In fact, it has been demonstrated that an average intake of 1g/day of polyphenols is between 10 and 100 times better than the consumption of vitamin C, vitamin E and carotenoids in protecting body tissues against oxidative stress agents (Correa, 2016; Gonzalez-Manzano et al., 1996).

From a chemical point of view, these compounds can be classified into two main groups: flavonoids (flavonols, anthocyanins and flavanols) and non-flavonoids (stilbenes and phenolic acids) (Garrido and Borges, 2013; Gonzalez-Manzano et al., 2009; Moreno and Peinado, 2012). The latter are characterized by a structure of a single ring of 6 carbon atoms, while flavonoids have two rings of 6 carbon atoms.

Grapes are an excellent source of different polyphenols (~2-3 mg GAE/g). Most of these compounds are concentrated in the skin (flavonols and anthocyanins) and seeds (flavanoles) of grapes berries (Gómez-Míguez et al., 2006). In addition to their health benefits, grape polyphenols play a key role in the sensory quality of wines, especially in reds, due to their impact on color and astringency (Belancic and Agosin, 2007; Fulcrand et al., 2004; Gawel, 1998). Polyphenols are a highly heterogeneous and complex family

of compounds (monomers, oligomers and polymers) that present several interactions with other organic compounds. These interactions include hydrogen bonds, esterification, glycosylation and hydrophobic interactions (Garrido and Borges, 2013). All of them explain their biological activities in the human body and their sensorial effects in foods.

Several grape varieties have been identified to produce high quality wines with a specific polyphenol profile. In this sense, *Carménère* is the emblematic grape of Chile; its long maturation period (~170 days) after flowering favors the accumulation of polyphenols in the skin and reduces tannin levels in the seed of the grape berry (Fredes et al., 2010; Jones and Davis, 2000; Belancic and Contreras, 2003; Pszczólkowski, 2015).

Between 1997 and 2015, the surface planted with *Carménère* grape in Chile has increased from 330 to 10,732 ha (Pszczólkowski, 2015). The production of red wine from this variety has reached 70 million L/year (~9% of the Chilean wine production) (SAG, 2016). Consequently, it is estimated that ~30,000 TM of grape pomace of Carménère is generated each year in Chile (SAG, 2016). Wines derived from this variety have high concentrations of flavanols, anthocyanins and flavonols such as quercetin and myricetin, malvidin and epigallocatechin, respectively (Obreque-Slier et al., 2012; Obreque-Slier et al., 2010). These polyphenols are related to the distinctive characteristics of the *Carménère* wine (Fulcrand et al., 2004; Gawel, 1998). This paper compiles information about the most abundant polyphenolic compounds identified to date in the *Carménère* grape. in addition, some technological, sensory and bioactive properties associated with them are discussed.

2.2. Polyphenols of *Carménère*

In *Carménère* grapes, the molecular weight of polyphenols varies according to their degree of polymerization (Moreno and Peinado, 2012). For example, the tannins or flavanols that confer the bitterness and astringency to wine have molecular weights between 500 and 3,000 Da (Gawel, 1998; Khanbabaee and Ree, 2001; Obreque-Slier et al., 2011; Moreno and Peinado, 2012). Similarly, anthocyanins with molecular weights

higher than 500 Da have been reported (Martinez, 2010). Polyphenols are distributed on the *Carménère* grape's skin and seed, as can be observed in Table 2.1. Anthocyanins are the major components in the skin (~ 42%), while flavanols are major compounds in the seeds (~ 52%).

Table 2.1. Distribution of polyphenols in *Carménère* grapes.

Location	Compounds
Skin	Anthocyanins
	Flavonols
	Flavanols
	Phenolic acids
Seed	Flavanols
	Phenolic acids

Distribution of polyphenols in skin and seeds of *Carménère* grapes (Fernández, Kennedy and Agosin, 2007; Mattivi, Vrhovsek, Masuero and Trainotti, 2009; Obreque-Slier et al., 2012, 2010).

The average content of total polyphenols in the skin and seeds of the *Carménère* grape is 1.1 ± 0.2 and 16.6 ± 2.8 mg GAE/g respectively (Obreque-Slier et al., 2012, 2010). In addition, wines from this variety contain between 2.23 and 2.86 g GAE/L (Zúñiga et al., 2014). The content of total polyphenols, anthocyanins and tannins distinguish *Carménère* from other commercial varieties like *Cabernet Sauvignon* (Table 2.2) (Price et al., 1995; Spayd et al., 2002). However, it is important to note that the wine polyphenolic profile depends also on the agricultural and oenological practices, as well as on the intrinsic characteristics of the environment (Price et al., 1995; Spayd et al., 2002). Therefore, it is difficult to compare the polyphenolic content of a variety with respect to

another. Here we consider the relative concentration of the various compounds (polyphenol profile) to highlight the benefits of a particular variety.

Given the complexity of grape polyphenols, previous studies with *Carménère* have focused on its monomers to differentiate and relate its polyphenolic profile to the particular characteristics of *Carménère* wine (Obreque-Slier et al., 2012, 2010; Zúñiga et al., 2014). Although this variety has wide polyphenolic diversity between flavonols, anthocyanins and flavanols, this review will focus only on the compounds that differentiate Carménère from other grape varieties

Table 2.2. Polyphenols content comparing *Carménère* (CM) with *Cabernet Sauvignon* (CS).

Description	**Skin**		**Seed**	
	CM	**CS**	**CM**	**CS**
Total polyphenols (mg GAE/g)	1.1±0.2	0.8 ±0.3	16.6±2.8	17.5±4.3
Total tannins (mg GAE/g)	2.8±0.4	3.0±0.1	32.9±3.9	36.9±9.1
Total Anthocyanins (mg ME/g)	0.9±0.2	0.5±0.1	- -	--

GAE: gallic acid equivalent, ME: malvidin-3-*O*-glucoside equivalent, adapted from Mattivi et al. (2009); Obreque-Slier et al. (2012); Obreque-Slier et al. (2010).

2.2.1 Flavonols

These compounds (Figure 2.1 a-f) are located in the *Carménère* grape skin and accumulate during the ripening period (Obreque-Slier et al., 2010; Pszczólkowski, 2004). The most common flavonols conjugates in grapes are the 3-*O*-glycosides as glucoside, galactoside and rutinoside (Castillo-Muñoz, Gómez-Alonso, García-Romero and

Hermosín-Gutiérrez, 2007; Jeffery, Parker and Smith, 2008). In wine, flavonols are in the form of aglycones or conjugates with free anthocyanins (Vergara et al., 2011).

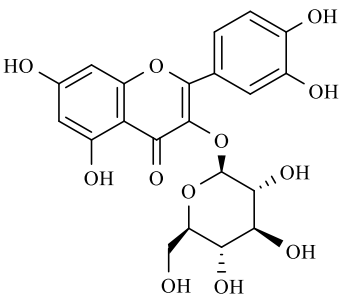

Figure 2.1a. Quercetin-3-O-glucoside

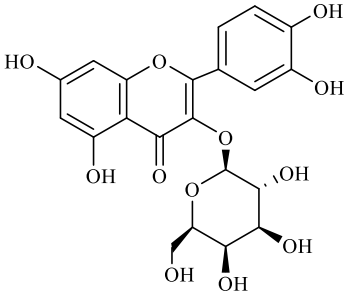

Figure 2.1b. Quercetin-3-O-galactoside

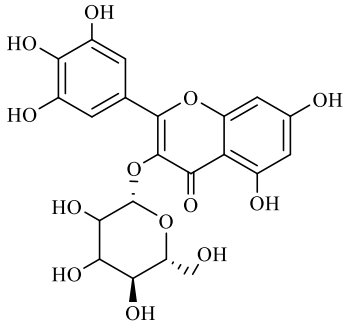

Figure 2.1c. Myricetin-3-O-glucoside

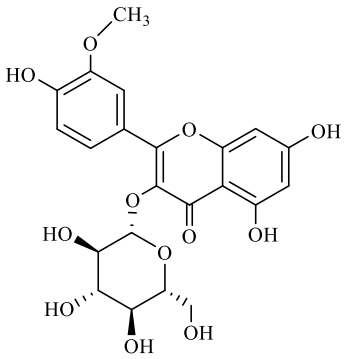

Figure 2.1d. Isorhamnetin-3-O-glucoside

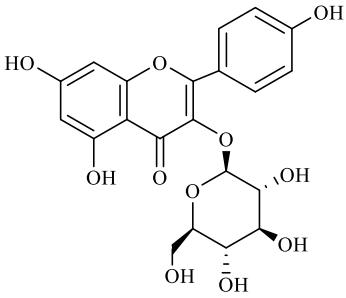

Figure 2.1e. Kaempferol-3-O-glucoside

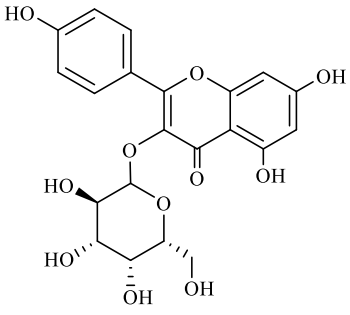

Figure 2.1f. Kaempferol-3-O-galactoside

Figures 2.1(a-f). Chemical structures of flavonols

Figure 2.2 shows the concentrations of flavonols which are found in *Carménère* grape's skin such as quercetin-3-*O*-glucoside, myricetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-galactoside (Ciudad and Valenzuela, 2002; Liang et al., 2014). These polyphenols are the most important antioxidants at a cellular level, especially quercetin (Wolfe and Liu, 2008).

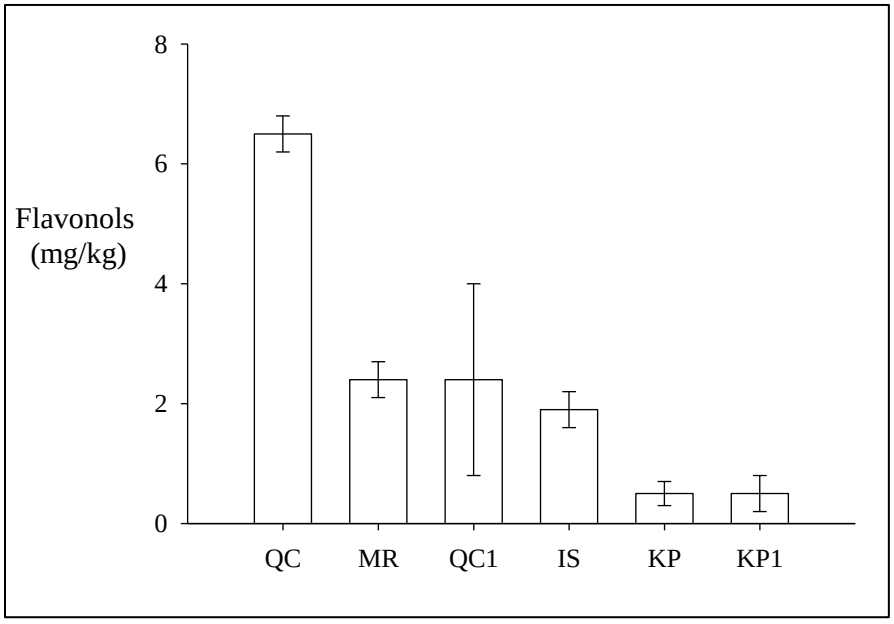

Figure 2.2. Flavonols content in *Carménère* grape skin. QC: quercetin-3-*O*-glucoside, MR: myricetin-3-*O*-glucoside, QC1: quercetin-3-*O*-galactoside, IS: isoramnethin-3-*O*-glucoside, KP: kaempferol-3-*O*-galactoside, KP1: kaempferol-3-*O*-glucoside (Ciudad and Valenzuela, 2002; Liang et al., 2014; Obreque-Slier et al., 2010)

The *Carménère* grape's skin has high concentrations of quercetin-3-*O*-glucoside (6.5 ± 1.6 mg/kg) and myricetin-3-*O*-glucoside (2.4 ± 0.3 mg/kg) compared with other varieties of grapes such as *Merlot* and *Cabernet Sauvignon* (Figure 2.3).

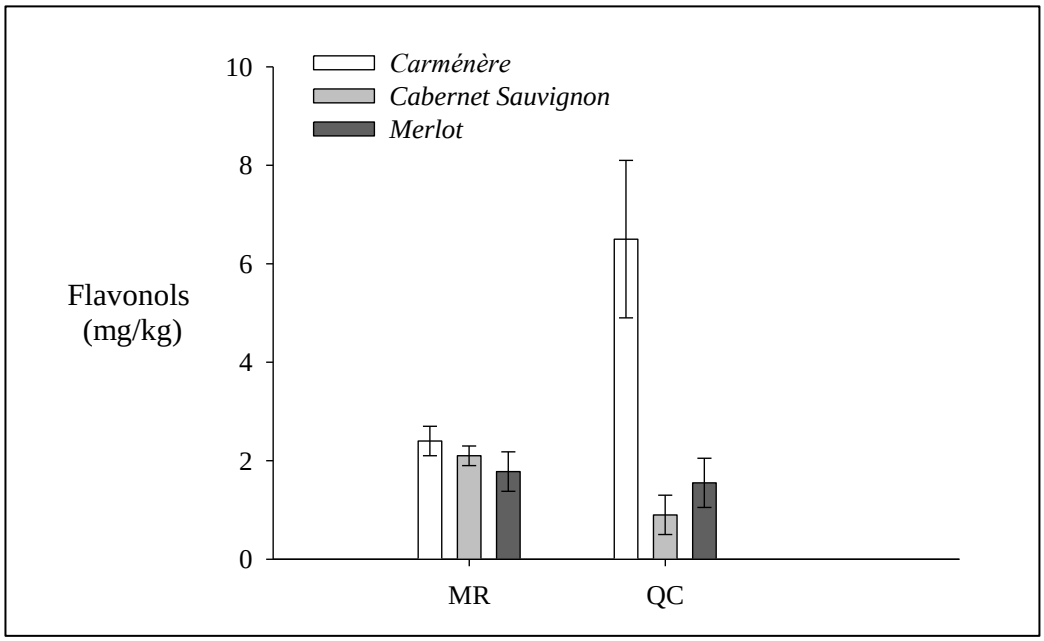

Figure 2.3. Flavonols content in grape skins of *Carménère, Cabernet Sauvignon* and *Merlot.* QC: quercetin-3-*O*-glucoside, MR: myrecetin-3-*O*-glucoside (Ciudad & Valenzuela, 2002; Liang et al., 2014; Obreque-Slier et al., 2010).

Red *Carménère* wines have high concentrations of quercetin and myricetin (Fanzone et al., 2015; Kobori, 2013). A recent study found that in red wines the ratio of total quercetin and total myricetin in *Carménère* is higher than in *Cabernet Sauvignon* (Vergara et al., 2011). The concentration and type of flavonols present in grapes are important factors to improve and stabilize the color in red wines, due to their ability to interact with anthocyanins through copigmentation reactions (hydrophobic interactions) (Carola Vergara, Von Baer, Mardones, and Gutiérrez, 2011). The characteristic of the color in red wines can be assessed through the CIELAB analysis. This method expresses color in terms of brightness, hue, and saturation, where L represents the brightness, and a and b are chromatic coordinates (Pérez-Magariño and González, 2003; Zhang and Wandell, 1997). For example, the copigmentation in red wine as a result of the interaction between malvidin-3-*O*-glycoside and quercetin-3-*O*-glucoside, reduces brightness by

25%, with chromatic parameters for a and b of -3.43 and +7.64 respectively; this generates an intense bluish red in the wine (Fanzone et al., 2015).

2.2.2 Anthocyanins

Anthocyanins are found in the skin of *Carménère* grapes. They consist of anthocyanidins (aglycones) glycosylated with different sugars (glucose, galactose, arabinose and xylose) and esterified by different acids (acetic, coumaric and caffeic) (Gómez-Míguez et al., 2006; Liang et al., 2014). Malvidin, cyanidin, petunidin, delphinidin and peonidin are the most abundant anthocyanidins (Figure 2.4 a-n) found in *Carménère* anthocyanins (Castillo-Muñoz et al., 2007; Jeffery et al., 2008; Pszczólkowski, 2015). So far 18 anthocyanins have been identified in *Carménère* grape skin, including monoglucoside anthocyanins, acetyl monoglucosides, coumaryl monoglucosides, caffeoyl monoglucosides and feruloyl monoglucosides (Figure 2.5).

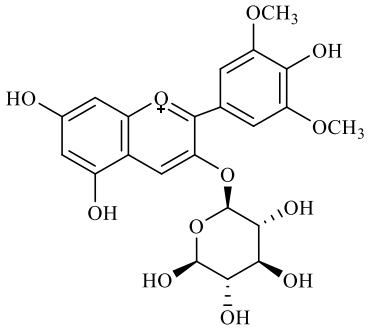

Figure 2.4a. Malvidin-3-O-glucoside .

