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RODRIGO DE SOUSA TRÊPA ALVES NETO

PROANTOCIANIDINAS DE ELEVADO PESO MOLECULAR: EXTRAÇÃO COM SOLVENTES EUTÉTICOS A PARTIR DE BAGAÇO DE UVA E SUAS APLICAÇÕES

HIGH MOLECULAR WEIGHT PROANTHOCYANIDINS: EXTRACTION WITH EUTECTIC SOLVENTS FROM GRAPE POMACE AND ITS APPLICATIONS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica do Doutor Armando Jorge Domingues Silvestre, Professor Catedrático do Departamento de Química da Universidade de Aveiro, da Doutora Joana Alexandra da Silva Oliveira, Investigadora Auxiliar do REQUIMTE e da Doutora Sónia Andreia Oliveira Santos, Investigadora do CICECO.

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Dedico esta tese às três mulheres da minha vida, os três pilares que me fizeram capaz de concluir este e todos os outros desafios.

o júri

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palavras-chave

Proantocianidina, solvente eutético, bagaço de uva, extração, micro-ondas, grau de polimerização, atividade antioxidante, síntese de nanoparticulas

resumo

As proantocianidinas (PACs) são compostos fenólicos poliméricos formados por catequina e seus derivados, sendo normalmente obtidas a partir de culturas agroflorestais dedicadas como o quebracho (*Schinopsis balansae*) e que encontram aplicação na produção de cabedal, aglomerados de madeira e maturação de vinho.

Os métodos mais comuns de extração de PACs são caracterizados por uma baixa especificidade e eficiência, o que limita a sua utilização quer em aplicações convencionais quer em novas aplicações de maior valor acrescentado. Assim o desenvolvimento de métodos de extração inovadores e baseado em fontes de biomassa mais sustentáveis torna-se imperativo.

Os solventes eutéticos (ESs), normalmente descritos como sendo constituídos por um par de compostos, um dador e um aceitador de pontes de hidrogénio, têm vindo a ser propostos como uma alternativa viável aos solventes convencionais e são uma das opções mais promissoras para o melhoramento dos processos de extração baseados em solventes orgânicos. Isto deve-se ao seu baixo custo, simplicidade de preparação, biocompatibilidade e capacidade de serem preparados “à medida” para aplicações específicas.

Incluído no conceito de economia circular tem havido um interesse crescente no uso de subprodutos agroflorestais como fonte de biomassa para a obtenção de compostos com valor acrescentado e as PACs não são exceção. De entre as opções disponíveis, o bagaço de uva, um subproduto da indústria do vinho, é um dos subprodutos mais relevantes para a obtenção de extratos ricos nestes compostos devido à elevada produção anual a nível mundial, e em particular em Portugal, e também devido à elevada abundância de PACs nestes subrpodutos.

Assim, o principal objetivo desta tese é o melhoramento do processo de extração de PACs a partir do bagaço de uva com a utilização de ESs com vista à obtenção de frações mais puras e com propriedades bem definidas, bem como a exploração de aplicações de elevado valor acrescentado para as PACs.

resumo

Boa parte do trabalho apresentado é focado no estudo do efeito de diferentes combinações de SEs na extração de PACs a partir de bagaço de uva. Neste contexto explorou-se o processo de maceração simples ou em combinação com aquecimento por irradiação com micro-ondas otimizando-os com recurso à metodologia de superfície de resposta (RSM).

Relativamente ao processo de extração por maceração simples, concluiu-se que os melhores resultados foram obtidos com um sistema de ES quaternário composto por cloreto de colina, glicerol, etanol e água (frações mássicas de 0,5, 0,0, 0,2 e 0,3, respetivamente) com o qual foi possível obter um rendimento de 126 mg_{PAC}/g_{BM} (grau de polimerização médio (mDP)=6,5). Os melhores resultados da extração com aquecimento por irradiação com micro-ondas foram obtidos com um sistema de SE ternário composto por cloreto de colina, ácido lático e água (frações mássicas de 0,36, 0,39 e 0,25, respetivamente). Neste caso, o tempo de extração foi consideravelmente menor quando comparado com o processo de maceração simples (3,56 min ao invés de 1h) e o rendimento foi de 135 mg_{PAC}/g_{BM} (mDP=7,2). Por fim, o sistema otimizado de maceração simples foi utilizado na uniformização de extratos a partir de diferentes bagaços de uva do qual foi possível obter extratos com características específicas, nomeadamente valores de mDP de 6,5 ou 7,5, utilizando para o efeito solventes com uma composição específica a cada bagaço de uva e valor de mDP.

Finalmente foram estudadas algumas aplicações das PACs, nomeadamente a avaliação da atividade antioxidante na presença de SE e o efeito do mDP no processo de síntese de nanopartículas de ouro.

Destes ensaios conclui-se que, em ensaios celulares *in vitro* a atividade antioxidante das PACs pode ser melhorada cerca de 45% quando combinadas com uma solução a 2 g/L de betaína e ureia, em proporções mássicas iguais. Demonstrou-se também que PACs de elevado mDP podem ser utilizadas no processo de síntese de nanopartículas core-shell de ouro-PAC em que estas desempenham simultaneamente os papéis de agente redutor e estabilizador.

keywords

Proanthocynidin, eutectic solvent, grape pomace, extraction, microwave, degree of polymerization, antioxidant activity, nanoparticle synthesis

abstract

Proanthocyanidins (PACs) are polymeric phenolic compounds composed by catechin and its derivatives that are normally obtained from dedicated crops such as quebracho (*Schinopsis balansae*) and used in the production of leather, wood agglomerates and wine maturation.

The most commonly employed PAC's extractions methods are characterized by the low specificity and efficiency which limits their use in conventional and novel applications with added value. Therefore, the development of innovative extraction methods based on more sustainable biomass sources becomes imperative.

Eutectic solvents (ESs) are normally described as being composed of a pair of compounds, a hydrogen bond donor and a hydrogen bond acceptor, have been proposed as a viable alternative to conventional solvents and are one of the most promising solutions in the improvement of extraction processes based on conventional organic solvents. This is due to their low-cost, easiness of preparation, biocompatibility and ability of being tailor-made to a specific application.

Within the circular economy concept there has been a growing interest in the use of agroforestry by-products as biomass source for the production of added value compounds and PACs are no exception. Among the available options, grape pomace, a by-product from the wine industry, is one of the most relevant by-products for the obtention of PAC rich extracts due to the high annual production at a global scale, and specifically in Portugal, and because of the high contents of PACs in these byproducts.

Hence, the main objective of this thesis is the improvement of the PAC's extraction from grape by-product using ESs aiming at obtaining fractions with increased purity and with well-defined properties, as well as to explore PAC's utilization in added value applications.

abstract

A large part of the information presented here is focused on the study of the effect of ESs different combinations in PACs extraction from grape pomace. In this context, the processes of simple maceration or in combination with microwave irradiation induced heating were optimized by response surface methodology (RSM). Relatively to the simple maceration process it was concluded that the best results were obtained with a quaternary ES system based on choline chloride, glycerol, ethanol and water (mass fractions of 0.5, 0.0, 0.2 e 0.3, respectively) from which was possible to obtain an extraction yield of 126 mg_{PAC}/g_{BM} (mean degree of polymerization (mDP)=6.5). The best results for microwave-assisted extraction were obtained with a ternary ES system based on choline chloride, lactic acid and water (mass fractions of 0.36, 0.39 e 0.25, respectively). In this situation, the extraction time was considerably shorter when compared with the simple maceration process (3.56 min instead of 1h) and the extraction yield was 135 mg_{PAC}/g_{BM} (mDP=7.2). Lastly, the optimized simple maceration system was used in the normalization of extracts obtained from different sources from which was possible to obtain extracts with specific characteristics, namely, mDP values of 6.5 or 7.5, using for that purpose specific solvent compositions for each grape pomace and mDP value.

Finally, some PACs' applications were explored, namely, evaluation of their antioxidant activity in the presence of PACs and mDP's effect in the synthesis of gold nanoparticles.

From these experiments it was concluded that, in *in vitro* cellular assays, PACs' antioxidant activity could be increased by 45% by combining them with betaine and urea solution in equal mass fractions at a concentration of 2 g/L. Furthermore, it was demonstrated that PACs with high mDP can be utilized in the synthesis of gold-PAC core-shell nanoparticles in which PACs play a simultaneous role of reducing and stabilizing agent.

Abbreviations

PAC – Proanthocyanidin

mDP – Mean degree of polymerization

%Gal – Galloylation percentage

DMACA - 4-dimethylaminocinnamaldehyde

RP-HPLC – Reverse phase-High performance Liquid Chromatography

DP – Degree of polymerization

NP-HPLC – Normal phase-High performance Liquid Chromatography

PL – Phloroglucinolysis

TL – Thiolysis

MS – Mass spectrometry

C – Catechin

EC – Epicatechin

GC – Gallocatechin

EGC – Epi-gallocatechin

IS – Internal standard

ESI – Electrospray ionization

APCI – Atmospheric pressure chemical ionization

MS/MS – Tandem mass spectrometry

QM – Quinone methide

RDA – Retro-Diels-Alder

HRF – Heterocyclic ring fission

MALDI – Matrix-Assisted Laser Desorption/Ionization

TOF – Time of flight

LC – Liquid chromatography

SLE – Solid-liquid extraction

Y_{PAC} – Proanthocyanidin extraction yield

GP – Grape pomace

MAATPE – Microwave-assisted aqueous two-phase extraction

MAE – Microwave-assisted extraction

UAE – Ultrasound-assisted extraction

ASE – Accelerated solvent extraction

IL – Ionic liquid

ES – Eutectic solvent

HBA – Hydrogen bond acceptor

HBD – Hydrogen bond donor

DES – Deep eutectic solvent

NADES – Natural deep eutectic solvent

RSM – Response surface methodology

ChCl – Choline chloride

Bet – Betaine

Pro – Proline

Ur – Urea

MalA – Malic acid

Glu – Glucose

Glyc – Glycerol

%BM – Percentage of biomass

Y_{CH} – Carbohydrate extraction yield

LacA – Lactic acid

CitA – Citric acid

TMSP – Trimethylsilyl propanoic acid

CH – Carbohydrate

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

DCFDA – Dichlorofluorescein diacetate

PRM – Proanthocyanidin reference material

ROS – Reactive oxygen specie

NP – Nanoparticle

AuNP – Gold nanoparticle

TEM – Transmission electron microscopy

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1. Introduction

Adapted from: Neto, R.T.; Santos, S.A.O.; Oliveira, J.; Silvestre, A.J.D. Biorefinery of high polymerization degree proanthocyanidins in the context of circular economy. *Ind. Crops Prod.* **2020**, *151*, 112450

1.1. Structural Features of Proanthocyanidins

Proanthocyanidins (PACs) are a subgroup of phenolic compounds which are the largest class of plant secondary metabolites, consisting of aromatic compounds with one or more hydroxyl groups. The most common scheme used for the classification of phenolic compounds is based on the molecule's carbon skeleton (table 1.1) and can range from simple phenols (C_6), such as catechol and phloroglucinol, up to flavonoids (C_6-C_3-C_6), such as catechin and quercetin, with oligomers and polymers of the mentioned molecules also occurring. At the moment, more than 10000 different phenolic compounds have been identified, which is indicative of the structural and functional diversity found within the phenolic compound family [1].

Flavonoids represent over half of the phenolic compound's structural diversity, accounting for more than 7000 structures identified so far [2], and are based on the flavan skeleton that has a general structure composed of 15 carbon atoms, with two benzenic rings (A and B) and a heterocyclic ring (C) (figure 1.1). They are present in almost all plant tissues but besides color the role of flavonoids in leaves, seeds and other vegetative organs is still not fully elucidated [3]. Nevertheless, several connections have been established between this type of metabolites and plant defense mechanisms against external stress factors, such as protection against UV radiation [4], extreme temperatures, both high [5] and low [6] and heavy metals tolerance, e.g. aluminum [7]. As most phenolic compounds, flavonoids present high antioxidant potential and are believed to be involved in the *in vivo* elimination process of H_2O_2 [8]. In addition, they are also involved in the biological defense mechanism against fungi, such as *Rhizoctonia solani* [9] and herbivore animals, such as insects [10] or mammals [11].

Apart from conferring plants tolerance against several stress factors, flavonoids have also been associated with regulatory functions such as sexual reproduction [12], facilitating agrobacterium infection [13] and nodulation [14] in leguminous plants, mycorrhiza development in superior plants [15], auxin modulation [16] and seedling development [17]. In fact, flavonoids and especially PACs, are so important for seeds that when they are missing, seeds show significant lower resistance and longevity [18]. In addition, as mentioned above, color is an obvious characteristic of flavonoids and it plays an important role in pollinators signaling, both in the visible range [19] and also in the UV [20] ranges.

Flavonoids can be divided into five categories (figure 1.1), namely, flavones, flavanones, flavonols, flavanols and anthocyanidins that are based on the presence or absence of a carbonyl group at C-4, a hydroxyl group at C-3 and on the saturation degree of the C ring.

Table 1.1. Phenolic compounds molecular skeleton and respective classes [21]

No. of carbon atoms	Molecular skeleton	Class	Examples
6	C_6	Simple phenols Benzoquinones	Catechol, Phloroglucinol 2,6-Dimethoxybenzoquinone
7	C_6-C_1	Phenolic acids Phenolic aldehydes	Gallic acid 2-Hydroxybenzaldehyde
8	C_6-C_2	Acetophenones Phenylacetic acids	Acetophenone 4-Hydroxyphenylacetic acid
9	C_6-C_3	Hydroxycinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic acid Eugenol Umbelliferone Thunberginol A Eugenin
10	C_6-C_4	Naphthoquinones	Juglone
13	C_6-C_1-C_6	Xanthonoids	Xanthone
14	C_6-C_2-C_6	Stilbenoids Anthraquinones	Resveratrol 1,3,8-trihydroxyanthraquinone
15	C_6-C_3-C_6	Chalconoids Flavonoids Isoflavonoids Neoflavonoids	Chalcone Quercetin Genistein Dalbergin
18	$(C_6$-$C_3)_2$	Lignans Neolignans	Pinoresinol Eusiderin
30	$(C_6$-C_3-$C_6)_2$	Biflavonoids	Amentoflavone
Polymeric	$(C_6$-$C_3)_n$ $(C_6)_n$ $(C_6$-C_3-$C_6)_n$ C_6-$(C_6$-$C_1)_n$	Lignins Catechol melanins Proanthocyanidins Hydrolysable tannin	

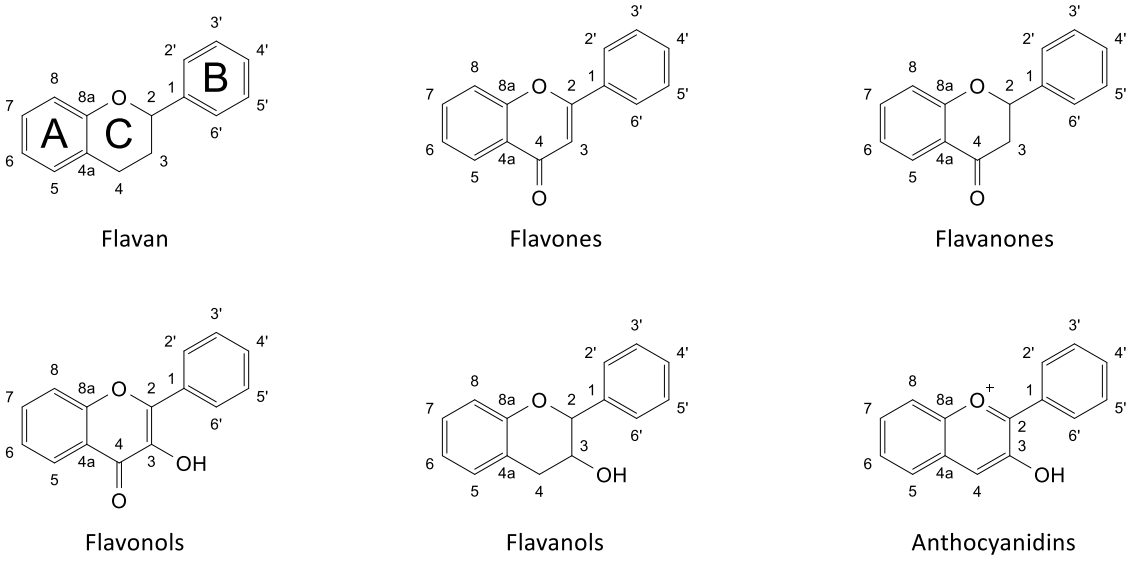
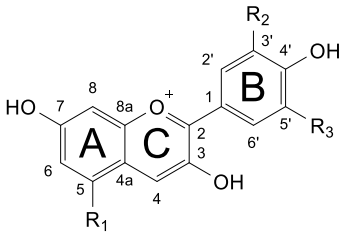


Figure 1.1. General chemical structure of the different flavonoid families

PACs are oligomers or polymers of flavanols that upon acid-catalyzed depolymerization give rise to anthocyanidins monomers and are the most abundant flavonoid type of compounds found in plants. Their classification depends on the anthocyanidins obtained upon depolymerization, i.e. procyanidins, prodelphinidins, profisetinidins, propelargonidins, prorobinetinidins or proguibourtinidins when cyanidins, delphinidins, fisetinidin, pelargonidins, robinetinidins or guibourtinidins are obtained, respectively (figure 1.2) [22].



Anthocyanidins	R1	R2	R3
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Fisetinidin	H	OH	H
Pelargonidin	OH	H	H
Robinetinidin	H	OH	OH
Guibourtinidin	H	H	H

Figure 1.2. Structures of anthocyanidins found in PACs

In PACs, the linkage between monomeric units occurs mostly between carbon C-4 of the upper unit and carbon C-8 of the lower one (B-type) (figure 1.3C), although linkages between C-4 and C-6 are also considered B-type (figure 1.3B). A mixture of both B-type linkages can also be observed in the same molecule as in the case of crown PACs (figure 1.3D). In some cases, an extra ether linkage is found between C-2 and O-5 or O-7 (A-type) (figure 1.3A). Additionally, esters of gallic acid with the

hydroxyl group at C-3 can also occur in both types of PACs, further contributing to their high structural diversity.

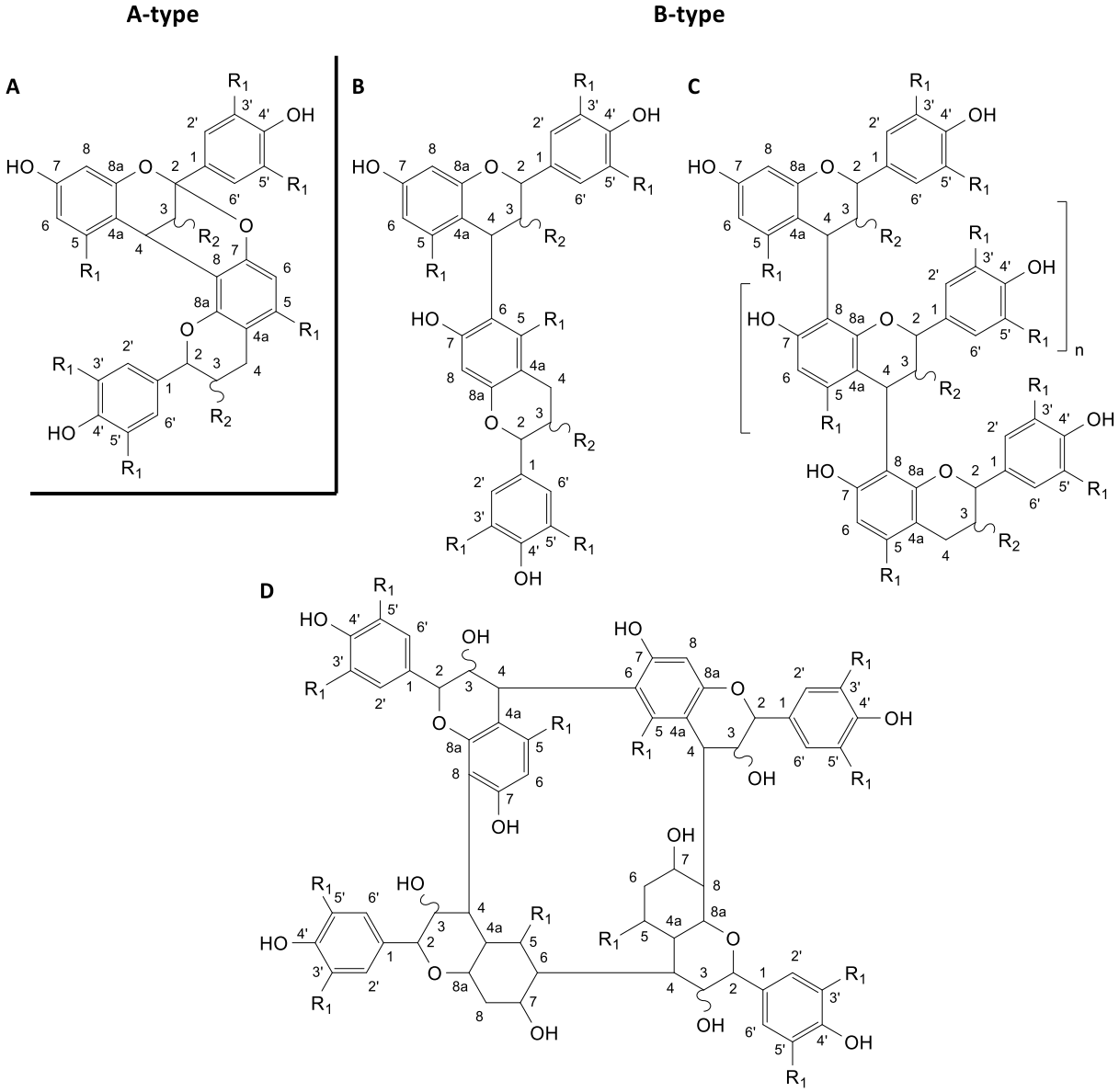


Figure 1.3. A and B-type PAC's structures (R_1 = H or OH; R_2 = OH or galloyl)

As mentioned above for flavonoids, the function of PACs in plants is still largely discussed but there is evidence that they represent the first line of defense against microbial pathogens, either by inhibiting the germination of some fungal spores [23] or by depleting the media from iron which inhibits bacteria growth [24], and against herbivory from insects (due to toxicity [25]) or from mammals (due to nutrient sequestration [26]). These are related with well-known properties of PACs, namely, protein precipitation/agglomeration due to phenyl groups stacking and hydrogen bonding [27], metallic ions complexation which is believed to rely on chelation by the phenolic

hydroxyl groups of the B ring [28] and enzymatic inhibition through a mixed, non-competitive mechanism in which it binds to the enzyme at a non-specific sites [29].

1.2. Characterization and Quantification of Proanthocyanidins

PACs' molecular characterization and quantification are often laborious tasks due to their structural diversity. Nevertheless, these are important steps without which correct assessments concerning PACs' content and their molecular characteristics are impossible. In general, molecular characterization of PACs consist in the determination of mean degree of polymerization (mDP), galloylation percentage (%Gal), and percentage of each type of monomer (%monomer). Analytical methods for characterization and quantification of PACs can be divided in three major groups of techniques, namely, colorimetry, chromatography, and mass spectrometry.

1.2.1. Colorimetry

Colorimetric methods were the first to be developed for PAC's analysis and are still largely used in modern labs. These methods are mostly used for PAC quantification and have the advantage of being extremely cheap, simple, and fast and therefore, are often used for the preliminary analysis of PACs. Nevertheless, these techniques are not specific and have an array of interfering compounds that make them poorly reproductible between distinct types of samples and purity degrees [30]. The methods referred to as "acid butanol", "4-dimethylaminocinnamaldehyde (DMACA)" and the "vanillin" assays are the most frequently employed.

Acid butanol assay is based on the acid-catalyzed cleavage of PACs into their terminal and extension units, flavan-3-ol and anthocyanidin, respectively, from which the resulting red color of anthocyanidins can be measured at 550 nm as described by Porter et al. [31] (figure 1.4). The addition of Fe^{3+} ions was also described by Porter et al. as being fundamental in acid butanol assay due to the fact that transition metals work as catalysts in the autoxidation process of acidic depolymerization of PACs, contributing to higher sensibility and linearity range of the method, along with lower reaction times, minimizing the influence of structural differences that result from using different PACs sources [31].

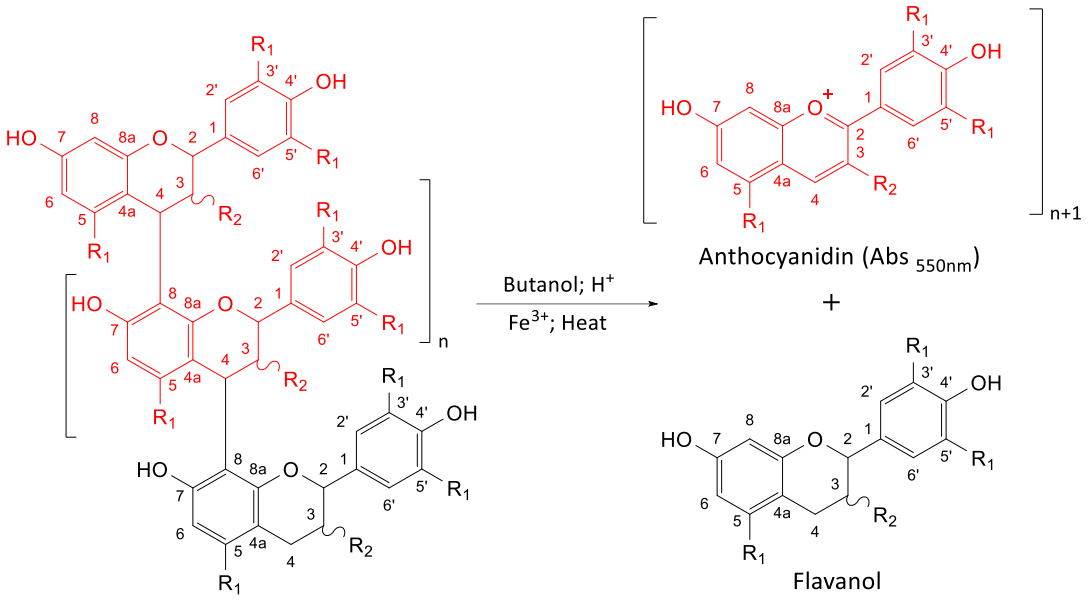


Figure 1.4. Acid butanol assay chemical reaction (R_1 = H or OH; R_2 = OH or galloyl)

There are no studies regarding the effect that PAC's mDP have on the relationship between total PACs content and the intensity of the resulting absorbance measurements. This is particularly critical if the material used as standard has a low mDP, since most of the PACs mass will not contribute to color production (figure 1.4) leading to overestimated results. Unfortunately, there is no commercial polymeric PAC reference material that might be used without the risk of under or over estimating PAC content. Therefore, the use of PACs isolated from the same source is recommended so that the structural interferences mentioned above are minimized.

4-Dimethylaminocinnamaldehyde (DMACA) assay is based on the preferential reaction of DMACA with the carbon C-8 of the A-ring in acidic conditions (normally performed at room temperature for 20 min), forming a colored compound detectable at 640 nm [32] (figure 1.5). In most cases, C-8 is only available for reaction in the last extension unit (one reaction site per molecule) resulting in a molar-based quantification [33] that does not take into account the PAC's degree of polymerization (DP). Nonetheless, PACs containing interflavanic bonds between C-4 and C-6 have an additional free C-8 which contributes to inconsistencies in quantification.

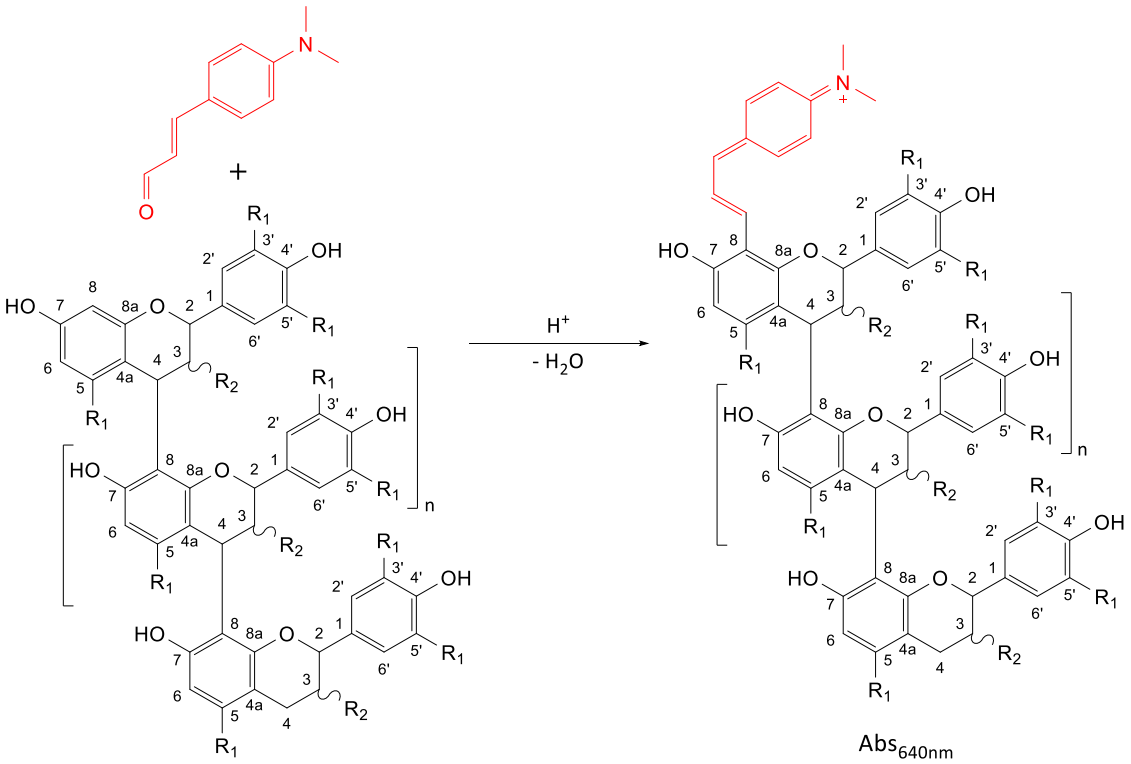


Figure 1.5. DMACA assay chemical reaction (R_1 = H or OH; R_2 = OH or galloyl)

Similarly, the vanillin assay is also carried out in acidic conditions, but the reaction occurs between vanillin and the carbon C-6 of the A-ring and therefore is not limited to the last extension unit (figure 1.6). However, due to steric hindrance, the reaction does not occur in all the positions of the PAC polymeric chain contributing to some inconsistency of the analytical results [34]. This reaction is also highly affected by temperature and therefore it should be carefully controlled [35]. The absorbance maximum of the resulting compound is observed at 500 nm making it susceptible to anthocyanins interference.

Similarly, to acid butanol assay, these last two methods are affected by structural differences and consequently, PACs isolated from the same source should be used as reference [33,36].

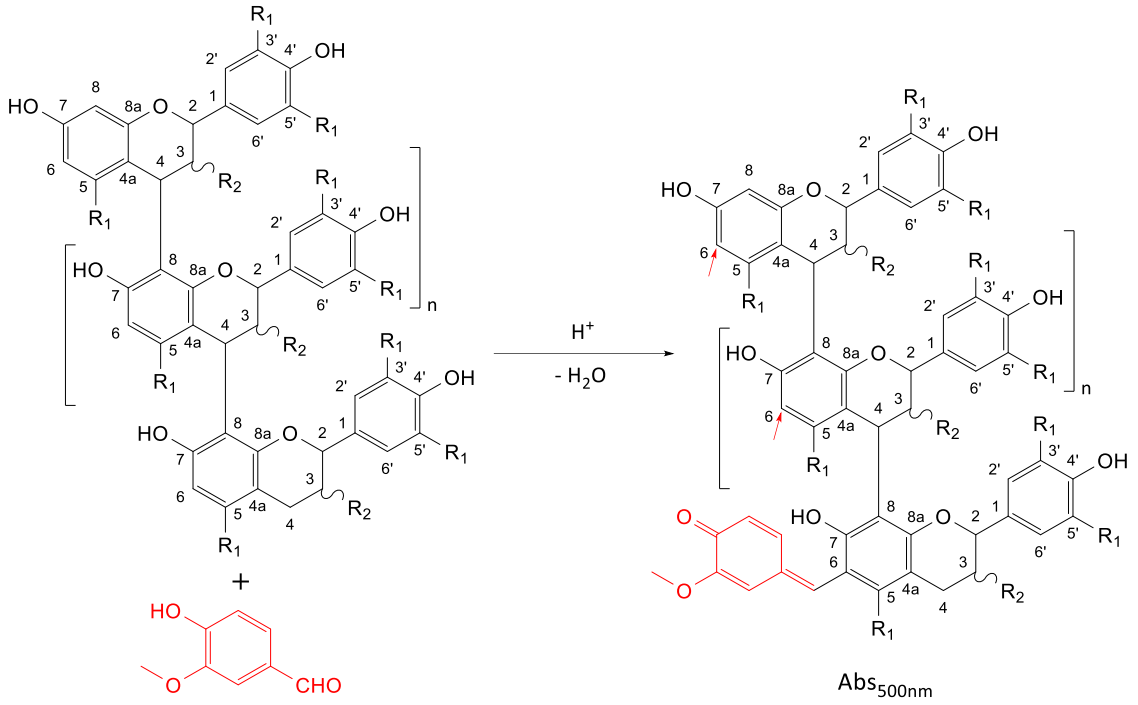


Figure 1.6. Vanillin assay chemical reaction (arrows indicate possible reaction sites; R_1 = H or OH; R_2 = OH or galloyl)

1.2.2. Chromatography

If in addition to PAC quantification one intends to do the molecular characterization of the PAC extract, chromatographic techniques are often employed. These are particularly helpful in the analysis of complex mixtures such as PACs extracts and help in the obtention of a clear picture of their profile.

Reverse phase-HPLC (RP-HPLC) with C-18 columns is the most used analytical chromatographic technique and is capable of easily analyzing PACs with low DP, being capable of quickly separate and identify monomers, dimers, and trimers and correctly assigning type of monomers and linkages upon coupling with adequate spectroscopic techniques or comparison with analytical standards [37] (figure 1.7).

Nevertheless, its application to the analysis of PACs with high DP is limited because separation in RP-HPLC columns is based on compounds' polarity which varies between isomers with the same molecular weight. This is problematic because, as mentioned above, the number of isomers increases exponentially with the DP, resulting in reduced amounts of each specific compound and in an increase in compounds with similar polarities. The same is true to proanthocyanidins

containing different linkages, between C-4 and C-6 instead of the more common ones between C-4 and C-6 and for PACs containing different types of monomers.

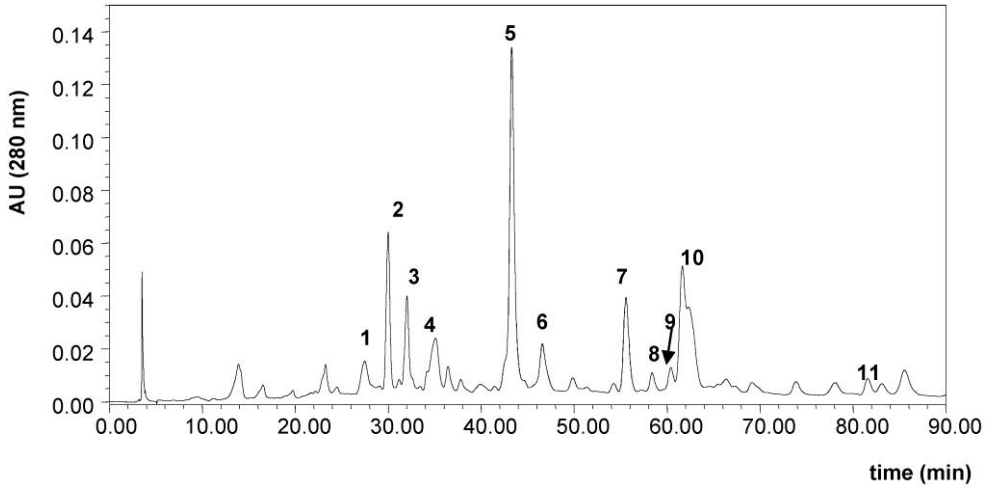


Figure 1.7. Representative chromatogram of low DP proanthocyanidins analysis by RP-HPLC; 1 - procyanidin dimer B3; 2 - procyanidin dimer B1; 3 - procyanidin trimer T2; 4 - procyanidin dimer B4; 5 - procyanidin dimer B2; 6 - procyanidin dimer B2-3-O-gallate; 7 - procyanidin dimer B2-3'-O-gallate; 8 - (−)-epicatechin; 9 - procyanidin dimer B1-3-O-gallate; 10 - procyanidin trimer C1; 11 - (-)-epicatechin-3-O-gallate (reproduced from [37])

In order to obtain a separation based on DP, normal phase-HPLC (NP-HPLC) should be used. This chromatographic process can be carried out using a diol-type HPLC columns (silica functionalized with dihydroxypropyl groups) [38] or a bare silica columns [39]. The utilization of the latter has fallen out of favor when compared with the former as it requires the use of dichloromethane as an eluent.

In NP-HPLC, PACs elute with increasing DP and low isomer separation but with decreasing resolution, as depicted in figure 1.8 where PACs with higher DP present broader peaks. Hence, the maximum separable DP varies depending on the column manufacturer [40] and eluents used and it can go as high as 14 [41], with co-elution for higher DP.

Quantification with this method is difficult due to the inexistence of commercial standards for calibration curve construction. Nevertheless, it is the only method that allows for a direct detection of each PACs' DP and for their separation and subsequent recovery, if preparative scale is used [38].

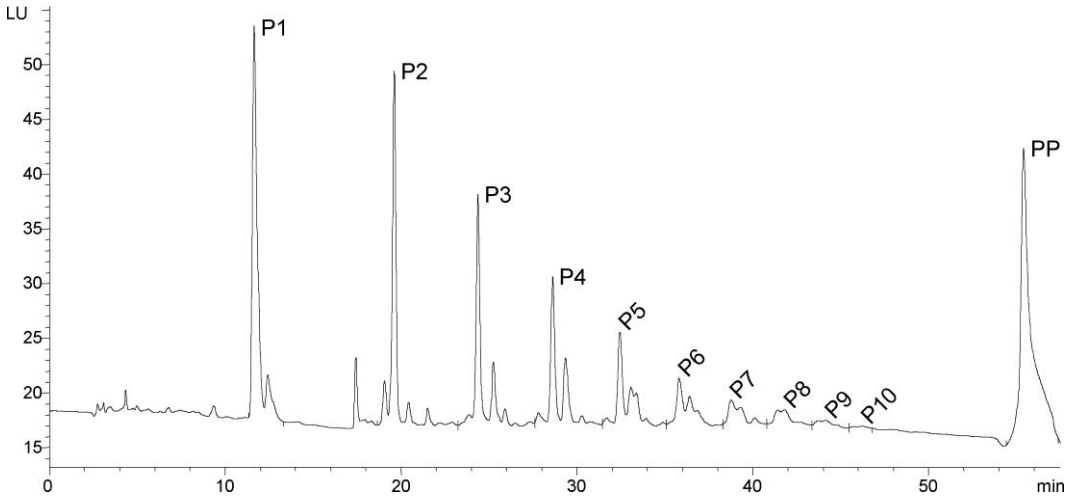


Figure 1.8. Representative chromatogram of oligomeric PACs analysis by NP-HPLC; P1-P10 - proanthocyanidin with specific degrees of polymerization; PP - polymeric proanthocyanidins (reproduced from [42])

Regardless of the chromatographic/elution system used, there will be a maximum DP at which separation is no longer possible and PACs will coelute. To overcome this situation, methods were developed in which the complete acid-catalyzed depolymerization of PACs is achieved in the presence of a nucleophile. These allow for the differentiation of terminal and extension units either by the reaction with the phloroglucinol (figure 1.9) or benzylmercaptan (figure 1.10), in a process known as phloroglucinolysis (PL) [43] or thiolysis (TL) [44], respectively. More specifically, terminal units will remain unaltered while extension units will react with the respective nucleophile.

