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Revisitando a química do bagaço de maçã – o subproduto da indústria de concentrados de sumo

Revisiting the chemistry of apple pomace – the byproduct of juice concentrates industry

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Revisiting the chemistry of apple pomace – the byproduct of juice concentrates industry

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência e Tecnologia Alimentar e Nutrição, realizada sob a orientação científica da Doutora Susana Maria Almeida Cardoso, Investigadora Doutorada do Departamento de Química da Universidade de Aveiro, do Professor Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação da Universidade de Aveiro e da Professora Doutora Dulcineia Maria de Sousa Ferreira Wessel, Professora Adjunta do Instituto Politécnico de Viseu.

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Aos meus pais, família e amigos.

À Andreia pelo seu incansável apoio.

o júri

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agradecimentos

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palavras-chave

Polifenol, oxidação, políssacarideos pécticos, arabinanas, xiloglucanas, extração, interação, secagem.

resumo

Durante a extração do sumo de maçã é gerado um resíduo insolúvel denominado bagaço de maçã. O bagaço de maçã representa o material da parede celular do fruto, identificado como fonte de polissacarídeos e polifenóis. No entanto, o bagaço de maçã é maioritariamente descartado pelas indústrias. Por um lado, pelo facto de alguns polissacarídeos e compostos fenólicos corresponderem a moléculas de aplicações e estruturas desconhecidas, resultantes de fenómenos de oxidação que ocorrem durante a extração de sumo. Por outro, as indústrias carecem de metodologias de secagem capazes de corresponder às suas necessidades para valorização dos compostos do bagaço de maçã.

A adoção de diferentes metodologias de extração e fracionamento permitem obter uma visão global das propriedades e estruturas dos compostos passíveis de serem recolhidos do bagaço após implementação de um processo de secagem para preservação do subproduto. Com base nesta hipótese, o bagaço de maçã foi submetido a extrações com água, metanol e acetona:água (60:40; v:v), sem ou com ureia, e água superaquecida por micro-ondas com o objetivo de desvendar a existência de outras estruturas fenólicas além daquelas que ocorrem naturalmente no fruto. Adicionalmente, para identificação de carbohidratos quimicamente modificados como consequência da oxidação de polifenóis, foram realizadas extrações com água quente seguidas de fracionamento por diálise (12-14 kDa) e precipitação em etanol.

A extração com água quente, metanol e acetona seguida de análise por cromatografia líquida permitiu identificar a presença de flavan-3-óis, flavonóis, di-hidrocalconas e ácidos hidroxicinâmico, totalizando 5 g/kg de bagaço seco. Também foram detectados produtos de oxidação de di-hidrocalconas e ácidos hidroxicinâmicos no bagaço de maçã. A análise por fusão alcalina do material insolúvel em água demonstrou a ocorrência de procianidinas oxidadas não-extractáveis, cuja prevalência foi 4 vezes superior aos compostos fenólicos nativos da maçã. A extração com água superaquecida por micro-ondas seguida de precipitação em etanol dos extratos obtidos mostrou que estas se encontravam covalentemente ligadas aos polissacarídeos pécticos, xiloglucanas e celulose presentes no material insolúvel em água, podendo corresponder até 40% dos compostos fenólicos possíveis de serem obtidos a partir do bagaço de maçã.

A extração em fase sólida do material solúvel em água quente, usando cartuchos C_{18}, mostrou que, juntamente com os polifenóis, foram extraídos polissacarídeos, alguns dos quais apresentando um comportamento hidrofóbico a pH 7 e/ou a pH 3. O fracionamento por diálise e precipitação em etanol, e a análise das ligações glicosídicas por cromatografia em fase gasosa, ressonância magnética nuclear, cromatografia de exclusão molecular e fusão alcalina, permitiu inferir que os polifenóis ligados covalentemente aos polissacarídeos são responsáveis pela sua hidrofobicidade, constituindo complexos de xiloglucanas-polifenóis-polissacarídeos pécticos e complexos de arabinanas-polifenóis.

Para compreender a formação dos complexos polissacarídeos-polifenóis, estabelecidos por ligações covalentes, foram estudadas as interações de arabinanas com o ácido 5-*O*-cafeoilquinico e floridzina. Através da sua difusão ao longo de membranas de diálise na presença ou ausência de polissacarídeo, verificou-se que as arabinanas lineares apresentaram 10 a 2 vezes maior retenção do ácido 5-*O*-cafeoilquinico e da floridzina do que as arabinanas ramificadas sugerindo que um maior grau de ramificação dos polissacarídeos limita a sua interação com os compostos fenólicos. A mesma tendência foi observada para a interação de arabinanas com procianidinas por calorimetria de titulação isotérmica, onde arabinanas lineares apresentaram maiores constantes de afinidade (K_a 540 M^{-1}) do que arabinanas ramificadas (K_a 391 M^{-1}). Esta abordagem demonstrou também que a ocorrência de polifenóis ligados covalentemente a arabinanas restringe possíveis interações adicionais com os polifenóis (K_a 85 M^{-1}). A análise do material que precipitou após interação entre as arabinanas e as procianidinas mostrou que as procianidinas de maior grau de polimerização e os polissacarídeos menos ramificados são as estruturas que tendem a formar os agregados insolúveis.

Com o objetivo de garantir a estabilidade do bagaço de maçã para posterior extração de compostos, este subproduto foi secado via tecnologia de micro-ondas por hidrodifusão e gravidade (MHG), utilizando potências de 300-900 W. A secagem por MHG mostrou-se um processo composto por três fases: aquecimento, secagem e ponto de queima. Esta metodologia apresentou uma estimativa de 80% de eficiência energética, bem como tempos de secagem (1-2.6 h) e fluxos de água (5,1-13,9 mL/min) até 4 vezes superiores às verificadas por secagem com ar quente (3,6-9,9 h e 1-3,5 mL/min). Pela técnica MHG foi possível obter em simultâneo com a secagem, extratos constituídos por polifenóis e polissacarídeos. Através da adição de etanol durante o processo de secagem, a massa de extrato foi incrementada. O bagaço secado mostrou-se estável por pelo menos 2 anos, após os quais foi possível obter extratos ricos em compostos fenólicos e polissacarídeos.

Em conclusão, a adoção de diferentes mecanismos de extração e de fracionamento permitiu uma maior compreensão das estruturas e propriedades químicas dos compostos presentes no bagaço de maçã, especialmente aquelas derivadas de reações de oxidação dos compostos fenólicos. Isto, em conjunto com a eficiente e rápida secagem verificada pela tecnologia de MHG abre novas perspetivas de investigação e aplicações que podem culminar na possível mitigação do bagaço de maçã como um resíduo agroindustrial.

keywords

Polyphenol, oxidation, pectic polysaccharides, arabinans, xyloglucans, extraction, interaction, drying.

abstract

During the extraction of apple juice an insoluble residue, called apple pomace, is generated. Apple pomace represents the fruit cell wall material, identified as a source of polysaccharides and polyphenols. However, apple pomace is mostly discarded by the industries. In the one hand, this occurs because some polysaccharides and polyphenols still correspond to molecules of unknown applications and structures resulting from oxidation phenomena that occur during juice extraction. On the other hand, the industries lack drying methodologies capable of meeting their needs for valorization of apple pomace compounds.

The adoption of different extraction and fractionation methodologies gives an overview of the properties and structures of the compounds that can be available from apple pomace after the implementation of a drying process to preserve the byproduct. Based on this hypothesis, apple pomace was subjected to extractions with water, methanol and acetone:water (60:40; v:v), without or with urea, and microwave superheated water extractions to unravel the existence of phenolic structures other than those naturally occurring in the fresh fruit. Additionally, for identification of chemically modified carbohydrates due to polyphenol oxidation, hot water extractions were performed followed by dialysis (12-14 kDa) and ethanol precipitation.

Extraction with hot water, methanol and acetone followed by analysis by liquid chromatography allowed to observe the presence of flavan-3-ols, flavonols, dihydrochalcones and hydroxycinamic acids, totaling 5 g/kg of dry apple pomace. Oxidation products of dihydrochalcones and hydroxycinnamic acids were also detected in apple pomace. Alkaline fusion analysis of water-insoluble material demonstrated the occurrence of non-extractable oxidized procyanidins, whose prevalence was 4-fold higher than in native apple polyphenols. Extraction with microwave superheated water followed by ethanol precipitation of the obtained extracts showed that procyanidins were covalently linked to the pectic polysaccharides, xyloglucans and cellulose present in the water-insoluble material and could account for up to 40% of the polyphenols that can be obtained from apple pomace.

Solid phase extraction of the hot water-soluble material using C_{18} cartridges showed that, along with polyphenols, polysaccharides were also extracted, some of them exhibiting a hydrophobic behavior at pH 7 and/or pH 3. Dialysis followed by ethanol precipitation, alongside with glycosidic linkages analysis by gas chromatography, nuclear magnetic ressonance, size-exclusion chromatography, and alkaline fusion, led to the conclusion that polyphenols covalently linked to polysaccharides are responsible for their hydrophobicity, constituting xyloglucan-polyphenols-pectic polysaccharide and arabinan-polyphenol complexes.

To understand the formation of polysaccharide-polyphenol complexes established by covalent bonds, the interactions of arabinans with 5-*O*-caffeoylquinic acid and phloridzin were studied. Through their diffusion along dialysis membranes in the presence or absence of polysaccharide, linear arabinans showed 10-fold and 2-fold greater retention of 5-*O*-caffeoylquinic acid and phloridzin than branched arabinans suggesting that a higher degree of branching limits polysaccharides interaction with polyphenols. The same trend was observed for the interaction of arabinans with procyanidins by isothermal titration calorimetry, where linear arabinans presented higher affinity constants (K_a 540 M^{-1}) than branched arabinans (K_a 391 M^{-1}). This approach also demonstrated that the occurrence of covalently linked polyphenols to arabinans restricts further interactions with polyphenols (K_a 85 M^{-1}). The analysis of the material that precipitated after interaction between arabinans and procyanidins showed that procyanidins of higher degree of polymerization and less branched polysaccharides tended to form insoluble aggregates.

In order to ensure the stability of apple pomace for further extraction of compounds, this byproduct was dried *via* microwave hydrodiffusion and gravity (MHG) technology using delivery powers of 300-900 W. MHG drying proved to be a three phases process: heating, drying and burning point phase. This methodology presented an estimated 80% energy efficiency, as well as drying times (1-2.6 h) and water flows (5.1-13.9 mL/min) up to 4-fold higher than those verified by hot air drying (3.6-9.9 he 1-3.5 mL/min). Using MHG drying, it was also possible to obtain extracts composed by polyphenols and polysaccharides. The addition of ethanol during the drying process can increase the obtained extract mass. The dried apple pomace was stable for at least 2 years, after which was possible to obtain extracts rich in polyphenols and polysaccharides.

In conclusion, the adoption of different extraction and fractionation procedures allowed a better understanding of the structures and chemical properties of the compounds present in apple pomace, especially those derived from oxidation reactions of polyphenols. This, together with the efficient and rapid drying verified for MHG technology, opens new research perspectives and applications that should culminate in the possible mitigation of apple pomace as an agro-industrial residue.

Publications and communications

Along the development of this thesis, several publications in international scientific peer-reviewed journals, oral and poster comunnications in both national and international meetings were performed.

Publications in peer-reviewed journals

- Fernandes, P. A. R., Le Bourvellec, C., Renard, C. M. G.C., Nunes, F. M., Bastos, R., Coelho, E., Wessel, D. F., Coimbra, M. A., Cardoso, S. M. "*Revisiting the chemistry of apple pomace polyphenols*". Food Chemistry, **2019**.

- Fernandes, P. A. R., Silva, A. M. S., Evtuguin, D. V., Nunes, F. M., Wessel, D. F., Cardoso, S. M., Coimbra, M. A. "*The hydrophobic polysaccharides of apple pomace*". Carbohydrate Polymers, **2019**.

- Fernandes, P. A. R., Le Bourvellec, C., Renard, C. M. G.C., Wessel, D. F., Cardoso, S. M., Coimbra, M. A., "*Interaction of arabinan rich pectic polysaccharides with polyphenols*". Carbohydrate Polymers, **2019**..

- Fernandes, P. A. R., Bastos, R., Calvão, J., Coelho, E., Neto, F., Wessel, D. F., Coimbra, M. A., Cardoso, S. M., Passos, C. P. "*Microwave assisted drying of apple pomace*". In preparation.

Oral communications

- Fernandes, P. A. R., Bastos, R., Passos, C. P., Calvão, J., Wessel, D. F., Cardoso, S. M., Coimbra, M. A. "*Microwave Hydrodiffusion and gravity for the extraction of sugars and polyphenols from apple pomace*". Microbiotec15, 10th – 12th December 2015, Évora, Portugal.

- Fernandes, P. A. R., Wessel, D. F., Coimbra, M. A., Cardoso, S. M. "*Subprodutos Agroindustriais – uma nova fonte de ingredientes funcionais*". III Seminário "Alimentos e Manifestações Culturais Tradicionais" e II Simpósio Internacional "Alimentação e Cultura: Tradição e Inovação na Produção e Consumo de Alimentos", 24th – 26th May 2016, Vila Real, Portugal.

- Fernandes, P. A. R., Wessel, D. F., Coimbra, M. A., Cardoso, S. M.; "*Apple pomace arabinan phloridzin binding capacity*", Glupor12 – 12º Encontro dos Glúcidos, 11th – 13th September 2017, Aveiro, Portugal.

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Oral communications

- Fernandes, P. A. R., Le Bourvellec, C., Renard, C. M. G. C., Nunes, F. M., Coimbra, M. A., Wessel, D. F., Cardoso, S. M., "*Deconstructing apple pomace chemistry*", XXIV Encontro Luso-Galego de Química, 21st-23rd November 2018, Porto, Portugal.

- Fernandes, P. A. R., Le Bourvellec, C., Renard, C. M. G. C., Wessel, D. F., Coimbra, M. A., Cardoso, S. M., "*Flavan-3-ols in apple pomace: role of their interactions with arabinans*", XX EuroFoodChem - EFCXX, 17th-19th June 2019, Porto, Portugal.

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Poster communications

- Fernandes, P. A. R., Nunes, F. M., Wessel, D. F., Coimbra, M. A., Cardoso, S. M., "*Apple pomace arabinan – uncovering its apparent hydrophobicity*". 29th International Carbohydrate Symposium, 14th-19th July 2018, Lisboa, Portugal.

Other communications

- Passos, C. P., Calvão, J., Bastos, R., Fernandes, P. A. R., Neto, F., Wessel, D. F., Cardoso, S. M., Coelho, E., Coimbra, M. A. "*Recovery of dried apple pomace after juice preparation: conventional vs microwave hydrodiffusion and gravity drying processes*". Microbiotec15, 10th – 12th December 2015, Évora, Portugal.

- Passos, C. P., Calvão, J., Ferreira, S. S., Jorge, R., Fernandes, P. A. R., Martins, V., Correia, E., Wessel, D. F., Cardoso, S. M., Nunes, C., Coimbra, M. A. "*Microwave Hydrodiffusion and gravity: an emergent technology for green extraction of non-volatile compounds*". Microbiotec15, 10th – 12th December 2015, Évora, Portugal.

- Fernandes, P. A. R., Ferreira, S. S., Cruz, M. T., Coimbra, M. A., Wessel, D. F., Cardoso, S. M.; "*Apple pomace aqueous extract as a food ingredient*"; 11th World Congress on Polyphenols Applications, 29th of June – 1st July 2016, Porto, Portugal,

- Fernandes, P. A. R., Ramoa, H., Elvas, B., Pinto, A., Gonçalves, F., Coimbra, M. A., Wessel, D. F., Cardoso, S. M.; "*Composição química e características microbiológicas de bagaço de maçã proveniente da indústria agroalimentar*", XIII Encontro de Química dos Alimentos, 14th – 16th September 2016, Porto, Portugal.

- Ferreira, S. S., Fernandes, P. A. R., Wessel, D. F., Cardoso, S. M., Coimbra, M. A. "*O perfil de compostos antioxidantes de subproductos das agroindústrias*". II Congresso Nacional de Escolas Superiores Agrárias, 16th – 18th November 2017, Elvas, Portugal.

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Abbreviation List

60% Ac – 60% acetone
60% AcU – 60% acetone 8 M urea
ABD – Average branching degree
ABTS – 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AG -II – Type II arabinogalactan
AG-I – Type I arabinogalactan
Ara – Arabinose
Asn – Asparagine
Asp – Aspartic acid
Cys – Cysteine
DAD – Diode array detector
DHA – 3-deoxy-lyxo-2-heptulosaric acid
DMSO – Dimethyl sulphoxide
Et50Pp – 50% ethanol precipitate
Et80Pp – 80% ethanol precipitate
Et80Sn – 80% ethanol supernatant
Fru – Fructose
GAE – Gallic acid equivalents
Gal – Galactose
GalA – Galacturonic acid
GC-FID – Gaseous chromatography coupled to a flame ionization detector
GC-MS – Gaseous chromatography coupled to a mass spectrometer
Glc – Glucose
HG – Homogalacturonan
Hyp – 4-L-hydroxyproline
ITC- Isothermal titration calorimetry
K_a – Association constant
KDO – 3-deoxy-manno-2-octulosonic acid
MeOH – Methanol
MHG – Microwave hydrodiffusion and gravity

Mn – Number-average molecular weight
MS – Mass spectrometry
Mw – Weighted average molecular weight
MWE – Microwave superheated water extraction
n – Molarity
NMR – Nuclear magnetic resonance
NRC – Normalized retention coefficient
PCA – Procyanidin fraction
PD – Polydispersity
PDO – Protected designation of origin
PG – Polygalacturonase
PGI – Protected geographical indication
PLZE – Phloridzin equivalents
PMAA – Partially methylated alditol acetate
PME – Pectin methylesterase
PPO – Polyphenol oxidase
RC – Retention Coefficient
RG-I – Type I rhamnogalacturonan
RG-II – Type II rhamnogalacturonan
Rha – Rhamnose
RV-UHPLC – Reverse phase ultra-high-pressure liquid chromatography
SB Ara – Sugar beet arabinan
SB dAra – Sugar beet debranched arabinan
SEC – Size exclusion chromatography
Ser – Serine
TDC – Thermal conductivity detector
TE – Trolox equivalents
Thr – Threonine
WPp -Water precipitate at 4 ºC
XGA – Xylogalacturonan
Xyl – Xylose
ΔBD – Branching degree variation

ΔDP – Degree of polymerization variation

ΔG – Gibbs energy variation

ΔH – Enthalpy variation

ΔS – Enthropy variation

Motivation

1. Apple fruit and its economic relevance

Malus domestica, the apple tree, is one of the oldest and most economically relevant plants of *Rosaceae*. For thousands of years, apple trees were grown in Europe and Central Asia and disseminated all over the world after the 16th century. As a result, today more than 7,500 cultivars are available worldwide, each one producing apples with distinct characteristics **(Janick, Cummins, Brown, & Hemmat, 1996)**.

Apple is a pome (**Figure 1**), i.e. a pseudo fruit as it does not derive from the ovary, but instead from the adjacent tissues' exterior to the carpel. Other examples of this kind of fruits include pear, quince and medlar. The average apple size and weight is 6.5 cm of diameter and 200 g, although variations may exist according to apple variety, edaphoclimatic conditions and, most of all, the tree fruit load **(Janick et al., 1996)**. Apples may present flat, obtlate or oblong shapes with colors ranging from green to pale cream or yellow, depending on the content and balance between chlorophyll and carotenoid pigments in the exocarp (skin) **(Delgado-Pelayo, Gallardo-Guerrero, & Hornero-Méndez, 2014)**. If anthocyanins are present, the fruit may also acquire different shades of red. In contrast, the flesh is generally white or pale yellow due to presence, or even absence, of very low content of pigments **(Janick et al., 1996)**.

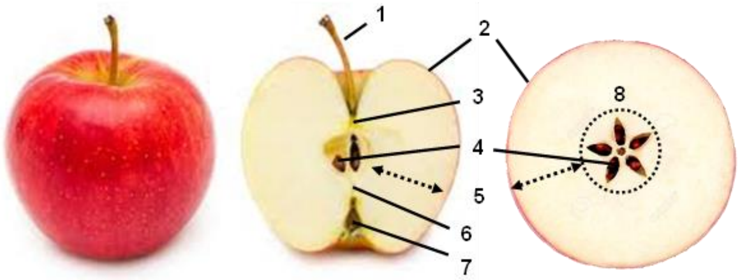


Figure 1 – Apple fruit general morphology: 1 – peduncle; 2- exocarp (skin); 3-endocarp; 4-seeds; 5- mesocarp (parenchyma); 6-style; 7-stamen and sepals; 8 – outer limit of 5 carpels (core).

According to Food and Agriculture Organization, just in 2017 more than 83 million tons of apples were produced worldwide **(FAOSTAT, 2019)**. Of these, 300,000 were produced in Portugal, mainly in the regions of Ribatejo and Oeste (40%), Trás-os-Montes (30%) and in Beira Litoral and Beira Interior, where this activity is part of the cultural and economic heritage. In fact, in addition to Golden Delicious, Royal Gala, Red

Delicious/Starking, Jonagold, Jonagored and Reineta cropped worldwide (**Table 1**), these regions present also characteristic and unique apple varieties such as Bravo de Esmolfe, presently classified as protected designation of origin (PDO), and apple of Beira Alta, Cova da Beira, Alcobaça and Portalegre, which are classified as protected geographical indication (PGI) **(GPP, 2007)**.

Table 1 – Relative prevalence of apple varieties in the Portuguese producing regions. Data adapted from (GPP, 2007).

Region	**Apple variety (%)**					
	Golden Delicious	Red delicious/ Starking	Gala	Bravo de Esmolfe	Reineta	Others
Trás-os-Montes	54	34	5	3	2	2
Beira Litoral	48	22	17	4	2	7
Beira Interior	40	57	0	3	0	0
Ribatejo e Oeste	15	1	48	0	14	22

The high levels of production and available varieties are a result from the huge popularity of apple. This is because apple is perceived as a healthy food, as demonstrated by the very popular aphorism “An apple a day keeps the doctor away”. In fact, apple present polyphenols (**Table 2**) **(Sanoner, Guyot, Marnet, Molle, & Drilleau, 1999)** but carbohydrates are the major components of these fruits. These include simple sugar molecules like fructose (Fru), glucose (Glc) and sucrose, to more complex carbohydrates like pectins, hemicelluloses and cellulose **(Feliciano et al., 2010)**. Minerals as K, Ca, Mg and Na, as well as trace elements such as Mn, Zn, B, F, Cu, Fe and Mo are also found in the fruit. All these components have been related, at some extent, to the health benefits attributed to apple consumption including in the prevention of cardiovascular diseases and various cancers **(Hyson, 2011)**. The convenience and durability of this fruit, for up to a full year, also contributes to apple popularity **(Janick et al., 1996)**.

Table 2 – Chemical composition of apple **(Feliciano et al., 2010; Sanoner et al., 1999)** and of apple pomace **(Bhushan, Kalia, Sharma, Singh, & Ahuja, 2008; Kennedy et al., 1999)**.

Parameter/100 g	Apple	Apple pomace
Water (g)	78-84	79-81
Lipids (g)	tr	0.2-0.8
Total sugars (g)	10-13	10-12
***Fru* (g)**	4-6	3-6
***Glc* (g)**	1-2	1-2
***Sucrose* (g)**	1-3	0.6-1
***Fiber* (g)**	1-4	1-10
Protein (g)	0.01-0.09	0.6-1
Polyphenols (g)	0.1-0.7	0.1-0.2
Minerals		
K (mg)	70-115	90-170
Ca (mg)	1-4	12-20
Mg (mg)	3-4	4-72
Na (mg)	1-2	4-6
Mn (µg)	20-50	20-40
Zn (µg)	20-30	40-160
Cu (µg)	30-150	40-240
Fe (µg)	120-180	800-1600

2. Apple processing

Approximately 37% of the harvested apples are processed into juice or cider (19%), canned apples (14%), dried fruits (3%) or vinegar and jellies (1%). The process of apple juice extraction initiates with the selection of the fruit (**Figure 2**). In fact, different apple varieties have distinct profiles of aroma compounds, acids, sugars, polysaccharides and polyphenols, thus affecting the sensorial properties of the juice **(Downing, 2012)**. Furthermore, the ripening stage is a relevant issue as immature fruits, for instance, are more astringent, have less aroma than mature apples, and contain higher levels of starch, which hardens the clarification of apple juice. On the other hand, overripe fruits lead to low juice extraction yields and to more difficult processing **(Downing, 2012)**. Golden Delicious and Royal Gala are the preferred apple varieties for juice production **(Downing, 2012; Root & Barret, 2004)**.

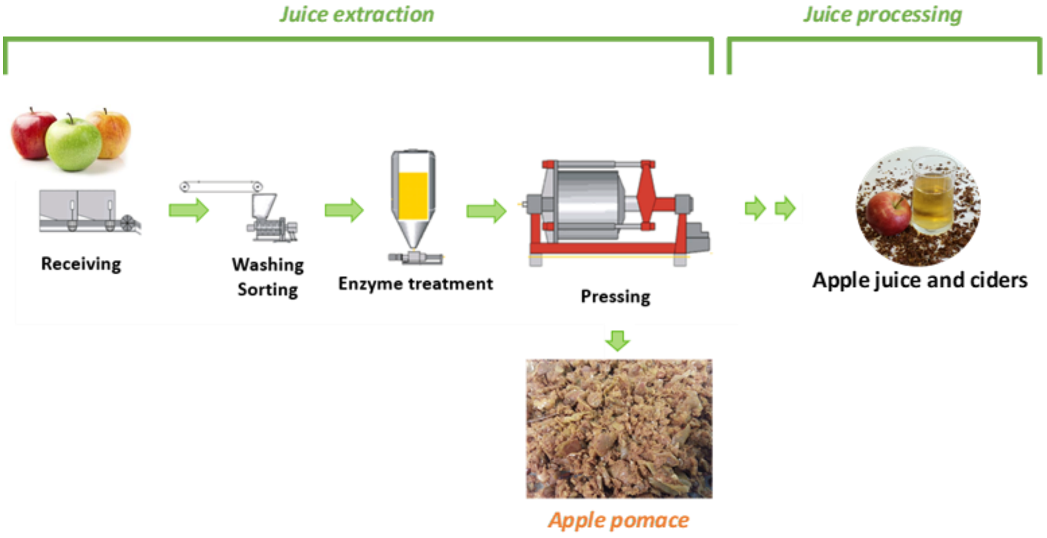


Figure 2 – Overall schematic representation of apple juice extraction as divided in two distinct phases: juice extraction which yields the solid residue called apple pomace and juice processing which includes different processes to yield a juice for consumption or cider production.

After fruit selection, a washing and sorting step is performed to remove any foreign materials or degraded apples, protecting the flavor, but also ensuring the absence of patulin in the juice **(Root & Barret, 2004)**. Afterwards, apples are milled into smaller particles and then subjected to a pressing procedure for juice extraction. This process can be applied in batch or continuous, but for small companies, the first is more common by using vertical hydraulic presses **(Rabetafika, Bchir, Blecker, & Richel, 2014)**. Due to pressing, pectic degrading enzymes naturally present in apple are also released. These include pectin methylesterase (PME), polygalacturonase (PG), pectinlyase, and rhamnogalacturonacetylesterase **(Downing, 2012; Philippe, Pelloux, & Rayon, 2017).** PME deesterifies the galacturonic acid residues of pectic polysaccharides. As a result, pectic polysaccharides become more susceptible to hydrolysis by PG, inducing cell disruption and reducing their water binding capacity which causes the leak of the juice **(Alkorta, Garbisu, Llama, & Serra, 1998; Kashyap, Vohra, Chopra, & Tewari, 2001)**. Enzymes also play a key role on the transfer of apple polyphenols, contributing to the color of the juice **(Bump, 1989)**. Commercial enzymatic systems are also available for this purpose allowing to perform an enzymatic digestion even before the pressing procedure. After this step, the juice is processed in different ways, depending on the aimed final product. This can be a clarified apple juice, natural apple juice, pulpy apple juice, apple juice concentrate or apple juice blends **(Downing, 2012)**.

After juice extraction, the obtained insoluble residue may be washed with cold or warm water, to recover soluble solids that are then reintroduced in the juice production system **(Downing, 2012)**. Alternatively, a secondary enzymatic treatment with the aid of cellulases, for a complete liquefaction of the fruit, can be performed to recover additional juice, characterized by having a lower commercial value than the crude one.

3. Apple pomace

Apple pomace represents the heterogeneous insoluble material remaining after apple juice extraction, corresponding to about 25% of the fruit. It comprehends the pulp, skin, seeds and stalks of the fruit **(Bhushan et al., 2008; Kennedy et al., 1999)**, although its specific composition depends on the variety and stage of maturity of the apples being processed, and mostly, on the extent of processing. Excessive enzymatic digestion affects the amount of pectin remaining in the pomace while water washing step decreases the free sugars and soluble solids **(Kennedy et al., 1999; Root & Barret, 2004)**.

Water is the major component of apple pomace, accounting for 800 g/kg (**Table 2**). This, alongside with the organic compounds, especially carbohydrates, turns apple pomace easily fermentable and a cause of environmental pollution. Although rich in dietary fiber and minerals, a highly relevant trait for animal feed and compost, only a small fraction is valued. One important/relevant factor is that its transportation to far distances is financially unviable due to the pomace water content **(Kennedy et al., 1999; Rabetafika et al., 2014)**. As a result, most of the apple processing industries direct this byproduct to wastelands, but still face high costs associated with the pomace disposal. In this context, over the last 20 years, apple processing industries and food chemists have been trying different approaches for apple pomace valuation (**Figure 3**), ranging from the use for recovery **(Perussello, Zhang, Marzocchella, & Tiwari, 2017)** or production **(Vendruscolo, Albuquerque, Streit, Esposito, & Ninow, 2008)** of high added value products and test of different drying processes for its use as animal feed **(Joshi & Sandhu, 1996).**

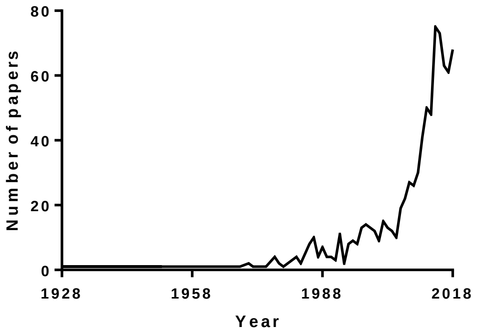


Figure 3 – Number of published papers published over the years focused on apple pomace. Information retrieved from Scopus using “apple pomace” as key word.

3.1 Valuation strategies

One of the most studied approaches include the valuation of apple pomace as a substrate for the microbial production of acids (e.g citric acid), aroma compounds, enzymes (e.g. pectinases) and ethanol **(Vendruscolo et al., 2008)**. However, this approach requires a huge initial investment that most apple processing industries are unavailable to perform. Furthermore, the production yields are low and use only a fraction of the whole pomace, impairing the economic feasibility of this approach **(Kennedy et al., 1999)**.

Pectic polysaccharides are probably the most relevant compounds in apple pomace, given its prevalence in apple pomace and those most easily valued. The recovery of pectic polysacchaarides is in fact already performed at industrial level, usually using diluted acids, followed by concentration and precipitation **(Perussello et al., 2017)**. To improve the extraction yields of pectic polysaccharides, several technologies have been considered including microwave-assisted extraction **(Wang et al., 2007)** and superheated water extraction **(Wang, Chen, & Lü, 2014)**. Alongside with pectic polysaccharides, secondary metabolites such as polyphenols are recovered from the pomace, efficiently separated from each other by using food-grade absorber resins **(Schieber et al., 2003)**. Alternative approaches including microwave-assisted extraction **(Bai, Yue, Yuan, & Zhang, 2010)**, ultrasound-assisted extraction **(Pingret, Fabiano-Tixier, Bourvellec, Renard, & Chemat, 2012)** and supercritical fluid extraction **(Ferrentino, Morozova, Mosibo, Ramezani, & Scampicchio, 2018)** or enzymatic assisted extraction **(Oszmiański, Wojdyło, & Kolniak, 2011)** have been shown to improve the polyphenol extraction yields when compared to conventional hot-water extractions. This is attributed to the capability of these approaches to disrupt hydrogen bonds, increase the penetration of the solvent in the matrix, increase the pressure thus inducing cell wall disintegration followed by cell rupture, and change of the water dielectric constant towards that of some organic solvents **(Perussello et al., 2017)**. Nevertheless, these strategies still yield a residue that is often discharged, even though pectic polysaccharides and polyphenols are still relevant components **(Le Bourvellec, Guyot, & Renard, 2009; Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013)**. This has been attributed to polyphenol oxidation reactions which yield new phenolics with unknown structures and properties, differently from those initially present in the fruit **(Guyot, Bernillon, Poupard, & Renard Catherine, 2009)**. Furthermore, these reactions have also been linked to modulate polysaccharide properties and extractability **(Le Bourvellec et al., 2009)** and due to that, they are generally perceived as a negative feature in apple and its subsidiary products **(Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994)**.

3.2 Preservation

Storage at sub-zero temperatures is one of the possible approaches to prevent apple pomace spoilage, but it demands high energy inputs as well as storage space **(James & James, 2010)**. To overcome these limitations, drying has been suggested as an alternative as it yields a low moisture content pomace, which prevents its spoilage, besides a significant volume reduction for storage and transportation **(Vadivambal & Jayas, 2007)**. Hot-air drying is the most commonly applied drying technology for apple pomace, given its low capital and operational costs **(Motevali, Minaei, & Khoshtagaza, 2011; Wang et al., 2007)**. However, as it requires air circulation, control of humidity, high temperatures, and long drying times, the cost/benefit ratio for apple processing industries is still high, leading most industries to dispose their pomaces **(Périno-Issartier, Zill, Abert-Vian, & Chemat, 2011).**

Microwave hydrodiffusion and gravity (MHG) is a novel technology designed by **Vian, Fernandez, Visinoni, and Chemat (2008),** perceived as an "upside down" alembic (**Figure 4 a**) that combines microwave heating and gravity at atmospheric pressure. The principle of MHG is based on the heating of the internal water within the plant material, thus resulting in the destruction of plant cell. This results in the release of the internal water of the cell alongside with other cellular compounds, defined as the hydrodiffusion phenomenon. In this way, essential oils **(Jacotet-Navarro et al., 2016; Santos et al., 2019),** polyphenols **(Virot, Tomao, Le Bourvellec, Renard, & Chemat, 2010; Zill, Abert Vian, Maingonnat, & Chemat, 2009)**, glucosinolates **(Ferreira, Passos, Cardoso, Wessel, & Coimbra, 2018)** and polysaccharides **(Boukroufa, Boutekedjiret, Petigny, Rakotomanomana, & Chemat, 2015; Ferreira et al., 2018)** can be recovered in the water that drops out of the microwave reactor by gravity while the remnant material is dried inside the vessel.

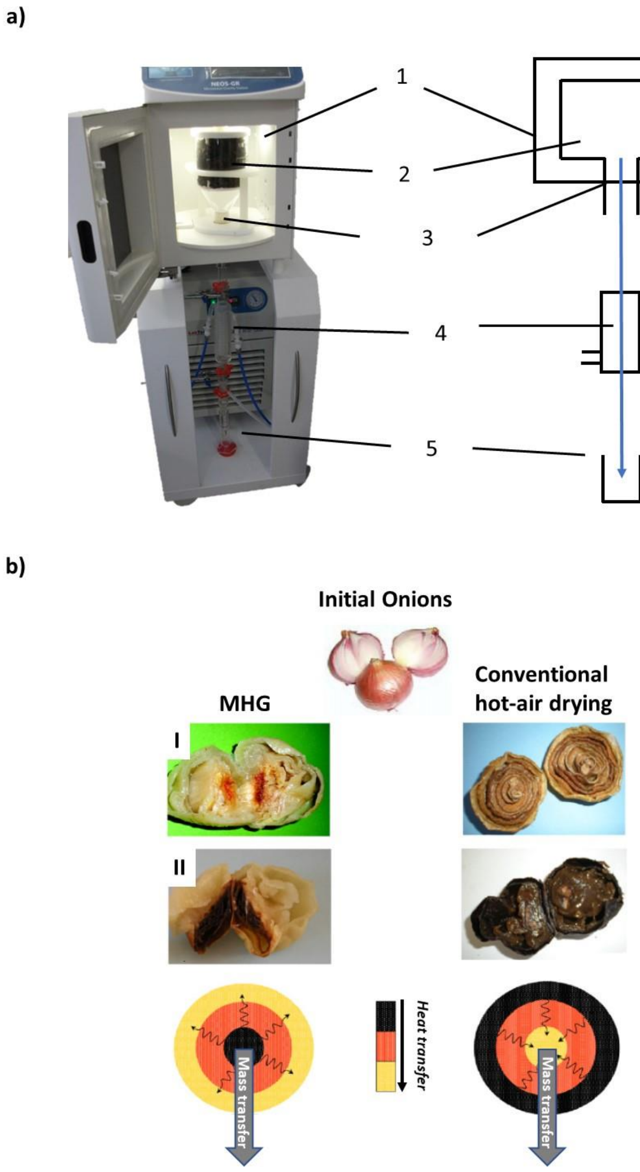


Figure 4 – Representation of a) NEOS-GR apparatus from the Microwave Hydrodiffusion and Gravity (MHG) technology: 1) Microwave vessel, 2) Reactor, 3) Porous disk, 4) condenser, 5) Collector. In b) is represented a schematic process of MHG and conventional hot-air drying heating and mass transfer processes based on the empirical data obtained from onion drying. The figures were adapted from **Zill et al. (2009)** and **Périno-Issartier et al. (2011)**.