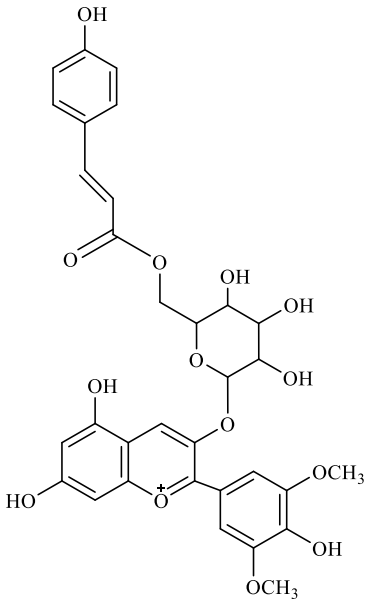

Figure 2.4b. Malvidin-3-O-(6-coumaroyl)glucoside

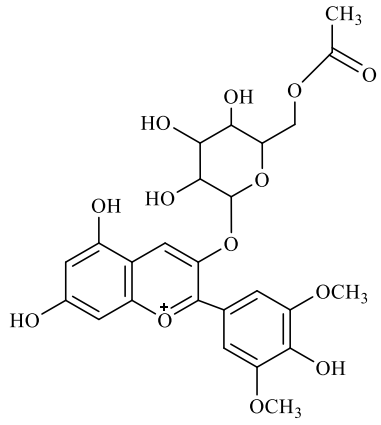

Figure 2.4c. Malvidin-3-O-(6-O-acetyl)glucoside

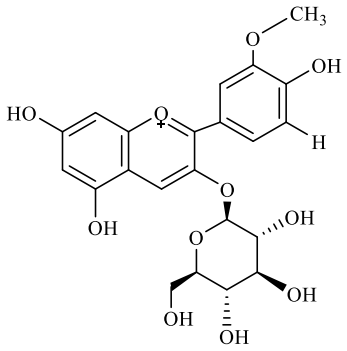

Figure 2.4d. Peonidin-3-O-glucoside

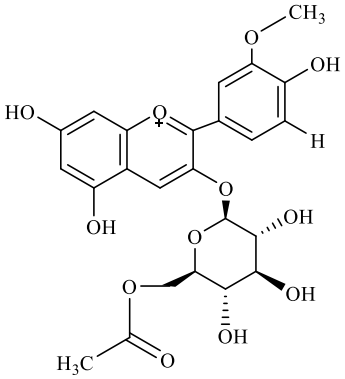

Figure 2.4e. Peonidin-3-O-(6-O-acetyl)glucoside

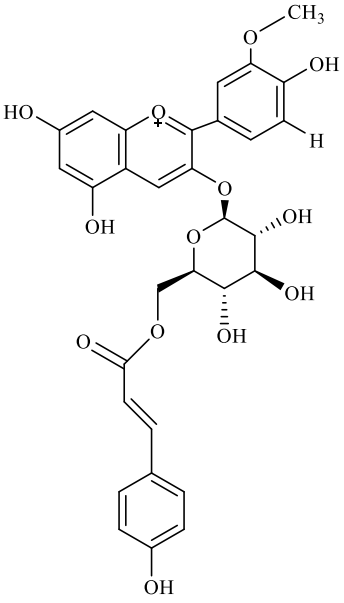

Figure 2.4f. Peonidin-3-O-(6-O-coumaroyl)glucoside

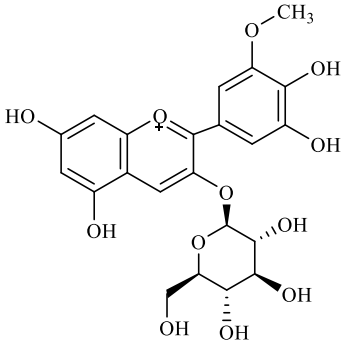

Figure 2.4g. Petunidin-3-glucoside

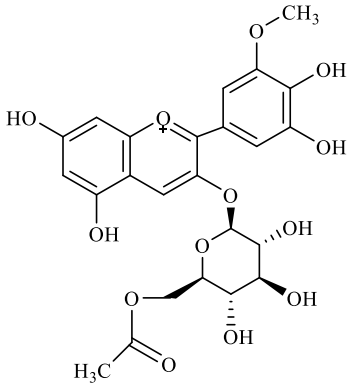

Figure 2.4h. Petunidin-3-(6-O-acetyl)glucoside

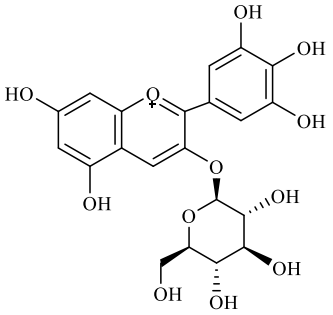

Figure 2.4i. Delphinidin-3-O-glucoside

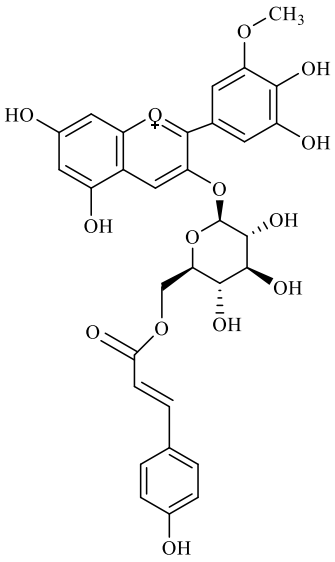

Figure 2.4j. Petunidin-3-(6-O-coumaroyl)glucoside

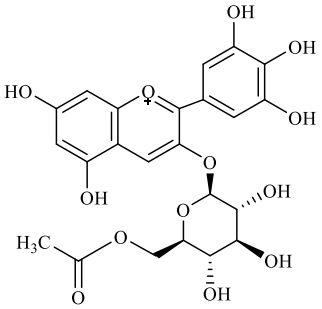

Figure 2.4k. Delphinidin-3-O-(6-O-acetyl)glucoside

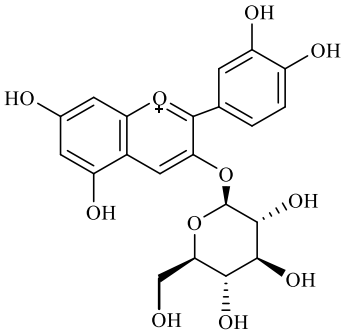

Figure 2.4l. Cyanidin-3-O-glucoside

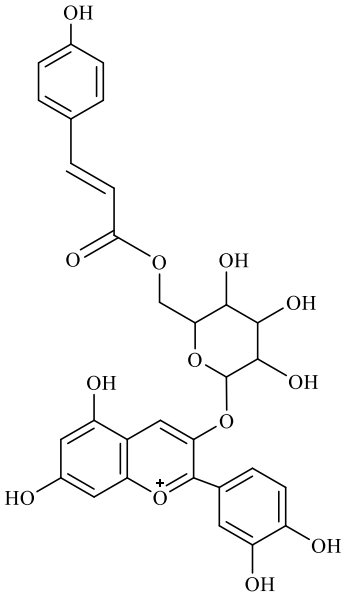

Figure 2.4m. Cyanidin-3-O-(6-O-coumaroyl)glucoside

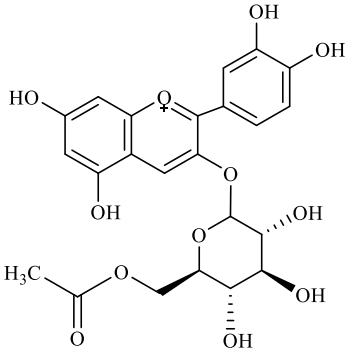

Figure 2.4n. Cyanidin-3-O-(6-O-acetyl)glucoside

Figure 2.4a-n. Chemical structures of anthocyanins

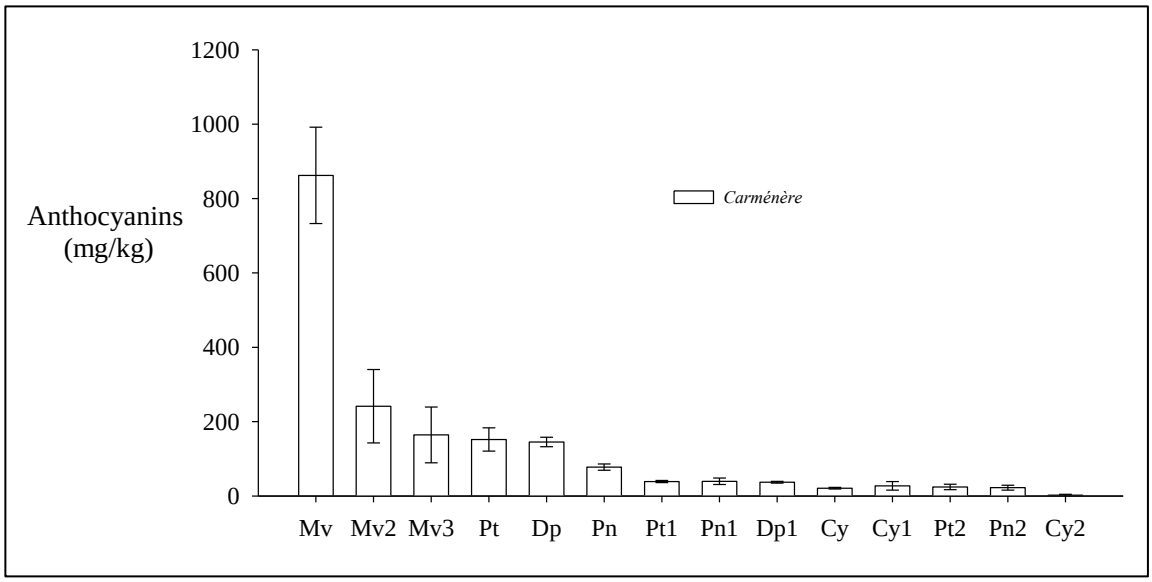

Figure 2.5. Anthocyanins content in *Carménère* grape skin. Mv: malvidin-3-*O*-glucoside, Mv2: malvidin-3-*O*-(6-*O*-acetyl)glucoside, Pt: petunidin-3-*O*-glucoside, Dp: delphinidin-3-*O*-glucoside, Pn: peonidin-3-*O*-glucoside, Mv3: malvidin-3-*O*-(6-*O*-coumaroyl)glucoside, Pt1: petunidin-3-*O*-(6-*O*-acetyl)glucoside, Pn1: peonidin-3-*O*-(6-*O*-acetyl)glucoside, Cy: cyanidin-3-*O*-glucoside, Dp1: delphinidin-3-*O*-(6-*O*-acetyl)glucoside, Cy1: cyanidin-3-*O*-(6-*O*-coumaroyl)glucoside, Cy2: cyanidin-3-*O*-(6-*O*-acetyl)glucoside, Pn2: peonidin-3-*O*-(6-*O*-coumaroyl)glucoside, Pt2: petunidin-3-*O*-(6-*O*-coumaroyl)glucoside (Liang et al., 2014; Obreque-Slier et al., 2010; Pinto, 2008).

Anthocyanins are the pigments responsible for the color in red wines (Garrido and Borges, 2013). The color of these compounds depends on the number of hydroxyl and methoxy groups in their chemical structure. For example, higher hydroxylation degrees produce displacements towards blue hues while higher methoxylation degrees produce red colorations (He et al., 2010). Therefore, the color in red wines is related to six anthocyanidins such as cyanidin (red-orange), peonidin (red), delphinidin (bluish red), pelargonidin (orange) petunidin and malvidin (bluish red) (He et al., 2010; Koponen, Happonen, Mattila and Törrönen, 2007).

The *Carménère* grape, unlike other commercial varieties such as *Cabernet Sauvignon* and *Merlot*, has high concentrations of malvidin-3-*O*-glucoside (862 mg/kg) (Figure 2.6). This compound is the major anthocyanin in grapes and wines. Hence, several studies assessed the co-pigmentation in red wines through interaction between malvidin-3-O-glucoside and other polyphenols (flavanols and flavonols) (Fanzone et al., 2015; Lambert et al., 2011; Zhang et al., 2016). This type of interaction enhances between 30 and 50% the color intensity in aged red wines that can be quantified by the copigmentation constant (K_1). This constant measures the absorbance (nm) as a result of the association between two compounds (Lambert et al., 2011). Several studies have found that the interaction between malvidin and quercetin has high levels of copigmentation (K_1 = 2900 ± 1300 L/mol) compared to other polyphenols such as catechin (K_1 = 90 ± 20 L/mol) and procyanidins (K_1 = 330 ± 30 L/mol) (Boulton, 2001; Fanzone et al., 2015; Lambert et al., 2011).

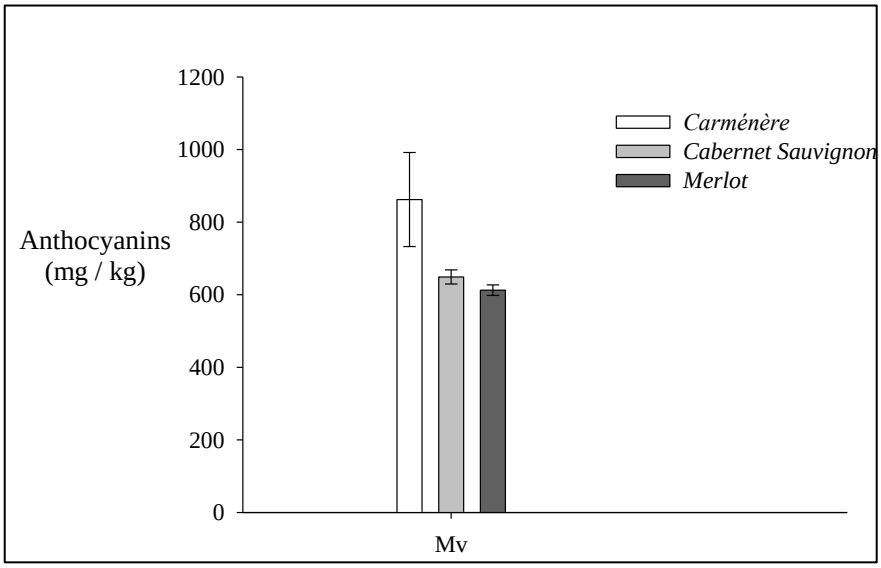

Figure 2.6. Mv: Malvidin-3-*O*-glucoside content in grape skins of *Carménère*, *Cabernet Sauvignon* and *Merlot* (Liang et al., 2014; Obreque-Slier et al., 2013; Pinto, 2008).

2.2.3 Flavanols

Flavanols (Figure 2.7a-j) are present in the *Carménère* grape's skin and seeds, as monomers and condensed tannins (proanthocyanidins) in the form of simple dimers, or complex molecules (oligomers and polymers) (Obreque-Slier et al., 2012, 2010). The major proportion of these compounds is found in the seed (~75%) (Mattivi et al., 2009).