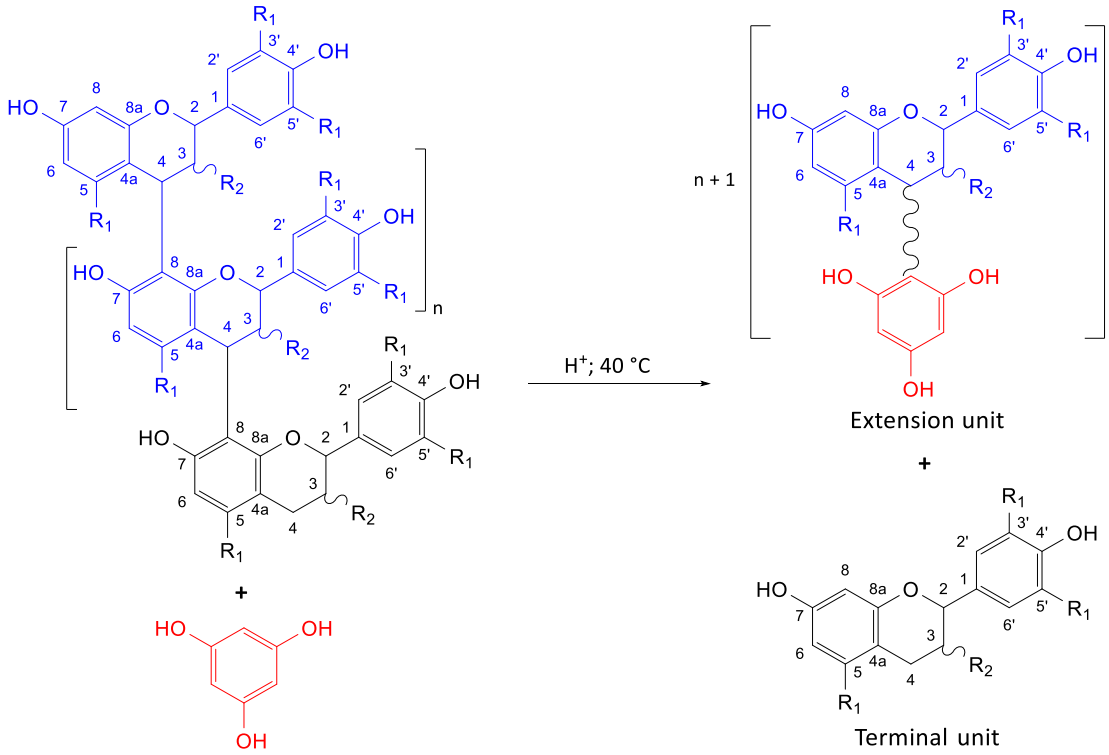


Figure 1.9. Phloroglucinolysis assay chemical reaction (R_1 = H or OH; R_2 = OH or galloyl)

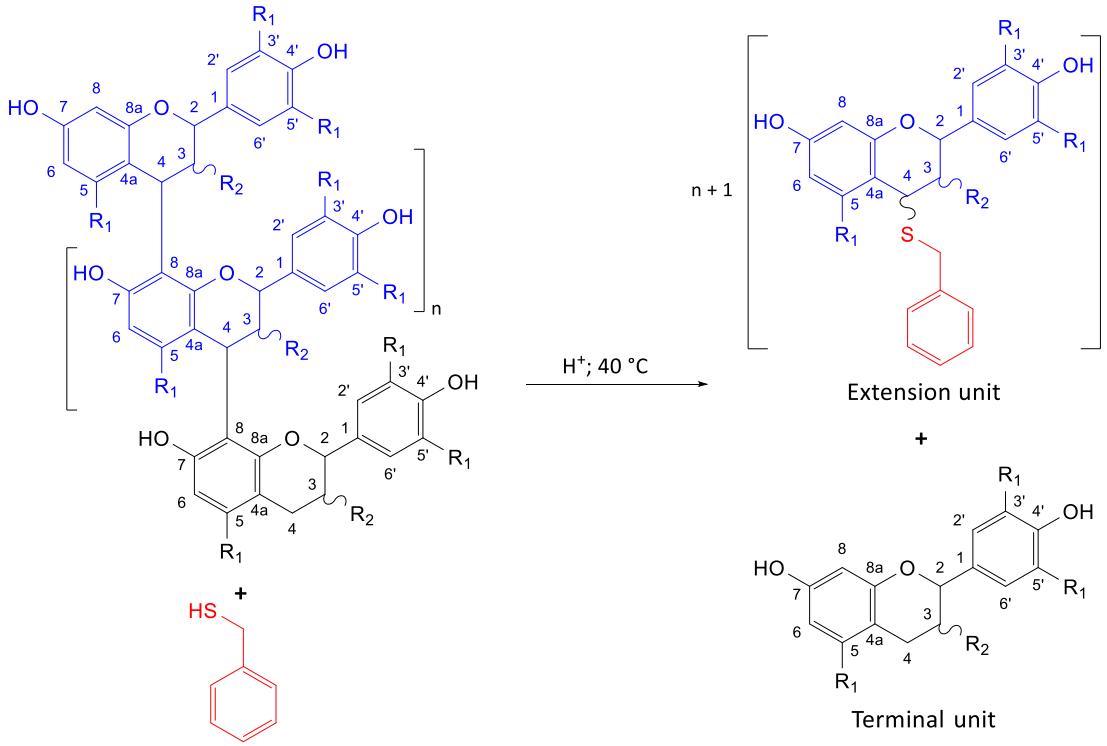


Figure 1.10. Tiolysis assay chemical reaction (R_1 = H or OH; R_2 = OH or galloyl)

The resulting depolymerization mixture is resolved by RP-HPLC (figure 1.11) and peak identification is made by comparison with analytical standards for terminal units and by mass spectrometry (MS)

in negative mode for extension units [45] since the expected masses of the reaction products between the extension units and the nucleophiles can be calculated (table 1.2). Quantification of terminal and extension units is normally made with a calibration curve made with (+)-catechin from which is possible to calculate the mDP (equation 1.1), %Gal (equation 1.2) and %monomer (equation 1.3).

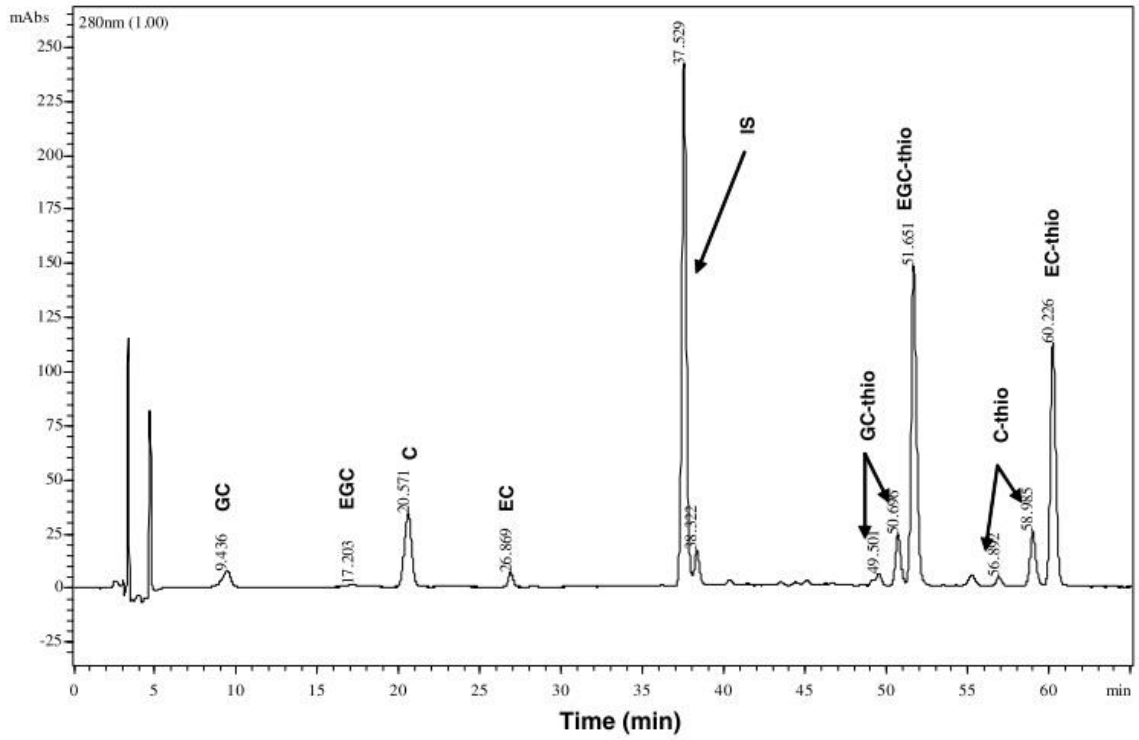


Figure 1.11. Representative RP-HPLC chromatogram of thiolysis products; C - catechin; EC – epicatechin; GC - gallocatechin; EGC - epigallocatechin; IS - internal standard, dihydroquercetin; C-thio - catechin benzylthioether; EC-thio - epicatechin benzylthioether; GC-thio - gallocatechin benzylthioether; EGC-thio - epigallocatechin benzylthioether (reproduced from [46])

Table 1.2. Molecular weight (MW) of naturally occurring grape catechins and respective molecular ions of derivatives resulting from phloroglucinolysis (PL) / thiolysis (TL)

Molecule	MW	PL MW $[M-H]^-$	TL MW $[M-H]^-$
(+)-catechin	290.08	413.09	411.09
(+)-gallocatechin	306.07	429.08	427.09
(-)-epicatechin	290.08	413.09	411.09
(-)-epigallocatechin-3-gallate	458.08	581.09	579.10
(-)-epicatechin-3-gallate	442.09	565.10	563.10

Equation 1.1. $mDP = \frac{\sum monomer^{terminal} + \sum monomer^{extension}}{\sum monomer^{terminal}}$

Equation 1.2. $\%Gal = \frac{\sum monomer^{galloylated}}{\sum monomer^{galloylated} + \sum monomer^{non-galloylated}} \times 100$

Equation 1.3. $\%monomer^{x} = \frac{\sum monomer^{x}}{\sum monomer^{x} + \sum monomer^{non-x}} \times 100$

1.2.3. Mass Spectrometry

RP and NP-HPLC can be coupled with mass spectrometers composed by several combinations of ionization sources and type of analyzers that enable the molecular characterization of chromatographic peaks even in the case of badly resolved ones. The most commonly used ionization source is Electrospray Ionization (ESI) [47] although Atmospheric Pressure Chemical Ionization (APCI) has also been described for the same purpose [48].

In general, PACs are analyzed in negative mode and due to the utilization of soft ionization techniques such as ESI and APCI, low levels of fragmentation are observed. In addition, the molecular ions that are preferentially formed are $[M-H]^-$ ions for PACs with lower molecular weight (up to tetramers) [42], while higher molecular weight PACs having the tendency to form multiple charged ions , such as $[M-4H]^{-4}$ for hexadecamers [49].

A typical MS spectrum of a PAC will consist of a molecular ion in which the PAC's molecular weight is described by the equation 1.4, that will have one or more charges, depending on DP, resulting in the correspondent m/z value. Multiple charged ions can be identified by analyzing the mass differences of the resulting isotopic pattern.

Equation 1.4. $MW\ PAC = (\sum_{i=1}^{n} MW\ monomer^{n}) - 2(n-1)$

The mass differences between PACs that are most commonly observed are 16 Da or multiples of it corresponding to the presence or absence of hydroxyl groups in the A and/or B rings of a given monomer (figure 1.12) [50]. Additionally, differences of 152 Da or multiples of it can also be found corresponding to the presence of one or more gallate moieties esterified at C-3 of the C ring (figure 1.12) [50].

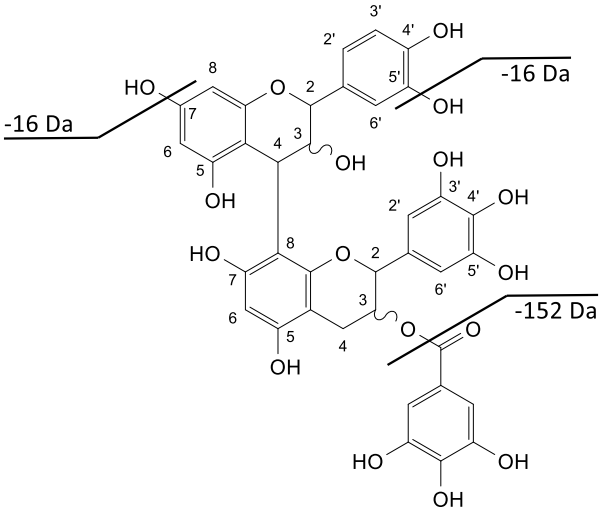


Figure 1.12. Commonly observed mass differences in PACs

Despite the large amount of information that can be obtained just by employing LC-MS, in some situations further information regarding chemical structure is needed. This comes from the fact that some compounds, such as catechin and robinetinidol or procyanidin dimer with two galloylations and procyanidin trimer with one galloylation have the same molecular weight, 290 and 882 g/mol, respectively, making them indistinguishable. To overcome this situation tandem mass spectrometry (MS/MS) must be employed, a technique in which molecular ions with specific m/z values are isolated and fragmented giving rise to a fragmentation pattern that can be correlated with the molecular structure.

PAC's MS/MS analysis follows three major fragmentation mechanisms, namely quinone methide (QM), retro-Diels-Alder (RDA) and heterocyclic ring fission (HRF) [51] (figure 1.13). The QM cleavage results in two molecular ions with m/z values corresponding to $[M\text{-monomer-2-H}]^-$ and $[monomer\text{-2-H}]^-$ from which PACs with different DPs but the same molecular weight can be distinguished. The RDA and HRF fractionation mechanisms result in the fission of the C ring with the difference that in the former the B ring is removed from the main structure while in the latter the A ring is the one removed. The RDA fractionation gives rise to a molecular ion with a m/z value of [M-(120+16n)-H]- where n equals to the number of hydroxyl groups in B ring and the HRF fractionation gives rise to a molecular ion with a m/z value of [M-(94+16n)-H]-. With this information is possible to assess in which ring the hydroxyl groups are present and distinguish between monomers with the same molecular weight. This methodology has already been applied in the analysis of several types of PACs such as procyanidins [42], prodelphinidins [52], propelargonidins [53] and profisetinidins [54]. Despite the absence of scientific literature of this type of analysis applied to prorobinetinidins and

proguibourtinidins this is most likely related to the low number of studies on these specific types of PACs and not to the impossibility of applying this methodology to them.

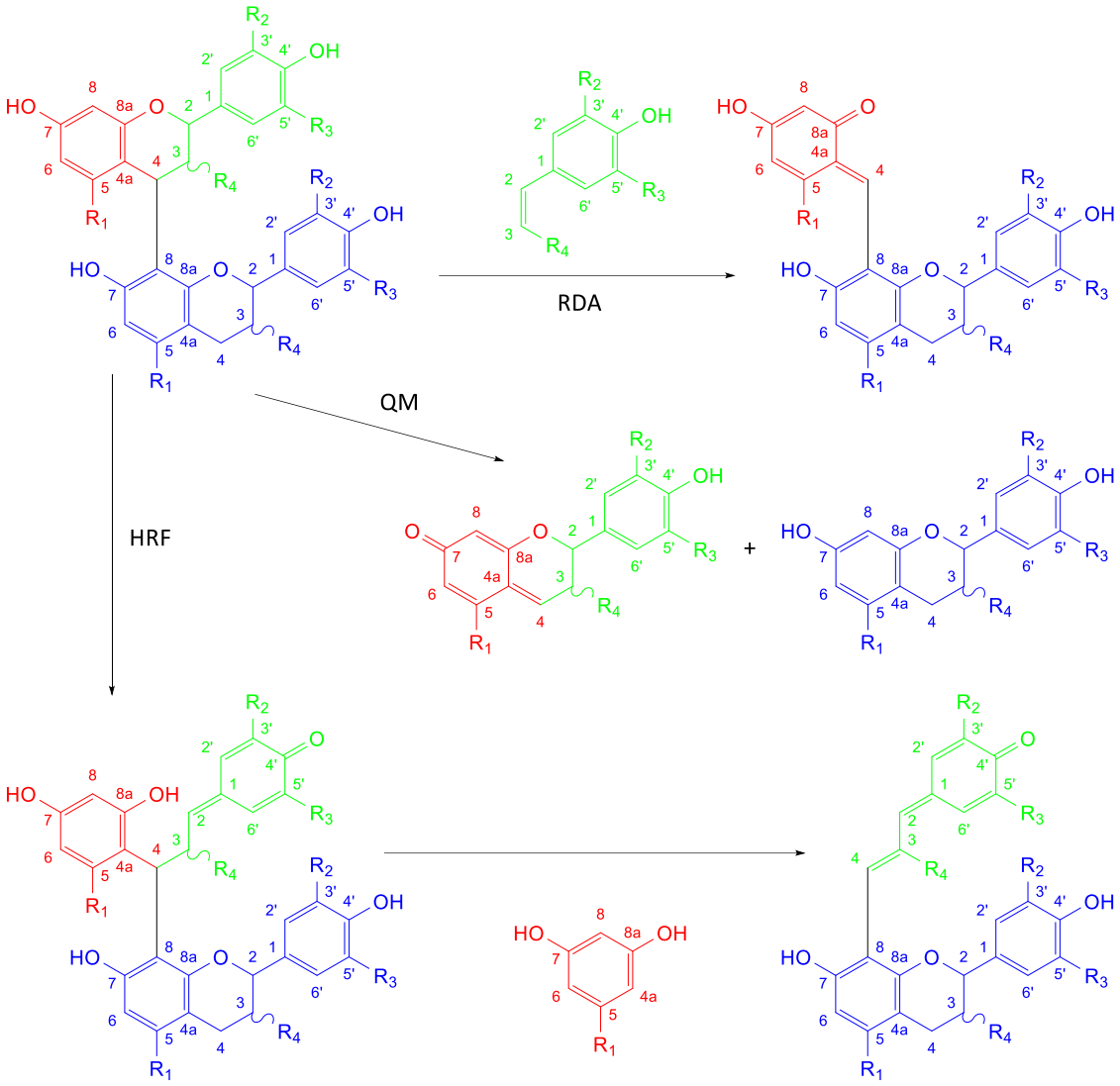


Figure 1.13. Fragmentation mechanisms of PACs; RDA - retro Diels-Alder, QM - quinone methide, HRF - heterocyclic ring fission (R_1, R_2 and R_3 = H or OH; R_4 = H or galloyl)

When dealing with samples that have a high mDP, PAC analysis with ESI may become difficult due to the increasing number of ionization sites that comes with it, resulting in a more complex and difficult signal to interpret which derives from the multiple m/z signals that can be attributed to the same molecule. In these situations, Matrix-Assisted Laser Desorption/Ionization (MALDI) is a more reliable ionization technique because of its ability to ionize PACs with up to 25 monomers and by the fact that it, usually, only produces a single type of ion for each molecule allowing for quick results with no prior chromatographic separation [50]. In addition, Time of Flight (TOF) analyzers that are usually coupled with MALDI, are also able to separate ions with m/z ratios of up to 7000Da

which further enables the use for this type of PACs. The major disadvantage of this technique is the inability of running it on-line which is related with the necessity of mixing samples with a matrix and placing them in a plate in which samples and matrix will be ablated through the use of a laser resulting in a charge transfer from the latter to the first. As far as molecular ions m/z values are concerned, they have a similar behavior as ESI/MS, including fragmentation mechanisms [55], with the only exception being the absence of multiple charged ions (figure 1.14). Interestingly, there are no studies on the use of LC coupled with MALDI ionization which could facilitate the access to further characterization of some PAC samples.

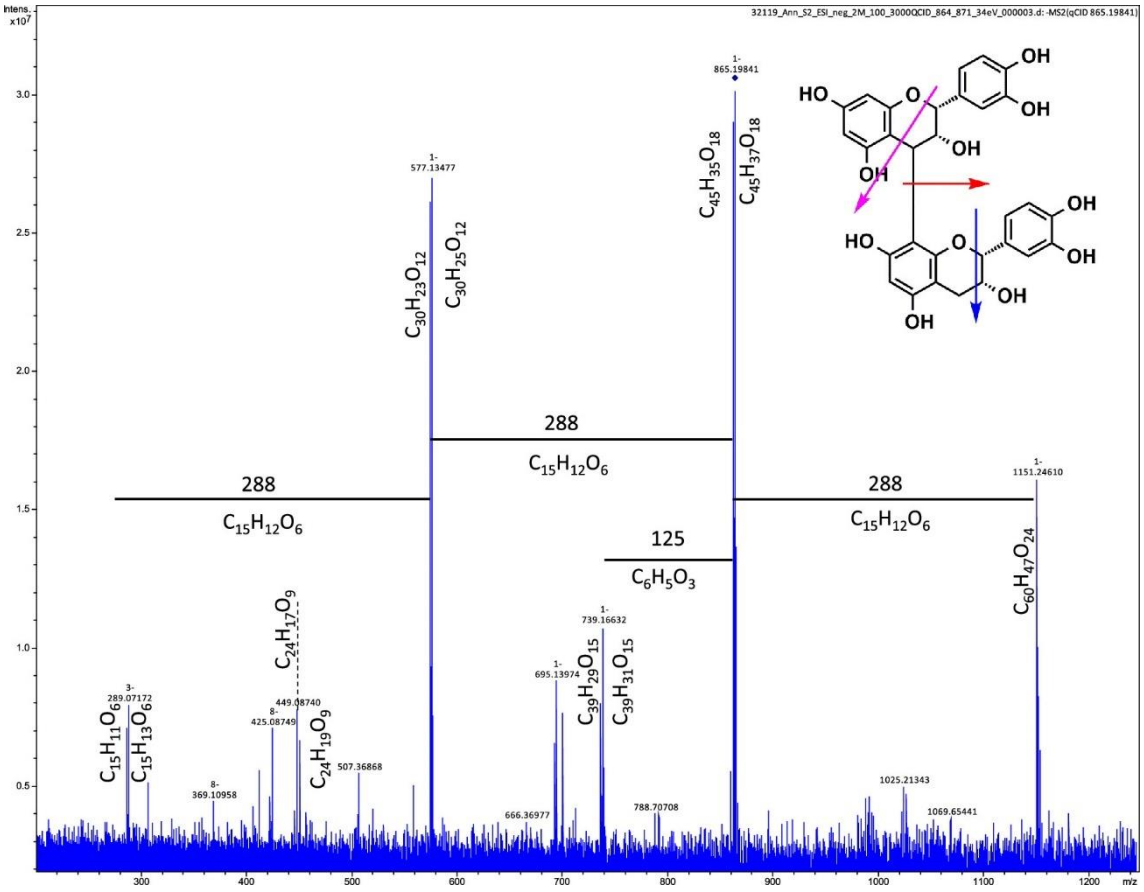


Figure 1.14. Mass spectrum obtained with MALDI-TOF (reproduced from [55])

1.3. Industrial Applications of Proanthocyanidins

Physical chemical properties of PACs, such as their ability to interact non-covalently (but strongly) with proteins and their multiple chemically reactive sites, enable their applications in several areas of modern industry. The majority of the annual tannin production is used in leather manufacture (62.5%), followed by wood adhesives (17.2%), wine production (13.6%), anti-corrosive primes (3.9%) and other niche applications, such as human and cattle nutrition (2.8%) [56].

Leather production is a process in which hides or skins are submitted to a series of treatments in which they become resistant to putrefaction. The great majority of hides/skins utilized for leather manufacture are by-products of the meat industry, without which leather production would not be economically viable, and would represent themselves a great environmental problem if not used for leather production.

The tanning process has several steps, namely, soaking, liming, deliming, pickling, tanning, post-tanning and finishing processes. The steps preceding tanning are mostly used for improving chemicals mobility and to remove non-collagenous components of the hide/skin while tanning is the key step in which the tanning agent will react and stabilize the collagenic component of the hide/skin, transforming it into leather. The tanning agent's binding site on the collagen sequence depends on the type of agent that is used (figure 1.15). When mineral tanning agents, such as chromium or aluminum are used, binding will occur through complexation with carboxylic groups, if the process is made with an aldehyde agent binding will occur through covalent binding between tanning agent and amine groups of the amino acids' side chains, and when PACs are used as tanning agent, phenyl group stacking and hydrogen bonding with carbonyl groups on the main chain are established producing a chemically and biologically stable material.

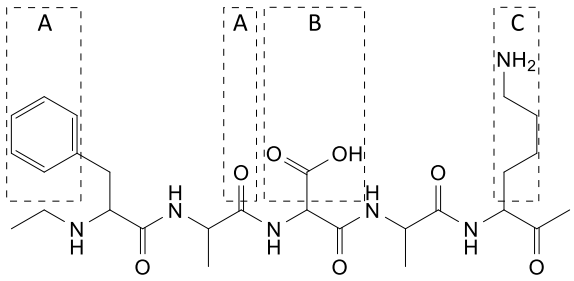


Figure 1.15. Collagen binding sites for different tanning agents. A – PACs; B – mineral; C – aldehyde (adapted from [57])

Despite being an industry that is based on the use of a by-product, leather production is considered to have a high environmental impact associated with the high volume of effluents that are produced, especially with the chromium-based process. As a consequence, and due to the increasing environmental consciousness of law makers and the general public, an increase in the demand of tannin-based leather was verified [58]. This was demonstrated by the release in the market of PAC-based tanning agent extracted from olive leaves¸ with the commercial name wet-green® that is used to produce high-end leather products.

PACs use as a tanning agent, despite being desired has several limitations that derive from the optimum average molecular weight and existence of monomers that are more reactive than others, especially in hybrid tanning processes in which more than one tanning agent is used. The best example is the combination of PACs with oxazolidine, an aldehydic crosslinker, in which the best shrinkage temperatures are obtained when mimosa PACs are used [59] due to the preferable reaction of oxazolidine with monomers in which the C-5' position is hydroxylated such as prodelphinidins and prorobinetinidin [60]. Additionally, the presence of galloylated moieties has also been shown to contribute to the increase the reaction rates between flavonoids and oxazolidine.

In summary, it can be concluded that the successful implementation of PACs in the leather industry depends on the development of extractive methods that allow the obtention of PAC extracts with well-defined characteristics such as %Gal and mDP.

PACs application in wood composites has been steadily increasing and consequently so have the use of wood binders and adhesives. The most commonly used adhesives are based on urea-formaldehyde, melanine-formaldehyde, melanin-urea-formaldehyde and phenol-formaldehyde that represent risks for the environment and human health since formaldehyde has been described as a probable human carcinogenic [61].

PACs implementation in the formulation of wood adhesives has been made by several routes, namely by replacing formaldehyde as a hardening compound by others, such as lignin-based vanillin and vanillin derivatives [62], tris(hydroxymethyl)nitromethane [63] or lignosulfonates [64]. Additionally, it was also showed that PACs obtained from pine bark lead to similar results whether formaldehyde or glyoxal are used as hardening agents [65].

Another strategy for the implementation of PACs is to couple them with other compounds that can improve their binding ability, namely lignin [66] or soy protein [67] coupled with glyoxal/hexamine or urea/hexamine hardeners, respectively. Hexamine, in special as received a lot of attention as a hardening compound due to the ability of establishing tannin-CH_2-NH-CH_2-tannin bonds that are capable of absorbing formaldehyde emissions [62] enabling the use of some formaldehyde in the formulation without resulting in its emission on the latter stages of the wood agglomerate life cycle.

Despite the increasing demand for greener wood binders the low global PAC production still limits their implementation [68] and therefore, steps should be taken in the direction of developing extraction methods that can either improve on the extraction yield or expand the extraction to raw materials derived from agricultural by-products.

Wine industry is one of the areas from which PAC consumption is expected to increase more in the future [56]. PACs are employed in the vinification and wine aging processes resulting in a product with higher sensory qualities and therefore with a higher market value. In fact, the segment of premium wines in United States was estimated to grow between 9 and 13% in 2016 while the lower price range wines market seems to be in decline [69]. Consequently, there is an opportunity to explore the development of new tannin-based products that match this sector specifications and that can further contribute to the improvement of the intrinsic wine characteristics.

Currently, PACs are applied to wines with the intent of protecting them against oxidation [70], improve wines with deficiencies in flavanols and pigments contents [71] and address specific vintages problems with low color stability [72]. PAC's role in color stability is firstly dominated by π-stacking with anthocyanins in a process denominated copigmentation [73]. At later stages of wine maturation the main mechanism of color stability is the direct and indirect reaction of anthocyanins with PACs via aldehyde, resulting in new pigments that are orange or purple, respectively [74] (figure 1.16). In addition, PACs can also be employed to protect the wine against *Botrytis cinerea* damage by inhibiting laccase activity [75].

Despite the lack of studies on the impact of the PAC's fine structure in the aforementioned wine applications it is plausible, as in the other PAC's industrial applications, that differences in terms of monomer type, mDP and %Gal can have a great impact on the intended result.

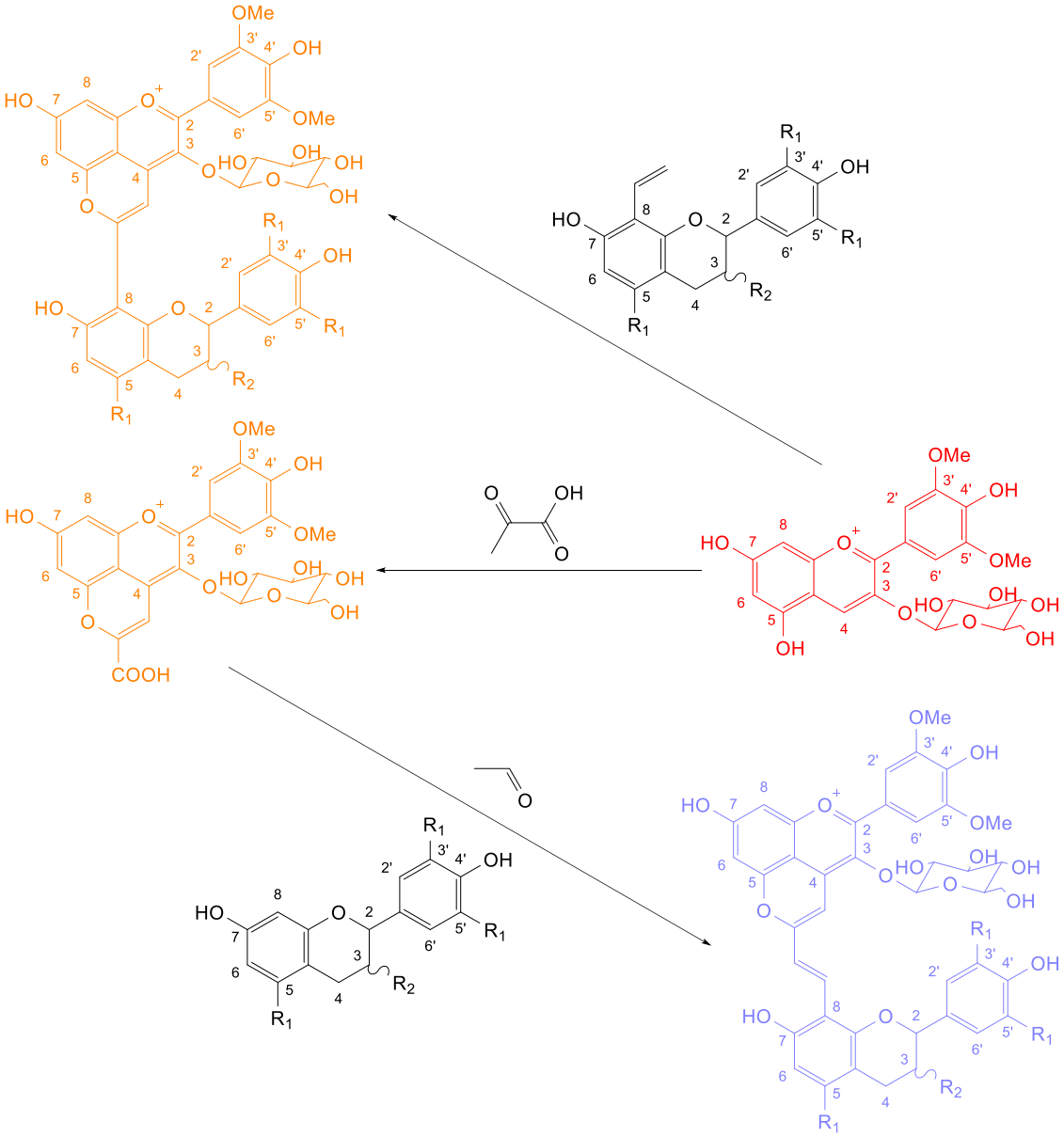


Figure 1.16. Possible reactions of anthocyanin (malvidin 3-o-glucoside) with proanthocyanidin in wine (R_1 = H or OH)

1.4. Novel Applications of Proanthocyanidins

Plant's phenolic compounds have been frequently described as human health promoters [76–81], good candidates for the replacement of synthetic food additives [82,83] and in the development of functional foods [84]. In the same vein, PACs have also been described as having similar properties, namely as anticarcinogenic [85,86], anti-inflammatory [87], antioxidant [88–92] and antimicrobial activities [46,91,93–97].

PACs are characterized by having a high antioxidant potential [88] which is largely related, as in other phenolic compounds, with the presence of phenolic hydroxyl groups. This results in a synergistic effect that makes, e.g. grape seed extract (composed mostly by oligomeric PACs and low molecular weight phenolic compounds), more effective than other commercial antioxidants by themselves, such as vitamin E and C, twenty and fifty times higher, respectively [89]. DP also appears to positively influence the antioxidant potential of PACs, as the increase in the number of monomers leads to an increased antioxidant activity [92, 93]. In a similar way, galloylation also increases antioxidant activity as verified by the comparison of PACs from grape seeds (more galloylated) and skins (less galloylated) [92] which is mostly related to the increase in phenol moieties.

Antimicrobial activity of PACs has also been studied due to the high demand for natural alternatives for microbiological control in food and cosmetic products. In fact, their effect has already been shown against bacteria such as *Escherichia coli* [93], *Staphylococcus aureus* [91], *Peptostreptococcus anaerobius* [46] and *Listeria monocytogenes* [96, 97]. In addition, the inhibitory effect against yeasts was also verified for *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* [96]. Part of the antimicrobial potential appears to be related with their ability of complexing metals that relies on chelation with phenolic hydroxyl groups [28]. In addition, PAC's high DP allows for a better enveloping of the metal ion producing stabler complexes when compared with other phenolic compounds [28]. As with the antioxidant potential, antimicrobial activity appears to be related, at least to some extent, to the DP since no activity is observed for monomers [81], nevertheless, the detailed mechanisms by which antimicrobial activity occurs are still largely unknown.

PAC's anti-inflammatory activity has been demonstrated for cell lines of different types of tissues, namely, macrophages (RAW 264.7 from murine) [98], colon epithelium (Caco-2 and HT-29 from human) [87], skin fibroblast (HDFa from human) [99], lung epithelium (A549 from human) [100] and gastric epithelium (AGS from human) [101]. Interestingly, anti-inflammatory activity was shown to be dependent on DP either by showing maximum activity at a specific mDP value after which the activity decreased [98], or by showing that polymeric PACs were more effective than oligomeric ones [87,101], or by showing that PACs are more effective than other phenolic compounds [99,100].

Due to the antioxidant, antimicrobial and anti-inflammatory effect of PACs, they have been tested in several pathologies in which this type of effects can ameliorate or solve certain conditions. It has been tested for the treatment of breast cancer [85], diabetes [102], oesophageal cancer [86] and

prevention of cataract formation [103] to give some examples in which not only the beneficial effect of PACs is shown but the positive relationship with the DP as well as.

1.5. Conventional Extraction of Proanthocyanidins

Currently, there are several commercially available PACs extracts obtained from different sources such as maritime pine (*Pinus pinaster*), monterey pine (*Pinus radiata*), black wattle (*Acacia mearnsii*), quebracho (*Schinopsis lorentzii*) and grape (*Vitis vinifera*). The general conventional extraction and purification process is depicted in figure 1.17 in which after biomass is dried and milled, it is submitted to a solid:liquid extraction (SLE) with a aqueous solution. Filtration is then used for solids removal and the final solution is concentrated with low-pressure evaporation.

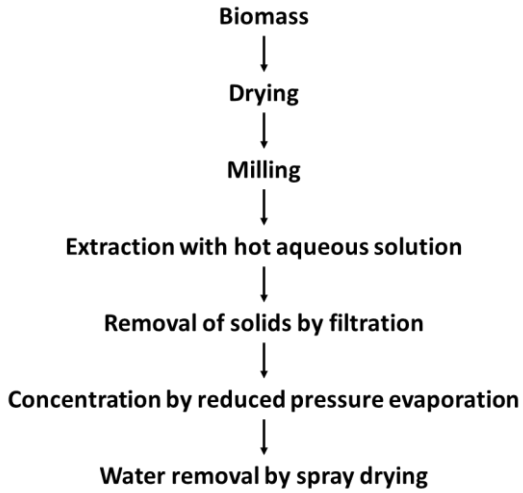


Figure 1.17. Schematic representation of the PACs industrial extraction procedure [104]

The extraction media is most often composed of hot aqueous basic solutions of sodium hydroxide [105] or sodium carbonate and sodium sulfite [106] resulting in extracts that are then purified to different extents depending on the final application. These extraction media have been extensively used due to their ability to either dissolve lignocellulosic matrix as in the case of sodium hydroxide or by promoting PACs solubilization as in the cases of sodium carbonate and sodium sulfite. Nevertheless, these also have a negative impact on the final extracts since PACs can degrade at high pH values [107] and are chemically altered when treated with sodium sulfite [108].

The obtained extracts, despite being easy to produce have low purity, as described by Kardel et al. [109] that observed a PAC content in quebracho and mimosa commercial extracts of 12.3 and 23.5%, respectively. Shirmohammadli et al. [110] reported 33, 29 and 15% for commercial extracts

of mimosa, quebracho and pine, respectively, and Obreque-Slíer et al. [111] reported 7.3% for a grape commercial extract. In addition, these procedures do not take advantage of the whole PACs content present in the biomass that, as shown by Hellström et al. [112], can have varying contents of unextracted PACs depending on the raw material, ranging from 4.3% in 'Valkeakuulas' apple to 62.5% in white grapes.

When dealing with classic SLEs there are parameters that have a higher impact on the final PACs yield (Y_{PAC}), namely, type of solvent, sample to solvent ratio and temperature, which ultimately are related with the solubility of PACs in a specific combination of solvents and temperature.

When considering by-products as PACs source, in general the total extraction yield does not vary too much with values ranging from 16.5% (m/m) from hazelnut skin with 80% aqueous acetone [113] to 24.3% (m/m) from oak bark with 70% aqueous acetone [114], passing through 17.8% (m/m) from black spruce bark with water at 100 °C [115], 19.2% (m/m) from grape marc using ethanol [116] and 20.9% (m/m) from Norway spruce bark with water at 75 °C [117], which might be related with similar lignocellulosic content that by-products possess and that are not solubilized unless harsher solvents/conditions are employed. Nevertheless, the corresponding PAC content varies considerably (41 [113], 277 [114], 230 [115], 50 [116] and 501 $mg_{PAC}/g_{extract}$ [117]) which might be related with the lower PAC content of the original by-product [113], emphasizing the need of properly selecting the raw materials considering their PAC content, and avoiding the use of ineffective solvents such as pure ethanol [116] which, as shown by Pedan et al. [118] and Bosso et al. [119], benefits considerably from the addition of water to the extraction media.

Another very relevant aspect, when extracting PACs with conventional SLEs, and especially when using water as solvent, is temperature. In the literature temperature is always kept above 75 °C [115,117,120,121], while when using organic solvents [116,122] or mixtures of water with organic solvents [113,114,123–130] it is kept much lower and frequently at room temperature with often higher Y_{PAC}s. Therefore, one must always have to choose between the environmental impact of employing organic solvents or the decreased yield and possible sample thermal degradation that comes with employing water-based solvents. In addition, and regardless of the solvent employed, temperature appears to have a negative impact on the mDP of PACs as demonstrated by Ramos et al. [131], who observed an approximately 50% decrease in mDP when increasing the extraction temperature from 40 to 120 °C that, despite the steep increase in overall extraction yield, did not contribute to an increased PAC's purity in the final extract.

Despite the increasing interest in PACs, as discussed in the previous section, they are still very understudied in large part due to difficulties in the extraction and isolation process, especially when compared with other phenolic compounds [132]. Furthermore, obtaining them in a pure form is a complex process with costs that, in many cases, limit their industrial application or even the search for new applications. The price of PACs extracted from grapes can range from 300-400€/kg for a low-purity industrial grape extract, passing through 900€/g for a mixture of oligomers and reaching costs as high as 100€/mg for a pure trimmer.

The conventional SLE methods described above, although largely used in industry, are frequently time consuming and require large volumes of solvent. In addition, PACs with high DP are difficult to extract [35,133,134] and therefore, one must consider alternative paths for the development more efficient and scalable extraction methods. Bearing this, some innovative and hopefully more sustainable extraction methods that might contribute to overcome the mentioned limitations will be discussed in the coming sections.

1.6. The Importance of Circular Economy

The modern agroforestry industry is based on a High Volume-Low Value economical model which requires the processing of large amounts of raw material in order to be profitable. This in turn results in considerable amounts of by-products that, at the moment, have low economical value and are often disposed in a way that has high environmental impact [135]. Due to the increasing environmental awareness of the general population as well as national governments, a lot of pressure has been put in the reduction of the total amount of agroforestry by-products as well as in their valorization with the goal of developing a biorefinery based economy in which, ideally, no waste would be produced [136].

The European Commission [136] defines waste as any substance or object which the holder discards or intends or is required to discard. On the other hand, a substance or object can be classified as a by-product if:

- its further use is certain,
- it can be used directly without any further processing other than normal industrial practice,
- it is produced as an integral part of a production process,
- fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts.

In addition, the same directive [136] defines the hierarchy for prevention and management of wastes, starting for prevention followed by re-using, recycling, other recovery (e. g. energy recovery or compounds extraction) and lastly disposal.

Biorefinery is defined as the processing of biomass with the objective of replacing the use of non-renewable fossil resources to obtain energy and chemical compounds [137]. The use of biorefinery methodologies has been more focused in the valorization of by-products, especially from agroforestry activities, as they can be considered more sustainable [138,139] than the use of dedicated crops since these will produce their own by-products. In addition, biorefinery can meet the goal of reducing waste amount if by-products are used as raw material.

Despite being possible to include the current PACs production model into the biorefinery definition since they are derived from biomass, they also fit into the linear economy logic (from resource to product to waste) which should be replaced by a circular economy-based process in which all the by-products are kept for as long as possible in the utilization cycle [140,141]. Extraction of PACs from industrial by-products fits into this definition and therefore, it makes sense to discuss the available extraction methodologies in terms of by-products valorization.

With this in mind and since PACs are mostly present, and often in high concentrations in plant parts that are not commercially exploited such as wood bark or fruit skins and seeds [142], these could and should be used as the main raw material for PAC production. This has the advantage, when compared to conventional sources, of not contributing to the depletion of natural resources while actively contributing to the reduction of the total amount of waste in the industries from which the by-product is derived.

1.7. Wine Industry and its By-products

Wine is an alcoholic beverage that results from the fermentation of grape juice by yeast and its history is intertwined with the human civilization almost since its inception. The first records related to the wine production were discovered at Hajji Firuz in the Iranian Zagros Mountains dating back to 5400 BC [143]. Since then, wine production was disseminated across Europe during the Roman Empire expansion which settled the first wine regions that still exist today, e.g. Champagne, Bordeaux or Rhineland.

Industrial wine production (figure 1.18) starts by harvesting grapes at their optimum maturation point (sugar content, acidity, phenolic content) that are then transferred from the vineyard to the

cellar where stems are separated from the berries, and these are crushed to produce the must. Depending on the type of wine to be made, white or red, there are two possible processes, whereas for the former the grapes will be pressed before fermentation for must release, and in the latter, they will be pressed after fermentation and maceration. In both situations the amount of by-products produced in these two steps represent around 15% of the initial mass and consist in stems and grape pomace (seeds and skins) (GP) [144]. At the end of fermentation, the yeast dies and settles at the bottom of the fermenting vat being removed as lees. Malolactic fermentation will occur if desired by the wine maker producing a second batch of lees. The last step of the process will consist in the wine stabilization that can be performed using several oenological products depending on the intended result, e.g. bentonite will be used for protein stabilization of white wine and gelatin will be used for color stabilization of red wine.