So far, samples as mushrooms **(Rodríguez-Seoane et al., 2019)** and algae **(López-Hortas, Conde, Falqué, Domínguez, & Torres, 2019)** and by-products from broccoli **(Ferreira et al., 2018)**, orange **(Boukroufa et al., 2015)** and ginger **(Jacotet-Navarro et al., 2016)** were tested using MHG as a drying approach. This whole process takes at most about 2 h, a consistently faster approach than conventional hot-air drying. This results from the fact that in MHG heat water transfer occurs in the same direction: from the inside of the material to the bulk reactor, contrarily to what happens with conventional hot-air drying (**Figure 4 b**). Due to that, a pilot-scale apparatus, called MAC-75, was developed in order to allow the implementation of this technology at industrial level **(Périno, Pierson, Ruiz, Cravotto, & Chemat, 2016)**.

4 Hypothesis

Considering the levels of apple production in Portugal, it is estimated that on average, 60,000 tons of apples are directed for juice production. This means that every year 15,000 tons of apple pomace are disposed by apple processing industries despite of knowing the environmental issues that this action raises and the existence of several options for apple pomace valuation. Two major reasons can be identified for this behavior: 1) oxidation, which occurs during apple processing, is perceived as a negative feature that yields compounds of unknown structure and properties, and 2) the absence of a technology capable to meet the needs of industries with regards the stability of the by-product, the amounts of pomace that are industrially generated and the economic feasibility of the process. However, the adoption of different extraction and fractionation procedures allows to obtain a global vision regarding the properties and structures of the different compounds that constitute apple pomace, especially those derived from oxidation reactions, and that are potentially recoverable after an efficient and economically feasible drying process for pomace preservation. Based on this hypothesis and following a holistic approach that so far, has not been explored, four different objectives are proposed:

1. To isolate and characterize the polyphenols present in apple pomace taking into consideration the chemical changes induced by oxidation. This approach is essential as polyphenols are high value compounds present in apple and are

modified during processing due to polyphenol oxidation reactions thus presenting different properties from the initial ones **(Guyot, Serrand, Le Quéré, Sanoner, & Renard, 2007; Le Guernevé, Sanoner, Drilleau, & Guyot, 2004)**.

2. To isolate and characterize polysaccharides chemically changed by polyphenol covalent bonding. So far, for apple, only indirect evidences of the occurrence of such complexes were gathered being attributed to polyphenol oxidation reactions **(Le Bourvellec et al., 2009).** It is suggested that these features possibly yield polysaccharides with distinct chemical and biological properties from those of native compounds **(Saura-Calixto, 2011)** and which may culminate in innovative approaches for apple pomace valuation.

3. To assess the capability of the polysaccharides involved in the covalent complexes to interact with polyphenols through non-covalent linkages. Such approach is essential to understand the polyphenol covalent bonding in polyphenol oxidation reactions as, very probably, they represent the first step of the covalent bonding mechanism. Furthermore, the establishment of these interactions may explain the retention of polyphenols in apple pomace limiting polyphenol transfer to the juice fraction.

4. To disclose the possibility of using MHG as an alternative and economic feasible drying process in relation to conventional hot-air drying. Drying is in fact the first step required for apple pomace valuation by inhibiting its spoilage and to allow cost reduction related to its storage and transportation.

To achieve this, different strategies were used for polyphenol extraction from apple pomace including hot water extraction, aqueous/methanol and aqueous/acetone solutions. The polyphenols were analyzed by reverse-phase ultra-high-pressure liquid chromatography (RV-UHPLC) coupled to a diode-array detector (DAD) and a mass spectrometer (MS) complemented by analysis through alkali fusion for the determination of the non-extractable ones. For polysaccharides isolation, hot water extractions were performed, followed with fractionation by solid-phase extraction using C_{18} cartridge, dialysis and ethanol precipitation.

Gaseous phase chromatography with a flame ionization detector (GC-FID), gaseous chromatography coupled to a mass spectrometer (GC-MS) and nuclear magnetic resonance (NMR) were used for polysaccharide characterization. For the interaction studies between pectic polysaccharides and polyphenols, pure standards or isolated phenolic fractions were assayed either by their diffusion performance along a dialysis membrane or by the determination of the interaction thermodynamic patterns by isothermal titration calorimetry (ITC). In order to overcome apple pomace perishability and, ultimately, to allow its valuation, the pomace drying was studied by MHG and compared with the conventional hot-air drying with respect to drying kinetics, extracted components and energy requirements upon drying.

Chapter 1- Introduction

1.1 Phenolic compounds

Phenolic compounds, also commonly referred as polyphenols, are secondary metabolites of plants which, although not essential for their survival, have a relevant role in their protection against pathogens, herbivores and radiation. Among other functions, polyphenols have a role in plant reproduction acting as pigments and therefore, attracting pollinator agents. In Human nutrition, polyphenols have also gathered attention, particularly due to their relation in the prevention of several chronic diseases **(Del Rio et al., 2012)**.

Polyphenols are characterized by an aromatic backbone constituted by at least one aromatic ring and a hydroxyl group. These molecules may present distinct molecular weights, ranging from simple monomers to highly polymerized structures. As a result, polyphenols encompass a chemical family composed by several thousands of distinct structures usually divided in non-flavonoids and flavonoids **(Crozier, Jaganath, & Clifford, 2009; Crozier et al., 2007)**. Both these two categories can be found in apple (**Figure 1.1**): non-flavonoids are comprised by chlorogenic acids while flavonoids are represented by monomeric flavan-3-ols, polymeric flavan-3-ols, flavonols, dihydrochalcones and anthocyanins.

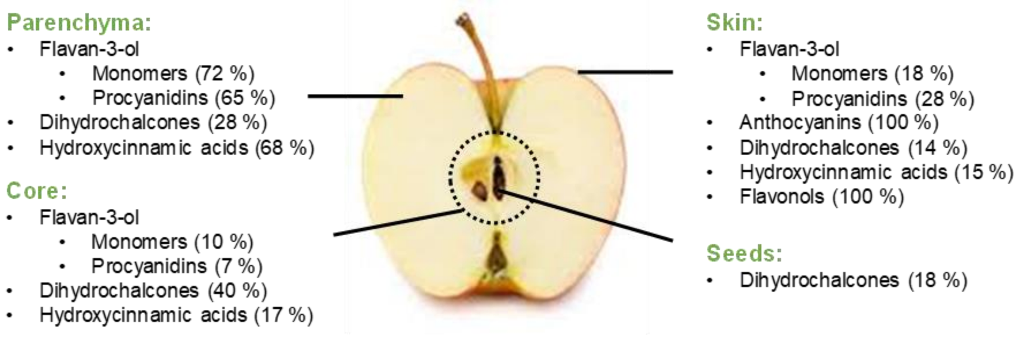
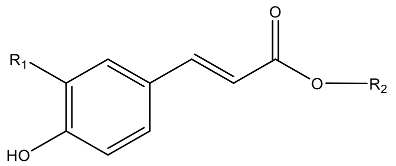


Figure 1.1 - Relative distribution of polyphenols along the different apple tissues according to **Guyot, Marnet, Laraba, Sanoner, and Drilleau (1998)**.

1.1.1 Chlorogenic acids

Chlorogenic acids are characterized by a phenolic ring linked to a propionic chain establishing a C6-C3 backbone (**Figure 1.2**). Among them, caffeic acid and *para*-coumaric acid, which differ on the hydroxylation pattern of the aromatic ring, are found in apple, mainly as esters of quinic acid **(Clifford, 1999)**.

In apple, hydroxycinnamic acids are mostly found in the parenchyma, although they are also present in the skin and core of the fruit (**Figure 1.1**). The amount of hydroxycinnamic acids found in apple depends on several factors, especially the apple variety. For instance, the parenchyma of cider apples tend to contain higher levels of hydroxycinnamic acid (300-1000 mg/kg fresh weight) than dissert apples (60-150 mg/kg fresh weight) **(Feliciano et al., 2010; Sanoner et al., 1999)**. Of these, 75-95% correspond to esters of caffeic acid (5-*O*-caffeoylquinic acid), and 5-20% to esters of *para*-coumaric acid (4-*O*-*p*-coumaroylquinic acid) **(Amiot, Tacchini, Aubert, & Nicolas, 1992)**. After processing, these compounds can be found in apple pomace at values ranging from 60 to 100 mg/kg fresh weight **(Lu & Foo, 1997; Schieber et al., 2003)**. However, as these compounds are substrates of polyphenol oxidase (PPO) **(Nicolas et al., 1994)**, their value tends to decrease along processing and storage time.



Compound	**R_1**	**R_2**
p-coumaric acid	H	H
4-*O*-*p*-coumaroylquinic acid	H	Quinic acid
Caffeic acid	OH	H
5-*O*-caffeoylquinic acid	OH	Quinic acid

Figure 1.2 - Structure of the hydroxycinnamic acids found in apple.

1.1.2 Flavonoids

Flavonoids are characterized by a fifteen-carbon skeleton (C6-C3-C6) delineated by two benzene rings, A and B, and a heterocyclic bridge that forms a third ring, ring C (**Figure 1.3)**. Within this basic structure, variations occur at the level of hydroxylation, double bounds, keto groups, and B ring position yielding a wide structural diversity. Thus, flavonoids are divided in five major subgroups: flavonols, flavones, isoflavones, flavanones, flavan-3-ol and anthocyanins. Other flavonoids such as chalcones, dihydrochalcones, coumarins, and others also take part of this group, but are less frequently found in Human diet **(Crozier et al., 2009; Crozier et al., 2007; Gonzales et al., 2015)**. In apple, the flavonoids pool includes flavan-3-ols, flavonols, dihydrochalcones and anthocyanins **(Guyot, Le Bourvellec, Marnet, & Drilleau, 2002; Schieber et al., 2003)**.

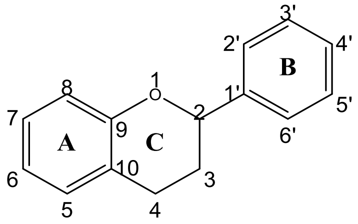
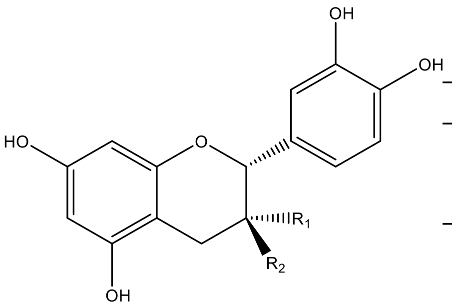


Figure 1.3 - Schematic representation of flavonoids backbone.

1.1.2.1 Flavan-3-ols

Flavan-3-ols represent the major group of polyphenols structurally characterized by two chiral carbons (C2 and C3) and a hydroxyl group at C3 (**Figure 1.4**). The A ring is a phloroglucinol structure, with hydroxyl groups at C5 and C7 and an ether at C9. The B-ring is a catechol structure, hydroxylated at C3' and C4' **(Crozier et al., 2009)**. In apples, only (+)-catechin and (-)-epicatechin are found, being present at relatively higher concentrations in the skins. In apple parenchyma, these compounds account, on average, for 65 to 130 mg/kg fresh weight and from 150 to 660 mg/kg fresh weight in dessert and cider apples respectively. Of these, (-)-epicatechin generally represents 88% **(Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997; Guyot et al., 2002; Pires et al., 2018)**.



Compound	R_1	R_2	Configuration
(+)-catechin	H	OH	2R 3S
(-)-epicatechin	OH	H	2R 3R

Figure 1.4 - Structure of flavan-3-ol monomers found in apple.

Flavan-3-ols can also be found in apples as polymers, structures called proanthocyanidins or procyanidins (**Figure 1.5**). This denomination results from the fact that these polymers, under hot acidic solutions, yield anthocyanidins, which correspond to red pigments structurally like anthocyanins. These polymers are also called condensed tannins and differ from the hydrolysable ones, which in turn are polymers of gallic and/or ellagic acid ester linked to a polyol **(Hemingway, Karchesy, & Branham, 1989)**. The structure of procyanidins is defined by three distinct structural features: 1) the constitutive monomeric units; 2) the type of linkages and 3) the degree of polymerization **(Aron & Kennedy, 2008; Hemingway et al., 1989)**.

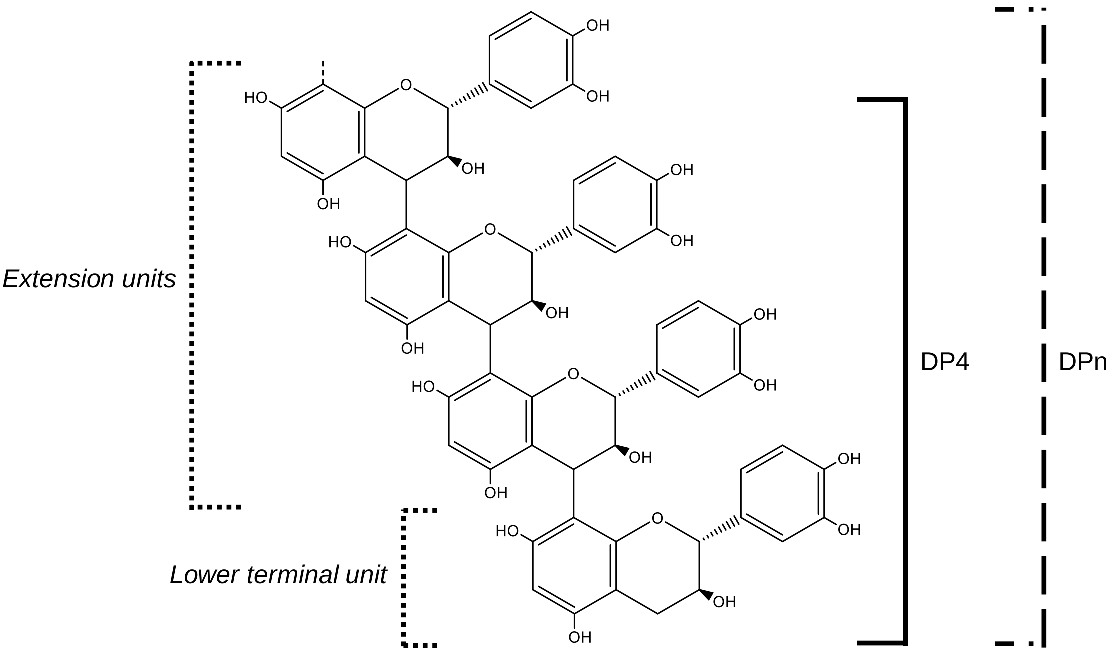


Figure 1.5 – Schematic representation of procyanidins structure. Image adapted from **Guyot et al. (1997).** DP means for degree of polymerization.

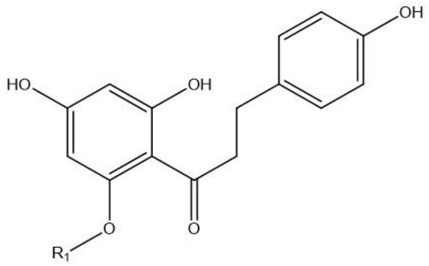
(-)-epicatechin and (+)-catechin represent the monomeric units of the procyanidins found in apple. (-)-epicatechin can be found either as a terminal or extension unit while (+)-catechin is solely found as a terminal unit **(Guyot, Marnet, & Drilleau, 2001)**. The different units of procyanidins can be linked by type B and A linkages. Type B linkages comprise flavan-3-ols linked via C-C, generally between carbons 4 and 8 (C4-C8 linkage) and less frequently in between carbons 4 and 6 (C4-C6 linkage). In contrast, type A linkages present C-O-C (ether linkages) englobing carbons 2 and 7 (C2-O-C7) or carbon 2 and 5 (C2-O-C5), in addition to the C-C linkages **(Aron & Kennedy, 2008; Hemingway et al., 1989)**. This additional ether linkage is thought to be as not being native from apple, but instead to result from type B procyanidin oxidation **(Poupard, Sanoner, Baron, Renard, & Guyot, 2011)**. Furthermore, the formation of interflavanic linkages increase the number of stereoisomers that may occur, as the C4 becomes chiral. In apple, three procyanidins of B type can be found: procyanidins B1 ((-)-epicatechin-(4β→8)-(+)-catechin), B2 ((-)-epicatechin-

(4β→8)-(-)-epicatechin, and B5 ((-)-epicatechin-(4β→6)-(-)-epicatechin **(Lea & Arnold, 1978; Nicolas et al., 1994; Pérez-Ilzarbe, Martínez, Hernández, & Estrella, 1992)**. Moreover, a trimer, procyanidin C1 ((-)-epicatechin residues linked via C4-C8) were identified to occur in apple **(Shoji et al., 2003)**.

By taking advantage of procyanidins depolymerization under hot acidic medium, several methods were developed for the analysis of procyanidins. These methods are based on the cleavage of the interflavanic linkage, resulting in the conversion of the C4 linked residues (extension units) into carbocations, while the terminal residues are released as intact flavan-3-ols monomeric units. The carbocations then react with a nucleophile (benzylthioether for instance), yielding compounds that can then be quantified by RP-HPLC **(Aron & Kennedy, 2008; Guyot et al., 1998)**. This type of approach allowed the estimation of the average degree of polymerization of procyanidins (DP), i.e., the average number or flavan-3-ols units that constitute the procyanidins. The degree of polymerization of procyanidins in apple depends on several factors, including the apple tissue, variety and ripening degree. Studies on Kermerrien cider apple variety showed that procyanidins of higher degree of polymerization are present (DP of 11) in the seeds, followed by the epidermis zone (DP of 6), and parenchyma and core zones (DP of 5) **(Guyot et al., 1998)**. When taking the parenchyma tissues as a reference, differences can also be observed between apple varieties. In dessert apples as Golden delicious or Granny smith, the DP usually range from 4 to 8 **(Guyot et al., 2002; Sanoner et al., 1999)**, but for some cider apples such as Avrolles, a procyanidin might possess more than 50 monomeric units **(Sanoner et al., 1999)**. Along ripening, it is generally perceived that the DP of procyanidins decreases **(Alonso-Salces et al., 2005)**, but for some apple varieties, the opposite might be observed as well **(Renard, Dupont, & Guillermin, 2007)**. Procyanidins, (+)-catechin and (-)-epicatechin, all together may represent on average up to 45-90% of the total polyphenolic pool found in apple **(Guyot et al., 2002; Sanoner et al., 1999)**. However, a low percentage (about 30%) of them are transferred to the juice fraction along apple processing, in comparison with hydroxycinnamic acids (65%) and dihydrochalcones (79%) **(Guyot, Marnet, Sanoner, & Drilleau, 2003; Le Bourvellec, Le Quere, & Renard, 2007; Renard et al., 2011)**. This is attributed to the capability of apple cell wall material to interact with procyanidins, thus limiting their transfer to the juice fraction **(Le Bourvellec et al., 2007)**. In apple pomace huge amounts of procyanidins, in some cases surpassing 600 mg/kg fresh weight, can be found **(Birtic, Régis, Le Bourvellec, & Renard, 2019; Diñeiro García, Valles, & Picinelli Lobo, 2009; Pingret et al., 2012)**.

1.1.2.2 Dihydrochalcones

Dihydrochalcones represent a class of flavonoids in which the C-ring structure is opened (**Figure 1.6**). Therefore, in contrast to other flavonoids the A and B ring of dihydrochalcones are linked through a three-carbon bridge. In apples, phoretin, i.e., the aglycone characterized by an OH group at C5, is frequently present. In addition, phloretin glucosides, namely phloridzin and phloretin-xyloglucoside, occur. Analysis on Karmerien apple variety showed that these compounds are heterogeneously distributed along the apple tissues, being found at high concentration in the seeds (about 3400 mg/kg fresh weight), followed by the core zone (600 mg/kg fresh weigh), epidermis (140 mg/kg fresh weight) and parenchyma (75 mg/kg fresh weight) **(Guyot et al., 1998)**. The global amount in apple fruit is, however, dependent on the apple variety, overall ranging from totaling 40 to 280 mg/kg fresh weight **(Feliciano et al., 2010; Guyot et al., 1998; Vrhovsek, Rigo, Tonon, & Mattivi, 2004).** As dihydrochalcones are also substrates of PPO **(Nicolas et al., 1994)** their content in apple pomace might be affected if oxidation is not prevented. Values around 160-500 mg/kg fresh weight have been described to occur in apple pomace **(Diñeiro García et al., 2009; Pingret et al., 2012; Schieber et al., 2003)**.

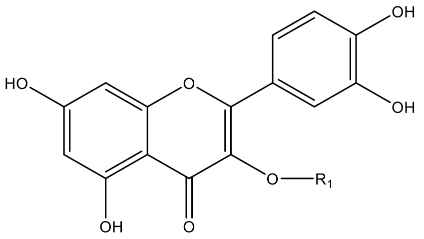


Compound	R_1
Phloretin	H
Phloridzin	Glc*p*
Phloretin-xyloglucoside	Glc*p*-Xyl*p*

Figure 1.6 - Schematic structure of the dihydrochalcones found in apple.

1.1.2.3 Flavonols

Structurally, flavonols are characterized by a double bond between C2 and C3, a OH group at C3 and a carbonyl group at C4. It is generally considered that flavonoids are hydroxylated in positions 2, 3, 5, 7, 3’, 4’ or 5’ and some of them can even be methylated or acetylated, ascribing a more hydrophobic nature to the molecule. However, in apple, flavonols are only represented by quercetin (**Figure 1.7**), either as aglycone or conjugated with sugars at the position C3 **(Crozier et al., 2007)**. The typical sugars found on quercetin glycosylated derivatives comprise Glc, Rha, Gal, Xyl and Ara **(Guyot et al., 2002; Vrhovsek et al., 2004)**.



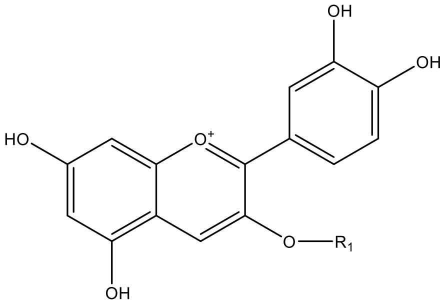
Compound	R_1
Rutin	Glc*p*-Rha*p*
Hyperoside	Gal*p*
Isoquercitrin	Glc*p*
Reinoutrin	Xyl*p*
Guajaverin	Ara*p*
Avicularin	Ara*f*
Quercitrin	Rha*p*
Quercetin	OH

Figure 1.7 – Structure of flavonols found in apple.

In the whole fruit, flavonols content ranges from 35 to 85 g/kg fresh weight **(Vrhovsek et al., 2004)**, being almost exclusively found in apple skin (150-540 mg/kg of fresh weight) **(Guyot et al., 2002; Guyot et al., 1998)**. Due to that, flavonols can be abundantly found in apple pomace (80-300 mg/kg fresh weight) **(Diñeiro García et al., 2009)**.

1.1.2.4 Anthocyanins

Anthocyanins present a C6-C3-C6 backbone characteristic of flavonoids, but with a charged heterocyclic ring (**Figure 1.8**). In addition to hydroxyl groups, anthocyanins may also appear as methylated structures. Glycosylation with Rha, Xyl, Ara, Gal and Glc may be also present at C3 or C5. Phenolic acids may also be found attached to anthocyanins backbone **(Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Santos-Buelga, Mateus, & De Freitas, 2014)**. In plants, anthocyanins may act as pigments proving red, blue, purple and black color, depending on the pH. At acidic pH (<2) anthocyanin contribute to the acquisition of a reddish color as they are majorly found as flavylium cations. As the pH increases to 4, the quinoidal base starts to appear (purple color) and over 5, the carbinol pseudobase (blue) and the chalcone (yellow) form appear causing shift the different colors **(Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Santos-Buelga et al., 2014)**.



Compound	R_1
Cyanidin-3-*O*-galactoside	Gal*p*
Cyanidin-3-*O*-glucoside	Glc*p*
Cyanidin-3-*O*-xyloside	Xyl*p*
Cyanidin-3-*O*-arabinoside	Ara*p*

Figure 1.8 – Structure of anthocyanins found in apple.

In apple, anthocyanins are only found in the skin of red apples being responsible for its red color. At the pH of apple (3.5-4.0), the predominating molecular forms are the colorless hemiketal, followed by the colored flavylium cation and the bluish quinoidal base, this later in much lower concentrations **(Février, Le Quéré, Le Bail, & Guyot, 2017)**. Their content ranges from 17 to 200 mg/kg fresh weight in the skin being mostly represented by cyanidin-3-*O*-galactoside **(Tsao, Yang, Young, & Zhu, 2003)**, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-xyloside **(Février et al., 2017; Mazza & Velioglu, 1992; Vrhovsek et al., 2004)**.

1.2 Polyphenol oxidation products

Oxidation reactions are essential in cell homeostasis being involved in processes such as respiration. In fruits and vegetables, oxidation reactions are, however, perceived as the alteration of color, generally a browning one. This results from the enzymatic oxidation of polyphenols by several enzymes, namely PPO and to a lesser extent, peroxidases. For this reason, these reactions are commonly called as “enzymatic browning”, in opposition to other browning reactions catalyzed by very high temperatures (caramelization and Maillard reactions) **(Guyot et al., 2009)**. Although the pH of apple, around 3.5-4, does not correspond to the optimal pH of this enzyme (4 to 7.5 depending on the type of PPO), the huge amounts of PPO that are present in the apple pomace turn polyphenol oxidation reactions a relevant phenomenon governing the quality of this byproduct **(Nicolas et al., 1994; Rabetafika et al., 2014)**.

1.2.1 The formation of quinones

Enzymatic browning initiates during apple processing, immediately after tissue disruption, i.e., during crushing and pressing. This is because, PPO, initially restrained in plastids, and its substrates, restrained in the vacuole, come together in the presence of oxygen. In the case of apple, 5-*O*-caffeoylquinic acid is the preferential substrate, being converted into the corresponding quinone with the reduction of oxygen to water (**Figure 1.9**) **(Guyot et al., 2009; Nicolas et al., 1994)**.

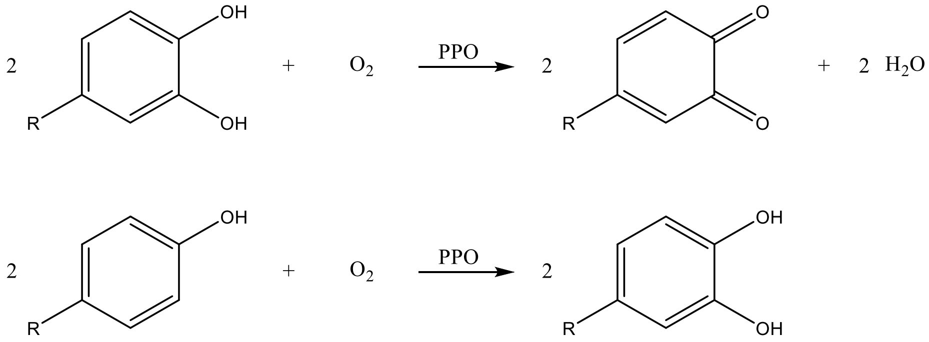


Figure 1.9 – Reactions catalyzed by polyphenol oxidase (PPO): the first represent the oxidation of a *O*-diphenol to the respective quinone and the second corresponds to the conversion of a monophenol to a *O*-diphenol (cresolase activity).

The formed quinones correspond to compounds in which the aromatic ring, instead of presenting the hydroxyl have a ketonic groups. This compound is denominated *O*-quinone and results from the loss of two electrons by diphenolic compounds **(Guyot et al., 2009)**. In addition to 5-*O*-caffeoylquinic acid, PPO can use (+)-catechin and (-)-epicatechin as substrates, but not flavonols neither anthocyanins nor polymeric flavan-3-ols. Phloridzin and phloretin-xyloglucoside may also be substrates of PPO, firstly by the conversion of the monophenol to a *O*-diphenol and then oxidation to the respective quinone **(Nicolas et al., 1994)**.

1.2.2 Quinone reduction

As 5-*O*-caffeoylquinic acid is the main substrate of PPO in apple, its corresponding *O*-quinone is the major driver in the initial stages of polyphenol oxidation reactions. This newly formed *O*-quinone intervenes as an oxidant, reacting with other reducing molecules **(Guyot et al., 2009; Nicolas et al., 1994)**. The reaction with other polyphenolic structures involves a coupled redox reaction (**Figure 1.10**). This means that two electrons of the *O*-diphenol are transferred to the *O*-quinone leading to the reduction of the *O*-quinone to the corresponding *O*-diphenol and the oxidation of the other *O*-diphenol to the corresponding *O*-quinone. This is the proposed mechanisms for the oxidation of apple polyphenols that are not substrate of PPO (flavonols, procyanidins and anthocyanins) to the respective quinones **(Oszmianski & Lee, 1990)**. Nevertheless, in most of the times, instead of being reduced, the *O*-quinone, is incorporated in the structure of the *O*-diphenolic compounds resulting in the formation of different oxidation products **(Amiot, Forget-Richard, & Goupy, 1996; Poupard et al., 2011)**.

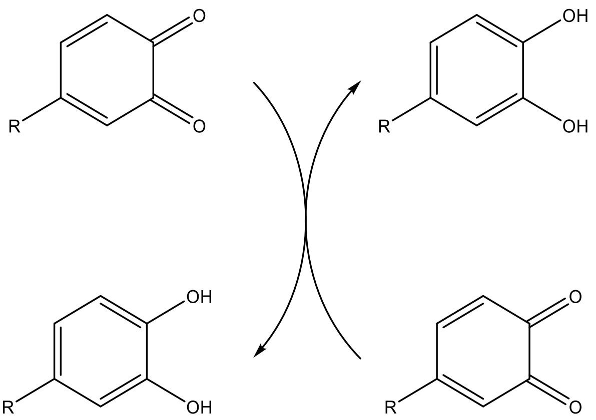


Figure 1.10 – Schematic representation of the principle of coupled oxidation reactions.

Dismutation and inverse disproportionation is a equilibrium where an *O*-diphenol reacts with an *O*-quinone leading to the formation of semiquinones, an intermediary state that instead of two, has one unpaired electron (**Figure 1.11**). This exchange of electrons has been identified to be one of the mechanisms of polyphenol oxidation in apple products **(Tazaki, Taguchi, Hayashida, & Nabeta, 2001)** relevant for flavonols and for monomeric or polymeric flavan-3-ol oxidation **(Guyot et al., 2009; Poupard et al., 2011)**.

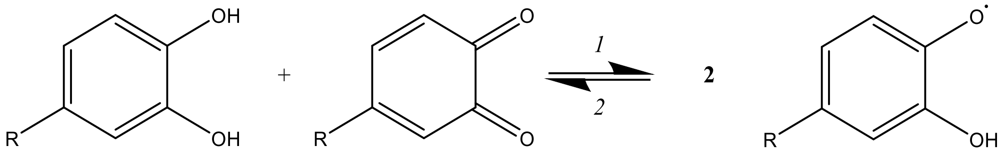


Figure 1.11 – Equilibrium reaction between the *O*-diphenol and *O*-quinone with the semi-quinone. The reaction 1 correspond to a dismutation while reaction 2 corresponds to a reverse disproportionation.

The pK_a of apple native polyphenols is high, generally higher than 8, and therefore they mostly occur in their protonated form in the fruit **(Sauerwald, Schwenk, Polster, & Bengsch, 1998)**. However, the pK_a values of semiquinones are close to the pH of apple. For instance, the pK_a of (+)-catechin semiquinone is 4.6 at 20 ºC **(Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994)**. Therefore, they occur in equilibrium between the protonated and non-protonated forms thus affecting the type of oxidation products formed. Additionally, semiquinones, in their anionic form, might play a role in the generation of novel reactive oxygen species. This process involves its reactions with oxygen yielding the anion superoxide which in turn can react with oxygen producing hydrogen peroxide **(Mochizuki, Yamazaki, Kano, & Ikeda, 2002)**. This means that, at the pH of apple, polyphenol semiquinones may generate reactive oxygen species that further increase the number of oxidation side reactions and products formed. This is because the superoxide radical might react with other phenolic structure starting a new chain of events **(Guyot et al., 2009; Nicolas et al., 1994)**. The formation of hydrogen peroxide might also promote the occurrence of Fenton reactions given the presence of metals in apple (Cu and Fe) **(Simões et al., 2016)**.

1.2.3 Linkages formation

As a result of polyphenol oxidation reactions, new type of linkages between molecules can be formed. Two mechanisms are involved in the formation of these linkages: non-radical mechanisms and radical mechanisms in which *O*-quinones and semiquinones intervene, respectively. Michael reactions are characteristic of the reactions between a ketone or aldehyde and a carbanion or another nucleophile. In the case of polyphenols, the *O*-quinone acts as a ketone and reacts with a nucleophile by a reductive addition, which results in the regeneration of the phenolic ring (**Figure 1.12**). Alternatively, the *O*-quinone might be in equilibrium with a quinone methide causing the nucleophilic to occur on the carbon α of the aromatic ring, allowing its re-aromatization. Another proposed reacting mechanism is the Diels-Alder reaction, which is characterized for involving a diene, having four π electrons and a dienophile with two π electrons.

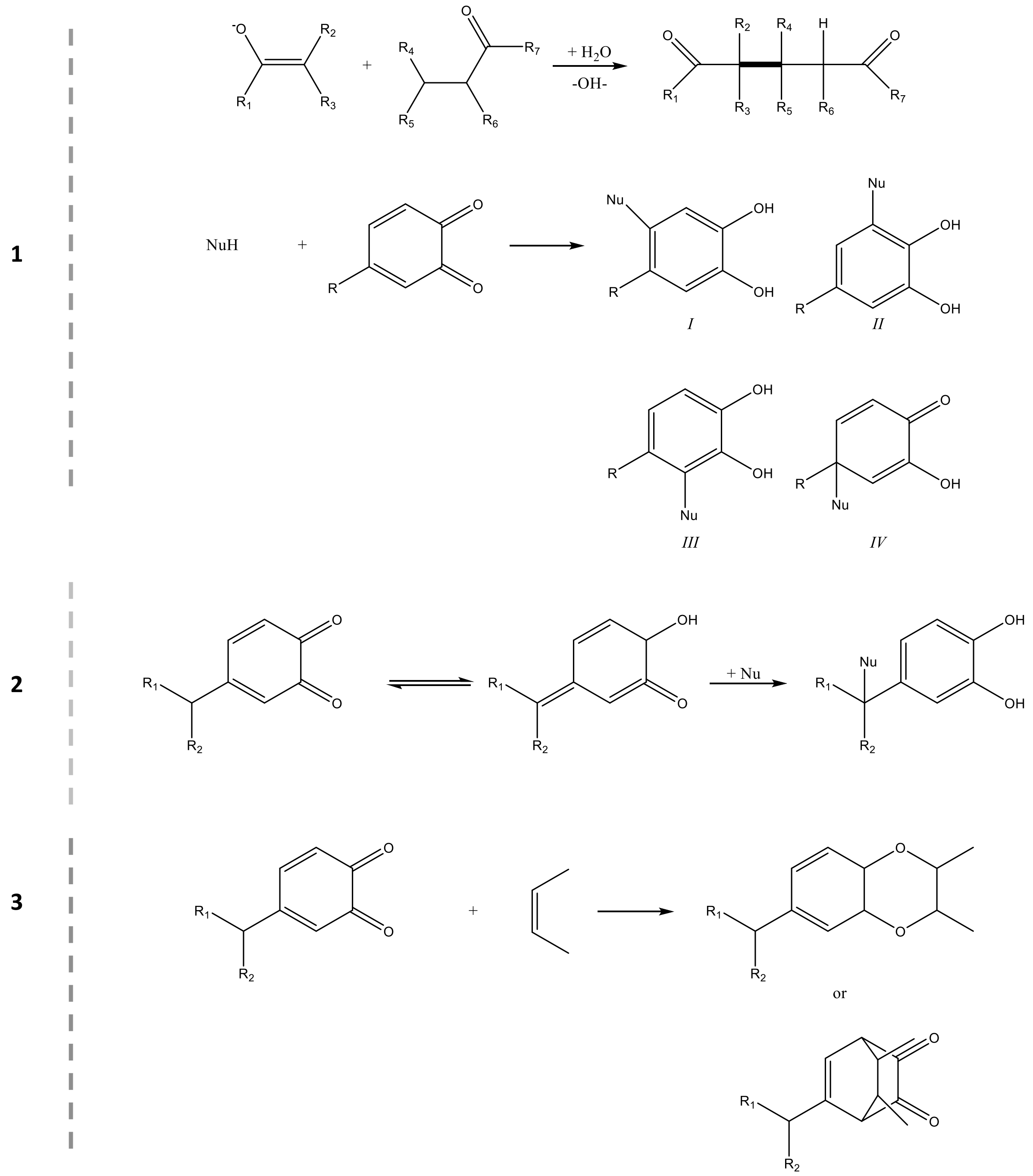


Figure 1.12 – Non-radical mechanisms involved on the formation of linkages in polyphenol oxidation reactions: 1) Michael reactions, 2) nucleophilic addition over quinone methide and 3) Diels-Alder reaction type. Images adapted from **Bernillon (2005)**. Nu means for nucleophile.