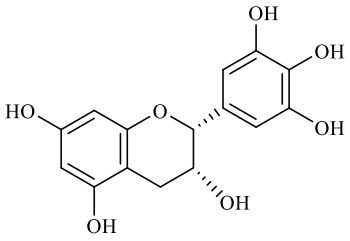

Figure 2.7a. (-)-Epigallocatechin

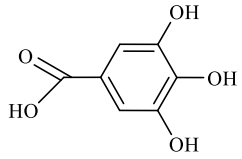

Figure 2.7b. Gallic acid

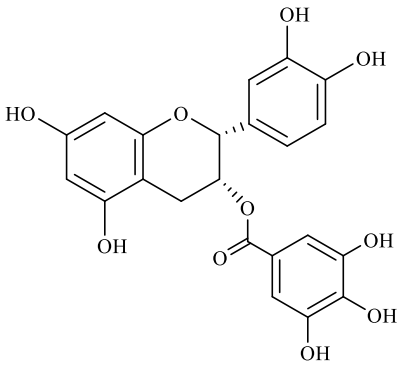

Figure 2.7c. (-)-Epicatechin-3-O-gallate

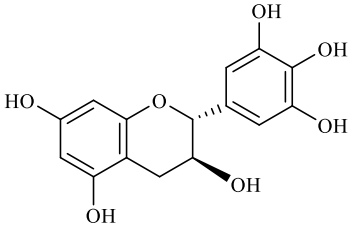

Figure 2.7d. (+)-Gallocatechin

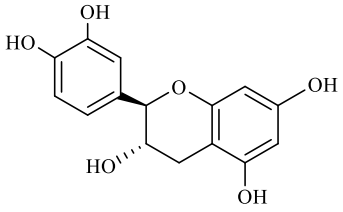

Figure 2.7e. (+)-Catechin

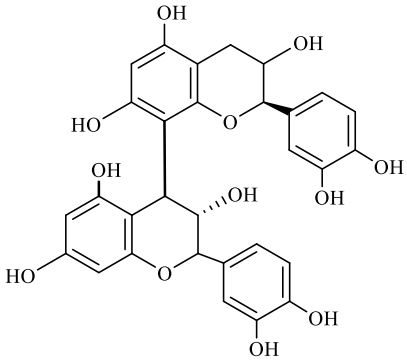

Figure 2.7f. Catechin-(4,8)-catechin

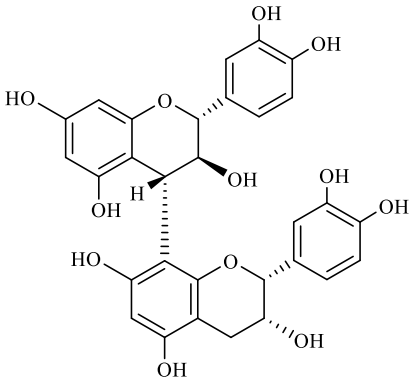

Figure 2.7g. Catechin-(4,8)-epicatechin

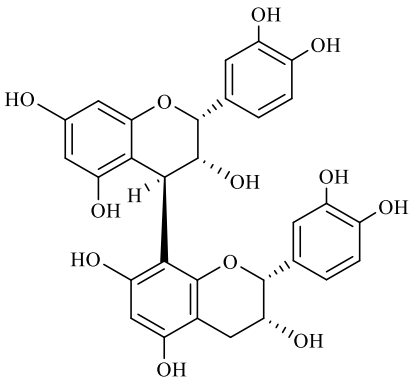

Figure 2.7h. Epicatechin-(4,8)-epicatechin

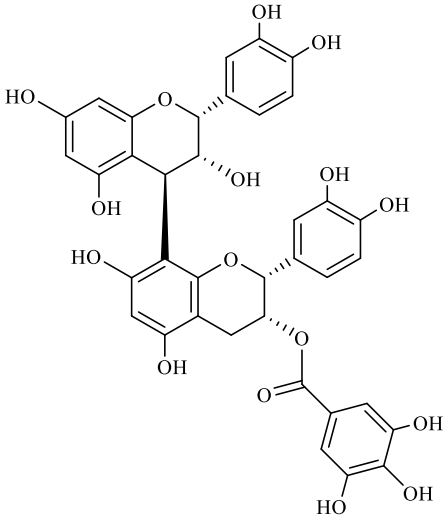

Figure 2.7i. Epicatechin-(4,8)-epicatechin-3-O-gallate

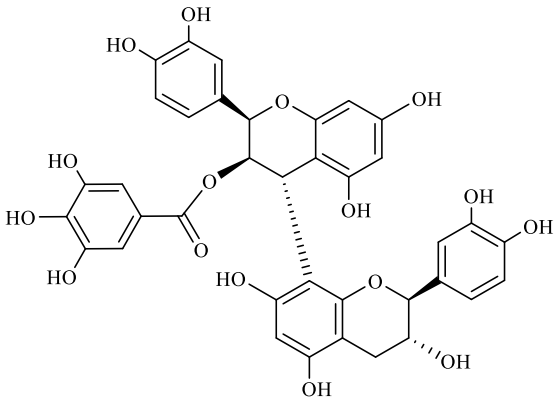

Figure 2.7j. Epicatechin-3-O-gallate-(4,8)-catechin

Figure 2.7 (a-j). Chemical structure of flavanols

The most abundant monomers in the *Carménère* grape's seed are (+)-catechin and (-)-epicatechin; whose concentrations are shown in Figure 8. Several studies have reported the presence of procyanidins in the form of dimers and trimers in the seed such as epicatechin-(4β-8)-epicatechin, catechin-(4α-8)-catechin, catechin-(4α-8)-epicatechin-3-O-gallate, among others (Figure 2.8) (Mattivi et al., 2009; Obreque-Slier et al., 2012, 2010). These compounds are generally dimers and trimers of catechin and epicatechin

(Vidal et al., 2003). The flavanols in *Carménère*'s seed are galloylated with gallic acid (~13.8%) (Fernández et al., 2007; Vidal et al., 2003). This particular interaction between flavanol and gallic acid is responsible for the astringency and sensory perception in red wines (Del Rio & Kennedy, 2006; Fernández et al., 2007). It has been shown that these galloylated flavanols interact with the proline reach proteins of human saliva, forming aggregates that contribute to wine astringency and bitterness (Lesschaeve and Noble, 2005; Obreque-Slier, Peña-Neira and López-Solís, 2011; Vidal et al., 2003).

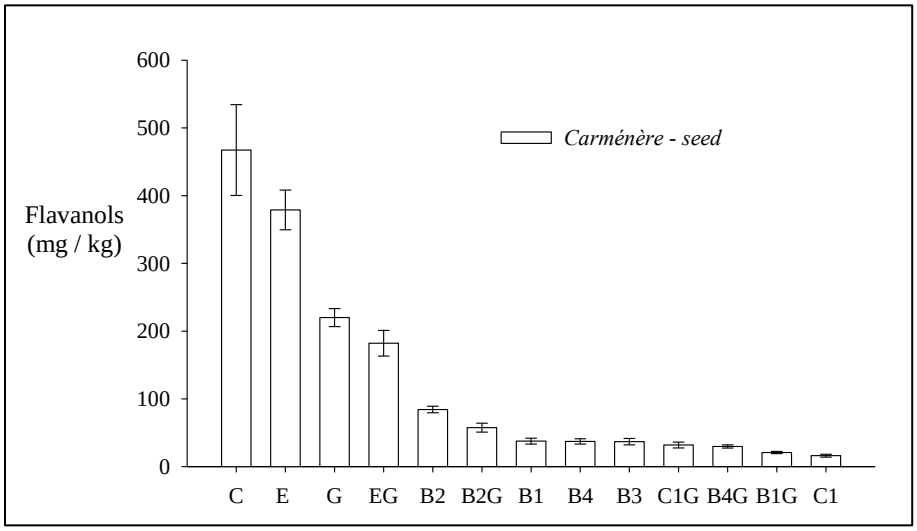

Figure 2.8. Flavanols content in *Carménère* grape seed; C: (+)-catechin, EC: (-)-epicatechin, G: gallic acid, B4: catechin-(4α-8)-epicatechin, B2: epicatechin-(4β-8)-epicatechin B3: catechin-(4α-8)-catechin, B1: epicatechin-(4β-8)-catechin, B4G: catechin-(4α-8)-epicatechin-3-*O*-gallate, B1G: epicatechin-3-*O*-gallate-(4β-8)-catechin, B2G: epicatechin-(4β-8)-epicatechin-3-*O*-gallate, C1: epicatechin-(4β-8)-epicatechin-(4β-8)-catechin, C1G: epicatechin-(4β-8)-epicatechin-(4β-8)-catechin-3- *O*-gallate (Mattivi et al., 2009; Obreque-Slier et al., 2012, 2010).

Skin flavanols include (-)-epigallocatechin, (+)-gallocathechin and (+)-catechin, as well as procyanidins with high degree of polymerization (Figure 2.9). These procyanidins are generally formed by catechin and epigallocatechin monomers (Souquet,

Cheynier, Brossaud and Moutounet, 1996). Unlike seed flavanols, skin flavanols have a low percentage of galloylation (~1.9%) (Fernández et al., 2007; Souquet et al., 1996).

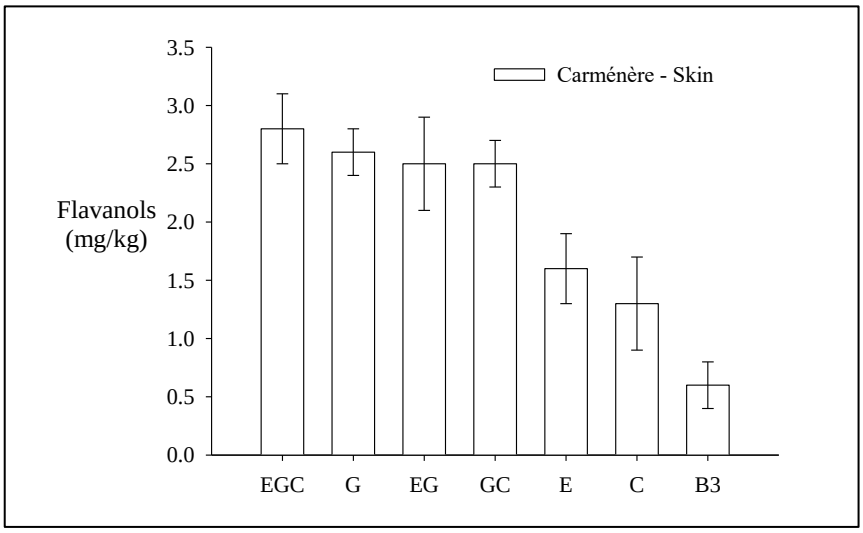

Figure 2.9. Flavanols content in *Carménère* grape skin; EGC: (-)-epigallocatechin, G: gallic acid, EG: (-)-epicatechin-3-O-gallate, GC: (+)-gallocathechin, C: (+)-catechin, B3: catechin-(4α-8)-catechin (Mattivi et al., 2009; Obreque-Slier et al., 2010).

Compared to other commercial varieties such as *Merlot* and *Cabernet Sauvignon*, the *Carménère* grape's skin has high concentrations of epigallocatechin rich procyanidins (Figure 2.10). Hence, the ratio of procyanidins between seeds and skin is around 2, while in other commercial varieties such as *Merlot* and *Cabernet Sauvignon*, these ratios vary between 3 and 10 (Fernández et al., 2007). This particular characteristic distinguishes the sensory properties of *Carménère* wines from other varieties. A recent study found, through a scale of 0 to 10, that the level of astringency in *Carménère* wines is less than that of *Cabernet Sauvignon*, with average scores of 5.3 and 6.0 respectively ($p < 0.03$, LSD 5%) (Fernández et al., 2007). Red wines with high concentrations of tannins rich in epigallocatechin present low astringency, since these tannins do not form aggregates with the proteins of the human saliva (Bandyopadhyay, Ghosh and Ghosh, 2012; Gibbins and Carpenter, 2013; Vidal et al., 2003).

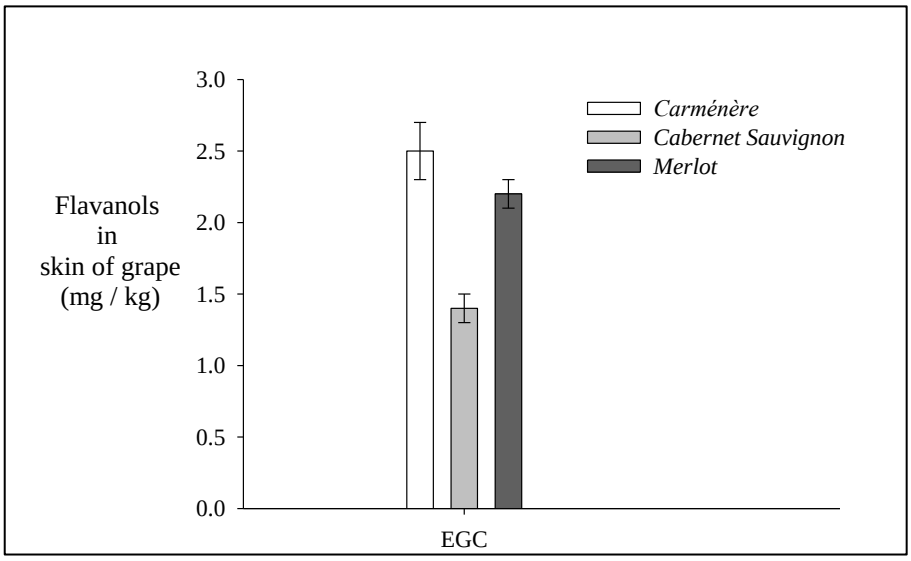

Figure 2.10. EGC: Epigallocatechin content in grape *Carménère*, *Cabernet Sauvignon* and *Merlot* (Mattivi et al., 2009; Obreque-Slier et al., 2013, 2010).

2.3. Bioactive polyphenols found in *Carménère*

The chemical structure of polyphenols would determine some biological properties such as antioxidant activity and specific interactions with cell receptors that can be identified as the mechanisms responsible for their potential health benefits (Table 2.3).

Several *in vivo* and *in vitro* studies have shown that most abundant *Carménère* polyphenols (e.g. malvidin, quercetin, myricetin and epigallocatechin) possess important biological activities (Table 2.3). For example, *in vitro* studies performed in rat hearts have shown that malvidin, the major *Carménère* polyphenol, reduces mammalian myocardial contractility and induced coronary vasodilation (Quintieri et al., 2013). These results evidence the potential health benefits of malvidin, as a human cardioprotective agent like resveratrol and cyanidin. Similarly, epigallocatechin and myricetin have been associated not only with the promotion of bone regeneration but also with the inhibition of cancer at *in vitro* and *in vivo* assays (Huang et al., 2016; Ko et al., 2011).

Table 2.3. Potential health benefits of *Carménère* polyphenols. Main biological effects *in vitro* and *in vivo*

Compounds	Potential health benefits	Biological activity
Malvidin	Cardio-protective effects:	Modulates myocardial and coronary performance. Regulates the activity of nitrous oxide synthetase enzymes. Positive effect against lipid oxidation.
	Anti-inflammatory effects:	Inhibits macrophage activation in the blood. Attenuates the TNF-α-induced inflammatory responses in endothelial cells.
	Anti-carcinogenic effects:	Mediate the cytotoxicity through the arrest of the G2/M phase of the cell cycle and by induction of apoptosis. Inhibited the growth of HL60 human leukemia cells through the induction of apoptosis.
Myricetin	Anti-inflammatory effects:	Inhibits the production of pro-inflammatory mediators through the suppression of NF-κB in LPS-stimulated RAW264.7 macrophages.
	Anti-carcinogenic effects:	Exerts potent anti-proliferative and pro-apoptotic effects on K562 human leukemia cells.
Quercetin	Anti-allergenic effects:	Inhibits the release of histamine, IgE-mediated.
	Anti-inflammatory effects:	Antioxidant and protective effect against gastric ulceration. Ability to suppress NO production in LPS-stimulated macrophage RAW 264.7 cells. Interact synergistically with resveratrol contributing to counteract inflammation of the skin and resulting in tissue repair and wound healing.
	Anti-carcinogenic effects:	Inhibits cell growth and induction of apoptosis in H460, A549 and H1299 lung cancer cells. Inhibits the growth of HCT116 cancer cells which caused apoptosis in the cells. Interact synergistically with resveratrol and ellagic acid in the induction of apoptosis and reduction of cell growth in human leukemia cells (MOLT-4).
Epigallocatechin	Anti-carcinogenic effects:	Inhibits strongly the growth of breast cancer cell lines (MCF-7 and MDA-MB-231) due to an induction of apoptosis.
	Bone effects:	Effective in promoting osteogenic differentiation in bone formation.

Adapted from Quintieri et al. (2013); Van Acker et al. (1996); Decendit et al. (2013); Huang, Wang, and Li (2013); Huang et al. (2004); Hyun and Chung (2004); Katsube et al. (2003); Cho et al. (2016); Lee and Lee (2016); Pan et al. (2016); Kimata et al. (2000); Choi et al. (2012); Youn et al. (2013); Kuo, Liu, and Chao (2004); Mertens-Talcott and Percival (2005); Vergote, Toillon, Hondermarck, and Bourhis, 2002); Ko et al. (2011); Ko, Lau, Choy, and Leung (2009).

The biological activity responsible for these benefits seems to be specific for each polyphenol, as can be observed in Table 2.3. Additionally, all these compounds exhibit anti-inflammatory effects both for *in vitro* and *in vivo* studies. Therefore, the extraction of these polyphenols from *Carménère* pomace could be considered a good option to develop nutraceutical and functional food products.

2.4. *Carménère* grape pomace as a source of valuable polyphenol extracts

Carménère grape is produced especially for the elaboration of red wine; the remnants of this process, such as skin, stems and seed, are known as grape pomace. This agroindustrial residue contains between 60 and 65% of the grape polyphenols after red wine production (Balík et al., 2008). The concentration of phenolic compounds in the grape pomace depends on the wine processing details (maceration, fermentation, clarification and aging) (Balík et al., 2008; Kennedy, 2008). *Carménère* grape's pomace has high concentrations of anthocyanins, flavanols and flavonols (Fig. 2.11) (de la Cerda-Carrasco et al., 2014). Consequently, this biomass is a reach source to produce bioactive extracts.

The demand for polyphenols has grown significantly in recent years (Goldberg, 1994). The price of these compounds varies with the degree of purity. For example, the price of epigallocatechin, quercetin and malvidin with 95% purity is $ 30,000, $ 30 and $ 7 per gram respectively (SIGMA, 2016). In addition, the prices of concentrated extracts of polyphenols with concentrations between 20 and 40%, range between $ 50 and $ 75 per kilogram (Alibaba, 2016).

Extracting these polyphenols from an agroindustrial biomass such as grape pomace can be performed through conventional techniques using organic solvents (solid-liquid extraction). These techniques however, are characterized by long processing times and the consumption of high amounts of solvents. Moreover, solvent recovery, extract safety and environmental impact are growing concerns. Furthermore, this type of

extraction causes oxidation and hydrolysis of polyphenols (Fontana, Antoniolli, & Bottini, 2013; Wang & Weller, 2006).