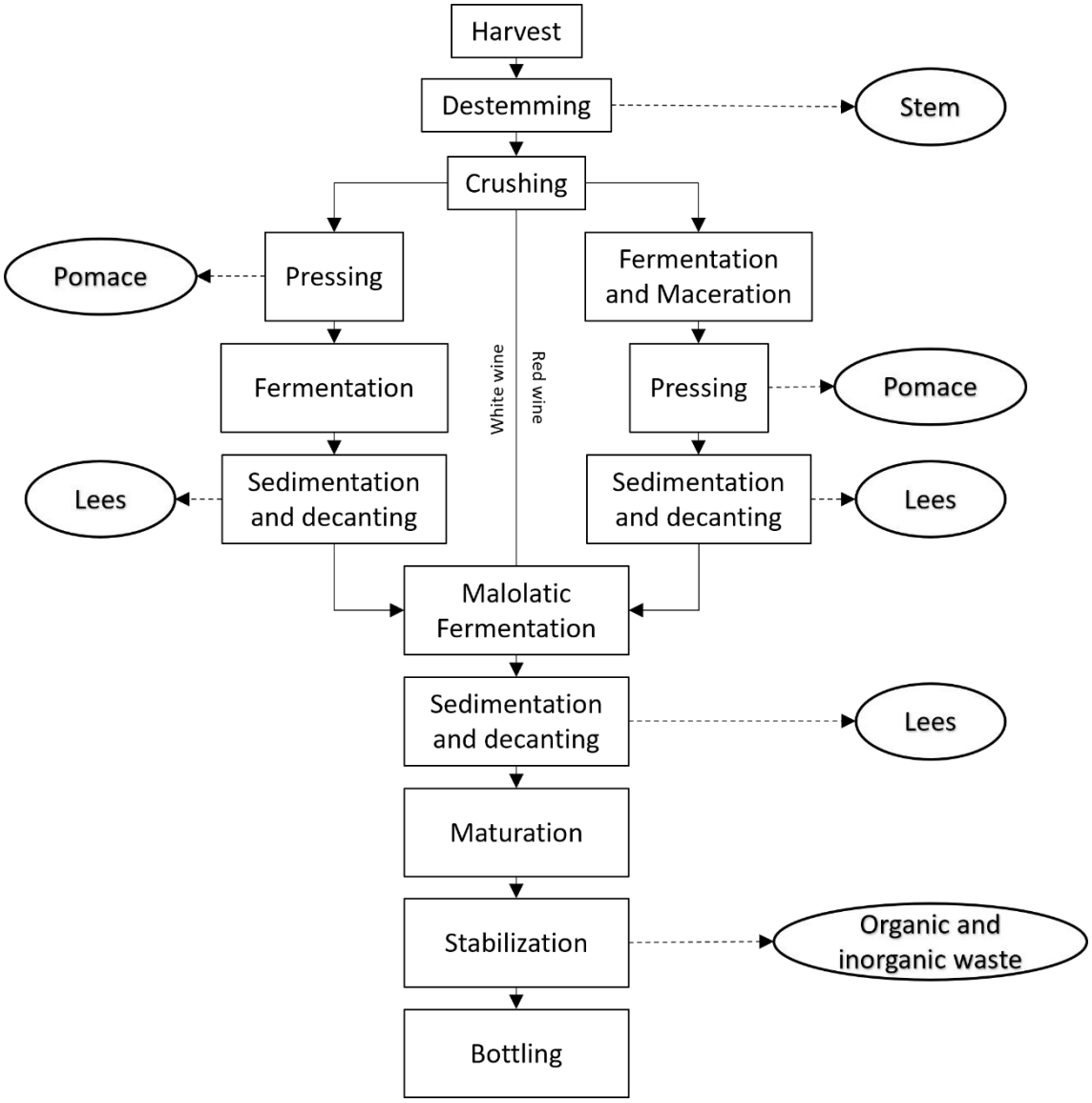


Figure 1.18. Vinification process and by-products produced at each step

Nowadays, wine industry is one of the most important agricultural activities worldwide and produced 292 million hL of wine and 44 million tons of grape for that purpose in 2018 [31]. This led to the production of approximately 6.6 million tons of GP (considering that 1 kg of fresh grapes results in 0.15 kg of pomace). Ideally, the totality of produced GP should be converted into a by-product, meaning that all of it should be used as a raw material for the obtention of other relevant compounds. This way, there would be no need for disposal, or considerably less, and further value would be added to the value chain. As it stands GP has little to no commercial value and often represents additional costs for the producer related to their disposal.

Nowadays there are several options for valorization of by-products although the value that wineries obtain from them is low consisting mostly in the removal of disposal costs [145]. GP is normally sold to distilleries for spirits production, stems and other vegetable residues are usually composted or used as energy source. Unfortunately, in many occasions GP not suitable for composting due to its high contents in heavy metal and phenolic compounds [146].

A large amount of research has been dedicated to the development of new applications for wine by-products, especially in the field of added value products that may become a more economically interesting option for wineries.

The application of wine lees as a nutritional supplement for the growth of other microorganism was successfully applied in the production of xylitol [147], lactic acid [148] and poly(3-hydroxybutyrate) [149] where its major advantage is the replacement of expensive growth medium components. Another approach for the valorization of wine lees is the extraction of compounds with economical value, such as squalene [150], β-glucan [151], *n*-caprylate and *n*-caproate [152], anthocyanins [153] or extracts containing different types of phenolic compounds [154,155].

As far as stems are concerned, a lot of attention has been given to stilbenoids extraction and its application as a natural preservative [156–158] and to production of extracts containing phenolic compounds [159]. Other studies focused on the use of the whole stem either as a substrate for production of lignocellulolytic enzymes [160] or for removal of heavy metals from effluents [161,162].

GP has been the most studied wine industry by-product which is related with the fact that it is the most abundant and that it presents a considerable amount of easily extractable biologically active compounds. Some examples are the production of anthocyanins-concentrates for dying purposes [163,164], tannin-based wood adhesives [165], multi-purpose biorefinery processes in which phenolic compounds, volatile fatty acids and polyhydroxyalcanoates were obtained [166]. GP has also been used as fermentation substrate for production of hydrolytic enzymes [167], lactic acid and biosurfactants [168].

1.8. Extraction of Proanthocyanidins from By-products

Several references were collected in order to assess the potential of different by-products as a source of PACs. This survey was made on Web of Science database before January 31st of 2022 and the key words used were combinations of ‘proanthocyanidin’, ‘condensed tannin’, ‘grape’, ‘bark’,

'fruit', 'by-product' and 'waste'. The article selection was made based on following the criteria listed below:

- The raw material had to be an agroforestry by-product (dedicated crops were excluded),
- Calculation of yield on a dry weight basis of raw material had to be possible,
- Only colorimetric and chromatographic quantification methods were considered.

From the analysis of literature data, the most relevant information concerning Y_{PAC}, extraction procedure and quantification method for by-products resulting from three main categories, namely, wine making, forestry activities and fruits, nuts and legumes processing were systematized in tables 1.3, 1.4 and 1.5, respectively, and will be discussed in the following sections.

In those tables a large variability of Y_{PAC}s are observed, which can be attributed not only to the use of different types of raw materials but to the different extraction techniques and quantification methods. Due to the different conditions used, comparisons are not straightforward, and thus, must be done critically, taking into account the aforementioned differences.

Concerning the quantification methods as discussed previously, considerable differences between colorimetric [22,159,169,170] and chromatographic [125,171–173] methodologies are observed. In general, colorimetric methods despite being less expensive and easier to implement, are more prone to PAC's overestimation.

1.8.1. Grape By-products as a Source of Proanthocyanidins

In table 1.3 the extractable PACs content of by-products from several grape varieties is compared. Amongst those, it is noteworthy the considerable amount of readily available PACs in several grape pomace components with values that can go as high as 92.1 $mg_{PAC}/g_{sample\ dw}$ for whole pomace [169], 60.9 $mg_{PAC}/g_{sample\ dw}$ in seeds [124], 24.1 $mg_{PAC}/g_{sample\ dw}$ in skins [174] and 202 $mg_{PAC}/g_{sample\ dw}$ in stems [159].

The large differences in Y_{PAC}s are attributed to the influence that the plant part, namely, seed, skin and stem, as well as the grape variety have on the final yield. This was assessed by Sá et al. [123] who showed that different parts of Fernão Pires grape variety result in different Y_{PAC}s, with seeds giving rise to extracts with Y_{PAC}s three times higher than stems and skins. Similar observations were made by Bordiga et al. [126] for Nebbiolo grapes. They demonstrated the possibility of obtaining a 10-fold increase in Y_{PAC}s by using seeds instead of skins as raw material.

Even if the same plant part is used, grape variety may have a great impact on the amount of extractable PACs as shown by González-Centeno et al. [159] that compared stems from ten different grape varieties obtaining values that could be as low as 79.1 $mg_{PAC}/g_{sample\ dw}$ for Chardonnay and as high as 202.3 $mg_{PAC}/g_{sample\ dw}$ for Callet variety.

Similar observations were made in grape skins by Deng et al. [174] that described values ranging from 8.0 to 24.1 $mg_{PAC}/g_{sample\ dw}$ for Morio Muscat and Merlot grape varieties, respectively. A similar effect was observed for seeds, as described by Khanal et al. [172] that obtained values of 3.13, 13.8 and 23.0 $mg_{PAC}/g_{sample\ dw}$ for Merlot, Chardonnay and Riesling, respectively. As expected, mixtures of several parts of the same plant such as grape pomace is also affected by grape variety as shown by González-Centeno et al. [169] which reported PACs contents of 71.9, 50.8, 92.1 and 73.2 $mg_{PAC}/g_{sample\ dw}$ for Chardonnay, Macabeu, Parellada and Prensal Blanc varieties, respectively.

Moreover, the wine-making process has also been shown to have a direct impact on the amount of PACs that is possible to obtain from grape processing by-products [124]. More specifically, seeds of red grapes used for white wine production allow to obtain a higher amount of extractable PACs than the ones used for red wine, which is probably related with the longer contact time that seeds experience during red wine production.

When grape varieties are compared there are only minor differences in terms of PACs composition, namely in the proportion and type of monomer, mDP and %Gal [159,169]. In opposition, the different grape parts showed to be an important factor for the PACs composition, with seeds having highly galloylated procyanidins [175] while grape skins have less galloylated procyanidins and prodelphinidins [176].

Table 1.3. Y_{PAC}, mDP, extraction procedure and quantification method of by-products resulting from several grape varieties

Grape variety	Biomass type	Y_{PAC} ($mg_{PAC}/g_{sample\ dw}$)	mDP	Extraction conditions[1]	Quantification Method	Ref.
Fernão Pires	Skin	15.0	nd	Methanol 80% + Acetone 75% (1:10); stirring; 3h + 3h; RT	Vanillin	[123]
	Seed	60.0	nd			
	Stem	18.0	nd			
Savaliano	Seed	26.5	nd	Ethanol 50% (1:20); stirring; 3h; RT	Butanol	[124]
Moschofilero		61.0	nd			
Agiorgitiko		29.7	nd			
Riesling	Seed	30.4	nd	Acetone:ammonium acetate (ns); MAATPE (650W); 80s; RT	Butanol	[177]
Chardonnay	Pomace	71.9	4.5	8xAcetone 80% + 3xMethanol 60% (1:4); ASE (1500Psi); 4min; 40 °C	Butanol; Phloroglucinolysis	[169]
Macabeu		50.8	7.1			
Parellada		92.1	5.0			
Premsal Blanc		73.2	10.1			
Cabernet Sauvignon	Stem	124.9	5.9	8xAcetone 80% + 3xMethanol (ns); ASE (1500psi); 4min; 40 °C	Butanol; Phloroglucinolysis	[159]
Callet		202.3	4.7			
Manto Negro		165.3	6.0			
Merlot		84.0	5.8			
Syrah		161.4	6.1			
Tempranillo		147.3	6.9			
Chardonnay		79.1	4.6			
Macabeu		108.8	6.2			
Parellada		165.2	5.0			

Premsal Blanc		181.4	8.5			
Frankovka	Seed	23.3	nd	Ethanol 50% (1:40); stirring; 3,3h; 80 °C	Butanol	[178]
Nebbiolo	Seed	14.3	8.7	Acetone 70% (1:50); stirring; 3x2h; RT	Phloroglucinolysis	[126]
	Skin	1.28	19.0			
Morio Muscat	Skin	8.0	nd	Acetone 70% (1:4); UAE; 1h; 45 °C	Butanol	[174]
Muller Thurgau		19.4	nd			
Cabernet Sauvignon		17.2	nd			
Pinot Noir		11.9	nd			
Merlot		24.1	nd			
Plavac Mali	Skin	137	nd	2.5 M C_4min Br solution (1:25); stirring; 4h; RT	Vanillin	[179]
Portogizac	Pomace	30.5	nd	Ethanol 50% (1:50); stirring; 2h; 80 °C	Butanol	[180]
Merlot	Seed	3.13	nd	Acetone 70% (2:15); UAE; 3x15min; 50 °C	NP-HPLC	[172]
Chardonnay		13.8	nd			
Riesling		23.0	nd			
Sunbelt	Pomace	3.73	nd			
Sunbelt	Pomace	57.2	nd	Ethanol 70% (1:30); ASE (1000psi); ns; 120 °C	NP-HPLC	[173]
Tempranillo	Pomace	52.5	nd	Ethanol 40% (1:8); soxhlet; 8h; 83.1 °C	Vanillin	[181]

Notes: [1] Solvent (solid:liquid ratio); Method; Duration; Temperature, mDP – mean degree of polymerization, MAATPE – Microwave-Assisted Aqueous Two-Phase Extraction, UAE – Ultrasound Assisted Extraction, ASE – Accelerated Solvent Extraction, RT – Room Temperature, NP-HPLC – Normal Phase-High Performance Liquid Chromatography, C_4min Br - 1-butyl-3-methylimidazolium bromide, nd – not determined, ns – not specified

1.8.2. Forestry By-products as a Source of Proanthocyanidins

Other very common type of by-product that are frequently explored for the production of PACs extracts are forestry by-products (table 1.4). This type of by-products can reach high contents of extractable PACs such as the case of *Picea mariana* bark (60.6 $mg_{PAC}/g_{sample\ dw}$) [182], *Pinus taeda* needles (103 $mg_{PAC}/g_{sample\ dw}$) [130] and *Betula nana* leaves (140 $mg_{PAC}/g_{sample\ dw}$) [22].

Similarly to grapes, the total content of extractable PACs in wood by-products is highly dependent on tree species as shown by Bianchi et al. [183] that described Y_{PAC}s from barks varying from 16.0 $mg_{PAC}/g_{sample\ dw}$ in *Abies alba* to 1.7 $mg_{PAC}/g_{sample\ dw}$ from *Pinus sylvestris*, which represents a 9.4 fold difference while using the same extraction procedure. The part of the tree from which the bark is removed was also showed to have a minor impact on the overall PACs content as shown by Bianchi et al. [184] that reported a difference in the PACs content of 8.6 to 9.9 $mg_{PAC}/g_{sample\ dw}$ from bark extracted from 1 m and 10 m high, respectively. Additionally, a considerable decrease in both values, to 1.3 and 1.7 $mg_{PAC}/g_{sample\ dw}$, respectively, are observed when barks are exposed to weathering for 18 months, which shows the importance of a short processing time after by-product collection. Karonen et al. [22] also showed the importance of tree species selection, by comparing different species of the genus *Betula* and obtaining PAC extraction values of 44, 102 and 145 $mg_{PAC}/g_{sample\ dw}$ for *B. ermanii*, *B. pubescens* and *B. nana*, respectively.

In the case of forestry by-products, wood debarking, a process in which the bark is separated from the wood, becomes extremely important since this process does not separate both parts perfectly and some wood will be present in the final by-product. The impact of this was demonstrated by Kemppainen et al. [117] who observed the inexistence of PACs in the wood fraction of the *Picea abies* by-product which lead to a decrease in the total Y_{PAC} if the separation was not done properly. Similarly, Balaban et al. [185] quantified the PAC content in the bark, sapwood and heartwood of *Quercus vulcanica* and concluded that not only the amount of extractable PACs was considerably lower in sapwood and heartwood but also the latter presented a high content of ellagitannins, a chemically different type of tannin, that could lead to a decrease in the overall yield and contamination with unintended substances.

Tree species also impacts on the type and proportion of PACs obtained in the final extract [183] that can either be composed by a mixture of procyanidins and prodelphinidins as in the case of *Abies alba* and *Larix decidua* or only by procyanidins as in the cases of *P. abies*, *Pseudotsuga menziesii* and *P. sylvestris*.

Table 1.4. Extraction procedures, Y_{PAC}, mDP and quantification method of by-products resulting from the forestry activity

Plant	Biomass type	Extraction conditions[1]	Y_{PAC} ($mg_{PAC}/g_{sample\ dw}$)	mDP	Quantification Method	Ref.
Picea mariana (Black spruce)	Bark	Hot water (1:10); soxhlet; 1h; ns	60.6	7.6	Butanol; Thiolysis	[182]
		Hot water (1:10); soxhlet; 1h; 100 °C	30.0	nd	Butanol	[115]
Picea abies (Norway spruce)	Bark	Tap water (1:10); stirring; 2h; 75 °C	107	nd	Butanol	[117]
		Water (ns); ASE (1500psi); 10min; 60 °C	9.9	5.5	Thiolysis	[184]
		Water (ns); ASE (1500psi); 10min; 60 °C	3.60	6.2	Thiolysis	[183]
Abies alba (Silver fir)	Bark	Water (ns); ASE (1500psi); 10min; 60 °C	16.0	3.5	Thiolysis	[183]
Pinus taeda (Loblolly pine)	Needles	Acetone 70% (1:15); stirring; 4x30min; 25 °C	103	nd	Butanol	[130]
Pinus pinaster (Maritime pine)	Bark	Ethanol 80% (1:10); MAE (100W); 3min; ns	37.1	nd	Vanillin	[186]
Pinus sylvestris (Scots pine)	Bark	Water (ns); ASE (1500psi); 10min; 60 °C	1.70	6.7	Thiolysis	[183]
Quercus sideroxyla (Oak)	Bark	Acetone 70% (1:10); stirring; 24h; RT	67.0	nd	Vanillin	[114]
Quercus vulcanica (Kasnak oak)	Bark	Methanol 80% (1:20); stirring; ns; ns	3.22	nd	Butanol	[185]
Larix decidua (European larch)	Bark	Water (ns); ASE (1500psi); 10min; 60 °C	11.0	5.6	Thiolysis	[183]
Larix gmelinii (Dahurian larch)	Bark	1.25M C4min Br solution (1:20); MAE (230W) + stirring; 2x(10min+4h); RT	115	nd	Vanillin	[170]
Pseudotsuga menziesii (Douglas fir)	Bark	Water (ns); ASE (1500psi); 10min; 60 °C	4.60	4.4	Thiolysis	[183]
Betula nana (Dwarf birch)	Leaves	Acetone 70% (1:30); stirring; 4x40min; ns	140	nd	Butanol	[22]
Betula ermanii (Erman's birch)	Leaves	Acetone 70% (1:30); stirring; 4x40min; ns	44.0	nd	Butanol	[22]
Betula pubescens (White birch)	Leaves	Acetone 70% (1:30); stirring; 4x40min; ns	102	nd	Butanol	[22]
Pinus sylvestris (Scotch pine)	Bark	Methanol 80% (1:20); UAE (60W); 20min; RT	4,27	5.8	Butanol; Thiolysis	[187]

Notes: [1] Solvent (solid:liquid ratio); Method; Duration; Temperature, mDP – mean degree of polymerization, MAE – Microwave-Assisted Extraction, UAE – Ultrasound Assisted Extraction, ASE – Accelerated Solvent Extraction, RT – Room Temperature, nd – not determined, ns – not specified

1.8.3. Fruits, Nuts and Legumes By-products as a Source of Proanthocyanidins

In addition to the more commonly studied by-products, other PACs sources have also been investigated, namely nuts, legumes and fruits (excluding grapes) (table 1.5). As in the case of grape and forestry by-products, some candidates such as Macadamia nuts skin [120] and peanut seed coat [129] showed high potential, with Y_{PAC}s of 70.0 and 84.7 $mg_{PAC}/g_{sample\ dw}$, respectively. Others, such as skins from hazelnut [113] and mango [122] and hulls from chickpea and soy [128], resulted in low Y_{PAC}s (6.76, 5.80, 7.38 and 7.91 $mg_{PAC}/g_{sample\ dw}$, respectively).

However, it should be mentioned that the extraction of PACs from many of these types of by-products is still largely unexplored and information concerning the effect that different species or varieties have on the Y_{PAC}s and type of PACs is still missing.

Table 1.5. Extraction procedures, Y_{PAC}, mDP and quantification method of by-products resulting from the fruits, nuts and legumes

Plant	Biomass type	Extraction conditions[1]	Y_{PAC} ($mg_{PAC}/g_{sample\ dw}$)	mDP	Quantification Method	Ref.
Macadamia tetraphylla (Macadamia nuts)	Skin	Water (1:5); stirring; 20min; 90 °C	70.0	nd	Vanillin	[120]
Corylus avellane (Hazelnut)	Skin	Acetone 80% (1:10); stirring; 12h; RT	6.76	nd	DMACA	[113]
Arachis hypogaea (Peanut)	Seed coat	Acetone 70% (1:10); stirring; 15h; ns	84.7	nd	Vanillin	[129]
Cicer arietinum (Chickpea)	Hull	Methanol 80% (1:50); stirring; 2h; RT	7.38	nd	Vanillin	[128]
Glycine max (Soybean)	Hull	Methanol 80% (1:50); stirring; 2h; RT	7.91	nd	Vanillin	[128]
Oryza sativa (Rice)	Bran	Acetone 70% (1:20); stirring; 2x2h; RT	22.6	nd	Vanillin	[127]
Punica granatum (Pomegranate)	Peel	Water (1:50); stirring; 20min; 90 °C	45.0	nd	Vanillin	[121]
Manguifera indica (Mango)	Skin	Methanol 100% (1:50); stirring; 1h; 25 °C	5.80	nd	Butanol	[122]
Euterpe oleracea (Açai)	Seed	Acetone 60% (ns); ultrasound bath; 3x10min; RT	18.0	nd	Butanol	[188]
Pinus koraiensis (Pine nuts)	Skin	Ethanol 60% (1:44); stirring; 2h; 51 °C	3.80	nd	Vanillin	[189]
Ceratonia siliqua (Carob)	Pods	Ethanol 45% (1:30); stirring; 2h; 50 °C	4.22	nd	Vanillin	[190]
Actinidia chinensis (Kiwi)	Leaves	Acetone 70% (1:30); UAE (ns); 15min; 70 °C	122.2	nd	Vanillin	[191]
Carya illinoinensis (Pecan nut)	Pomace	Ethanol 50% (1:50); UAE (40kHz, 300W); 15min; 30 °C	13.8	nd	Vanillin	[192]

Notes: [1] Solvent (solid:liquid ratio); Method; Duration; Temperature, mDP – mean degree of polymerization, DMACA – 4-dimethylaminocinnamaldehyde assay, RT – Room Temperature, nd – not determined, ns – not specified

1.9. Novel Extraction Methods for Proanthocyanidins

The most frequent extraction methods used as alternatives to conventional ones are physical methods. More specifically, Microwave (MAE) and Ultrasound (UAE) assisted extraction and Accelerated Solvent Extraction (ASE), which have been frequently proposed in scientific literature as good complementary techniques since they are able to considerably reduce extraction time and solvent consumption.

MAE is based on the use of electromagnetic radiation between 300 MHz and 300 GHz that leads to an increase in the temperature due to ionic conduction and dipole rotation which in turn leads to evaporation of water inside the cells or other cellular structure resulting in their rupture followed by leaching of target compounds [193].

MAE potential for the extraction of PACs was first described by Li et al. [194] who were able to obtain a maximum Y_{PAC} of 149 $mg_{PAC}/g_{sample\ dw}$ from purple cabbage at 260 W in 1 min. In addition, it was also verified that an increase in microwave power caused the Y_{PAC} to decrease. Similar observations were made by Bhuyan et al. [195] and Huma et al. [190] but no explanation for that phenomenon was proposed.

As far as the application of MAE in the valorization of by-products through PACs extraction is concerned, Dang et al. [177] was able to obtain 30.4 $mg_{PAC}/g_{sample\ dw}$ with Microwave Assisted Aqueous Two-Phase Extraction (acetone and ammonium acetate buffer) from grape seeds within 1.3 min. Similar results were observed by Chupin et al. [186], obtaining 37.1 $mg_{PAC}/g_{sample\ dw}$ from *Pinus pinaster* bark using 80% aqueous ethanol within 3 min, and by Bhuyan et al. [195] that obtained 16.7 $mg_{PAC}/g_{sample\ dw}$ from *Eucalyptus robusta* leaves using water within 2 min. Despite the differences in the raw materials and solvents used, resulting in slightly different Y_{PAC}s, these studies have in common the extremely short extraction times, ranging from 1.3 to 3 min, which are considerably shorter than the 2 to 24 hours normally employed when using conventional maceration [109,113,114,123,126]. In terms of overall Y_{PAC}, the differences between MAE and conventional SLE, when using the same raw material, are small but as mentioned before the considerable decrease in operation time end up compensating for the extra energy consumption as verified by Huma et al. [190] that compared the two approaches in the extraction of PACs from carob.

UAE is based on the use of ultrasonic energy with a frequency higher than 20 kHz that, when applied to the sample causes cavitation bubbles that rapidly collapse leading to high shear forces that are able to completely disrupt cells [196]. In addition, it is also proposed that ultrasounds

are able to disaggregate PACs from other biomass compounds such as proteins and polysaccharides [197].

UAE potential for the extraction of PACs was first described by Gu et. al [171] who obtained 35.3 $mg_{PAC}/g_{sample\ dw}$ from grape seeds using 70% aqueous acetone as solvent and UAE as pretreatment for 10 min at 37 °C followed by 50 min of maceration at room temperature. Khanal et. al [172] also used 70% aqueous acetone to extract grape pomace three times for 15 min with solvent renewal at 50 °C obtaining 3.73 $mg_{PAC}/g_{sample\ dw}$.

A different approach was used by Deng et. al [174] who extracted grape skins during 1 h at 45 °C using also 70% aqueous acetone and obtained 24.1 $mg_{PAC}/g_{sample\ dw}$. Similarly, Ma et. al [198] also used a single-step extraction of 30 min using an aqueous poly(ethylene glycol) 200 solution at 40% (m/m) and obtained 32.5 $mg_{PAC}/g_{sample\ dw}$ from almond skin. Despite the differences that arise from the use of different source materials it is noteworthy that UAE is also able to reduce the extraction time, although not as much as MAE but still considerable shorter than conventional maceration, as mentioned before. The optimal amount of ultrasound power to be employed as also been a subject of research with Liu et al. [199] and Chu et al. [200] showing that 660 and 330 W were the optimum power values to extract PACs from *Cinnamomum longepaniculatum* leaves and *Zizania latifolia* seeds, obtaining 7.88 and 6.33 $mg_{PAC}/g_{sample\ dw}$, respectively.

As in the case of conventional SLE, MAE and UAE are also characterized by the existence of an optimum extraction time and power above which a decrease in Y_{PAC} is observed. This was confirmed by Aspé et al. [201] that postulated the possibility of thermal degradation in the case of MAE, an aspect that was also observed in conventional extraction [131], and oxidative degradation due to the radicals produced during sonolysis for UAE. The studies in which MAE or UAE are employed have not addressed their impact on the DP distribution of the obtained extracts.

Finally, ASE takes advantage of the use of pressure to go above solvents boiling point at ambient pressure, contributing to an increased solubility of target compounds as well as reduced solvent viscosity, making mass transfer and sample impregnation faster [202]. This technique has the disadvantage of being hard to upscale to an industrial level and therefore has been exclusively used at a laboratory level. Nevertheless, its compatibility with automatization and short contact times that allow a high number of extraction cycles enable the easy determination of the total amount of PACs, as well as mDP, that can be obtained with one or more solvent systems.

Additionally, with this technique, comparisons between the maximum Y_{PAC} of different species can be simultaneously made as shown by Bianchi et al. [183] where the PACs contents of water extracts from barks of different European wood species were compared, concluding that, among the tested samples, *Abies alba* was the best choice in terms of Y_{PAC} (16.0 $mg_{PAC}/g_{sample\ dw}$) in opposition to *Pinus sylvestris* (1.7 $mg_{PAC}/g_{sample\ dw}$) making the former a better raw material for production of PACs rich extracts. However, if an extract with a high mDP is intended, a swap in roles is verified since the *A. alba* extract has a mDP of 3.5 while the latter has a mDP of 6.1. Interestingly, an almost perfect inverse relationship between total Y_{PAC} and mDP on the extracts obtained from different species was observed, supporting the idea that PACs with higher DP are in fact more difficult to extract.

Similar studies were made by González-Centeno et al. for grape stems [159] and pomace [169] in which it was shown that certain grape varieties, such as Callet and Parellada, respectively, are more suitable for PACs extraction as far as Y_{PAC} is concerned with 202.3 and 92.1 $mg_{PAC}/g_{sample\ dw}$. In terms of mDP, despite not being possible to observe an almost perfect inverse relationship with the Y_{PAC}, as the one reported above for barks, it is still possible to observe that the Callet extracts had the lowest mDP with 4.7 and the Parellada ones had the second lowest with 5.0.

In order to avoid the degradation verified in conventional extraction techniques and in MAE and UAE, alternative approaches that enable the use of milder conditions such as the use on enzymes has been suggested.

For instances, the use of enzymes such as pectinases, cellulases and tannases in grape seeds and skins, led in general to a 30% increase in overall phenolic content when compared with conventional SLE with 33% aqueous ethanol, and an increase in mDP for the first two enzymes and a decrease in the last one [203]. This occurs due to enzymatic depolymerization of cell wall components, namely, pectin and cellulose, as well as to the depolymerization of PACs by tannase, which, overall, facilitates their release from the lignocellulosic matrix.

More recently, a similar procedure was applied to litchi pericarp where a considerable increase in the Y_{PAC} (from 20.7 to 46.3 $mg_{PAC}/g_{sample\ dw}$) with 50% aqueous ethanol was observed using pectinase and β-glucosidase [204]. Contrarily to the previous example, the addition of tannase led to a decrease in the overall Y_{PAC}. In both examples, the use of pectinase appears to be the most effective treatment while the effect of the combination with other enzymes depends on the type of biomass used as raw material, i.e. cellulase for grape skin and seed [203] and β-glucosidase for litchi pericarp [204].

1.10. Extraction of Proanthocyanidins with Eutectic Solvents

Neoteric solvents such as ionic liquids (ILs) and eutectic solvents (ESs) have also been proposed for the extraction of PACs. The main advantage of these solvents is their ability to efficiently solvate target compounds through electrostatic interactions and hydrogen bonding, leading to increased extraction efficiency.

ILs are organic asymmetric salts that have a melting point below 100 °C and have been extensively studied in the processing of biomass [205]. Studies on PACs extraction using ILs have been conducted for cinnamomi cortex in which it was possible to obtain 45 $mg_{PAC}/g_{sample\ dw}$ by using a MAE with a 1.25 M aqueous solution of 3-methylimidazolium bromide, corresponding to a 125% Y_{PAC} increase when compared to conventional MAE process with water as solvent [206].

The same IL aqueous solution was used to extract PACs from *Larix gmelini* bark with a Y_{PAC} of 115 $mg_{PAC}/g_{sample\ dw}$, corresponding to an Y_{PAC} increase of 100% when compared to conventional extraction with water and 20% increase when compared to a MAE with 80% aqueous ethanol [170].

More recently ILs were also applied in the extraction of PACs from grape skins by Ćurko et al. [179] who concluded that a 2.5 M aqueous solution of 3-methylimidazolium bromide was the best option for the extraction process. However, the obtained Y_{PAC}, 137 $mg_{PAC}/g_{sample\ dw}$, was lower than the one obtained by maceration with 80% aqueous acetone followed by 60% aqueous methanol (218 $mg_{PAC}/g_{sample\ dw}$) which might be explained by the fact that conventional extraction was made with a two-step process. None of these studies addressed the effect of the extraction conditions on the PAC's mDP.

ESs are mixtures of two or more compounds that present a decrease in the melting temperature when compared to the individual compounds due to a decrease in the lattice energy, and that in the correct proportions can give rise to mixtures that are liquid at temperatures suitable for biomass processing (figure 1.19). In general, these are described as being composed of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) that give rise to a hydrogen bond network capable of effectively solvating solutes. This concept derived from the work of Abbot et al. who described for the first time the properties of the deep eutectic solvent (DES), cholinium chloride:urea (1:2) [207]. DESs differentiate from ESs from the fact that in the former the decrease in melting temperature is more pronounced than what is expected from an ideal mixture while the latter follows the behavior of an ideal mixture [208] (figure 1.19). Nevertheless, both can be employed in different areas of applications and a considerable amount of review articles are available, such as, nanoparticles synthesis and materials

functionalization [209], biodiesel synthesis [210], organic synthesis [211], biocatalysis [212], and on extraction and separation processes [213].

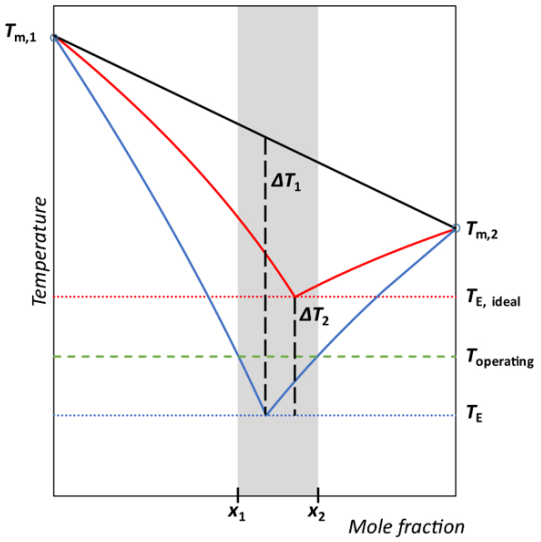


Figure 1.19. Schematic representation of the comparison of the phase diagram of a simple ideal eutectic mixture (red line) and a deep eutectic mixture (blue line); (T_m – melting temperature, T_E – eutectic melting temperature) (extracted from [208])

ESs can be prepared from almost any type of compound which include but are not limited to inorganic or organic compounds, synthetic or naturally occurring, chemically synthesized or biorefined. Naturally, these include compounds with varying costs and levels of toxicity that when selected appropriately can have several advantages when compared to ILs, namely, easiness of preparation, lower cost, higher sustainability and lower toxicity [214]. Furthermore, ESs can also present advantages over traditional organic solvents due to their renewable origin, tailoring ability, non-flammability, enzyme compatibility and lower overall environmental impact [215]. Nevertheless, ES also present some disadvantages when compared with traditional solvents related to their high viscosity, that complicates handling and mass transfer (but that can be overcome by the use of water). Low vapor pressure often considered an advantage (due to the absence of volatiles emissions), in some situation might be a drawback as it complicates the downstream process.

Recently, ESs application in PACs extraction was assessed by Cao et. al [216] who reported an Y_{PAC} of 22.1 $mg_{PAC}/g_{sample\ dw}$ from *Ginkgo biloba* leaves using a mixture of choline chloride and malonic acid at a molar ratio of 1:2 and with a water content of 55% (m/m). This Y_{PAC} is higher than those reported with conventional solvents such as aqueous acetone 70% (v/v) (13.26 $mg_{PAC}/g_{sample\ dw}$), aqueous methanol 70% (v/v) (7.87 $mg_{PAC}/g_{sample\ dw}$) or aqueous ethanol 70% (v/v) (7.84 $mg_{PAC}/g_{sample\ dw}$). Unfortunately, no further characterization of the resulting extracts

was made and therefore, information of the effect that different ESs have on the type of monomer and mDP is still missing.

ESs have been described as viable alternatives for the extraction of (+)-catechin from different sources such as red grape skins [217] and tea [218,219]. Other types of flavonoid compounds were also extracted from biomass, more specifically, anthocyanins from red grape skin [217], apigenin from tomato by-products [220], myricetin from *Chamaecyparis obtusa* [221] and quercetin from *Flos sophorae* [222] or even complex phenolic compounds with higher molecular weight such as carthamin from *Carthamus tinctorius* [223]. From these papers some general conclusions concerning the extraction of phenolic compounds with ESs can be drawn, more specifically, that the addition of water leads to an increase in catechin derivatives' extractability from biomass, which might be related to either the decrease in viscosity and consequent higher mass transfer, or to a better solvation of target compounds. In addition, it can also be concluded that the most commonly used compounds for ES preparation include choline chloride, organic acids such as lactic or oxalic acid, sugars such as glucose or fructose and alcohols such as glycerol or 1,4-butanediol.

Other factor that might contribute to the promising characteristic of ES in flavonoids extraction is the fact that they can also work effectively in the solubilization of the lignin fraction of biomass, contributing to the degradation of the lignocellulosic matrix and promoting the release of compounds of interest. In addition, in some cases, it is possible that same ES formulation will effectively extract both since most of the compounds mentioned for flavonoids extraction have also been described as good extractors for lignin such as organic acids, although with a higher content of organic acid, choline chloride:oxalic acid (1:20) [224] and choline chloride:lactic acid (1:10) [225], that resulted in 90% and 70% recuperation of lignin, respectively. In addition to the fact that the described ES are very effective in the dissolution of lignin they perform very poorly with cellulose [226], which might be used advantageously in a downstream process as suggested by Liu et. al [224] in which wood lignocellulose was successfully fractionated into lignin and cellulose fractions suitable for other applications such as aromatic compounds production, and by Kim et. al [227] that efficiently removed lignin from switchgrass and achieved high enzymatic saccharification levels using ES prepared with lignin derived phenolic compounds such as 4-hydroxybenzyl alcohol, catechol, vanillin or *p*-coumaric acid, that could themselves be synthesized from the same lignin fraction they are solubilizing (figure 1.20). Similar observations were made for ES prepared from hemicellulose derived compounds by Yu et. al [228] where formic, acetic, glucuronic, glycolic and levulinic acids were successfully employed in the delignification of Akebia residues, adding to the sustainability of natural DES (NADES) industrial

use. The solubility of lignin monomers in aqueous solutions of ES were already accessed by Soares et. al [229] that showed the possibility of increasing the solubility of compounds such as syringic acid or ferulic acid as high as 50-fold when compared to pure water using 75% and 50% (m/m) of propionic acid:urea (2:1) in water, respectively.

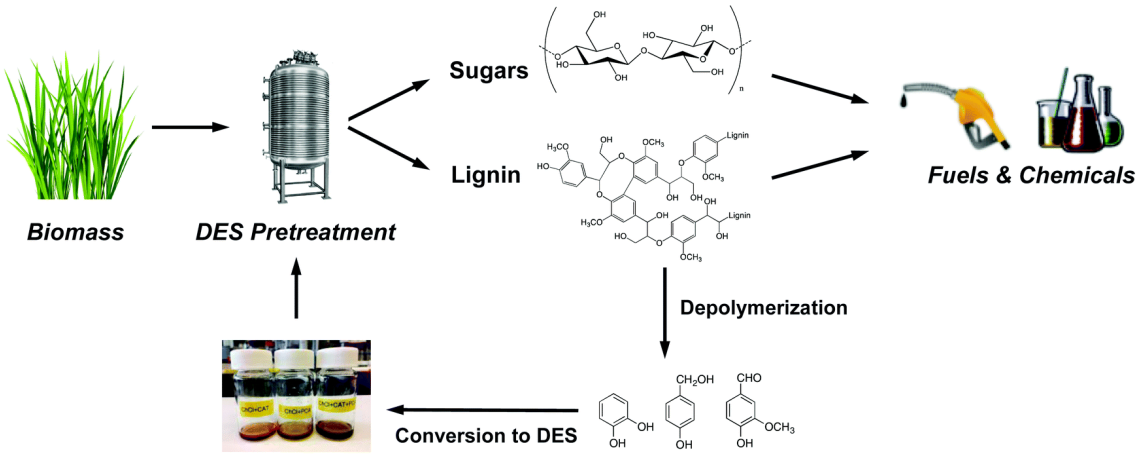


Figure 1.20. Future biorefinery concept using renewable ESs derived from lignocellulosic biomass (reproduced from [227])

1.11. Objectives of the Thesis and Work Plan

As thoroughly discussed in the **chapter 1** of the present document, PACs have several promising added value applications that depend on the extract's final characteristics, namely, mDP and %Gal. Furthermore, the search for new application is, in large part, limited by the existing extraction methodologies that render PAC's extracts expensive and with poor final characteristics. In this context, this thesis is focused on the improvement of PAC's extraction using grape pomace as raw material and ESs as extraction media. Additionally, exploratory assays were made with the intent of developing the field of PACs practical application (figure 1.21).

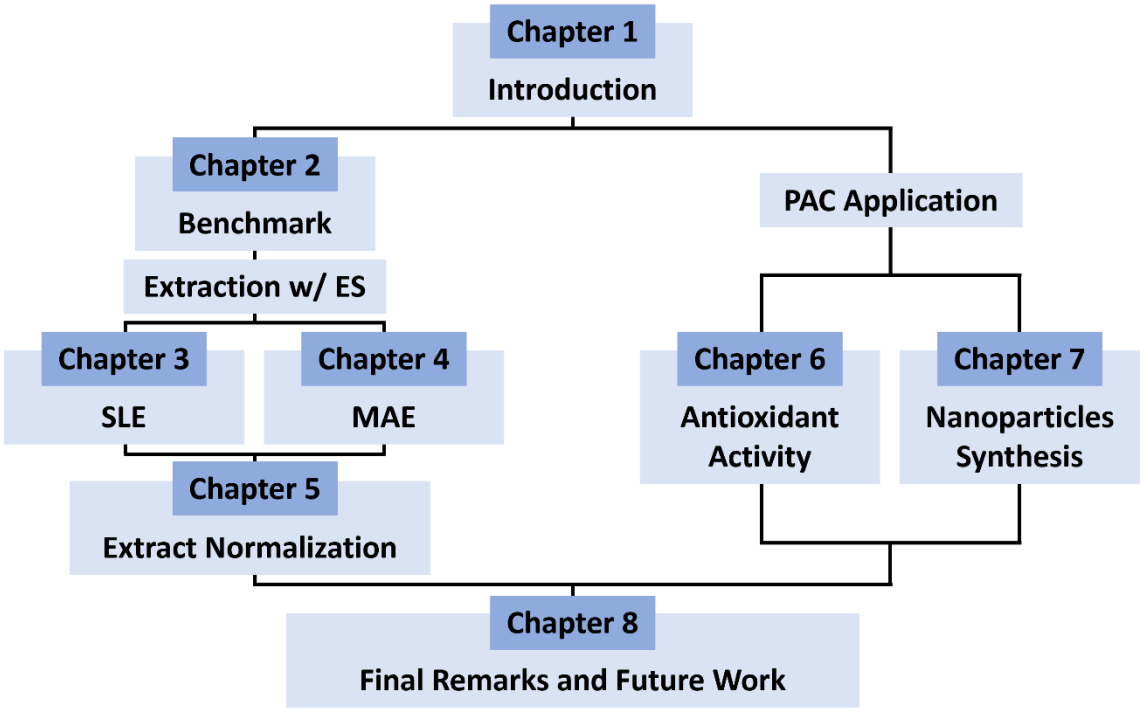


Figure 1.21. Schematic representation of thesis work plan

More specifically, on **chapter 2** a benchmark of the existing commercially available PAC extracts from grape were assessed with the intent of asserting their molecular characteristics, namely, purity, mDP and %Gal. Furthermore, these were compared with PAC extracts obtained at laboratory scale using conventional solvents.

On **chapter 3**, the employment of ES on the extraction of PACs by maceration was explored. The effect of HBA:HBD combinations, impact of water and ethanol addition, and extraction conditions, namely, temperature, biomass content and extraction time were assessed by response surface methodology (RSM) resulting in the development of a quaternary ES system.

On **chapter 4**, the effect that the microwave-assisted extraction of PACs with different HBA:HBD combinations had on PAC's integrity and the extraction conditions, namely, temperature, biomass content and extraction time were optimized by RSM.

Using the information gathered in the previous chapters, in the **chapter 5**, a proof-of-concept of the feasibility of obtaining PACs extracts with normalized mDP values was performed using the aforementioned quaternary ES system and RSM for the determination of the necessary extraction media composition.

Finally, exploratory studies were made with the goal of testing two hypothesis, namely, if ES composition can have a positive impact on PAC's antioxidant activity that was made by screening HBA:HBD combinations in molecular and cellular antioxidant assays on **chapter 6**, and if PACs

with high mDP values can improve on the synthesis of gold-PAC core-shell nanoparticles when compared with catechin, **chapter 7**.