In radical mechanisms, mesomerism, i.e., delocalization of the unpaired electron along the molecule, is a relevant aspect. In the case of caffeic acid, the double bound allows the impaired electron to delocalize over the molecule allowing the formation of 10 types of caffeic acid radicals **(Bakalbassis, Nenadis, & Tsimidou, 2003)**. For (+)-catechin semiquinones, the radical is limited to the catechol B ring, interchangeable between the O3’ and O4’ (**Figure 1.13**). However, 4’-phenoxyl is more stable and yet the more easily oxidable **(van Acker et al., 1996)**.

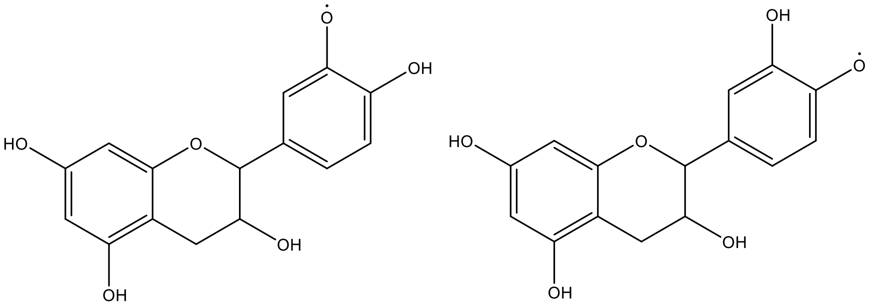


Figure 1.13 – Mesomeric forms of (+)-catechin semiquinone.

At acidic pH, radical addition reaction is prone to occur. Therefore, this type of reaction most probably correspond to one of the mechanisms occurring in apple polyphenol oxidation. Three distinct mechanisms are expected to occur (**Figure 1.14**): I) direct radical coupling between two unpaired electrons, yielding a covalent linkage; II) addition of the radical towards a neutral molecule followed by the loss of an electron and proton, generating a stable dimer; and III) reaction between the radical and a phenoxy cation. Studies on the formation of a dimer of coniferyl alcohol in lignin suggests however that the first two are the most probable mechanisms involved. From all these reactions, it is expected the formation of aryl, diaryl-ether, peroxide, alkyl, aryl-ether and alkyl-aryl linkages **(Bernillon, 2005)**.

1.2.4 Oxidation products

Currently very little is known regarding the structure of the compounds that are formed during polyphenol oxidation. However, it is known that the products formed affect the final quality of the product, including color, turbidity, bitterness and astringency **(Février et al., 2017; Millet, Poupard, Guilois-Dubois, Zanchi, & Guyot, 2019)**. Furthermore, if the newly formed compounds preserve the phenolic structure, they would even also contribute to the antioxidant activity of the product **(Wong-Paz, Muñiz-Márquez, Aguilar, Sotin, & Guyot, 2015)**. In this section, polyphenol oxidation products will be discussed, with emphasis on those already reported to occur in apple derived products.

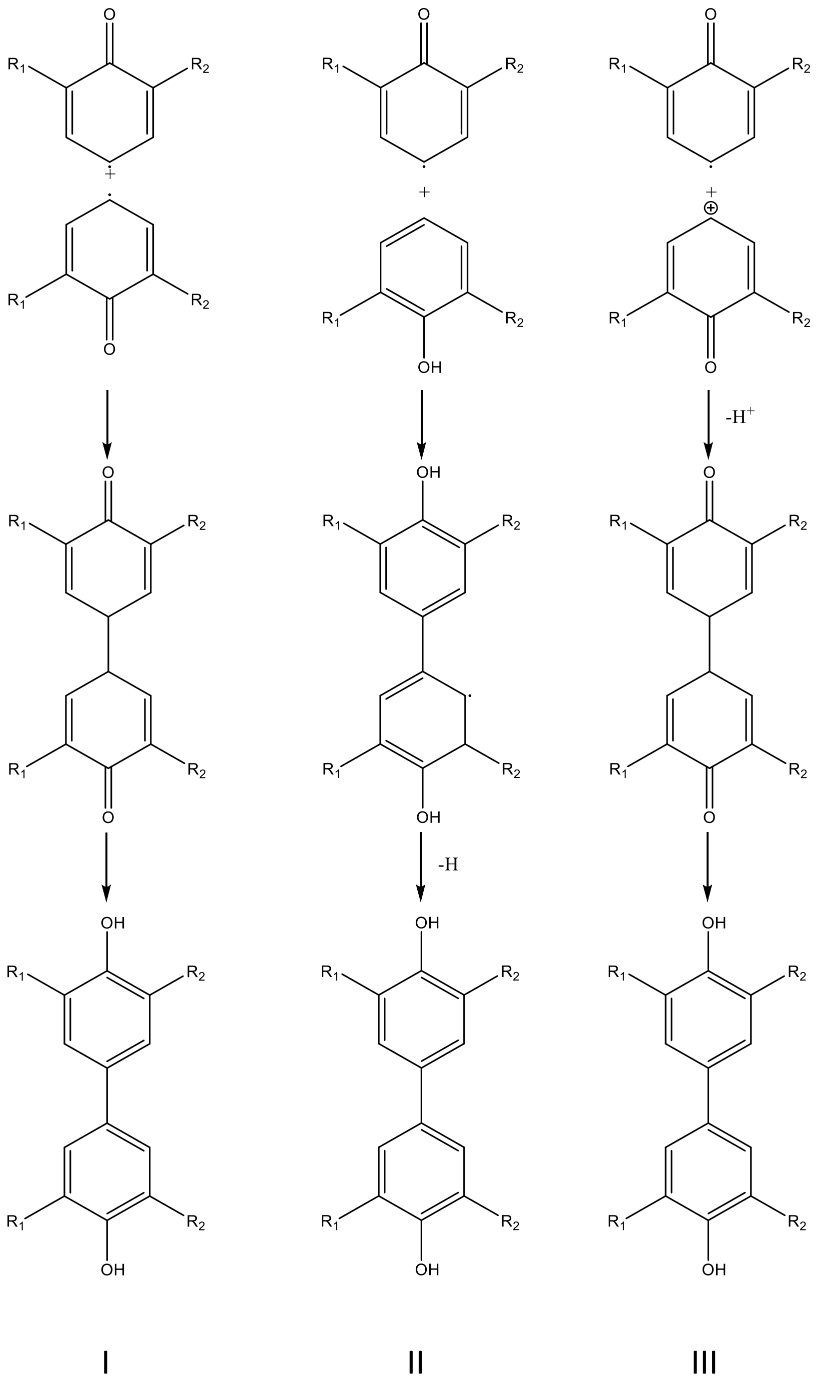


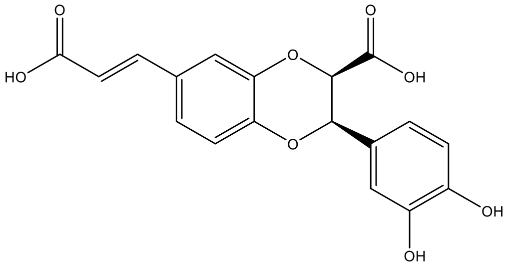
Figure 1.14 – Radical mechanisms involved in the formation of dimers. Adapted from **Bernillon (2005)**.

1.2.4.1 Hydroxycinnamic acid oxidation products

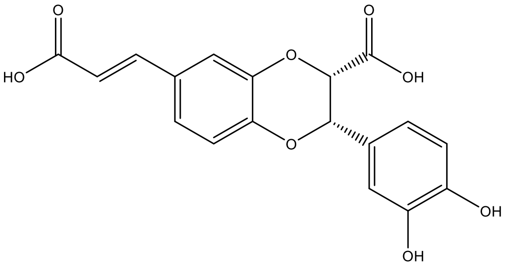
The formation of hydroxycinnamic acid oxidation products derives mostly from their semiquinones. Studies performed with caffeic acid demonstrated that dimers and trimers of hydroxycinnamic acids may be formed, presenting a more hydrophobic nature than the parent compound **(Oszmianski & Lee, 1990)**. The dimers are denominated caffeicins. Caffeicins A to D correspond to stereoisomers of two caffeic acid residues linked via the hydroxyl groups of one unit and the propionic chain of another constituting oxidation products of dehydrobenzodioxan type (**Figure 1.15**). Caffeicin of type E (dihydronaphthalene type) and F (dihydrobenzofuran-type), and tetrahydrofuran were also observed to be formed **(Cilliers & Singleton, 1991; Fulcrand, Cheminat, Brouillard, & Cheynier, 1994)**. These structures preserve their catechol group being able to be further oxidized to *O*-quinones, which may further react intramolecularly forming novel oxidation products **(Guyot et al., 2009)**. Studies with 5-*O*-caffeoylquinic acid demonstrated that these kinds of products are also formed when hydroxycinnamic acids are ester-linked to quinic acid **(Bernillon, Guyot, & Renard, 2004; Castillo-Fraire, Poupard, Guilois-Dubois, Salas, & Guyot, 2019)**. In fact, all these products were already found in apple juices **(Bernillon et al., 2004)** and ciders **(Guyot et al., 2009)**.

1.2.4.2 Flavan-3-ol oxidation products

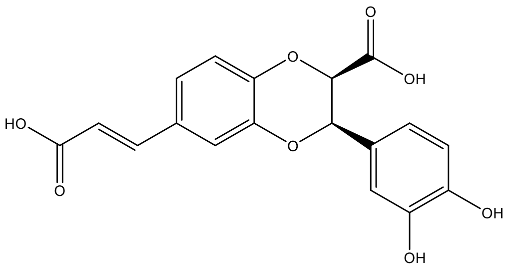
Flavan-3-ols, both (+)-catechin, (-)-epicatechin and procyanidins, can be considered one of the main targets for quinones as they represent the major phenolic groups found in apple. Radical-induced oxidation by peroxidase in the presence of H_2O_2 was shown to result in the conversion of (+)-catechin into 8-hydroxycathecin, through the electrophilic addition of the OH radical to C8 **(Bernillon, 2005)**. Alternatively, by the action of PPO, (+)-catechin polymerization might occur, thus yielding dihydrocatechins **(Guyot, Cheynier, Souquet, & Moutounet, 1995; Guyot, Vercauteren, & Cheynier, 1996)**. As for flavan-3-ols classifications, the formed dihydrocatechins are classified as of type B and type A depending if they have C-C or C-O-C interflavanic linkages **(Mouls & Fulcrand, 2012; Poupard et al., 2011)**. It has also been reported that due to oxidation, trimeric (-)-epicatechin structures might be formed due to two oxidative coupling reactions involving three (-)-epicatechin molecules and one intramolecular addition, as was the case for dehydrodicatechin A **(Guyot et al., 2009)**.



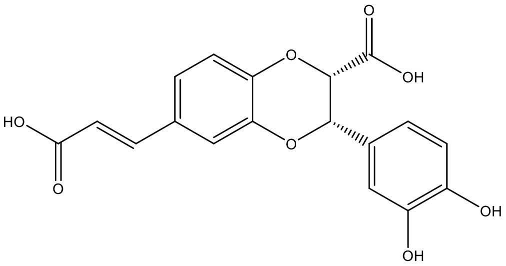
Caffeicin A



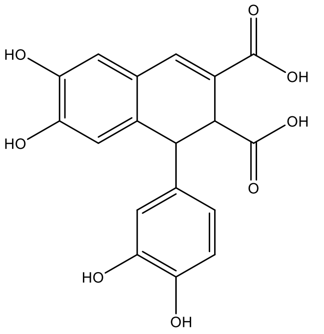
Caffeicin B

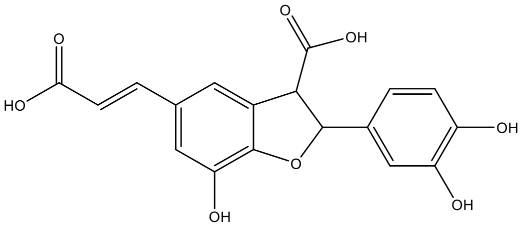


Caffeicin C



Caffeicin D





Caffeicin E **Caffeicin E**

Figure 1.15 – Dimeric oxidation products derived from caffeic acid.

For polymeric flavan-3-ols, the mechanisms of oxidation are quite like those reported for (+)-catechin and (-)-epicatechin. This means that by coupled oxidation reactions, the monomeric units of procyanidins can be oxidized to the respective quinones. These quinones will then react both at intra or intermolecular level leading to significative structural changes. For instance, procyanidin B2 oxidation induced by 5-*O*-caffeoylquinic acid quinone led to its conversion into procyanidin A2 **(Poupard et al., 2011)**. The suggested mechanism behind this reaction is based on the formation of the *O*-quinone on the extension unit that further suffers tautomerism to quinone methide (**Figure 1.16**). Afterwards occurs a nucleophilic attack on the O7 of the terminal unit resulting in the ether bond **(Bernillon, 2005)**. This process was also confirmed to occur in procyanidins of higher degree of polymerization **(Chen, Yuan, Chen, Jia, & Li, 2014)**. Polymerization as a result of procyanidins oxidation was also reported, although at limited extent. For instance, from procyanidin B2, oxidation products composed of two oxidized procyanidin B2 units were detected by **Poupard et al. (2011).**

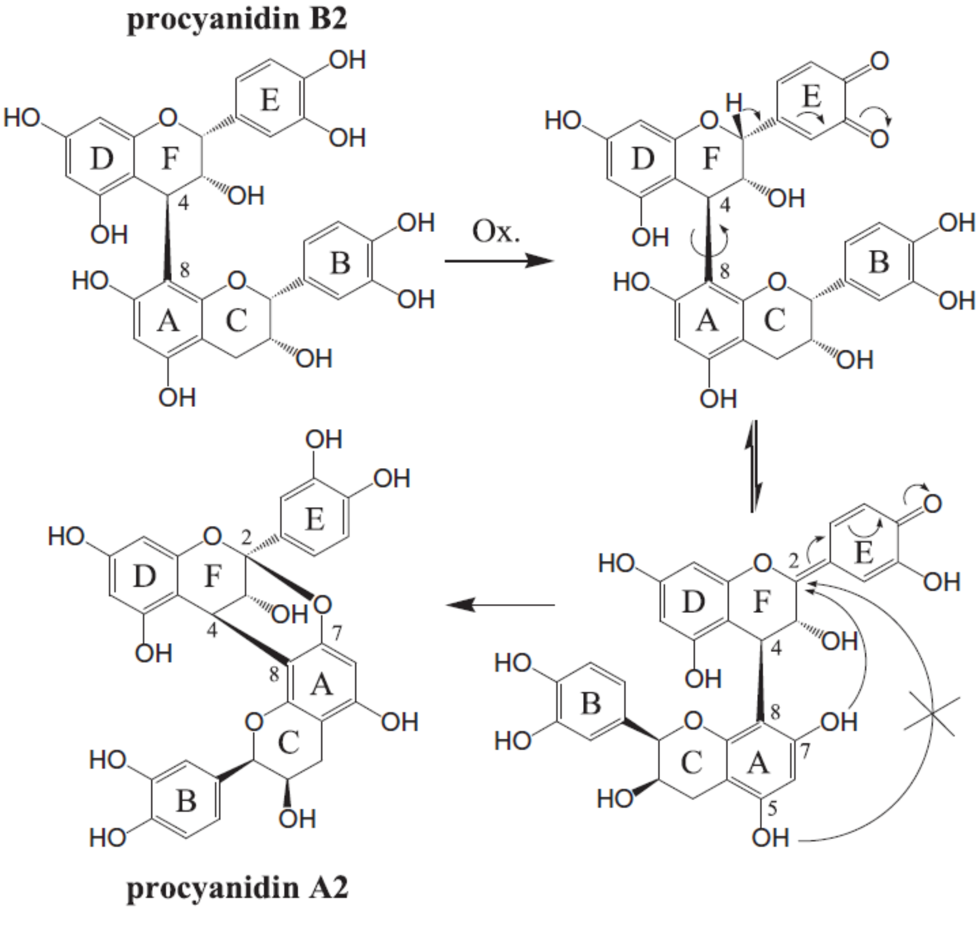


Figure 1.16 – Proposed mechanism for procyanidin A2 formation from procyanidin B2 oxidation. Figure adapted from **Poupard et al. (2011).**

Analysis of procyanidins of higher degree of polymerization showed that, as a result of oxidation, procyanidins quantification is affected. This has been attributed to the fact that some of the newly formed linkages are resistant to the usual depolymerization conditions, contrarily to the original interflavanic linkages **(Le Bourvellec et al., 2009; Mouls &**

Fulcrand, 2012, 2015). Mass spectrometry analysis of the obtained residues showed that these resistant units mostly comprised dimers and trimers, more or less oxidized, and some with additional hydroxyl groups. This suggests a competition between intramolecular and intermolecular reactions with impact on the size, branching and conformation and, consequently, on procyanidins properties **(Mouls & Fulcrand, 2012)**. Therefore, it can be concluded that from flavan-3-ol oxidation, two distinct phenomena occur: oxidative polymerization of flavan-3-ols and re-oxidation of the products formed, thus increasing the number of potential products. Some of these products, especially those presenting the type A oxidation linkages may present λ_{max} at 391 and 385 nm, being suggested to contribute to the yellowish color of apple juice and ciders **(Guyot et al., 2009)**.

1.2.4.3 Flavonol oxidation products

Flavonols are not substrates of polyphenol oxidase, and therefore, their oxidation results from alternative oxidation mechanisms. Chemical oxidation of quercetin and kaempferol showed that showed that C-ring cleavage might occur, thus leading to the formation of several A and B-ring derived products, including for instance, 3,4-dihydroxybenzoic acid **(Hvattum, Stenstrøm, & Ekeberg, 2004)**. Tyrosinase oxidation of flavonols allowed to conclude that dihydrobenzofuranne products might also be formed, attributed to the addition of water on quinone methide, opening of the heterocycle ring and cyclization **(Kubo, Nihei, & Shimizu, 2004)**. Moreover, during flavonols oxidation, dimeric and trimeric structures might also be formed between the C3-O-C4’ or C3-O-C3’ **(Ghidouche, Es-Safi, & Ducrot, 2008)**. In addition, dimeric structures of dioxane type were also reported to occur from flavonol oxidation by radicalar mechanisms **(Krishnamachari, Levine, & Paré, 2002)**.

1.2.4.4 Dihydrochalcone oxidation products

As dihydrochalcones are flavonoids with an opened C-ring and monohydroxylated in the B ring, the first effect of PPO is of cresolase activity resulting in the addition of a hydroxyl group in the C3’ carbon of the B-ring. The dihydroxylated dihydrochalcone is then oxidized to the respective *O*-quinone, becoming able to react with other dihydrochalcones (phloridzin and phloretin-xyloglucoside). It is observed that after oxidation, the formed quinone is hydrated forming a diaryl structure with a carboxylic acid (**Figure 1.17**). This structure can then be further oxidized and lead to the formation of a heterocyclic compound, which preserves the carbohydrate moiety **(Le Guernevé et al., 2004)**. This type of product is formed either with phloridzin or phloretin-3-*O*-xyloglucoside being characterized by a λ_{max} = 410 nm at the pH of apple **(Guyot et al., 2007)**. As a result, these compounds are recognized to be responsible for the color of both apple juices **(Bernillon et al., 2004; Wong-Paz et al., 2015)** and ciders **(Le Deun, Van der Werf, Le Bail, Le Quéré, & Guyot, 2015)**.

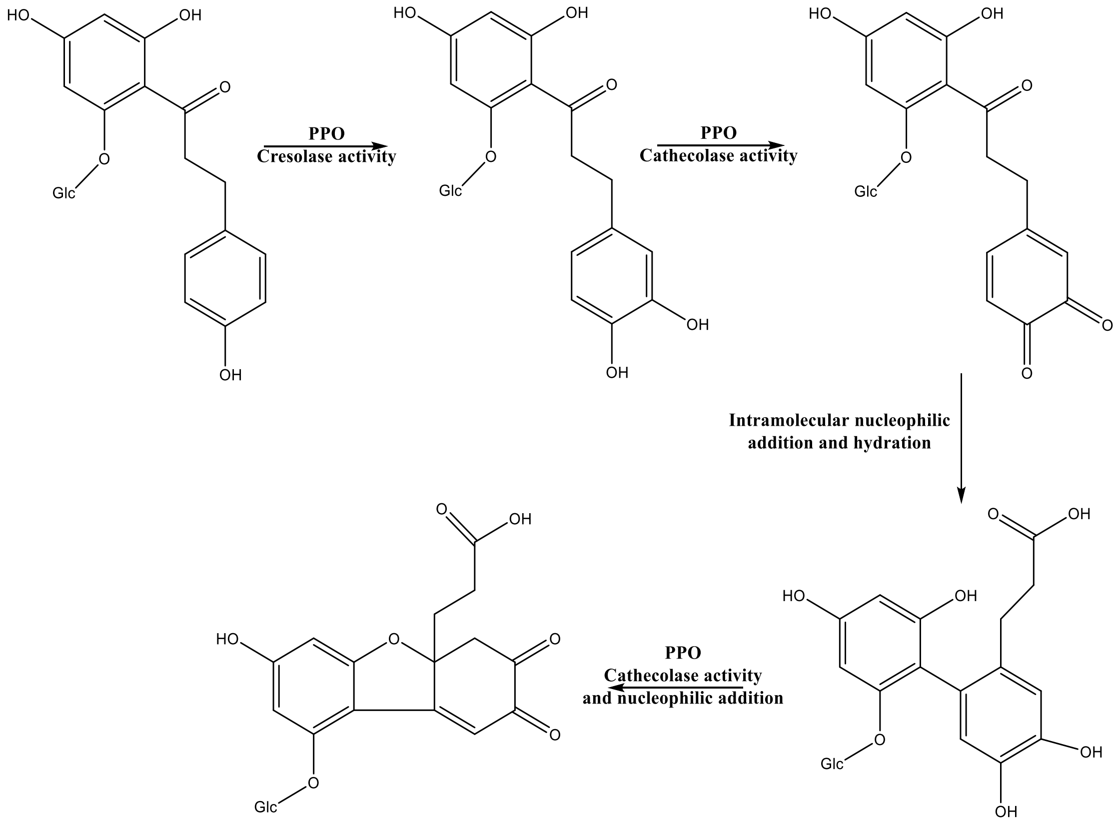


Figure 1.17 – Reaction pathway for the formation of polyphenol oxidation products derived from phloridzin. Scheme adapted from **Le Guernevé et al. (2004)**.

1.2.4.5 Anthocyanin oxidation products

Anthocyanins are not substrates for PPO and their oxidation is mainly governed by coupled oxidation reactions withy 5-*O*-caffeoylquinic acid quinones **(Sarni-Manchado, Cheynier, & Moutounet, 1997)**. As these polyphenols present different structures depending on the pH, all of them in equilibrium, the type of products generated can be more diversified than those observed for other phenolic classes. However, the higher instability of anthocyanins at pH 5.5 suggests that the hemiacetal form is the most relevant with regard to the formation of oxidation products **(Février et al., 2017)**.

In apples, anthocyanin oxidation has been recognized to play a role in red apple juices color, but so far no oxidation products were identified **(Février et al., 2017)**. Studies based on radical oxidation of anthocyanins in their flavylium form suggest that some of the structures formed might be of coumarin type **(Dangles, Fargeix, & Dufour, 2000)**. Malvidin-3-*O*-glucoside was previously shown to be oxidized by caftaric acid quinone yielding various colored and colorless oxidation products **(Sarni-Manchado et al., 1997)**.

1.2.4.6 Hetero-oligomeric oxidation products

As apple represent a food matrix composed by different polyphenols classes and given the different mechanisms expected for polyphenol oxidation, other products, in addition to those that are strictly originated from structures of the same class, are expected to occur. In fact, it was reported in model systems, using 4-methylcatechol and phloroglucinol, that 5-caffeoylquinic acid quinone might covalently attach to flavonoids A or B ring. Due to that, in apple juices and cider, oxidation products with 5-*O*-caffeoylquinic acid covalently attached to the phloroglucinol of (-)-epicatechin, procyanidins or onto their oxidized derivatives can be found by means of coupled oxidation-reduction reactions **(Bernillon et al., 2004; Poupard, Guyot, Bernillon, & Renard, 2008; Poupard et al., 2011)**. Hydroxycinnamic acids/dihydrochalcones **(Bernillon, 2005; Oszmianski & Lee, 1991)** and hydroxycinnamic acids/anthocyanins **(Sarni-Manchado et al., 1997)** dimers have also been identified as a result of oxidation**.**

Like hydroxycinnamic acids, flavonoids may also form dimers with other polyphenolic classes present in the matrix. This was observed in wine where anthocyanins were described to react with flavan-3-ols, a process mediated by acetaldehyde **(Oliveira, Ferreira, De Freitas, & Silva, 2011).** Given that most of these oxidation products may preserve their phenolic structure, additional oxidation reactions are expected to occur between and within different polyphenolic classes thus increasing the number of structures present in the matrix.

1.3 The composition of apple cell wall

The plant cell presents as main cellular components the nucleus, chloroplast, lysosomes and other vacuoles, mitochondria, ribosomes, enzymes, and its cell wall. The latter is responsible for the size and shape of the cells and provides resistance to the turgor pressure induced by the diffusion of molecules **(Cosgrove, 2005)**. Additionally, the cell wall protects the protoplast against pathogens, acts as an energy storage and is involved in the communication between neighboring cells **(Malinovsky, Fangel, & Willats, 2014)**.

The outer layer of the vegetable cell wall is called middle lamella (**Figure 1.18**). The middle lamella is its thinnest layer (<30 nm of thickness) and is responsible for the cohesiveness between adjacent cells. This is achieved due to the presence of polysaccharides deposited during cell division and maintained along the cell's life cycle **(Daher & Braybrook, 2015)**. As the cell division finishes, other polysaccharides start to deposit between the middle lamella and the cell membrane, a process that continues along cell growth, forming the primary cell wall. In parenchyma cells, which represent about 90% of the apple tissues, the primary cell wall is thin. However, in cholenchyma cells and epidermal cells present in the apple skin, the primary cell wall is thick and may reach up to 1 µm thick, providing additional strength and resistance to the fruit **(Knee, 2002)**. Even so, these still represent soft tissues allowing apples to easily suffer bruising **(Mitsuhashi-Gonzalez, Pitts, Fellman, Curry, & Clary, 2010; Videcoq et al., 2017)**. In more specialized cells, such as those in the xylem present in the stalks of the fruit, a secondary wall starts to deposit after the cell division process. This secondary wall provides additional resistance to the tissues allowing them to act as support or vessels for the transport of water and solutes **(Lazar, 2003)**.

The properties and functions attributed to cell wall result from the presence of polysaccharides, protein, polyphenols and other molecules, all with distinct physical and chemical properties. As a result, cell wall material also represents a relevant role in food science and nutrition acting as thickeners or as functional ingredients due to their role in the prevention of several chronic diseases, properties highly dependent on the polysaccharide structures **(Lovegrove et al., 2017)**.

1.3.1 Pectic polysaccharides

Pectic polysaccharides represent a collective group of carbohydrate structures composed of 17 different monosaccharides, mostly galacturonic acid (GalA), arabinose (Ara), galactose (Gal), rhamnose (Rha) and xylose (Xyl), and at least 20 different types of glycosidic linkages **(Ridley, O'Neill, & Mohnen, 2001; Voragen, Coenen, Verhoef, & Schols, 2009)**. These polysaccharides are the major constituents of the middle lamella but are also present in both the primary and secondary cell walls, thus being involved in cell growth, translocation of solutes and strength of the cell wall. In dicotyledonous plants, as apple, it is estimated that pectic polysaccharides account for about 35% of the primary cell walls **(Fry, 1988)**.

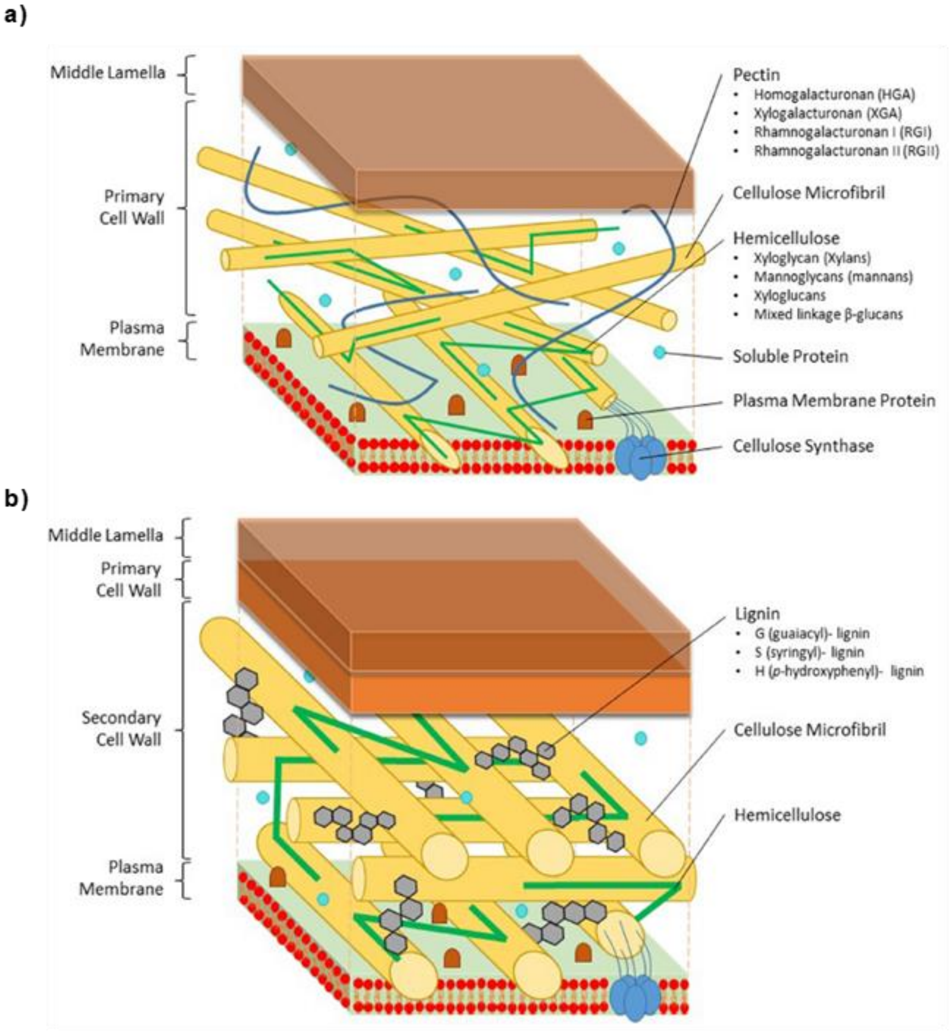


Figure 1.18 – Structure and composition of the primary a) and secondary b) plant cell wall. Adapted from **Loix et al. (2017)**.

Pectic polysaccharides are characterized for being soluble in hot water solution with diluted acids, Na_2CO_3, chelating agents as oxalate or cyclohexane diamine tetraacetic acid, also known as CDTA **(Renard & Thibault, 1993; Schols, Vierhuis, Bakx, & Voragen, 1995; Selvendran, 1985).** Cold diluted solutions of NaOH **(Schols et al., 1995)** or enzymes **(Renard, Voragen, Thibault, & Pilnik, 1991; Schols, Posthumus, & Voragen, 1990)** can also be used for the extraction of these compounds. All these methods yield polysaccharides fractions with different structural features, but the stage of maturity of the fruit is also relevant **(Michael Knee, 1973)**. Nevertheless, regardless of the extraction methodology employed, GalA residues are always found, as they constitute the backbone of pectic polysaccharides. The GalA might be methoxylated or acetylated while its chains can be branched with neutral sugars constituting the hairy regions of pectic polysaccharides. The non-branched chains are called smooth regions **(de Vries, Rombouts, Voragen, & Pilnik, 1984; Renard et al., 1991)**. However, enzymatic hydrolysis with PG, an enzyme that cleaves the linkages between non-methyl esterified GalA residues, have shown that pectic polysaccharides can be divided in three major groups: Homogalacturonans (HG), Rhamnogalacutronans I (RGI) and Rhamnogalacturonans II (RGII) (**Figure 1.19**), all with different types and patterns of substitution **(Willats, McCartney, Mackie, & Knox, 2001)**.

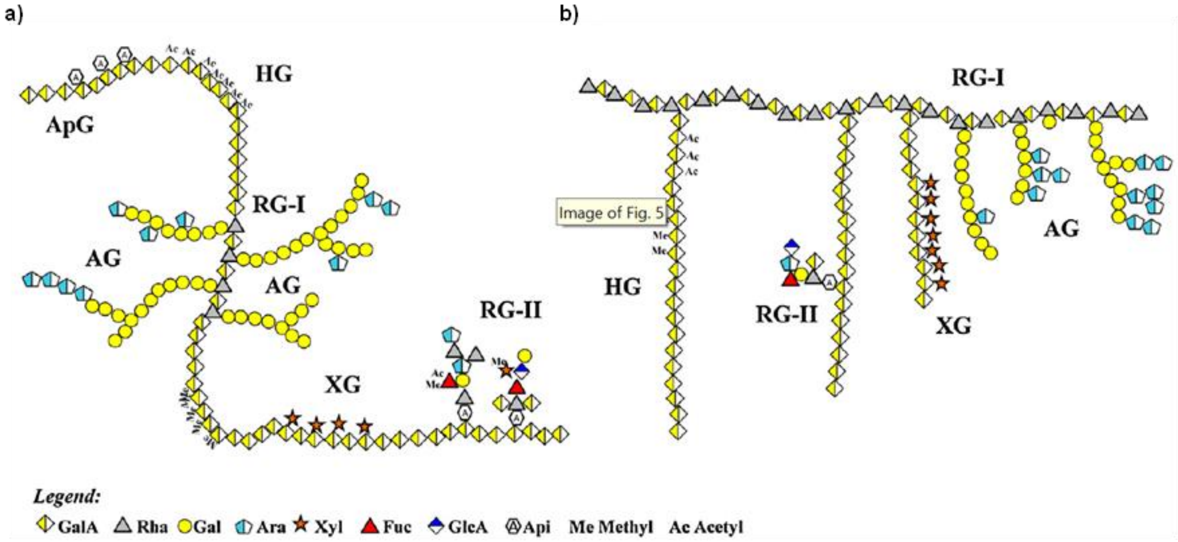


Figure 1.19 – Schematic representation of pectic polysaccharides structure according to **Pérez, Rodríguez-Carvajal, and Doco (2003)** and **Voragen, Voragen, Schols, Visser, and Visser (2003)**.