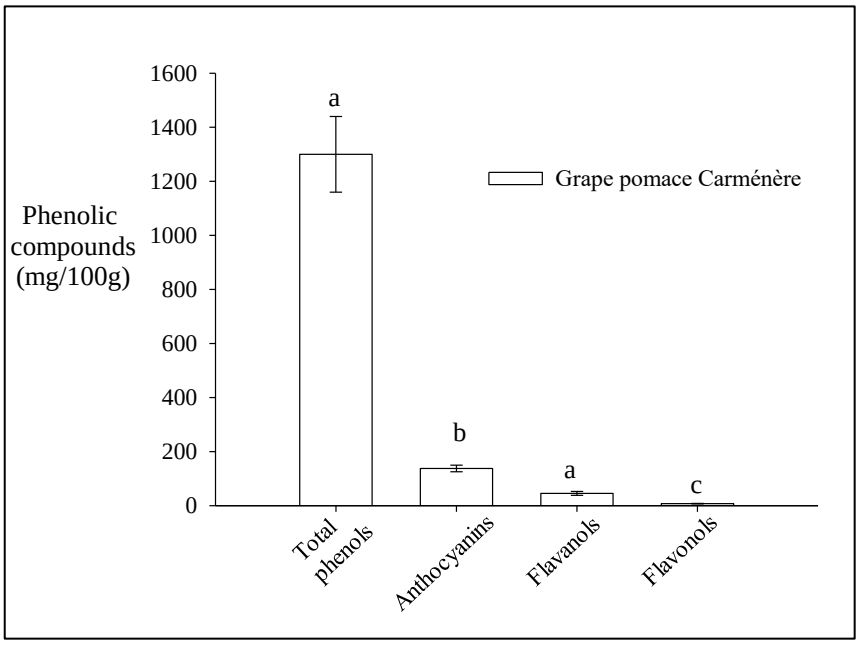

Figure 2.11. Content of phenolic compounds present in *Carménère* grape pomace. a: expressed as gallic acid equivalent, b: expressed as malvidin equivalent, c: expressed as quercetin equivalent (de la Cerda-Carrasco et al., 2014).

Hot pressurized liquid extraction (HPLE) is an alternative technique that overcomes many of the limitations of conventional extraction. In this method, the liquid solvent (normally pure water or hydroalcoholic mixtures) is subjected to pressures between 10 and 15 MPa and temperatures between 50 and 200°C (Vergara et al., 2013). Under these conditions, the solvent remains in the liquid state, enhancing the extraction of polyphenolic compounds (Fontana et al., 2013; Vergara-Salinas et al., 2013).

There are several agroindustrial natural sources for producing polyphenol extracts (Figure 2.12), although their polyphenol content varies depending on the type of processing to which the biomass has been subjected. Hence, the recovery of polyphenols from *Carménère* grape pomace is an economically attractive option considering the high

price of polyphenol extracts, the high concentration of polyphenols in the grape pomace after wine fermentation and the application of alternative extraction technologies.

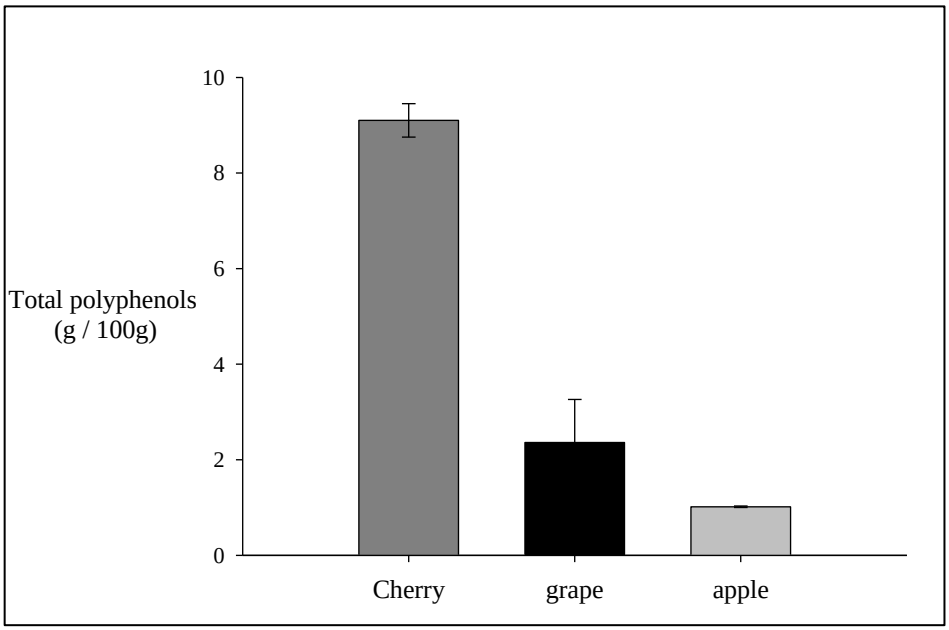

Figure 2.12. Total polyphenol content in agroindustrial biomass. (Chamorro et al., 2012; Kolodziejczyk et al., 2013; Sudha et al., 2007)

2.5. Conclusion

Carménère is the emblematic grape of Chile; it presents a particular polyphenolic profile with significant concentrations of malvidin, quercetin, myricetin and epigallocatechin. These compounds are responsible for the characteristics that distinguish its wines from other commercial wines. The polyphenols identified in *Carménère* have bioactive properties in the treatment and prevention of diseases associated with oxidative stress. Given the particularity of the *Carménère* polyphenols' profile, research regarding extraction, concentration and purification of their polyphenols should be encouraged. Obtained extracts could be used to produce functional foods, nutraceutics and enological additives to moderate wine astringency and stabilize color in aged wines.

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3. IMPACT OF AN INTEGRATED PROCESS OF HOT PRESSURIZED LIQUID EXTRACTION-MACROPOROUS RESIN PURIFICATION OVER THE POLYPHENOLS, HYDROXYMETHYLFURFURAL AND REDUCING SUGARS CONTENT OF *CARMÉNÈRE* POMACE EXTRACTS.

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3.1. Introduction

The Chilean wine industry generates large amounts of *Carménère* pomace (~30,000 TM/year) (ODEPA 2016; Pszczólkowski 2015) a winery residue which presents high phenolic contents (18 – 58 mg GAE/g) (Kabir, Sultana and Kurnianta, 2015; Lavelli et al., 2016).

Carménère pomace is an excellent source of malvidin, quercetin and epigallocatechin which have exhibited a positive influence over distinctive red wine attributes such as color and astringency (Huamán-Castilla, Mariotti-Celis and Pérez-Correa, 2017). Additionally, it contains proanthocyanidins that have been associated with the prevention of various oxidative stress-related diseases like cancer, and several cardiovascular and neurodegenerative diseases (Pedan et al., 2017).

Usually, the extraction of polyphenols from plant material is carried out using organic solvents, with the subsequent environmental and human risks. In this scenario, it is desirable to use "clean green technologies" for polyphenols extraction in order to maximize the safety of the functional ingredient and minimize the environmental impact (Kumar et al., 2017).

Hot pressurized liquid extraction (HPLE) is a liquid-solid extraction process carried out at elevated temperatures (50-200 °C) and pressures (3 - 20 MPa) during short time periods (5 to 10 min) which overcomes many of the limitations of conventional extraction. It is relatively inexpensive, minimizes or totally avoids the use of objectionable

solvents, and provides a facile means for supplying concentrated polyphenols (Janghel et al., 2015).

Previous studies have evaluated the use of ethanol solutions (80%) combined with high temperatures (120-200 °C) in HPLE for the recovery of polyphenols from grape pomace. However, the high temperatures applied during extraction produce thermal deterioration of polyphenols and enhance the extraction of other undesirable compounds (e.g. reducing sugars) which can affect the stability of extracts when used as food ingredients. Additionally, high extraction temperatures favor the formation of potential human carcinogens (e.g. hydroxymethylfurfural) (Plaza and Turner, 2015).

Consequently, a further purification step which improves the purity of the extracts should be applied. The method of adsorption/desorption in columns packaged with synthetic macroporous resins (e.g. Diaion HP-20) is one of the most frequently used due to its simpler operation, higher efficiency, less-yielding cost, environmentally friendly, and easier regeneration (Shin and Kim, 2016). During the adsorption step, polyphenols present in the raw extract are retained in the packed column. Then, after a washing step with water, polyphenols are normally recovered using an ethanol/water solution (Yongliang et al., 2017).

However, the content of ethanol in the raw extract may affect the recovery of polyphenols because the same solvent is used to desorb these compounds from the resin Hence, a desolventizing process is necessary before purification, with the subsequent increase in the process complexity and cost (Wu et al., 2014). Taking into consideration these demands, it is highly advantageous to define optimal operating conditions of a combined process of extraction and purification.

We conducted a comprehensive study to efficiently produce purified polyphenol extracts from *Carménère* pomace, free of reducing sugars and hydroxymethylfurfural (HMF) using an integrated process of hot pressurized liquid extraction-resin purification (HPLE-RP).

3.2. Materials and methods

3.2.1 Chemicals and analytic reagents

Analytical grade reagents, standards and solvents were used in chemical analyses. Folin-Ciocalteu reagent, sodium carbonate, gallic acid, glucose, fructose, HMF standards Dimethylaminocinnamaldehyde (DMAC; F.W. 175.23), solvents (acetone, methanol, acetonitrile, formic acid, hydrochloric acid, acetic acid and ethanol), Carrez solution I, Carrez solution II and sodium hydroxide were purchased from Sigma Aldrich (Steinheim, Germany). Procyanidin A2 (HPLC; purity >99%) was from Extrasynthèse (Genay, France).

3.2.2 Grape pomace

Carménère pomace samples (Concha y Toro Vineyard, Region del Maule, Chile) were taken after the wine process had finished and they were immediately frozen (-20°C) considering a storage period no longer than 2 months. Each sample was reduced to a particle size lower than 1 mm diameter prior to extraction.

3.2.3 Liquid extraction of Carménère pomace polyphenols (HPLE)

Carménère pomace was subjected to HPLE in an Accelerated Solvent Extraction device (ASE 150, Dionex) according to the methodology described by Vergara-Salinas et al. (2013). It was carried out varying extraction temperature (60, 75 and 90°C) and ethanol content (0%, 5%, 10% and 15 %). *Carménère* pomace was also extracted by HPLE at 130, 150 and 200°C without ethanol. Additionally, maceration was performed at 30°C with an extraction solution of acetone (60%) (Naczk and Shahidi 2004). All extracts were

collected and stored in amber vials at −20°C prior resin purification (RP) and chemical analysis.

3.2.4 Macroporous resin purification of Carménère pomace extracts

Carménère pomace extracts were purified using a polystyrene column packed with ~10g of HP-20 resin (Diaion, Tokyo, Japan). For polyphenols adsorption, 150 mL of Carménère pomace extract were passed through the resin with a flow rate of 5 mL/min. Then, polyphenol desorption was carried out using different ethanol solutions as eluents (80%, 70% and 60%) at a rate of 5 mL/min. The RP polyphenol purifications were performed at 30°C. Additionally, the extracts obtained by maceration were purified at the same RP conditions but using acetone solutions (60%, 70%, and 80%) as eluents. Finally, purified extracts were stored at -20°C until chemical analysis.

3.2.5 Determination of Total Polyphenols (TPF)

TPF of raw and purified extracts were determined by Folin−Ciocalteu assay (Singleton and Rossi, 1965). Results were expressed as gram of gallic acid equivalent (GAE) per gram of dried pomace.

3.2.6 Quantification of HMF

HMF concentrations of raw and purified extracts were measured according to the methodology of Toker et al. (2013). Analyses were performed in triplicate and results were expressed in mg of HMF per gram of dried pomace.

3.2.7 Quantification of fructose and glucose

The reducing sugar contents of raw and purified extracts were quantified by HPLC-IR. Samples were mixed with MiliQ water (3:2) and centrifuged (4025 x g, 10 min, 4°C). The supernatant was filtered and of mixed with acetonitrile (3:7) to be injected in a HPLC-IR (Thermo Scientific Dionex Ultimate 3000, Massachusetts, USA) equipped with a normal phase Li ChroCART ® 250-4 Purospher ® STAR (5 µm) at 40°C. Chromatographic separation was carried out at isocratic conditions. The mobile phase, flow rate and injection volume were: acetonitrile solution (70 % v/v), 1 mL/min and 20 µL, respectively. Under these operating conditions the fructose and glucose retention time were 4.6 and 5 min, respectively. Analyses were performed in triplicate and results were expressed in mg of reducing sugar (fructose/glucose) per gram of dried pomace.

3.2.8 Proanthocyanidin analysis

The proanthocyanidins content of the best raw and purified extract was quantified according to the methodology of Prior et al. (2010). Additionally, the oligomeric distribution of proanthocyanidins was established by HILIC-FLD. 10 mL of the sample were concentrated and reconstituted with 5 mL of formic acid solution (1 % v/v) to be added in 500 mg Sep-Pak C-18 cartridges (Waters) previously washed with the same solvent. Then, the C-18 columns were washed with 1 mL of formic acid solution (1 % v/v) and recovered with 2 mL of methanol solution (70%) acidified with formic acid (5%). The sample was filtered in a PTFE filter of 0.45 µm and injected into an HILIC-FLD system (Waters Alliance 2695 HPLC, Milford, USA). Chromatographic separation was carried out using a mobile phase consisting of A (acetic acid/water; 98:2) and B (methanol/water/acetic acid; 95:3:2) in a gradient elution analysis programed as follow: 0-35 min, 0-60% A; 35-55 min, isocratic 60% A; 55-60 min, 60-20 A; with a column re-conditioning period of 30 min, at a flow rate of 0.8 mL/min. The detection wavelength

was set at 230 nm (excitation) and 321 nm (emission).

3.2.9 Statistical Analyses

Extraction-purification processes and chemical analysis were performed in triplicate with the data presented as mean and SD. Statgraphics Plus for Windows 4.0 (Statpoint Technologies, Inc., Virginia, USA) was used for statistical analyses. To study the effects of the temperature, co-solvent and eluent on purification performance as well as the interaction between these three factors, analysis of variance (ANOVA) and least significant difference tests were applied to the response variables with a significance of $p \leq 0.05$.

3.3. Results and discussion

3.3.1 Changes occurred in the chemical composition of Carménère pomace extracts during HPLE.

Carménère pomace extracts obtained by HPLE at different extraction conditions were chemically characterized. An increase in the extraction temperature (from 60 to 90°C) and the co-solvent concentration (from 0 % to 15%) improved the recovery of polyphenols from 9.57 to 23.99 mg GAE/g dried pomace (Table 3.1). The use of higher temperatures enhances the mass transfer and extraction solubility of several polyphenols; however, increased temperatures (T° > 50°C) can also degrade thermo-labile polyphenols such as anthocyanins (Monrad et al., 2010). Therefore, these disadvantages should be taken into consideration when optimizing the process (Syahariza et al., 2017). Additionally, using hydroethanolic mixtures, advantages of both solvents can be utilized. While ethanol improves the solubility of polyphenols, water easily assists the desorption of these compounds by breaking matrix and matrix–analyte bonding (Mustafa and Turner,

2011). Moreover, the use of non-polar co-solvents reduces the boiling point and affects the dielectric constant of water obtaining high polyphenol extraction yields at lower working temperatures (Wijngaard and Brunton, 2009).

According to our results, the addition of small amounts of ethanol (15%) is an alternative for reducing the HPLE temperature (from 130°C to 90°C) without decreasing the total polyphenol content of the extracts (130 °C - 0%: 26.88 mg GAE/g dried pomace). High anthocyanin recoveries (~90%) were reported by Plaza and Turner (2015) under similar processing conditions, suggesting that moderate temperatures (80-100 °C) and small amounts (15%-25%) of ethanol are the best operating conditions for batch extraction of this polyphenol family. Nevertheless, Vergara-Salinas et al. (2013) found that tannin extraction with subcritical water is favored at high temperatures (130 °C≤ T° ≤150 °C). Tannins which are mainly located in the seeds of grapes are strongly associated to the polysaccharides of cell wall, requiring higher temperatures for their extraction compared to anthocyanins which are present in the skin of grapes weakly associated to pectin (Kennedy et al., 2001; Hanlin et al., 2010).

Converse to polyphenol's behavior, the extraction of glucose and fructose was favored at higher temperature and reduced at higher ethanol concentrations (Table 3.1).

HMF was generated only at HPLE performed at temperatures higher than 130°C and without co-solvent (Table 3.1). During the extraction of polyphenols at high temperatures (~120 °C), reducing sugars are transformed into furfural compounds by Maillard reactions (Plaza and Turner, 2015).

Table 3.1. Chemical characterization of *Carménère* pomace extracts obtained under different operating conditions.