2. Benchmarking of Grape Proanthocyanidin's Extracts

Abstract

Improvements of PAC's extraction process in terms of yield and characteristics are necessary so that their use in conventional and novel applications can be further explored. Therefore, is important to benchmark of what is currently available and what kind of improvement can be achieved by changing the extraction process.

From the available options, PACs from grape, and its by-products, are the most relevant due to the importance of that specific culture in the local economy.

Herein, the mDP and %Gal values of commercially available grape extracts, two from grape seed (**PAC Sig** and **PAC Sd**) and one from skin (**PAC Sk**), were compared with the ones obtained at a laboratory scale from white grape pomace using two conventional solvents, aqueous acetone 70% (v/v) (**PAC Ace**) and hot water (**PAC HW**). From these assays was possible to conclude that the respective mDP (3.3, 5.5 and 4.0) and %Gal (29.5, 27.9 and 33.2%) values of the commercial extracts were in line with the ones obtained for **PAC HW** (3.9, 26.5%), indicating that this method's extraction yield should be considered the base line for the development of a new one.

2.1. Introduction

To better understand how to improve the PACs extraction process one must firstly benchmark the industry existing options against which the developed processes can be compared. As previously discussed, there are several commercially available extracts from different sources such as quebracho, mimosa or grape, among others. Herein, different commercially available PAC's extracts from grape were assayed, namely, two oenological products obtained from grape seed and grape skin, and a purified grape seed extract from a lab supplier. In addition, the effectiveness of conventional extraction methods, such as extraction with aqueous acetone 70% (v/v) at room temperature (**PAC Ace**) [126] and extraction with distilled water in pressurized containers at 110 °C (**PAC HW**) [230] were also assayed and compared with the available commercial products.

Extracts characterization was made with acid butanol assay for PAC's colorimetric quantification, with the phloroglucinolysis assay for mDP determination and with NP-HPLC-MS for detailed identification of PACs.

The choice fell on acid butanol assay for colorimetric quantification due to its sensitivity towards every type of monomer as long as they are extension units, making it exceptionally effective and reliable in the detection/quantification of PACs with high mDP [31].

Phloroglucinolysis assay was chosen due to the lower toxicity of its reagents when compared to the ones used in the thiolysis assay, especially benzyl mercaptan, contributing to a greener method as far as environment and operator safety are concerned.

NP-HPLC-MS was selected since is the only available analytical method that allows for the effective chromatographic separation of extracts containing PACs with a wide molecular weight distribution and accomplish the intended molecular identification [40].

Herein, the characteristics of each extract were assessed, and conclusions were drawn with the intention of defining the standard process in the obtention of PACs from GP.

2.2. Materials and Methods

2.2.1. Commercial Proanthocyanidin Extracts

Commercial oenological tannin extracts obtained from seeds (**PAC Sd**) (Premium Uva) or skins (**PAC Sk**) (Premium Vinacciolo) of grapes were purchased from Inoser (Peso da Régua, Portugal) and an oligomeric PAC extract from grape seed (**PAC Sig**) was purchased from Sigma Aldrich (St. Louis, Missouri, USA) (table 2.1). All extracts were characterized without further processing.

Table 2.1. Commercial PAC extracts price per kilogram

Extract	Designation	Price (€/kg)
Premium Uva (Grape Seed)	PAC Sd	335.71
Premium Vinacciolo (Grape Skin)	PAC Sk	422.38
Grape Seeds Oligomeric Proanthocyanidins (Sigma Aldrich)	PAC Sig	608 000

2.2.2. Grape Pomace Preparation

GP was composed by a mixture of white grape varieties from the Douro region (Portugal) collected during the 2019 harvest after pressing for must extraction and was kept frozen at -20 °C until freeze-drying after which it was kept tightly closed at room temperature protected from light until use.

2.2.3. Extraction of Proanthocyanidins from Grape Pomace with Conventional Solvents

To better understand the impact of conventional solvents on the extraction of PACs, these were extracted from GP with aqueous acetone 70% (v/v) (**PAC Ace**) and distilled water (**PAC HW**). GP was composed by a mixture of white grape varieties from the Douro region (Portugal) collected during the 2019 harvest after pressing for must extraction and was kept frozen at -20 °C until

freeze-drying after which it was kept tightly closed at room temperature and protected from light until use.

PAC Ace was obtained following the method described by Alwerdt et al. [231] with slight modifications. Briefly, freeze-dried GP was firstly defatted with dichloromethane in a Soxhlet apparatus at a solid:liquid ratio of 1:10 for 6 hours. The defatted GP was then extracted three times with aqueous acetone 70% (v/v) acetone for 2 h at room temperature under continuous agitation at 100 rpm and with 10% (m/m) of biomass. After extraction, the supernatants were pooled together, the acetone was removed in a rotary evaporator at 40 °C and the resulting residue was resuspended in distilled water, freeze-dried and kept in a desiccator until use.

PAC HW was obtained following the method described by Francezon et al. [230] with slight modifications. Briefly, GP was extracted twice in a pressure cooker at 110 °C for 90 mins with 10% (m/m) of biomass. After extraction, the supernatants were pooled together, freeze-dried and kept in a desiccator until use.

2.2.4. Fractionation of Proanthocyanidins Based on Degree of Polymerization

High mDP PACs were isolated following the method described by Neto et al. [232]. Briefly, 2 g of **PAC Ace** were dissolved in 20 mL of methanol and, after vortexing thoroughly for 5 min, the suspension was centrifuged for 10 min at 6000 rpm. The supernatant was then loaded into a glass column with an internal diameter of 16 mm packed with 100 mm of Toyopearl HW-40 resin, previously equilibrated with methanol. The flow was kept at 1.5 mL/min with the extract being firstly eluted with 300 mL of methanol (**PAC F1**), followed by the elution with 250 mL of methanol with 30% (v/v) acetone (**PAC F2**) and lastly with 100 mL of aqueous acetone 70% (**PAC F3**). The solvents were completely removed in a rotary evaporator under vacuum at 40 °C, the dry residues were resuspended in a minimum amount of distilled water, freeze-dried and kept in a desiccator until further use.

2.2.5. Acid Butanol Assay for Proanthocyanidin Quantification

PACs extract's purity ($mg_{PAC}/g_{extract}$) and Y_{PAC} (mg_{PAC}/g_{GP}) were determine following the acid butanol assay described by Porter et al. [31] using **PAC F3** fraction obtained in the section 2.2.4 as standard for calibration curve construction. Briefly, 82.5 µL of standard/sample dissolved in methanol were mixed with 500 µL of butanol reagent (butanol with 5% (v/v) of concentrated hydrochloric acid) and 18 µL of ammonium iron (III) sulfate dodecahydrate solution (20 mg/mL prepared in aqueous hydrochloric acid (2 M)) in pressure and temperature resistant tubes that

were then incubated at 100 °C for 50 min. The absorbance of the resulting solutions was measured at 520 nm.

2.2.6. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PAC's mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] with slight modifications. Briefly, 10 to 100 mg of sample, depending on PAC concentration, were dissolved in 1.0 mL of a freshly prepared methanol solution containing 50 g/L of phloroglucinol, 10 g/L of ascorbic acid and 0.1 M of hydrochloric acid. Insolubilized material was removed by centrifugation, after which 400 µL of supernatant were transferred to pressure resistant vials and incubated at 50 °C for 1 h followed by the addition of 2 mL of 40 mM sodium acetate aqueous solution to stop the reaction.

Depolymerization products were quantified by RP-HPLC with a Hypersil™ GOLD RP C18 column (100 x 2.1 mm; 1.9 µm) kept at 40 °C following the elution program described in table 2.2 with eluents A - acetonitrile with 0.1% (v/v) of formic acid, and B - water (99% (v/v)) and acetonitrile (1% (v/v)) with 0.1% (v/v) of formic acid at a flux of 0.4 mL/min. A representative chromatogram is depicted in figure 2.1 and peak attribution was made by Electrospray Ionization Mass Spectrometry (ESI-MS) on negative mode in a LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Nitrogen sheath and auxiliary gas flow rates were 50 and 10, respectively, and voltages of capillary and lens were set to -28 V and -115 V, respectively [234]. Data acquisition was carried out with Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA).

mDP and %Gal were calculated following equations 1.1 and 1.2, respectively.

Table 2.2. LC-MS elution program for quantification of phloroglucinolysis products

t (min)	% A	% B
0	1	99
3	1	99
30	31	69
32	100	0
36	1	99
40	1	99

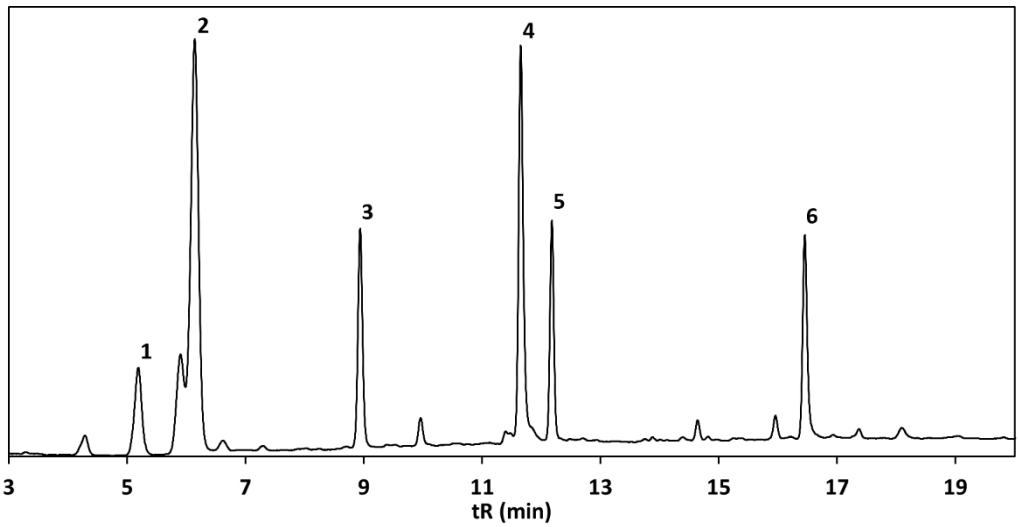


Figure 2.1. Representative chromatogram of phloroglucinolysis products quantification by LC-MS. 1 – phloroglucinol derivative with catechin/epicatechin; 2 - phloroglucinol derivative with catechin/epicatechin; 3 – catechin; 4 - phloroglucinol derivative with catechin gallate/epicatechin gallate; 5 – epicatechin; 6 - catechin gallate/epicatechin gallate

2.2.7. Normal Phase-HPLC-MS of Proanthocyanidins

PACs were analyzed by NP-HPLC using a Luna® HILIC (150 x 2 mm; 3 µm) kept at 35 °C following the elution program described in table 2.3 with eluents A - acetonitrile with 0.1% (v/v) of formic acid and B - methanol (97% (v/v)) and water (3% (v/v)) with 0.1% (v/v) of formic acid at a flux of 0.45 mL/min. Peak attribution was made with ESI-MS using the conditions detailed in section 2.2.5.

Table 2.3. LC-MS elution program for identification of PACs by NP-HPLC-MS

t (min)	% A	% B
0	93	7
3	93	7
15	70	30
40	51	49
45	93	7
60	93	7

2.3. Results

2.3.1. Characterization of Commercial Proanthocyanidin Grape Extracts

Commercial PAC extracts obtained from grape can vary considerable depending on the source or extraction process, as demonstrated in the previous chapter. With that in mind, a full

characterization of the purchased extracts was made, namely, determination of purity, mDP and %Gal.

As it can be observed in figure 2.2 the PAC extract purchased from Sigma (**PAC Sig**) was the one with the highest purity (971 $mg_{PAC}/g_{extract}$), being composed almost exclusively by PACs, which is to be expected considering its considerably higher price when compared with the remaining assayed commercial extracts (table 2.1) that have lower purities, 740 and 438 $mg_{PAC}/g_{extract}$ for **PAC Sd** and **PAC Sk**, respectively.

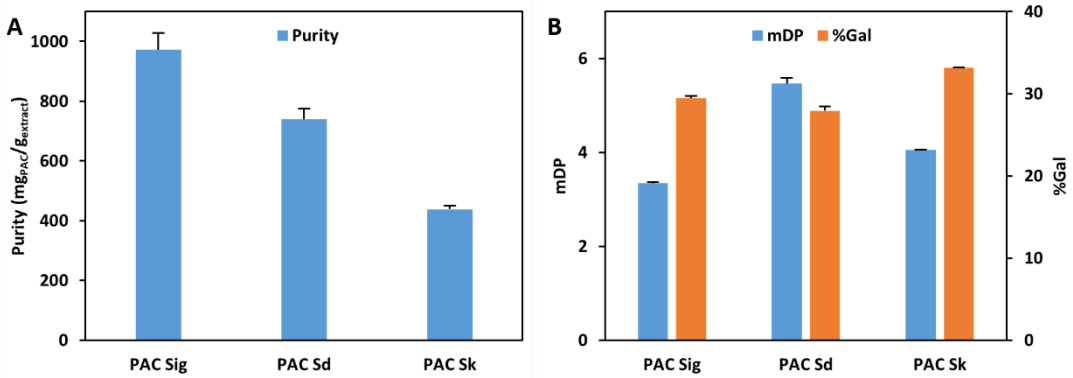


Figure 2.2. Characterization of commercial PAC grape extracts (A - Purity, B - mDP and %Gal)

Interestingly, among the tested commercial samples, **PAC Sig** extract has the lowest mDP, 3.3, followed by **PAC Sk** with 4.0 and **PAC Sd** with 5.5. As far as the %Gal is concerned the highest values were obtained for **PAC Sk** (33.2%) followed by **PAC Sig** (29.5%) and **PAC Sd** (27.9%). These results are difficult to interpret without having additional information from the manufacturer concerning the type of raw material, extraction method and downstream process. Nevertheless, from these it can be concluded that, in general, if the correct raw material is used a relatively high purity (740 $mg_{PAC}/g_{extract}$) can be obtained at a cost of 300-400 €/kg (table 2.1). Unfortunately, the observed mDP values, with the exception of **PAC Sd**, are low when compared with the literature values measured for different types of grape PACs extracts that in general present values upwards from 4.5 for Chardonnay pomace up until 10.1 for Prensal Blanc [169] and upwards from 4.6 for Chardonnay stems up until 8.5 for Prensal Blanc [159]. These differences can be attributed to low extraction efficiency of PACs with higher DP or due to degradation of PACs during the employed extraction/downstream processes.

2.3.2. Extraction of Proanthocyanidins from Grape Pomace with Conventional Solvents

As far as extraction with conventional solvents is concerned, **PAC HW** gave rise to a higher Y_{PAC} when compared with **PAC Ace**, 193 and 144 $mg_{extract}/g_{GP}$, respectively (figure 2.3A).

Nevertheless, **PAC Ace** presents a considerably higher purity, 582 $mg_{PAC}/g_{extract}$, in opposition to the 33.0 $mg_{PAC}/g_{extract}$ observed for **PAC HW** which resulted in a 13-fold increase in Y_{PAC}, from 6.35 to 83.7 mg_{PAC}/g_{GP}, despite the lower Y_{PAC} of **PAC Ace** (figure 2.3A). Even though the **PAC Ace** extraction process has an additional defatting step it can be concluded that aqueous acetone 70% (v/v) is a solvent capable of extracting PACs more selectively than distilled water. Despite being a volatile organic solvent, acetone presents several risks and the need for a recovery circuit that might lead to increased operational costs [235].

Additionally, the mDP value obtained with **PAC Ace** is twice as high as the one obtained with **PAC HW**, 8.1 and 3.9, respectively, while the %Gal followed a similar trend, 46.4% and 26.5%, respectively (figure 2.3B).

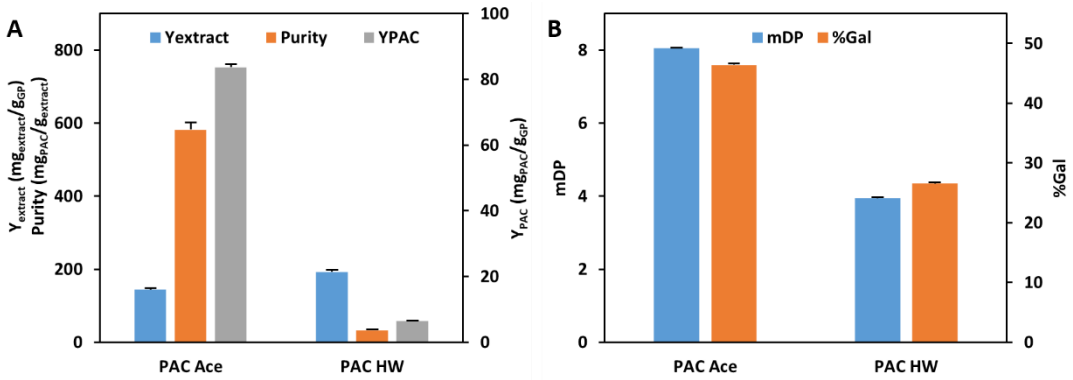


Figure 2.3. Characterization of PAC grape extracts obtained with conventional solvents (A - $Y_{extract}$, purity and Y_{PAC}, B - mDP and %Gal)

Based on these results and considering the similarity of mDP and %Gal values between **PAC HW** (figure 2.3B) and the tested commercial PAC extracts, **PAC Sig**, **PAC Sd** and **PAC Sk** (figure 2.2B), it is likely that the solvent used in the obtention of these extracts was hot water, to which an additional purification step was added, in order to bring the extract's purity to acceptable levels. However, and as mentioned in the previous section, it is hard to pinpoint the exact method employed in the purification of PACs without further information from the manufacturer. Nevertheless, based on registered patents [236,237] and scientific literature [238] this process is most likely made through a filtration process.

Regardless of purification method, assuming that the industrial scale extraction is made with hot water, it is possible to conclude that this process has a low Y_{PAC}, 6.35 mg_{PAC}/g_{GP}, which represents 7.6% of the total PACs obtained with aqueous acetone 70% (v/v). The low efficiency of the extractive process, as demonstrated here, is the most likely reason for PACs extract's high prices (table 2.1). Furthermore, as shown by Ding et al. [238] the use of additional extraction steps reduces the environmental performance of the process and therefore improvements must be

made in the available extraction methodologies, more specifically, by bettering the solubilization of PACs in a single-step SLE.

2.3.3. Fractionation of Proanthocyanidins Based on Degree of Polymerization

Despite the importance that PACs' high or specific DPs have on chemical and biological activities there are no commercially available extracts that address this necessity, even though the fractionation process is frequently described in scientific literature.

Purification of PACs with high mDP is frequently an expensive and labor-intensive process and therefore it is important to assess the gains that can be achieved with such methodology. Herein the fractions' characteristics (**PAC F1**, **PAC F2** and **PAC F3**) are compared with the initial reference material (**PAC Ace**).

From the analysis of the fractionation process (figure 2.4) it can be concluded that it is possible to recover 61,8% of the initial PAC material with the losses being related to the incomplete solubilization of **PAC Ace** in methanol, which is the loading solvent, and to the irreversible adsorption of PACs to the resin employed in the fractionation. Furthermore, more than half of the total amount of recovered PACs are eluted in the first fraction (**PAC F1**), although with a considerably lower purity, 225 $mg_{PAC}/g_{extract}$, when compared with the remaining fractions, **PAC F2** and **PAC F3**, that were mostly composed of PACs, 987 and 972 $mg_{PAC}/g_{extract}$, respectively.

In terms of %Gal (figure 2.4B) the values obtained for the different fractions were close to **PAC Ace**, 46.2%, but with a slight increase for each fraction varying from 47.9% for **PAC F1** to 51.2% for **PAC F3**. As far as mDP is concerned, **PAC F1** resulted in an extract with a mDP value of 9.0 which is higher than the initial extract, **PAC F2** resulted in an extract that despite having high purity had a mDP value of 7.2 which is lower and **PAC F3** that resulted in a pure extract with a mDP value of 12.0.

From these values it can be concluded that with this fractionation method despite being possible to obtain pure fractions for analytical purposes, the resulting Y_{PAC}s for **PAC F2** and **PAC F3** (13.5 and 11.1 mg_{PAC}/g_{GP}, respectively) are too low to be considered an effective method for large scale application and therefore alternative methods must be considered. Nevertheless, these fractions were used as reference material in the quantification of PACs due to their high purity and high mDP values.

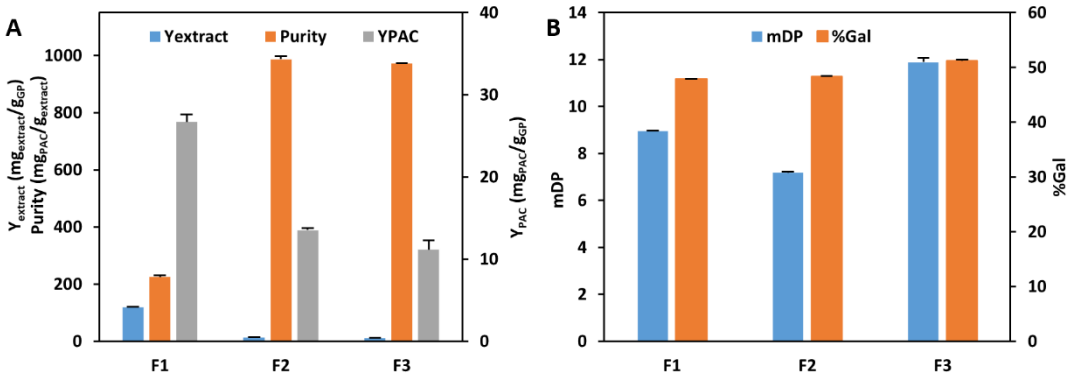


Figure 2.4. Characterization of PAC Ace fractions obtained with Toyopearl HW-40 resin (A - $Y_{extract}$, purity and Y_{PAC}, B - mDP and %Gal)

2.3.4. Normal Phase-HPLC-MS of Proanthocyanidins

From the chromatograms on figure 2.5 is possible to verify that the extract's mDP can be correlated with the behavior presented by the chromatogram i.e., lower mDP will lead to chromatograms that have comparatively higher concentration of compounds with lower retention time (lower DP) than with higher retention times (high DP) even when chromatographic separation is low, which is the case of the latter.

This is especially visible for **PAC Ace** and the derived fractions in which **PAC F1** is composed almost exclusively of PACs with low retention time (up until 25 min) while **PAC F2** is composed of PACs with medium retention time (from 20 to 40 min) and **PAC F3** content which elutes in the end of the chromatographic run (after 40 min).

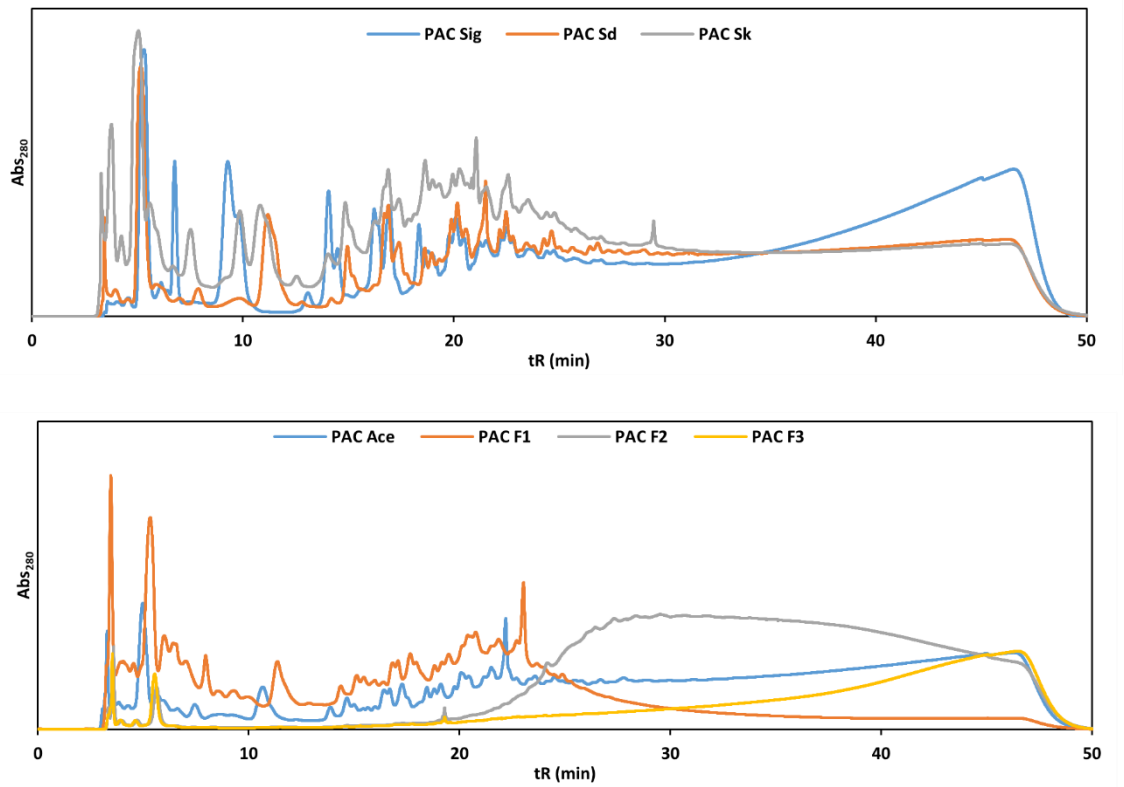


Figure 2.5. Normal phase-HPLC chromatograms of commercially available PAC extracts (PAC Sig, PAC Sd and PAC Sk) and PAC extracts obtained in the laboratory (PAC Ace, PAC F1, PAC F2 and PAC F3) acquired at 280 nm and at a concentration of 2.0 mg_{PAC}/mL

Despite being possible to effectively separate PACs up until a DP of 5 (figure 2.6) it was not possible to obtained resolved peaks above that point as demonstrated by the information obtained by Abs_{280} and MS (figure 2.6). In addition, it was not possible to detect molecular ions above the DP of 10 in any of the PAC extracts analyzed. Based on colorimetric quantification (figures 2.2A, 2.3A and 2.4A), phloroglucinolysis assay (figures 2.2B, 2.3B and 2.4B) and the NP-HPLC chromatograms (figure 2.5) it is fair to assume that nevertheless, PACs with DP higher than 10 are present in the extracts. There are two possible explanations for the inability of detecting PACs with higher DPs.

Firstly, the increasing number of stereoisomers associated with the high molecular weight decreases the absolute amount of each molecule which consequently leads to a decrease in absorbance and MS signal. This is corroborated by the signal widening that can be observed both in the absorbance signal (figure 2.5) and MS signal (figure 2.6) for PACs with higher DP.

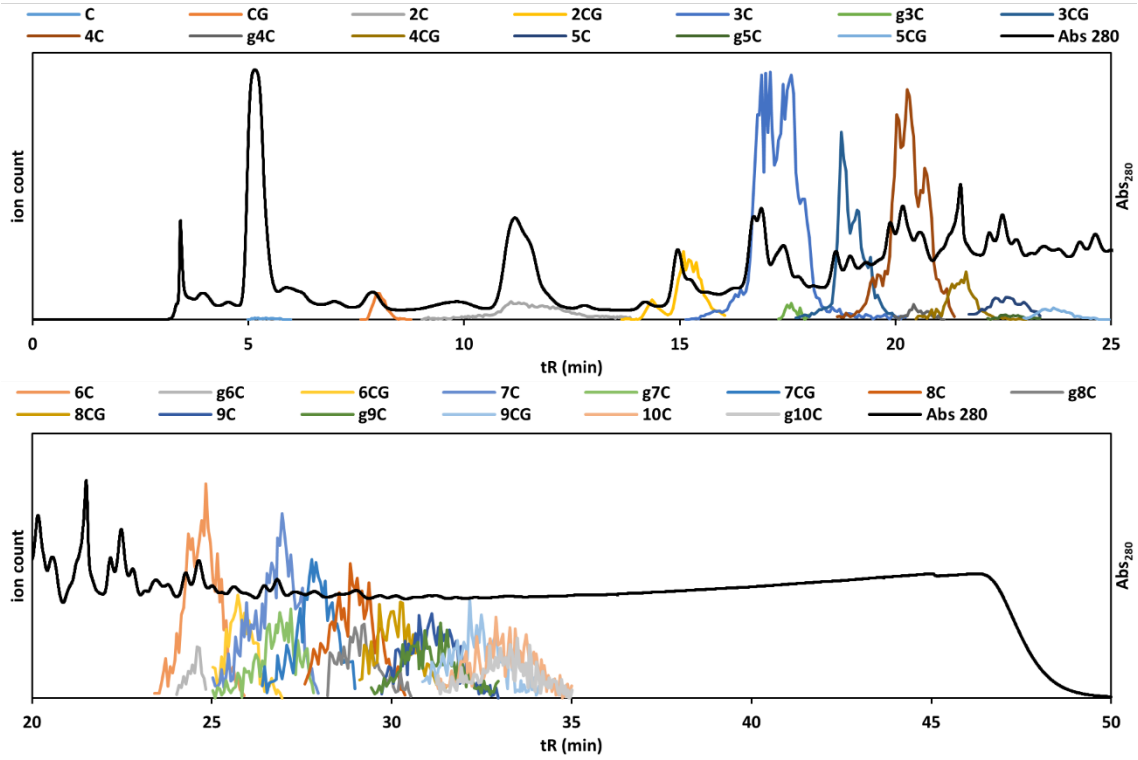


Figure 2.6. Normal phase-HPLC chromatogram of PAC Sd acquired at 280 nm, at a concentration of 2.0 mg_{PAC}/mL and the ions counts of PACs (numbers 1 to 10 followed by C represent DP, g = additional OH, G = additional gallate)

Secondly, the ionization efficiency also appears to be affected by DP which is corroborated by the fact all extracts, regardless of mDP, had a maximum ion count at PACs with DP value of three, and by the fact that extracts with higher mDP had considerably lower total ion count (figure 2.7).

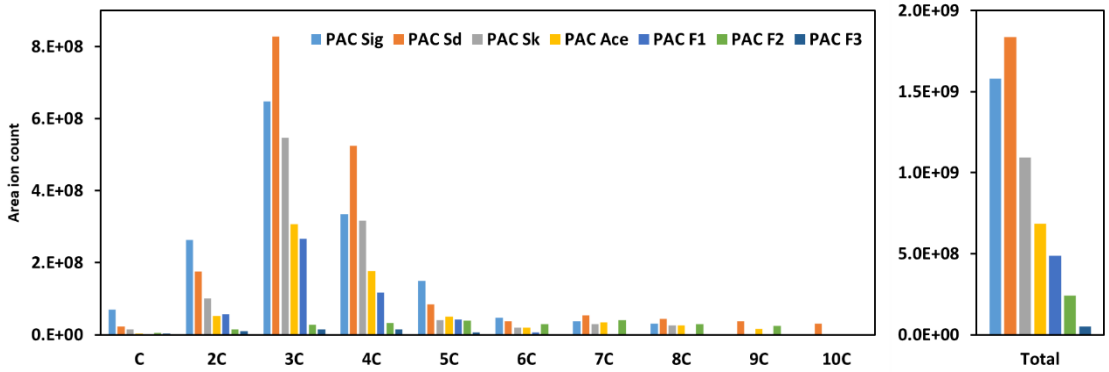


Figure 2.7. Ion count for each DP and the total for each sample

2.4. Conclusion

Considering the similar mDP and %Gal values obtained for commercial PAC extracts (**PAC Sig**, **PAC Sk** and **PAC Sd**) and the ones obtained with hot water extraction (**PAC HW**) is possible to conclude that the former is obtained with a similar method to the latter. Therefore, **PAC HW**

was used as reference in the following optimization processes as far as Y_{PAC}, mDP and Gal% are concerned.

Furthermore, the NP-HPLC method employed here was also shown to be limited when dealing with PACs with high DP due to low resolution and ionization efficiency. Nevertheless, it was also demonstrated that NP-HPLC enables the rapid distinction between samples with low and high mDP with no prior treatment.

3. Extraction of Proanthocyanidins by Maceration with Eutectic Solvents

Adapted from: Neto, R.T.; Santos, S.A.O.; Oliveira, J.; Silvestre, A.J.D. Tuning of Proanthocyanidin Extract's Composition through Quaternary Eutectic Solvents Extraction. *Antioxidants* **2020**, *9*, 1–17

Abstract

Currently available PAC extraction methods rely on dedicated crops and have low specificity and yield which limits their industrial application. Consequently, the development of novel methodologies and the use of sustainable sources is of great importance.

ESs have been proposed has good alternatives for conventional solvents due to their low price, easiness of preparation, biocompatibility, and ability of being custom made to a specific application.

Herein the effective extraction of PACs from GP and the possibility of tuning the extract's characteristics such as mDP and %Gal is explored by means of varying the composition of a quaternary ES composed by choline chloride, glycerol, ethanol and water. It was found that mDP values can vary from 6.0 to 7.37 and %Gal can vary from 32.5 to 47.1% while maintaining extraction yield above 72.2 mg_{PAC}/g_{GP}. Furthermore, the increase of temperature up to 100 °C has showed a significant effect on the extraction yield being possible to increase it by 238% when compared to the conventional extraction method.

3.1. Introduction

PACs, also known as condensed tannins, are secondary metabolites ubiquitous to all plant kingdom [239]. PACs are polymeric phenolic compounds comprising of flavan-3-ols monomers such as catechin and its derivatives (figure 1.3). PACs are believed to play essentially two roles in plants, namely, as defense against microbial pathogens [23,24] and as deterrents against herbivory [25,26]. The mechanism by which PACs are able to achieve these functions come from their complexation ability of metal ions in the former case [240], and protein aggregation [27] and enzyme inhibition [29] in the latter.

Currently, PACs are mostly used in the production of high-end leather [241] and wood agglomerates [242] as well as in wine maturation [243]. More recently, PACs have also been proposed as viable alternatives for the replacement of synthetic food grade antioxidant [244,245] and antimicrobial [246] agents. In addition, PACs have also been reported for their beneficial properties for human health, more specifically, in the inhibition of enzymes related to high blood pressure [204] and carbohydrate metabolism [247–249], as well as anti-cancer [86,250] and anti-inflammatory activities [87].

The most common source of PACs for commercial use is Quebracho (*Schinopsis lorentzii*) heartwood which can have PACs contents up to 43% (m/m) [251]. Nevertheless, Quebracho

trees are only present in South America and their use as raw material implies the transportation of the resulting extract across the globe. In addition, PACs obtained from Quebracho come from a dedicated crop, specifically grown for that purpose which is not the optimal use of water and arable land in terms of economic value since they can be found in high concentration in agroforestry by-products [252].

Agroforestry by-products could be used as alternative raw materials for the obtention of PACs which would allow for a more sustainable and efficient process as far as limited resources, such as water and arable land, are concerned. In addition, this approach is also valuable for the decrease of overall amount of waste as described by the European commission directive (2008/98/EC) [136] as well as to increase economic value of agroforestry by-products such as fruit peels [121] and wood barks [183] by taking advantage of their high PAC content as summarized elsewhere [252].

Grape pomace in particular, is a by-product of wine production which has been the subject of several dedicated reviews that explored its potential as a source of antimicrobial agents [253], human health promoter [254] and animal nutrition [255] in part due to its high PACs content.

Wine industry is one of the most important agricultural activities worldwide and produced 292 million hL of wine and 44 million tons of grape for that purpose in 2018 [256], which led to the production of approximately 6.6 million tons of grape pomace (considering that 1 kg of fresh grapes results in 0.15 kg of pomace). Currently, grape pomace has little to no commercial value and often represents additional costs for the producer related to their disposal.

Presently, the industrial use of PAC extracts is often limited by their high price, especially when compared to the available synthetic (yet more harmful) alternatives. In general, these extracts are obtained through the use of a hot pressurized sulfite aqueous solution [106]. Unfortunately, the amount of unextracted PACs can be as high as 62.5% in white grapes [112] and 62.3% in Norway spruce bark [184], resulting in a low yield process and a final extract that is mostly composed of PACs with lower DP that are not as effective as the ones with higher DP. Additionally, in order to achieve the intended mDP further downstream processing is needed making its final price prohibitive for most applications.

Therefore, to further increase the extraction efficiency and added value of PACs extracts it is essential to combine the use of by-products rich in these compounds with innovative extraction procedures. Some innovations come from the combination of ultrasound assisted or microwave assisted extraction with aqueous mixtures of organic solvents as discussed elsewhere [252].

Nevertheless, these approaches still rely on the use of harmful organic solvents and new greener alternatives must be found.

More recently, ESs have been proposed as very promising media for the extraction of bioactive compounds from biomass [257]. ESs can be described as a mixture of two compounds (HBA and HBD) that have a decreased melting point when compared to the individual components and were first proposed as a solvent in the context of DES by Abbot *et al.* [207]. These differentiate from ESs by the fact that the decrease in the melting point is greater than what would be expected in an ideal mixture [208]. (D)ESs are characterized by their biocompatibility, low toxicity, easiness of preparation and ability of being custom made to a specific application.

The application of ESs in the extraction of PACs is still limited, however they have been studied in the extraction of PACs from *Gingko biloba* leaves with satisfactory results [216]. A mixture of choline chloride and malonic acid at a molar proportion of 1:2 with 55% (m/m) of water at 65 °C was used and an improvement of 67% when compared with aqueous 70% (v/v) acetone was obtained.

Due to (D)ESs high viscosity, water addition is frequently employed, as shown in the example presented before [216]. Nevertheless, the use of ethanol for that purpose, a solvent that is naturally sourced and safe for human consumption, is still unexplored. In addition, it is also a more effective solvent at room temperature on the extraction of PACs [181] when compared to water and therefore, could represent a valuable option in the development of new solvent systems.

One aspect that is frequently overlooked in the extraction of PACs is the possibility of tuning the extract's final characteristics such as mDP and %Gal. mDP has been shown to positively correlate with the inhibition of α-glucosidase and pancreatic lipase [200], cellular antioxidant [258] and anti-inflammatory [87] activities, and protein precipitation [38,259]. %Gal has been shown to influence antiviral activity [260] and protein precipitation [261] as well. In addition, both characteristics appear to play an important role in antiproliferation of human colon cancer cells [262].

Herein, the use of mixtures of ethanol, water and ESs in the obtention of PACs from white grape pomace was explored for the first time. Several combinations of HBAs and HBDs were screened, and the best candidate was selected. The effects of mass fraction of HBA, HBD, water and ethanol were optimized using response surface methodology (RSM) to determine the best solvent composition in the tuning of the final extract characteristics. A similar approach was used to optimize the extraction conditions of PACs, namely the temperature, solid:liquid ratio

and extraction time. Y_{PAC} was quantified using the acid butanol method and the mDP and %Gal were determined by phloroglucinolysis.

3.2. Materials and Methods

3.2.1. Grape Pomace Preparation

GP was prepared following the method detailed in section 2.2.2.

3.2.2. Proanthocyanidin Reference Material

PAC reference material was isolated from GP following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention.

3.2.3. Purification of Proanthocyanidins with High Degree of Polymerization

High DP PACs were isolated following the method described by Neto et al. [232] that is detailed in section 2.2.4.

3.2.4. Acid Butanol Assay for Proanthocyanidin Quantification

PACs extract's purity ($mg_{PAC}/g_{extract}$) and Y_{PAC} (mg_{PAC}/g_{GP}) were determine following the acid butanol assay described by Porter et al. [31] that is detailed in section 2.2.5.

3.2.5. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PAC's mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] that is detailed in section 2.2.6.

3.2.6. Extraction of Proanthocyanidins with Ethanol Solutions

Prior to exploring the effect that ethanol addition has ES solvent systems, the combination ethanol and water at different percentages was assayed (**PAC $EtOH_x$**; x=15, 30, 50 or 70) in a single step SLE for 4 h at room temperature with 10% (m/m) of GP, followed by centrifugation at 10 000 rpm for 10 min. The supernatants were collected and stored at -20 °C until characterization.

3.2.7. Preparation of Eutectic Solvents with Water/Water:Ethanol

ESs mixtures were prepared by adding the appropriate mass of each component to a flask with a magnetic bar and placing it in a magnetic stirrer at room temperature or at 40 °C, if needed, until a continuous liquid phase was obtained.

3.2.8. Screening of Hydrogen Bond Acceptor and Hydrogen Bond Donor Combination

Different combinations of HBAs (choline chloride (ChCl), betaine (Bet), proline (Pro)) and HBDs (urea (Ur), malic acid (MalA), glucose (Glu) and glycerol (Glyc)) were screened at a 3:1 (HBA:HBD) molar proportion with a 50% (m/m) water content. GP samples were incubated for 4 h at 30 °C with 5% (m/m) of GP under continuous stirring at 100 rpm, followed by centrifugation at 10 000 rpm for 10 min. The supernatants were collected and stored at -20 °C until characterization. In the back on these results the most suitable HBA candidate was selected based on Y_{PAC}.

HBD selection was made using a HBA:HBD molar ratio of 3:1 at a final concentration of 25 and 75% (m/m) with the remainder being composed of either water or a water:ethanol mixture at a mass proportion of 1:1. The extractions were made as described previously and the most suitable HBD candidate was selected based on Y_{PAC}, mDP and solvent characteristics.

3.2.9. Determination of Optimal Solvent Composition

After a preliminary selection of the most suitable HBA and HBD candidates, namely ChCl and Glyc, respectively, and having verified the positive impact of water and ethanol addition, the optimal extraction media composition was determined using RSM for mixtures using D-optimal design in Expert Design v12 from StateEase. The experimental design consisted of 20 experimental points in which the assayed variables were the mass fractions of choline chloride (x_{ChCl}), glycerol (x_{Glyc}), water (x_{water}) and ethanol (x_{EtOH}), and the experimental constraints were $x_{ChCl} < 0.7$, $x_{Glyc} < 0.95$, $0.05 < x_{water} < 0.5$, $x_{EtOH} < 0.4$ and $x_{ChCl} + x_{EtOH} < 0.7$. Experimental solvent compositions are detailed in table 3.1 and all extractions were performed at 30 °C under continuous agitation at 100 rpm with 10% (m/m) of grape pomace. Validation runs were performed in triplicate for the solvent compositions that resulted in the best Y_{PAC} and best %Gal.