1.3.1.1 Homogalacturonans

Homogalacturonans (HG) represent a significant polysaccharide fraction (≈60%) of pectic polysaccharides found in apple and are the major polysaccharides comprising the pectin obtained by hot water extractions **(Voragen et al., 2009)**. Structurally (**Table 1.1)**, HG consist on →4)-D-Gal*p*A-(α1→ with possible methyl esterified groups at O-6 **(Gee, Reeve, & McCready, 1959; Renard & Thibault, 1993)** and acetylation at O-2 and/or O-3 **(Schols et al., 1990)**. In apples, the acetylation level of the GalA residues is very low, >1%, being localized mostly in the residues near neutral sugar side chains **(Schols et al., 1990)**. In contrast, GalA methyl esterification occur at relatively high values allowing the classification of pectic polysaccharides in three different groups: 1) those of high degree of methyl esterification where at least 50% of the HG residues are methyl esterified; 2) those of low degree of methyl esterification, where 10 to 50% of the GalA are methy esterified; and 3) pectic acids where GalA present <10% of methyl esterification. Some HG may be substituted with Xyl*p*-(β1→ at O-3 yielding xylogalacturonans (XGA). In apple, this substitution may reach 75% of the GalA residues including those presenting methyl esterified groups **(Schols, Bakx, Schipper, & Voragen, 1995)**.

1.3.1.2 Rhamnogalacturonan I

Type I rhamnogalacturonans (RG-I) are constituted by →2)-L-Rha*p*-(α1→4)-D-Gal*p*A-(α1→ repeating units (**Table 1.1)**. In apple, GalA residues of RGI can be methyl esterified as well as acetylated **(Schols et al., 1990)**. On the other hand, the rhamnosyl residues can be substituted at O-4 with neutral sugar side chains mainly composed of Gal (galactans) and Ara (arabinans) **(Colquhoun, de Ruiter, Schols, & Voragen, 1990; Schols et al., 1990)**. These side chains are very frequently long, but short chains, 1 to 3 units long, can also be found **(Colquhoun et al., 1990)**. RG-I also differs structurally, depending on its localization among the cell wall. RG-I present in the middle lamella present less Rha, shorter side chains and higher GalA methyl esterification than those occurring in the primary cell wall **(Brett & Waldron, 1996)**.

Table 1.1 - Main structural features of pectic polysaccharides according to **Voragen et al. (2009)**.

Polysaccharide	**Carbohydrates**		
	Backbone	**Lateral chains**	**Observations**
Homogalacturonan (HG)	→4)-D-Gal*p*A-(α1→	D-Xylp-(β1→	When xylose is present as branching unit the polysaccharide is called xylogalacturonan
Rhamnogalacturonan (RG-I)	→2)-L-Rha*p*-(α1→4)-D-Gal*p*A-(α1→	→5)-L-Ara*f*-(α1→ →4)-D-Gal*p*-(β1→ L-Fuc*p*-(α1→	The Rha may be branched at O-4.
Arabinans	→5)-L-Ara*f*-(α1→	→2)-L-Ara*f*-(α1→ →3)-L-Ara*f*-(α1→ →5)-L-Araf-(α1→ L-Ara*f*-(α1→	Present as lateral chains of RG-I. Branched at O-2 and/or O-3.
Galactans	→4)-D-Gal*p*-(β1→		Some Gal units may also be present as →6)-D-Gal*p*-(β1→.
Arabinogalactans (AG-I)	→4)-D-Gal*p*-(β1→	→5)-L-Ara*f*-(α1→ L-Ara*f*-(α1→	Present as lateral chains of RG-I. Branched at O-3.
Arabinogalactans (AG-II)	→3)-D-Gal*p*-(β1→	→6)-D-Gal*p*-(β1→ D-Gal*p*-(α1→ L-Ara*f*-(α1→ L-Ara*p*-(α1→	Present as lateral chains of RG-I or part of glycoproteins. Branched at O-6.
Rhamnogalacturonan (RG-II)	→4)-D-Gal*p*A-(α1→	Unusual sugars as apiose, aceric acid and others.	Lateral chains linked to Gal*p*A.

1.3.1.3 Arabinans

Arabinans are polymers of →5)-L-Ara*f*-(α1→ with branches at the O-2 and/or O-3 positions (**Table 1.1**). As branches, are commonly found →2)-L-Ara*f*-(α1→, →3)-L-Ara*f*-(α1→ and/or →3)-L-Ara*f*-(α1→3)-L-Ara*f*-(α1→ sequences. These polysaccharides are highly abundant in apples **(Schols et al., 1990; Voragen, Rombouts, Searle-van Leeuwen, Schols, & Pilnik, 1987)** and present L-Ara*f*-(α1→ as terminal units. However in olives **(Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007; Cardoso, Silva, & Coimbra, 2002)** and European spruce (*Picea abies*) **(Shakhmatov, Belyy, & Makarova, 2018)**, unusual L-Ara*f*-(β1→ were found to occur.

1.3.1.4 Galactans

Galactans are polymers constitutive of pectic polysaccharides composed by →4)-D-Gal*p*-(β1→ (**Table 1.1**). Ara residues can also be found attached to the O-3 of galactans thus constituting the type I arabinogalactans (AG-I), as reported for apples **(de Vries, den Uijl, Voragen, Rombouts, & Pilnik, 1983; Schols et al., 1990)**. Studies in potato and soybean also suggest that type I arabinogalactans may possess internal →5)-L-Ara*f*-(α1→ and →3)-D-Gal*p*-(β1→ **(Hinz, Verhoef, Schols, Vincken, & Voragen, 2005; Huisman et al., 2001)**.

Type II arabinogalactans (AG-II) are also found in apple **(de Vries et al., 1983; Schols et al., 1990)**. Structurally, these galactans are characterized by having →3)-D-Gal*p*-(β1→ backbone, branched with →6)-D-Gal*p*-(β1→. These side chains might be extended with →3)-L-Ara*f*-(α1→ residues and present commonly L-Ara*f*-(α1→ as terminal units. AG-II are commonly associated with proteins, thus being also designated as arabinogalactan proteins. The protein moiety is known to be particularly rich in proline/hydroxyproline, alanine, serine, and threonine, globally accounting for 10% of the structure **(Gaspar, Johnson, McKenna, Bacic, & Schultz, 2001; Immerzeel, Eppink, De Vries, Schols, & Voragen, 2006)**.

1.3.1.5 Rhamnogalacturonan II

Rhamnogalacturonan II (RG-II) correspond to highly preserved structural domains of pectic polysaccharides in plant kingdom (**Table 1.1)**. These polysaccharides are released by treatment with pectic polysaccharides by endo-PG thus being a distinct region of HG. This structure is characterized by having four different side chains composed of unusual sugar residues including apiose, 3-deoxy-lyxo-2-heptulosaric acid (DHA), aceric acid, and 3-deoxy-manno-2-octulosonic acid (KDO) but also by methylated sugars as 2-*O*-metil-L-fucose e 2-*O*-metil-D-xylose. These chains may also contain L-Rha, L-Ara, D-Gal and D-GlcA. The side chains rely over a backbone composed of about 9 GalA residues, some of which are methyl esterified, directly linked to HG **(Doco, Williams, Vidal, & Pellerin, 1997; Tadashi Ishii & Matsunaga, 2001)**. RG-II commonly exist as cross-linked dimers by borate-diol esters involving the apiofuranosyl residues **(Ishii et al., 1999)**.

1.3.2 Hemicelluloses

Hemicelluloses correspond to cell wall polysaccharides that are extractable when using concentrated alkaline solutions (NaOH or KOH), generally at concentration from 1 to 4 M. The nature and structure of hemicelluloses depend on the plant species and overall contain Glc, Xyl, Ara, Man, Gal and/or Fuc (**Table 1.2**). These polysaccharides are believed to be relevant in cell wall elongation process.

Table 1.2 - Main structural features of hemicellulosic polysaccharides and cellulose.

Polysaccharide	Carbohydrates		
	Backbone	**Lateral chains**	**Observations**
Xyloglucans	→4)-D-Glc*p*-(β1→	D-Xyl*p*-(α1→ →2)-D-Xyl*p*-(α1→ D-Gal*p*-(α1→ →2)-D-Gal*p*-(α1→ L-Fuc*p*-(α1→	Branching at O-6.
Xylans	→4)-D-Xyl*p*-(β1→	L-Ara*f*-(α1→	Branching at O-2 or O-3.
Mannans	→4)-D-Man*p*-(β1→		Might be O-2 and/or O-3 acetylated. If 4)-D-Glc*p*-(β1→ is present at the backbone the polysaccharide is called glucomannan.
Cellulose	→4)-D-Glc*p*-(β1→		Polysacchrides oh high degree of polymerization (2000-15,000).

1.3.2.1 Xyloglucans

Xyloglucans are the most abundant hemicellulose present in the primary cell wall of apples, accounting for 18% of the polysaccharides **(Renard, Lomax, & Boon, 1992)**. These polymers are characterized by a →4)-D-Glc*p*-(β1→ backbone (**Table 1.2).** Of the Glc residues, about 75% are branched at O-6 with D-Xyl*p*-(α1→, D-Gal*p*-(α1→2)-D-Xyl*p*-(α1→ and D-Fuc*p*-(α1→2)-D-Gal*p*-(α1→2)-D-Xyl*p*-(α1→ **(Renard, Lemeunier, & Thibault, 1995; Voragen, Schols, & Pilnik, 1986)**. Xyloglucans might be acetylated in Gal, Xyl or Glc **(Vierhuis, Korver, Schols, & Voragen, 2003)** but it is not common in apple **(Renard et al., 1992)**.

1.3.2.2 Xylans

Xylans are polysaccharides characteristic of the secondary wall of angiosperms. These polysaccharides are characterized by having a →4)-ᴅ-Xyl*p*-(β1→ backbone (**Table 1.2)**. In apple, the xylans backbone have been described to possess side chains composed of ʟ-Ara*f* as terminal unit in the positions O-2 and/or O-3, constituting arabinoxylans **(Selvendran, 1985; Stevens & Selvendran, 1984)**. According to **Selvendran (1985)**, these arabinoxylans are part of a crosslinked complex composed of arabinans and protein or arabinans and xyloglucans.

1.3.2.3 Mannans

Mannans are some of the linear structures constituting the cell wall of cotyledons **(Selvendran, 1985)**. These polysaccharides are characterized by having a →4)-ᴅ-Man*p*-(β1→ backbone (**Table 1.2)** and have been suggested to occur in apple **(Stevens & Selvendran, 1984; Voragen et al., 1986)**. In apple, glucomannans, polymers that present ᴅ-Glc*p* and ᴅ-Man*p* linked via β-(1→4) linkages, have also been suggested to occur. According to other plant sources, mannans might be acetylated at the O-2 and/or O-3 **(Parente, Adão, da Silva, & Tinoco, 2014)**.

1.3.2.4 Cellulose

Cellulose is another major component of the primary and secondary cell walls, accounting for 20-30% and 40-90% of these structures, respectively. This polysaccharide is characterized by having a linear →4)-ᴅ-Glc*p*-(β1→ structure that allows it to acquire a linear conformation. As a result, different cellulose chains are able to interact with each other via hydrogen bonding and Van der Waals forces **(Nishiyama, Langan, & Chanzy, 2002)**. This results in the formation of microfibrils which may account for 36 glucans chains and 30 nm in width, visible by electron microscopy **(Herth, 1983)**. The cellulose chains of the primary cell wall have a lower degree of polymerization (2000-6000) than those occurring in the secondary cell wall (15000) **(Brown, 2004)**.

1.3.3 Polyphenols

Polyphenols in cell wall can be found attached to polysaccharides. Ferulic acid is the major phenolic structure associated to polysaccharides, being mostly linked at the O-2 and/or O-5 of Ara residues or O-6 of Gal by means of ester linkages **(Fry, 1988; Levigne et al., 2004)**. However, apple cell wall extensive studies have not identified these types of linkages suggesting that it is not a common feature in apple fruits.

Lignin is defined as a network of aromatic compounds crosslinked via ether or carbon-carbon linkages. This polymer is mostly found in the secondary wall of cells, but is also present, although at very low amount, in the primary cell wall, including that of parenchyma cells. Studies on dicotyledons lignin show that this polymer is mostly composed of hydroxyphenyl, guaicyl and syringyl units **(Barros, Serk, Granlund, & Pesquet, 2015)**. However, it has been reported that apple lignin, as determined as Klaison lignin, mostly results from polyphenol oxidation reactions **(Renard et al., 1995)**

1.3.4 Proteins

Plants primary cell wall present about 5-10% of protein, most of which with unknown function. Some proteins occur freely in the intercellular space, others are covalently or non-covalently bonded (hydrophobic and/or ionic interactions and hydrogen bonding) to the cell wall components **(Brett & Waldron, 1996)**. The plant cell wall proteins are well known to be particularly rich in 4-*trans*-L-hydroxyproline (Hyp), especially extensins, arabinogalactan-proteins and other hydroxyproline rich proteins. Other classes of proteins include proline or glycine rich proteins. These proteins are generally N-glycosylated in Asn-X-Thr/Ser sequences while *O*-glycosylation occurs mostly in Ser or Hyp. As observed for arabinogalactan proteins, glycosylation often occurs with Ara and/or Gal residues which may account till 90% the whole structure. Phosphorylation has also been reported to occur in cell wall proteins **(Brett & Waldron, 1996; Jamet et al., 2008)**.

About 13% of the known plant cell wall proteins have an unknown function. Of those with known function, 26% act on polysaccharides (glycosilhydrolases, glycosiltransferases, carbohydrate esterases, carbohydrate lyases, and expansins), 12.4% are oxidoreductases (peroxidases, multicopper oxidases, and germins), 11.9% are proteases (subtilisins, Asp proteases, Cys proteases, and Ser carboxypeptidases), 10.6% are proteins with interacting domains through leucine-rich repeat domains (lectins, enzyme inhibitors for PG, PME, and proteases), 7.4% are involved in signaling (e.g. arabinogalactan proteins), 5.4% are involved in lipid metabolism (e.g. lipases), 1.8% possess a structural function and 11.9% are classified as "miscellaneous proteins given their multifunctions **(Jamet et al., 2008; Nguema-Ona et al., 2014)**.

1.3.5 Primary cell wall organization

Two types of primary cell walls have been identified. In dicotyledons, as apples, the primary cell wall (type I) is mainly composed of pectic polysaccharides (40%), followed by cellulose (35%), hemicelluloses (15%), protein (5%) and polyphenols (5%). In contrast, in type II primary cell walls (characteristic of Gramineae), xyloglucans are substituted by glucuronoarabinoxylans and the pectin contents are much lower.

Over the years, several models have been proposed to understand the organization of the cell wall components **(Carpita & Gibeaut, 1993; Keegstra, Talmadge, Bauer, & Albersheim, 1973)**, but the most accepted model was proposed by **Carpita and Gibeaut (1993).** This model suggests that the primary cell wall comprises three independent domains. The first domain is established by the cellulose-xyloglucan framework that accounts for 50% of the cell wall mass. This framework is characterized by the interaction between the xyloglucan network and cellulose, by means of hydrogen bonds, thus coating the microfibrils. The fact that part of xyloglucans can be obtained by enzymatic treatment with endoglucanase and/or strong alkali treatments, while other remains unextractable, suggest that xyloglucans are attached, at different extents to cellulose microfibrils **(Caffall & Mohnen, 2009; Wang & Hong, 2015)**.

The xyloglucan-cellulose framework is embedded in a second domain composed of pectic polysaccharides (30% of the cell wall mass) whose distribution along the cell wall is not uniform. In fact, studies performed with monoclonal antibodies demonstrated that HG chains predominate at the level of the middle lamella establishing calcium bridges due to the presence of PME along the cell wall. On the other hand, RG-II are mostly located near the cell membrane, crosslinked via borate diester linkages **(Cosgrove, 2015; Somerville et al., 2004)**. RG-I neutral sugar side chains can be cross-linked between them or even with xylans, xyloglucans and cellulose **(Grones, Raggi, & Robert, 2019),** with the possible involvement of polyphenols as linking bridges **(Fry, 1983; Levigne et al., 2004)** by means of ether linkages **(Selvendran, 1985)**. Each one of these cross-linkages appears to have a specific function along the cell wall. For instance, it is believed that arabinan cross-linking through ferulic acids act as a mechanism for the inhibition of HG calcium complexes, thus providing stiffness to the cell wall **(Jones, Milne, Ashford, & McQueen-Mason, 2003)**.

The third domain of type I primary cell wall involves mostly proteins, especially extensins. These proteins appear to interact very strongly with polysaccharides via hydrogen bonding. However, the occurrence of covalent linkages between proteins and polysaccharides is not discarded given that some pectic polysaccharides are resilient to enzymatic treatment and alkali extractions, but recoverable after protease treatment **(Keegstra et al., 1973)**. Ether linked polyphenols could also be detected in cell wall proteins involving the tyrosine residues **(Brett & Waldron, 1996)**, establishing cross-linkages with other cell wall polymers. All these domains are relevant for the assembly, strength and functionality of the pectic network in the cell wall. However, it is not known how the polysaccharides come together to yield a functional primary cell wall, turning this topic the focus of cell wall research **(Grones et al., 2019; Keegstra, 2010)**.

1.4 Polysaccharide/polyphenol interactions

The original structure of the plant cell is stablished by several compartments limited by lipidic membranes and an external cell wall mainly composed by polysaccharides. Nevertheless, when the cell is disrupted either by mechanical or thermal procedures, polyphenols that were initially circumscribed to the cytoplasm or vacuoles become into contact with proteins and polysaccharides by means of non-covalent interactions **(Razmkhab et al., 2002; Renard, Baron, Guyot, & Drilleau, 2001)**. Additionally, as a result of the reaction between polyphenol quinones and cell wall molecules, covalent interactions might also be established **(Renard, Watrelot, & Le Bourvellec, 2017)**.

The mechanisms behind the interactions of polyphenols with either proteins or polysaccharides have been elucidated over the past years to assess eventual implications at nutritional and technological levels. In the case of fruit pomaces, the elucidation of these interactions might be relevant for their valuation as it shows to affect the extractability of polysaccharides and polyphenols **(Le Bourvellec et al., 2007)**. Given the much higher prevalence of polysaccharides over proteins in fruits, this topic will discuss the interactions of polysaccharides with polyphenols in more detail by uncovering the mechanism and relevant structural features.

1.4.1 Non-covalent interactions

1.4.1.1 Mechanisms of interaction

From the first experiments conducted by **McManus et al. (1985)** to the studies carried out by **Taira, Ono, and Matsumoto (1997)** the gathered information pointed out to the involvement of non-covalent interaction between polysaccharides and polyphenols. However, with the works developed by **Renard et al. (2001)** e **Le Bourvellec, Guyot, and Renard (2004),** the mechanisms behind these interactions started to be decoded.

The adsorption of polyphenols to polysaccharides is almost an instantaneous process since most of the polyphenols (90%) adsorb in less than 5 min. As the time of contact increases, the adsorption rate decreases, leading to a plateau phase due to the saturation of the polysaccharide **(Renard et al., 2001)**. This adsorption results from the establishment of hydrophobic interactions and hydrogen bounds between polyphenols and polysaccharides. Structurally, this means that the polyphenols may interact with polysaccharides by different mechanisms. Studies on α-cyclodextrin, consisting of six glucopyranose units linked by α-(1–4) glycosidic bonds, suggest that polysaccharides might form hydrophobic cavities (**Figure 1.20**) that enable polyphenol incorporation by means of Van de Waals interaction and/or hydrogen bounds between the hydroxyl groups of the polyphenols and the oxygen of the glycosidic linkage or between the C-ring oxygen and the hydroxyl of polysaccharides **(Hostettmann, Lederer, Marston, & Leipzig-Pagani, 1997; Saura-Calixto, 2011).** Another mechanism is the adsorption to the surface of polysaccharides mediated by reorganization of the CH groups of the carbohydrates and the aromatic rings of polyphenols, stabilized by hydrogen bounding **(Renard et al., 2017; Saura-Calixto, 2011)**. The occurrence of ionic interactions has also been suggested to occur between oppositely charged polyphenols and polysaccharides **(Fernandes, Brás, Mateus, & de Freitas, 2014; Gonçalves et al., 2018; Lin, Fischer, & Wicker, 2016)**.

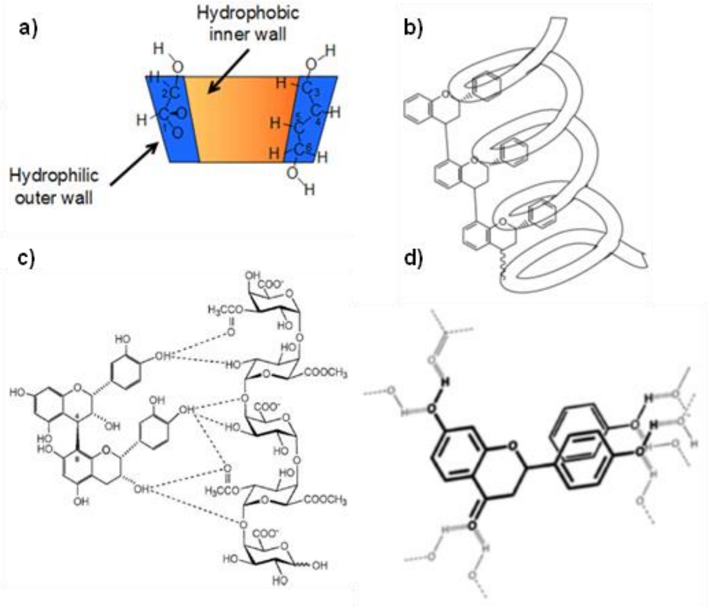


Figure 1.20 – Schematic representation of the possible mechanisms involved on polysaccharide/polyphenol interactions. In a) is represented a hydrophobic cavity formed by polysaccharides and in b) a probable inclusion complex formed in these cavities. In c) is represented the possible hydrogen bond established between polysaccharides (homogalacturonan) and polyphenols (procyanidin) and in d) a visual representation of the stacking phenomenon established between polyphenols. Figure adapted from **Bordenave, Hamaker, and Ferruzzi (2014)** and **Amoako and Awika (2019)**.

Simultaneously with the mechanisms described above, a stacking phenomenon appears to occur as a result of inter and intra molecular interactions between polyphenols **(Poncet-Legrand, Cartalade, Putaux, Cheynier, & Vernhet, 2003; Riou, Vernhet, Doco, & Moutounet, 2002)**. When a polyphenol adsorbs to a polysaccharide, it forms a possible site of nucleation which allow the retention of an increasing number of polyphenols through hydrophobic and hydrogen bounds **(Renard et al., 2001)**. This phenomena, known as π-stacking, may occur in three ways: 1) sandwich, 2) T-shaped or 3) Parallel-displaced stacking being the latter two the most thermodynamically stable **(Berké & de Freitas, 2005; Galland, Mora, Abert-Vian, Rakotomanomana, & Dangles, 2007; Teixeira et al., 2013)**. The occurrence of this phenomenon during the interaction between polysaccharide/polyphenols is supported by the evidence of **Renard et al. (2001)**, who observed a biphasic elution process of procyanidins from the cell wall material. Initially, buffers eluted a large amount of procyanidins, which were possibly held by weak interaction, and secondly, urea caused a second elution phase possibly as a result of the strong interaction with the cell wall material. Furthermore, polyphenols ability to self-associate also support polyphenol stacking in polyphenol/polysaccharide interactions **(Poncet-Legrand et al.,**

2003; Riou et al., 2002). A recent study developed by **Chirug, Okun, Ramon, and Shpigelman (2018)** suggested an additional mechanism where iron ions might also be involved in the interaction between polyphenols and pectic polysaccharides.

As a result of these mechanisms, the interactions of polysaccharides with polyphenols, when in solution, may result in the formation of soluble complexes **(Fernandes et al., 2014; Gonçalves et al., 2018; Gonçalves, Rocha, & Coimbra, 2012)** or colloidal aggregates that precipitate or remain in solution, as described for proteins (**Figure 1.21**) **(Mekoue Nguela, Poncet-Legrand, Sieczkowski, & Vernhet, 2016; Riou et al., 2002)**.

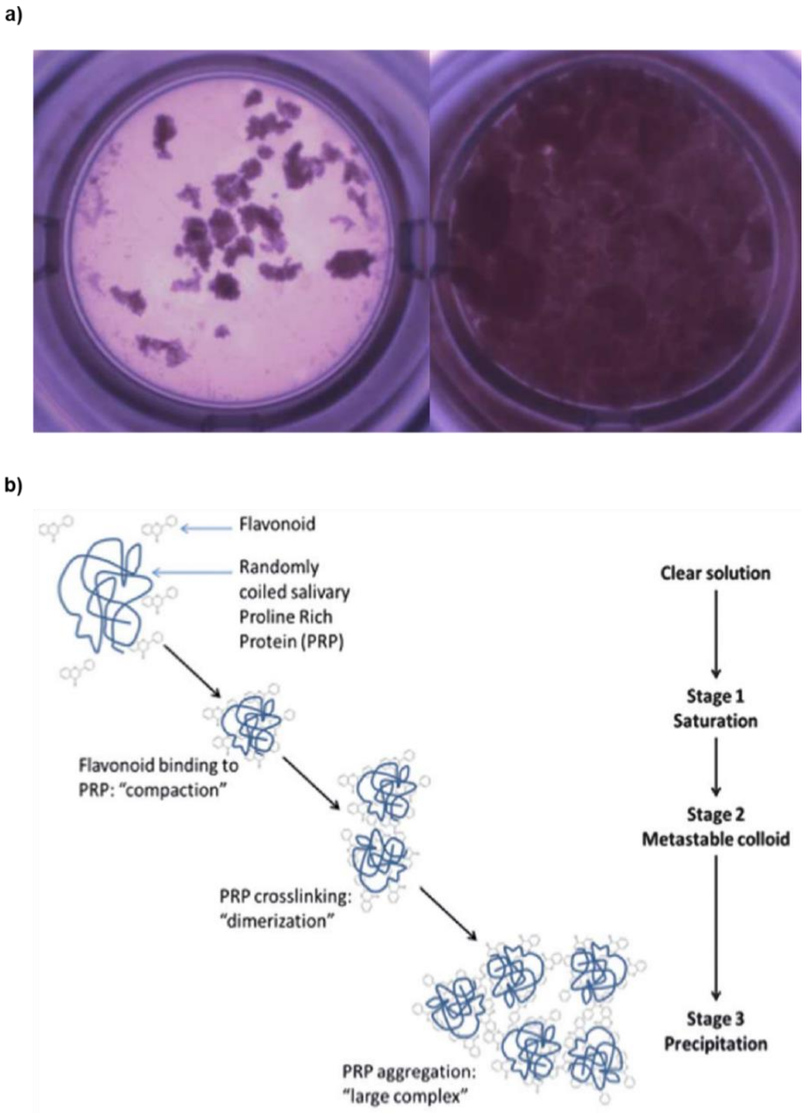


Figure 1.21 – Image of a 70% methylated homogalacturonans solution in the presence of low polymerized procyanidins (DP9) (left) or highly polymerized procyanidins (DP30) (right) **(Renard et al., 2017)**. In b) is represented the purposed mechanism of protein precipitation when interacting with procyanidins **(Bordenave et al., 2014)**.

1.4.1.2 Influence of the polyphenolic structure

High molecular weight polyphenols

Polyphenols present a wide structural diversity, which consequently modulates their propensity to interact with polysaccharides **(McManus et al., 1985; Renard et al., 2001).** High molecular weight polymers, such as proanthocyanidins, are generally perceived as rigid and linear structures **(Bindon, Smith, & Kennedy, 2010).** However, they strongly interact with cell wall polysaccharides (**Table 1.3**), a phenomenon that is selective for polymers of higher degree of polymerization **(Bautista-Ortín, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Bindon, Smith, & Kennedy, 2010; Le Bourvellec, Bouchet, & Renard, 2005; Le Bourvellec et al., 2009; Renard et al., 2001)**. In other words, in a complex mixture of polyphenolic polymers, those presenting higher molecular weight will more readily adsorb to the cell wall polysaccharides whereas the smaller molecules will remain free. This results from the fact that the polyphenolic polymer presents a higher tanning effect, i.e., present many rings and hydroxyl groups which act as multiple anchors capable of connecting simultaneously to several molecules and points of the polysaccharide structure, conferring a greater stability to the complex. In contrast, low molecular weight polymers perform less interactions, which cause them to adsorb to polysaccharides with lower affinity. However, for more complex structures such as the cell wall, differences in porosity and chemical composition may limit the accessibility and interaction of larger molecules. In the case of low porous structures, very high molecular weight polyphenols adsorb less than their low molecular weight counterparts **(Bindon, Smith, Holt, & Kennedy, 2010; Renard et al., 2001)**.

In addition, the monomeric constituents of polyphenolic polymers have an impact on the adsorption towards polysaccharides. For instance, polymers formed of (+)-catechin are linked to right helical structures whereas polymers of (-)-epicatechin are associated to left helical structures. In this context, procyanidins with different proportions of (+)-catechin / (-)-epicatechin may yield twists that affect the structural conformation of the molecule and thus its ability to form hydrophobic interactions or hydrogen bounds with different polysaccharides **(Fletcher, Porter, & Haslam, 1976; Le Bourvellec et al., 2004)**. Furthermore, the presence of substituent groups may have effects on the extent of these interactions. In the case of procyanidins of similar degree of polymerization, those presenting galloyl groups exhibit higher interactions towards polysaccharides **(Bindon,**

Smith, Holt, et al., 2010; Le Bourvellec et al., 2004; Le Bourvellec & Renard, 2005). However, when these galloyl groups are involved in intermolecular covalent linkages, as in the case of ellagitannins, the molecule flexibility can be strictly restricted, negatively affecting the hydrophobic interaction **(Tang, Covington, & Hancock, 2003)**.

Table 1.3 – Effect of procyanidins structure towards their interaction with cellulose, starch, xyloglucans and pectin isolated from apple. Maximum number of binding sites (Qmax) and affinity constant (*K_L*) of the interactions, determined by the Langmuir isotherms, are presented. Data from **Le Bourvellec et al. (2005)**.

Polysaccharide	**Procyanidins**	**Langmuir**	
		Q_{max} (g/g)	K_L (L/g)
Cellulose	Adp3	0.76±0.35	0.1±0.07
	Adp10	0.77±0.24	0.05±0.02
	Adp70	1.03±0.73	0.04±0.04
	Pdp35	0.95±0.17	0.05±0.02
	Gdp 8 Gal 22%	0.92±0.35	0.05±0.03
Starch	Adp3	1.91±0.99	0.05±0.04
	Adp10	1.63±0.85	0.05±0.04
	Adp70	1.42±0.20	0.13±0.04
	Pdp35	1.41±0.14	0.12±0.02
	Gdp 8 Gal 22%	1.41±0.50	0.07±0.04
Pectin	Adp3	0.86±0.12	0.58±0.28
	Adp10	0.83±0.09	0.70±0.33
	Adp70	0.20±0.02	2.20±0.79
	Pdp35	0.57±0.03	0.90±0.20
	Gdp 8 Gal 22%	0.76±0.05	1.22±0.38
Xyloglucan	Adp3	-	0.003
	Adp10	2.17±0.94	0.13±0.10
	Adp70	3.25±1.04	0.20±0.12
	Pdp35	2.83±1.04	0.19±0.12
	Gdp 8 Gal 22%	2.49±0.88	0.18±0.13

Oxidised polymeric polyphenols are also able to establish interactions with polysaccharides **(Mekoue Nguela et al., 2016)**. As the structural changes resulting from oxidation are still poorly understood, it is difficult to establish structure/interaction relationships. However, there is some evidence that oxidation may affect tannins DP and conformation (linear versus branched polymers) and the hydrophobic/hydrophilic character of the polyphenols. Such changes probably modify the accessibility to the interaction sites of polysaccharides and the mechanisms involved.

Low molecular weight polyphenols

Low molecular weight polyphenols also interact with polysaccharides but 10-fold lower when compared with high molecular weight polyphenols **(Phan et al., 2015; Renard et al., 2001).** As observed for high molecular weight polyphenols, this interaction is highly dependent on polyphenol structural features (**Table 1.4**).

Table 1.4 – Overall balance of the phenolic structure towards the interaction with cellulose and apple cell all material (CWM). Maximum number of binding sites (Q_{max}) and affinity constant (K_L) of the interactions. determined by the Langmuir isotherms are also presented **(Phan, Flanagan, D'Arcy, & Gidley, 2017; Phan et al., 2015)**.

Polysaccharide	**Polyphenol**	**Charge (pH<4)**	**Langmuir**	
			Q_{max} (μg/mg)	K_L *(L/g)*
Cellulose	(+)-Catechin	neutral	1488	0.006
	Ferulic acid	negative	1409	0.002
	Gallic acid	negative	388	0.010
	Chlorogenic acid	negative	1060	0.011
	Cyanidin-3-Glc	positive	1109	0.055
Apple CWM	(-)-epicatechin	neutral	240	0.05
	phloridzin	neutral	244	0.108
	Chlorogenic acid	neutral	347	0.067
	(+)-Catechin	neutral	611	0.015
	Ferulic acid	negative	421	0.008
	Cyanidin-3-Glc	positive	325	0.15

The number of aromatic rings is an important structural feature, as demonstrated by the affinity constants observed for cyanidin-3-*O*-glucoside and (+)-catechin towards cellulose or apple cell wall, when compared to that of phenolic acids **(Phan et al., 2015)**. Moreover, the presence of charged groups seems to be an important feature for the adsorption phenomenon having in mind the electronegative charge of the polysaccharides.

For instance, at pH<5, cyanidin-3-*O*-glucoside, positively charged, presented higher constant affinities for cellulose than (+)-catechin (non-charged) and phenolic acids (negatively charged), a fact that was attributed by the authors to the slightly negative surface cellulose **(Phan et al., 2015)**. This effect is more marked when these polyphenols interact with pectin in acidic pH where the carboxylic groups of GalA are negatively charged. Cyaniding-3-*O*-glucoside, a positively charged polyphenol, has higher interaction with pectin than negatively charged ferulic acid **(Phan et al., 2017)**.

Studies on wine showed that anthocyanins interact at different extents with polysaccharides. Higher interactions were reported for coumaroyl and acyl derivatives, over non-acylated ones, which highlights that polyphenols of higher hydrophobicity further interact with polysaccharides **(Gonçalves et al., 2018; Gonçalves et al., 2012; Morata et al., 2003).** Notably, these appear to be more prone to occur when anthocyanins are in their flavylium form, rather than in their hemiketal form and to increase with the number of hydroxyl groups present in the B-ring **(Fernandes et al., 2014).** Glycosylation of polyphenols has been suggested to impair the interactions with polysaccharides **(Gómez-Mascaraque, Dhital, López-Rubio, & Gidley, 2017).**

1.4.1.3 Effect of polysaccharide structure

As mentioned by **Carpita and Gibeaut (1993)**, cellulose, xyloglucan and pectic polysaccharides are polymers with a relevant role in the supramolecular arrangement of the cell walls. In this context, works have been performed using apple **(Le Bourvellec et al., 2005; Le Bourvellec et al., 2004; Le Bourvellec et al., 2007; Renard et al., 2001)** and grape cell wall **(Bautista-Ortín et al., 2014; Bautista-Ortín, Martínez-Hernández, Ruiz-García, Gil-Muñoz, & Gómez-Plaza, 2016; Bindon, Bacic, & Kennedy, 2012; Bindon, Smith, Holt, et al., 2010)** or even simpler models such as composites of cellulose/pectin **(Padayachee et al., 2012)**, in order to understand the mechanisms of interaction between polyphenols and the cell wall material. In fact, by performing sequential extractions with ammonium oxalate (chelating agent for pectin extraction), pectin lyase and 4 M KOH, **Le Bourvellec et al. (2009)** proposed a model for polyphenol interactions with cell wall material by specifying which polysaccharide families appeared to be involved (**Figure 1.22**). Nevertheless, the complexity of these systems did not provide direct evidences of which cell wall structures were responsible for this interaction phenomenon. In this context, single type of cell wall material started to be employed.