Temperature (°C) – co-solvent (%)	Total polyphenol (mg GAE / g)		Fructose (mg / g)		Glucose (mg / g)		HMF (mg / g)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
60 - 0	9.57^{a}	±1.05	10.99^{a}	1.10	8.31^{a}	±0.03	ND	
60 - 5	11.55^{ab}	±0.23	10.47^{a}	0.73	7.53^{a}	±0.03	ND	
60 - 10	14.21^{b}	±0.85	9.67^{a}	0.87	6.65^{b}	±0.07	ND	
60 - 15	14.97^{b}	±1.80	8.53^{b}	0.51	6.37^{b}	±0.06	ND	
75 – 0	12.88^{ab}	±1.29	11.40^{b}	1.03	9.08^{ac}	±0.02	ND	
75 – 5	14.32^{b}	±0.72	10.21^{ab}	1.12	7.95^{a}	±0.03	ND	
75 – 10	15.41^{b}	±2.46	9.27^{ab}	2.04	7.42^{ab}	±0.01	ND	
75 – 15	16.87^{b}	±2.19	8.67^{ab}	1.04	7.378	±0.04	ND	
90 – 0	13.23^{b}	±0.40	11.27^{c}	1.01	9.69^{c}	±0.04	ND	
90 – 5	16.92^{b}	±1.35	10.91^{ac}	0.55	8.58^{ac}	±0.01	ND	
90 – 10	21.70^{c}	±0.87	9.59^{c}	1.15	8.39^{a}	±0.05	ND	
90 – 15	23.99^{c}	±0.96	9.26^{ab}	0.93	7.37^{b}	±0.09	ND	
130 – 0	26.88	±0.27	10.06^{ab}	0.10	8.49^{a}	±0.06	0.05^{a}	±0.01
150 – 0	38.42	±1.15	7.65^{d}	0.15	6.51^{b}	±0.12	0.09^{b}	±0.01
200 – 0	61.39	±4.28	3.80^{e}	0.04	3.59^{d}	±0.02	2.49^{c}	±0.02

*Polyphenol, fructose and glucose contents are expressed as mg per g of dried pomace; ND: HMF was not detected at all operating conditions analyzed. CV: Coefficient of variation (n=3.).
**Different letters between files, show significant differences ($p \leq 0.05$) between the extraction treatments applied.

3.3.2 Impact of an integrated process of HPLE-RP over chemical composition of Carménère pomace extracts.

The use of ethanol during HPLE improved the polyphenol extraction at lower extraction temperatures; however, reducing sugars remained in the crude extracts. Therefore, the effect of integrating a subsequent purification step over the chemical composition of the extracts was evaluated. For all HPLE conditions studied in this research, unexpectedly, an increase in the ethanol concentration did not reduce the overall

polyphenols recovery, independent of the ethanol content in the eluent in the desorption stage. Additionally, a higher content of ethanol in the eluent favored the overall polyphenols recovery (Table 3.2).

Table 3.2. Chemical characterization of *Carménère* pomace extracts obtained using an integrated process of HPLE-RP.

Hot pressurized liquid extraction (HPLE)						**Resin Purification (RP)**					
Operating conditions Temperature – Ethanol	Total polyphenol (mg GAE / g) Mean	SD	Fructose (mg / g) Mean	SD	Glucose (mg / g) Mean	SD	**Eluent %**	PTF (mg GAE /g) Mean	CV	Fructose (mg/g) Mean	SD	Glucose mg/g Mean	SD
							60	5.28[a]	±0.16	ND		ND	
60 °C – 0 %	9.58[a]	±1.05	10.99[a]	±1.05	8.31[a]	±0.00	**70**	5.61[a]	±0.11	ND		ND	
							80	6.66[b]	0.27	ND		ND	
							60	6.74[b]	± 0.20	ND		ND	
75 °C – 10 %	15.41[b]	± 2.31	9.27[ab]	± 2.31	7.42[ab]	±0.07	**70**	7.30[b]	±0.37	ND		ND	
							80	8.95[c]	±0.45	ND		ND	
							60	6.48[b]	±0.07	ND		ND	
90 °C – 0 %	13.23[b]	±0.40	11.27[c]	±0.40	9.69[c]	±0.39	**70**	7.04[b]	±0.08	ND		ND	
							80	7.77[c]	±0.07	ND		ND	
							60	7.45[b]	±0.17	ND		ND	
90 °C – 5 %	16.92[b]	±1.35	10.90[ac]	±1.35	8.59[ac]	±0.09	**70**	7.85[b]	±0.16	ND		ND	
							80	8.71[c]	±0.17	ND		ND	
							60	9.33[c]	±0.19	ND		ND	
90 °C – 10 %	21.70[c]	±0.87	9.59[ab]	±0.87	8.39[a]	±0.42	**70**	10.50[d]	±0.32	ND		ND	
							80	10.69[d]	±0.11	ND		ND	
							60	10.56[d]	±0.32	ND		ND	
90 °C – 15 %	23.99[c]	±0.96	9.26[ab]	±0.93	7.37[b]	±0.22	**70**	10.64[d]	±0.21	ND		ND	
							80	11.71[e]	±0.12	ND		ND	

*Polyphenol, fructose and glucose contents are expressed as mg per g of dried pomace.
**ND: Both fructose and glucose were not detected after the purification process for all evaluated conditions. CV: Coefficient of variation (n=3).
***Different letters between files, show significant differences ($p \leq 0.05$) between the treatments applied.

After RP, there were no significant differences between the polyphenol content of extracts obtained at different extraction conditions. Purified extracts obtained using the proposed HPLE-RP process presented only a 10% less of total polyphenols than those obtained using maceration-purification. Moreover, the RP step significantly reduced the HMF content (~ 98%) of the extracts obtained at 200°C (Figure 3.1).

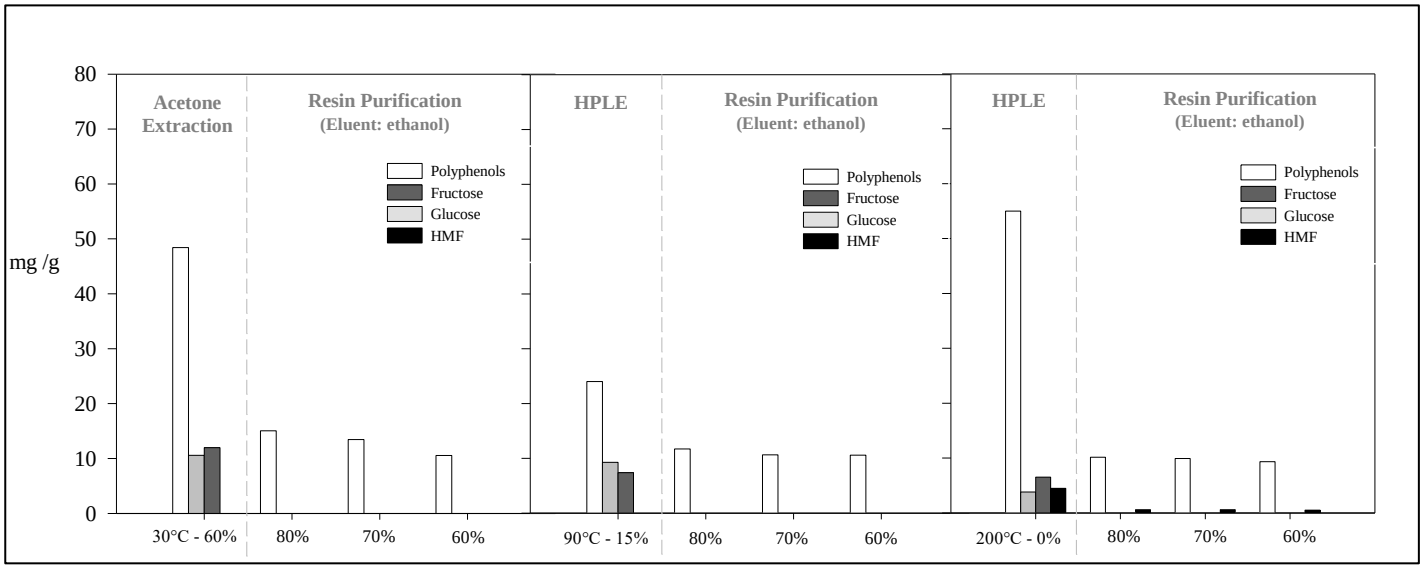

Figure 3.1. Impact of different integrated processes on the chemical composition *Carménère* pomace extracts

All HPLE-RP extracts were free from glucose and fructose (Table 3.2), since reducing sugars do not interact with the resin (Shin and Kim 2016; Yang et al. 2016).

Total content of proanthocyanidins was significantly different depending on the processing conditions applied. Maceration with acetone yields extracts with higher total proanthocyanidin content (98 mg GAE/g) compared to the best HPLE process (65.05 mg GAE/g). Maceration was performed at a lower temperature (20°C); hence, proanthocyanidins were not thermally deteriorated (García-Marino et al., 2006; Khanal, Howard and Prior, 2009). The total proanthocyanidin content of the best HPLE-RP extract was reduced (27.14 mg GAE/g); however, its oligomeric distribution (DPn) was mainly preserved (Figure 3.2). More research is necessary to find HPLE-RP operating conditions that maximize the overall recovery of polyphenols.

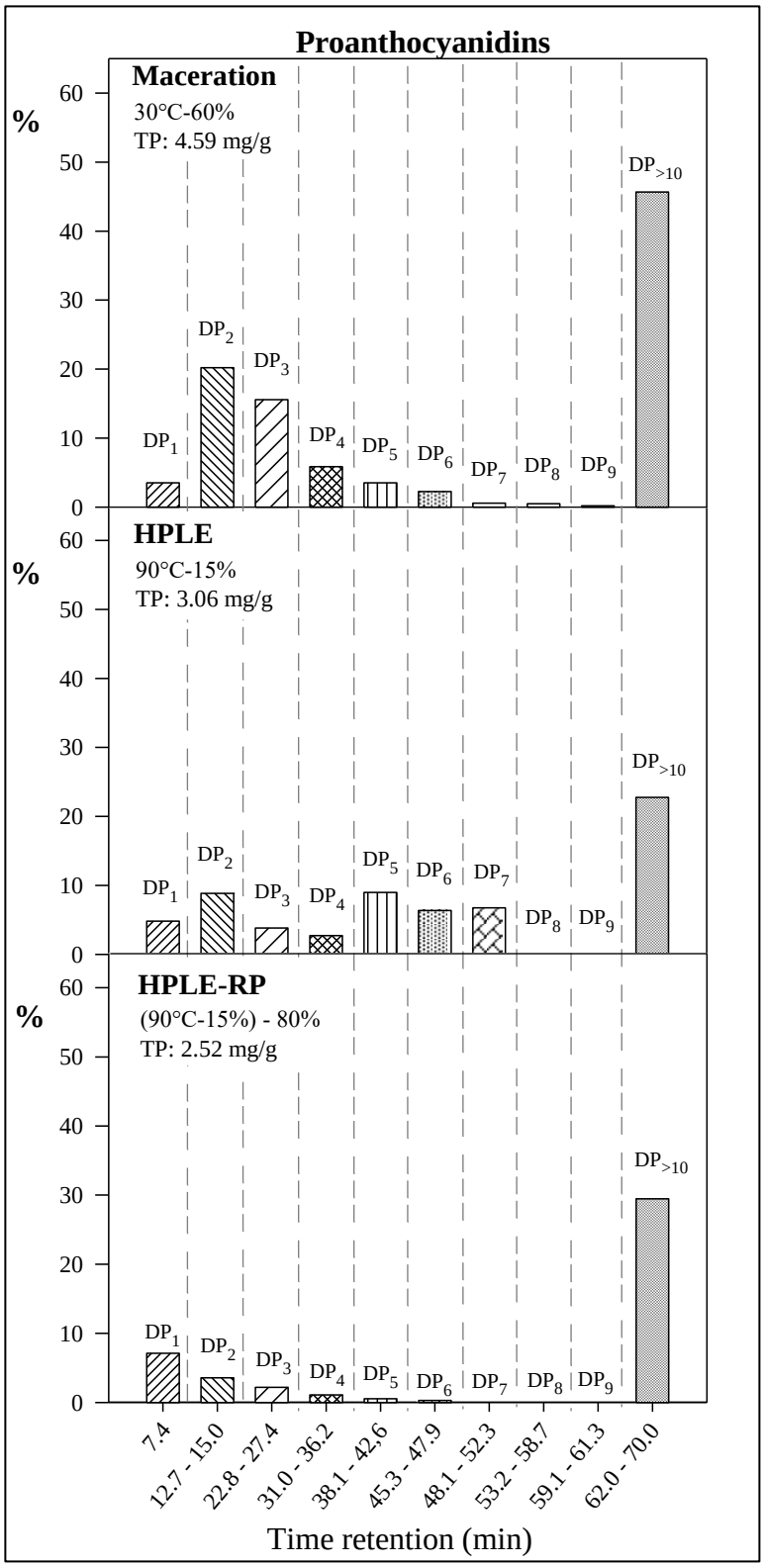

Figure 3.2. Changes produced by the integrated process of HPLE-RP on the oligomeric distribution (DPn) of the total proanthocyanidins content. TP: Total polyphenol content. DPn: Distribution of proanthocyanidins, where n indicates the number of monomers detected in the corresponding retention time. HPLE: Hot pressurized liquid extraction. HPLE-RP: Integrated process of hot pressurized liquid extraction–resin purification.

3.4. Conclusions

It is advisable to use ethanol as co-solvent during HPLE to limit the extraction of reducing sugars and to operate at lower temperatures, decreasing the thermal degradation of polyphenols. We have verified that ethanol addition up to 15% in the extraction stage; does not reduce the overall polyphenols recovery of the HPLE-RP integrated process. Purified Carménère extracts obtained using the combined HPLE-RP process, present similar polyphenol content and proanthocyanidins oligomeric distribution as those obtained using conventional maceration and RP with acetone, without reducing polyphenols yield significantly. In addition, the obtained purified extracts were free from HMF.

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4. THE IMPACT OF TEMPERATURE AND ETHANOL CONCENTRATION ON THE GLOBAL RECOVERY OF SPECIFIC POLYPHENOLS IN AN INTEGRATED HPLE/RP PROCESS ON CARMÉNÈRE POMACE EXTRACTS.

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4.1. Introduction

Carménère wine production generates ~80.000 t/year of grape pomace (skin and seed residuals) (ODEPA, 2019). This agroindustrial waste has low economic value, but is an excellent natural source of polyphenols (18 - 56 mg GAE/g) (de la Cerda-Carrasco et al., 2015). Flavanols, flavonols, phenolic acids and stilbenes are the most representative polyphenolic compounds present in *Carménère* wine pomace (Huaman-Castilla, Mariotti-Celis and Perez-Correa, 2017). This pomace is rich in flavanols, such as epigallocatechin and catechin, that show a high antioxidant activity and partly explain the distinctive astringency of *Carménère* wines (Huaman-Castilla et al., 2017; Xia, Deng, Guo and Li, 2014). In addition, *Carménère* grapes contain large amounts of the flavonol quercetin, which favor the co-pigmentation process in red wines, and have been related to the prevention of obese-related diseases (Huaman-Castilla et al., 2017; Rodríguez-Pérez, Segura-Carretero and del Mar Contreras, 2017). Abundant phenolic acids in these grapes, such as gallic and caffeic acid, have shown bioactivity against skin cancer (Dzialo et al., 2016). Resveratrol is the main stilbene present in grape´s skins and represent another important group of *Carménère* polyphenols, which has demonstrated anticancer effects (De Filippis et al., 2017; Pugajeva, Perkons and Górnaś, 2018). Consequently, efficient and food grade extraction processes to obtain extracts rich in specific polyphenols are desirable to commercially produce functional ingredients for a wide spectrum of food and nutraceutical applications.

Conventional extraction (solid-liquid) uses organic solvents to obtain bioactive extracts from vegetable matrices (Babbar, Oberoi, Sandhu and Bhargav, 2014). Commonly these solvents are chosen according to their dielectric constant ($\mathcal{E}$) that is assumed to be correlated with the solvent polarity (Katritzky et al., 2004). However, the ability of solvents to form hydrogen-bonds with the metabolite of interest should also be considered when choosing the right solvent, because both properties significantly impact the solvation capacity of solvents (Jessop, 2011; Jessop, Jessop, Fu and Phan, 2012).

Water mixtures with acetone and methanol present intermediate polarity and they are able to form hydrogen-bonds. These elements have been shown to be favorable in recovering a wide range of polyphenols from plant materials (Dai and Mumper, 2010). According to the structure of specific polyphenols, some mixtures are more appropriate (Galanakis, Goulas, Tsakona, Manganaris, and Gekas, 2013). For example, a conventional extraction (28°C) with an acetone solution (65%) is efficient to obtain flavanols. While a methanol solution (85%) under the same operating conditions is more efficient to obtain flavonols (Alberti et al., 2014). Interestingly, when the temperature of this methanol solution is increased to 50°C, the extraction is more selective for stilbenes (Soural et al., 2015). Consequently, the extractability of polyphenols in conventional extraction depends on many factors, such as the solvation properties of the solvent, the extraction temperature and the polyphenol´s structure (Ameer, Shahbaz and Kwon, 2017; Jessop et al., 2012; Katritzky et al., 2004).