Table 3.1. Mass fractions of choline chloride, glycerol, water, and ethanol used in optimization of solvent composition and respective measured and predicted values of Y_{PAC}, mDP and %Gal

Run	x_{ChCl}	x_{Glyc}	x_{water}	x_{EtOH}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal	
					Measured	Predicted	Measured	Predicted	Measured	Predicted
1	0.000	0.100	0.500	0.400	69.6	68.3	6.2	6.3	39.7	39.8
2	0.263	0.316	0.050	0.371	75.7	74.5	6.5	6.8	40.7	40.9
3	0.237	0.446	0.317	0.000	59.7	60.9	6.5	6.4	33.6	34.1
4	0.000	0.500	0.500	0.000	48.7	49.2	6.3	6.2	34.8	34.5
5	0.000	0.100	0.500	0.400	66.8	68.3	6.3	6.3	39.9	39.8
6	0.540	0.250	0.050	0.160	72.5	74.2	6.8	6.6	38.3	38.2
7	0.000	0.500	0.500	0.000	50.6	49.2	6.2	6.2	34.9	34.5
8	0.300	0.040	0.260	0.400	84.1	84.0	6.5	6.3	36.5	36.1
9	0.000	0.526	0.282	0.192	73.7	72.4	7.0	6.8	42.9	42.4
10	0.000	0.950	0.050	0.000	57.4	59.8	7.7	7.7	44.9	45.1
11	0.700	0.000	0.300	0.000	81.3	76.9	5.7	5.7	29.1	29.4
12	0.104	0.699	0.050	0.147	68.5	68.0	7.2	7.4	43.9	44.5
13	0.000	0.550	0.050	0.400	72.2	71.5	7.2	7.2	47.3	46.6
14	0.000	0.950	0.050	0.000	61.5	59.8	7.5	7.7	44.5	45.1
15	0.401	0.000	0.500	0.099	72.4	71.5	5.6	5.5	28.9	28.1
16	0.378	0.552	0.070	0.000	60.5	58.6	7.0	7.0	39.4	38.7
17	0.000	0.550	0.050	0.400	69.7	71.5	7.4	7.2	46.5	46.6
18	0.147	0.231	0.500	0.122	61.9	63.2	5.7	6.0	31.8	33.4
19	0.459	0.235	0.306	0.000	65.5	66.7	6.0	6.1	31.6	31.2
20	0.700	0.000	0.300	0.000	73.3	76.9	5.7	5.7	29.3	29.4

3.2.10. Determination of Optimal Extraction Conditions

Optimal extraction conditions were determined using RSM with Box-Behnken Design in Expert Design v12 from StateEase. The experimental design consisted of 15 experimental points in which the assayed variables were temperature (temp), biomass percentage (%BM) and extraction time (time) with minimum (-1) and maximum (1) values ranging from 70 to 110 °C, 5 to 20% (m/m) and 1 to 5 h, respectively. Experimental conditions for each run are detailed in table 3.2 All extractions were performed under continuous agitation at 100 rpm and solvent composition was the one from which the highest Y_{PAC} values were obtained in the previous section. Validation runs were performed in triplicate for the experimental conditions that resulted in the best Y_{PAC} and best %Gal.

Table 3.2. Temperature, biomass percentage and time used in the optimization of extraction conditions and respective measured and predicted values of Y_{PAC}, mDP and %Gal

Run	T (°C)	%BM	t (h)	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal	
				Measured	Predicted	Measured	Predicted	Measured	Predicted
1	90	20.0	1	111.9	116.5	5.8	5.9	30.6	29.9
2	90	12.5	3	131.8	133.8	5.8	5.7	28.8	29.2
3	90	12.5	3	134.0	133.8	5.5	5.7	29.0	29.2
4	90	5.0	5	130.4	127.4	5.1	5.0	26.0	26.6
5	110	20.0	3	130.4	128.7	5.4	5.4	28.2	28.7
6	90	20.0	5	133.1	134.3	5.7	5.6	29.0	29.9
7	110	12.5	5	133.6	141.0	4.8	4.9	28.4	27.9
8	70	12.5	1	102.3	102.9	5.7	5.6	30.5	30.5
9	70	12.5	5	113.2	110.6	5.7	5.7	30.4	30.5
10	70	20.0	3	102.4	98.3	5.7	5.7	32.0	31.2
11	90	5.0	1	129.5	129.8	5.3	5.3	27.1	26.1
12	70	5.0	3	95.6	101.6	5.1	5.1	26.6	27.3
13	90	12.5	3	138.7	133.8	5.6	5.7	28.7	29.2
14	110	5.0	3	135.2	131.9	4.8	4.8	24.5	24.8
15	110	12.5	1	135.8	133.3	5.7	5.7	28.3	27.9

3.3. Results

3.3.1. Evaluation of Ethanol Content Effect on the Extraction of Proanthocyanidins

When extracting with aqueous solutions of ethanol it is possible to improve the results comparing to the ones that are obtained with hot water, increasing the Y_{PAC} by more than 4-fold by simply adding 15% (v/v) of ethanol. Interestingly, Y_{PAC} only increases up until 50% (v/v) of ethanol with which is possible to obtain 67.1 mg_{PAC}/g_{GP}, decreasing to 58.2 mg_{PAC}/g_{GP} when 70% (v/v) aqueous ethanol is used (figure 3.1A). A direct correlation is verified between ethanol content in the extraction mixture and %Gal that reaches its maximum value at 70% (m/m) ethanol with 41.6% of galloylated monomers (figure 3.1B). This trend was not verified for mDP which resulted in a constant value above 30% (m/m) ethanol (5.9) (figure 3.1B).

As far as the use of mixtures of ethanol and water is concerned, it is clear that the variation of ethanol concentration allows for a good control in the %Gal without considerably affecting mDP which occurs in detriment of Y_{PAC} for ethanol percentages higher than 50% (m/m), being indicative that ethanol is not an efficient system by itself for the extraction of PACs.

Nevertheless, considering the straight correlation that was verified between ethanol content and %Gal it is possible that ethanol could be used to tightly control this characteristic in an extract obtained with a complex solvent system.

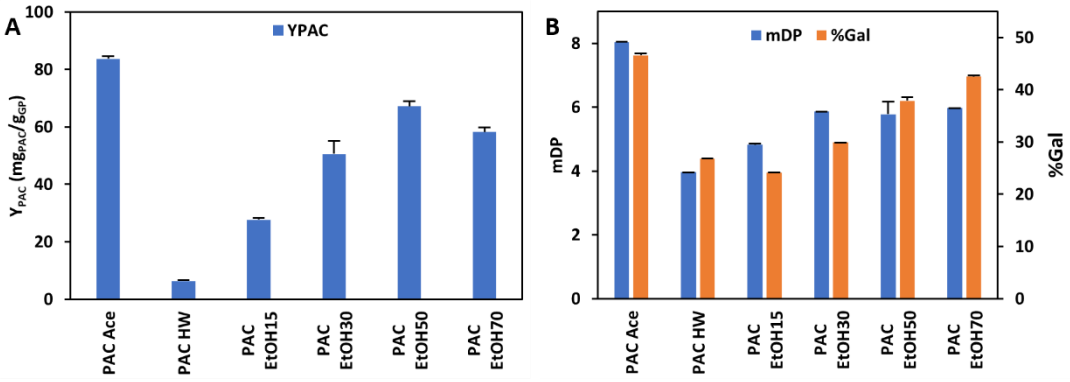


Figure 3.1. Comparison of Y_{PAC} (A) and mDP and %Gal (B) obtained with conventional solvents and aqueous ethanol solutions (PAC Ace - aqueous acetone extraction; PAC HW - hot water extraction; PAC EtOH x - aqueous ethanol extraction)

3.3.2. Screening of Hydrogen Bond Acceptor and Hydrogen Bond Donor Combination

The selected HBAs and HBDs were combined and tested at a molar proportion of 3:1 (HBA:HBD) and with 50% (m/m) of added water (figure 3.2) from which is obvious that when using ChCl as HBA the obtained results are higher than the ones obtained with Bet or Pro, regardless of HBD. Consequently, Bet or Pro were not further explored in the context of extraction of PACs.

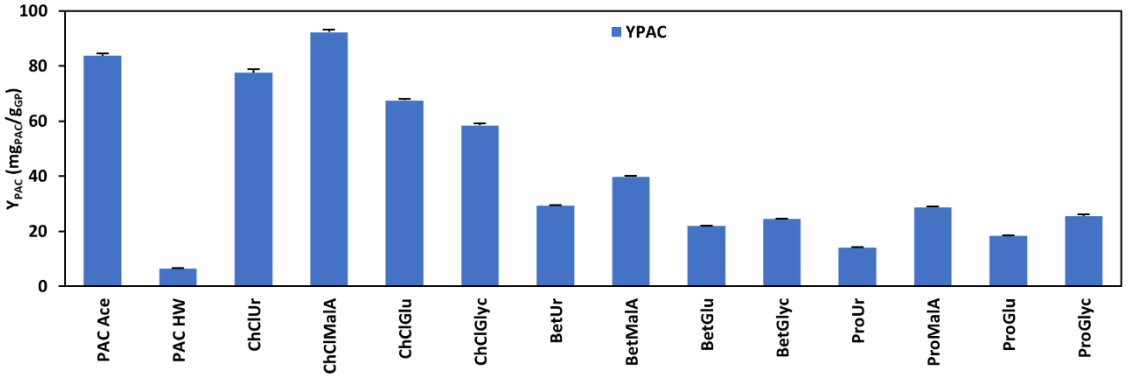


Figure 3.2. Screening of the effect of HBA:HBD combinations on Y_{PAC} (PAC Ace - aqueous acetone extraction; PAC HW - hot water extraction; ChCl - choline chloride, Bet - betaine, Pro - proline, Ur - urea, MalA - malic acid, Glu - glucose, Glyc - glycerol)

The effect of different HBDs in combination with ChCl on the Y_{PAC} at a molar proportion of 3:1 (HBA:HBD) is compared in figure 3.3A and it is possible to observe that at a concentration of 75% of ES, the best results were obtained with Ur and MalA (80.7 and 81.4 mg_{PAC}/g_{GP}, respectively) followed by Glu and Glyc (65.9 and 66.4, respectively). Additionally, if the water content is increased to 75%, a decrease in Y_{PAC} of approximately 50% is observed for all candidates except

for MalA which only decreases 30%. Nevertheless, this effect can be overcome if a mixture of water and ethanol is used instead of adding only water to ESs. This is especially true for ESs containing Glu and Glyc (75.0 and 76.2 mg_{PAC}/g_{BGP}, respectively) which led to Y_{PAC}s higher than the ones obtained with 75% ES and less accentuated for Ur and MalA (72.3 and 85.0 mg_{PAC}/g_{GP}, respectively).

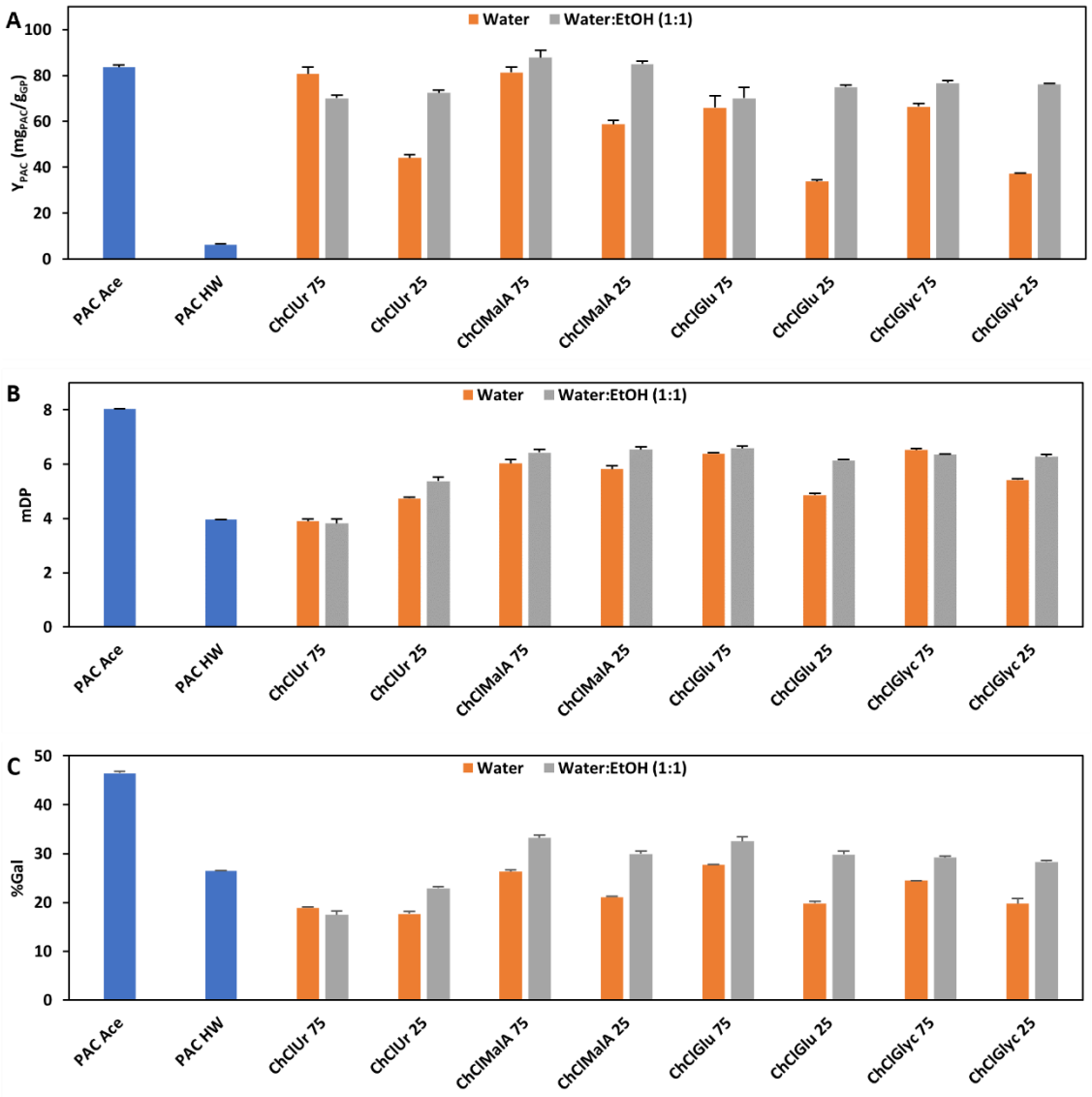


Figure 3.3. Comparison of Y_{PAC} (A), mDP (B) and %Gal (C) between conventional solvents (HSE, AAE and AEE50) and mixtures of ES with water or water and ethanol. (ChCl - choline chloride; Ur - urea; MalA - malic acid; Glu - glucose; Glyc - glycerol; the number following ES represents its percentage)

In terms of mDP (figure 3.3B), the best results were obtained with Glu and Glyc (6.4 and 6.5, respectively) followed by MalA (6.0) and Ur (3.9). As for Y_{PAC}, the increase on the water content has a negative effect on the overall results, except for Ur, and the addition of ethanol overcomes the reduction in the mDP. Similar observations were made for %Gal (figure 3.3C) concerning the

effect of HBD and water content. Nevertheless, the addition of ethanol has a more noticeable effect on the final result when compared to the effect on mDP which is corroborated by the results depicted in figure 3.1B.

In order to select the better HBA:HBD combination for the intended application, ESs stability and possible interactions with PACs must also be considered.

More specifically, if only Y_{PAC} is considered, the combination of ChCl and MalA would be considered the best candidate, as shown in figure 3.3A. Especially, considering that this specific mixture and others alike have been extensively characterized and proposed as valid alternatives for biomass processing [263]. Unfortunately, and as described elsewhere ESs composed by ChCl and MalA are not long term stable at room temperature and are negatively affected by temperature even in short incubation times [264]. These would limit considerably not only the process optimization by means of increased temperature but the possibility of recycling the solvent which might be necessary in order to develop a feasible industrial process.

Despite resulting in Y_{PAC} that in some conditions are comparable to the ones obtained with MalA, ESs containing Ur give rise to extracts with low mDP and %Gal. In addition, contrary to what happens with the other candidates, Ur does not work synergistically with ethanol that as shown before is an important factor for %Gal tuning.

The use of Glu mixtures resulted in lower Y_{PAC} with little effect on mDP and %Gal. Nevertheless, Glu [265] and carbohydrate based [266] ESs have in general very high viscosity values which require the addition of considerable amounts of water in order to achieve reasonable viscosity levels. This in turn results, as discussed previously, in a reduction of the extraction efficiency and in a decrease in microbiological stability which makes solvent recycling not feasible.

The mixture of ChCl with Glyc presents comparable values of Y_{PAC} and mDP to the ones obtained with Glu mixtures but lower %Gal values. As in the case of MalA, Glyc-based solvents have been extensively described for biomass processing both in [267] and out [268] of the ES context. Additionally, Glyc is a cheap and abundant by-product obtained from biodiesel production that can be sustainably sourced [269] and is already largely used in food, medical and cosmetic industries due to its safety for human consumption. Recently, promising results were observed in the use of Glyc-based ES for the extraction of other flavonoids, namely apigenin, luteolin and quercetin from *Satureja thymbra* [270].

Based on these results, ChCl and Glyc mixtures were chosen as the best candidates for solvent composition optimization due to low price, chemical and microbiological stability. Additionally,

water and ethanol content were also considered due to their effect on solvent viscosity reduction and %Gal content tuning, respectively.

3.3.3. Determination of Optimal Solvent Composition

For solvent composition optimization a 20-run mixture RSM experiment was performed where x_{ChCl}, x_{Glyc}, x_{water} and x_{EtOH} were varied as specified in table 3.1 and Y_{PAC}, mDP and %Gal were determined.

The resulting polynomials are presented in equations 3.1., 3.2. and 3.3. for Y_{PAC}, mDP and %Gal, respectively, from which contour plots were derived and presented in figure 3.4 where x_{ChCl}, x_{Glyc} and x_{water} are varied and x_{EtOH} is kept constant at 0.2.

Equation 3.1. $$Y_{PAC} = 72.2x_{ChCl} + 58.3x_{Glyc} - 13.8x_{water} + 8.13x_{EtOH} - 38.0x_{ChCl}x_{Glyc} + 145x_{ChCl}x_{water} + 145x_{ChCl}x_{EtOH} + 108x_{Glyc}x_{water} + 129x_{Glyc}x_{EtOH} + 278x_{water}x_{EtOH}$$

Equation 3.2. $$mDP = 6.21x_{ChCl} + 7.87x_{Glyc} + 4.49x_{water} + 6.46x_{EtOH} + 3.31x_{water}x_{EtOH}$$

Equation 3.3. $$\%Gal = 39.1x_{ChCl} + 46.3x_{Glyc} + 22.7x_{water} + 32.7x_{EtOH} - 12.8x_{ChCl}x_{Glyc} - 22.7x_{ChCl}x_{water} + 27.0x_{Glyc}x_{EtOH} + 48.3x_{water}x_{EtOH}$$

The performed ANOVA analysis revealed that the resulting models for Y_{PAC}, mDP and %Gal all have *p*-values < 0.0001, adjusted r^2 values of 0.92, 0.94 and 0.98, respectively and predicted r^2 values of 0.82, 0.92 and 0.97, respectively, indicating the statistical robustness of the resulting quadratic models.

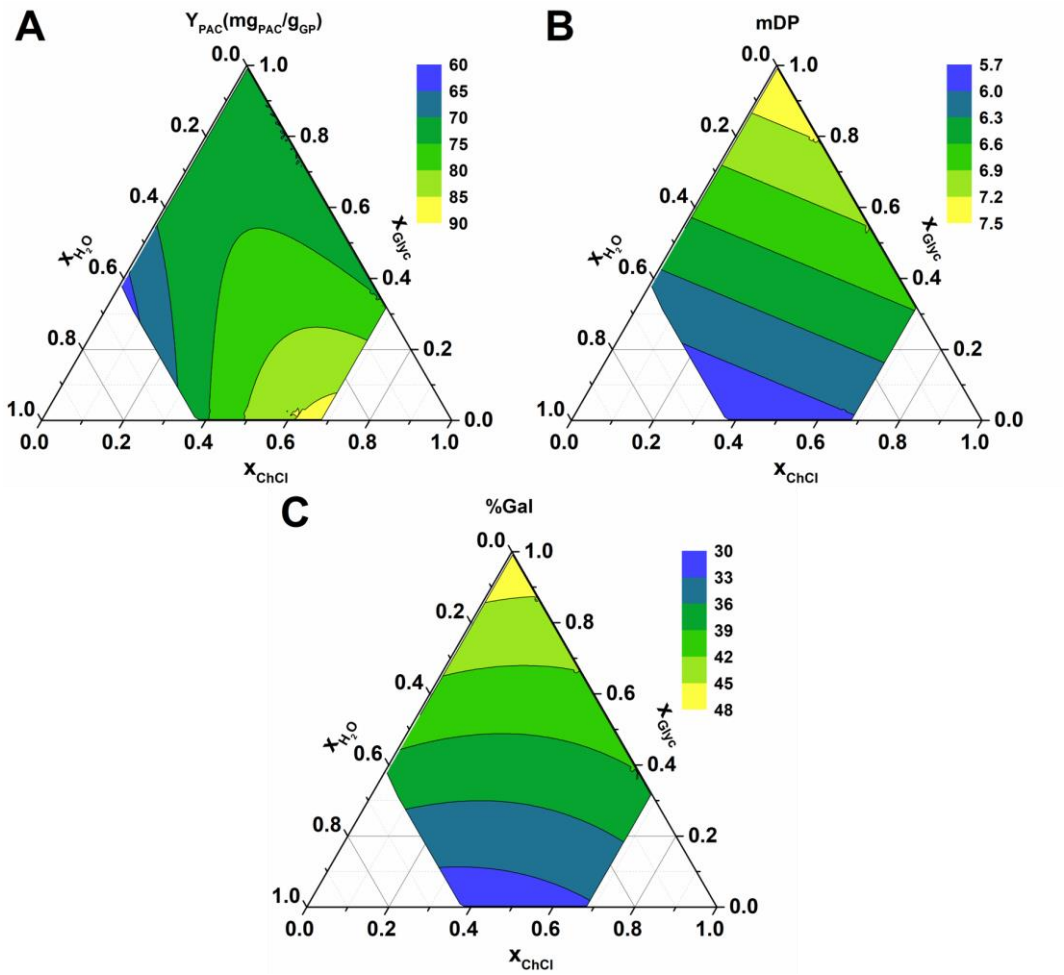


Figure 3.4. Contour plots obtained for solvent compostiion optimization at x_{EtOH} = 0.2 for A - Y_{PAC}, B - mDP and C - %Gal

From these models it can be concluded that, with respect to Y_{PAC} (figure 3.4A), x_{ChCl}, x_{water} and x_{EtOH} have an optimal value at 0.5, 0.3 and 0.2, respectively and that x_{Glyc} has a detrimental effect on it. With this combination, the predicted values of Y_{PAC}, mDP and %Gal are 86.6 mg_{PAC}/g_{GP}, 6.0 and 32.5%, respectively which represents 102.4% of the Y_{PAC} obtained with **PAC Ace**, a decrease in mDP, 8.1, and a lower %Gal, 46.6%. The validation runs resulted in a Y_{PAC} of 72.4 mg_{PAC}/g_{GP}, mDP of 5.9 and %Gal of 30.6% which are in a reasonable agreement with the predicted values, validating the models. Based on the results obtained for ESs, it is possible to conclude that **PAC Ace** is a more suitable method for laboratory scale extraction high DP PACs, especially if solvent removal is considered.

Nevertheless, when compared to the conventional extraction process, **PAC HW**, a 13-fold increase in Y_{PAC} and higher mDP values, 6.0 in opposition to 4.0, are observed, which indicates the potential of the proposed system in replacing conventional methods.

If other aspects of the final extract are prioritized, namely mDP (figure 3.4A) and %Gal (figure 3.4C), it is possible to achieve, with this system, values that can go as high as 7.37 and 47.1%, respectively, while maintaining a Y_{PAC} of 72.2 mg_{PAC}/g_{GP}. This can be achieved with x_{Glyc}, x_{water} and x_{EtOH} values of 0.68, 0.05 and 0.27, respectively, which still represents an 11-fold increase in Y_{PAC} when compared with the conventional extraction process, **PAC HW**. The validation runs resulted in a Y_{PAC} of 67.2 mg_{PAC}/g_{GP}, mDP of 7.5 and %Gal of 47.2% which are in a reasonable agreement with the values predicted by the model.

Considering the different mDP and %Gal that were obtained with different component proportions of the proposed quaternary system, it can be inferred that by varying ESs composition it is possible to tune the final extract characteristics to specific values of mDP and %Gal. More specifically, to values ranging from 5.95 to 7.37 for mDP and from 32.5 to 47.1% for %Gal, whilst maintaining an acceptable Y_{PAC}. Additionally, if the temperature difference between the conditions used in the solvent composition optimization assay (30 °C) and conventional extraction method, **PAC HW** (110 °C), is considered a further increase in Y_{PAC} is to be expected.

3.3.4. Determination of Optimal Extraction Conditions

For the optimization of the extraction conditions, the solvent composition that resulted in a higher Y_{PAC} (x_{ChCl} = 0.5, x_{water} = 0.3 and x_{EtOH} = 0.2) was chosen since mDP and %Gal are still above what is obtained with HSE.

Consequently, a 15-run RSM experiment with Box-Behnken design was made where temperature, %BM and extraction time were varied as specified in table 3.2 and Y_{PAC}, mDP and %Gal were determined.

The resulting polynomials are presented in equations 3.4., 3.5. and 3.6. for Y_{PAC}, mDP and %Gal, respectively, from which contour plots were derived and presented in figure 3.5A-F where %BM was kept constant at 14.4% in A, C and E, and extraction time was kept at 5 h in B, D and F.

Equation 3.4. $$Y_{PAC} = -184 + 6.10\text{temp} + 1.81\%_{BM} - 2.30\text{time} + 0.338\%_{BM}\,\text{time} - 0.0297\text{temp}^2 - 0.122\%_{BM}^2$$

Equation 3.5. $$\text{mDP} = -0.0559 + 0.0996\text{temp} + 0.140\%_{BM} + 0.430\text{time} - 0.00571\text{temp time} - 0.000505\text{temp}^2 - 0.00407\%_{BM}^2$$

Equation 3.6. $$\%\text{Gal} = 28.3 - 0.0629\text{temp} + 0.794\%_{BM} - 0.0214\%_{BM}^2$$

The performed ANOVA analysis revealed that the resulting models for Y_{PAC}, mDP and %Gal have *p*-values of 0.0002, 0.0007 and < 0.0001, respectively, adjusted r^2 values of 0.89, 0.88 and 0.89, respectively and predicted r^2 values of 0.73, 0.67 and 0.82, respectively, indicating the statistical robustness of the resulting quadratic models.

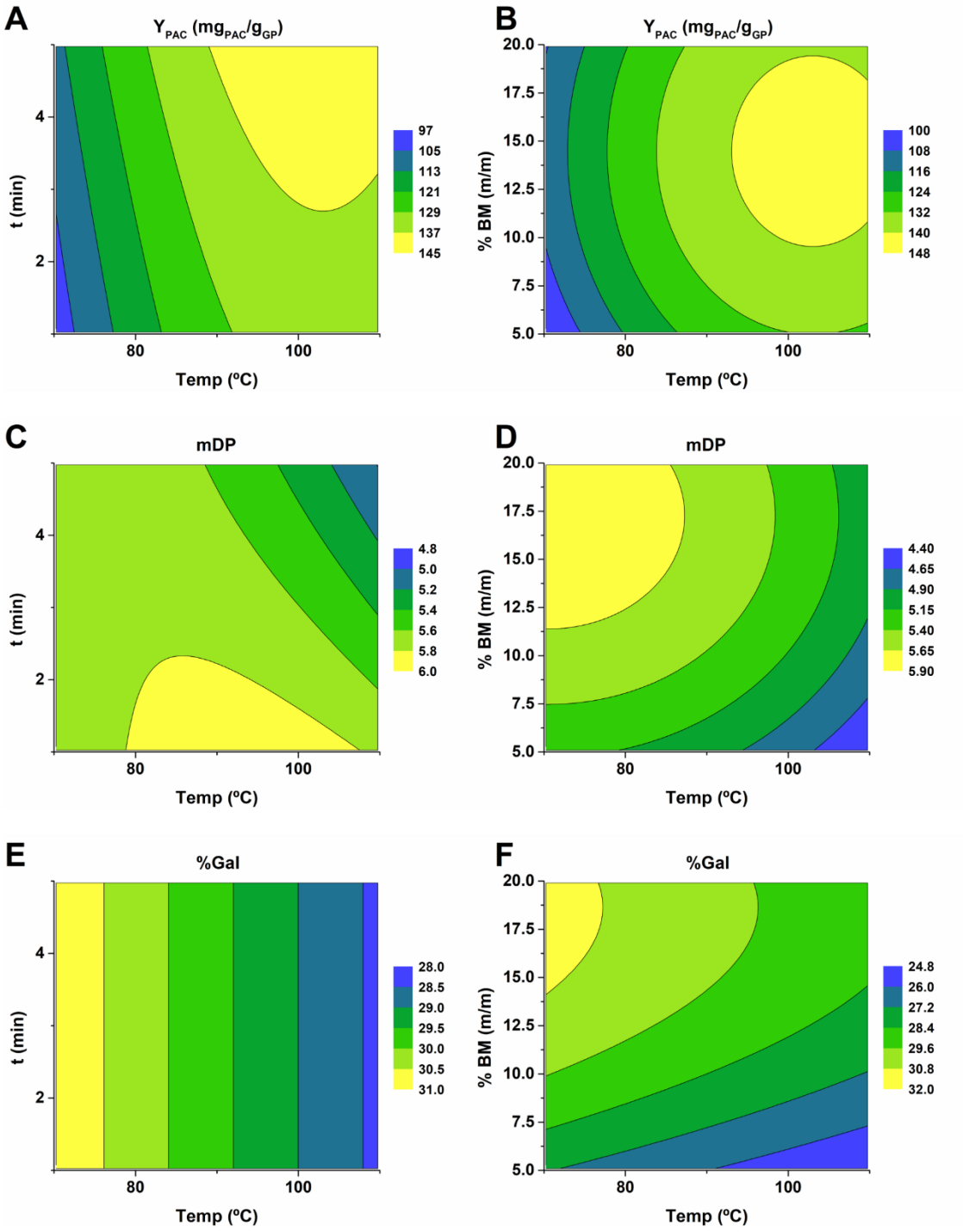


Figure 3.5. Contour plots obtained for extraction conditions optimization for A and B - Y_{PAC}, C and D - mDP and E and F - %Gal

From these models it can be concluded that the optimal Y_{PAC} values are obtained with 14.4% (m/m) of GP, at 102.8 °C for 5 h (figure 3.5A and B). This resulted in predicted Y_{PAC} of 143.0 mg_{PAC}/g_{GP}, mDP of 5.2 and %Gal of 28.8% which despite representing a 65% increase on the Y_{PAC} from what is obtained at 30 °C and 22-fold increment when compared to **PAC HW**, also represents a decrease in mDP compared to the value obtained at 30 °C (6.0). The validation runs resulted in a Y_{PAC} of 144.1 mg_{PAC}/g_{GP}, mDP of 6.0 and %Gal of 28.3% which are in agreement with the model predictions.

In addition, and even though the best results are obtained with 5 h of extraction time, this factor has a small impact on the overall Y_{PAC}. Furthermore, if mDP is considered (figure 3.5C) it becomes clear that at temperatures above 90 °C, the increase in extraction time causes a decrease in mDP, indicating thermal degradation of the PACs, which is in line with the results published by Ramos *et al.* [131].

With this in mind, the results can be improved considering both Y_{PAC} and mDP in which case the optimal conditions would be 99 °C with 13.4% of biomass for 1 h. In this situation the extraction process would have an Y_{PAC} of 133.6 mg_{PAC}/g_{GP} and would result in a final extract with a mDP of 5.9 and %Gal of 28.8%. These conditions are not optimal for Y_{PAC} but would enable the obtention of an extract more similar to the one obtained at 30 °C in terms of mDP. The confirmation runs resulted in a Y_{PAC} of 125.9 mg_{PAC}/g_{GP}, mDP of 6.5 and %Gal of 29.6% which are in a reasonable agreement with what should be expected.

mDP also relates with the %BM (figure 3.5D), being possible to obtain higher mDP values at higher biomass concentrations which further indicates the selectivity of the quaternary solvent system towards PACs with higher DP. This conclusion is based on the fact that with an increase in biomass concentration one should expected an increase in compounds that are more easily extracted in detriment of others, in this case PACs with higher DP.

As far as %Gal is concerned (figure 3.5E and F), extraction time has no effect on the final result and, similarly to mDP, an increase in %Gal can be achieved with higher %BM and lower temperatures, although this effect is less accentuated.

3.4. Conclusion

The models developed here, in addition to allowing for the optimization of the process in terms of Y_{PAC}, mDP and %Gal, can also be used to minimize compositional differences in the final extract that derive from differences in the grape variety used as raw material [271] or different plant parts from the same variety [176], enabling the compositional normalization of the final

extract and facilitating its implementation in industrial processes. If taken to its full potential these models could also be employed to minimize differences between two completely different sources [252] further facilitating the industrial implementation of PAC extracts in the context of circular economy.

ESs have recently increased their popularity in biomass processing and the work here presented further demonstrates the potential that this type of solvents has on the improvement of conventional methodologies. More specifically, herein it is described for the first time, at the best of our knowledge, the possibility of selectively improving the final extracts' content in PACs with higher degree of polymerization by means of a quaternary solvent system composed of choline chloride, glycerol, water and ethanol. Furthermore, it was shown that it is possible to tune the percentage of galloylated PACs to specific values by varying the mass fractions of the four components whilst not significantly compromising on the overall Y_{PAC}.

In addition, the feasibility of using grape pomace as raw material for the extraction of PACs is further demonstrated reinforcing the role of by-products in the context of a circular economy.

4. Extraction of Proanthocyanidins by Microwave-Assisted Extraction with Eutectic Solvents

Adapted from Neto, R.T.; Santos, S.A.O.; Oliveira, J.; Silvestre, A.J.D. Impact of Eutectic Solvents Utilization in the Microwave Assisted Extraction of Proanthocyanidins from Grape Pomace. *Molecules* **2022**, *27*, 1–15.

Abstract

The extraction of PACs despite being an important and limiting aspect of their industrial application is still largely unexplored. Herein the possibility of combining eutectic solvents (ESs) with MAE in the extraction of PACs from GP is explored aiming to improve not only the extraction yield but also the mDP.

The combination of choline chloride with lactic acid was shown to be the most effective combination for PACs extraction yield (135 mg_{PAC}/g_{GP}) and, despite the occurrence of some depolymerization, also enabled to achieve the highest mDP (7.13). Additionally, the combination with MAE enabled the process to be completed in 3.56 min, resulting in a considerable reduced extraction time.

4.1. Introduction

PACs, or condensed tannins are polymeric phenolic compounds that consist of flavan-3-ols monomers and its derivatives, and can be found throughout the plant kingdom [239]. Their function in plants is believed to be mostly related with defense against microbial pathogens [24] and herbivores [26]. Beyond that, PACs have several industrial applications such as tanning agents in leather production, as adhesives in wood agglomerates and as additives in wine maturation [272].

PACs obtention process for industrial applications is based on the extraction from dedicated crops, such as quebracho heartwood, with a hot aqueous sulfite solution [106]. Despite being widely used, this method has a low extraction yield and results in extracts with low mDP [109], rendering PAC extracts very expensive, which limits their use in industrial applications.

Considering the role that PACs might have in the replacement of hazardous chemicals in the production of leather or wood agglomerates, the development of new, sustainable, and more efficient extraction methodologies is of the utmost importance. To address the current limitations, sustainable PAC sources need to be found and more efficient extraction methodologies must be developed.

As far as raw materials are concerned, there is increasing evidence of the agroforestry by-products potential as reliable, low-cost alternative sources for PACs due to their high PAC content, availability and possibility of being locally and sustainably sourced [252]. In addition, the use of by-products from other agroforestry activities fits into the circular economy concept in which by-products are reused and extracted from as much as possible before being discarded

as waste [141]. From the available options, GP, a by-product from the wine industry, comes out as one of the most interesting source of PACs, since in 2018 alone 292 million hL of wine were produced [256], corresponding to 11 million tons of grape pomace that have no added value application, despite presenting high PAC content [169].

The PACs extraction process, particularly from GP, can be improved by exploring two routes, namely, neoteric solvents such as ESs and auxiliary extractive techniques such as MAE.

ESs use as a biomass processing solvent has increased in popularity due to their low price and toxicity, ease of preparation and possibility of being tailor-made for a specific purpose [257]. ESs are generally described as binary systems composed by a HBA and HBD that have an eutectic point (composition at which the melting temperature is the lowest) and differ from DES for following the behavior of an ideal mixture [208].

PACs extraction with ESs was previously explored for different raw materials such as *Ginkgo biloba* leaves from which it was possible to extract 22.1 mg_{PAC}/g using a mixture of choline chloride and malonic acid at a molar ratio of 1:2 and with a water content of 55% (m/m) [216], and white grape pomace from which it was obtained 125.9 mg_{PAC}/g using a mixture with mass fractions of 0.5, 0.3 and 0.2 of choline chloride, water and ethanol, respectively [232]. In addition, it was also shown that by varying the composition of a quaternary eutectic system (choline chloride, glycerol, water and ethanol) the characteristics of the final grape pomace extract, such as mDP and galloylation percentage (%Gal), can be controlled with values ranging from 5.9 and 30.6%, respectively, with the ES composition mentioned previously to 7.5 and 47.2%, respectively, using an ES mixture of glycerol, water and ethanol with mass fractions of 0.68, 0.05 and 0.27, respectively [232].

MAE has been described as a good candidate in the extraction of PACs from agroforestry by-products in combination with conventional solvents such as ethanol:water mixtures in the extraction of PACs from maritime pine bark [186]. Additionally, neoteric solvents, such as ionic liquids, have also been suggested in the PACs extraction from cortex cinnamomic with a 1.25 M aqueous solution of 3-methylimidazolium bromide that resulted in an yield improvement of 125% when compared to the conventional MAE combined with water [206]. To the best of our knowledge the use of ESs in the extraction of PACs with MAE has not yet been explored.

Herein we explore the stability and extractability of PACs in ESs, as well as the stability of ESs when subjected to MAE. After selecting the combination of choline chloride with lactic acid or glycerol as the best candidates in MAE of PACs, the impact that the content of each ES component has on relevant parameters of the final extract such as Y_{PAC}, mDP and %Gal was

assessed. Additionally, the carbohydrate yield (Y_{CH}) obtained for each condition was also determined in order to understand possible contaminations with other macroconstituents. Similarly, the impact that MAE conditions, namely temperature (T), biomass percentage (%BM) and extraction time (t) have on the mentioned parameters of the final extract was also assessed.

4.2. Materials and Methods

4.2.1. Grape Pomace Preparation

GP was prepared following the method detailed in section 2.2.2.

4.2.2. Proanthocyanidin Reference Material

PAC reference material was isolated from GP following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention.

4.2.3. Purification of Proanthocyanidins with High Degree of Polymerization

High DP PACs were isolated following the method described by Neto et al. [232] that is detailed in section 2.2.4.

4.2.4. Acid Butanol Assay for Proanthocyanidin Quantification

PACs extract's purity ($mg_{PAC}/g_{extract}$) and Y_{PAC} (mg_{PAC}/g_{GP}) were determine following the acid butanol assay described by Porter et al. [31] that is detailed in section 2.2.5.

4.2.5. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PAC's mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] that is detailed in section 2.2.6.

4.2.6. Phenol Sulfuric Assay for Carbohydrate Quantification

Carbohydrate content was determined by the phenol-sulfuric assay [273] using D-glucose as standard. Samples were prepared by incubating a known amount of extract in 1 mL of distilled water and incubating for 1 h at 80 °C followed by centrifugation at 10000 rpm for 10 min and supernatant recovery. 100 µL of distilled water were mixed with 100 µL of sample/standard and

5 µL of phenol solution (90% (m/m)) followed by the quick addition of 500 µL of concentrated sulfuric acid. After cooling the absorbance was measured at 490 nm.

4.2.7. Screening of Proanthocyanidin Stability in Eutectic Solvents

The thermal stability of PACs in a MAE process was evaluated by dissolving 80 mg of PAC reference material in 4 g of selected ESs and agitated under magnetic stirring at room temperature until complete dissolution. The prepared solutions were then divided into four aliquots of 1 g with one serving as control and the remaining ones incubated for 10 min at 70, 100 and 130 °C in a Microwave Synthesis Reactor (Monowave 300 from Anton Paar (Madrid, Spain)). PAC content was determined as detailed in section 2.2.4.

4.2.8. Screening of Proanthocyanidin Extractability with Eutectic Solvents

PAC extractability with ESs in a MAE process was evaluated by mixing 300 mg of GP with 2.7 g of the different ESs and incubating it at 100 °C and 600 rpm in a microwave extractor for 10 min. PAC content was determined as detailed in section 2.2.4.

4.2.9. Screening of Eutectic Solvents Stability

The thermal stability of ESs in a MAE process was evaluated by incubating 1 g of each ES in a microwave extractor for 10 min at 130 °C.

4.2.10. Eutectic Solvent Composition Optimization

ES solvent composition was optimized with Response Surface Methodology (RSM) using the mixtures function with D-optimal design in Expert Design v12 from StateEase (Minneapolis, MN, USA). The experiment consisted of 16 experimental points in which the mass fractions were varied as follow: ChClLacA - 0 to 0.7 for ChCl, 0 to 0.9 for LacA and 0 to 0.7 for water; ChClGlyc - 0 to 0.7 for ChCl, 0 to 1 for Glyc and 0 to 0.5 for water. Experimental solvent compositions and experimental results are detailed in tables 4.1 and 4.2. Extractions were performed under continuous agitation at 600 rpm with 10% (m/m) of GP at 100 °C and confirmation runs were performed in triplicate for the solvent compositions that resulted in the best Y_{PAC} and mDP. PAC content was calculated by Acid Butanol Assay and PAC mDP was determined by phloroglucinolysis.

Table 4.1. Solvent composition and experimental results of solvent composition optimization of ChCl-LacA-water.

Run	x_{ChCl}	x_{LacA}	x_{H2O}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal		Y_{CH} (mg_{CH}/g_{GP})	
				Meas	Pred	Meas	Pred	Meas	Pred	Meas	Pred
1	0.490	0.408	0.102	101.7	113.7	9.9	10.6	38.11	38.32	60.9	66.2
2	0.248	0.602	0.150	113.0	115.1	9.5	10.2	38.03	38.19	68.8	63.6
3	0.492	0.201	0.307	114.7	116.4	10.8	10.4	34.86	35.54	82.0	79.1
4	0.700	0.240	0.060	72.8	77.2	9.2	8.7	33.58	35.73	52.0	55.8
5	0.698	0.000	0.302	70.8	71.0	7.6	7.7	31.00	31.52	62.5	60.0
6	0.505	0.000	0.495	73.2	80.5	7.5	7.8	31.37	31.49	65.3	73.3
7	0.502	0.000	0.498	74.5	80.3	7.4	7.8	31.24	31.47	66.8	73.5
8	0.700	0.237	0.063	89.4	77.5	8.8	8.7	38.04	35.69	56.8	56.0
9	0.000	0.405	0.595	58.3	63.4	6.0	6.2	30.72	31.27	95.0	89.5
10	0.000	0.399	0.601	60.6	62.1	5.8	6.2	31.47	31.17	87.8	89.7
11	0.000	0.800	0.200	75.2	71.5	7.5	7.0	34.28	34.59	54.3	45.01
12	0.000	0.599	0.401	78.4	86.3	7.2	7.8	33.34	33.58	63.3	76.2
13	0.306	0.381	0.313	132.2	127.7	11.0	10.9	37.41	36.83	83.1	83.9
14	0.700	0.000	0.300	73.7	70.7	7.7	7.7	32.27	31.51	67.2	59.8
15	0.000	0.800	0.200	75.1	71.4	7.4	7.0	35.10	34.59	39.9	45.01
16	0.247	0.216	0.537	119.2	97.6	10.1	8.7	34.04	33.37	101.0	90.2

Table 4.2. Solvent composition and experimental results of solvent composition optimization of ChCl-Glyc-water.