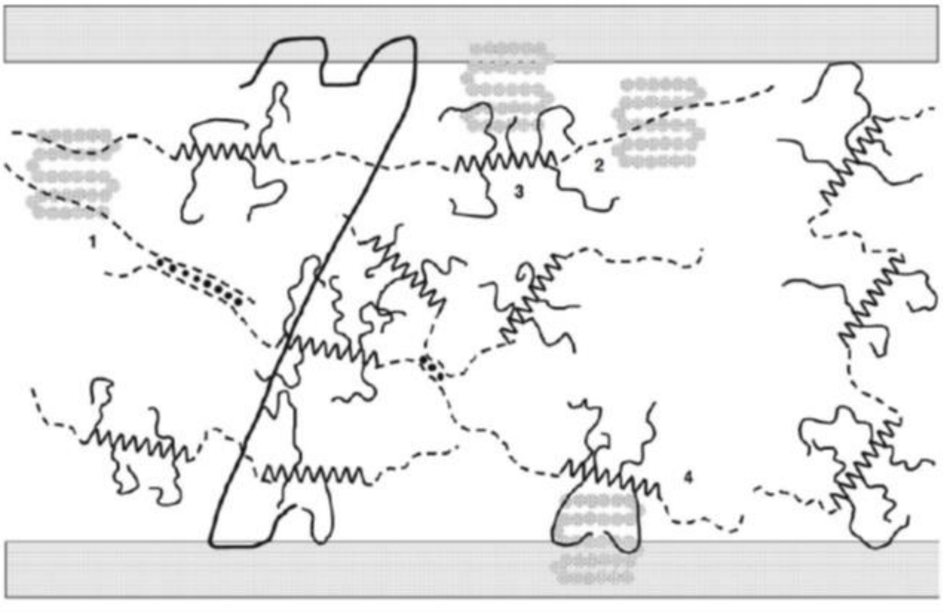


Figure 1.22 - Potential binding sites of procyanidins along apple cell wall. Shaded solid bands. cellulose microfibrils; jagged line. rhamnogalacturonan; dotted line. homogalacturonan; thin solid line. neutral sugar side chain; thick solid line. xyloglucan; grey shaded chain. procyanidin. (1) Highly methylated pectins. (2) Pectin smooth regions. (3) Pectin hairy regions. (4) Reinforced interactions between pectin neutral side chains and cellulose. Figure adapted from **Le Bourvellec et al. (2009)**.

Pectic polysaccharides

All pectic polysaccharides present the capability to interact with polyphenols **(Watrelot, Le Bourvellec, Imberty, & Renard, 2013)**. Increasing the degree of methylesterification results in stronger interactions (**Table 1.5**), attributed to increased hydrophobic effects between methyl groups of pectins and dihydropyran heterocycles (C-rings) of polyphenols, but also to the increased flexibility of polysaccharide chain **(Watrelot et al., 2013)**. The only exception is observed for pectic polysaccharide interaction with anthocyanins given the relevance of GalA charged carboxylic groups on the interactions **(Fernandes et al., 2014; Lin et al., 2016; Padayachee et al., 2012).**

Table 1.5 – Effect of pectic polysaccharide structure on their interaction with procyanidins. The results are expressed in terms of affinity constant, *K* (M^{-1}). Data adapted from **Watrelot et al. (2013)** and **Watrelot, Le Bourvellec, Imberty, and Renard (2014)**.

Polysaccharide	**Procyanidins**	**Concentration PS/PCA (mM)**	***K*** **(M^{-1})**
Apple pectin	DP9	30/30	1240
	DP30	30/30	1460
Citrus Pectin	DP9	30/30	1085
	DP30	30/30	1225
HG 0% ME	DP30	30/30	-
HG 30% ME	DP30	30/30	6220
HG 70% ME	DP30	3/60	421
RG-I	DP9	3.75/7.5	2530
	DP30	3.75/7.5	2620
Arabinan	DP9	3.75/7.5	1955
	DP30	3.75/7.5	574
RG-I with monomeric galactans	DP9	3.75/7.5	1850
	DP30	3.75/7.5	5390
Arabinan Type I galactan Xylogalacturonan	DP9	3.75/7.5	2105
	DP30	3.75/7.5	1835
Arabinan Type II galactan	DP9	3.75/7.5	872
	DP30	3.75/7.5	875
Type I galactan	DP9	3.75/7.5	2225
	DP30	3.75/7.5	1170
Galactan Xylogalacturonan	DP9	3.75/7.5	-
	DP30	3.75/7.5	-
mRG-II	DP9	3.75/7.5	-
	DP30	3.75/7.5	72
RG-II	DP9	3.75/7.5	-
	DP30	3.75/7.5	62
Galactan Xylogalacturonan	DP9	3.75/7.5	44
	DP30	3.75/7.5	112
AG-II	DP9	3.75/7.5	57
	DP30	3.75/7.5	165

PS – polysaccharide concentration; PCA- Procyanidin concentration.

It is also suggested that the Rha content increases pectin interactions with polyphenols, also attributed to the higher flexibility of the polysaccharide chain. In fact,

Watrelot et al. (2014) observed that RG-I present much higher affinity constants towards procyanidins when compared to other pectic polysaccharides. In contrast, RG-II associated to the conserved structures of pectin, present very low levels of interactions with procyanidins but are still able to form insoluble aggregates **(Riou et al., 2002; Watrelot et al., 2014)**.

Pectin neutral sugar sidechains, arabinans, galactans and arabinogalactans have low affinity constants towards procyanidins thus suggesting that the occurrence of branches might limit pectic polysaccharides/polyphenol interactions **(Brahem, Eder, Renard, Loonis, & Le Bourvellec, 2017; Riou et al., 2002; Watrelot et al., 2014)**. Arabinogalactans have been also shown to interact with anthocyanins, a property that has been attributed to the flexibility of these polysaccharides to form hydrophobic domains **(Gonçalves et al., 2018)**.

Glucans

Cellulose and starch are the most common glucans in plants differing on the conformation of their anomeric carbon: β conformation for cellulose and α for starch. Due to that, cellulose present lower interaction capacity towards polyphenols than starch. This is attributed to the compact structure of cellulose, provided by its β-linkage conformation, allowing only a surface adsorption of polyphenols. For starch, in addition to this, an encapsulation phenomenon as observed for cyclodextrins is suggested to occur, given its more porous structure that yields hydrophobic cavities **(Amoako & Awika, 2019; Le Bourvellec et al., 2005)**. It is suggested that some of these cavities consists on the inclusion of polyphenols within the amylose single helix or in the inter-helical space **(Amoako & Awika, 2019)**.

Xyloglucans were identified as being the second major class capable of interacting with polyphenols after pectin **(Le Bourvellec et al., 2005)**. Such property was attributed to their conformation in the wall, i.e., an extended and relatively rigid conformation **(Park & Cosgrove, 2015)**, allowing the interaction with polyphenols by a stacking phenomenon. However, when in solution, xyloglucans conformation may also acquire a coiled shape, characteristic of most polymers, but stiffer than highly flexible chains such as pectin **(Park**

& Cosgrove, 2015). As a result, encapsulation in more hydrophobic domains as described for cyclodextrins **(Saura-Calixto, 2011)** is also considered.

Beta-glucans, a polysaccharide present in cereals, with a →3)- D-Glc*p*-(β1→ and →4)-D-Glc*p*-(β1→ backbone, is characterized for being organized in random coil, yet semi-flexible structure when in solution. Because of that, beta-glucans are able to establish intermolecular interactions between different polysaccharide chains leading to their aggregation **(Cui & Wang, 2009)**. When in the presence of polyphenols, this phenomenon is enhanced, a fact that is presumed to result from beta-glucan/polyphenol interactions **(Li et al., 2019)**. Laminarins, with →3)-D-Glc*p*-(β1→ backbone with intrachain links with →6)-D-Glc*p*-(β1→ and branching at C-6 showed also the capability to interact with tannins, although at very low extent **(Mekoue Nguela et al., 2016)**.

Other polysaccharides

In addition to the polysaccharides naturally occurring in plant cell walls, other polysaccharides from non-conventional sources have also been studied regarding their interaction with polyphenols. The most studied polysaccharides are mannoproteins, characterized by a →6)-D-Man*p*-(α1→ decorated by →2)-D-Man*p*-(α1→ and →3)-D-Man*p*-(α1→ side chains. These polysaccharides have been shown to interact with anthocyanins **(Gonçalves et al., 2018),** explaining the observed retention of these by yeast cell walls **(Mazauric & Salmon, 2006; Mekoue Nguela, Vernhet, Sieczkowski, & Brillouet, 2015; Morata et al., 2003).** This has been attributed to the occurrence of phosphate groups in mannoproteins, which provides them a surface negatively charged, as well as an increased hydrophilicity **(Gonçalves et al., 2018)**. Additionally, mannoproteins have the capability to interact with wine tannins **(Mekoue Nguela et al., 2015),** resulting in the inhibition of haze by tannin aggregation **(Riou et al., 2002)**. Likewise, hyaluronan, a polysaccharide with a backbone composed of repeating units of →4)-D-Glc*p*A-(β1→3)-D-Glc*p*NAc-(β1→, has also been shown to interact with tannins forming stable colloidal suspensions **(Carn et al., 2012).**

Another survey on polysaccharide/polyphenol interactions is based on the capability of carbohydrates to inhibit the interactions of proteins with polyphenols. In addition to pectic polysaccharides, xanthan, carrageenan, arabic gum have been shown to effectively inhibit protein/polyphenol interactions while glucose, dextran and β-cyclodextrin are ineffective **(de Freitas, Carvalho, & Mateus, 2003; Gonçalves, Mateus, & De Freitas, 2011; Mateus, Carvalho, Luís, & de Freitas, 2004; Soares, Mateus, & de Freitas, 2012)**. This effect is proposed to result from the ability of polysaccharides to form a ternary complex protein–polyphenol–carbohydrate, thereby enhancing its solubility (**Figure 1.23**), or by a competing with proteins towards polyphenols **(Mateus et al., 2004)**.

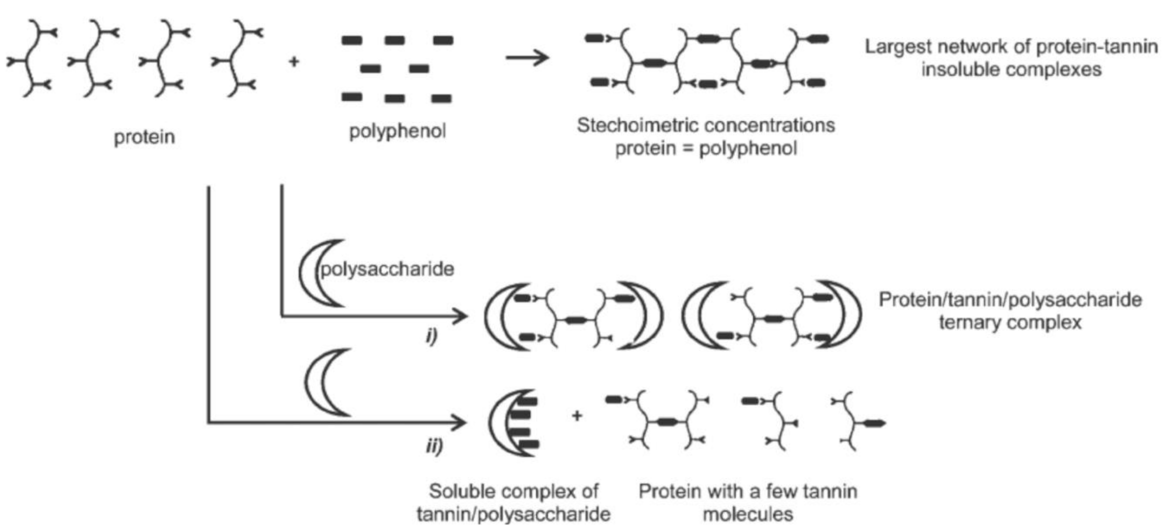


Figure 1.23 - Possible mechanisms (i and ii) involved in the inhibition of the aggregation of tannins and proteins by polysaccharide. Adapted from **Mateus et al. (2004).**

1.4.1.4 Effect of the environmental parameters

Varying the pH between 2.5 and 6 was shown to have no significant effect on the affinity of procyanidins and other non-charged polyphenols because these molecules are weak acids with pKa of 9–10 **(Le Bourvellec et al., 2004; Renard et al., 2001).** However, for charged polyphenols as chlorogenic acid (pKa of 3.3), increasing the pH from 3.5 to 5 results in a decreased interaction towards the cell wall material, given the electrostatic repulsion between their charged carboxylic groups **(Liu, Lopez-Sanchez, Martinez-Sanz, Gilbert, & Gidley, 2019)** and those of GalA (pKa = 3.5) of pectins. A similar phenomenon is observed for neutral phenolic structures at very alkali conditions. In the case of tannic acid (pKa about 6) for instance, for a pH superior to its pKa value, hydroxyl groups become deprotonated decreasing its interaction with beta-glucans due to electrostatic repulsion **(Li et al., 2019).** For anthocyanins, the loss of charge as a result of increasing the pH may negatively affect their interactions with negatively charged polysaccharides **(Fernandes et al., 2014),** but appears to enhance towards the neutral ones by favouring the establishment of hydrophobic interactions **(Phan, D'Arcy, & Gidley, 2016).**

The ionic strength is another parameter with a huge diversity among food products and may impact on polysaccharide/polyphenols interactions. In theory, in case hydrophobic interactions represent the major mechanism, the interactions between polyphenols and polysaccharides are strengthened with ionic increments **(Le Bourvellec et al., 2004; Li et al., 2019; Renard et al., 2001)**. However, when the interactions do not increase with ionic strength, the involvement of hydrophobic interaction becomes unlikely to be relevant in comparison to hydrogen bonding and electrostatic interactions **(Le Bourvellec et al., 2004)**.

Temperature influences the interactions between polysaccharides and polyphenols. This results from the relevance of hydrogen bonds, which formation is an exothermic process, and hydrophobic interactions, which are endothermic, in the stabilization of polyphenol/polysaccharide complexes. It is commonly reported to occur a decrease of interactions with the increase of temperature due to hydrogen bonding disruption, but depending on the structures involved, different susceptibilities to temperature variations might be observed **(Le Bourvellec et al., 2004; Phan et al., 2016)**. For instance, low molecular weight procyanidins are more susceptible to temperature increments than those of higher degree of polymerization **(Le Bourvellec et al., 2004)** thus suggesting that hydrogen bonding represent the most relevant mechanism of interaction in the latter while hydrophobic interactions are dominant for the former.

The occurrence of stronger hydrogen bond acceptors, such as urea, may negatively impact the stability of polysaccharide/polyphenol interactions due to hydrogen bonding disruption. On the other hand, amphiphilic molecules as sodium dodecyl sulphate (SDS) inhibit polysaccharide/polyphenol interactions by affecting the hydrophobic interactions **(Le Bourvellec et al., 2004; Li et al., 2019)**. Solvents such as ethanol, also result in a decreased interaction. In the case of procyanidins for instance, the smaller polyphenolic polymers interaction with the cell wall material is more affected by the presence of ethanol than those of higher molecular weight **(Le Bourvellec et al., 2004)**.

1.4.1.5 Effect of processing

One of the most common processing procedures is drying, a process that may affect among other parameters, the surface areas of the material. In this context, hot air drying appears to decrease the surface area of cell wall material, i.e., porosity resulting in half of the affinity towards polyphenols of the original cell wall material (**Table 1.6**) **(Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012; Liu et al., 2019)**. Freeze-drying has even a more pronounced negative effect on cell wall material porosity, and therefore, on its interactions with polyphenols. This is attributed to the smoother and compact structure that restricts the number of hydroxyl groups available for polyphenol binding **(Le Bourvellec et al., 2012; Liu et al., 2019; Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert, & Gidley, 2017)**. In contrast, solvent exchange drying resulted in a structure of higher porosity compared to that resulting from hot-air and freeze-drying, thus yielding a structure with superior retention of polyphenols **(Le Bourvellec et al., 2012)**.

Boiling is one of the most common processing procedures and is known to cause leakage of cell wall components. As a result, cell wall material capacity to interact with polyphenols after boiling is reduced, attributed to the leakage of highly methylated pectic polysaccharides **(Le Bourvellec et al., 2012)**. On the other hand, boiling causes starch jellification, which changes from a compact semi-crystalline to a swollen structure. As a result, starch becomes able to interact with polyphenols **(Gómez-Mascaraque et al., 2017)**. Contrastingly, boiling or even autoclaving has no effect on polyphenol bonding to cellulose **(Liu et al., 2017)**.

Enzymatic treatments towards polysaccharides have also been shown to affect the interaction between the cell wall and polyphenols. Treatments with commercial preparations of PG, pectin lyase and PME and also β-glucanase and proteases do not cause the disruption of polysaccharide/polyphenol complexes as polyphenols impair the access of enzymes to polysaccharides (their substrates) **(Castro-López, Gómez-Plaza, Ortega-Regules, Lozada, & Bautista-Ortín, 2016; Le Bourvellec et al., 2009)**. However, when enzymatic digestion of the cell wall material is performed before contact with polyphenols, the interactions between the cell wall material are reduced due to the leakage and degradation of key polysaccharides **(Castro-López et al., 2016)**. Pectin extraction using chelating agents followed by hemicellulose extractions with KOH were also been shown to decrease the affinity of the cell wall material towards polyphenols **(Ruiz-Garcia, Smith, & Bindon, 2014)**.

Table 1.6 - Effect of processing on apple cell wall material interaction with procyanidins. Maximum number of binding sites (Qmax) and affinity constant (K_L) of the interactions, determined by the Langmuir isotherms, are presented. Data from **Le Bourvellec and Renard (2005)** and **Le Bourvellec et al. (2012)**.

Polysaccharide	**Procyanidins**	**Langmuir**	
		Q_{max} (g/g)	K_L (L/g)
Apple CWM	DP3	1.48±0.82	0.04±0.03
	DP10	1.05±0.14	0.25±0.07
	DP70	0.93±0.07	0.85±0.22
	DP35	0.93±0.09	0.61±0.17
	DP7 Gal 5%	1.18±0.20	0.14±0.04
	DP 8 Gal 22%	0.96±0.07	0.62±0.14
Apple CWM dried at 100ºC	DP3	1.77±0.42	0.04±0.01
	DP10	1.59±0.19	0.11±0.02
	DP70	1.25±0.18	0.25±0.09
	DP35	1.32±0.16	0.24±0.06
	DP7 Gal 5%	2.14±0.64	0.06±0.02
	DP8 Gal 22%	1.56±0.15	0.23±0.06
Apple CWM	DP9	0.62	0.40
Freeze Drying		0.67	0.21
Solvent drying		0.79	0.22

1.4.2 Covalent interactions

Polyphenols covalently linked to polysaccharides can be found as a result of biosynthetic processes, being reported to occur in arabinogalactans linked to ferulic acid derivatives in spinach **(Fry, 1983),** sugar beet **(Levigne et al., 2004; Rombouts & Thibault, 1986)** and arabinoxylans in bamboo **(Ishii, 1991)**. However, covalent linkages between cell wall material and polyphenols may also occur as a result of polyphenol oxidation reaction initiated by tissue disruption during processing **(Ferreira et al., 2002; Le Bourvellec et al., 2009; Nicolas et al., 1994)**.

Covalent bonding between polyphenols and polysaccharides originated by oxidation reactions has been demonstrated only by indirect evidences in olives **(Coimbra, Waldron,**

& Selvendran, 1995), cauliflower **(Femenia, Rigby, Selvendran, & Waldron, 1999)**, wine **(Saura-Calixto & Díaz-Rubio, 2007)**, pear **(Ferreira et al., 2002)** and apple **(Stevens & Selvendran, 1984)**. These evidences were focused on the resilience of polyphenols to be extracted with aqueous/organic solvent solutions following oxidation reactions **(Ferreira et al., 2002; Le Bourvellec et al., 2005; Pérez-Jiménez et al., 2013).** The incapacity to separate polyphenols from polysaccharide-rich fraction using several chromatographic techniques also supports the occurrence of these covalent linkages **(Stevens & Selvendran, 1984)**. The mechanisms behind their formation remains to be elucidated but it is known that polyphenol quinones play a relevant role in the process by direct reaction with carbohydrates **(Le Bourvellec et al., 2005).** Quinones may also produce reactive oxygen species able to react with polysaccharides **(Faure, Sánchez-Ferrer, Zabara, Andersen, & Nyström, 2014; Simões et al., 2016)** causing their oxidation and thus, the formation of several compounds possessing carbonyl groups **(Boulos & Nyström, 2016; Mäkelä et al., 2017)** (**Figure 1.24**).

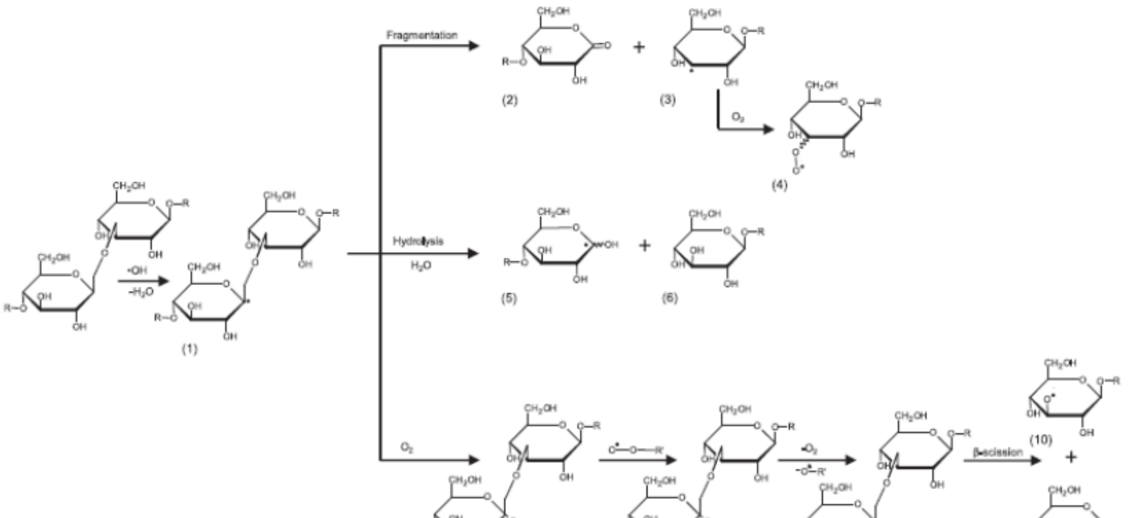


Figure 1.24 – Schematic representation of oxidative cleavage of →3)-D-Glc*p*-(β1→. Adapted from **Faure et al. (2014)**.

In contrast to polysaccharides, direct evidences of the covalent bonding of polyphenols to protein as a result of oxidation reactions have been demonstrated. For instance, 5-*O*-caffeoylquinic acid and (-)-epicatechin (and therefore procyanidins) may form oxidation products with sulphur containing amino acids as cysteine and methionine **(Cao, Zhang, Zhang, Zhong, & Qian, 2009; Ishii et al., 2011)**. Amine groups (NH_2) might also be involved in the formation of covalent adducts involving amino acids as in the case of arginine yielding Schiff bases (compounds with C-N double bond). Lysine, histidine, tryptophan and, tyrosine appears to be those of higher reactivity with quinones and arginine, asparagine and glutamine the lower **(Prigent et al., 2008)**.

As a result of an acidic medium and high temperatures, the interflavanic linkages might be cleaved yielding carbocations. These carbocations are prone to react with other nucleophiles, a property considered for procyanidin quantification by using toluene-α-thiol **(Guyot et al., 1997)**. However, other nucleophilic agents as cysteine might be involved resulting in the formation of covalent adduct with proteins **(Herrero-Martínez et al., 2003)** or even polysaccharides **(Le Bourvellec et al., 2013)**. This phenomenon, together with oxidation of some of the carbocations was attributed to pear pinking (**Figure 1.24**) during canning **(Le Bourvellec et al., 2013)**.

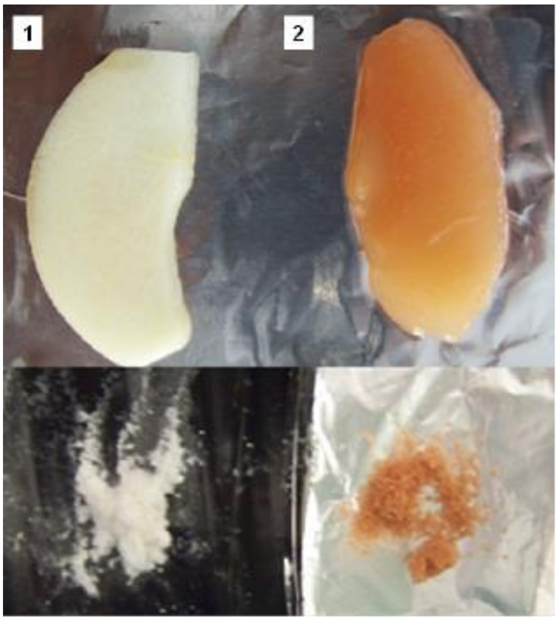


Figure 1.25 – Pinking of William pear after heat treatment in acidic conditions **Le Bourvellec et al. (2013)**. 1) raw pear and 2) pear processed at 95°C and pH 2.7 for 418 min. Below are present the cell wall powders.

The formation of covalent adducts between carbohydrates and polyphenols can also be achieved at very high temperature (<175 ºC) and dry conditions as those observed during coffee roasting **(Moreira et al., 2015; Moreira et al., 2017)**. In a model system composed of arabinotriose and 5-*O*-caffeoylquinic acid, it was observed that arabinogalactans might form hybrid molecules composed by one or two phenolics, bearing a sugar moiety composed by quinic or caffeic acid of 5-*O*-caffeoylquinic acid **(Moreira et al., 2015)**. The release of some of these compounds by alkaline fusion suggests that polyphenol can be linked to the polymeric material by aryl-ether, stilbene type, and/or biphenyl linkages **(Coelho et al., 2014).** Glycosidic linkages have also been observed to be established between polyphenols and polysaccharides via non-enzymatic transglycosylation reactions during coffee roasting **(Moreira et al., 2017).**

1.4.3 Consequences of interactions

Polysaccharide/polyphenols interactions have been shown to be dependent of processing, environmental parameters, but most of all, of the structural features of the compounds involved. In this context, consequences can be expected as a result of the establishment of polysaccharide/polyphenol interactions.

1.4.3.1 Juice extraction

During fruit processing, two phenomena modulate the transfer of polyphenols to the juice fraction: polyphenol oxidation catalysed by PPO and the establishment of non-covalent interactions between the fruit polyphenols and the cell wall material. In the case of apple juice this is reflected on the transfer of at least of 80% of low molecular weight polyphenols (hydroxycinnamic acids, flavonols, and dihydrochalcones) and less than 50% of those of higher molecular weight (procyanidins) **(Guyot et al., 2003; Le Bourvellec et al., 2007)**. In grape juice extraction for winemaking, the same process is observed where the polymeric polyphenols are strongly retained in the pomace thus affecting the final quality of the juice **(Bindon et al., 2012; Bindon, Smith, Holt, et al., 2010)**. From a technological point of view, studies have demonstrated that increasing the pressing temperature during juice extraction, the transfer of polyphenols from the fruit to the juice fraction can be increased in about 30% **(Renard et al., 2011).** Enzymatic treatment, especially before pressing, has also been suggested as an approach to maximize polyphenol transfer to juice **(Castro-López et al., 2016).** Alternatively, the use of ripe fruits instead of overripen ones is suggested **(Brahem et al., 2017; Garrido-Bañuelos et al., 2019).**

1.4.3.2 Winemaking

In addition to the modulation of polyphenol transfer to grape juice fraction for winemaking, another approach has been considered by taking advantage of polysaccharide/polyphenol interactions. This approach is based on the use of polysaccharides as substituents of proteins for wine finning **(Bautista-Ortín et al., 2014; Mekoue Nguela et al., 2016)**. These include the use of yeast lees, rich in mannoproteins **(Mazauric & Salmon, 2006; Mekoue Nguela et al., 2016; Razmkhab et al., 2002)** or fruit cell walls. Alternatively, isolated polysaccharide fractions can be used to inhibit the haze formation resulting from tannins aggregation **(Riou et al., 2002)**. The disadvantage of this approach is the higher amounts of polysaccharides required for an effective finning when compared to proteins **(Bindon et al., 2012)**.

Another approach explored in wine is the use of polysaccharides to compete with proteins, modulating the perceived astringency of wine **(de Freitas et al., 2003; Mateus et al., 2004)**. The precise mechanisms of this phenomenon are not yet very well known, but it is accepted to result from the inhibition of the interaction between salivary proteins (especially proteins rich in proline) and polyphenols causing the sensation of astringency **(Soares et al., 2012)**. Furthermore, these interactions can be explored in the scope of wine colour stabilization **(Razmkhab et al., 2002; Rodrigues, Ricardo-Da-Silva, Lucas, & Laureano, 2012)**. Due to that, industries can take advantage of such interactions for tailored high-quality wines regarding their astringency or colour.

1.4.3.3 Extraction of components from byproducts

Over the last few years several efforts have been made to reduce agro-industrial wastes by their byproduct's valuation. A very common approach consists on the recovery of remnant polyphenols that were retained in the solid residue remaining after juice extraction.

Conventional water or hydroethanolic extractions are commonly performed for a food-industry perspective **(Fernandes et al., 2019)**. However, such approach fails to disrupt most of the interactions established between polysaccharides and polyphenols thus leading to the non-recovery of such compounds. To overcome this, methanol and acetone solutions are suggested given their chaotropic nature **(Le Bourvellec et al., 2004; Li et al., 2019)**. Given the occurrence of polyphenol oxidation reactions, part of the polyphenolic pool may form covalent adducts with polysaccharides and proteins constituting the residue remaining after juice extraction **(Le Bourvellec et al., 2009)**. In such cases, the polyphenols become non-extractable, presenting resilience to the commonly used aqueous/organic solvents **(Pérez-Jiménez et al., 2013)**. Studies using new emerging technologies such as ultrasound assisted extraction **(Pingret et al., 2012)** or microwave superheated water extraction **(Bai et al., 2010)** have been shown to increase polyphenol extraction yields, which can be attributed to cell wall disintegration and recovery of the polysaccharide/polyphenol covalent complexes. Another consequence related to oxidation reactions is the limitation regarding the extractability of polysaccharides due to polysaccharide/polyphenols interactions, as well as the accessibility of polysaccharide-cleaving enzymes **(Le Bourvellec et al., 2009)**.

1.4.3.4 Bioavailability of molecular components

The nutritional consequences of polysaccharide/polyphenol interactions can be estimated in terms of their effect on the bioaccessibility of polyphenols. Studies with wine showed that wine polysaccharides may retard anthocyanins diffusion along a dialysis membrane, a phenomenon that reflects that dietary fibre is able to limit the amount of polyphenols available for absorption **(Gonçalves et al., 2018; Gonçalves et al., 2012).** In fact, studies with carrot plant cell walls showed that along simulated gastric digestion, the release of polyphenols (anthocyanins and phenolic acids) is minimal thus suggesting that most of the polyphenols are transported to the colon where they would be expected to be released by the action of cell wall degrading bacteria **(Padayachee et al., 2013).** Once in the colon, polyphenols are then jointly metabolized with polysaccharides by gut microbiota, yielding metabolites capable of exerting several biological properties. For apple, when bonded to polysaccharides, procyanidins metabolization is reduced, and when free, polysaccharide fermentability e negatively affected. Nevertheless, both yield metabolites with close anti-inflammatory properties in gut cells **(Le Bourvellec et al., 2019)**. For carob pod and grapes, it was shown that the colonic fermentation of polyphenols in the presence of polysaccharides enhanced the yield of polyphenol metabolites **(Fulgencio et al., 2010)**. Nevertheless, it has been generally perceived that polysaccharide/polyphenol complexes exhibit potential health benefits, more precisely at the gastrointestinal level, such as increased antioxidant and antiproliferative properties, modification of gene expression, among others **(Pérez-Jiménez et al., 2013)**. Further research is however required for a better understanding of their health effects of dietary antioxidants in general.

Chapter 2- Materials and methods

2.1 Samples

Agro-industrial apple pomace samples used in this work were provided by Indumape, S.A., Pombal, Portugal following milling, enzymatic digestion (amylase, pectin lyase, and PG), and pressing procedures of a mixture of apples for a minimum period of 3 hours. Royal Gala was the major apple variety composing the pomaces. The apple pomaces were transported to the lab at 4 ºC in less than 1 h, in order to minimize PPO activity, and stored at -20 ºC until freeze-drying and further analysis **(Nicolas et al., 1994)**.

The pomace used for the extraction and characterization purposes (API) was provided in September 2015 while those used for the drying experiments, APII and APIII, were provided in September and November 2014, respectively.

2.2 Chemistry of apple pomace polyphenols

2.2.1 Preparation of apple pomace extracts

API was frozen in liquid nitrogen, milled and stored at -20 ºC until freeze-drying and further analysis. Afterwards, water/acetic acid (99/1; v/v), pH 2.5, was added to API at a ratio of 1:5 (w/v) and submitted to hot water extraction (100 ºC) for 1 h (**Figure 2.1**). The soluble fraction was then separated from the insoluble material by filtration in a glass microfibre filter (MFV3, Filter Lab) followed by a G3 sintered funnel. The collected insoluble material was then submitted to three additional aqueous extractions to recover all water-soluble material. The water-soluble fractions were combined, concentrated on a rotary evaporator at 45 ºC, frozen and freeze-dried, yielding a hot water extract (HWE).

The residue remaining after hot water extraction was sequentially extracted following a similar procedure than before, first using five times acetone/water/acetic acid (60/39/1; v/v/v) mixture, and then acetone/8 M urea/acetic acid (60/39/1; v/v/v) mixture. The filtrates from the 60% acetone extraction were combined, evaporated under reduced pressure at 40 ºC to remove acetone and freeze dried thus yielding a 60% acetone extract (60% Ac). In turn, the acetone/8 M urea/acetic acid filtrate was rotary evaporated to remove the acetone, resuspended in water/acetic acid (98/2; v/v) and subjected to solid-phase extraction with C18 sep-pak cartridges (SPE-C18, Supelco-Discovery, 20g) to remove urea. For this purpose, the column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of water/acetic acid (98:2; v/v). The sample was loaded into the cartridge, followed by washing with 140 mL of water/acetic acid (98/2; v/v). The retained fraction was then obtained using acetone/acetic acid (98/2; v/v), concentrated under reduced pressure at 40 ºC to remove acetone, frozen and freeze-dried, yielding the 60% acetone 8 M urea extract (60% AcU). Urea was removed from the hot water/acetone/acetone 8 M urea insoluble cell wall material by dialysis (12-14 kDa cut-off membrane, Medicell), followed by freezing and freeze-drying.

The freeze-dried hot water/acetone/acetone 8 M urea insoluble cell wall material was submitted to three sequential cycles of microwave superheated water (MWE) extractions using a MicroSYNTH Labstation (Milestone srl., Bergamo, Italy) equipment with high pressure teflon-coated vessel of 100 mL capacity. A control reactor with pressure and temperature sensors was used for monitoring the extraction experiments. The maximum output delivery power was 1000 W until reaching the set point temperatures for the desired time:

1. 140 ºC for 5 min at a liquid/solid ratio of 14:1 and continuous stirring for Cycle 1 (optimal conditions for pectin extraction established by **Wang and Lü (2014)**). The reactor was cooled at room temperature, and the unsoluble material obtained after filtration was subjected to a second extraction under the same conditions (Cycle 2).
2. 180 ºC for 2 min at a liquid/solid ratio of 28:1 and continuous stirring for the Cycle 2 insoluble cell wall material (Cycle 3), the optimal condition for xylan extraction established by **Coelho, Rocha, Saraiva, and Coimbra (2014)**. After cooling, the slurry was filtrated, the final residue frozen and freeze-dried.

The freeze-dried replicates resulting from cycle 1, 2 and 3 were combined and solubilized in water at a concentration of 10 mg/mL at 4 ºC. The insoluble material (WPp) was recovered by centrifugation at 20,000*g* at 4 ºC. To the water-soluble material, absolute ethanol was added to reach 50% (v/v) and centrifuged yielding a precipitate (Et50Pp). To the 50% (v/v) soluble material, absolute ethanol was added to reach 80% (v/v). The solution was then centrifuged yielding a precipitate (Et80Pp) and a supernatant (Et80Sn). All fractions were evaporated under reduced pressure to remove ethanol at 45 ºC, frozen and freeze-dried.