Nevertheless, conventional extraction processes are slow and consume large volumes of solvents which are usually not food-grade and not friendly to the environment (Soquetta, Terra and Bastos, 2018). Hot pressurized liquid extraction (HPLE) is an alternative food grade and clean technology that allows reducing the extraction time and solvent volumes as well as improving the extraction yield of polyphenols (Ameer et al., 2017; Plaza and Turner, 2015). High pressures keep the solvent in liquid state and favor its diffusion into the plant matrix (Plaza and Turner, 2015). Like in conventional extraction, the extractability of polyphenols in HPLE is also influenced by the solvation properties of the solvent, extraction temperature and the polyphenol´s structure (Ko,

Cheigh and Chung, 2014; Plaza and Turner, 2015). As illustrated in the supplementary material (Figure S1), high pressure increases the dielectric constant of pure water, while at elevated temperatures the dielectric constant is reduced (Floriano, 2004). In turn, both pressure and temperature decrease the polarity/polarizability Kamlet-Taft parameter (π^*) and the polarity/acidity Reichardt dye ($E_{T(30)}$) parameter for water (Alghoul, Ogden and Dorsey, 2017; Lu, Brown, Boughner, Liotta and Eckert, 2002). Since choosing the right solvent to preferentially recover a given polyphenol using HPLE is challenging, experimental measurements can be complemented with computational tools. These have been successfully used for calculating the dielectric constant of solvent mixtures (Haworth, Wang and Coote, 2017; Zhang et al., 2017) and for establishing the molecular interactions between the solvent and the metabolite of interest.

Normally, the content of total polyphenols and antioxidant capacity of the extracts increase with temperature; however, some specific polyphenols could be degraded during HPLE (Ko, Cheigh, Cho and Chung, 2011). In grape pomace extraction, pure water at high temperatures ($T° \geq 120°C$) increases the degradation rate of low and high molecular weight polyphenols such as kaempferol, quercetin, catechin, epicatechin and procyanidins (García-Marino, Rivas-Gonzalo, Ibáñez and García-Moreno, 2006; Vergara-Salinas, Vergara et al., 2015). In addition, the recovery of reducing sugars and the generation of Maillard undesirable compounds are favored at high temperatures (Plaza, Abrahamsson, and Turner, 2013), complicating the handling of the extracts in the subsequent operations (e.g., atomization) and the formulation of functional foods and nutraceuticals (Muzaffar, Nayik, and Kumar, 2015; Tuomilehto et al., 2017).

Previous research studies of HPLE have analyzed the effects of temperature (60°C - 200°C) and ethanol as co-solvents (0 – 100%) in the recovery of total polyphenols, their degradation as well as the generation and recovery of undesirable compounds (hydroxymethyl furfural (HMF) and sugars) (Monrad et al., 2010; Otero-Pareja et al., 2015; Wijngaard and Brunton, 2009). However, few studies have analyzed the effect of these conditions on the profile of the polyphenols (Mariotti-Celis et al., 2018; Mauromoustakos et al., 2009; Wijngaard and Brunton, 2009). These studies show that it

is difficult to predict the effects of co-solvent and temperature on the recovery of specific polyphenols; hence, optimal extraction conditions should be obtained by experimental design.

To purify polyphenol extracts, macroporous resin processes are widely used since they are easily scalable and relatively low cost; in addition, resins are reusable and non-toxic (Buran et al., 2014). With this technique, unwanted compounds are eliminated and specific polyphenols with a given chemical structure and molecular weight are adsorbed selectively, depending on the resin and the characteristics of the solvent (Buran et al., 2014; Jampani, Naik, and Raghavarao, 2014). The purification process consists of two stages: adsorption, where some polyphenols are retained in the resin, and desorption where the retained polyphenols are liberated using a water/ethanol solution as eluent (Mariotti-Celis et al., 2017). In aqueous extracts of grape pomace, catechin, epicatechin, quercetin and kaempferol were successfully separated with the FPX-66 resin using a 70% ethanol solution as eluent (Sandhu and Gu, 2013). Sun et al. (2013) found that the X5 macroporous resin (70% ethanol solution) allowed the selective separation of chlorogenic acid from aqueous extracts of apple pomace. In previous research, we observed that the use of an ethanol solution (15%) in HPLE reduced the adsorption of total polyphenols in the subsequent purification stage, affecting their global recovery; however, some specific polyphenols like epicatechin (flavanols) and feruloylquinic acid (phenolic acid) increased their global yield (Mariotti-Celis et al., 2017). Thus, the ethanol content in HPLE also affects the recovery of total and specific polyphenols during the purification process.

Normally, the extraction stage in HPLE is designed to maximize the recovery of total polyphenols. Then, after applying these optimal extraction conditions, the purification stage is optimized independently to get rid of unwanted compounds (Lima et al., 2017; Wang, Zhang, Chi and Chen, 2018). Only a few studies have optimized the extraction and purification simultaneously to recover specific compounds (Mariotti-Celis et al., 2017; Sun et al., 2013; Wang et al., 2018). Previously, we have demonstrated that by using relatively low concentrations of ethanol (15%) at 90°C in HPLE, the global recovery (after purification) of total polyphenols and of some specific polyphenols of low

and high molecular weight is reduced (Mariotti-Celis et al., 2017; Mariotti-Celis et al., 2018). By using quantum chemical calculations, we concluded that the molecular dimensions of some polyphenols did not affect their selectivity in this integrated HPLE/RP process (Mariotti-Celis et al., 2017).

The behavior of specific polyphenols in different solvents should be assessed using both empirical and mathematical methods since they are complementary (Hansen, Rasmussen, Schiller and Gmehling, 1979). In this sense, the implicit solvation models allow us to understand the physicochemical behavior of intermolecular interactions between the metabolite of interest and the solution, where the solvent is represented as a polarizable dielectric continuum (Klamt, 1995; Tomasi, Mennucci and Cammi, 2005). These continuum models are frequently used in quantum mechanics calculations (Jorgensen, 1989; Mobley et al., 2009). Additionally, quantum mechanics, which incorporate the electronic structure, improve the characterization of the polarization of the solute by the solvent and allow for a more accurate description of the molecular shape and charge distribution (Haworth et al., 2017; Zhang et al., 2017).

In this study, we analyzed the effect of varying HPLE conditions of temperature (90 – 150°C) and ethanol concentration (15 – 50%) on the global recovery of some specific polyphenols in an integrated HPLE/RP process. Additionally, we applied computational chemical calculations to understand the selective recovery of some polyphenols and the solvent characteristics under HPLE conditions. Hence, we will be able to define optimum extraction conditions depending on the target polyphenol to recover.

4.2. Materials *and Methods*

Carménère grape pomace was extracted by HPLE with several water-ethanol mixtures and subsequently purified using a water/ethanol solution as desorption eluent. The crude and purified extracts were chemically characterized through (i) specific

polyphenols profiles; (ii) fructose and glucose content and (iii) 5- hydroxymethylfurfural (HMF) concentration.

4.2.1 Chemicals and analytic reagents

Ethanol (Sigma Aldrich, St. Louis, USA) was used for both the extraction and purification stages. For the chemical analysis, the following analytical grade reagents were used: Folin-Ciocalteu reagent, Carrez solution I, Carrez solution II, dimethylaminocinnamaldehyde (DMAC; F.W. 175.23), sodium carbonate, sodium chloride, sodium hydroxide, ammonium hydroxide, HMF (Sigma Aldrich, St. Louis, USA). The standards used in the polyphenol profiles, such as gallic acid (≥99%), catechin (≥98%), epigallocatechin (≥98%), epicatechin (≥98%), kaempferol (≥98%), resveratrol (≥98%), quercetin (≥97%), caffeic acid (≥99%), chlorogenic acid (≥98%), vanillic acid (≥99%) and ferulic acid (≥98%) were purchased from Xi'an Haoxuan Bio-Tech Co., Ltd. (Baqiao, China).

4.2.2 Wine pomace

Carménère wine pomace was obtained from Concha y Toro Vineyard, Region del Maule, Chile. Wine pomace samples were taken after the Carménère wine process had finished; the samples were then immediately frozen (-20°C). Subsequently, samples were ground with an Oster blender (Sunbeam Products, Inc., Boca Raton, FL, USA) to a particle size smaller than 1 mm diameter.

4.2.3 Hot pressurized liquid extraction (HPLE) of Carménère pomace

This process was developed according to the methodology of Mariotti-Celis et al. (2018) with some modifications. *Carménère* pomace samples of 5 g (dry weight) were extracted at 90, 120 and 150°C using different amounts of ethanol (15, 32.5 and 50%) as co-solvents in an accelerated solvent extraction device (ASE 150, Dionex) applying pressurized nitrogen (~10.2 atm). Raw extracts were frozen (−20°C) in amber vials until the chemical analysis.

4.2.4 Purification process (RP) of Carménère pomace raw extracts

Carménère pomace raw extracts were purified at 30°C using a glass column (Ø: 25 mm; h: 100 mm) packed with ~18g of HP-20 resin (Diaion, Tokyo, Japan) according to the methodology of Mariotti-Celis et al. (2018) with some modifications. First, 50 mL of raw extract were passed through the resin with a flow rate of 3 mL/min; then, after the column was washed with 100 mL of distilled water, polyphenols were desorbed with a water/ethanol solution (80%) with a flow rate of 3 mL/min. Finally, the column was regenerated using 100 mL of distilled water, 100 mL of HCl (2N) and 100 mL of NaOH (1N).

4.2.5 Total polyphenols content (TPC)

TPC levels of the Carménère pomace extracts were determined by Folin−Ciocalteu assay (Singleton, Rossi and Rossi, 1965). A volume of 3.75 mL of distilled water, 0.5 mL of Carménère pomace extract and 0.25 mL of Folin−Ciocalteu reactive (1N) were mixed with 0.5 mL of a sodium carbonate solution (10% w/v). Absorbance was measured at 765

nm (Spectrometer UV 1240, Shimadzu, Kioto, Japan) after a reaction time of 1 h at 20°C. Results were expressed as mg of gallic acid equivalent (GAE) per gram of dried pomace.

4.2.6 Antioxidant capacity

The antioxidant capacity of the extracts was determined using the DPPH radical scavenging method (Brand-Williams, Cuvelier and Berset, 1995). In summary, first 0.1 mL of extract was mixed with 3.9 mL of DPPH solution (0.1 mM), then the solution was mixed by vortex for 10 s and incubated at room temperature in the dark for 30 min. The reduction of DPPH was measured at 517nm (Spectrometer UV 1240, Shimadzu, Kioto, Japan). The IC50 (mg/L), defined as the effective extract concentration needed to inhibit 50% of DPPH radical absorption, was calculated and compared with Trolox, using the Trolox equivalent antioxidant capacity (TEAC) equation (TEAC = IC50 Trolox/IC50 sample) (Vergara-Salinas et al., 2013). Antioxidant capacity values were expressed as µM of Trolox equivalent (TE) per gram of dry mass of pomace.

4.2.7 Quantification of 5- hydroxymethylfurfural (HMF) concentration

HMF content was determined according to the methodology of Mariotti-Celis et al. (2018), using a high-performance-liquid-chromatography with a diode-array detector (HPLC-DAD) (Thermo Scientific Dionex Ultimate 3000, Waltham, MA, USA) equipped with a reverse phase Acclaim TM 120 C18 column. Results were expressed as mg of HMF per g of dry mass of pomace.

4.2.8 Quantification of fructose and glucose concentration

Glucose and fructose concentrations of the extracts were measured according to the methodology of Mariotti-Celis et al. (2018). Analyses were performed by HPLC-Infrared (IR) (Thermo Scientific Dionex Ultimate 3000, Massachusetts, USA) equipped with a normal phase Li ChroCART ® 250-4 Purospher ® STAR (5 µm). Results were expressed as mg of glucose or fructose per g of dry mass of pomace.

4.2.9 Quantification of target polyphenols

The presence of gallic acid, catechin, epigallocatechin, epicatechin, kaempferol, resveratrol, quercetin, caffeic acid and chlorogenic acid was quantified according to the methodology of Liu et al. (Liu et al., 2012) with some modifications. Samples of 1 mL were diluted with distillated water (1/10) and filtered through a 0.22 mm membrane. Then, 5 µL of filtered sample was injected into a UPLC-MS (Dionex Ultimate 3000 with Detector MS Orbitrap Exactive plus, Thermofisher, Massachusetts, USA) equipped with a reverse phase Acquity UPLC BEH C18 column (1.7 µm x 2.1 x 100 mm) at 35°C.

Chromatographic separation was carried out using a mobile phase consisting of A (acetonitrile and formic acid 0.1%) and B (water and formic acid 0.1%) in a gradient elution analysis programmed as follows: 80% A – 20% B for 6 min, then 15% A – 85% B for 18 min and 80% A – 20% B was maintained for 30 min, at a flow rate of 0.2 mL/min. Calibration curves were obtained by plotting peak areas versus nine different concentrations of standard solutions between 0.05 and 1.5 µg/L. Analyses were performed in triplicate and results were expressed in µg of the specific polyphenol. A good fit ($R^2 >$ 0.999) was found in the given concentration range (Table 4.1) with a relatively low limit of detection (LOD: 0.03 µg/L) and limit of quantification (LOQ: 0.1 µg/L) for all of the quantified polyphenols. The relative standard deviation (RSD, %) was taken as a measure of precision for quantitative determination of nine components.

Table 4.1. Analytical features of Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) method for polyphenol quantification in grape pomace extracts.

Target polyphenols	m/z	Regression equation	R^2
Epigallocatechin	305.066	Y = 49243.6*X	0.9995
Gallic acid	169.015	Y = 1.57814e+006*X	0.9971
Chlorogenic acid	353.087	Y = 3.10744e+006*X	0.9996
Vanillic acid	167.044	Y = 41264.3*X	0.9742
Ferulic acid	193.050	Y = 740734*X	0.9999
Catechin	289.071	Y = 3.67625e+006*X	0.9990
Epicatechin	289.071	Y = 4.67949e+006*X	0.9994
Caffeic acid	179.034	Y = 4.54778e+006*X	0.9998
Resveratrol	227.071	Y = 112818*X	0.9998
Quercetin	301.035	Y = 14919.2*X	1.0000
Kaempferol	285.040	Y = 2.26042e+006*X	0.9990

4.2.10 Computational Chemistry calculations

The calculations were carried out using the Gaussian 09 (Frisch et al., 2009) program package. The Solvation Model based on Density (SMD) (Marenich, Cramer and Truhlar, 2009) was employed. Following a gas-phase optimization at density functional theory (DFT) M062x/6-311+G(d,p) level of theory, the solvation free energy was calculated from the single-point energy difference between the gas phase and the liquid phase. No imaginary vibrational frequencies were found at the optimized geometries, which indicates that they are true minima of the potential energy surface.

With the aim of incorporating specific intermolecular interactions, such as hydrogen bonds, within our quantum mechanics calculations, we used a discrete-continuum approach. Four solvent explicit molecules were incorporated representing the first solvation sphere, while the continuum model solvent (SMD) was represented varying the dielectric constant value. For the system containing 25%/75% ethanol/water, one

ethanol and three water molecules were explicitly considered. While for 50%/50% ethanol/water, two ethanol and two water molecules were explicitly considered. Except for the dielectric constant, the default parameters were used; the weighted average of the values of the dielectric constant of water and ethanol (3:1 and 1:1, respectively) were used to represent the ethanol/water mixtures (25%/75% and 50%/50% respectively) (Lide, 2004). We compared ΔG_{solv} of chlorogenic and gallic acids in ethanol/water systems with increasing ethanol contents. Specifically, we evaluated the change in the ΔG_{solv} ($\Delta\Delta G_{solv}$) of chlorogenic and gallic acids in a system in which the ethanol/water content was increased from 25%/75% to 50%/50%.

4.2.11 Statistical analysis

A factorial experimental design was applied to determine the effect of extraction temperature and co-solvent during extraction on the global recovery of specific polyphenols; this follows the methodology proposed by Mariotti-Celis et al. (2017). In addition, mean and coefficient of variation (CV) results were presented. Analysis of variance (ANOVA) and least significant difference tests were applied to the response variables ($p \leq 0.05$). The statistical analyses of data were carried out using the software Statgraphics Plus for Windows 4.0 (Statpoint Technologies, Inc., Virginia, USA).

4.3. Results and discussion

4.3.1 Effect of ethanol as co-solvent in HPLE

In order to find the optimal operational conditions in HPLE for the recovery of flavanols, flavonols, stilbenes (resveratrol) and phenolic acids from Carménère pomace, the extraction temperature (90, 120 and 150°C) and different water-ethanol mixtures (15,

32.5 and 50%) were assessed, considering the relevant role of the characteristics of the solvent in the extraction of some specific polyphenols.