Run	x_{ChCl}	x_{Glyc}	x_{H2O}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal		Y_{CH} (mg_{CH}/g_{GP})	
				Meas	Pred	Meas	Pred	Meas	Pred	Meas	Pred
1	0.000	1.000	0.000	63.3	62	7.6	7.6	39.80	39.48	69.9	63.2
2	0.321	0.479	0.200	74.2	71.1	7.4	7.4	34.90	34.28	70.8	66.0
3	0.000	0.758	0.242	70.0	72.6	7.6	7.7	35.58	36.18	81.1	79.1
4	0.501	0.000	0.499	71.7	76.0	7.1	7.1	31.21	31.97	71.2	66.2
5	0.699	0.000	0.301	66.2	70.7	7.8	8.0	31.00	32.21	69.1	69.5
6	0.242	0.259	0.499	68.2	70.8	6.6	6.7	32.49	31.93	61.5	67.3
7	0.488	0.229	0.283	74.5	73.3	7.4	7.5	33.26	32.92	65.8	67.3
8	0.659	0.281	0.060	47.87	46.94	7.1	7.2	32.33	32.10	54.5	51.1
9	0.502	0.498	0.000	47.11	45.78	6.7	6.8	31.85	33.55	44.03	44.57
10	0.000	0.502	0.498	60.7	62.5	6.6	6.6	31.89	32.24	70.6	74.1
11	0.000	1.000	0.000	63.3	62.0	7.7	7.6	40.00	39.48	58.1	63.2
12	0.000	0.502	0.498	66.1	62.5	6.6	6.6	32.28	32.24	77.2	74.1
13	0.657	0.283	0.060	48.02	47.04	7.3	7.2	33.88	32.11	53.6	51.1
14	0.500	0.000	0.500	81.4	76.0	7.1	7.1	32.83	31.97	69.1	66.1
15	0.246	0.754	0.000	51.5	55.8	7.0	7.0	35.77	36.40	44.86	51.2
16	0.699	0.000	0.301	73.6	70.7	7.8	8.0	32.18	32.21	62.0	69.5

4.2.11. Extraction Conditions Optimization

Extraction conditions were optimized with RSM following Box-Behnken Design in Expert Design v12 from StateEase (Minneapolis, MN, USA). The experiment consisted in 15 experimental points in which T, %BM and t assayed values were ranging from 70 to 130 °C, 2.5 to 17.5% (m/m) and 2 to 10 min, respectively. Experimental extraction conditions and experimental results are detailed in table 4.3. Extractions were performed under continuous agitation at 600 rpm and the solvent composition was the one from which the highest Y_{PAC} and mDP values were obtained and confirmation runs were performed in triplicate for the experimental conditions that resulted in the best Y_{PAC} and mDP. PAC content was calculated by Acid Butanol Assay and PAC mDP was determined by phloroglucinolysis.

Table 4.3. Extraction conditions and experimental results of extraction conditions optimization of ChCl-LacA-water.

Run	T (°C)	%BM	t (min)	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal		Y_{CH} (mg_{CH}/g_{GP})	
				Meas	Pred	Meas	Pred	Meas	Pred	Meas	Pred
1	130	9,84	10	112.8	109.8	3.3	3.4	63.8	61.0	144.1	145.3
2	100	17,51	10	127.8	135.1	7.1	6.7	50.7	53.2	103.3	94.4
3	100	2,621	10	121.7	122.8	3.0	3.5	44.89	42.88	135.3	149.4
4	100	9,99	6	135.8	137.5	8.2	8.7	44.27	43.93	113.0	110.8
5	100	2,471	2	201.4	202.1	3.9	4.3	41.24	38.90	142.6	156.2
6	100	10,22	6	139.3	136.8	9.1	8.8	43.43	44.09	111.4	109.8
7	70	10,63	2	77.4	87.3	6.8	8.0	34.91	37.50	76.3	76.4
8	130	17,72	6	53.9	61.7	3.0	4.5	58.1	57.0	84.9	92.9
9	130	10,30	2	141.3	123.1	5.2	4.4	58.6	56.6	137.4	138.0
10	100	10,30	6	150.2	136.5	8.9	8.8	44.06	44.14	105.6	109.5
11	100	17,47	2	84.3	90.5	8.2	7.6	47.10	49.41	90.4	81.3
12	70	10,01	10	79.8	72.3	7.3	7.0	37.09	39.43	82.3	81.1
13	70	17,46	6	90.3	85.8	8.8	8.1	41.68	36.77	72.0	81.6
14	70	2,569	6	73.8	76.0	5.0	4.9	26.84	26.40	109.2	95.9
15	130	2,626	6	158.0	171.5	2.2	1.4	41.73	46.68	223.3	208.6

4.2.12. NMR analysis

Solvent stability was evaluated by ^{13}C NMR with spectra being obtained with a Bruker Avance 300 at 75.47 MHz, using trimethylsilyl propanoic acid (TMSP) as internal reference and deuterated water as solvent.

4.3. Results and Discussion

4.3.1. Screening of Proanthocyanidin Stability in Eutectic Solvents

PACs have been described to undergo depolymerization under heat and acidic conditions [31], and to degrade under mild alkaline conditions [107]. Therefore, it is important to assess the impact that each ES have on PACs under normal operating MAE conditions. In order to determine such, solutions of 20 mg_{PAC}/g_{ES} of PAC reference material obtained from white GP were prepared in ESs composed of 37.5% (m/m) of HBA and HBD, and 25% (m/m) of water.

PAC reference material was then incubated at 70, 100 or 130 °C for 10 min in the selected ESs, and the PAC content was determined by the acidic butanol assay with the results being presented in figure 4.1.

As it can be seen on figure 4.1 when Ur is used as HBD, PAC degradation can be observed above 100 °C especially when paired with ChCl with this effect being even more evident at 130 °C where the total PAC content is reduced to one third of the initial amount for ChClUr and BetUr while ProUr experiences only a decrease of 37%. This is in line with what would be expected considering the alkaline character of these Ur-based ESs [107].

When using MalA as HBD no degradation was detected up to 100 °C except when combined with ChCl (figure 4.1A) and all HBAs led to the degradation of half of the PAC content at 130 °C, which is in agreement with what was previously described for strong acid media [31]. Comparing the combination of ChCl with other organic acids such as LacA and CitA (figure 4.1A) it can be observed that despite presenting similar degradation values at 100 °C, LacA leads to significantly less degradation at 130 °C when compared to the other tested organic acids. This might be related with the fact that LacA is a monocarboxylic acid while MalA and CitA are dicarboxylic and tricarboxylic acids, respectively, which in turn leads an increasing molar concentration of carboxylic groups per unit mass of ES, 4.16x10-3, 5.60x10-3 and 5.85x10-3 mol/gES, respectively.

A wide range of outcomes can be observed when using Glu as HBD. Particularly that no degradation is observed when paired with ChCl regardless of temperature (figure 4.1A), mild degradation at 130 °C when paired with Bet (figure 4.1B), and complete degradation at 130 °C when paired with Pro (figure 4.1C). This is probably mostly related with degradation induced by the by-products resulting from the Maillard reactions that are expected to occur in ESs containing amino acids and sugars when exposed to heat [274]. Similarly, when using Glyc as HBD no degradation is observed up to a temperature of 100 °C for all HBAs and with ChCl at 130 °C (figure 4.1A), mild degradation at 130 °C with Bet (figure 4.1B), and PAC reduction to half of the initial amount with Pro (figure 4.1C).

In summary, it can be concluded that in general the use of ChCl-based ESs is less prone to PAC degradation, from which the combination with Glu or Glyc have the best results (no degradation). Additionaly, the use of LacA was shown to be less prone to degradation at high temperatures when compared with the remaining organic acids.

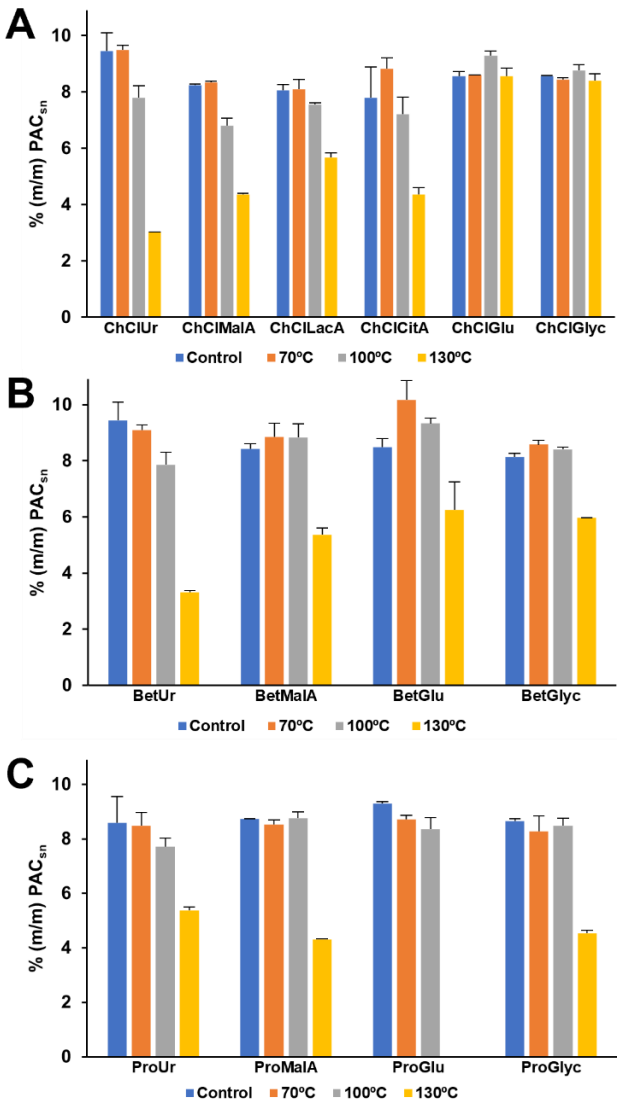


Figure 4.1. Quantification of PACs solubilized in different ESs and incubated in MAE at different temperatures. A – ChCl-based; B – Bet-based; C – Pro-based

4.3.2. Screening of Eutectic Solvents Stability

In addition to assessing the PAC stability in the tested ESs, it is important to also determine the stability of the ESs themeselves during the MAE process. Therefore, all the ESs were incubated for 10 min at 130 °C and the relevant results are presented in figure 4.2.

As it can be observed the combinations of all HBAs with organic acids are stable as far as visual analysis is concerned. Nevertheless, side reactions between ChCl and organic acids have been previously reported, especially for MalA with which is possible to achieve 17% (mol%) of ChCl esterification after 2 h at 100 °C and to a lesser extent with LacA that achieves 7% in the same conditions [264]. When using Glu as HBD some color development is always present especially when combined with Bet and Pro, most likely due to the formation of melanoidins during

Maillard reactions [274]. Similar observations were made with Glyc although not as prevalent and were considered to be nonexistent when paired with ChCl.

To sum up, the use of ChCl-based ESs appears to give rise to more stable solvents when compared to Bet and Pro, and HBDs such as Glu seem to be very thermal sensitive and therefore should be avoided in situations in which temperature in necessary.

Figure 4.2. ESs after incubation in MAE at 130 °C for 10 min

4.3.3. Screening of Proanthocyanidin Extractability with Eutectic Solvents

PAC extractability, or the ability to remove the compound of interest from the matrix in which it is present and effectively keep it in solution, is of the utmost importance in the development of an extraction process. To screen the effectiveness of each candidate for this purpose 10% (m/m) of GP was suspended in the selected ESs and were subjected to MAE for 10 min at 100 °C, followed by centrifugation and recovery of the supernatant.

In figure 4.3 it can be observed that, in general, the screened ESs follow the same trend, with the ones containing ChCl as HBA leading to higher Y_{PAC}s than the remaining ones which is in general agreement with the findings of Cao et al. [216] that compared several ChCl and Bet-based ESs. Nevertheless, when Ur is used as HBD the best results are obtained with Pro as HBA (43.7 mg_{PAC}/g_{GP}) followed by Bet and ChCl.

The best extractability results were obtained by combining MalA with ChCl (130.5 mg_{PAC}/g_{GP}) doubling from what is obtained with Bet or Pro. When analyzing the effect of combining different organic acids with ChCl, namely, LacA and CitA, it becomes clear that, for this system, no significant differences are observed with very similar values being obtained, 135.0 and 129.1 mg_{PAC}/g_{GP}, respectively. The use of ESs based on ChCl and organic acids although not widely explored for the extraction of PACs, is frequently found to be the most effective combination

when compared to others not containing organic acids for the extraction of phenolic compounds from biomass, such as chlorogenic acid from *Morus alba* L. leaves using ChClCitA [275], anthocyanins and catechin from grape skin using ChCl with oxalic acid [263] and pelargonidin-3-glucoside from strawberry extrudate using ChCl with glycolic and oxalic acids [276]. In the case of PACs this may be explained by two factors, namely due to PAC depolymerization that reduces their affinity for the pectin and hemicellulose fractions of the cell wall [277] or by dissolving the hemicellulose or lignin components of the cell wall contributing to the PAC release into solution [224].

Similarly, when using Glu or Glyc the best results are also obtained by combining them with ChCl, resulting in Y_{PAC}s of 88.8 and 51.8 mg_{PAC}/g_{GP}, respectively. The use of polyols in ESs, such as Glu or Glyc, has been extensively explored and are considered good solvents for phenolic compounds due to high number of hydroxyl groups [278]. Nevertheless, the Y_{PAC}s obtained here are considerably lower than those obtained with carboxylic acids. Interestingly, the use of Glu is considerably more affected by the combination of different HBAs than Glyc which, as shown previously, could be related with its degradation when combined with Bet or Pro.

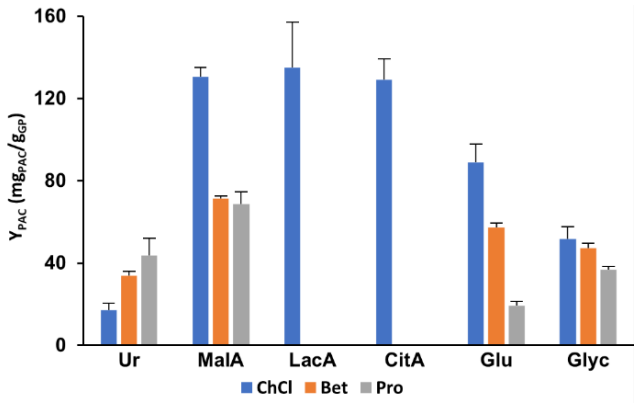


Figure 4.3. Extractability of PACs from GP with different ESs using MAE at 100 °C for 10 min

Based on the results obtained previously, ChClLacA and ChClGlyc were chosen as the best candidates for solvent composition optimization. This is justified by the thermal stability exhibited by both ESs, by the high extractability of ChClLacA and comparatively low PAC degradation at high temperatures when compared with the other organic acids, and by the inexistence of PAC degradation at all tested temperatures with ChClGlyc.

4.3.4. Solvent Composition Optimization

Although ChClLacA and ChClGlyc presented good extraction characteristics, it should be noted, as described in our previous work [232], that the variation of each ES component's content has

a great impact on the characteristics of the final extract, affecting not only Y_{PAC} but mDP and %Gal as well.

In order to determine the effect of each component on the variables mentioned before, RSM was employed with 16 experimental points in which the mass fractions were varied from 0 to 0.7 for ChCl, 0 to 0.9 for LacA and 0 to 0.7 for water as specified in table 4.1. For each experimental condition Y_{PAC}, mDP, %Gal and Y_{CH} were determined. The corresponding polynomials are depicted in equations 4.1., 4.2., 4.3. and 4.4. and the respective contour plots shown in figure 4.4.

Equation 4.1. $$Y_{PAC} = -31.83x_{ChCl} + 17.69x_{LacA} - 102.3x_{water} + 414.6x_{ChCl}x_{LacA} + 589x_{ChCl}x_{water} + 486.1x_{LacA}x_{water}$$

Equation 4.2. $$mDP = 2.402x_{ChCl} + 3.875x_{LacA} - 4.181x_{water} + 26.45x_{ChCl}x_{LacA} + 34.81x_{ChCl}x_{water} + 29.75x_{LacA}x_{water}$$

Equation 4.3. $$\%Gal = 27.67x_{ChCl} + 34.20x_{LacA} + 22.18x_{water} + 32.57x_{ChCl}x_{LacA} + 26.14x_{ChCl}x_{water} + 17.50x_{LacA}x_{water}$$

Equation 4.4. $$Y_{CH} = 20.63x_{ChCl} - 3.584x_{LacA} + 64.2x_{water} + 178.3x_{ChCl}x_{LacA} + 124.5x_{ChCl}x_{water} + 218.9x_{LacA}x_{water}$$

The performed ANOVA analysis revealed that the resulting models for Y_{PAC}, mDP, %Gal and Y_{CH} all have *p*-values < 0.002, adjusted r^2 values of 0.80, 0.82, 0.82 and 0.75, respectively and predicted r^2 values of 0.70, 0,73, 0.67 and 0.57, respectively, indicating the statistical robustness of the resulting quadratic models.

As it can be observed in figure 4.4A and B the optimal solvent composition for Y_{PAC} and mDP are very similar (Y_{PAC} - x_{ChCl}=0.34, x_{LacA}=0.39, x_{water}=0.27; mDP - x_{ChCl}=0.38, x_{LacA}=0.39, x_{water}=0.23). This result is important because it demonstrates that the two variables can be maximized under the same conditions. The result is also interesting considering that under these conditions the mixture develops a slight red color normally associated with PAC depolymerization [31] which could be indicative of a decrease in the final extract's mDP. Nevertheless, the reduction in mDP is not observed which might be related to the high acidic character of the solvent that leads to the depolymerization of PACs with very high mDP (20 or higher, especially the ones present in grape skin [279]), resulting in the release of PACs with mDP around 10 into solution that as mentioned before, can be explained by the reduction of PAC's affinity for the pectin and

hemicellulose fractions of the cell wall [277]. Another possible explanation mentioned before is the solubilization of the hemicellulose or lignin components of the cell wall contributing to the PAC release into solution [224] which would contribute in a similar way to the release of high mDP PACs.

By maximizing the resulting models for Y_{PAC} and mDP the best solvent composition is x_{ChCl}=0.36, x_{LacA}=0.39 and x_{water}=0.25 and the expected results are 128.5 mg_{PAC}/g_{GP} and a mDP of 11.1. Nevertheless, the confirmation runs results were 152.4 mg_{PAC}/g_{GP} and a mDP of 8.4 which might be indicative that when using this solvent system additional PAC depolymerization should be expected to occur if extraction conditions are not carefully controlled.

The effect of the ES composition in other characteristics, such as %Gal and Y_{CH}, can also be observed in figure 4.4C and D from which one can conclude that the highest %Gal values are obtained without water addition to the ES and that the opposite is observed for Y_{CH}, respectively. This could be explained by the fact that when water is added to the ES dissociation and ionization of ChCl and LacA, respectively, occurs leading to an increase in solvent polarity that contributes to the solubilization of non-galloylated PACs and carbohydrates. Interestingly, the maximum Y_{PAC} obtained (figure 4.4A) seems to be a combination of the high carbohydrate solubilization obtained with high x_{water} and a x_{LacA} between 0.2 and 0.4 (figure 4.4D), and high %Gal obtained with low x_{water} and x_{ChCl} between 0.3 and 0.5 (figure 4.4C).

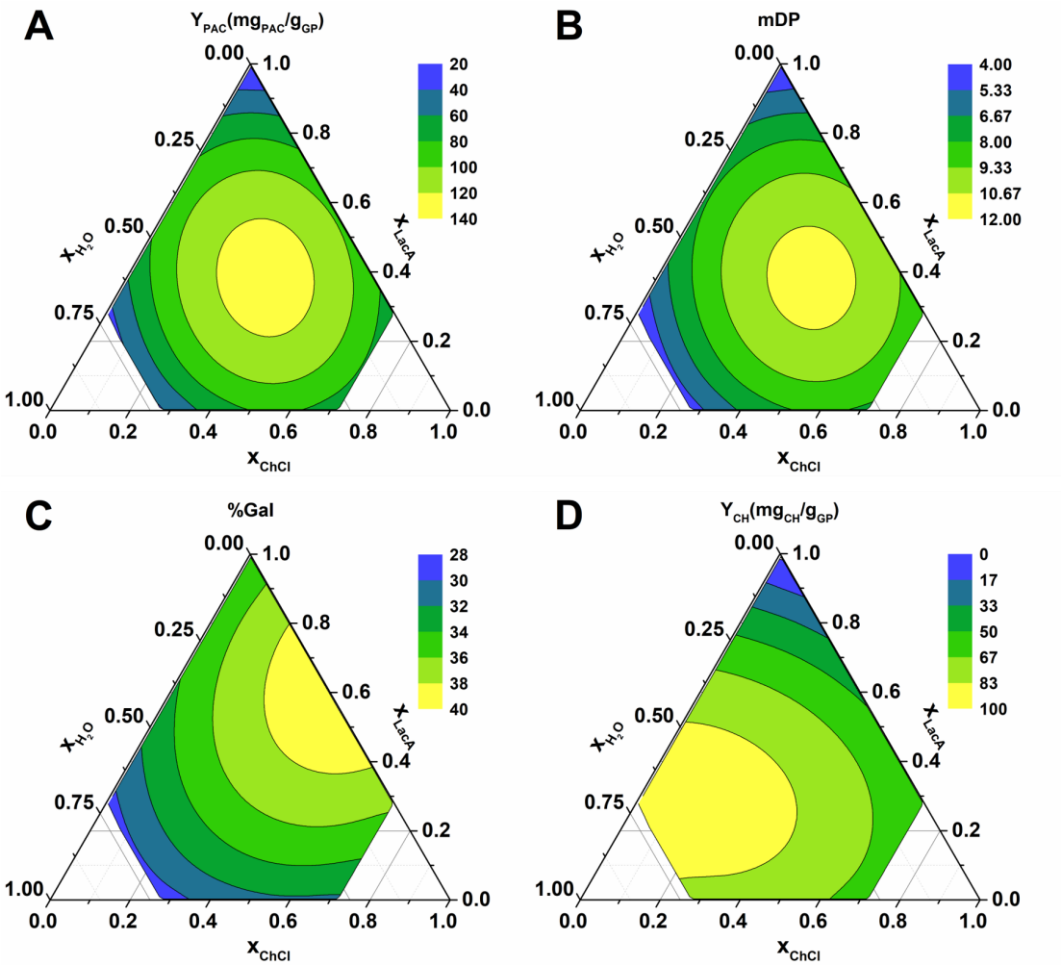


Figure 4.4. Contour plots obtained for solvent composition optimization of ChClLacA. A – Y_{PAC}; B – mDP; C - %Gal; D – Y_{CH}

Similar experiments were conducted for ChClGlyc in which the mass fractions were varied from 0 to 0.7 for ChCl, 0 to 1 for Glyc and 0 to 0.5 for water as specified in table 4.2. For each experimental condition Y_{PAC}, mDP, %Gal and Y_{CH} were determined. The corresponding polynomials are depicted in equations 4.5., 4.6., 4.7. and 4.8. and the respective contour plots shown in figure 4.5.

Equation 4.5. $$Y_{PAC} = 15.65x_{ChCl} + 62.0x_{Glyc} - 20.60x_{water} + 28.21x_{ChCl}x_{Glyc} + 313.7x_{ChCl}x_{water} + 166.7x_{Glyc}x_{water}$$

Equation 4.6. $$\%Gal = 7.53x_{ChCl} + 7.65x_{Glyc} + 1.205x_{water} - 3.177x_{ChCl}x_{Glyc} + 11.08x_{ChCl}x_{water} + 8.60x_{Glyc}x_{water}$$

Equation 4.7. $$mDP = 29.06x_{ChCl} + 39.48x_{Glyc} + 23.22x_{water} - 2.805x_{ChCl}x_{Glyc} + 23.32x_{ChCl}x_{water} + 3.451x_{Glyc}x_{water}$$

Equation 4.8. $$Y_{CH} = 49.18x_{ChCl} + 63.2x_{Glyc} - 1.290x_{water} - 46.41x_{ChCl}x_{Glyc} + 168.6x_{ChCl}x_{water} + 171.8x_{Glyc}x_{water}$$

ANOVA analysis showed that the resulting models for Y_{PAC}, mDP, %Gal and Y_{CH} all have p-values < 0.002, adjusted r^2 values of 0.79, 0.95, 0.86 and 0.73, respectively and predicted r^2 values of 0.61, 0,90, 0.77 and 0.53, respectively, which indicates the statistical robustness of the resulting quadratic models.

As it can be observed in figure 4.5A and B, Y_{PAC} and mDP are both negatively affected by increasing Glyc mass fraction, with the optimal solvent compositions not including it (Y_{PAC} - x_{ChCl}=0.56, x_{Glyc}=0.0, x_{water}=0.44; mDP - x_{ChCl}=0.70, x_{Glyc}=0.0, x_{water}=0.30). Interestingly, and contrary to what happens with ChClLacA, the observed behavior for Y_{PAC} has a maximum that follows a ridge with roughly constant water content and with varying mass fraction values of ChCl and Glyc (figure 4.5A). This is in agreement with our previous findings [232]. This allows for the obtention of PAC extracts with different values of mDP and %Gal with minimal loss of Y_{PAC}. Contrary to what is observed for ChClLacA, no PAC depolymerization is visually observed (based on the absence of red color). Despite that fact, the resulting Y_{PAC} and mDP values are considerably lower, further sustaining the importance of PAC depolymerization/lignin and hemicellulose solubilization in their extraction process.

If the results are maximized for Y_{PAC} and mDP the ideal solvent composition is x_{ChCl}=0.65, x_{Glyc}=0.0 and x_{water}=0.35 and the expected results are 74.0 mg_{PAC}/g_{GP} and a mDP of 7.85. Interestingly, the confirmation runs returned values that are much closer to what is expected from the models than what is observed for LacA further sustaining the advantage of using ESs that do not contribute to PAC depolymerization.

As far as %Gal and Y_{CH} are concerned (figure 4.5C and D), water content appears to have a similar effect to what is observed with ChClLacA with small differences in %Gal that can be attributed to Glyc. More specifically, Glyc, having several hydroxyl groups, has a great effect on the extraction of PACs with galloylated subunits, as shown by our previous work [232] in which it was demonstrated that the increase in ethanol content is directly proportional to the %Gal.

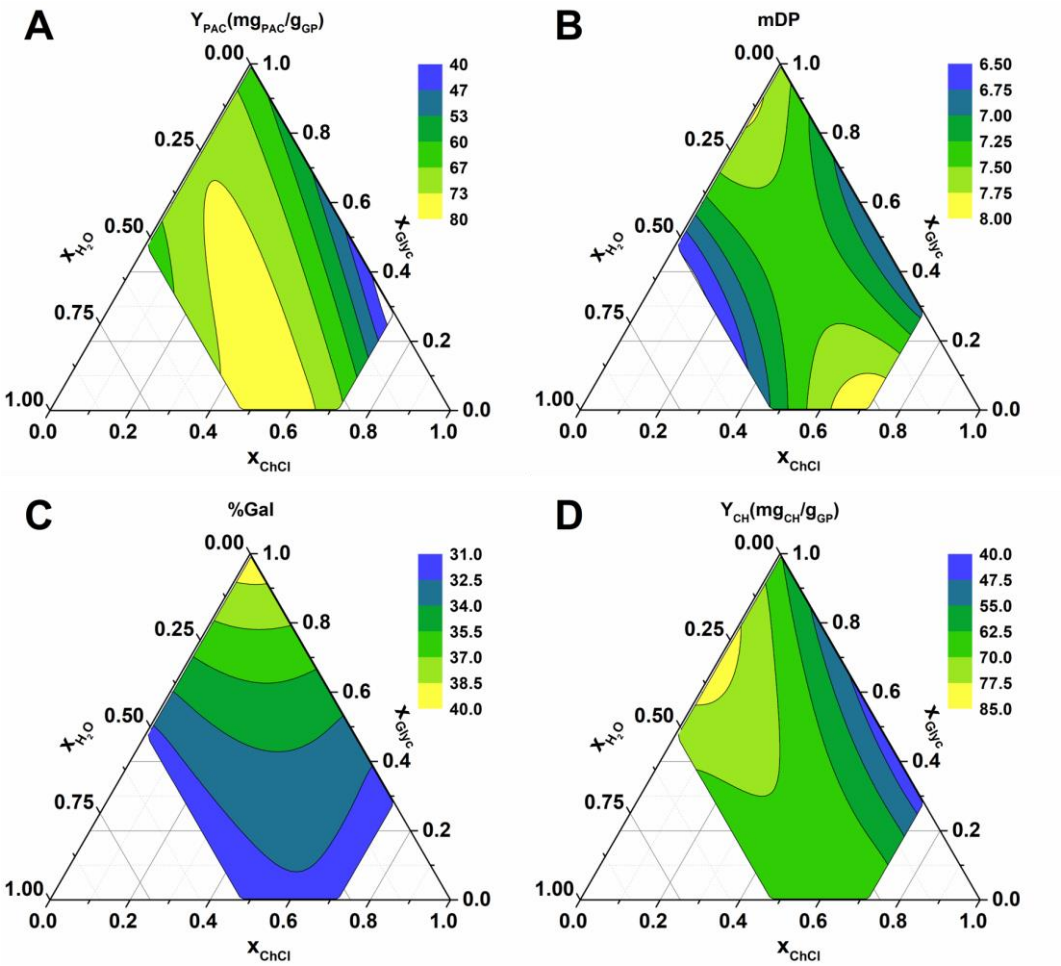


Figure 4.5. Contour plots obtained for solvent composition optimization of ChClGlyc. A – Y_{PAC}; B – mDP; C - %Gal; D – Y_{CH}

One aspect that is also relevant in the development of new extraction processes is the presence of other compounds in the final extract that might interfere with the downstream process. In this case, the Y_{CH} was determined (figures 4.4D and 4.5D) and it was shown that in the conditions used for the confirmation runs the ratio between PAC and CH content was 1.47 and 1.33 for ChClLacA and ChClGlyc, respectively.

Due to the higher Y_{PAC}, mDP and Y_{PAC}/Y_{CH} ratio that was possible to obtain with ChClLacA this was chosen as the best candidate for the optimization of extraction conditions. Nevertheless, it is noteworthy that if PAC depolymerization is completely undesired the use of ChClGlyc might be beneficial in spite of the substantially lower Y_{PAC}.

4.3.5. Extraction Conditions Optimization

MAE provides, as main advantage, the considerable reduction in extraction time [280] which leads to considerable savings in energy consumption. When using MAE there are three main

parameters that can be optimized, namely, temperature (T), extraction time (t) and biomass percentage (%BM). To determine the effect of each parameter on the variables mentioned before, while using ChClLacA, RSM was employed with 15 experimental points in which the parameters were varied from 70 to 130 °C for temperature, 2 to 10 min for extraction time and 2.5 to 17.5 for %BM as specified in table 4.3. For each experimental condition Y_{PAC}, mDP, %Gal and Y_{CH} were determined. The corresponding polynomials are depicted in equations 4.9., 4.10., 4.11. and 4.12. and the respective contour plots shown in figure 4.6.

Equation 4.9. $$Y_{PAC} = -372.0 + 10.41T + 3.717\%BM - 12.48t - 0.1322T\%BM + 1.033\%BMt - 0.04235T^2$$

Equation 4.10. $$mDP = -14.42 + 0.3721T + 0.955\%BM + 0.742t - 0.00215T^2 - 0.03668\%BM^2 - 0.0709t^2$$

Equation 4.11. $$\%Gal = 9.89 + 0.3010T + 1.511\%BM - 3.439t + 0.00597Tt - 0.04068\%BM^2 + 0.276t^2$$

Equation 4.12. $$Y_{CH} = -39.55 + 2.177T + 2.525\%BM - 1.129t - 0.1126T\%BM + 0.1590\%BMt + 0.1717\%BM^2$$

ANOVA analysis revealed that the resulting models for Y_{PAC}, mDP, %Gal and Y_{CH} all have *p*-values < 0.003, adjusted r^2 values of 0.86, 0.86, 0.84 and 0.90, respectively and predicted r^2 values of 0.64, 0,68, 0.42 and 0.62, respectively. This indicates that despite the high adjusted r^2 that is possible to obtain the predicted r^2 were lower than what should be expected, especially for %Gal which might be indicative of some statistical fragility of the resulting quadratic models.

In figure 4.6 it can be observed the impact that these parameters have on Y_{PAC}, mDP, %Gal and Y_{CH}. In terms of Y_{PAC}, the maximum value is obtained at 120 °C with 2.5% BM (figure 4.6A). Interestingly, by increasing the % BM the temperature at which maximum Y_{PAC} is obtained decreases. In addition, for any given temperature, the increase in extraction time leads to a decrease in Y_{PAC} (figure 4.6B) demonstrating that thermal degradation is present regardless of the temperature employed, further sustaining the necessity of carefully controlling temperature and extraction time in order to preserve Y_{PAC}.

As far as mDP is concerned, the maximum values were obtained with a temperature of 86 °C and a % BM of 13%, becoming clear the negative effect that higher temperatures have on mDP (figure 4.6C). In fact, using the conditions at which the maximum Y_{PAC} is obtained the resulting

mDP is 2.4 which is proof of almost complete PAC depolymerization at higher temperatures and under high acid content. Furthermore, extraction time has a positive effect on mDP up until 5.23min after which a decrease is verified (figure 4.6D).

The highest values for %Gal were obtained with the main values tested for all parameters which overlap with low values of Y_{PAC} and mDP that might indicative of a higher resistance of galloylated monomers to thermal degradation (figure 4.6E and F). The results obtained for Y_{CH} were in line with the expected, increasing considerably with temperature and decreasing with higher % BM (figure 4.6G). Extraction time had no effect on the overall Y_{CH} (figure 4.6H).

Considering that the most important characteristics are Y_{PAC} and mDP the best results are obtained while using 99.2 °C, 8.3% BM and 3.56 min which should result in Y_{PAC} of 152 mg_{PAC}/g_{GP} and a mDP of 8.13. The confirmation runs resulted in Y_{PAC} of 135 mg_{PAC}/g_{GP} and a mDP of 7.19 which is in reasonable agreement with the expected values, validating the models used.

This is a slight improvement on the Y_{PAC} obtained in our previous work, from 126 mg_{PAC}/g_{GP} in which extraction was made through conventional maceration using the same raw material [232]. Despite the inexistance of considerable improvements on Y_{PAC} it is noteworthy that the extraction time was drastically reduced from 1 h to 3.56 min.

Furthermore, the use of MAE has been described as one of the best candidates for reduction of environmental impact of extraction processes while improving overall Y_{PAC} even after scale-up to pilot scale extraction [281].

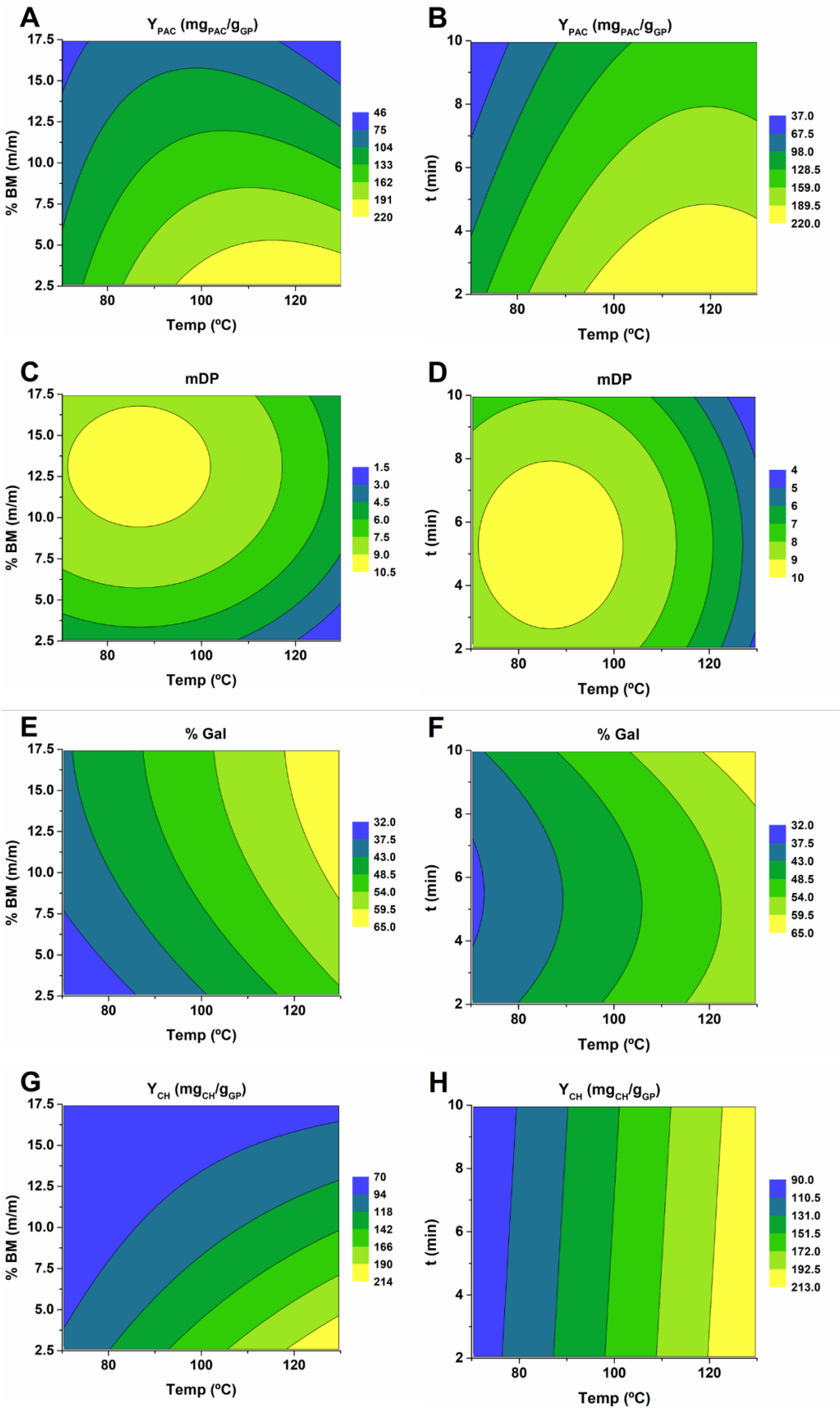


Figure 4.6. Contour plots obtained for extraction conditions optimization of ChClLacA. A – Y_{PAC} for t = 2 min; B – Y_{PAC} for %BM = 2.5%; C – mDP for t = 5.23 min; D – mDP for %BM = 13%; E – %Gal for t = 10 min; F - %Gal for %BM = 17.5%; G – Y_{HC} for t = 2 min; H – Y_{HC} for %BM = 2.5 min

Considering the importance of the solvent stability throughout the MAE process and taking into account that the possibility of reusing it is of the utmost importance as far as sustainability is concerned, the effect of using the optimized extraction conditions twice on the solvent were assessed by ^{13}C NMR spectroscopy comparing the ES spectra with literature data [282].

As can be observed on figure 4.7 no differences were observed in the corresponding ^{13}C NMR spectra even after the solvent has been submitted twice (figure 4.7C) to an incubation at 99.2 °C for 3.56 min on a microwave extractor.

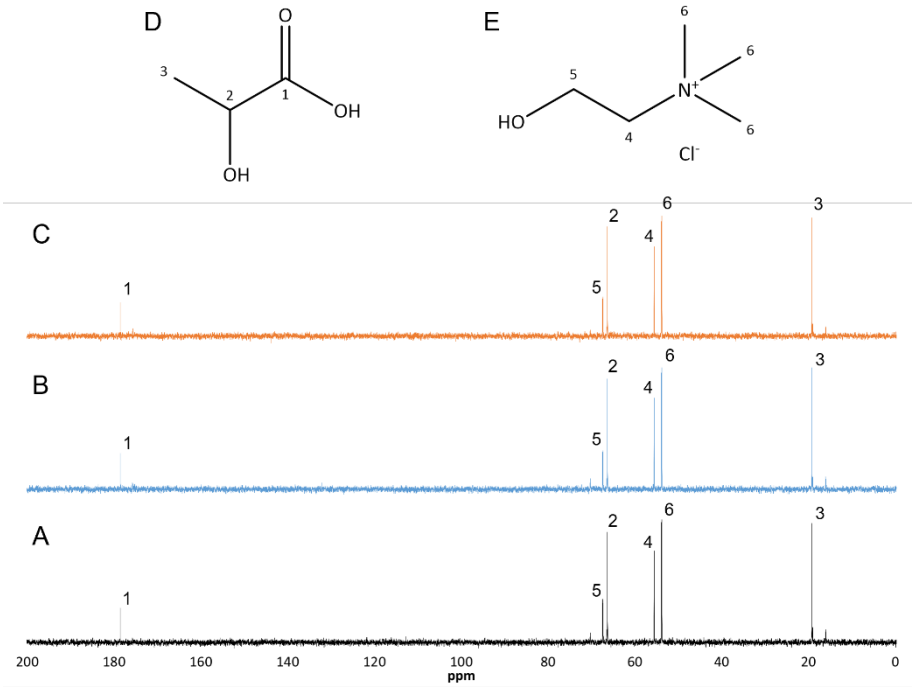


Figure 4.7. ^{13}C NMR spectra of ChCl:LacA:H_2O (36:39:25) (m:m:m). A – control; B – incubated once; C – incubated twice; D – lactic acid molecular structure; E – choline chloride molecular structure. 1-6 – ^{13}C NMR peak attribution.

4.4. Conclusion

Direct comparisons with published work is always problematic since each type of biomass has its own characteristics which have a direct impact on the characteristics of the final extract. Neverthless, in our previous work [232] that used the same GP as raw material it was already showed the improvement that could be achieved by replacing conventional solvents with ESs, namely, mixtures of ChClGlyc with ethanol. In this work it was shown that by using an ES composed of x_{ChCl}=0.36, x_{LacA}=0.39 and x_{water}=0.25 in combination with MAE at 99.2 °C, with 8.3% BM it was possible to considerably reduce the extraction time from 1 h to 3.56 min while obtaining slightly higher Y_{PAC} (135 vs 126 mg_{PAC}/g_{GP}) and mDP (7.2 vs 6.5).

Herein the possibility of combining ESs with MAE in the extraction of PACs from GP was achieved, being demonstrated that the combination of choline chloride with lactic acid was the most effective for Y_{PAC} and, despite the occurrence of some depolymerization, also enabled to achieve the highest mDP. Despite the initial necessary investiment, this should be offseted by the reduction in the energy necessary for the extraction process and the higher Y_{PAC}.