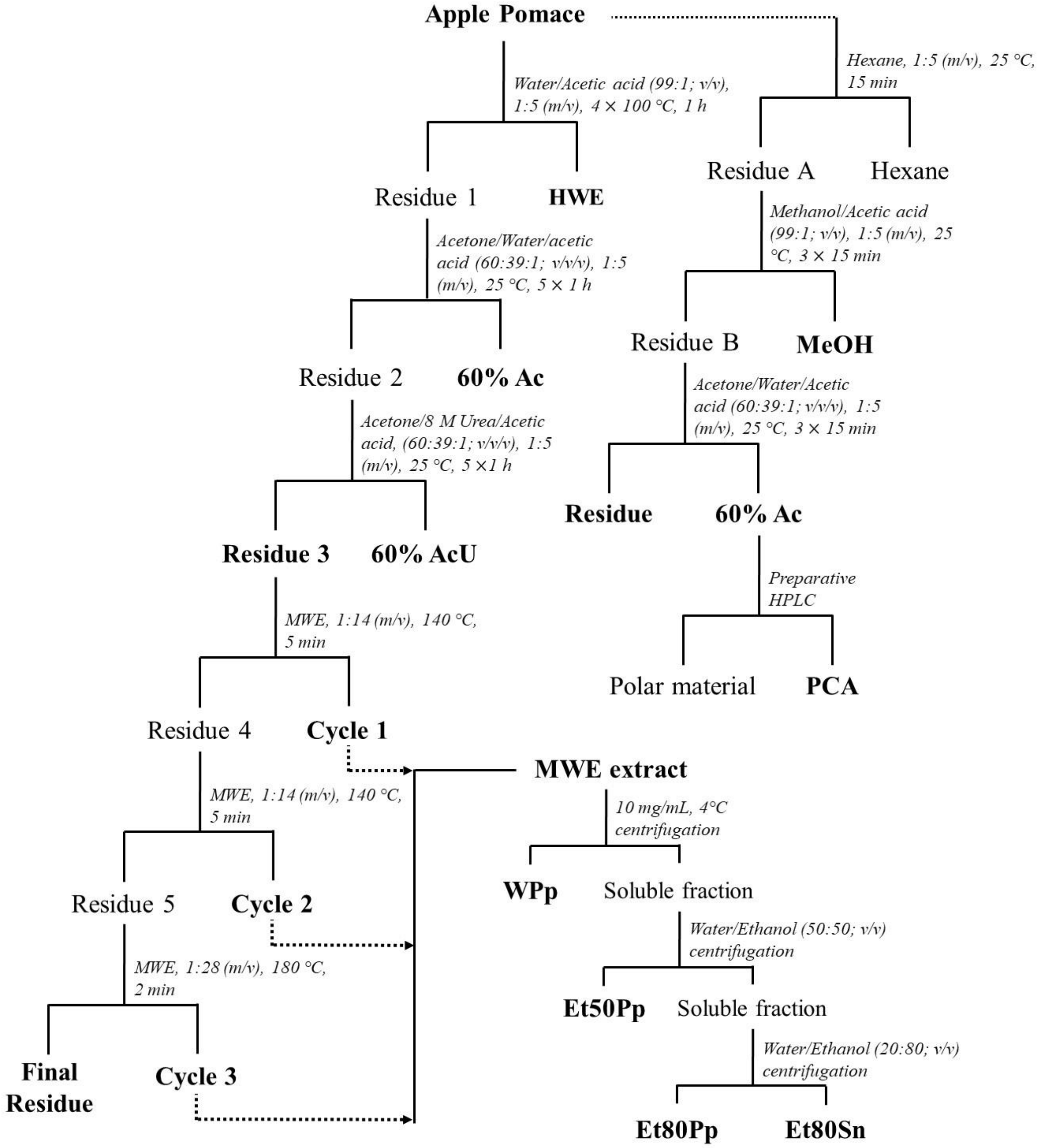


Figure 2.1 - Schematic representation of the extraction and fractionation procedures performed for apple pomace (API). Fractions studied in this work are highlighted in bold.

2.2.2 Isolation of procyanidin rich fractions from apple and apple pomace

Procyanidin were isolated following the general procedure preciously described by **Guyot et al. (1997)**. Briefly, about 150 g of freeze-dried API or Golden Delicious apple (90% of parenchyma and 10% of skins) were defatted with 750 mL of hexane for 15 min and filtered in a G3 funnel (**Figure 2.1**). The filtrate was discharged while the residue was extracted with 750 mL of methanol/acetic acid (99/1; v/v) for 15 min, followed by filtration of the suspension in a sintered glassware G3 funnel. The resulting residue was reextracted two additional times following the same procedure. At the end, the filtrates from the methanolic suspensions were combined, rotary-evaporated at 40 ºC and freeze dried. The final residue of the methanolic extractions was extracted with 750 mL of acetone/water milliQ/acetic acid (60/39/1; v/v/v) for 15 min for three times. The obtained hydroacetonic suspensions were filtered, evaporated at 40 ºC, frozen a freeze-dried.

The isolation of a procyanidin rich-fraction from the 60% acetone extract was performed by high performance liquid chromatography using a Hibar 205x25 mm Lichrospher 100 RP-18 12 µm (Merck, Darmstadt, Germany) column at preparative scale. The system was composed by two high pressure pumps PU-2077 plus, one degasser, one interface LC-NetII/ADC, one injection valve rheodyne 3752i-038 (Rhonert Park, CA, USA) and a detector UV-visible UV-2077 plus, controlled by the software ChromNav (Jasco, Tokyo, Japan). The column was pre-conditioned with water/acetic acid (97.5/2.5; v/v) for 30 min at a flow of 35 mL/min and the detection performed at 280, 320 and 520 nm. The supernatant obtained after 60% acetone extract (5 g) dissolution in 100 mL of water milliQ/acetic acid (97.5/2.5; v/v), and centrifugation (16800g, 15 min), was then injected in the column and rinsed with the same solvent for 1 h for sugars removal. Procyanidins were eluted at a flow of 25 mL/min using a gradient of water milliQ/acetic acid (97.5/2.5; v/v) (eluent A) and acetonitrile (eluent B). The gradient started with 0-30% B (0-5 min); 30-30 % B (5-20 min); 30-90% B (20-22 min); 90-90% B (22-27 min); 90-50% B (27-30 min). Polyphenols were collected by monitoring the absorbance at 280 nm. Following, phenolic fractions were rotary evaporated at 40 ºC, frozen and freeze-dried, yielding the procyanidins fractions for apple (Apple PCA) and apple pomace (AP PCA).

2.3 Fractionation of the hot water extract (HWE)

The HWE was fractionated as summarized in **Figure 2.2** in order to separate hydrophilic from the hydrophobic material. Briefly, the HWE was subjected to solid-phase extraction with C_{18} sep-pak cartridges (SPE-C_{18}, Supelco-Discovery, 20 g) by a two-step fractionation process. First, the column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of phosphate buffer (10 mM, pH 7). The sample, dissolved in the same buffer solution, was added to the cartridge and washed with 140 mL of phosphate buffer followed by water/acetic acid solution (98:2; v/v) to remove salts. The retained fraction at pH 7 (Fr7) was then eluted using methanol/water/acetic acid (70/28/2; v/v/v), concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried.

The pH of the solution containing the non-retained material in the C_{18} cartridge at pH 7 was adjusted to 3 with acetic acid. This fraction was then submitted to solid-phase extraction using the same eluents as before, except for phosphate buffer. The use of water/acetic acid (98/2; v/v) and methanol/water/acetic acid (70/28/2; v/v/v) as eluents allowed to obtain a hydrophilic (non-retained neither at pH 7 nor at pH 3 (NrFr)) and hydrophobic materials at pH 3 (Fr3). Both fractions were concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried. The NrFr, the Fr7 and Fr3 fractions were then sequentially dialysed in a 12-14 kDa cut-off membrane (Medicell) and 1 kDa cut-off membrane (Medicell), yielding fractions with >12 kDa and 1< x <12 kDa, respectively. All fractions were concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried. This procedure was also performed for sugar beet arabinan used as standard. The polysaccharide was solubilized in water/acetic acid (98:2; v/v) and submitted to solid-phase extraction. The obtained fractions were dialysed in a 12-14 kDa cut-off membrane.

The Fr7 and NrFr retained in the 12 kDa membranes were additionally suspended in water at a concentration of 10 mg/mL at 4 ºC and centrifuged at 20,000g, yielding a water insoluble (WPp) and soluble fractions. The soluble fraction was then rotary evaporated until 1/6 of its initial volume followed by the addition of absolute ethanol until a concentration of 50% (v/v) and centrifugation yielding a precipitate (Et50Pp). To the 50% (v/v) soluble material, absolute ethanol was added to reach 80% (v/v) and centrifuged, yielding a precipitate (Et80Pp) and a supernatant (Et80Sn). All fractions were evaporated under reduced pressure to remove ethanol, frozen, and freeze dried.

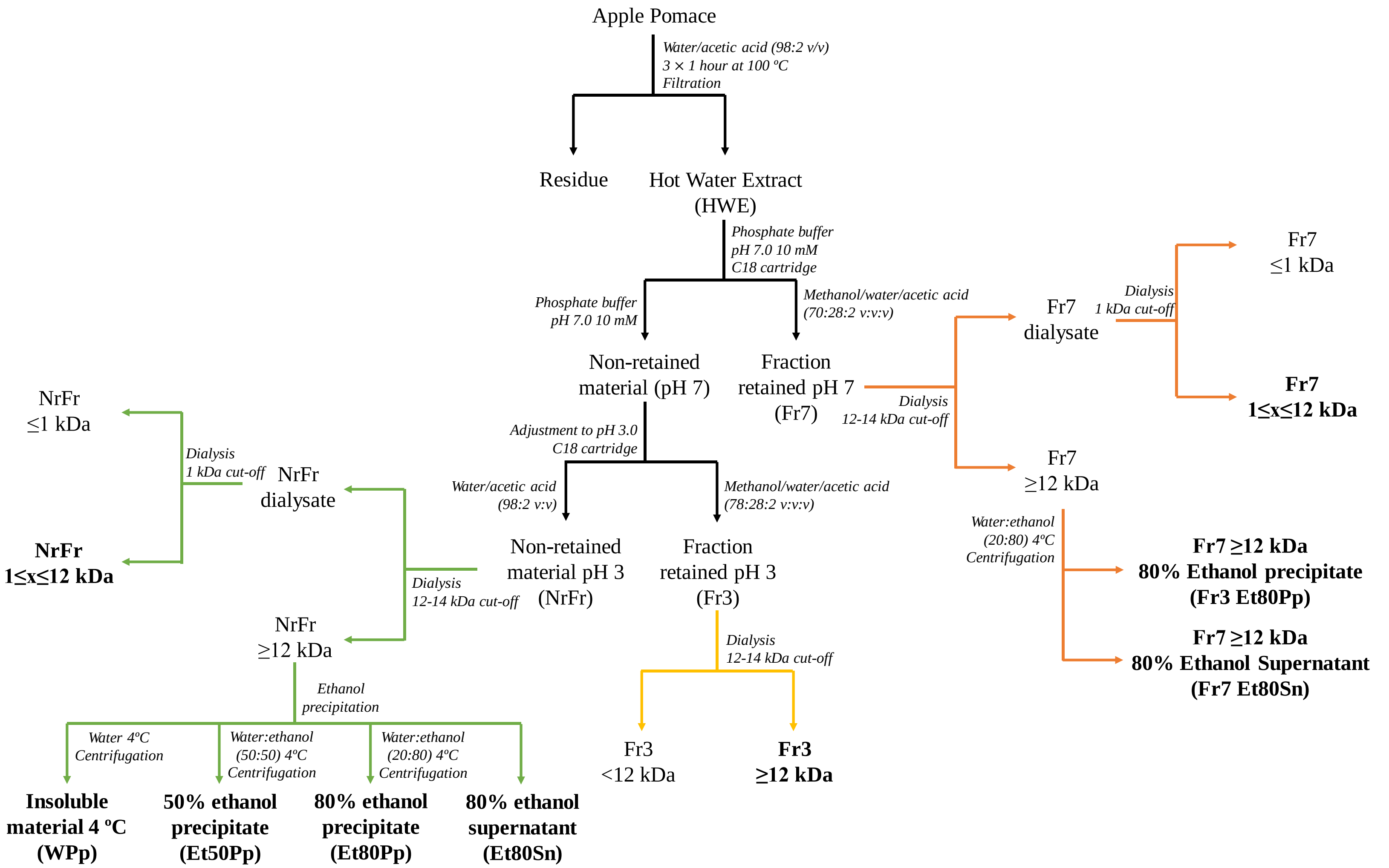


Figure 2.2 - Schematic representation of the fractionation procedure adopted for the different apple pomace carbohydrate fractions. The additional fractionation procedures adopted for the material retained in C18 cartridges at pH 7 (Fr7), pH 3 (Fr3) and non-retained (NrFr) are highlighted in orange, yellow and green, respectively. The fractions studied in this work are highlighted in bold.

2.4 Interaction of apple polysaccharides with polyphenols

2.4.1 Interactions with 5-*O*-caffeoylquinic acid and phloridzin

To determine possible interactions between arabinans and low molecular weight polyphenols, 1 mL of sugar beet arabinan (SB Ara) or debranched arabinan (SB dAra) solution (2 mg/mL) were introduced in dialysis membranes filled with 1 mL of a 5-*O*-caffeoylquinic acid:phloridzin mixture (100 µg/mL of each polyphenol). This proportion of polysaccharide/polyphenols was selected to determine their interactions by 1) the retention coefficient in the first dialysis and 2) observe the asymptote of the diffusion behaviour with the minimum number of dialysis **(Gonçalves et al., 2018)**. The dialysis experiments were performed using malate buffer (20 mM, pH 3.8) during 10 h, with buffer renewal (20 mL) renewal every 2 h, yielding five dialysate solutions for each experiment: Ara1-Ara5 for SB Ara trial, and dAra1-dAra5 for SB dAra trial. The blank assay consisted in the dialysis of the phenolic solutions in the absence of arabinan, also resulting in five dialysate solutions, B1-B5. For 5-*O*-caffeoylquinic acid and phloridzin, a retention coefficient (RC) was calculated using the **Equation 2.1** and based on the percentage of released polyphenol in the first dialysate solution.

$$\mathrm{RC} = 1 - \frac{\%Ps1}{\%B1}$$ **Equation 2.1**

%Ps1 corresponds to the relative amount of each polyphenol released to the first dialysate solution in the presence of polysaccharides (SB Ara or SB dAra) and %B1 is the relative amount of polyphenol released to the first dialysate solution in the absence of polysaccharide. A RC=0 means that SB Ara or SB dAra did not have any capacity of retention, i.e, absence of interactions, while RC=1 represents 100% of retention, i.e, that all polyphenols were retained by the polysaccharides. The normalized retention capacity (NRC) was also calculated for each polyphenol using **Equation 2.2**.

$$\mathrm{NRC} = \frac{Ps_5 - B_5}{PS}$$ **Equation 2.2**

where PS5 and B5 corresponds to the amount (µg) of each polyphenol retained after the dialysis experiments in the presence and absence of polysaccharides, respectively. PS corresponds to the amount (mg) of polysaccharide used in the experiment.

2.4.2 Interactions with procyanidins

2.4.2.1 Isothermal titration microcalorimetry

The enthalpy changes associated with polysaccharide−procyanidin interactions at 25 °C, were measured on a TAM III microcalorimeter (TA Instruments, New Castle, USA). Isolated procyanidins from apple Golden delicious variety (Section 2.2.2), at a 93% level of purity and a DP of 9 were used (**Section 2.2**). (-)-Epicatechin was the major constituent of procyanidins either as extension (89%) or terminal units (9%) while (+)-catechin was only present as terminal unit (2%).

Procyanidins (50 mmol/L in (-)-epicatechin equivalents) and polysaccharide fractions (7.5 mmol/L and 15 mmol/L in GalA equivalents for arabinans and apple pomace pectic polysaccharides, respectively) were prepared in citrate/phosphate buffer (pH 3.8, ionic strength 0.1 mol/L) and degassed prior to measurements. The reference cell was filled with buffer while the titration cell of the calorimeter was loaded with 850 µL of polysaccharide solution. The procyanidin solution was loaded into an injection syringe and titrated (5 µL aliquots with an injection time of 5 s) into the titration cell of the calorimeter. A total of 50 injections were performed with separating delay of 20 min. The content of the titration cell was constantly stirred throughout the experiment at 90 rpm to ensure mixing. As recommended for low affinity systems, to obtain a hyperbole curve, different concentrations of procyanidins and polysaccharides were tested. Raw data obtained as a plot of heat flow (microjoules per second) against time (seconds) were then integrated peak-by peak and normalized to obtain a plot of observed enthalpy change per mole of injectant (ΔH, kJ.mol−1) against the molar ratio. The ratio was represented as (-)-epicatechin/GalA in order to be comparable with previous experiments using pectic polysaccharide **(Watrelot et al., 2013, 2014)**. Peak integration was performed using NanoAnalyze v3.7.5 (TA Instruments, New Castle, USA). Control experiments consisted on the titration of procyanidin fractions into buffer without polysaccharides and was subtracted to titration experiments. The experimental data was fitted to a theoretical titration curve using NanoAnalyze v3.7.5, with

ΔH (enthalpy change), *K*a (association constant), and *n* (number of binding sites per molecule) as adjustable parameters, from the relationship:

$$Q_i = \frac{nP_t\Delta HV_0}{2}\left[1 + \frac{A_t}{nP_t} + \frac{1}{nk_aP_t} - \sqrt{\left(1 + \frac{A_t}{nP_t} + \frac{1}{nk_aP_t}\right)^2 - 4\frac{A_t}{nP_t}}\right]$$

Equation 2.3

where Pt is the total GalA concentration, A_t is the total concentration of the ligand, V_0 is the volume of the cell, and Q_i is the total heat released for injection. The thermodynamic parameters ΔG and ΔS were calculated from the van't Hoff equation:

$$\Delta G = -RT\ln(K_a) = \Delta H - T\Delta S$$

Equation 2.4

where ΔG is free enthalpy, K_a is the association constant, ΔH is the enthalpy, and ΔS is the entropy of interaction. After titration, the solutions present in the titration cell were collected and centrifuged at 8000 g and 4 ºC for 15 min. The supernatant and precipitate were collected and analyzed to determine variations in procyanidins degree of polymerization (ΔDP) in solution and polysaccharides branching degree (ΔBD), respectively.

2.4.2.2 Phase diagram

To identify aggregates formation due to polysaccharide-procyanidin interactions, polysaccharide/procyanidin mixed solutions (volume of 100 µL) were prepared on citrate/phosphate buffer (pH 3.8 and ionic strength 0.1 mol/L) using a 96-wells "microplate" at 25 °C. Different concentration of procyanidins (0, 0.03, 0.06, 0.117, 0.23, 0.47, 0.94, 1.875, 3.75, 7.5, 15, and 30 mM (−)-epicatechin equivalent) were tested along rows, while different concentrations of polysaccharides (0, 0.03, 0.06, 0.117, 0.47, 1.875, 7.5, and 30 mM GalA equivalent) were tested along columns. The formation of aggregates was detected by measuring the optical density at 650 nm using a SAFAS flx-Xenius XM spectrofluorimeter (SAFAS, Monaco). Wells containing only pectic compounds or procyanidins in buffer were used as control.

2.5 Drying of apple pomace

APII and APIII were used in the drying experiments. Given the limited amounts of available APII, this pomace was only used for replication of microwave-hydrodiffusion and gravity (MHG) assisted drying at 400 W with or without ethanol addition.

2.5.1 Hot-air drying

APIII was dried by hot-air using a convective hot air oven composed by a centrifugal blower (to control the air velocity within the drying chamber), electric heating elements of 2 kW (for temperature control), a humidifier by steam and a cooler/dehumidifier (for air moisture control). The system was controlled using the Matlab⁄Simulink® from Mathworks® (Natick, Massa-chusetts, USA) platform and a National Instruments® (Austin, Texas, USA) data acquisition board model 6025E. A micro-manometer Furness, model FC0150, with a Pitot tube was used for air velocity monitored. Air temperature and the relative humidity were monitored using a digital thermo-hygrometer Omega model RH411. For APIII drying, about 500 g of apple pomace were placed in the drying chamber over a digital balance at a mass/area ratio of 0.48g/cm^2. This ratio corresponded to the maximum amount of apple that could be processed in order to assure a uniform and complete dried product. Drying was performed at different temperatures, ranging from 40 to 100 ºC, and a relative air humidity of 35 and 60%. The mass was recorded every 5 min, thus providing information of the instantaneous moisture (**Equation 2.5**).

$$X_t = \frac{m_x - m_d}{m_x}$$ **Equation 2.5**

Where X_t represents the moisture content at time (t in min), m_x the instantaneous mass, and m_d is the apple pomace dry mass. From this information it was possible to estimate the relative moisture content of the pomace along drying using **Equation 2.6**:

$$X_{rel} = 1 - \frac{(X_i - X_{t})}{X_i}$$ **Equation 2.6**

Where X_i corresponds to the initial moisture content of apple pomace and X_t the instantaneous moisture content of the product.

2.5.2 Microwave Hydrodiffusion and Gravity (MHG) assisted drying

APIII microwave assisted drying was performed with a MHG apparatus (Milestone NEOS-GR microwave laboratory oven). The reactor with 2.45 GHs had a maximum delivery power of 900 W, variable in 10 W increments. An external infrared sensor was used to monitor temperature. The vessel, made of Pyrex, had a volumetric capacity of 1.5 L. The "easy-WAVE" software package was used for time, temperature, and power control. For the optimization, powers of 300, 400, 500, 600 and 900 W per 1.2 kg of plant material were selected. Hot water and steam (*in situ* water) move by earth gravity downwards to a spiral condenser outside the microwave cavity. The condensate was collected continuously in 50 mL aliquots. The microwave drying was continued until achieving burning point (no water). This was performed to obtain a full dehydration kinetics. Due to water losses as vapor, the relative water content of apple pomace was expressed in relation to the total volume of water collected as condensate following **Equation 2.7**:

$$X_{rel} = \frac{(V_t - V_x)}{V_t} \qquad \textbf{Equation 2.7}$$

Where V_t is the total volume of water collected by MHG and Vx is the cumulative volume of water collected along fractions.

By determining the energy required for heating the water in the pomace ($E_{Heating}$), **Equation 2.8** and the energy required for water vaporization ($E_{vaporization}$), **Equation 2.9**, it was possible to determine the total theoric energy (E_{Total}) required for water removal from apple pomace (**Equation 2.10)**.

$$E_{Heating} = \frac{w_{H2O} \times C_p \times (100 - T_i)}{w_{dAP}} \qquad \textbf{Equation 2.8}$$

$$E_{Vaporization} = \frac{w_{H2O} \times \Delta H_{vap}}{w_{dAP}} \qquad \textbf{Equation 2.9}$$

$$E_{Total} = E_{Heating} + E_{vaporization}$$ **Equation 2.10**

Where w_{H2O} corresponds to the mass (kg) of water in the processed pomace, C_p is the water heating capacity ($kJ.kg^{-1}.^{o}C^{-1}$), T_i is the initial temperature of the pomace (ºC), ΔH_{vap} is the enthalpy of vaporization ($kJ.kg^{-1}$) and w_{dAP} is the dry weight of processed pomace (kg).

From the applied delivery power, P (W), it was possible to determine the provided energy by the NEOS-GR according to **Equation 2.11:**

$$E_{provided} = \frac{P \times (t_{heating} + t_{vaporization}) \times 3.6}{w_{dAP}}$$ **Equation 2.11**

Where $t_{heating}$ and $t_{vaporization}$ correspond to the heating and vaporization time (h).

Thus, the energy efficiency (E_{eff}), attributed to MHG drying was determined as % according to **Equation 12**:

$$E_{Eff} = \frac{E_{total}}{E_{provided}} \times 100$$ **Equation 2.12**

Fractions obtained by MHG assisted drying were frozen and freeze-dried. The obtained dried pomace was stored at variable room temperature for a period of 2 years. APII was also dried by MHG at 400 W following the general procedure described for APIII. An additional drying process, at the same power, was performed to APII in order to promote the extraction of compounds. This process consisted on the adding of absolute ethanol (150, 400 and 500 mL) when the cumulative amount of liquid fraction reached 200 mL, 600 mL and 1050 mL, respectively.

2.5.3 Extractions from MWG-dried pomaces

Microwave dried APIII was submitted to hot water extraction with 1% acetic acid (pH 2.5), at a solid (dry weight) to solvent ratio of 1:5 (g/mL) for 1 h. The obtained extracts were filtered (Whatman filter paper nº 4 and G3 sintered funnel) and the residue was re-extracted two more times following the same procedure. The obtained extracts were concentrated under reduced pressure at 45 °C and freeze dried yielding a hot water extract (HWE), assigned as HWEI, HWEII and HWEIII, according with the extraction set.

2.6 General chemical characterization

2.6.1 Protein analysis

Protein was estimated by determining total nitrogen in a Truspec 630-200-200 elemental analyzer with a thermal conductivity detector (TDC) and using the conversion factor of 5.72, established for apples **(Sosulski & Imafidon, 1990)**.

2.6.2 Carbohydrate analysis

Neutral sugars were determined by a pre-hydrolysis step in 0.2 mL of 12 M H_2SO_4 for 3 h at room temperature, followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 ºC **(Selvendran, March, & Ring, 1979)**. Afterwards, 2-deoxyglucose was added as internal standard and the mixture submitted to a reduction step with $NaBH_4$ (15% in 3 M NH_3) during 1 h at 30 ºC. The excess of $NaBH_4$ was then destroyed by adding glacial acetic acid and the alditols formed were subsequently acetylated with acetic anhydride (3 mL) in the presence of 1-methylimidazole (0.450 mL) during 30 min at 30 ºC. The resulting alditol acetate derivatives were then isolated with dichloromethane and submitted to gaseous chromatography with a flame ionization detector (GC-FID) equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA, USA) with internal diameter and film thickness of 0.25 mm and 0.15 µm, respectively. The oven temperature program used: initial temperature 200 ºC, a rise in temperature at a rate of 40 ºC/min until 220 ºC, standing for 7 min, followed by a rate of 20 ºC/min until 230 ºC maintaining this temperature for 1 min. The injector and detector temperatures were, respectively, 220 and 230 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min. Free sugars were determined without the hydrolysis step. Given the absence of free mannose in apple pomace **(Dhillon, Kaur, & Brar, 2013)**, fructose was quantified from the ratio that epimerized to mannitol (43%) during the reduction step **(Brunton, Gormley, & Murray, 2007)**. Free glucose was determined by difference between the total glucitol detected and the glucitol yielded by fructose reduction.

Uronic acids were quantified by the *m*-phenylphenol colorimetric method **(Blumenkrantz & Asboe-Hansen, 1973)**. Samples were previously submitted to pre-hydrolysis in 0.2 mL of 12 M H_2SO_4 for 3 h at room temperature followed by hydrolysis for 1 h in 1 M H_2SO_4 at 100 ºC. To the 0.5 mL of diluted hydrolyzed sample (1:4), 3 mL of of boric acid 50 mM H_2SO_4 98% (w/w) were added. The mixture was shaken and heated at 100 ºC for 10 min. After cooling, 100 µL of *m*-phenylphenol was added, left to react for 30 min in dark after which the absorbance was measured at 520 nm. D-galacturonic acid was used as standard and the results were expressed as GalA, the representative uronic acid reported to occur in apple pectins **(Renard et al., 1991; Schols et al., 1990; Stevens & Selvendran, 1984)**. Sugars associated to polysaccharides were estimated by difference between the total sugars determined after acid hydrolysis and free sugars. Total carbohydrates were determined by the sum of free sugars and sugars attributed to polysaccharides.

2.6.3 Glycosidic-linkage composition of polysaccharides

Glycosidic-linkage analysis was performed as described by **Coimbra, Delgadillo, Waldron, and Selvendran (1996)**, by gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu) of partially methylated alditol acetates (PMAA). Derivatization was performed by weighting 1-2 mg of sample and solubilization in anhydrous dimethyl sulfoxide (DMSO). The solution was left at room temperature and constant stirring until total solubilization of the sample. Afterwards, NaOH (30 mg) added to the solution and kept stirring during 30 min. Methylation was carried out by adding three times 80 µL of methyl iodide (total of 240 µL) with reaction intervals of 20 min under stirring. The obtained mixture was dissolved in 3 mL of methanol/dicloromethane (50/50; v/v) and dialyzed 4 times against water/ethanol (50/50; v/v mixtures). The obtained retentate was then submitted to centrifugal evaporation (Univapo 100 ECH, UniEquip, Germany) until complete removal of the solvents. The methylation was repeated. The obtained permethylated polysaccharides were then suspended in 0.5 mL of 2 M trifluoroacetic acid, submitted to hydrolysis for 1 h at 121 ºC and dried by centrifugal evaporation. The obtained partially methylated monosaccharides were then reduced with 20 mg of $NaBD_4$ during 1 h at 30 ºC in 300 µL of 2M NH_3. The reaction was terminated by the addition of glacial acetic acid being followed by an acetylation step with 3 mL of acetic anhydride and 450 µL of 1-methylimidazole (30 min at 30 ºC). Then, 3 mL of distilled water were added to decompose the acetic anhydride, and the acetylated sugars were extracted with 5 mL of CH_2Cl_2. The organic phase was washed three times with water and then dried by centrifugal evaporation. The PMAA were dissolved in acetone and analyzed by gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu). The oven temperature was programmed as follows: hold 5 min at 45 ºC, to 140 at 10 ºC/min (hold 5 min at 140 ºC), to 170 at 0.5 ºC/min (hold 1 min at 170 ºC) and then to 280 at 15 ºC/min (hold 5 min at 280 ºC). Helium carrier gas had a flow of 1.7 mL/min and a column head pressure of 2.8 psi. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 40-500 m/z, in a full scan acquisition mode. Identification was achieved comparing the standard mass spectra and other spectra with a laboratory made database.

For arabinans, the average branching points (ABP) was determined by the ratio of total arabinose with branching points according to **Equation 2.12**:

$$\mathrm{ABP} = \frac{\text{Total Ara}}{(1\rightarrow3,5)\text{-Ara}f + (1\rightarrow2,5)\text{-Ara}f + (1\rightarrow2,3)\text{-Ara}f + (1\rightarrow2,3,5)\text{-Ara}f \times 2} \qquad \textbf{Equation 2.12}$$

2.6.4 Size exclusion chromatography of polysaccharides

Samples were suspended in a 0.1 M NaNO3 aqueous solution at a concentration of about 8-10 mg/mL and stirred until complete dissolution at 20 ºC for 12h. The solution was then filtered in a 0.4 µm PVDF filter. The size exclusion chromatography (SEC) analysis was performed by injecting 100 µL of sample on a PL-GPC 110 system (Polymer Laboratories, UK) with two PLaquagel-OH MIXED 8 µm (300 mm × 7.5 mm) columns protected by a PL aquagel-OH Guard 8 µm pre-column **(Mendes, Xavier, Evtuguin, & Lopes, 2013)**. The temperature of the injector system, columns, and detector (RI) were kept at 36 ºC. $NaNO_3$ at 0.1 M was used as eluent at a flow rate of 0.9 mL/min. Pullulans (Polymer Laboratories, UK) in the range 0.7–48.0 kDa were used for columns calibration.

2.6.5 NMR spectroscopic analysis

^{1}H and ^{13}C NMR spectra were recorded in a Bruker DRX 500 spectrometer operating at 500.13 and 125.77 MHz, respectively, using D2O as solvent and TSS as external reference; the chemical shifts were expressed in ppm. 2D (^{1}H-^{1}H) homonuclear shift correlation with presaturation during relaxation delay (COSYPR) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz. The recycle delay was 1.0 s. These data were processed in the absolute-value mode. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) heteronuclear single quantum coherence, using gradient pulses for selection (gHSQC) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz in F2 and 21739 Hz in F1. The recycle delay was 1.5 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant ^{1}J(CH) of 149 Hz. The heteronuclear multiple quantum coherence, using gradient pulses for selection (gHMBC) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz in F2 and 27777 Hz in F1. The recycle delay was 1.5 s. A sine multiplication was applied in both dimensions. The low-pass J-filter of the experiment was adjusted for an average coupling constant ^{1}J(CH) of 149 Hz and the long-range delay utilised to excite the heteronuclear multiple quantum coherence was optimised for 7 Hz.

2.6.6 Folin-Ciocalteu method for total polyphenol quantification

The total polyphenolic content of the samples was determined by Folin-Ciocalteu method **(Singleton & Rossi, 1965)**. Each sample (15 µL) were mixed with 60 µL of deionized water and 15 µL of Folin-Ciocalteu reagent (Sigma). After 5 min, 150 µL of 7% solution of sodium carbonate. The mixture was left 60 min at room temperature in the dark and the absorbance at 760 nm was measured. The results were expressed both as phloridzin equivalents (PLZE), one of the polyphenols found in apple, as well as in gallic acid equivalents (GAE) in g/kg. A concentration range of 50 to 500 mg/L was used.

2.6.7 UHPLC-DAD analysis of polyphenols

Individual polyphenolic composition was determined by UHPLC-DAD with or without thioacidolysis, method described by **Guyot, Marnet, and Drilleau (2001)**. Analyses were performed using an Ultra-Fast Liquid Chromatography Shimadzu Prominence system (Kyoto, Japan) controlled by a LC Solution software (Shimadzu, Kyoto, Japan). Separation were achieved using a Kinetex® column (100 x 4.6 mm, 2.6 µm, C_{18}, 100 Å) at 30 °C. The mobile phase consisted of water/formic acid (99:1; v/v) (eluent A) and acetonitrile (eluent B). The flow rate was 1 mL/min. The elution program was as follows:

2–2% B (0–1 min); 2–90% B (1–20 min); 90–90% B (20-25 min); 90–2% B (25–30 min); 2–2% B (30–35 min). Individual compounds were quantified by comparisons with external standards at 280 nm for (+)-catechin, (-)-epicatechin, phloretin xyloglucoside (quantified as phloretin), phloridzin, (-)-epicatechin benzylthioether (quantified as (-)-epicatechin); at 320 nm for 5-*O*-caffeoylquinic acid, 4-*p*-coumaroylquinic acid (quantified as *p*-coumaric acid) and their methylated derivatives obtained during thioacidolysis reaction quantified as their respective non-methylated equivalents, and at 350 nm for quercetin glycosides (quantified as quercetin). For procyanidins quantification, thiolysis was performed. Briefly, 25 to 100 mg of sample were mixed with 800 µL of benzylthioether 5% (v/v) and 400 µL of HCl 0.4 N in anhydrous methanol. The mixture was then vortexed and place in water bath at 40 ºC for 30 min, with stirring every 10 minutes. The reaction was stopped in an ice bath for 5 min, centrifuged, and the supernatant filtrated in 0.45 µm syringe filters. The thioether adducts plus terminal units were determined by UHPLC as mentioned above. The procyanidins average degree of polymerization (DPn) was determined by the molar ratio of all the flavan-3-ol units from thiolysis (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin terminal units.

2.6.8 UHPLC-DAD-ESI-MS^n analysis of polyphenol oxidation products

UHPLC-DAD-ESI-MS^n analysis of polyphenol oxidation products was performed on apple pomace procyanidins fractions using an Acquity Ultra performance LC apparatus from Waters (Milford, MA, USA), equipped with a photodiode array detector (detection at 280, 320, 350 and 520 nm) coupled with a Bruker Daltonics (Bremen, Germany) HCT ultra ion trap mass spectrometer with an electrospray ionization source. Separations were achieved using a Kinetex® column (100 x 4.6 mm, 2.6 µm, C18, 100 Å) operated at 30 °C, and using water/formic acid (99:1, v/v) (eluent A) and acetonitrile (eluent B) in the conditions previously described (**Section 6.8**). A capillary voltage of 2 kV was used in the negative ion mode for polyphenol analysis. Nitrogen was used as drying and nebulizing gas with a flow rate of 12 L/min. The desolvation temperature was set at 365 °C and the nebulization pressure at 0.4 MPa. The ion trap was operated in the Ultrascan mode from *m/z* 100 to 1000.

2.6.9 Quantification of polyphenols by alkaline fusion

Mixtures of 1 g of solid NaOH and 100 mg of zinc dust were weighed on a nickel crucible, and heated until 350 °C **(Coelho et al., 2014)**. After complete melting of the mixture, 10 mg of sample were added and left at 350 ºC for 10 s, followed by a rapid cooling on ice. The fusion cake was solubilized in 6 M HCl, and 200 µL of internal standard solution was added (3,4-dimethoxybenzoic acid, 1 mg/mL). After acidification to pH 1-2 with 6 M HCl, liquid/liquid extraction with 50 mL of diethyl ether was performed. The organic phase was then collected, rotary evaporated until complete dryness, redissolved in 2 mL of 50% methanol and analysed by HPLC (Dionex, Ultimate 3000) on a reversed-phase column (C_{18}-ACE; 25 cm length, 0.45 cm internal diameter, and 5 µm particle diameter). Water/formic acid (95:5; v/v) (eluent A) and methanol (eluent B) were used as eluents. The eluent program was as follows: 5% eluent B (0−5 min); 5-40% B (5−45 min); 40-70% B (45−65 min); 70-5% B (65−75 min). The column temperature was set at 25 °C, and the flow was 0.8 mL/min. The eluent was continuously monitored from 200 to 600 nm with a photodiode array detector (PDA-100, Dionex). Standards 3,4-dihydroxybenzoic acid, catechol, gallic acid and 4-hydroxybenzoic acid were used for their identification and quantification on samples submitted to alkaline fusion in order to detect interferences from phenolic structures of any residual lignified material occurring in apple pomace.