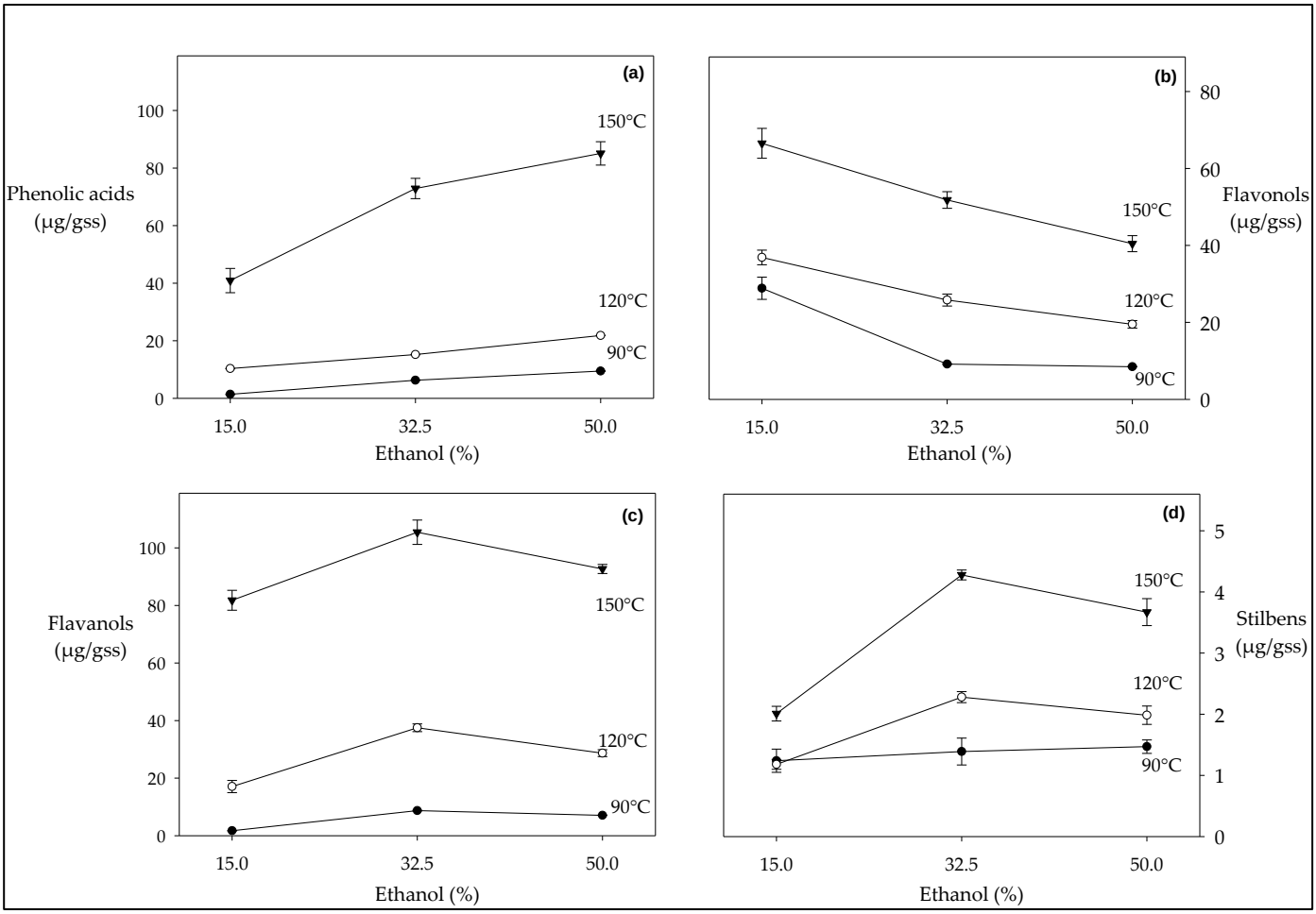

Figure 4.1. Effect of the temperature and ethanol concentration on recovery of specific polyphenols.

Table 4.2. Polyphenols profile of extracts obtained by HPLE process

Description	HPLE											
	90°C						120°C					
	15%		32.5%		50%		15%		32.5%		50%	
Acids (µg/gdp)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Gallic acid	0.97	0.07	3.99	0.05	4.73	0.06	5.80	0.04	10.06	0.04	13.87	0.09
Chlorogenic acid	0.77	0.05	0.23	0.08	0.19	0.04	0.84	0.06	0.39	0.07	0.28	0.07
Vanillic acid	ND		1.57	0.04	3.02	0.05	1.84	0.06	3.50	0.05	6.17	0.08
Caffeic acid	0.22	0.04	0.36	0.03	0.68	0.02	0.69	0.08	0.83	0.05	1.01	0.07
Ferulic acid	ND		0.14	0.07	0.26	0.09	0.30	0.04	0.43	0.08	0.49	0.04
Σ:	**1.96**	**0.05**	**6.29**	**0.06**	**8.88**	**0.05**	**9.47**	**0.06**	**15.21**	**0.06**	**21.82**	**0.07**
Flavanols (µg/gdp)												
Catechin	0.68	0.04	1.25	0.05	0.94	0.07	4.11	0.05	5.66	0.09	6.06	0.06
Epicatechin	0.71	0.04	1.32	0.11	1.35	0.07	3.22	0.07	5.49	0.06	6.17	0.07
Epigallocatechin	1.77	0.09	6.11	0.06	5.15	0.09	9.76	0.08	26.34	0.11	17.45	0.09
Σ:	**3.16**	**0.06**	**8.68**	**0.07**	**7.44**	**0.08**	**17.08**	**0.07**	**37.51**	**0.09**	**29.68**	**0.07**
Flavonols (µg/gdp)												
Quercetin	16.97	0.08	6.99	0.09	6.34	0.08	21.40	0.09	14.63	0.07	11.88	0.08
Kaempherol	10.67	0.07	1.08	0.10	0.77	0.07	14.28	0.08	7.39	0.09	5.26	0.07
Σ:	**27.64**	**0.08**	**8.07**	**0.10**	**7.18**	**0.08**	**35.68**	**0.09**	**22.03**	**0.08**	**17.14**	**0.08**
Stilbenes (µg/gdp)												
Resveratrol	1.24	0.07	1.39	0.09	1.07	0.08	1.18	0.05	2.28	0.06	1.94	0.08
Interfering (mg/gdp)												
Glucose	9.85	0.06	7.11	0.07	3.69	0.06	10.36	0.09	8.01	0.06	5.11	0.09
Fructose	7.84	0.08	6.25	0.08	2.94	0.07	9.47	0.08	7.61	0.08	4.50	0.07
HMF	ND		ND		ND		ND		ND		ND	

Description	HPLE					
	150°C					
	15%		32.5%		50%	
Acids (µg/gdp)	Mean	CV	Mean	CV	Mean	CV
Gallic acid	28.54	0.07	56.57	0.09	59.92	0.11
Chlorogenic acid	1.94	0.05	1.36	0.08	1.01	0.05
Vanillic acid	6.32	0.05	12.99	0.08	20.57	0.06
Caffeic acid	1.80	0.09	2.28	0.06	2.57	0.03
Ferulic acid	0.53	0.08	0.70	0.08	1.11	0.04
Σ:	**39.13**	**0.06**	**73.90**	**0.08**	**85.18**	**0.06**
Flavanols (µg/gdp)						
Catechin	15.41	0.08	23.39	0.10	21.05	0.06
Epicatechin	8.50	0.11	14.41	0.09	12.64	0.07
Epigallocatechin	57.89	0.06	67.66	0.10	58.99	0.11
Σ:	**81.80**	**0.08**	**105.46**	**0.10**	**92.68**	**0.08**
Flavonols (µg/gdp)						
Quercetin	43.14	0.08	34.12	0.10	30.95	0.08
Kaempherol	20.90	0.10	11.72	0.09	5.84	0.09
Σ:	**64.04**	**0.09**	**45.84**	**0.10**	**36.79**	**0.09**
Stilbenes (µg/gdp)						
Resveratrol	2.01	0.07	4.28	0.08	3.67	0.09
Interfering (mg/gdp)						
Glucose	12.63	0.07	10.86	0.05	6.45	0.06
Fructose	11.91	0.04	10.31	0.06	4.87	0.09
HMF	23.61	0.07	17.83	0.05	11.28	0.06

Specific polyphenols content is expressed as µg/g dry pomace. HMF: hydroxymethylfurfural is expressed as mg HMF/ g dry pomace. Fructose and glucose contents were expressed as mg/g dry pomace. CV: coefficient of variation. ND: not detected.

a) Phenolic acids

The yield of phenolic acids in HPLE was at its maximum (85.18 μg/gdp) at the highest temperature and highest ethanol concentration (Table 4.2 and Figure 4.1a). García et al. (2006) reported that extractability of phenolic acids was enhanced ~8 times when temperature was increased from 100 to 150°C, using pure water as an extraction solvent. In our study, when temperature is increased from 90 to 150°C, the extractability of phenolic acids enhanced ~9, ~12 and ~19 times with 15, 32.5 and 50% of ethanol respectively (Table 4.2). In HPLE (P: ~10 MPa, T: 150°C) pure water presents higher polarity (π*: 0.98) than ethanol (π*: 0.27) (Alghoul, Ogden and Dorsey, 2017; Lu et al., 2002). This difference could explain the higher recovery of phenolic acids with water-ethanol mixtures, in which an ethanol addition would decrease solvent polarity. Phenolic acids are organic molecules which have a greater affinity for organic solvents, such as ethanol, due to intermolecular interactions (dipole-dipole and dispersion forces of London) (Galanakis et al., 2013).

The recovery of specific phenolic acids such as gallic, vanillic, caffeic and ferulic increased with temperature and ethanol concentrations. The most extreme HPLE condition (50% - 150°C) recovered ~12 times more gallic acid compared to lower temperature extractions (Table 4.2). Grape pomace presents important concentrations of procyanidins, which are galloylated with gallic acid as terminal units (Huaman-Castilla et al., 2017). These compounds can be hydrolyzed at high temperatures (T> 120 ° C) with the subsequent release of gallic acid in the extracts (García-Marino et al., 2006; Plaza and Turner, 2015; Vergara-Salinas et al., 2013). Like the other phenolic acids, chlorogenic acid recovery increased with temperature, but it decreased (from 20% to 69%) with ethanol addition (Table 4.2). Wijngaard et al. (2009) reported similar behavior, arguing that the small size and the high number of carbonyl groups in chlorogenic acid are factors that improve solubility in pure water, when compared to water-ethanol mixtures (25% – 75%) at high temperatures (160 – 193°C). Chlorogenic acid was the largest phenolic acid analyzed in our extracts. In addition, carbonyl groups are better hydrogen bonding

acceptors than hydroxyl groups, and water is a better hydrogen bonding donor than ethanol. Hence, the interaction between the best acceptor and the best donor of hydrogen generates higher stabilization; this could explain that chlorogenic acid, which possesses the highest number of carbonyl groups among the polyphenols studied, decreases its solubility as the ethanol content increases in the water-ethanol mixture.

To verify if the number of carbonyl groups effectively explains the behavior of phenolic acids, we evaluated the $\Delta\Delta G_{solv}$ of chlorogenic and gallic acids in ethanol/water mixtures using chemical quantum calculations. As can be observed in Table 4.3, when the ethanol content of ethanol/water mixtures increased from 25% to 50%, a lower value of $\Delta\Delta G_{solv}$ was obtained for gallic acid (1.25 kJ/mol) compared to chlorogenic acid (1.52 kJ/mol). These results indicate that gallic acid was better solvated than chlorogenic acid at higher ethanol concentrations, which could explain the tendency observed in the extraction yields of these phenolic acids.

Table 4.3. Changes of the Gibbs free energies of solvation of chlorogenic and gallic acids in ethanol-water systems with increasing ethanol contents.

Phenolic acid	$\Delta G_{solv\ 25\%\ ethanol}$ [kJ/mol]	$\Delta G_{solv\ 50\%\ ethanol}$ [kJ/mol]	$\Delta\Delta G_{solv}$ [kJ/mol]
Gallic acid	-60.66	-59.41	1.25
Chlorogenic acid	-112.51	-110.99	1.52

b) Flavanols

Under subcritical conditions (P: ~10MPa), the highest recovery of flavanols was achieved at the highest temperature (150°C) and at the intermediate ethanol concentration (32.5%). When temperature was increased from 90 to 150°C, the flavanols content was

enhanced ~25 and ~12 times using ethanol at 15% and 32.5 % respectively (Table 4.2). Similar studies using pure water at high temperatures (from 100 to 200°C) enhanced the recovery of flavanols by ~2 times (Mauromoustakos et al., 2009; Vergara-Salinas et al., 2015).

The recovery of specific flavanols such as catechin, epicatechin and epigallocatechin increased with temperature and ethanol concentrations up to 32.5% (Table 4.2). Extracts obtained at 32.5% - 150°C presented a high epigallocatechin relative concentration (~64%) compared to other specific flavanols (Table 4.2). The skin of grape pomace contains procyanidins rich in epigallocatechin monomers (Huaman-Castilla et al., 2017) which can be released through hydrolysis reactions in HPLE (Vergara-Salinas et al., 2013), explaining the high epigallocatechin content in the 150°C extracts.

The recovery of flavanols decreased significantly (from 14% to 56%) for ethanol concentrations higher than 32.5% (Table 4.2 and Figure 4.1c). Downey et al. (2010) also reported that ethanol concentrations higher than 50% decreased the recovery of flavanols, although under atmospheric conditions (P: ~101.3 kPa, T: 23°C). Figure 1c shows that this effect is more noticeable at higher temperatures. This should be expected since the polarizability (π^*) of ethanol decreases significantly (from 0.51 to 0.35) when the temperature increases from 25°C to 150°C (Jessop et al., 2012; Lu, Boughner, Liotta and Eckert, 2002).

c) Flavonols

Extracts obtained at 15% - 150°C presented ~67% of quercetin and 33% of kaempferol (Table 4.2). Although high temperatures improved the extraction of flavonols, an increase in the ethanol concentration significantly decreased the recovery of these compounds (from 20% to 80%) (Figure 4.1b). Wijngaard et al. (2009) observed a similar trend in the recovery of flavonols at high concentrations of ethanol (≥ 50%) and high temperatures (≥ 100 ° C) in HPLE. This behavior could be explained by the competitive

interactions of ethanol, water and flavonol molecules. The presence of ketone type carbonyl groups in the structure of flavonols favors their solubility in water. As ethanol concentration increases in the solvent, ethanol-water interactions are more favored than ethanol-flavonol and flavonol-water interactions, which would decrease the solubility of flavonols, reducing their recovery during extraction.

d) Stilbenes (resveratrol)

Extractions at the highest temperature (150°C) and at the intermediate ethanol concentration (32.5%) maximized the recovery of resveratrol (4.28 μg/gdp), the only stilbene that we quantified. Ethanol concentrations higher than 32.5% significantly decrease its recovery (Table 4.2 and Figure 4.1d). This behavior is in agreement with the findings of Karacabey and Mazza (2008) who reported that ethanol concentrations higher than 60% decreased the recovery of resveratrol in extractions performed at atmospheric conditions (P: ~101.3 kPa, T: 20 – 80°C). Like flavanols, this effect is more pronounced at higher temperatures, and as discussed above, it is probably related to ethanol polarity being reduced at high temperatures.

e) Interfering compounds

Ethanol addition (15 - 50%) decreased both the glucose and fructose extraction (~60%) (Table 4.2). Reducing sugars are hydrophilic compounds that easily form hydrogen bonds with water molecules. Therefore, the presence of a low polar co-solvent such as ethanol disfavors their solubility during the extraction (Alves et al., 2007; Alavi, Pazuki and Raisi, 2014). In addition, high temperatures (T°≥ 120°C) favor the Maillard reaction, where reducing sugars transform into furfural compounds (Plaza et al., 2013).

We observed that ethanol addition reduced the formation of hydroxymethylfurfural (HMF) during HPLE performed at 120°C (Table 4.2) which could be attributed to the low content of reducing sugars (main precursors of HMF) in the crude extract. HMF was only detected in extracts obtained at 150°C (from 11 to 23 mg HMF/gdp) (Table 4.2). However, this concentration is significantly lower than the levels (80 mg/kg body weight/day) required to observe carcinogenic and genotoxic effects in laboratory animals (Abraham et al., 2011).

4.3.2 Global antioxidant properties

The obtained HPLE extracts presented the best global condition at 150°C with 32.5% of ethanol, in which the total quantified polyphenols families reached ~230 µg/gdp. It is in accordance with the total polyphenol content of the extracts which was 50% higher (~54 mg GAE/gdp) than the extracts obtained at the other evaluated conditions. Interestingly, regarding the antioxidant capacity, the highest value was also observed at the same conditions (~340 µM Trolox/gdp).