The importance of PACs for industrial applications has increased considerably in recent years due to their ability to replace harmful chemicals and therefore, its demand is also expected to increase. Herein it was shown that better and more efficient methods can be developed by combining ESs with MAE, more specifically, ChClLacA-based ESs.

5. Normalizing Proanthocyanidin Extract's Mean Degree of Polymerization

Adapted from article being prepared for publication

Abstract

PACs molecular characteristics, especially mDP, have a great impact on their anti-inflammatory and antioxidant activities. Nevertheless, PAC's mDP can change due to the use of plants from different varieties or grown in different conditions/locations leading to extracts with compositional variabilities that conventional extraction techniques cannot overcome.

ES are a new class of solvents that has the advantage of being possible to obtain by combining almost any type of compound in any proportion allowing for their customization for specific applications. In other words by adapting the ES composition is possible to obtain PACs with specific mDP regardless of raw material.

Herein, the use of a quaternary ES system composed by choline chloride, glycerol, water and ethanol that was previously described as an efficient media for the extraction of PACs was explored in the normalization of PAC extracts. By adjusting the composition of each ES component, it was possible to obtain PAC extracts with specific mDP values, namely, 6.5 and 7.5, from three different grape pomaces while maintaining the extraction conditions unchanged.

5.1. Introduction

PACs are secondary vegetable metabolites present throughout all plant kingdom [239]. These consist in polymeric phenolic compounds that have as monomers catechin and its derivatives and are generally characterized by their mDP and %Gal.

PACs industrial applications consist mostly on high-end leather production [60], resin formulation for wood agglomerates [65] and wine maturation [71]. In addition, PACs have been proposed as a natural food ingredient that can be used as preservative [246] or antioxidant [244,245] with lower toxicity than their synthetic counterparts [283].

One aspect that is frequently overlooked when exploring PACs application are the PAC's characteristics such as mDP and %Gal in the employed extract that, as shown by several authors can have a direct impact on PAC's activity. More specifically, higher mDPs have been associated with higher anti-inflammatory [87] and antioxidant [258] activities, higher enzymatic inhibition potential [200] and with antiproliferation of human colon cancer cells [262].

Most PAC extracts are obtained by hot water extraction from dedicated crops such as quebracho [284] but an increasing trend toward a circular economy in which by-products from one activity are used as raw materials for other applications in order to reduce or eliminate the total amount of waste has changed the focus to the obtention of PACs from agroforestry by-products [252].

GP which is a by-product of the wine industry obtained after grape pressing, has a high PAC content [169] and is produced in considerable amounts worldwide, more specifically, 11 million tons of GP as a result of the production of 292 million hL of wine in 2018 alone [256]. Nevertheless, GP utilization for PAC's extraction is still limited.

One of the limitations of using this type of biomass as a raw material is the compositional variability that is observed in plants and that leads to considerable differences in the final extract [159,285] which as mentioned before might have a high impact on the extract's activity and applicability.

Assuming that the same solvent is used, the final extract variability can be attributed to two major factors: firstly, the use of different varieties from the same plant species [159] and secondly, the use of material cultivated in different locations with variations in soil/water characteristics and edaphoclimatic conditions which also contributes to year-to-year variability [285].

An alternative to conventional solvents for the extraction of valuable compounds from plants that has received considerable attention in recent years are ESs. ESs were firstly described by Abbot et al. [207] and consist in a mixture of two or more components that possess a decreased melting point when compared with the individual components. Consequently, at the appropriate proportions ESs can be liquid at room temperature and can be used as solvent in biomass processing. ESs are normally described as a mixture of a HBA and a HBD but are nevertheless not limited to binary systems. In fact they have been employed with other compounds in the extraction of PACs, e.g. in combination with water [216] and ethanol [232].

Due to the possibility of preparing ESs by combining an endless number of compounds in virtually any proportion it is possible to prepare an extraction media that can be customized to a specific application, namely, to obtain a PAC extract with a specific mDP which is something that cannot be achieved with conventional solvents.

Recently, PAC's extraction process based on the use a quaternary ES system composed by choline chloride, glycerol, water and ethanol was explored [232]. The results demonstrated that the yield could be improved by 20-fold and that the extract's characteristics can be considerably improved, namely by increasing mDP values from 3.9 to 6.4 and %Gal from 26.5% to 29.6% when compared with the conventional hot water extraction.

Herein, the possibility of normalizing the mDP values of PAC extracts from different GPs is explored, i.e., consistently obtaining the same mDP value regardless of GP that is used as raw material. To achieve such a quaternary ES system composed by choline chloride, glycerol, water,

and ethanol was used as extraction media and the effect that the variation of the mass fraction of each component had on the characteristics of the final extract was evaluated. With this information predictive models were derived using RSM for each type of GP and validation runs were made to confirm that by adequately choosing the extraction media's composition is possible control the mDP value of the final PAC extract.

5.2. Materials and Methods

5.2.1. Grape Pomaces Preparation

Three different GPs were tested with GP1 being obtained during the 2019 harvest from the Douro region (Portugal), GP2 obtained during the 2020 harvest from the Vinho Verde region (Portugal) and GP3 obtained during the 2020 harvest from the Douro region (Portugal). All GPs were obtained after pressing for must extraction and were composed by a mix of several white grape varieties. After collection GPs were frozen, freeze-dried and store at room temperature, tightly closed and protected from light.

5.2.2. Grape Pomace Characterization

The different GPs were characterized by their PAC and CH contents. PAC content was defined as the amount of PACs that can be extracted from the GP with aqueous acetone 70% (v/v) following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention. CH content was defined as the amount of CH than can be extracted from the GP with two consecutive water extractions at 80 °C for 1h.

5.2.3. Purification of Proanthocyanidins with High Degree of Polymerization

PAC reference material was isolated from GP1 following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention. PAC reference material was then fractionated following the method described by Neto et al. [232] that is detailed in section 2.2.4 from which **PAC F3** was used as purified PAC for acid butanol quantification.

5.2.4. Acid Butanol Assay for Proanthocyanidin Quantification

Y_{PAC} (mg_{PAC}/g_{GP}) was determine following the acid butanol assay described by Porter et al. [31] that is detailed in section 2.2..

5.2.5. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PAC's mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] that is detailed in section 2.2.6.

5.2.6. Phenol Sulfuric Assay for Carbohydrate Quantification

CH content was determined following the method described by DuBois et al. [273] that is detailed in section 4.2.6.

5.2.7. Conventional Extraction of Proanthocyanidins from Grape Pomace

Conventional extraction methods based on hot water and aqueous ethanol were used as reference for the developed method. Hot water extraction was made for the different GPs following the method described by Francezon et al. [230] and is detailed in section 2.2.3 for **PAC HW**. Extraction with aqueous ethanol 60% (v/v) was made following the method detailed in section 3.2.6.

5.2.8. Eutectic Solvents Preparation

The ESs with different compositions were prepared by combining the appropriate amount of each compound (ChCl, Glyc, EtOH and water) and mixing them at 40 °C under continuous stirring until a continuous phase was obtained.

5.2.9. Effect of Grape Pomace's Characteristics on the Extraction with Eutectic Solvents

The different GPs were extracted following the optimal conditions described Neto et al. [232]. More specifically, at a GP concentration of 13.4% (m/m) and at 99 °C for 1h. ES's content of ChCl, Glyc, EtOH and water were varied according to a 18-point experiment based on RSM for mixtures using a D-optimal design on Expert Design v12 from StateEase. Mass fractions were varied between 0 and 0.7 for ChCl, 0 and 0.95 for Glyc, 0 and 0.4 for EtOH, and 0.05 and 0.5 for water. In addition, the sum of ChCl and EtOH mass fractions was kept bellow 0.7. Experimental ES's compositions and experimental results for Y_{PAC}, mDP and %Gal are presented in tables 5.1, 5.2 and 5.3. With this data, prediction models were constructed for the influence of ES composition on Y_{PAC}, mDP and %Gal.

Table 5.1. Solvent composition and experimental results of evaluation of GP effect on the extraction with ChCl-Glyc-EtOH-water ES system for GP1.

Run	x_{ChCl}	x_{Glyc}	x_{H2O}	x_{EtOH}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal	
					Meas	Pred	Meas	Pred	Meas	Pred
1	0.00	0.50	0.50	0.00	69.4	70.0	6.13	6.13	31.1	31.2
2	0.15	0.23	0.50	0.12	85.9	92.5	6.42	6.17	34.4	35.2
3	0.00	0.95	0.05	0.00	98.1	100.1	8.51	8.56	38.5	38.2
4	0.00	0.55	0.05	0.40	104.1	100.1	7.66	6.71	41.7	41.3
5	0.70	0.00	0.30	0.00	126.0	133.1	9.01	9.00	38.4	38.6
6	0.09	0.28	0.23	0.40	92.3	107.2	6.50	5.98	38.1	39.1
7	0.70	0.00	0.30	0.00	133.6	133.1	8.89	9.00	38.2	38.6
8	0.30	0.02	0.28	0.40	124.8	117.9	7.33	6.42	37.8	37.9
9	0.22	0.49	0.29	0.00	112.5	110.6	7.80	7.66	36.0	35.5
10	0.18	0.68	0.06	0.07	114.3	111.0	8.43	8.22	38.6	39.5
11	0.00	0.11	0.50	0.40	88.1	88.0	6.17	4.47	39.5	38.7
12	0.00	0.10	0.50	0.40	88.7	87.8	6.07	4.45	38.4	38.7
13	0.53	0.25	0.05	0.17	121.7	116.4	8.60	8.28	40.5	39.9
14	0.45	0.46	0.09	0.00	116.2	122.6	8.44	8.67	39.2	39.6
15	0.00	0.55	0.05	0.40	96.4	100.0	7.71	6.71	41.3	41.3
16	0.41	0.25	0.32	0.02	127.8	122.4	8.40	7.99	37.7	36.7
17	0.00	0.52	0.27	0.21	104.7	96.1	7.25	6.41	38.0	37.7
18	0.40	0.00	0.50	0.10	119.1	114.7	7.62	7.29	36.8	36.6

Table 5.2. Solvent composition and experimental results of evaluation of GP effect on the extraction with ChCl-Glyc-EtOH-water ES system for GP2.

Run	x_{ChCl}	x_{Glyc}	x_{H2O}	x_{EtOH}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal	
					Meas	Pred	Meas	Pred	Meas	Pred
1	0.00	0.50	0.50	0.00	66.3	64.1	6.46	6.34	29.6	29.6
2	0.15	0.23	0.50	0.12	71.9	89.6	6.41	7.09	33.0	34.3
3	0.00	0.95	0.05	0.00	86.0	88.5	9.00	9.01	35.2	35.1
4	0.00	0.55	0.05	0.40	90.0	100.9	8.04	8.37	40.8	40.1
5	0.70	0.00	0.30	0.00	116.1	114.4	8.96	8.99	36.0	36.6
6	0.09	0.28	0.23	0.40	87.0	100.1	7.25	7.70	35.6	37.8
7	0.70	0.00	0.30	0.00	107.8	114.4	8.75	8.99	36.4	36.6
8	0.30	0.02	0.28	0.40	120.2	113.7	7.98	7.94	36.6	36.2
9	0.22	0.49	0.29	0.00	95.1	98.5	8.22	8.26	34.0	34.0
10	0.18	0.68	0.06	0.07	99.5	101.6	8.83	9.01	36.8	37.7
11	0.00	0.11	0.50	0.40	73.8	76.7	5.98	6.23	37.2	36.9
12	0.00	0.10	0.50	0.40	83.5	76.6	6.68	6.21	37.5	36.9
13	0.53	0.25	0.05	0.17	103.8	105.3	8.83	8.7	38.6	38.1
14	0.45	0.46	0.09	0.00	105.9	105.2	9.01	9.04	37.2	37.6
15	0.00	0.55	0.05	0.40	111.8	101.0	8.84	8.37	40.3	40.2
16	0.41	0.25	0.32	0.02	126.8	111.4	9.05	8.58	37.5	35.5
17	0.00	0.52	0.27	0.21	98.5	83.3	7.86	7.58	37.0	36.2
18	0.40	0.00	0.50	0.10	117.7	116.3	8.40	8.15	35.8	35.7

Table 5.3. Solvent composition and experimental results of evaluation of GP effect on the extraction with ChCl-Glyc-EtOH-water ES system for GP3.

Run	x_{ChCl}	x_{Glyc}	x_{H2O}	x_{EtOH}	Y_{PAC} (mg_{PAC}/g_{GP})		mDP		%Gal	
					Meas	Pred	Meas	Pred	Meas	Pred
1	0.00	0.50	0.50	0.00	44.4	44.8	5.30	5.12	30.0	29.8
2	0.15	0.23	0.50	0.12	49.4	55.9	5.02	5.59	32.8	34.0
3	0.00	0.95	0.05	0.00	56.7	56.9	6.45	6.20	33.2	33.5
4	0.00	0.55	0.05	0.40	49.8	50.2	5.65	5.64	39.8	39.3
5	0.70	0.00	0.30	0.00	71.9	76.9	7.10	7.18	34.4	35.0
6	0.09	0.28	0.23	0.40	55.3	52.0	5.29	5.38	37.0	38.4
7	0.70	0.00	0.30	0.00	77.2	76.9	7.16	7.18	35.3	35.0
8	0.30	0.02	0.28	0.40	57.3	59.6	5.19	5.42	36.7	36.4
9	0.22	0.49	0.29	0.00	61.6	58.9	6.00	6.13	34.5	33.7
10	0.18	0.68	0.06	0.07	59.0	63.9	6.22	6.63	36.5	36.5
11	0.00	0.11	0.50	0.40	49.6	46.9	4.97	4.88	39.5	38.3
12	0.00	0.10	0.50	0.40	43.9	46.8	4.98	4.86	37.6	38.3
13	0.53	0.25	0.05	0.17	76.2	74.8	7.49	7.12	37.4	37.2
14	0.45	0.46	0.09	0.00	72.5	70.2	7.00	7.13	36.9	36.9
15	0.00	0.55	0.05	0.40	49.0	50.1	5.68	5.63	39.3	39.3
16	0.41	0.25	0.32	0.02	69.7	66.4	6.66	6.49	34.4	34.7
17	0.00	0.52	0.27	0.21	57.7	54.5	5.91	5.80	36.9	36.5
18	0.40	0.00	0.50	0.10	71.1	66.5	6.36	6.04	34.7	34.0

5.2.10. Normalization of PAC Extract's mDP

Based on the prediction models determined previously, the ES compositions for the obtention of extracts with specific mDPs and maximum Y_{PAC} were determined, more specifically to obtain mDP values of 6.5 and 7.5. Selected ES compositions were prepared and GPs were extracted and characterized following the procedures described previously.

5.3. Results and Discussion

5.3.1. Grape Pomace Characterization

Grape origin and variety have a great impact on the characteristics of the extract that can be obtained from it [159]. In addition, wine production method may also impact on the grape

pomace's final conditions [285], thus impacting the composition of PAC extracts obtained thereof.

GP characteristics were determined by using the extraction with aqueous acetone 70% (v/v) which was used to define the total content and characteristics of PACs present in each GP. Additionally, total CH content was determined by using water extraction as this is the most common type of compound that can interfere with the conventional water-based extraction of PACs.

In figure 5.1A it can be observed that PAC content varies considerably, namely from 81.8 mg_{PAC}/g_{GP} for GP2 to 109.7 mg_{PAC}/g_{GP} for GP1 which represents a 34% increase. In addition, the CH content also varies greatly (figure 5.1A) in which the GP3's CH content (441.5 mg_{CH}/g_{GP}) is 7.3 times higher than the ones observed for GP1 and GP2 (59.8 and 61.8 mg_{CH}/g_{GP}, respectively). This difference is most likely related to the fact that some wine producers add a washing step of the GP before discarding it with the intention of recovering additional fermentable CH.

In figure 5.1B it can be verified that the obtained mDP and %Gal values were of 7.4, 7.1 and 7.5 and 47.1, 45.7 and 45.4% for GP1, GP2 and GP3, respectively, which are similar values despite the differences in PAC and CH contents. From these it can be concluded that the use of aqueous acetone as solvent is not prone to interferences and that the PACs present in the tested GPs have similar characteristics.

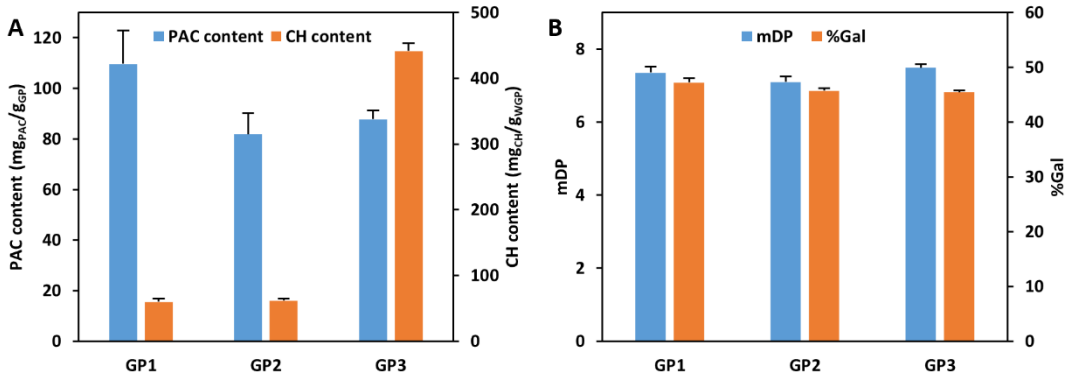


Figure 5.1. Characterization of different white grape pomaces (GP) extracts obtained with aqueous 70% (v/v) acetone and hot water extraction; A – PAC and CH content; B – mDP and %Gal

5.3.2. Extraction with Conventional Solvents

Despite being very effective at a laboratory scale, the use of acetone is not a viable option for industrial applications due to it being hazardous for the environment and for industrial operators. In general, PACs are extracted with water that, as shown in figure 5.2A, results in very

low Y_{PAC}, more specifically, in 5.3, 7.2 and 11.4% of what is obtained with aqueous acetone for GP1, GP2 and GP3, respectively. Interestingly, the high CH content of GP3 appears to have a positive effect on Y_{PAC} which is in accordance with what was previously described [232]. In addition, mDP (figure 5.2B) and %Gal (figure 5.2C) values are also considerably lower than the ones obtained with aqueous acetone, despite having low variability between them.

In alternative to the use of water as solvent, aqueous ethanol extraction (60EtOH) is a viable option since ethanol can be obtained from renewable sources by a fermentation process, only requiring a distillation process for its purification. Contrary to what is observed with water extraction, the use of aqueous ethanol as solvent appears to be negatively affected by the high CH content of GP3, as depicted in figure 5.2A. In it can be verified that the Y_{PAC} of GP3 (29.4 mg_{PAC}/g_{GP}) is less than half of what is obtained for GP1 and GP2 (63.6 and 68.0 mg_{PAC}/g_{GP}, respectively). Interestingly, the obtained %Gal values (figure 5.2C) were very similar to those obtained with aqueous acetone and with low variability between them which was not observed for the mDP values (figure 5.2B) that were slightly lower for GP1 and GP2 and considerably lower for GP3, indicating that the final mDP of the extract is also negatively affected by a high CH content.

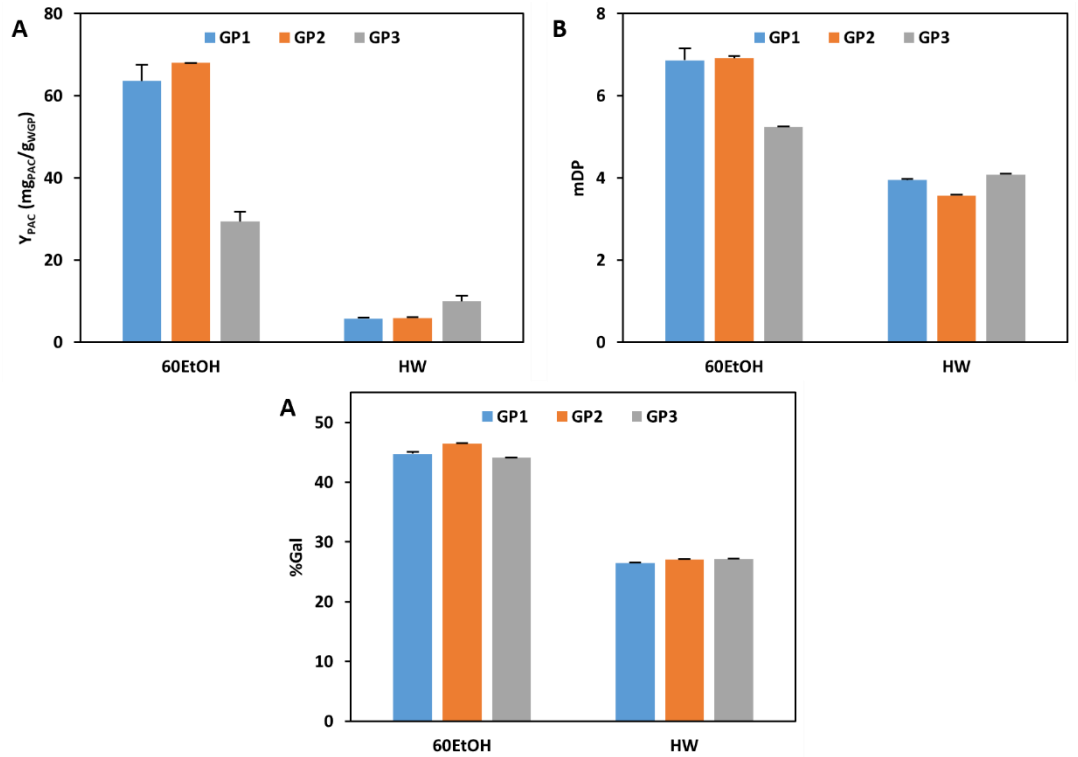


Figure 5.2. Characterization of the extracts obtained from the tested GPs with conventional solvents; 60EtOH - aqueous ethanol 60% (v/v), HW - hot water extraction; A - Y_{PAC}, B - mDP and C - %Gal

From these results it can be concluded that the use of water as extraction solvent, despite resulting in extracts that have mDP and %Gal values that are not affected by biomass differences, lead to mDP and %Gal values that are half of what is observed in figure 5.1B and to Y_{PAC}s that represent a fraction of the available PAC content, as determined with aqueous acetone extraction. In addition, the use of aqueous ethanol leads to high and constant %Gal values but results in mDP values that are dependent of biomass properties. Considering the importance of obtaining constant and high mDP values for PACs biological activities [87,200,258,262] these solvents present a limitation in the current application of PACs that must be addressed.

5.3.3. Effect of Grape Pomace's Characteristics on the Extraction with Eutectic Solvents

Quaternary ES systems have previously been suggested as a viable option in the improvement of PAC extraction, namely the combination of ChCl, Glyc, EtOH and water [232]. With that the in mind the effect that GP differences have on the Y_{PAC}, mDP and %Gal when using this solvent system was assessed. This was achieved by means of RSM from which the effect of the ES composition on Y_{PAC}, mDP and %Gal was modulated, resulting on polynomials described by equations 5.1 to 5.3 for GP1, 5.4 to 5.6 for GP2 and 5.7 to 5.9 for GP3, respectively, and the respective contour plots represented on figure 5.3.

Equation 5.1. $$Y_{PAC} = 104.8x_{ChCl} + 100.4x_{Glyc} - 21.6x_{H2O} + 95.1x_{EtOH} + 65.2x_{ChCl}x_{Glyc} + 315.6x_{ChCl}x_{H2O} + 121.6x_{Glyc}x_{H2O} + 222.0x_{H2O}x_{EtOH}$$

Equation 5.2. $$\mathrm{mDP} = 9.57\mathrm{x_{ChCl}} + 8.85\mathrm{x_{Glyc}} + 3.85\mathrm{x_{H2O}} + 4.20\mathrm{x_{EtOH}} - 1.20\mathrm{x_{ChCl}x_{Glyc}} + 5.47\mathrm{x_{ChCl}x_{H2O}} + 1.13\mathrm{x_{ChCl}x_{EtOH}} - 0.91\mathrm{x_{Glyc}x_{H2O}} + 3.37\mathrm{x_{Glyc}x_{EtOH}} + 7.10\mathrm{x_{H2O}x_{EtOH}}$$

Equation 5.3. $$\%\mathrm{Gal} = 42.2\mathrm{x_{ChCl}} + 39.4\mathrm{x_{Glyc}} + 30.4\mathrm{x_{H2O}} + 33.2\mathrm{x_{EtOH}} + 1.38\mathrm{x_{ChCl}x_{Glyc}} - 0.49\mathrm{x_{ChCl}x_{H2O}} - 7.59\mathrm{x_{ChCl}x_{EtOH}} - 14.7\mathrm{x_{Glyc}x_{H2O}} + 21.1\mathrm{x_{Glyc}x_{EtOH}} + 30.9\mathrm{x_{H2O}x_{EtOH}}$$

Equation 5.4. $$\mathrm{Y_{PAC}} = 58.9\mathrm{x_{ChCl}} + 91.2\mathrm{x_{Glyc}} + 36.8\mathrm{x_{H2O}} + 122.7\mathrm{x_{EtOH}} + 104.1\mathrm{x_{ChCl}x_{Glyc}} + 295.8\mathrm{x_{ChCl}x_{H2O}} + 77.2\mathrm{x_{Glyc}x_{EtOH}}$$

Equation 5.5. $$\mathrm{mDP} = 7.86\mathrm{x_{ChCl}} + 9.31\mathrm{x_{Glyc}} + 3.36\mathrm{x_{H2O}} + 6.63\mathrm{x_{EtOH}} + 2.10\mathrm{x_{ChCl}x_{Glyc}} + 11.79\mathrm{x_{ChCl}x_{H2O}} + 2.27\mathrm{x_{ChCl}x_{EtOH}} + 1.58\mathrm{x_{Glyc}x_{EtOH}} + 4.43\mathrm{x_{H2O}x_{EtOH}}$$

Equation 5.6. $\%Gal = 37.6x_{ChCl} + 36.0x_{Glyc} + 30.1x_{H2O} + 29.0x_{EtOH} + 8.10x_{ChCl}x_{Glyc} + 6.03x_{ChCl}x_{H2O} - 13.8x_{Glyc}x_{H2O} + 32.0x_{Glyc}x_{EtOH} + 30.7x_{H2O}x_{EtOH}$

Equation 5.7. $Y_{PAC} = 87.4x_{ChCl} + 58.2x_{Glyc} + 31.3x_{H2O} - 34.0x_{EtOH} + 30.3x_{ChCl}x_{H2O} + 118.5x_{ChCl}x_{EtOH} + 121.1x_{Glyc}x_{EtOH} + 170.2x_{H2O}x_{EtOH}$

Equation 5.8. $mDP = 8.59x_{ChCl} + 6.32x_{Glyc} + 3.90x_{H2O} - 0.39x_{EtOH} + 4.36x_{ChCl}x_{EtOH} + 8.67x_{Glyc}x_{EtOH} + 10.43x_{H2O}x_{EtOH}$

Equation 5.9. $\%Gal = 37.0x_{ChCl} + 33.9x_{Glyc} + 25.8x_{H2O} + 31.6x_{EtOH} + 9.87x_{ChCl}x_{Glyc} + 6.60x_{ChCl}x_{H2O} - 5.75x_{ChCl}x_{EtOH} + 26.8x_{Glyc}x_{EtOH} + 41.7x_{H2O}x_{EtOH}$

From figure 5.3 it can be observed that, similarly to what was observed for aqueous ethanol extraction (figure 5.2), GP1 and GP2 have a similar behavior as far as Y_{PAC}, mDP and %Gal are concerned. More specifically, in both cases the best Y_{PAC} is obtained by a solvent composed by only ChCl and water, x_{ChCl}=0.7 and x_{H2O}=0.3 for GP1 and x_{ChCl}=0.54 and x_{H2O}=0.46, resulting in 133 and 122 mg_{PAC}/g_{GP}, respectively. Contrarily, the best Y_{PAC} result for GP3 was 78 mg_{PAC}/g_{GP} and was obtained with x_{ChCl}=0.7, x_{Glyc}=0.25 and x_{H2O}=0.05.

In terms of mDP the predicted values varied greatly with the ES composition ranging from 6.1 to 9.0 for GP1, from 6.0 to 9.1 for GP2 and 5.0 to 7.8 for GP3 following the same trend observed for Y_{PAC}.

As far as %Gal is concerned the predicted values ranged from 31.1 to 41.7% for GP1, from 29.6 to 40.8% for GP2 and 30.0 to 39.8% for GP3 which are much more constant and in line to what was observed for the PAC extractions with conventional solvents (figure 5.2). Further confirming that as long as the solvent composition is kept constant the same %Gal is obtained, regardless of solvent system.

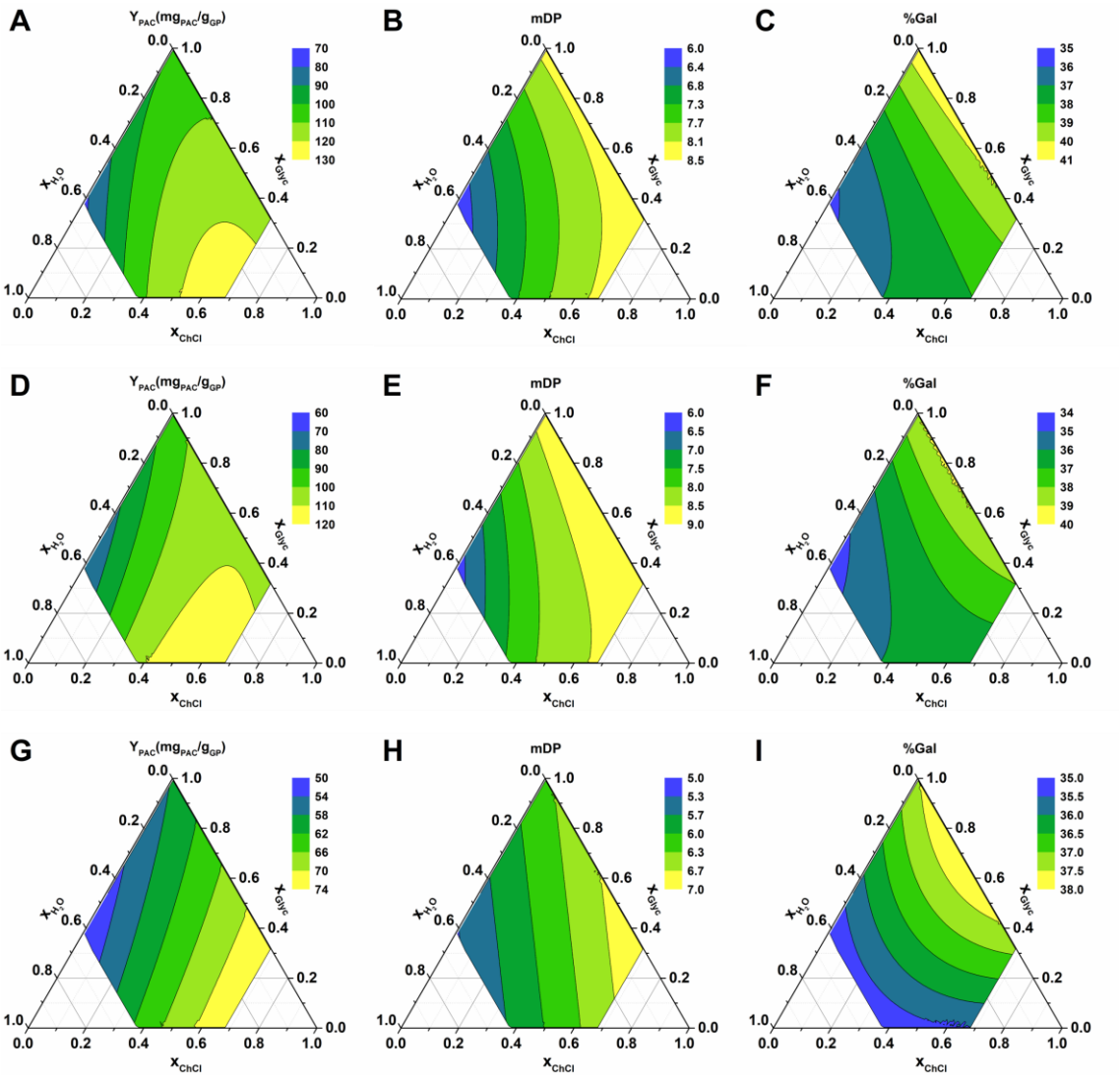


Figure 5.3. RSM contour plots of the GP (GP1 – A,B and C; GP2 – D, E and F; GP3 – G, H and I) effect on Y_{PAC} (A, D and G), mDP (B, E and H) and %Gal (C, F and I) in the extraction with ESs.

5.3.4. Normalization of PAC extracts Obtained from Different Grape Pomaces

From the results discussed above it can be postulated that by choosing the correct ES composition it is possible to obtain a specific mDP regardless of the initial GP composition which would result in a decreased Y_{PAC} but in the possibility of normalizing PAC's extracts. Taking into account the mDP values that are possible to obtain from the GPs that were used as raw material, the mDP values of 6.5 and 7.5 were arbitrarily chosen as proof of concept. With that in mind, and using the optimization tool in DesignExpert, the ES's compositions for the obtention of mDP values of 6.5 and 7.5, while maximizing for Y_{PAC} were determined for each GP (table 5.4).

Table 5.4. Eutectic solvent composition for the obtention of PAC extracts with specific mDPs

GP	x_{ChCl}	x_{Glyc}	x_{H2O}	x_{EtOH}
mDP=6.5				
GP1	0.137	0.000	0.463	0.400
GP2	0.060	0.040	0.500	0.400
GP3	0.511	0.000	0.300	0.189
mDP=7.5				
GP1	0.364	0.000	0.300	0.336
GP2	0.217	0.000	0.383	0.400
GP3	0.669	0.174	0.126	0.031

After extracting the three GPs with the ES compositions described on table 5.4, PAC extracts with very close mDP values were obtained (figure 5.4B), more specifically, 6.42, 6.41 and 6.41, and 7.61, 7.47 and 7.69 for predicted mDP values of 6.5 and 7.5, respectively, thus confirming the accuracy of the models used. In addition, the resulting Y_{PAC}s in mDP=6.5 (figure 5.4A) are comparable with the ones observed in the extraction of GP1 and PG2 with aqueous ethanol (figure 5.2A) and considerably higher for GP3. In the case of the Y_{PAC} in mDP=7.5 all values (figure 5.4A) are considerably higher than those obtained with aqueous ethanol (figure 5.2A). As far as %Gal is concerned, the values obtained with ESs (figure 5.4C) are lower than those verified with aqueous ethanol but higher than the ones obtained with water (figure 5.2C). The %Gal values are less constant between samples which is to be expected since different solvent compositions were used and as showed before solvent composition is the main factor influencing %Gal with GP characteristics having little effect on the final result.

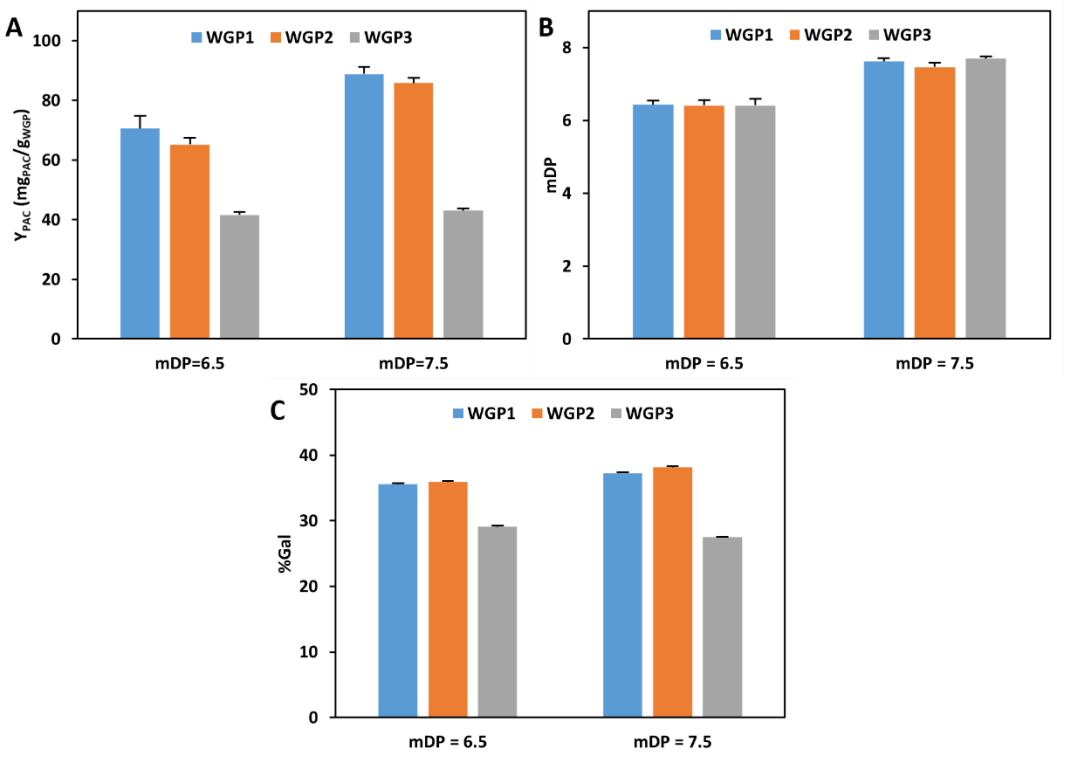


Figure 5.4. Characterization of the extracts obtained with the ES systems described on table 5.4; A - Y_{PAC}, B - mDP and C - %Gal

5.4. Conclusion

From the results presented here it can be concluded that by using this type of quaternary ES system, composed of ChCl, Glyc, EtOH and water is possible not only to normalize extracts obtained from different GPs but to produce extracts with a specific mDP in a reproducible way, independently from raw material characteristics. This type of solvent system represents a considerable improvement on Y_{PAC} values when compared with the conventional hot water extraction without representing a change in the overall procedure which makes it easy to implement at an industrial level. In addition, these findings open the possibility of obtaining PAC extracts similar to the ones obtained here but from other type of by-products, as long they possess the same type of PAC, further contributing to a circular economy by consolidating the local sourcing of PACs.

6. Improving Proanthocyanidin's Antioxidant Activity by Combination with Eutectic Solvents

Adapted from article being prepared for publication

Abstract

PACs' antioxidant activity has been described both *in vitro* and *in vivo*. Unfortunately, their large-scale extraction process is limited to a hot water process that results in a low yield.

ESs, a class of solvents with promising results in the extraction of PACs, which due to their nontoxic character can be kept with the extracts for subsequent applications.

In this perspective, herein the effect that the presence of ESs have on the antioxidant activities of PACs was evaluated. Molecular antioxidant activity was evaluated with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay (ABTS) from which it was concluded that the best results were obtained with the combinations of betaine and proline with urea (reductions of 20.4 and 27.4% in the IC50 value, respectively). Cellular antioxidant activity was evaluated with the dichlorofluorescein diacetate assay (DCFDA) from which was concluded that the best results were obtained with the combination of betaine and urea that suppressed the formation of the reactive oxygen species after UV irradiation by 45% in HaCaT cells.

6.1. Introduction

PACs are vegetable polymeric phenolic compounds composed by catechin and its derivatives that are used in the manufacture of leather [60] and wood agglomerates [65], and in wine maturation process [71]. In addition, they have also been proposed has good candidates for the replacement of synthetic food additives such as preservatives [246] or antioxidants [244,245]. Furthermore, PACs have also been described has possessing good *in vivo* antioxidant [286,287] and anti-inflammatory [87,98,101] activities. In addition, the use of PACs in the treatment of several ailments such as breast cancer [85] and diabetes [102] has also been proposed, in which the antioxidant and anti-inflammatory activities appear to play an important role. Despite PAC's potential its large scale isolation process is limited to hot water extraction that has a very low yield and leads to extracts with low quality namely with low purity and mDP values [232].

With the objective of overcoming the current limitations in the PACs extraction process, several studies were made in which the use of ESs as extraction medium was explored [216,232,288,289]. This was made either SLE [216,232] or by combining it with MAE [288] or UAE [289] which considerably reduced extraction time.

ESs are derived from the DES concept that was first described by Abbot et al. [207] and consist in a mixture of two or more components that possess a decreased melting point when compared with the individual components. In general, ES are defined as a combination of HBA and a HBD

but are not necessarily limited to binary systems being in most situations composed by tertiary [216] or even quaternary [232] systems.

The best possible results in terms of Y_{PAC} are dependent on the type of raw material and on the extraction procedure, resulting in optimal ES compositions that vary considerably from case to case, i.e. while choline chloride is frequently found to be the best option for HBA, HBD is more variable with glycerol [232], malonic acid [216] and lactic acid [232,289] being suggested. Additionally, the optimal water content is also quite variable, ranging from 18 to 55% (m/m).

Despite the wide use of ESs in the extraction of phenolic compounds from biomass [278], the inexistence of effective downstream processes and the relative biocompatibility of ESs have led many authors to suggest that they may be kept in the final formulation [290] or with the active component being part of the eutectic system [291].

One of the valuable properties that are frequently looked for in studies concerning extracts of phenolic compounds, PACs and others, is their antioxidant activity regardless of ESs being used as extraction media or not.

Nevertheless, extracts that are obtained with different ESs, i.e., with different HBA:HBD combinations and proportions or different water contents are effectively different in terms of quantitative and often qualitative compositions. Therefore, variations in antioxidant activity can either be attributed to differences in extract composition or the presence of specific ES's components.

To the best of our knowledge the effect of ES's presence on the antioxidant activity of PACs, while normalizing for extract composition, is yet to be described and is herein reported for the first time. This was achieved by dissolving a PAC reference material in aqueous solutions of different ES combinations that have been described in the extraction of PACs [288] and screening the antioxidant potential variability with molecular and cellular assays.

6.2. Material and Methods

6.2.1. Proanthocyanidin Reference Material

PACs reference material (PRM) was isolated from GP following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention.

6.2.2. Purification of Proanthocyanidins with High Degree of Polymerization

High DP PACs were isolated following the method described by Neto et al. [232] that is detailed in section 2.2.4.

6.2.3. Acid Butanol Assay for Proanthocyanidin Quantification

PRM's purity ($mg_{PAC}/g_{extract}$) was determine following the acid butanol assay described by Porter et al. [31] that is detailed in section 2.2.5.

6.2.4. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PRM's mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] that is detailed in section 2.2.6.

6.2.5. Eutectic Solvents Preparation

All ESs were composed by 32.5% (m/m) of HBA (choline chloride (ChCl), betaine (Bet) and proline (Pro)), 32.5% (m/m) of HBD (urea (Ur), malic acid (MalA), glucose (Glu) and glycerol (Glyc)) and 25% (m/m) of water. The different ES's combinations were prepared by combining the appropriate amount of each compound and mixing them at 40 °C under continuous stirring until a continuous phase was obtained.