2.6.10 UHPLC-DAD-ESI-MS^n analysis of polyphenols from MHG

Individual polyphenols present in the MHG fractions were determined by ultra-high-performance liquid chromatography (UHPLC) on an Ultimate 3000 (Dionex Co., USA) apparatus equipped with a Diode Array Detector (DAD) from Dionex Co., USA as described by **Fernandes et al. (2019)**. The chromatographic system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector and an automatic thermostatic column compartment. Hypersil Gold (Thermo Scientific, USA) C_{18} column (100 mm length; 2.1 mm i.d.; 1.9 µm particle diameter, end-capped) at 30 °C was used. The mobile phase was composed of (A) 0.1% (v/v) formic acid and (B) acetonitrile. The solvent gradient started with 5% of solvent (B), reaching 40% at 14 min and 100% at 16 min, followed by the return to the initial conditions. The flow rate was 0.1 mL.min^{-1} and UV–Vis spectral data for all peaks were accumulated in the range 200–500 nm while the chromatographic profiles were recorded at 280, 320 and 340 nm for polyphenol analysis. The mass spectrometer used was a Thermo LTQ XL (Thermo Scientific, USA) ion trap MS equipped with an ESI source. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific, USA). Nitrogen above 99% purity was used, and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 ºC. The full scan covered the mass range from *m/z* 100 to 2000. CID–MS/MS and MS^n experiments were acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units.

For quantitative analysis, calibration curves were performed by injection of 5 known concentrations of standard compounds. Detection (LOD) and quantification (LOQ) limits were calculated using the parameters of the calibration curves, defined as 3.3 and 10 times the value of the regression error divided by the slope, respectively. For rutin (ACROS), the tested range was 1.0–10.0 µg/mL, and the equation was y=12624x-953, with R^2 value of 0.999. The quantification limit (LQ) and detection limit (LD) for this compound were 1.29 and 0.43 µg/mL, respectively. For quercetin-3-*O*-glucoside (Sigma-Aldrich), the tested range was 2.4–12.2 µg/mL, the equation was y=16421x-879 with an R^2 value of 0.999. The LQ and LD were 1.07 and 0.35 µg/mL, respectively. The calibration curve of phloridzin (Sigma-Aldrich), y=20429x-456, were performed for ranges of 2.3–11.7 µg/mL, presenting and R^2 of 0.999. The determined LQ and LD were 1.35 and 0.45 µg/mL, respectively. The remaining quercetin derivatives were quantified as quercetin-3-*O*-glucoside equivalents.

2.6.11 Antioxidant activity by ABTS$^{\bullet+}$ method

The 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) was performed according to **Re et al. (1999)**. The ABTS radical cation (ABTS$^{\bullet+}$) was prepared by mixing an aqueous solution of potassium persulfate (660 mg/L) with ABTS stock solution (3.8 g/L) and leaving it to stand in the dark at room temperature for 12–16 h. The ABTS$^{\bullet+}$ solution was diluted with water to an absorbance of 0.700 at 734 nm. 250 µL of this solution were then mixed with 50 µL of sample or standard and left to react for 15 min in the dark. The decrease in absorbance was determined at 734 nm. The results were expressed µmol Trolox eq./g of sample using a concentration range of standard between 0 and 34 mg/L.

2.7 Statistics

All experiments were performed at least in triplicate unless otherwise stated. The reproducibility of the results is expressed as pooled standard deviation.

Chapter 3- Results and Discussion

3.1 Revisiting the chemistry of apple pomace polyphenols

This chapter accounted with the contributions of:

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3.1.1 Apple pomace composition

On a dry weight basis, carbohydrates represented 670 g/kg of the apple pomace (**Table 3.1**), of which 50 g/kg were free sugars, mostly Fru (67 mol%) and Glc (33 mol%). Polysaccharides represented 620 g/kg, being the major monosaccharides Glc (44 mol %), GalA (25 mol%), Ara (14 mol%), Gal (7 mol%) and Xyl (7 mol%). This carbohydrate composition was in accordance with the polysaccharides reported for apple pomace, including cellulose, xyloglucans and pectic polysaccharides **(Mehrländer, Dietrich, Sembries, Dongowski, & Will, 2002)**. Protein represented 56 g/kg of apple pomace.

Polyphenols, determined by UHPLC-DAD after thioacidolysis, represented 5 g/kg of dry apple pomace and could be divided in four classes: flavan-3-ols, flavonols, dihydrochalcones and hydroxycinnamic acids. Flavan-3-ols were the major class (2.88 g/kg of dry apple pomace), with a number-average DP of 4.7 (**Table 3.2**), comparable to those usually found in apples **(Guyot et al., 2002).** Flavonols (1.92 g/kg of dry apple pomace) were the second major class, being represented by hyperoside (58%), quercetin-pentosides (14%), quercitrin (13%) and isoquercetrin (9%). Quercetin and rutin represented less than 6% of the flavonol pool. Dihydrochalcones (0.14 g/kg of dry apple pomace) were the third polyphenolic class, only constituted by phloridzin. Flavonols and dihydrochalcones are generally abundant in apple skins and seeds, respectively **(Guyot et al., 2002)**, beyond pulp and stalks, constituting a significant part of apple pomace. Hydroxycinnamic acids, represented only by 5-*O*-caffeoylquinic acid, was the less abundant polyphenolic class with levels of 0.03 g/kg of dry apple pomace. This reflected a typical polyphenolic composition of apple pomace, although dihydrochalcones and hydroxycinnamic content were much lower than the values commonly reported **(Çam & Aaby, 2010; Virot et al., 2010)**. Such differences can be explained by oxidation reactions catalysed by PPO, as 5-*O*-caffeoylquinic acid, phloridzin and phloretin-xyloglucoside are substrates of this enzyme **(Wong-Paz et al., 2015)**.

Table 3.1 - Yield (%), carbohydrate composition (molar %), total carbohydrates (g/kg), protein (g/kg), total polyphenols (g phloridzin (PLZE) equivalents/kg) and antioxidant activity (mmol Trolox equivalents (TE)/kg) of the different apple pomace fractions. In parenthesis are presented data in gallic acid equivalents (g GAE/kg) for total polyphenols. Data are expressed as Mean and pooled standard deviation (*Pooled Std*) of three replicates. n.d means for not determined.

Type of Extraction	**Sample**	**Yield (%)**		**Carbohydrates (mol%)**									**Total Carbohydrates (g/kg)**	**Protein (g/kg)**	**Total Polyphenols (g PLZE/kg)**	**Antioxidant activity (mmol TE/kg)**
				Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Fru**	**Gal**	**Glc**	**GalA**				
Water/acetone	Apple Pomace		Polysac.	1	1	14	7	1	-	7	44	25	622	56	n.d	n.d.
			Free Sugars	-	-	-	-	-	67	-	33	-	49			
	HWE	37	Polysac.	1	tr	23	3	-	-		24	43	721	11	28 (9)	33
			Free Sugars	-	-	-	-	-	68	-	32	-	116			
	60%Ac	2.4	Polysac.	tr	tr	50	tr	-	-	13	17	20	103	36	78 (29)	95
	60%AcU	2.2	Polysac.	-	-	-	-	-	-	-	-	-	-	-	42 (15)	23
	Residue	50	Polysac.	1	1	4	12	4	-	7	60	11	615	85	n.d	n.d
Microwave superheated water	Cycle 1	4.8	Polysac.	2	2	20	8	1	-	14	26	26	906	n.d.	28 (11)	n.d.
	WPp	0.8	Polysac.	1	-	1	-	-	-	-	73	24	954	n.d	9 (3)	n.d
	Et50Pp	0.1	Polysac.	2	2	14	9	1	-	14	14	44	953	n.d	19 (7)	n.d
	Et80Pp	2.0	Polysac.	1	1	8	14	1	-	21	13	40	901	n.d	10 (3)	n.d
	Et80Sn	1.8	Polysac.	3	4	48	3	1	-	10	4	26	931	n.d	49 (18)	n.d
	Cycle 2	2.1	Polysac.	3	3	12	10	3	-	13	22	35	937	n.d.	53 (20)	n.d.
	WPp	0.2	Polysac.	1	-	1	1	-	-	1	78	17	866	n.d	21 (8)	n.d
	Et50Pp	0.1	Polysac.	2	2	4	10	1	-	13	28	40	901	n.d	24 (8)	n.d
	Et80Pp	1.0	Polysac.	2	1	5	15	3	-	19	15	39	878	n.d	13 (5)	n.d
	Et80Sn	0.8	Polysac.	3	8	42	5	4	-	12	7	20	699	n.d	98 (39)	n.d
	Cycle 3	6.0	Polysac.	6	6	5	24	11	-	15	19	16	820	n.d.	108 (43)	n.d.
	WPp	0.1	Polysac.	2	6	5	22	9	-	13	22	22	493	n.d	138 (55)	n.d
	Et50Pp	0.3	Polysac.	-	1	1	24	2	-	10	39	23	918	n.d	25 (10)	n.d
	Et80Pp	0.6	Polysac.	1	-	1	20	3	-	14	34	27	952	n.d	21 (9)	n.d
	Et80Sn	4.7	Polysac.	2	7	6	25	14	-	15	15	15	673	n.d	136 (56)	n.d
	Final Residue	35	Polysac.	-	-	-	9	3	-	3	78	6	534	n.d.	n.d.	n.d.
	Pooled std	*0.4*		*0*	*0*	*0*	*0*	*0*	*2*	*0*	*0*	*1*	*7*	*1*	*0 (0)*	*2*

Table 3.2 – Extraction yields (%), individual polyphenolic composition (g/kg) and total polyphenols (g/kg) of dry apple pomace and apple extracts, as determined by HPLC-DAD. Data are expressed as Mean and pooled standard deviation (Pooled Std) of three replicates.

		Sample	Yield	Flavan-3-ols				Dihydrochalcones		Hydroxycinnamic acids		Flavonols						Total
				CAT	EPI	PCA	DP	PLZ	XPL	CQA	PCQ	Ru	Hy	Iso	Pent	Quc	Qu	
Apple Pomace	**Sequential extractions**	Apple Pomace	-	-	-	2.9	4.7	0.1	-	tr	-	tr	1.1	0.2	0.3	0.3	0.1	4.9
		HWE	37	-	-	1.3	3.4	0.4	-	0.1	-	0.1	2.6	0.4	0.4	0.8	0.6	6.7
		60% Ac	2.4	-	-	1.1	16	0.2	-	0.1	-	0.1	1.6	0.3	0.2	0.5	4.3	8.3
		60% ACU	2.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		Residue	50	-	-	1.9	6.8	-	-	-	-	-	-	-	-	-	-	1.9
	Polyphenol isolation	MeOH	12	-	-	1.4	3.3	1.0	-	0.2	-	0.2	6.2	1.2	1.6	1.6	0.5	14
		60% Ac	5.9	-	-	17.6	5.7	0.6	-	-	-	0.2	4.0	0.7	0.7	0.8	0.2	25
		AP PCA	0.1	-	-	235	5.8	11	-	0.8	-	4.1	72	8.7	12	15	2.1	360
		Residue	77	-	-	2.5	5.4	-	-	-	-	-	-	-	-	-	-	2.5
Apple	**Polyphenol isolation**	Apple	-	tr	1.0	11	4.4	0.4	0.2	2.5	0.2	tr	0.2	0.1	0.1	0.2	-	16
		MeOH	57	0.1	1.3	7.7	2.5	0.6	0.2	2.7	0.3	tr	0.3	0.1	0.2	0.3	-	14
		60% Ac	6.9	-	1.8	79	5.5	0.6	0.1	3.9	0.3	tr	1.2	0.3	0.3	0.3	-	88
		Apple PCA	0.4	-	16	912	7.3	4.9	-	26	2.6	0.2	12	2.4	2.7	2.5	-	965
		Residue	35	-	-	2.3	9.9	-	-	tr	tr	-	-	-	-	-	-	2.3
		Pooled std		*0.0*	*0.2*	*1.6*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.1*	*0.0*	*1.6*

CAT - (-)-Catechin; EPI - (-)-Epicatechin; PCA – Procyanidins. DP – Flavan-3-ols average degree of polymerization; PLZ – Phloridzin; XPL – Phloretin-xyloglucoside; CQA - 5-cafeoylquinic acid; PCQ - Para-coumaroylquinic acid; RU – Rutin; HY – Hyperoside; ISO – Isoquercitrin; PENT – Quercetin pentoside (Reynoutrin+Guajaverin+Avicularin); QUC – Quercitrin; QU -Quercetin; AP PCA – Apple pomace procyanidins; Apple PCA – Apple procyanidins.

3.1.2 Effect of hot water extraction on apple pomace polyphenols

Hot water has been suggested for extraction of polyphenols as it is cheap, non-toxic, environmentally friendly and provides an easily implementable process **(Çam & Aaby, 2010)**. To prevent polyphenol oxidation diluted acetic acid solutions are usually used **(Ferreira et al., 2002)**. As represented in **Table 3.1**, 37% (w/w) of the apple pomace was recovered in the HWE which contained all free sugars and 43% of the polysaccharides present in the pomace. The occurrence of GalA (43 mol%) and Ara (23 mol%) showed that the extract was particularly rich in pectic polysaccharides. Total polyphenolic content, determined by the Folin-Ciocalteu method, was 28 g PLZE/kg (9 g GAE/kg). Such amounts were higher than those previously reported **(Çam & Aaby, 2010)**, probably resulting from differences in the apple cultivars that originated the pomace **(Diñeiro García et al., 2009)**. About 7 g/kg of polyphenols were detected by UHPLC-DAD after thioacidolysis (**Table 3.2** and **Figure 3.1**), represented mainly by flavonols (73%), flavan-3-ols (20%) with an average DP of 3.4, dihydrochalcones (6%), and hydroxycinnamic acids (1%). Their occurrence was associated to the observed antioxidant capacity, 33 mmol Trolox eq./kg as measured by the ABTS$^{\cdot+}$ method. Proteins corresponded to the minor component in the HWE, only accounting for 11 g/kg of extract.

To determine the polyphenols still retained in the water-insoluble cell wall material, 60% acetone extractions were performed. The material recovered represented 2.4% (w/w) of the initial apple pomace, composed by a low amount of carbohydrates 103 g/kg, particularly rich in Ara (50 mol%). Total polyphenols analysis revealed the additional presence of 78 g PLZE/kg (29 g GAE/kg) of polyphenols, of which 8 g/kg were identified by UHPLC-DAD. Flavonols represented 83% of the total identified polyphenols while flavan-3-ols, dihydrochalcones and hydroxycinnamic acid represented 14, 2 and 1%, respectively. In contrast, the following extraction with 60% acetone/8 M urea, did not allow to recover any carbohydrates nor polyphenols as determined by GC-FID and UHPLC after thioacidolysis, respectively. However, the observed 42 g PLZE/kg (15 g GAE/kg) by the Folin-Ciocalteu method as well as the measured antioxidant activity by the ABTS$^{\cdot+}$ method (23 mmol TE/kg) suggested the presence of polyphenols. This allows to infer the occurrence of oxidised structures with bonds resistant to thioacidolysis **(Le Deun et al., 2015; Mouls & Fulcrand, 2015)**.

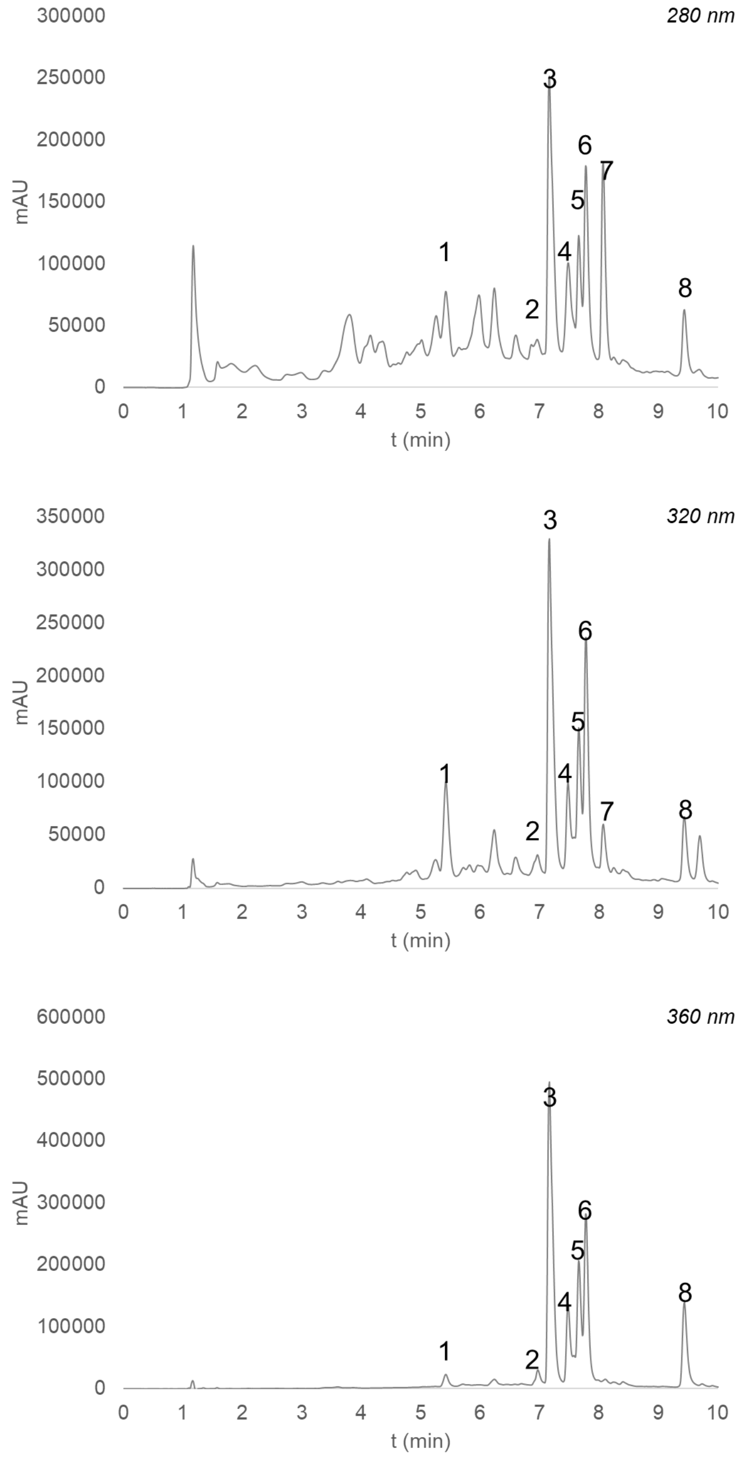

Figure 3.1 - Representative chromatogram (320 nm) of apple pomace hot water extract (HWE). 1- 5-*O*-caffeoylquinic acid; 2- Rutin; 3- Hyperoside; 4- Isoquercitrin; 5- Quercetin pentosides (Reynoutrin+Guajaverin+Avicularin); 6-Quercitrin; 7-Phloridzin; 8-Quercetin.

Carbohydrate analysis of the residue obtained after the extraction with 60% acetone/8 M urea, which represented 50% of the initial apple pomace, demonstrated a high prevalence of Glc (60 mol%), Xyl (12 mol%), GalA (11 mol %) and some Gal (7 mol%), Ara (4 mol%) and Man (4 mol%). Glycosidic linkages analysis (**Figure 3.2** and **Table 3.3**) revealed the occurrence of (1→4)-Glc*p* (54 mol%), typical of cellulose. Furthermore, (1→4)-Glc*p*, together with (1→4,6)-Glc*p* (12%), (1→2)-Xyl*p* (3.6%), (1→2)-Gal*p* (1.3%), t-Xyl*p* (5.7%), t-Gal*p* (2.4%) and t-Fuc*p* (1.1%), were diagnostic linkages of xyloglucan. In fact, the proportion the side chains residues ((1→2)-Xyl*p*, (1→2)-Gal*p*, t-Xyl*p*, t-Gal*p* and t-Fuc*p*) explained 9.3 out of 11.7% of the (1→4,6)-Glc*p*, supporting the xyloglucan structure **(Mehrländer et al., 2002; Stevens & Selvendran, 1984)**. The remaining 2.4% of (1→4,6)-Glc*p* could be explained by the t-Glc*p*, indicative that some residual starch might be present. Gal, represented by (1→4)-Gal*p* (3.1%) and (1→3,4)-Gal*p* (0.5%), pointed out also to the possible occurrence of galactans branched to a small extent at C-3 **(Stevens & Selvendran, 1984)**. The (1→5)-Ara*f* (2.4%), (1→3,5)-Ara*f* (1.5%), (1→2,5)-Ara*f* (0.4%) and t-Ara*f* (2.1%), characteristic of side chain arabinans of pectic polysaccharides indicated that these polysaccharides were also relevant in the residue. The proportion of (1→5)-Ara*f* to (1→3,5)-Ara*f*, (1→2,5)-Ara*f* and t-Ara*f* supported the occurrence of a highly branched arabinan structure **(Renard et al., 1991)**. UHPLC-DAD after thioacidolysis also demonstrated the presence of flavan-3-ols, 2 g/kg with a DP of 6.7, that were not able to be extracted.

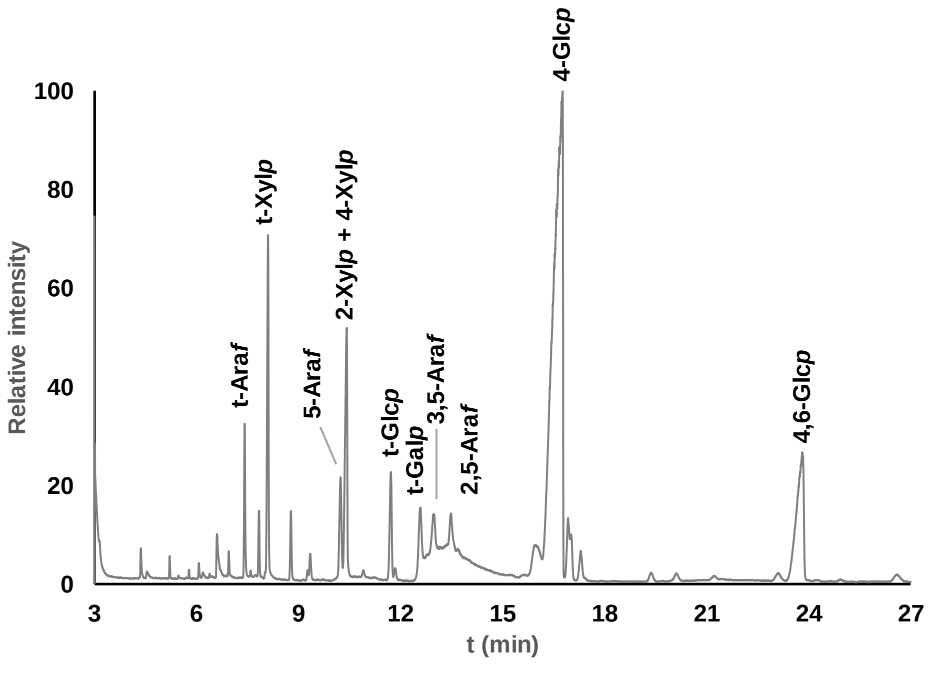

Figure 3.2 - Total ion chromatogram of the partially methylated alditol acetates (PMAA) of the residue resultant from the hot water/acetone sequential extraction

Table 3.3 – Main glycosidic linkages as partially methylated alditol acetates (PMAA) of the carbohydrates remnant in the residue resultant from the hot water/acetone sequential extraction.

Rt (min)	**PMAAs**	**Fragments** (diagnostic ions, m/z)	**Linkage**	**Average Mol %**
8.7	1,5-Di-O-acetyl-1-deuterio-6-deoxy-2,3,4-tri-O-methyl-L-galactitol	59, 72, 88, 89, 101, 102, 115, 118, 131, 175	t-Fuc*p*	1.1
			Total Fuc*p*	1.1
7.41	1,4-Di-O-acetyl-1-deuterio-2,3,5-tri-O-methyl-L-arabinitol	59, 71, 87, 101, 102, 118, 129, 161	t-Ara*f*	2.1
9.2	1,2,4-Tri-O-acetyl-1-deuterio-3,5-di-O-methyl-L-arabinitol	71, 87, 88, 100, 101, 129, 130, 161, 190	2-Ara*f*	0.1
9.3	1,3,4-Tri-O-acetyl-1-deuterio-2,5-di-O-methyl-L-arabinitol	59, 87, 99, 113, 118, 129, 160, 233	3-Ara*f*	0.4
10.2	1,4,5-Tri-O-acetyl-1-deuterio-2,3-di-O-methyl-L-arabinitol	87, 99, 102, 118, 129, 189	1,5-Ara*f*	2.4
12.9	1,3,4,5-Tetra-O-acetyl-1-deuterio-2-O-methyl-L-arabinitol	59, 85, 99, 118, 127, 261	3,5-Ara*f*	1.5
13.5	1,2,4,5-Tetra-O-acetyl-1-deuterio-3-O-methyl-L-arabinitol	87, 88, 129, 131, 189, 190	2,5-Araf	0.4
			Total Ara	6.9
8.01	1,5-Di-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-xylitol	58, 59, 87, 88, 101, 102, 117, 118, 161, 162	t-Xyl*p*	5.7
10.39	1,2,5-Tri-O-acetyl-1-deuterio-3,4-di-O-methyl-D-xylitol	88, 101, 117, 130, 190	2-Xyl*p*	3.6
10.39	1,4,5-Tri-O-acetyl-1-deuterio-2,3-di-O-methyl-D-xylitol	87, 99, 102, 118, 129, 189	4-Xyl*p*	3.6
			total Xyl*p*	12.9
11.8	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-mannitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	T-Man*p*	0.3
21.25	1,2,4,5-Tetra-O-acetyl-1-deuterio-3,6-di-O-methyl-D-mannitol	87, 88, 99, 113, 130, 190, 233	2,4-Manp	0.2
21.3	1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-D-mannitol	87, 88, 99, 113, 129, 130, 131, 190, 233	4,6-Manp	0.5
			Total Man	1.0
12.57	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-galactitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	t-Gal*p*	2.4
16.95	1,2,5-Tri-O-acetyl-1-deuterio-3,4,6-tri-O-methyl-D-galactitol	87, 88, 100, 101, 129, 130, 145, 161, 190, 205	2-Galp	1.3
16.00	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-galactitol	87, 99, 102, 113, 118, 129, 131, 162, 173, 233	4-Gal*p*	3.1
19.36	1,5,6-Tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-galactitol	87, 99, 102, 118, 129, 159, 162, 173, 189, 233	6-Gal*p*	0.4
20.1	1,3,4,5-Tetra-O-acetyl-1-deuterio-2,6-di-O-methyl-D-galactitol	59, 87, 118, 129, 143	3,4-Gal*p*	0.5
26.60	1,3,5,6-Tetra-O-acetyl-1-deuterio-2,4-di-O-methyl-D-galactitol	87, 101, 118, 129, 139, 160, 174	3,6-Gal*p*	0.5
			Total Gal	8.2
11.7	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-glucitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	t-Glc*p*	2.7
16.60	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-glucitol	87, 99, 102, 113, 118, 129, 131, 162, 173, 233	4-Glc*p*	54.6
17.3	1,5,6-Tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-glucitol	87, 99, 102, 118, 129, 162, 189, 233	6-Glc*p*	0.9
23.8	1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-D-glucitol	85, 99, 102, 118, 127, 261	4,6-Glc*p*	11.7
			Total Glc	69.9

RT – Retention time

The mass balance performed to the sequential extraction demonstrated a decrease in about 49% of the procyanidins (**Figure 3.3 a**) without impact on their average DP (4.7). A similar decrease is observed for pear procyanidins submitted to 95 ºC for 480 min **(Le Bourvellec et al., 2013)**, attributed to procyanidin depolymerization followed by thermal degradation caused by the hot acidic conditions. The levels of quercetin-glycosylated derivatives also presented an average loss of 15%. Nevertheless, as is described to occur with flavonol-glucosides of onion submitted to 180 ºC **(Rohn, Buchner, Driemel, Rauser, & Kroh, 2007)**, they were mainly converted to their aglycone form, as shown by the 5-fold increase in the amount of recovered quercetin, when compared to that initially present in apple pomace. Their molar balance demonstrated that they remained non-degraded alongside with dihydrochalcones and hydroxycinnamic acids (**Figure 3.3 a**). This resulted in an overall balance of 74% of the polyphenols in apple pomace and a shift of the polyphenolic pool towards the prevalence of flavonols (**Figures 3.2 b**).

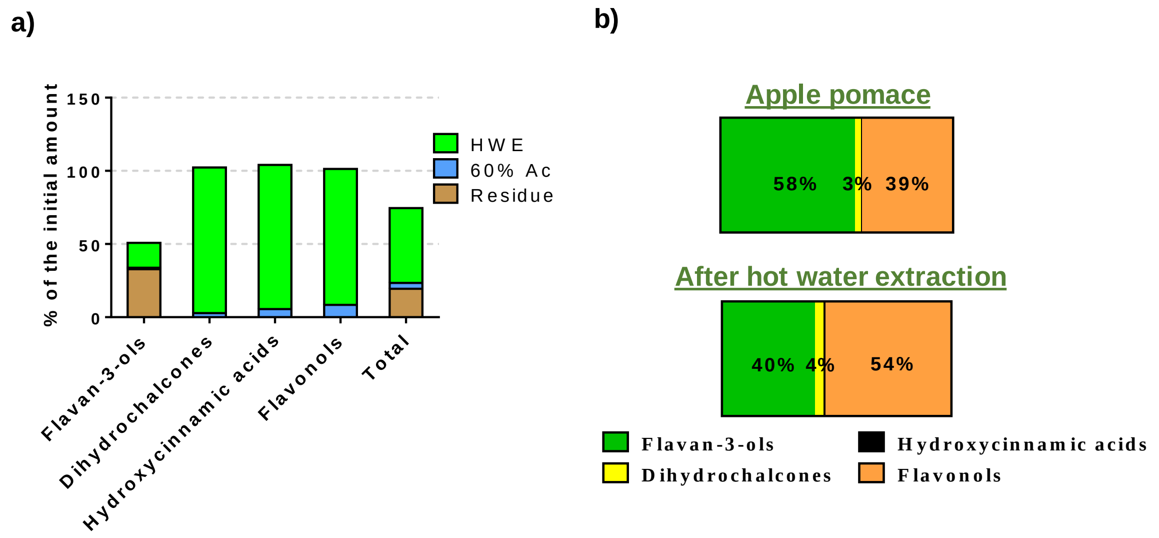

Figure 3.3 - Relative a) amount of the different polyphenolic classes after hot water/60%acetone/60%Acetone 8M urea extractions in relation to their initial amount in apple pomace, and b) proportion of apple pomace polyphenolic classes before and after the sequential extractions.

The difference in the polyphenolic content as determined by the Folin-Ciocalteu method and the UHPLC-DAD analysis, alongside the very low amounts of dihydrochalcones and hydroxycinnamic acids, suggested that oxidised polyphenolic structures could be present in the different fractions. To test this hypothesis, UHPLC-DAD-ESI-MS^n of purified polyphenol fractions was performed. Furthermore, as procyanidins oxidation may result in the formation of new linkages with resilience to thioacidolysis, alkaline fusion, a technique shown to be useful in the analysis of polyphenolic condensed structures in coffee melanoidins **(Coelho et al., 2014)** was performed to better estimate the polyphenols remaining in the residue obtained after hot water/acetone/acetone 8 M urea sequential extractions.

3.1.3 Occurrence of polyphenol oxidation products

3.1.3.1 Extractable polyphenol oxidation products

To study the existence of extractable polyphenol oxidation products, apple pomace was extracted with hexane, to remove fats and oils, methanol to remove most of the sugars and organic acids and 60% acetone. The latter fraction was then submitted to semi-preparative HPLC to remove remnant sugars and organic acids, yielding an extract composed of 360 g/kg of polyphenols of which 65% corresponded to procyanidins with and average DP of 5.8 (**Table 3.2**). Since oxidation reactions of polyphenol occur within and between polyphenol classes, leading to the formation of multiple oxidation products, each in very low concentration, HPLC-DAD-ESI-MS^n analysis of the purified fraction was performed covering *m/z* of known oxidation products formed in model solutions and known to occur in oxidised juice samples **(Bernillon et al., 2004)**.

The first eluting detected oxidation product, at RT of 5.8, presented a $[M-H]^-$ at *m/z* 597 (**Figure 3.4 a**), forming three major fragments (**Table 3.4**), at *m/z* 553, 303 and 259. The first fragment corresponded to the loss of 44 Da, justified by the elimination of a CO_2 molecule of a carboxylic group, while the second corresponded to the loss of 294, a typical loss of the Xyl-Glc moiety of phloretin-xyloglucoside found in apples **(Le Deun et al., 2015)**. The latter can be attributed to the loss of 338, outcoming from both CO_2 and Xyl-Glc moieties. This compound was identified as an oxidation product of phloretin-xyloglucoside (**Figure 3.4 d**), present in apple juices submitted to oxidative conditions **(Le Deun et al., 2015).**

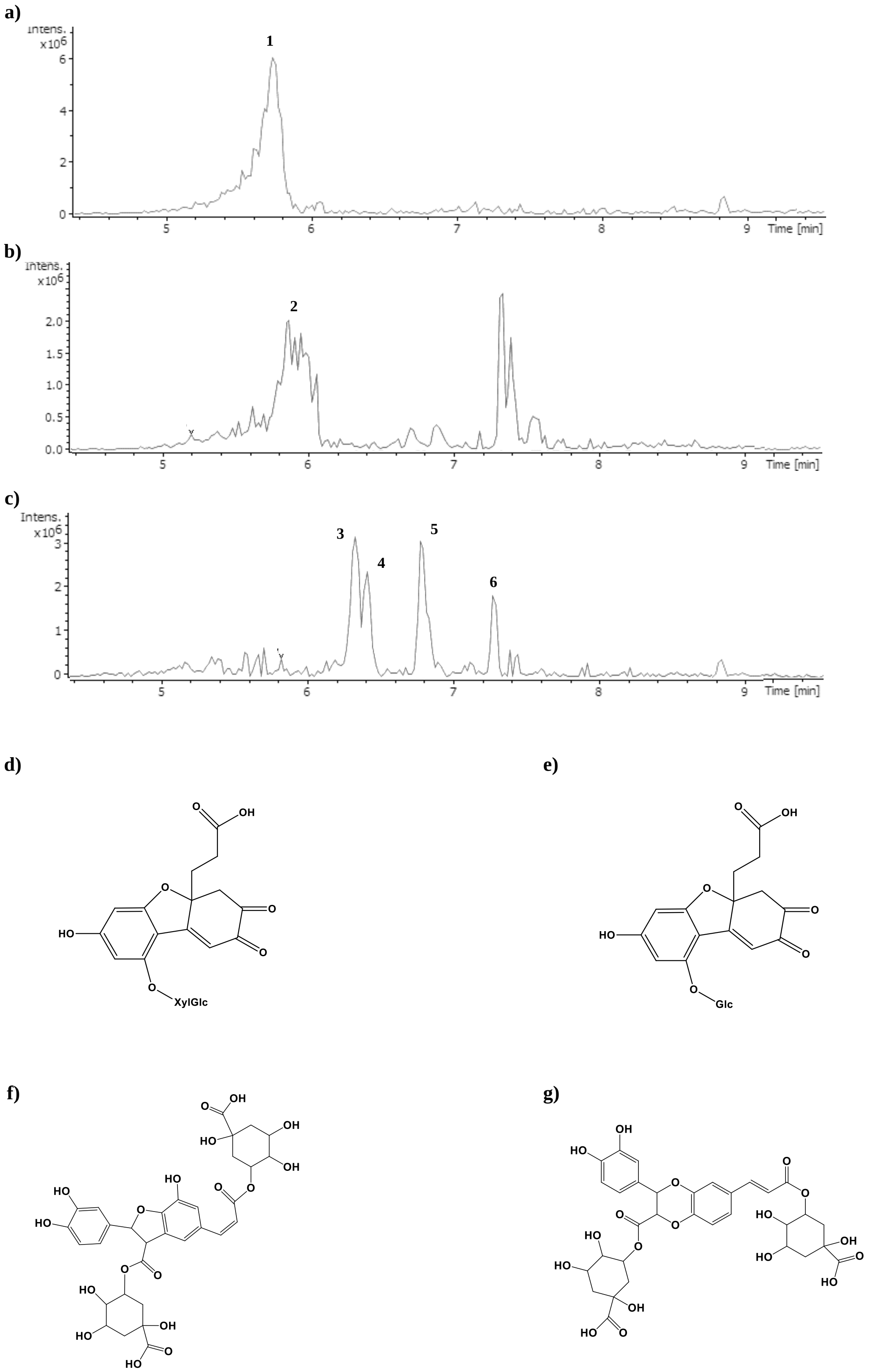

Figure 3.4 – Extracted ion MS chromatogram at a) *m/z* 597, oxidation product of phloretin-xyloglucoside, b) *m/z* 465, oxidation product of phloridzin and c) *m/z* 705, caffeoylquinic-acid oxidation products. The hypothetic structures are presented for compounds 1 d), 2 e), 3 and 4 f) and 5 and 6 g) as previously suggested by **Le Deun et al. (2015)**, **Guyot et al. (2007)** and **Bernillon (2005)**.