4.3.3 Purification with macroporous resin (RP)

After HPLE, the extracts were purified using RP to improve the specific separation (free of interfering compounds) of phenolic acids, flavanols, flavonols and stilbenes.

a) Purification of phenolic acids

An increase in the ethanol content of the extracts (15 - 50%) decreased the recovery of phenolic acids during RP (Table 4.4). For example, the extracts obtained at 150°C with

ethanol additions of 15%, 32.5% and 50% presented recoveries over ~65%, ~14% and ~8% of phenolic acids respectively, after RP (Figure 4.2a). The global HPLE-RP condition,15% - 150°C and 80%, allowed the highest recovery of phenolic acids, with gallic acid being the most abundant (~70%) (Table 4.4). Yang et al. (2018) observed a similar behavior during RP, arguing that probably the compounds of interest are more stabilized by the interactions with the solvent than by the interactions with the resin surface. Hence, the higher the ethanol content in the raw extracts, the lower the polyphenols content in the purified extracts (Table 4.4).

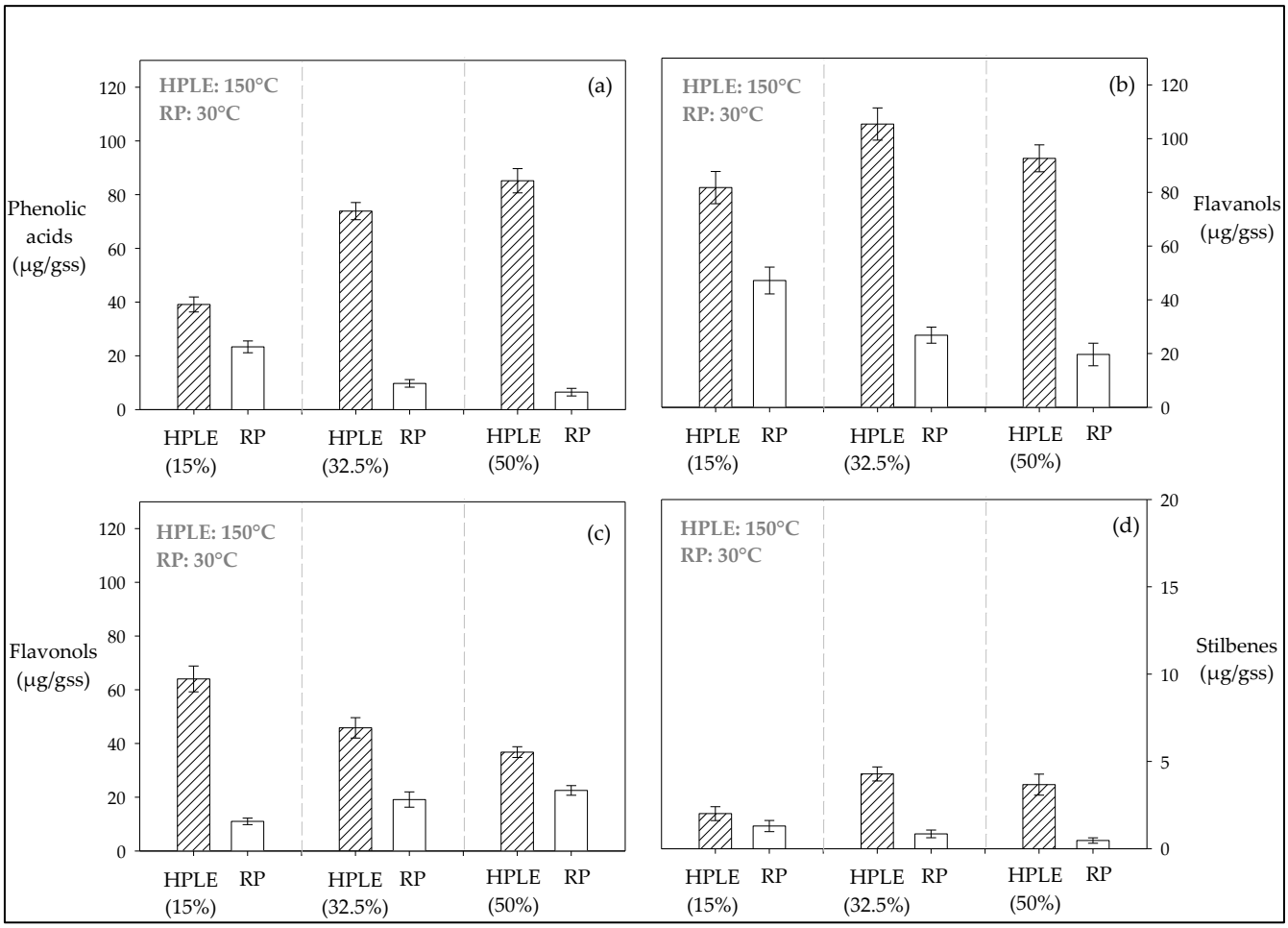

Figure 4.2. Effect of co-solvent addition on the recovery of specific polyphenols in an integrated HPLE-RP process.

a) Purification of flavanols

During RP, the adsorption of flavanols was enhanced significantly when the extracts presented a low ethanol content (Table 4.4); for example, in this stage, the extracts obtained at 150°C with ethanol concentrations of 15%, 32.5% and 50% presented recoveries of flavanols of ~57%, ~25% and ~21% respectively (Figure 4.2b). The global HPLE-RP condition, 15%-150°C and 80%, allowed a higher selectivity in the adsorption of epigallocatechin (~56%) compared to the other determined flavanols (Table 4.4). (Mariotti-Celis et al., 2017) observed a similar behavior during the RP of HPLE extracts obtained at low ethanol concentration (16%). The interactions between the polyphenols, the solvent and the resin would explain the selective recovery observed for epigallocatechin. HP-20 is a polyaromatic adsorbent resin without polar groups. Probably the stability between the van der Waals forces and π-π -stacking interactions, which occur between the aromatic rings of epigallocatechin and the aromatic rings in the surface of the resin, are more stable than the interactions between the ethanol/water mixture and the compound of interest after a determined polarity threshold (Mariotti-Celis et al., 2017).

b) Purification of flavonols

The recovery of flavonols after RP was considerably more efficient for the extracts with high ethanol concentrations (Table 4.4); for example, after RP, the extracts obtained at 150°C with ethanol concentrations of 15%, 32.5% and 50% presented recoveries of flavonols over ~17%, ~41% and ~61% respectively (Figure 4.2c). The global HPLE-RP, 50%-150°C and 80%, allowed the highest global recovery of quercetin (~62%). The structure of flavonols presents hydroxyl and ketonic groups which improve their stability in polar solvents (Cheigh et al., 2015). High ethanol levels in the raw extracts reduce the relative stability of flavonols in the solvent, favoring their adsorption in the resin surface.

c) Purification of stilbenes (resveratrol)

During RP, the recovery of resveratrol decreased when the ethanol concentration increased in the raw extracts (Table 4.4). For example, the extracts obtained at 150°C with ethanol concentrations of 15%, 32.5% and 50% presented recoveries of resveratrol over ~64%, ~19% and ~12% respectively (Figure 4.2d). Similar to flavanols and phenolic acids, stilbenes are probably more stabilized by the interactions with the solvent than by the interactions with the resin surface, which would explain the observed behavior (Table 4.4).

d) Interfering compounds

All purified extracts were free from reducing sugars, while HMF was only found in purified extracts obtained at the highest extraction temperature (150°C). However, it is worth noting that in these extracts HMF was significantly reduced (~95%) by RP (Table 4.4). The use of non-ionic resins favors interactions with non-polar compounds (e.g., polyphenols), whereas polar compounds (e.g., sugars and HMF) are not adsorbed, facilitating their elimination (Mariotti et al., 2017; Mariotti et al., 2018).

Table 4.4. Polyphenols profile of purified extracts obtained by integrated HPLE-RP process

HPLE	**90°C**						**120°C**						**150°C**					
	15%		32.5%		50%		15%		32.5%		50%		15%		32.5%		50%	
RP	80%		80%		80%		80%		80%		80%		80%		80%		80%	
Acids (µg/gdp)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Gallic acid	0.55	0.04	0.30	0.04	0.27	0.05	3.52	0.03	1.61	0.09	0.74	0.07	18.97	0.06	6.21	0.08	3.79	0.07
Chlorogenic acid	0.02	0.02	0.03	0.05	0.07	0.04	0.03	0.02	0.07	0.04	0.05	0.04	0.15	0.02	0.64	0.02	0.86	0.02
Vanillic acid	ND		2.65	0.06	0.58	0.06	1.48	0.06	0.66	0.08	0.35	0.09	4.64	0.09	2.05	0.11	1.54	0.09
Caffeic acid	0.13	0.05	0.14	0.03	0.08	0.03	0.60	0.04	0.57	0.03	0.36	0.04	1.24	0.06	0.61	0.06	0.20	0.06
Ferulic acid	ND		0.06	0.02	0.03	0.02	0.17	0.03	0.12	0.03	0.09	0.05	0.34	0.05	0.26	0.06	0.09	0.05
Σ:	**0.68**	**0.04**	**3.18**	**0.04**	**1.03**	**0.04**	**5.80**	**0.04**	**3.03**	**0.06**	**1.57**	**0.06**	**25.34**	**0.06**	**9.77**	**0.07**	**6.48**	**0.06**
Flavanols (µg/gdp)																		
Catechin	0.52	0.05	0.35	0.08	0.22	0.07	3.17	0.09	1.30	0.09	0.91	0.05	11.42	0.08	8.26	0.06	5.48	0.07
Epicatechin	0.63	0.06	0.44	0.05	0.21	0.08	2.26	0.08	1.51	0.06	0.88	0.07	6.38	0.09	3.84	0.09	2.79	0.08
Epigallocatechin	0.82	0.03	0.52	0.10	0.24	0.09	4.21	0.10	2.27	0.08	1.05	0.09	29.40	0.10	14.75	0.08	11.40	0.07
Σ:	**1.97**	**0.05**	**1.31**	**0.08**	**0.67**	**0.08**	**9.64**	**0.09**	**5.08**	**0.08**	**2.83**	**0.07**	**47.20**	**0.09**	**26.85**	**0.08**	**19.67**	**0.07**
Flavonols (µg/gdp)																		
Quercetin	0.65	0.07	2.81	0.09	3.99	0.09	0.98	0.09	6.56	0.08	8.16	0.10	9.86	0.09	15.28	0.08	19.09	0.08
Kaempherol	0.26	0.06	0.45	0.07	0.47	0.05	0.76	0.05	1.79	0.06	2.73	0.05	1.17	0.05	3.87	0.05	3.47	0.09
Σ:	**0.91**	**0.07**	**3.26**	**0.08**	**4.46**	**0.07**	**1.74**	**0.07**	**8.35**	**0.07**	**10.89**	**0.08**	**11.03**	**0.07**	**19.15**	**0.07**	**22.56**	**0.09**
Stilbenes (µg/gdp)																		
Resveratrol	0.69	0.05	0.42	0.06	0.13	0.06	0.88	0.05	0.70	0.04	0.68	0.06	1.30	0.05	0.85	0.06	0.47	0.05
Interfering (mg/gdp)																		
Glucose	ND		ND		ND		ND		ND		ND		ND		ND		ND	
Fructose	ND		ND		ND		ND		ND		ND		ND		ND		ND	
HMF	ND		ND		ND		ND		ND		ND		0.19	0.09	0.13	0.08	0.22	0.07

Specific polyphenols content is expressed as µg/g dry pomace. HMF: hydroxymethylfurfural is expressed as mg HMF/ g dry pomace. CV: coefficient of variation. ND: not detected.

4.4. Conclusions

The global recovery of the phenolic acids, flavanols, flavonols and stilbenes quantified in this study in an integrated HPLE-RP processes, significantly increase at 150°C. On the other hand, the optimal ethanol concentration used in HPLE is specific for each family of polyphenols (flavonols: 15%, flavanols and stilbenes: 32%; and phenolic acids: 50%), but also depends on the chemical structure of the compound of interest. For example, even though total phenolic acid recovery is at a maximum at the highest ethanol concentrations, chlorogenic acid shows lower recoveries at this condition. Using quantum chemical calculations, it was verified that this behavior is due to the high number of carbonyl groups in the compound's structure, compared to the other phenolic acids considered in this study. The obtained HPLE extracts presented the best global condition at 150 °C with 32.5% of ethanol in which the quantified polyphenol value (~230 µg/gdp), total polyphenol content (~54 mg GAE/gdp) and antioxidant capacity (~340 µM Trolox/gdp) were the highest. The ethanol concentration in HPLE significantly impacts the global yield of the integrated HPLE-RP process. The lower the ethanol content in the HPLE extracts, the higher the global recovery of phenolic acids, flavanols and stilbenes. However, flavonols present the opposite trend, which is attributed to the presence of polar functional groups in their structure. The integrated HPLE/RP separation process eliminates interfering compounds such as glucose and fructose. In addition, this process ensures the safety of the extracts, almost completely removing HMF from all purified extracts.

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5. GENERAL CONCLUSIONS AND FUTURE PROSPECTS

5.1. General conclusions

Carménère is the emblematic wine of Chile due to its particular polyphenols content. Production of this wine generates high amounts of grape pomace (skin and seed) (~80 000 tons per year), causing losses of potential economic benefits and generating environmental problems. After winemaking, an important fraction of the grape berry polyphenols is retained in the pomace, consisting mainly in malvidin (anthocyanin), quercetin (flavonol) and epigallocatechin (flavanol) as well as polymers of high molecular weight (procyanidins). These compounds present different technological and nutraceutical properties. Therefore, given the wide spectrum of applications of the polyphenols found in *Carménère* pomace, the development and optimization of sustainable extraction/purification technologies to recover them is a highly active research area.

Water-ethanol mixtures (0 - 15%) combined with moderate temperatures (60 – 90°C) is a promising alternative to obtain safe extracts rich in polyphenols during HPLE. The addition of ethanol as co-solvent up to 15% at 90°C allowed to recover 80% more total polyphenols compared to pure water under the same conditions. The effectiveness of water-ethanol mixtures can be explained by the high diffusion rate of water molecules into the solid matrix, releasing the polyphenols retained in the cell vacuoles, while ethanol molecules interact with polyphenols improving their solubility and extractability.

The use of ethanol as co-solvent at 15% allowed to decrease the recovery of glucose and fructose. Reducing sugars are hydrophilic molecules that easily form hydrogen bonds with water molecules. Therefore, the addition of a non-polar cosolvent like ethanol, disfavors the solubility of these sugars during extraction. In addition, water-

ethanol mixtures at 15% and 90°C achieved the same polyphenols yield than pure water at 130°C; hence, thermal degradation of high molecular weight polyphenols is significantly reduced.

Ethanol additions up to 15% in the extraction step at low temperatures (90 °C) allowed to recover ~60% of the total polyphenols during purification with macroporous resins, while undesirable compounds (sugars and HMF) were significantly reduced. In addition, purified extracts obtained using the integrated HPLE-RP process presented an increased in monomers concentration, while the distribution of dimers, trimers, tetramers and oligomers was preserved. Our results also indicate that Maillard reaction was inhibited using water-ethanol mixtures at 120°C. Therefore, integrated HPLE-RP process is a clean technology that allow to obtain safe polyphenols extracts from *Carménère* pomace.

Ethanol concentration at high temperatures (150°C) in HPLE modulated the solvent characteristics in order to obtain a selective extraction of polyphenols. The best ethanol concentrations were 15% for flavonols, 32.5% for flavanols and stilbenes, and 50% for phenolic acids. In this context, depending on the co-solvent content, different intermolecular interactions can be established with the functional groups of the polyphenols.

Finally, ethanol additions in the extraction impact significantly the global polyphenols yield of an integrated HPLE-RP process. A low ethanol content (15%) in HPLE allowed a higher global recovery of phenolic acids (65%), flavanols (57%) and stilbenes (64%) after RP. In contrast, the highest recovery of flavonols (61%) during RP was achieved when 50% of ethanol was used in HPLE. In addition, the integrated HPLE-RP process eliminates undesirable compounds such as sugars and HMF.

5.2. Future prospects

The present thesis studied in depth the effects of temperature and solvent characteristics on the extraction and selectivity of polyphenols in an integrated HPLE-RP process. Likewise, during the development of this research, new exploring avenues were opened, such as trying different solvents to improve even more the recovery and selectivity of specific polyphenols from agroindustrial discards. In this sense, it is proposed that future work should be focused on:

- Evaluate the use of green and low-cost alternative solvents like glycerol and deep eutectic solvents (DES), to improve the recovery of specific families of polyphenols in an integrated HPLE-RP process.

- Apply mass balances to define the fate of unrecovered polyphenols, and therefore add other green technologies such as membrane technology to minimize polyphenol losses in the HPLE-RP process.

- Evaluate the textural properties and adsorption/desorption dynamics of different macroporous resins in order to improve the recovery of polyphenols during purification.