6.2.6. Molecular Antioxidant Assay

Molecular antioxidant potential was determined by measuring the concentration of PRM necessary to reduce 50% (IC50) of the radical 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$). This was made following the method of Re et al. [292] with slight modifications. Briefly, the $ABTS^{\bullet+}$ solution was prepared by mixing aqueous solutions of ABTS and potassium persulfate to a final concentration of 7 and 2.45 mM, respectively, and incubating it at room temperature and in the dark, overnight.

ES solutions were prepared by dissolving them in water at a concentration of 200 mg/mL (150 mg_{solute}/mL, since 25% of the ES is water) or the individual components at a concentration of 75 mg/mL. Each ES solution was then used to prepare PRM stock solutions of 1 mg/mL. These PRM stock solutions were then diluted with the respective ES or individual component solution to PRM concentrations of 5, 20, 35, 50, 65, 80 and 100 µg/mL.

In a 96-well plate 200 µL of $ABTS^{\bullet+}$ solution were mixed with 40 µL of PRM solution with varying concentrations dissolved in solutions with different ES combinations. The plates were then incubated for 30 min in the dark at room temperature and the resulting absorbance was recorded at 730 nm. From the slope obtained for each ES combination the IC50 was determined.

6.2.7. HaCaT cell Culture

For the cellular evaluation of the PRM's antioxidant potential, spontaneously transformed aneuploid immortal keratinocyte cell lines from adult human skin (HaCaT) were chosen with the intent of exploring possible skin care applications. These were cultured in 22.1 cm^2 plates and maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum (FBS, CLS) and 1% of antibiotic/antimycotic solution (100 units/mL of penicillin, 10 mg/mL of streptomycin and 0.25 mg/mL of amphotericin B), at 37 °C in an atmosphere of 5% CO_2. Medium was renewed every two days and cells were harvested every two weeks.

6.2.8. Cytotoxicity of Proanthocyanidin Reference Material and Selected Eutectic Solvent *Components*

The cytotoxicity of ES's components and PRM were assessed in order to determine the maximum concentration of each component that can be used without causing cellular death. This was made by seeding cells at a concentration of $4x10^5$ cells/mL in a 96-well plate with the same conditions described previously and after 24 h, PRM ($3.235x10^{-4}$ to $6.47x10^{-1}$ µg/mL) and ES ($1.0x10^{-3}$ to 2.0 g/L) solutions were added and incubated for an additional 24 h. Cell survival was determined using the Alamar blue assay that consisted in adding 20 µL of Resazurin solution (0.15 mg/mL in PBS) to the 100 µL already present at the well and incubating it for 2h. The fluorescence intensity was then determined using excitation/emission pair 560/590 nm with fluorescence intensity being proportional to the number of viable cells. PRM and ES combinations were considered non-toxic when cell viability values were above 80% [293].

6.2.9. Cellular antioxidant assay

The suppression of reactive oxygen species formation (ROSs) was assessed using the dichlorofluorescein diacetate assay (DCFDA) in which cells were seeded at a concentration of $4x10^5$ cells/mL in a 96-well plate with the same conditions described previously and after 24 h, PRM (161 µg/mL) and ES (2 g/L) solutions were added and incubated for an additional 24 h. After that, the medium was removed and cells were washed twice with Hank's Buffered Saline

Solution and incubated with 100 µL of a 50 µM DCFDA solution for 15 min. Aggression was then induced using a UV light source at 365 nm for 15 min. The control was made by doing the same procedure but incubating the plate in the dark instead of subjecting it to UV radiation. The fluorescence intensity was determined using the excitation/emission pair 498/522 nm with fluorescence intensity being proportional to the formation of ROSs.

6.3. Results and discussion

6.3.1. Molecular Antioxidant Activity

The prepared PRM was characterized according to the previously described methods from which it was concluded that it possessed a purity of 582 $mg_{PAC}/g_{extract}$, a mDP value of 8.1 and a %Gal of 46.4%. PRM was then dissolved in water (control) and in solutions containing ES components as described previously. The IC50 of PRM dissolved in water was 70.1 $\mu g_{extract}$/mL and all IC50 values presented in figure 6.1 were measured against this value.

All ES as well as HBA and HBD solutions had no radical scavenging activity by themselves at the tested concentrations since no reduction in the Abs_{730} values were observed when PRM was not present. The only exception was ProGlu and consequently it was excluded from this assay. The most plausible explanation for this fact is the production of Mailard reaction products during the ES preparation [274] since this behavior was not observed for the individual components, Pro and Glu.

In figure 6.1, it can be observed the effect that using different ES, HBA or HBD solutions have on the PRM's IC50. In terms of individual compounds, ChCl, Glu and Glyc have residual effect on IC50 value with the first being slightly positive and the remaining ones slightly negative. The best results for individual compounds were obtained with Pro (IC50 reduction of 22.5%) followed by Bet (14.3% reduction) and Ur (7.1% reduction) while the worst results were obtained with MalA (50.6% increase).

When pairing ChCl with different HBDs an additive effect, i.e. equal to the sum of the individual components, is observed for ChClUr (8.5% reduction) and ChClGlu (2.9% increase), and a synergistic one, i.e. more than the sum of the individual components, is observed with ChClGlyc (10.0% reduction). In the case of Bet, additive effects are once again observed for BetUr (20.4% reduction) and BetGlu (13.7% reduction) but a detrimental effect in the combination with BetGlyc is observed resulting in an IC50 reduction of 3.5% in opposition to 14.3% of Bet by itself. In the cases of ProUr or ProGlyc additive effects are observed in both situations with IC50

reductions of 27.3 and 19.9%, respectively. Lastly, the combinations of MalA with all HBAs led to increases of 42.8, 45.2 and 40.0% in IC50 values for ChClMalA, BetMalA and ProMalA, respectively.

Based on these results Bet, Pro and Ur and their combinations were selected for cellular assays as they gave rise to the highest IC50 reductions in the molecular assays.

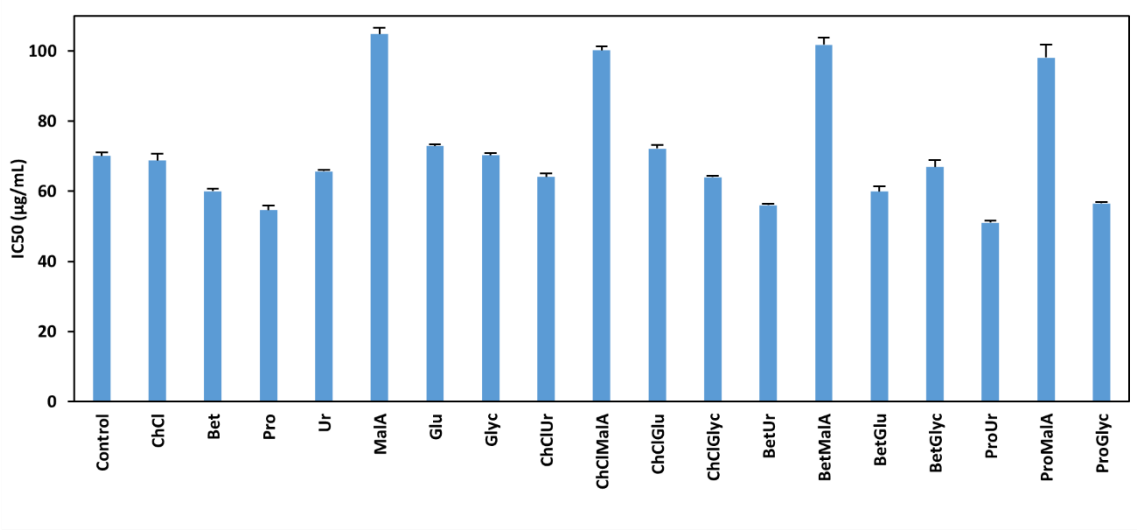


Figure 6.1. IC50 values in the ABTS radical scavenging activity of PRM dissolved in aqueous solutions of different ES, HBA or HBD

6.3.2. Cytotoxicity of Proanthocyanidin Reference Material and Selected Eutectic Solvent Components

After selection of Bet, Pro and Ur as the best candidates for improvement on the antioxidant activity of PRM the cytotoxicity of each of these components, individually and combined, was assessed with cell viability values above 80% being considered as safe.

As it can be observed on figure 6.2A, Bet, Pro and Ur had no toxic effect on HaCaT cells up to a concentration of 2.0 g/L while the combinations of Bet and Pro with Ur exhibited low toxicity, 8 and 13% of reduction in cell viability which was considered safe. This is in line with what has been described in literature where, with some exceptions, ES have been considered safe up to a concentration of 2.0 g/L [294].

Furthermore, the toxicity of PRM dissolved in water (control) or Bet, Pro, Ur, BetUr and ProUr at a concentration of 2.0 g/L was also assessed (figure 6.2B) from which it was verified that the cytotoxicity behavior was similar regardless of solvent and that no significant cytotoxicity was observed for values bellow 320 µg/mL. This in opposition with what was described by Kaplum et al. that described a PAC extract obtained from *Stryphnodendron adstringens* as having an IC50

of 55.5 µg/mL for the same type of cell [295]. Nevertheless, and since PACs are generally described as safe [283] it could be argued that these differences may be related to the presence of other compounds in the study of Kaplum et al. [295].

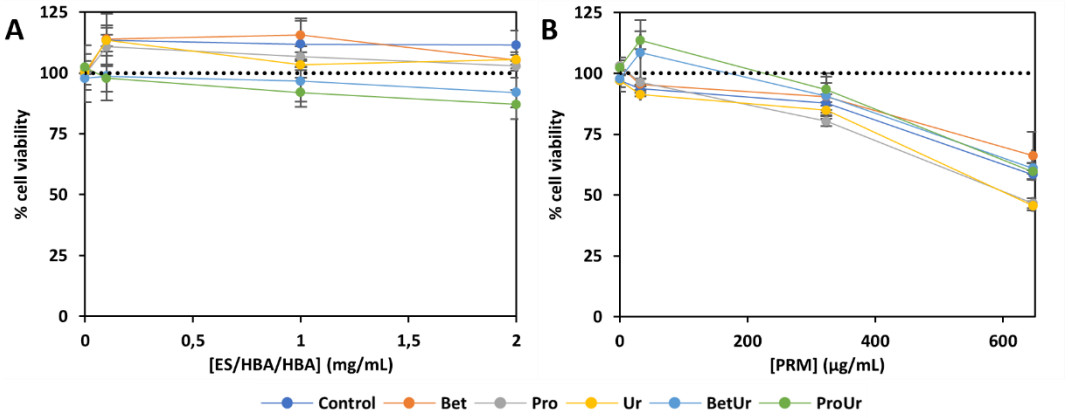


Figure 6.2. Effect of (A) ES, HBA and HBD concentration and (B) PRM concentration combined with 2.0 mg/mL of ES, HBA or HBD on cell viability

Based on the results reported above an ES, HBA and HBD concentration of 2.0 g/L and PRM concentration of 161 µg/mL were chosen due to the low toxicity levels at these concentration values that could interfere with the antioxidant activity measurement.

6.3.3. Cellular Antioxidant Activity

The amount of ROS that are produced with or without UV irradiation after HaCaT cells have been incubated with PRM dissolved in water or aqueous solutions of Bet, Pro, Ur, BetUr and ProUr is depicted in figure 6.3. As expected, considerable reductions in the produced ROS are observed when HaCaT are incubated with PRM by itself both with and without UV irradiation, as PACs have been extensively described for their antioxidant potential [286,287].

Nevertheless, different behaviors are observed in the presence of additional compounds. More specifically, despite Bet [296] and Pro [297] being described as having antioxidant capacity in specific situations, in the results expressed here it was shown that when they are applied by themselves in combination with PRM no reduction in ROS production was observed, effectively suppressing the positive effect of PRM.

In opposition, a considerable reduction in ROS production is observed when PRM is used in the presence of BetUr and ProUr combinations with the best results being obtained with BetUr which led to a reduction of 45% on the total amount of ROS produced.

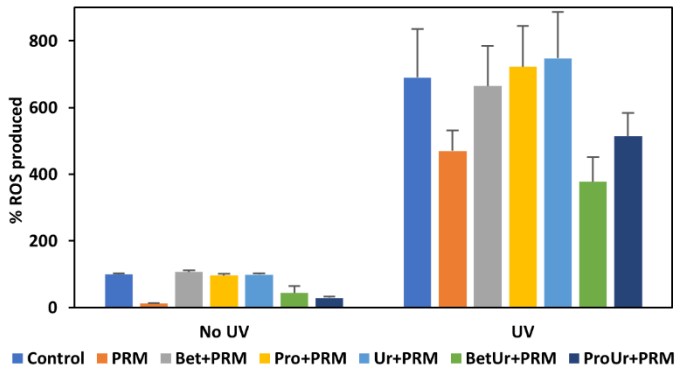


Figure 6.3. Effect of PRM (161 µg/mL) with aqueous solutions of ES, HBA or HBD (2.0 mg/mL) of the production of ROS before and after UV irradiation

6.4. Conclusion

ES represent a promising option in the improvement of PAC's extraction but the knowledge on how they can impact their molecular and cellular antioxidant activities is still limited and was explored here for the first time. The results presented here demonstrate the importance that the presence of specific components or their combinations with others may have on the biological activity of compounds such as PACs as these can improve their activity as in the case of BetUr or mitigate it as in the case of the individual use of Bet and Ur.

7. Synthesis of Core-Shell Gold-Proanthocyanidin Nanoparticles

Adapted from article being prepared for publication

Abstract

Metallic nanoparticles, particularly of gold, are becoming a common element of everyday applications. Consequently, an increasing attention has been given to their synthesis process and the hazardous compounds that it involves.

Based on green chemistry principles several efforts have been made with the intention of replacing hazardous compounds by safe and sustainable alternatives from which plant extracts and their constituents can be highlighted. Among those, a lot of attention has been given to phenolic compounds due to their strong reducing power. An example of such are the polymeric forms of catechin, PACs that despite of having been described in the synthesis of gold nanoparticles in the form of a crude extracts, their use as pure compounds is yet explored.

Herein the use pure PAC fractions with three different mean degree of polymerizations (low, medium, and high) in the synthesis of gold nanoparticles in a one-step process was compared with catechin. From which was possible to assess that by using PACs instead of catechin was possible to obtain smaller nanoparticles, with thinner organic shells that were stabler for longer time periods.

7.1. Introduction

Nanostructured materials and in particular nanoparticles (NPs) have been the focus of extensive studies in the past three decades [298,299]. Gold NPs (AuNPs) in particular were amongst the first ones to be investigated and are, to this day, still one of the main objects of research for an ever increasing number of applications such as in sensors [300], food packaging [301], cancer treatment [302] and biomedicine [303].

AuNPs synthesis is based in four components, namely, a gold precursor, a reducing agent, a stabilizing agent and a solvent. The most commonly used gold precursor is tetrachloroauric acid that can be reduced to elemental gold by several reducing agents such as sodium citrate [304], hydroxylamine [304], sodium borohydride [305] and hydrazine [306] with stabilizing agents being composed most often by alkanethiols [305]. Some of these components have high toxicity levels and a high environmental impact and therefore must be replaced by greener alternatives.

Green chemistry is a research trend in which the use of harmful or non-sustainable compounds is reduced or replaced by greener alternatives [307] and AuNPs synthesis is no exception [308]. Several methodologies have been suggested for the green synthesis of AuNPs that can be divided into three main groups: whole organisms such as bacteria [309], fungi [310] or plants

[311]; extracts from plants such as *Eucalyptus globulus* bark [312], *Memecylon umbellatum* leaves [313] or *Cassia auriculata* leaves [314]; and the use of sustainable and environmentally friendly reagents such as chitosan [315] or glucose [316].

From the available options, plant extracts have risen up as preferable options due to the high variability of compounds with reducing properties that are able to produce AuNPs with different sizes and morphologies [317] and the possibility of combining reduction and stabilization into a single step, with phenolic compounds being the main responsible for gold reduction [318].

Among the phenolic compounds family, PACs have demonstrated interesting properties in the field of AuNPs synthesis [319–322]. These polymeric compounds, based on catechin, and its derivatives, are widespread in the plant kingdom and are, in many cases, present in large amounts on agroforestry by-products such as grape pomace [169] or tree barks [183] that have low economical value and represent an additional cost to producers related with their disposal [145]. Therefore, the use of PACs in the synthesis of AuNPs would contribute to the valorization of these by-products, reducing the total amount of waste, while providing efficient chemicals for the replacement of hazardous ones, promoting the simultaneous implementation of circular economy and green chemistry values.

Up until this moment, PACs have been implemented in the synthesis of AuNPs in the form of crude extracts derived from *Vitis vinifera* [319], *Portulaca grandiflora* [322], *Galaxaura elongata* [320] and *Mimusops elengi* [321] that have in common the low reaction temperature, the poor size and morphology control, and the formation of an extract shell that encompasses several particles leading to a suspension with a purple color characteristic of AuNPs aggregation.

Additionally, pure catechin has also been studied as reducing and stabilizing agent in AuNPs synthesis [319,323–325] from which it can concluded that Au:catechin ratio has a relevant impact on the final size of AuNPs. More specifically, a Au molar excess leads to AuNPs with large size and morphology inconsistency [325], a molar ratio of 1:1 leads to monodispersed core-shell AuNPs [319,324] with a shell composed of different oligomeric products derived from the polymerization of oxidized catechin [323], and that an increase in the catechin:Au ratio from 1 to 3 leads to AuNPs with a smaller organic shell (22 nm to 4 nm), attributed to the faster reaction rate, and a decrease in zeta potential (-35 to -18 mV) [323].

Herein the main objective is to explore the potential of PACs with different mDP as reducing and stabilizing agent in the synthesis of core-shell Au-PAC NPs. This was made by comparing catechin with three pure PAC fractions with different mDPs that were either commercially available (low) or extracted from grape pomace (GP) (medium and high). In addition, the effect of Au:PAC

proportion and pH on AuNPs size and morphology were assessed by measuring the absorption spectrum, determining particle size distribution and zeta potential by dynamic light scattering (DLS), and transmission electron microscopy (TEM).

7.2. Material and Methods

7.2.1. Grape Pomace Preparation

GP was prepared following the method detailed in section 2.2.2.

7.2.2. Proanthocyanidin Reference Material

PAC reference material was isolated from GP following the method described by Alwerdt et al. [231] that is detailed in section 2.2.3 for **PAC Ace** obtention.

7.2.3. Fractionation of Proanthocyanidins Based on Degree of Polymerization

PACs fractions with medium and high mDP (**PAC F2** and **PAC F3**) were isolated following the method described by Neto et al. [232] that is detailed in section 2.2.4. **PAC F1** was excluded due to its low purity and a commercially available grape seed extract from Sigma-Aldrich (**PAC Sig**) was used instead.

7.2.4. Acid Butanol Assay for Proanthocyanidin Quantification

PAC fractions purities ($mg_{PAC}/g_{extract}$) were determine following the acid butanol assay described by Porter et al. [31] that is detailed in section 2.2.5.

7.2.5. Determination of Mean Degree of Polymerization and Galloylation Percentage by Phloroglucinolysis

PAC fractions' mDP and %Gal were determined following the phloroglucinolysis method described by Kennedy et al. [233] that is detailed in section 2.2.6.

7.2.6. Gold Nanoparticles Synthesis

AuNPs were synthesized by mixing 1.8 mL of a solution containing catechin (Cat) or PAC fractions (**PAC Sig**, **PAC F2** or **PAC F3**) to 0.2 mL of tetrachloroaurate solution corresponding to a final Cat/PAC concentration of 0.5 or 1.0 mM and a tetrachloroaurate concentration of 1.0 mM.

Before mixing, the tetrachloroaurate solution pH was kept unchanged (pH=2) or adjusted to 7.0 with a 10 M solution of sodium hydroxide which resulted, after mixing, in a final pH of 2.5 and 4.5, respectively.

The reaction media were kept at 30 °C for 14 h under continuous magnetic stirring after which the AuNPs were washed three times by centrifuging at 12000 rpm for 10 min and resuspending the pellet in distilled water.

7.2.7. Gold Nanoparticles Characterization

AuNPs were characterized by measuring the absorbance spectra in the range of 300 to 700 nm at 10 nm intervals in a plate reader from Biotek. The particle size distribution and the Zeta Potential were determined by dynamic light scattering (DLS) analysis using a Zeta sizer Nano-ZS (Malvern Panalytical). Transmission electron microscopy (TEM) images were obtained by using a Hitachi H-9000 operating at 200 kV. Samples were prepared by placing a colloid drop onto a carbon-coated copper grid and then allowing the solvent to evaporate.

7.3. Results and Discussion

7.3.1. Proanthocyanidin Fractionation

Currently there are no commercially available pure PACs extracts with high mDP. Consequently, herein the GP PAC extract fractionation was made to assess the possible impact of PAC's degree of polymerization on the synthesis of AuNPs.

In figure 7.1 the characteristics of **PAC Sig**, **PAC F2** and **PAC F3** were compared in terms of purity, mDP and %Gal. As it can be observed all tested extracts consisted almost exclusively of PACs (figure 7.1A) with purity values of 972, 987 and 972 $mg_{PAC}/g_{extract}$ for **PAC Sig**, **PAC F2** and **PAC F3**, respectively. The mDP values started at 3.3 for **PAC Sig** followed by 7.2 for **PAC F2** and 12.0 for **PAC F3**. %Gal were 29.4, 48.3 and 51.2% for **PAC Sig**, **PAC F2** and **PAC F3**, respectively (figure 7.1B).

Although a more consistent %Gal would be desirable the mDP values of the selected fractions are different enough and roughly equally spaced so that some relationship between PAC's mDP and AuNPs can be identified.

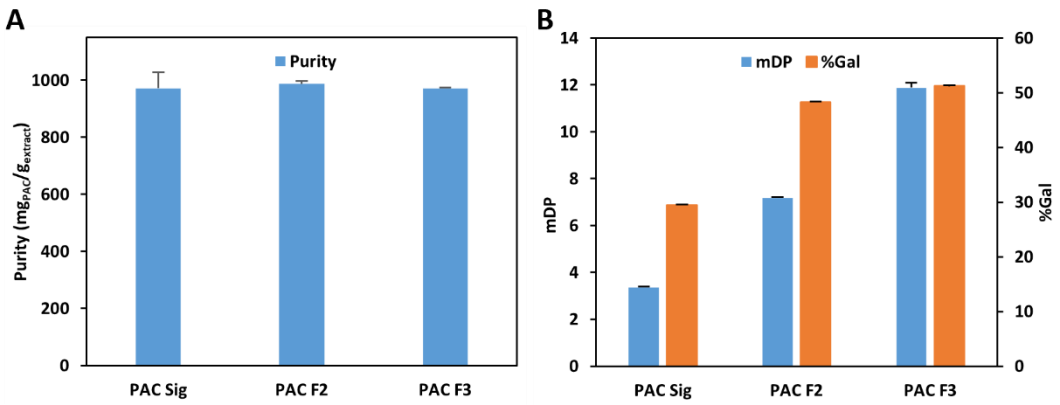


Figure 7.1. Characterization of PAC fractions used in the synthesis of AuNPs (A - purity, B - mDP and %Gal)

7.3.2. Gold Nanoparticles Characterization

Considering that PACs are oligomers/polymers of catechin and that the tested extracts were pure, a molecular weight of 290 g/mol was used for the calculation of the molar concentrations of all samples.

Based on the colloids' macroscopic aspect, shown in figure 7.2 it can be inferred that the AuNPs obtained with **PAC F2** and **PAC F3** at 0.5 mM, at a pH value of 2.5, presented a hazy aspect (figure 7.2) and a high dynamic particle size (when compared with the remaining experimental conditions) (table 7.1), and low yield of nanosized particles synthesis, indicated by the absence of strong red color, characteristic of AuNPs, (figure 7.2) and the respective low absorbance intensity (figure 7.3). This is probably related to the low concentration of reducing agent which promotes an excessive particle growth [312]. Furthermore, the AuNPs synthesized with 1.0 mM of Cat at a pH value of 4.5 resulted in a colloid with a purple hue (figure 7.2), associated with particle aggregation, and on an additional peak at 370 nm (figure 7.3) that can be attributed to a high concentration of products resulting from catechin oxidation [323].

The remaining samples had the characteristic absorbance peaks between 530 and 550 nm corresponding to the gold plasmon resonance with these variations being associated with differences in AuNP size [326].

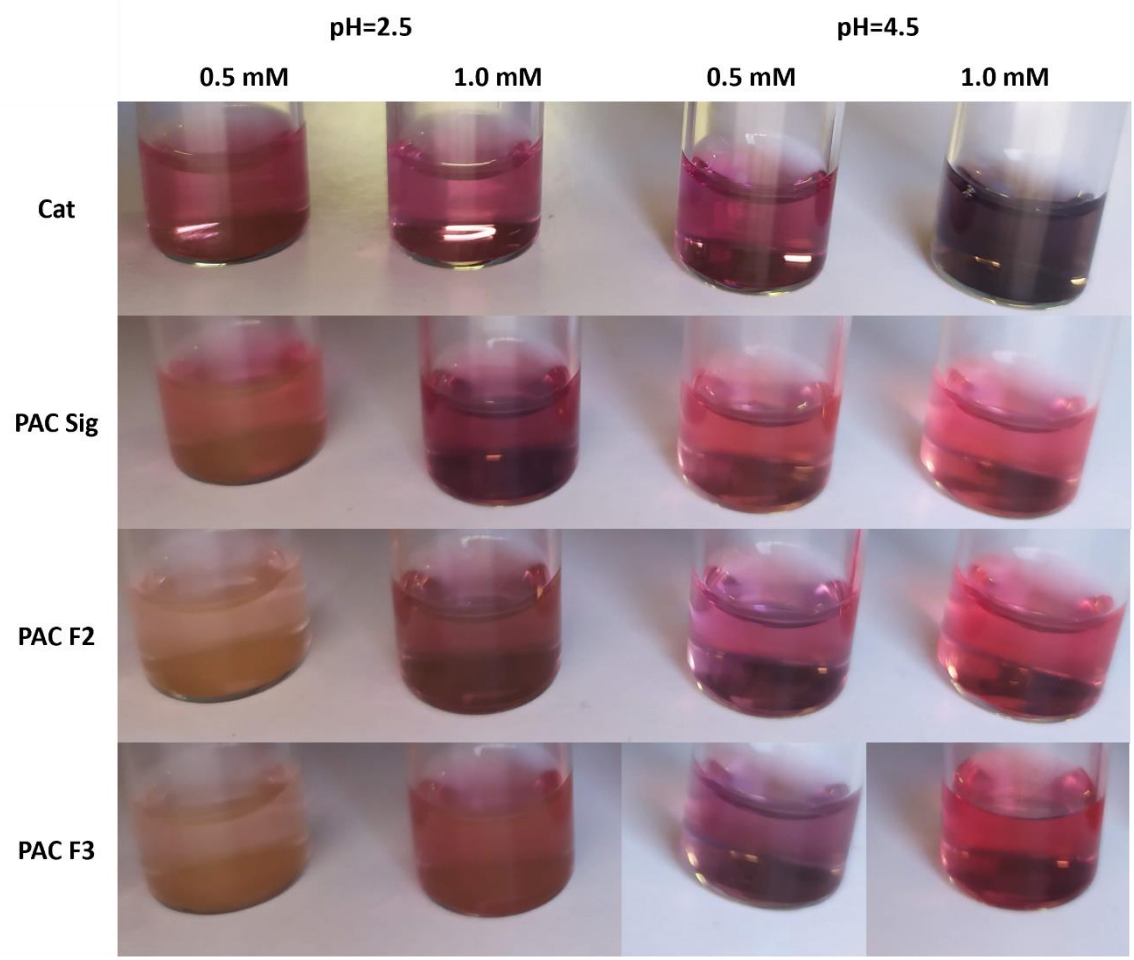


Figure 7.2. Macroscopic aspect of AuNPs synthesized using catechin or PAC fractions as reducing agents

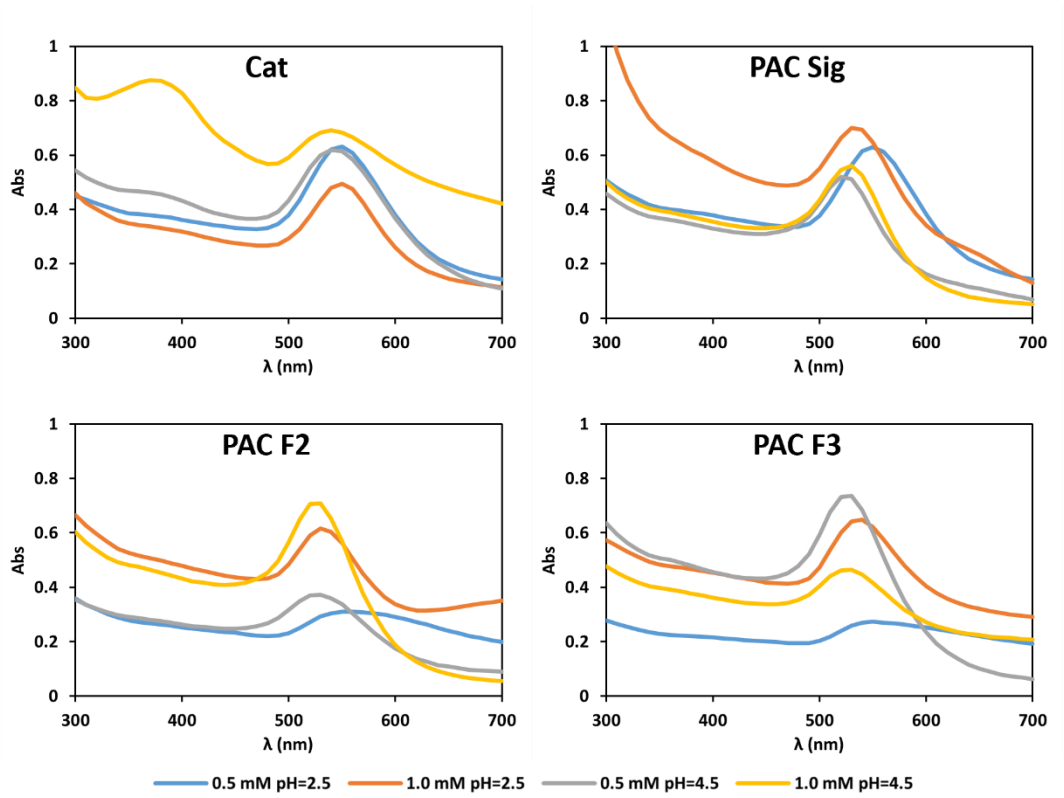


Figure 7.3. UV-Vis spectra of AuNPs synthesized using catechin or PAC fractions as reducing agents

The results from the DLS analysis are summarized in table 7.1 in which it can be observed that the increase in PAC concentration led to a decrease in the average particle size with the exception of catechin in which the opposite was observed. These findings are consistent with the visual inspection of the colloids (figure 7.2) in which the smaller particles are redder while the larger ones present a purple color. This can be explained by the fact that with lower concentrations of reducing agent less nucleation sites are formed in an initial stage and consequently more tetrachloroaurate salt will be available for particle growth at a later stage [312]. In addition, increasing the pH of the reaction media also leads to a decrease in the average particle size, regardless of reducing agent but especially for the PACs fractions in which the observed reduction was to less than half. This phenomenon has been largely described for AuNPs synthesis using sodium citrate [327,328] or extract from *Sargassum cymosum* [329] with NP size decrease being associated with the deprotonation of the hydroxyl groups. This leaves oxygen's electrons more easily available for the redox reaction with Au, effectively increasing the concentration of reducing agent which leads to a similar situation of increasing PAC concentration [312].

A lower polydispersity index (PDI) value indicates that the observed NPs sizes have a lower size distribution i.e., are monodispersed. Under the tested conditions, the use of Cat gives rise to

AuNPs that have less variation in terms of NP size (0.142 to 0.236) when compared to all PAC fractions (**PAC Sig** – 0.160 to 0.494; **PAC F2** – 0.170 to 0.514; **PAC F3** – 0.199 to 0.600). This can be explained by the fact that despite PAC fractions having high purity these are still mixtures of PACs with a range of DPs and %Gal which introduces variability to the system contrary to what is observed with pure catechin. In the case of PACs fractions although it can be observed that when the pH is 2.5 an increase in PAC concentration leads to an increase in PDI while at a pH value of 4.5 the same increase leads to the reduction in PDI no clear explanation can be found to describe these results.

With regards to zeta potential this value must be as distant as possible from zero which enables the particles to repel each other and therefore from a stable colloid. In the tested conditions there is no clear relationship between the experimental conditions and the resulting zeta potential value.

Table 7.1. Hydrodynamic particle size (HPS), polydispersity index (PDI) and zeta potential measured by dynamic light scattering (DLS) analysis for the AuNPs obtained with the different experimental conditions

Experimental Conditions			HPS (nm)	PDI	Zeta potential (mV)
Cat	0.5 mM	pH=2.5	98.0±0.5	0.231±0.011	-39.8±0.3
		pH=4.5	66.3±0.3	0.226±0.007	-46.2±1.7
	1.0 mM	pH=2.5	108.7±0.3	0.142±0.003	-38.0±0.8
		pH=4.5	76.1±0.7	0.236±0.010	-36.6±0.1
PAC Sig	0.5 mM	pH=2.5	86.7±0.2	0.164±0.002	-44.9±0.6
		pH=4.5	46.5±0.5	0.494±0.004	-43.6±1.6
	1.0 mM	pH=2.5	74.4±0.4	0.315±0.006	-40.2±0.5
		pH=4.5	33.4±0.3	0.160±0.003	-36.2±3.1
PAC F2	0.5 mM	pH=2.5	115.8±1.1	0.170±0.006	-32.5±0.7
		pH=4.5	42.7±0.9	0.514±0.037	-20.5±0.9
	1.0 mM	pH=2.5	75.6±0.7	0.408±0.006	-38.0±1.1
		pH=4.5	28.6±0.3	0.274±0.002	-46.0±2.7
PAC F3	0.5 mM	pH=2.5	141.2±1.7	0.199±0.008	-19.7±0.3
		pH=4.5	44.1±0.4	0.600±0.009	-30.2±0.3
	1.0 mM	pH=2.5	95.1±1.0	0.244±0.002	-34.8±0.4
		pH=4.5	40.6±0.9	0.436±0.025	-38.3±2.4

Considering that the best results were obtained when using a reducing agent concentration of 1.0 mM, these were selected to be analyzed by TEM so that further structural information can be assessed.

In figure 7.4 it can be observed that when using Cat as reducing agent at a pH of 2.5 the resulting AuNPs present a core-shell morphology with the Au core having different morphologies around 30 to 40 nm and the catechin shell presenting a constant thickness of 20nm regardless of core shape or size which is in line with was observed by Raula et al. [323] and Krishnaswamy et al. [319]. When the pH is changed to 4.5 the resulting AuNPs increase in size to 100 nm and the shell becomes irregular encompassing several NPs. This was not previously described but one could argue that the increase in pH promotes catechin oxidative polymerization [323] leading to the formation of bulk molecular structures that due to steric hindrance form less staple metal complexes resulting in an effective decrease in reducing agent concentration.

The use of **PAC Sig** showed that changing the pH value from 2.5 to 4.5 led to a reduction in NP size, from 30 to 20 nm, in addition to a higher uniformity (figure 7.4) which is corroborated by the DLS analysis (table 7.1). Nevertheless, no shell formation is observed with **PAC Sig** regardless of the pH value. In the case of **PAC F2** the same pH effect as for **PAC Sig** was observed, i.e. a reduction in NP size was observed from 30 to 20 nm, with the addition of having a shell of 5 nm when the pH is 4.5 (figure 7.4). Similarly, with **PAC F3** a reduction in NP is observed, although more pronounced, from 70 to 15/30 nm and a shell formation with 5 nm is also observed (figure 7.4).

Based on the fact that the concentrations of all reducing agents was similar and that when using PAC fractions, a smaller AuNP size was obtained it can postulated that the same mass of PAC fractions has a higher capacity for creating nucleation seeds [312] which may derive from PAC's polymeric character that allows for a better enveloping of the metal ion producing stabler complexes when compared with other phenolic compounds [28]. In addition, the thinner shell that is observed can be attributed to the lower tendency of the oxidized PACs to polymerize, in opposition to what is observed for catechin [323], which is most likely related to steric hindrance.

Moreover, after leaving the colloids undisturbed for four weeks (upper right inset in figure 7.4) is possible to observe the long-term stability that AuNPs synthesized with PAC fractions at a pH value of 4.5 exhibit when compared with the ones obtained with Cat, regardless of pH value. This phenomenon may also be associated with the aforementioned polymerization of oxidized material [323] that as postulated previously is more extensive in Cat than in PAC fractions.

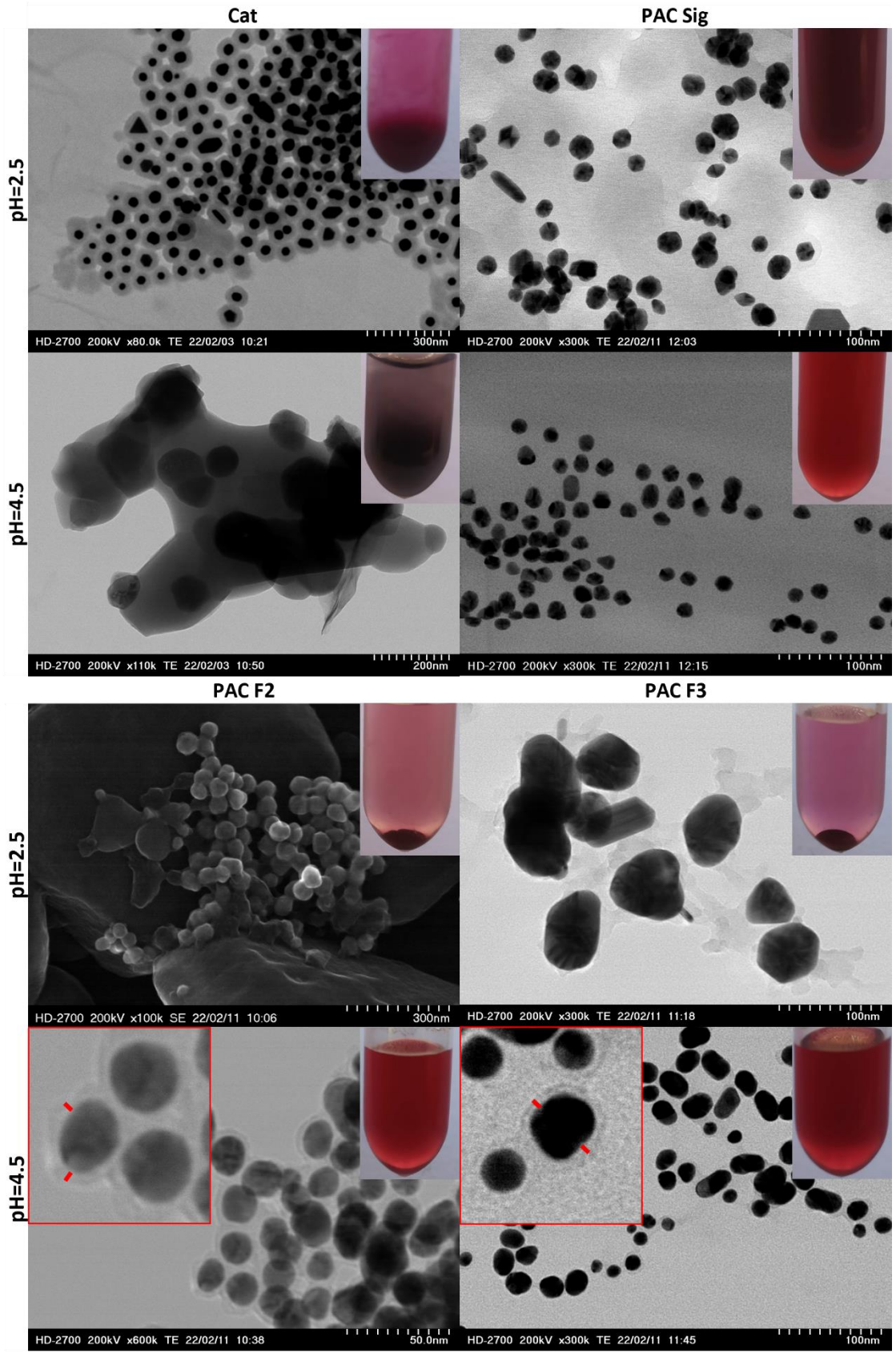


Figure 7.4. TEM micrographs of AuNPs synthesized using catechin or PAC fractions as reducing agents at a concentration of 1mM and pH values of 2.5 and 4.5 (upper right inset images correspond to the respective colloids left undisturbed for four weeks, upper left inset images correspond to detail of corresponding micrograph)

7.4. Conclusion

Similar to what was already described, and was shown here, catechin acts as a reducing and stabilizing agent producing AuNPs in a single-step at room temperature [319,323–325]. In addition, this method leads to a formation of a thick organic shell that might improve biocompatibility of AuNPs and provides hydroxyl phenolic groups that can be involved in the functionalization of AuNPs.

Furthermore, PAC fractions with well-defined mDPs were shown here, for the first time, to work as a reducing and stabilizing agent in the synthesis of AuNPs with the added advantage of producing AuNPs that are more stable than the ones synthesized with catechin. In addition, the use of PACs resulted in considerably smaller AuNPs which might improve its applicability due to the increase of surface area. Considering the lack of research in this field it is expectable that the morphology of the synthesized NPs can be improved by further exploring the use of different types of PACs from other sources and with specific DPs (instead of a range of DPs).

8. Final Remarks and Future Work

PACs could play an important role in the development of greener and more sustainable industrial processes and the improvement in their accessibility by making them cheaper and more abundant is of the utmost importance.

The present thesis has shed light on the improvement of extraction process of PACs from grape pomace, a by-product with great importance for the Portuguese economy, while using a solvent system, ES, that can be sustainably sourced. From the different studies develop herein it was possible to assess that an effective extraction can be achieved with conventional maceration using a mixture of choline chloride, glycerol, ethanol, and water that resulted in extracts in which no thermal degradation was observed. Alternatively, a microwave assisted extraction process can be used, using a solvent composed of choline chloride, lactic acid, and water, from which an increase in extraction yield with a considerably shorter extraction time could be observed associated with PAC depolymerization.

Based on this information, the normalization of extracts from different sources was made using the conventional extraction maceration since no PAC depolymerization was observed. This way, despite a lower extraction yield being observed, a more reliable process was achieved which allows for the obtention of extracts with reproducible and customizable characteristics, a step that might be of extreme importance for its industrial application.

Despite the successful development of an innovative PAC upstream process further work must made in the direction of improving the existing downstream processes, namely, by the application of filtration processes for this process, taking advantage the large difference in molecular weights between PACs and the solvent components. This methodology has the advantage of being easily scalable, energetically efficient and enabling solvent recovery allowing its reutilization.

In terms of PAC applications, in the preliminary results presented here it was shown that PAC antioxidant activity in cells could be improved by combining it with betaine and urea being imperative to move into in vivo assays in order to better understand the true potential of this methodology.

Lastly, the successful synthesis of core-shell gold-PAC nanoparticles achieved here demonstrate the necessity of moving past the use of plant extracts and dedicate efforts in the use of pure naturally occurring molecules such as PACs.

9. References

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