At RT of 5.9 min (**Figure 3.4 b**), the [M-H]$^-$ at *m/z* 465 was detected. The ESI-MS^2 spectrum of that ion (**Table 3.4**) revealed a similar fragmentation pattern with the one detected at 5.8 min. Namely, the main signal was obtained from the loss of 44 Da (ion at *m/z* 421), followed by the loss of 162 (ion at *m/z* 303) attributed to a hexose, probably the Glc unit of phloridzin **(Guyot et al., 2007)**. A loss of 206 (ion at *m/z* 259) was also observed, attributed to the loss of both molecules. These results agreed with the fragmentation patterns of a final oxidation product of phloridzin (**Figure 3.4 e**), reported by **Guyot et al. (2007)**. Both these molecules represent coloured oxidation products, being one of the contributors to yellowish/brownish colour of apple pomace and its extracts.

Table 3.4 – Retention time (RT) and mass spectrum (MS) of extractable polyphenol oxidation products found in apple pomace procyanidin rich fraction (AP PCA). MS^n fragments are expressed in decreasing order in relation to their intensity.

Nº	RT (min)	*m/z* [M-H]$^-$	MS^n	Tentatively identified as
1	5.8	597	MS^2 [597]: **553**, 303, 259	Phloretin-xyloglucoside oxidation product
2	5.9	465	MS^2 [465]: **421**, 303, 259	Phloridzin oxidation product
3	6.3	705	MS^2 [705]: **513** MS^3 [513]: **339**, 495, 321, 295	Caffeoylquinic acid oxidation product A_1
4	6.4	705	MS^2 [705]: **513** MS^3 [513]: **339**, 495, 321, 295	Caffeoylquinic acid oxidation product A_2
5	6.8	705	MS^2 [705]: **513** MS^3 [513]: **339**, 311, 161	Caffeoylquinic acid oxidation product B_1
6	7.3	705	MS^2 [705]: **513** MS^3 [513]: **339**, 311, 161	Caffeoylquinic acid oxidation product B_2

Four additional oxidation products were detected at RT 6.3, 6.4, 6.8 and 7.3 min (**Figure 3.4 c**), with a [M-H]$^-$ at *m/z* 705. Their ESI-MS^2 fragmentation pattern showed a strong ion signal at *m/z* 513, corresponding to the loss of 192 generally attributed to the loss of quinic acid (**Table 3.4**). ESI-MS^3 analysis showed that these four components could be divided in two groups according with their fragmentation pattern. ESI-MS^3 analysis of the compounds eluting at 6.3 and 6.4 min, group A, showed major ions at *m/z* 339, 495, 321 and 295. These corresponded to a loss of 174, 18, 192 and 218, attributed to the loss of deoxyquinic acid, H_2O, quinic acid and deoxyquinic acid jointly with CO_2, respectively. This fragmentation pattern was indicative that they represent isomers with probable structural similarities of previously identified dihydrobenzofuran type oxidation (**Figure 3.4 f**) products from 5-*O*-cafeoylquinic acid **(Bernillon et al., 2004)**. Compounds eluting at 6.8 and 7.3 min showed also similar fragmentation patterns yielding ions at *m/z* 339 and 311, corresponding to a loss of 174 attributed to deoxyquinic acid and 202 attributed to the loss of both deoxyquinic acid and CO. An ion at *m/z* 161, was also observed, attributed to the caffeic acid moiety after loss of quinic acid **(Bernillon, 2005)**. This fragmentation pattern was indicative that these components represent 5-*O*-caffeoylquinic acid oxidation products of dihydrobenzodioxan types (**Figure 3.4 g**) **(Bernillon, 2005; Bernillon et al., 2004)**. Their formation is proposed to result from a two electrons oxidative coupling with the simultaneous loss of two hydrogen atoms **(Wong-Paz et al., 2015)**.

3.1.3.2 Non-extractable polyphenol oxidation products

Alkaline fusion is a technique that can be used to provide structural features of unknown polyphenolic fractions **(Gramshaw, 1968)**. This is achieved by the capability of the strong alkaline medium to induce flavonoids C-ring opening and cleavage with formation of phloroglucinol (A-ring) and catechol (B-ring). In the latter, for instance, 3,4-dihydroxybenzoic acid and catechol might be formed **(Coelho et al., 2014; Gramshaw, 1968)**. These products, alongside with gallic acid and 4-hydroxybenzoic acid can also be formed by alkaline fusion of lignin due to the presence of syringyl, p-hydroxyphenyl and guaiacyl units found in lignin **(Batistic & Mayaudon, 1970)**. However, as most of the so-called apple pomace lignin (measured as “Klason lignin” i.e. material insoluble in concentrated sulfuric acid) is represented by polyphenol oxidation products formed during juice extraction **(Renard et al., 1995)**, 3,4-dihydroxybenzoic acid and catechol can be used as fingerprints for identification and quantification of non-extractable polyphenols.

Since (-)-epicatechin is known to be a precursor of coloured oxidation products **(Nicolas et al., 1994)** and the main monomeric unit of apple procyanidins, this compound was used as standard for assessment of the products resulting from alkaline fusion. Furthermore, as procyanidins were the major polyphenolic family present in the apple pomace final extraction residue, procyanidins from fresh fruits, structurally equivalent to those found in Royal Gala, were obtained and tested as representative of native, non-oxidized procyanidins. This fraction was particularly rich in procyanidins (930 g/kg), with a DP of 8, and residual amounts of hydroxycinnamic acids (3%), flavonols (2%) and dihydrochalcones (1%). Isolated apple pomace procyanidins were also tested as representative of oxidized procyanidin fractions given the detection of oxidation products already shown in this study. In this extract, procyanidins only accounted 235 g/kg, a much lower content than the one obtained from fresh Golden Delicious. This was attributed to polyphenol oxidation that impairs procyanidin quantification **(Mouls & Fulcrand, 2015)**.

As represented in **Table 3.5**, alkaline fusion of (-)-epicatechin and apple procyanidins produced 3,4-dihydroxybenzoic acid (0.67 mol/kg) and catechol (0.15 mol/kg) in similar amounts. As the formation of 3,4-dihydroxybenzoic acid and catechol is equimolar to the amount of (-)-epicatechin, it was estimated a yield of about 24% (w/w), possibly resulting from side reactions during alkaline fusion. In contrast, 0.12 mol/kg for both 3,4-dihydroxybenzoic acid and catechol were formed when performing alkaline fusion of apple pomace procyanidins. This represented a yield of 7% (w/w), indicative that during procyanidins oxidation new bonds and structural rearrangements occur, turning them less prone to yield 3,4-dihydroxybenzoic acid and catechol when submitted to alkaline fusion. This is supported by the formation of oxidation products of procyanidins involving the catechol B-ring **(Mouls & Fulcrand, 2015)**, as well as by the capability of the A-ring of one (-)-epicatechin unit to react with the B-ring via condensation reactions when submitted to extensive oxidative conditions **(Guyot et al., 1996)**.

Table 3.5 - Estimated non-extractable polyphenols (mol/kg) determined by alkaline fusion of (-)-epicatechin, apple procyanidins (Apple PCA), apple pomace procyanidins (Apple pomace PCA) and hot water/acetone/acetone 8 M urea extraction residue. In parentheses are presented the mass yields (g/kg) determined for the alkaline fusion of each sample.

Sample	3,4-dihydroxybenzoic acid	Catechol	Total
(-)-Epicatechin	0.67	0.15	0.82 (238)
Apple PCA	0.65	0.14	0.79 (229)
Apple Pomace PCA	0.14	0.14	0.28 (82)
Residue	0.04	0.08	0.12 (35)
Pooled Std	0.03	0.00	0.20 (8.59)

Alkaline fusion of the residue after hot water/acetone/acetone 8 M urea sequential extractions demonstrated the formation 3,4-dihydroxybenzoic acid and catechol, 0.04 and

0.08 mol/kg, respectively. This represented a yield of 3.7% (w/w), 19-fold higher than the amount observed by UHPLC-DAD after thioacidolysis. Additionally, extrapolating these non-extractable polyphenols to the initial apple pomace samples, they corresponded to at least 1.8% of the byproduct, more than 4-fold than those extracted with aqueous organic solvents and 17-fold than the non-extractable amount determined by UHPLC-DAD after thioacidolysis. This amount might be underestimated, given the yields obtained after alkaline fusion of (-)-epicatechin and procyanidins.

The ratio between the amount of 3,4-dihydroxybenzoic acid and catechol formed for (-)-epicatechin and apple procyanidins was about 4.4 while this was close to 1.0 and 0.5 for apple pomace procyanidins and residue, respectively. This, complemented by the data obtained by UHPLC-DAD after thioacidolysis, suggested that the polyphenols remaining in the residue are more closely related to oxidised procyanidins than to native apple (-)-epicatechin and procyanidins. However, it is important to note that such nature and amount may depend on the extent and processing conditions, more precisely the extension in which the pomace is exposed to oxygen involved in polyphenol oxidation reactions catalysed by PPO. Such conditions result in the formation of structures responsible for the observed brownish coloration **(Le Deun et al., 2015)** and that were found to be present in the pomace and its residue obtained after hot water extraction.

3.1.4 Carbohydrate structures associated to non-extractable oxidised polyphenols

Cellulose, xyloglucans, pectic polysaccharides and protein have been shown to interact with procyanidins by hydrogen bonding or hydrophobic interaction **(Renard et al., 2017)**. Their non-extractability was indicative that polyphenols were irreversibly bound to the polysaccharide and/or protein matrix that composes the residue, very probably by covalent linkages **(Pérez-Jiménez et al., 2013)**. In order to estimate to which polysaccharides, the non-extractable oxidised procyanidins were associated, microwave superheated water extraction (MWE) was performed on the hot water/acetone/acetone 8 M urea insoluble cell wall material in a three-cycles microwave sequential extraction. Two cycles were performed at 140 ºC for 5 minutes, the optimal conditions estimated for pectin extraction **(Wang & Lü, 2014)**, while the third cycle was performed at 180 ºC for 2 minutes, the optimal extraction conditions for xylans **(Coelho et al., 2014)**. Polyphenols were assessed by the Folin-Ciocalteu method in order to account both native and oxidized procyanidins.

As represented in **Table 3.1**, the two cycles of MWE at 140 ºC extracted additional 5 and 2% (w/w) of the apple pomace. The extracts were composed for more than 900 g/kg of carbohydrates and the high prevalence of GalA (26-35 mol%), Ara (12-20 mol%) and Gal (13-14 mol%) pointed out to the prevalence of pectic polysaccharides. In the MWE at 180 ºC, 6% of the initial pomace were obtained, composed mainly of carbohydrates (820 g/kg). The high prevalence of Xyl (24 mol%), Glc (19 mol%), Gal (15 mol%) and Fuc (6 mol%) indicated that xyloglucans corresponded to the main polysaccharides. The observed differences between the microwave extracts in terms of their polysaccharide composition is explained by the higher solubility of xyloglucans at temperatures above 150 ºC, through promotion of hemicellulose network degradation **(Wang et al., 2018)**. In all these fractions, significant amounts of polyphenols were detected, ranging from 28 to 108 g PLZE/kg. Of the total amount of recovered polyphenols, 77% were obtained in the xyloglucan rich-fractions, against the 23% recovered in the pectic-rich fractions (**Table 3.6)** suggesting that a relevant amount of non-extractable oxidised procyanidins were strongly associated to the xyloglucan matrix. In order to consolidate the occurrence of such carbohydrate/polyphenol complexes, the different microwave extracts were subject to graded ethanol precipitation with the principle that if they were not bond to polysaccharides, polyphenols would remain soluble in the ethanol supernatants.

Table 3.6 - Relative distribution of the total polyphenol between the different MWE extracts ethanol precipitates.

Fraction	MWE Cycle 1	MWE Cycle 2	MWE Cycle 3	Total
Ins 4ºC	0.75	0.44	1.15	2.34
Et50Pp	0.24	0.15	0.72	1.11
Et80Pp	2.05	1.42	6.73	10.2
Et80Sn	9.41	8.23	68.71	86.35
Total	12.45	10.24	77.31	100
Pooled Std	*0.04*	*0.05*	*0.02*	-

As shown in **Table 3.1**, ethanol precipitation of each of the MWE extracts yielded four distinct fractions corresponding to material insoluble in water at 4 ºC (WPp), insoluble in 50% ethanol (Et50Pp), insoluble in 80% ethanol (Et80Pp) and soluble in 80% ethanol (Et80Sn). Overall, these fractions presented a carbohydrate content ranging from 500-950 g/kg. Only 14% of the polyphenols were detected in the precipitates (**Table 3.6)**, mainly in the Et80Pp (10.2%), followed by the WPp (2.3%) and Et50Pp (1.1%), supporting the hypothesis that polyphenols were attached to the carbohydrate structures. Of these, it can be highlighted the WPp derived from the extract obtained at 180 ºC for 2 min that presented 138 g PLZE/kg of polyphenols along with Xyl (22 mol%), Glc (22 mol %), Gal (13 mol%) and Fuc (6 mol%), supporting that the polyphenols were attached to xyloglucans. The additional occurrence of GalA (22 mol%) in this fraction indicated that it may correspond to a pectic-xyloglucan complex, described to occur in apple **(Stevens & Selvendran, 1984)**, in which polyphenols may act as the linking bridges between pectic polysaccharides and xyloglucans, as suggested by **Le Bourvellec et al. (2009)**.

Most of the polyphenols, alongside with a significant fraction of the extracted polysaccharides, remained soluble in 80% ethanol. Given their non-extractability with the aqueous/acetone solutions, it is very likely that they also occurred as covalent polysaccharide/polyphenol complexes. The suggested capability of procyanidins to reinforce the interactions between pectic polysaccharides and cellulose **(Le Bourvellec et al., 2009)**, as well as the occurrence of polyphenols in the Glc-rich fractions obtained by MWE and WPp, indicated that in the final residue, composed of 534 g/kg of carbohydrates, mainly Glc (78 mol%) and residual Xyl (9 mol%) and GalA (6 mol%), some polyphenols may still remain to be extracted as a result of their involvement as cross-linkers between cell wall polymers.

So far, only indirect evidences of covalent adducts between polyphenols and polysaccharides, with exception for those occurring by biosynthesis as for ferulic acid in sugar-beet cell walls **(Levigne et al., 2004)**, have been demonstrated by irreversible polysaccharide/polyphenol complexes, as in sun-dried pear **(Ferreira et al., 2002)** or wine polymeric material **(Gonçalves et al., 2018).** These covalent adducts might be mediated by coupled oxidation/reduction reactions between procyanidins and caffeoylquinic acid *o*-quinones, in which the newly formed procyanidin *o*-quinones may nucleophilically react with other compounds, forming covalent adducts, as observed for proteins **(Trombley, Loegel, Danielson, & Hagerman, 2011)**. This mechanism seems also to occur with apple cell wall polysaccharides **(Le Bourvellec et al., 2009).** The type of linkages involved in these carbohydrate/polyphenol complexes still remains unknown but the increase in the yield of pectic material extracted from oxalate-depectinized apple, kiwi and onions by chlorite/acetic acid treatment, in comparison to alkali saponification, suggests also the presence of phenolic ether linkages involved in carbohydrate/polyphenol complexes **(Stevens & Selvendran, 1984)**. The apple pomace carbohydrate/polyphenol complexes may represent up to 40% of the total polyphenols (**Figure 3.5**) that can be obtained. It is expected that they present distinct properties of the compounds alone. For instance, the polyphenol/carbohydrate complexes present higher *in vitr*o fermentability, as measured by the increased short-chain fatty acid production, as well as polyphenol derived metabolites rather than free occurring polyphenols **(Pérez-Jiménez et al., 2013)**.

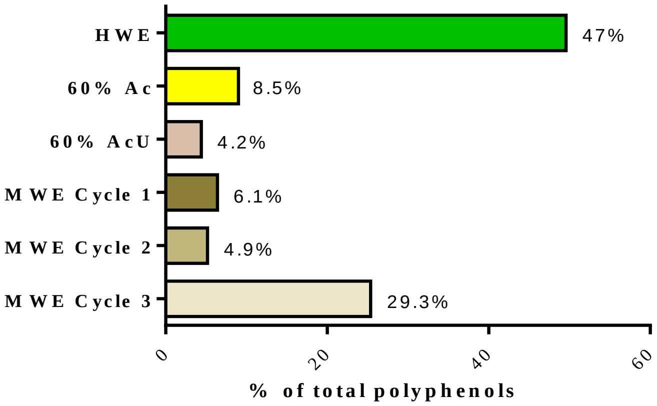

Figure 3.5 – Relative distribution of the overall extracted polyphenols and determined by the Folin-Ciocalteu method.

In order to determine the possible occurance of water-soluble polysaccharide-polyphenol covalent complexes, in Chapter 3.2, the HWE, rich in carbohydrates, was fractionated by solid-phase extraction using C_{18} cartridges. The obtained fractions were then dialyzed, ethanol precipitated and submitted to glycosidic linakge analysis, NMR, size-exclusion chromatography and alkaline fusion for characterization.

3.2 The hydrophobic polysaccharides of apple pomace

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3.2.1 Hot water extract fractionation

Hot water extraction has been suggested for extraction of pectin as it represents an easy industrially implementable process. Weakly acidic conditions are used to provide higher yields via β-elimination reactions **(Voragen et al., 2009)** while simultaneously preventing polyphenol oxidation **(Ferreira et al., 2002)**. Apple pomace acidified HWE contained free sugars (120 g/kg), mainly Fru (68 mol%) and Glc (32 mol%) (**Table 3.7**). However, it was mainly constituted by polysaccharides (72%) composed of GalA (43 mol%), Ara (23 mol%) and Gal (6 mol%), characteristic of pectic polysaccharides. Polyphenols represented 28 g PLZE/kg of dry extract (9 g GAE/kg) and proteins only accounted for 11 g/kg of extract.

To separate hydrophilic from the hydrophobic material, a C_{18} solid-phase extraction was performed yielding the hydrophobic fractions retained at pH 7 (Fr7) and at pH 3 (Fr3) and a non-retained hydrophilic fraction (NrFr). Alongside with polyphenols (61-63 g PLZE/kg of dry extract) and protein (8-11%), sugar analysis revealed that a large amount of carbohydrates, ranging from 400-420 g/kg of the extract, were present in Fr7 and Fr3. Fr7 was rich in neutral sugars, namely Ara (57 mol%), Glc (8 mol%) and Gal (6 mol%). GalA accounted for 20 mol%, part of which might be de-esterified **(Schols et al., 1990)**, conferring negative charges to the compounds that, even so, were hydrophobically retained in the C_{18} matrix. This allows to infer the occurrence of hydrophobic features that surpass the effect of the negative charges and hydroxyl groups of sugar residues components of these structures. When the pH of elution was 3, the amount of GalA in the fraction retained was 70 mol%. This increase in the relative proportion of GalA from Fr7 to Fr3 may be explained by the neutralization of the negative charges of the carboxylic acids from GalA (pK_a of GalA is 3.5 according to **Kohn and Kovac (1977)**), which prevented their retention at pH 7, although not at pH 3. Given that GalA does not take part of the glycosylated polyphenols found in apple **(Guyot et al., 2002; Lu & Foo, 1997)**, this data suggests that pectic polysaccharides were components of these hydrophobic fractions.

Table 3.7 – Yield (%), carbohydrate composition (mol %), total carbohydrates (g/kg), protein and total polyphenols (g PLZE/kg) of the different fractions derived from the water-soluble material obtained from apple pomace. In parenthesis are presented that data in gallic acid equivalents (g GAE/kg) for total polyphenols. Data are expressed as Pooled standard deviation (Pooled std) of three replicates. n.d means for not determined.

Fraction	**Yield (%)**		**Carbohydrate composition (molar %)**									**Total Carbohydrates (g/kg)**	**Total protein (g/kg)**	**Total PC (g PLZE/kg)**
			Rha	Fuc	Ara	Xyl	Fru*	Man	Gal	Glc	GalA			
HWE	100	Polysac.	1	tr	23	3	-	tr	6	24	43	720	11	28 (9)
		Sugars	-	-	-	-	68	-	-	32	-	116		
Fr7		Polysac.	2	1	57	4	-	2	6	8	20	422	105	61 (20)
>12 kDa	6.1	Polysac.	3	1	58	4	-	tr	6	10	16	647	9	62 (20)
Et80Pp	3.1	Polysac.	2	1	33	8	-	2	9	15	31	662	n.d	67 (25)
Et80Sn	2.7	Polysac.	2	tr	86	tr	-	2	1	2	7	638	n.d	79 (29)
1<x<12 kDa	1.9	Polysac.	2	tr	58	2	-	1	5	9	23	448	92	38 (16)
Fr3		Polysac	3	1	15	5	-	tr	4	3	70	394	80	63 (18)
>12 kDa	1.4	Polysac.	5	1	11	5	-	1	6	5	66	531	29	37 (13)
NrFr		.												
>12 kDa	26	Polysac	1	tr	20	4	-	tr	7	18	50	954	-	8 (3)
WPp	4.0	Polysac	tr	tr	2	tr	-	tr	1	92	4	833	-	16 (5)
Et50Pp	4.1	Polysac	2	tr	23	4	-	tr	8	12	51	966	-	8 (3)
Et80Pp	16	Polysac	2	tr	25	5	-	tr	8	7	52	928	-	5 (2)
Et80Sn	1.2	Polysac	1	tr	31	2	-	tr	10	2	54	897	-	25 (11)
1<x<12 kDa	2.0	Polysac	tr	tr	1	tr	-	tr	6	1	91	806	37	8 (4)
Pooled std	*0.3*		*0*	*0*	*1*	*0*	*4*	*0*	*0*	*0*	*1*	*9*	*1*	*1 (0)*

To obtain the high molecular weight material, all fractions were extensively dialysed using 12-14 kDa cut-off membranes. The polymeric material in Fr7 represented 6.1% (w/w) of the acidified HWE and was composed by 650 g/kg of carbohydrates, mainly Ara (58 mol%) and GalA (16 mol%) (**Table 3.7**). An extra 1.9% (w/w) of material containing 450 g/kg of carbohydrates and a similar sugar composition was obtained with a molecular weight ranging from 1-12 kDa. For the Fr3 fraction, it was also possible to recover high molecular weight material, representing 1.4% (w/w) of the acidified HWE. This material was composed by 530 g/kg of carbohydrates, mainly GalA (66 mol%). The sugars composition of these polymeric fractions confirmed the occurrence of pectic polysaccharides in apple pomace with hydrophobic properties.

The polymeric material with molecular weight higher than 12 kDa and not retained in the C_{18} cartridges neither at pH 7 nor pH 3 (NrFr) accounted for 26% (w/w) of the hot water extracted material. On the other hand, that ranging from 1-12 kDa accounted for 2% (w/w). Both fractions were rich in carbohydrates (>800 g/kg), but the former presented a high prevalence of GalA (50 mol%), Ara (20 mol%) and Glc (18 mol%) while the latter was composed majorly by GalA (91 mol%). Both extracts approached the sugars composition of Fr3 high molecular weight material. Overall, the Fr7, Fr3, and NrFr polymeric fractions represented 37% (w/w) of the hot water-soluble material, which is in agreement with the high occurrence of pectic oligosaccharides in apple pomace extracts **(Mehrländer et al., 2002)**. Based on the polymeric material recovered in Fr7, Fr3 and NrFr, it can be estimated that the amount of hydrophobic polymeric material recovered at pH 7 was 21% and 4% at pH 3. The remaining 75% corresponds to hydrophilic polymeric material. Together with the carbohydrates, Fr7, Fr3 and NrFr were also composed by a small amount of polyphenols (**Table 3.7**), which was higher for Fr7 and Fr3. Apple polyphenols reported in the literature correspond to single molecules or oligomeric structures with an average degree of polymerization of 5 **(Guyot et al., 2002),** which correspond to molecular weights much lower than the cut-off (12-14 kDa) of the dialysis membrane used in this study. As a result, their non-diffusion along the dialysis membrane might occur due to existing interactions with the polysaccharides of the Fr7, Fr3 and NrFr fractions, giving rise to polysaccharide-polyphenol complexes as reported to occur in wine **(Gonçalves et al., 2018; Gonçalves et al., 2012).**

To characterize the type of linkages between polysaccharides and polyphenols and their possible influence on the hydrophobicity of polysaccharides isolated from apple pomace, the Fr7 and NrFr fractions of high molecular weight were submitted to water/ethanol precipitations. This was based on the principle that free polyphenols i.e. not covalently bonded to polysaccharides, remain soluble in ethanol. The resultant fractions were characterized according to the sugar and linkage composition of the polysaccharides, size exclusion chromatography and alkali fusion to identify the polyphenolic features linked to the polysaccharides.

3.2.2 Characterization of the polysaccharide/polyphenol complexes

3.2.2.1 Ethanol precipitation fractions

The ethanol fractionation of the Fr7 high molecular weight material yielded a fraction that precipitated at 80% ethanol (Et80Pp), accounting for 3.1% (w/w), composed by 660 g/kg of carbohydrates, mainly Ara (33 mol%), GalA (31 mol%) and Glc (15 mol%) (**Table 3.7**). Glycosidic linkage analysis (**Table 3.8**) revealed the occurrence of (1→5)-Ara*f* (18.8%), (1→3,5)-Ara*f* (6.8%), (1→2,5)-Ara*f* (3.8%), (1→2,3)-Ara*f* (1.3%), (1→2,3,5)-Ara*f* (3.1%), and t-Ara*f* (13.4%), characteristic of arabinans **(Renard et al., 1991)**. The occurrence of (1→4)-Glc*p* (14.6 mol %), together with (1→4,6)-Glc*p* (4.1%), (1→2)-Xyl*p* (3.8%), t-Gal*p* (2.2%), (1→2)-Gal*p* (1.6%), t-Fuc*p* (1.6%) and t-Xyl*p* (0.4%), also suggests the occurrence of xyloglucans **(Cruz et al., 2018; Renard et al., 1995)**. The remaining 6.9% of *t*-Xyl*p* could be explained by the occurrence of xylogalacturonans **(Voragen et al., 2009)**. This fraction also contained polyphenols (67 g PLZE/kg), which represented 50% of the polyphenols in the Fr7 high molecular weight material. These structural features suggest the presence of pectic-polyphenol-xyloglucan complexes **(Selvendran, 1985)** in which polyphenols act as linking bridges between pectic polysaccharide and xyloglucans. The average molecular weight of this fraction was 43.4 kDa, with a polydispersity of 6.4 (**Table 3.9**) as determined by Size Exclusion Chromatography (SEC, **Figure 3.6**).

Table 3.8 – Main glycosidic linkages as partially methylated alditol acetates (PMAA), expressed as mol %, of the carbohydrates in the different fractions obtained by ethanol precipitation of the high molecular weight material of retained fraction at pH 7 (Fr7) and non-retained fraction (NrFr).

Linkage	**Fr7 >12 kDa**		**NrFr >12 kDa**			
	Et80Pp	Et80Sn	WPp	Et50Pp	Et80Pp	Et80Sn
t-Rha*p*	1.0	0.7		0.4	0.5	
2-Rha*p*		1.9		1.9	1.8	0.8
2,4-Rha*p*	0.7	0.6		1.1	1.7	0.6
Total Rha	1.7	3.2		3.4	4.0	1.4
t-Fuc*p*	1.6	0.8		0.2	0.3	
3,4-Fuc*p*	0.4	0.3				0.1
Total Fuc*p*	2.0	1.1		0.2	0.3	0.1
t-Ara*f*	13.4	7.7	0.6	14.0	14.6	15.9
2-Ara*f*	1.0	1.5		0.1	0.1	0.1
3-Ara*f*	3.9	8.2	0.2	2.1	2.7	3.9
5-Ara*f*	18.8	47.3	0.5	21.5	26.0	37.0
3,5-Ara*f*	6.8	13.1		10.7	11.1	14.7
2,3-Ara*f*	1.3	0.7				
2,5-Ara*f*	3.8	4.3		1.9	2.4	1.1
2,3,5-Ara*f*	3.1	2.1				
Total Ara*f*	52.0	84.9	1.3	50.3	56.9	72.7
t-Xyl*p*	6.3	1.0	0.3	2.2	1.9	1.3
2-Xyl*p*	3.8		0.2			
4-Xyl*p*			0.1	1.5	3.1	0.5
2,3-Xyl*p*		0.3				
2,4-Xyl*p*					0.7	0.4
Total Xyl*p*	10.1	1.3	0.6	3.7	5.7	2.2
t-Man*p*	1.3	0.7		0.2	1.6	0.3
6-Man*p*		1.0				
Total Man*p*	1.3	1.7		0.2	1.6	0.3
t-Gal*p*	2.2	1.0		1.9	2.9	1.4
2-Gal*p*	1.6					
3-Gal*p*				0.5	0.6	0.5
4-Gal*p*	10.2	1.8	1.3	9.5	10.7	16.1
6-Gal*p*				0.2	0.3	0.4
3,4-Gal*p*				0.9	0.6	
3,6-Gal*p*				0.4	0.7	1.4
4,6-Gal*p*		0.6		0.3	1.0	
Total Gal*p*	14.0	4.9	1.3	14.8	16.8	19.8
t-Glc*p*			4.7	2.2	0.2	0.1
4-Glc*p*	14.6	1.8	87.6	24.7	14.5	3.2
6-Glc*p*		0.1				
4,6-Glc*p*	4.1	1.1	4.5	1.6		0.2
Total Glc*p*	18.7	3.0	96.8	28.5	14.7	3.5

The material that remained solubilized in 80% ethanol (Et80Sn), accounted for 2.7% (w/w), contained 640 g/kg of carbohydrates, mainly Ara (86 mol%) with an average molecular weight of 4.9 kDa and a polydispersity of 2.0. Glycosidic linkage analysis (**Table 3.8**) demonstrated the prevalence of (1→5)-Ara*f* (47%) together with (1→3,5)-Ara*f* (13%), (1→2,5)-Ara*f* (4.3%), (1→2,3)-Ara*f* (0.7%), and (1→2,3,5)-Ara*f* (2.1%), characteristic of an arabinan with an average one branching point per 4 Ara*f* units. However, t-Ara*f* only explained 8% out of the 20% of branching. The determination of 79 g PLZE/kg of polyphenols in Et80Sn may explain this difference by the occurrence of bonded polyphenols to the arabinofuranosyl terminal units, similarly to biosynthetically linked ferulic acids in *Amaranthaceae* (spinach) **(Fry, 1983)** and *Poaceae* (bamboo) **(Ishii, 1991)**, respectively**.** This difference may also result from modifications of the *t*-Ara*f* as a result of polyphenol oxidative bonding. The number of branching points associated to polyphenols covalent linkage in Et80Sn (15 in 22) was higher than those observed in Et80Pp (5 in 18), although the total polyphenolic content (PLZE equivalents) was close between fractions. As Folin–Ciocalteu method follows a structure-activity relationship, in which the reaction is proportional to the number of hydroxyl groups present in the aromatic ring, the polyphenols structural diversity should result in different polyphenol response factors **(Everette et al., 2010)**. This polyphenol structural diversity, alongside with the possible arabinofuranosyl units structural modification due to polyphenol oxidative bonding, and arabinans lability to acid hydrolysis may also explain the lower content of compounds identified in the hydrophobic fractions (about 60%) when compared with the hydrophilic ones (>80%).

Table 3.9 - Estimated weighted-average (Mw), number-average (Mn) molecular weightsand polydispersity (PD) determined by size exclusion chromatography and alkaline fusion products of the different Fr7 >12-14 kDa fractions before and after 80% ethanol precipitation. Data from one replicate.

Sample	Size-exclusion chromatography			Alkaline fusion products (mol/kg)		
	Mw (kDa)	Mn (kDa)	PD (Mw/Mn)	3,4-dihydroxybenzoic acid	Catechol	Total
Fr7 >12-14 kDa	15.4	4.1	3.8	n.d.	n.d.	n.d.
Et80Pp	43.4	6.8	6.4	0.011	0.062	0.073
Et80Sn	4.9	2.5	2.0	0.008	0.060	0.068

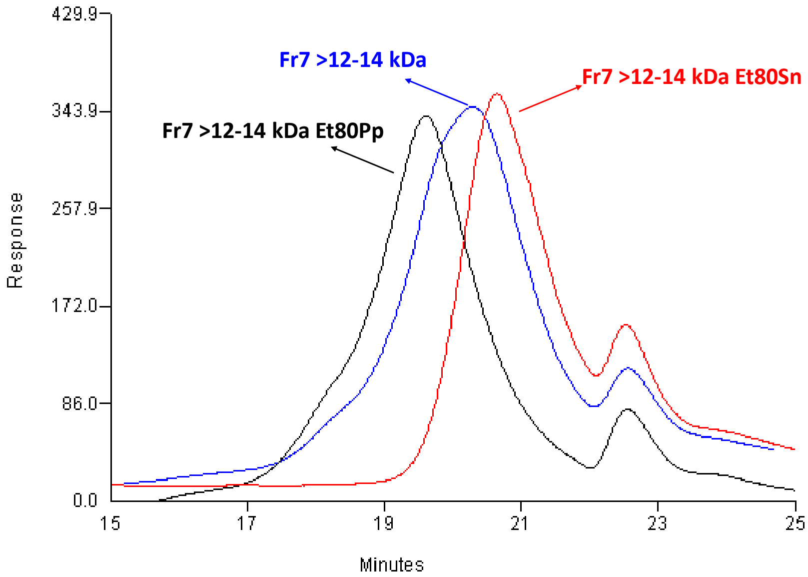

Figure 3.6 – Size exclusion chromatogram of Fr7 >12-14 kDa, Fr7 >12-14 kDa Et80Pp and Fr7 >12-14 kDa Et80Sn.

To access the contribution of polyphenols on the hydrophobic properties of Fr7, the hydrophilic material not retained in the C_{18} cartridges (NrFr), was also submitted to ethanol fractionation. Four distinct fractions, WPp (4.0% w/w), Et50Pp (4.1% w/w), Et80Pp (16% w/w) and Et80Sn (1.2% w/w) were obtained (**Table 3.7**). WPp was mainly composed of Glc (92 mol %), present as 1→4-Glc*p* (88 mol%), 1→4,6-Glc*p* (4.5 mol%) and t-Glc*p* (4.7 mol %) (**Table 3.8**), indicative of a highly linear glucan, possibly corresponding to glucan degraded material **(Cruz et al., 2018; Renard et al., 1995)**. This fraction only contained 16 g PLZE/kg of polyphenols. Et50Pp, Et80Pp and Et80Sn were mainly constituted by GalA (51-54 mol%), Ara (23-31 mol%) and Gal (8-10 mol%) characteristic of pectic polysaccharides with arabinan and galactan as side chains (representative chromatogram of the partially methylated alditol acetates of the neutral sugars in **Figure 3.7,** peak assignments in **Table 3.10**). These fractions only contained, on a weighted average, 7 g PLZE/kg of polyphenols (**Table 3.7**) which are 10-fold lower than that found for the Fr7 high molecular weight material. Protein, which could also contribute to the hydrophobicity of the Fr7, although absent in NrFr, accounted only for 1% in Fr7 emphasizing the relevance of polyphenols for the hydrophobic property.

To identify the possible structural polyphenolic features that may be associated to polysaccharides of Fr7, alkaline fusion was performed. As represented in **Table 3.9**, 3,4-

dihydroxybenzoic acid (0.008-0.011 mol/kg) and catechol (0.060-0.062 mol/kg) were detected in the two fractions derived from Fr7, demonstrating the presence of a catechol ring, possibly arising from oxidized procyanidins, the most prevalent polyphenols in apple **(Guyot et al., 2002; Lu & Foo, 1997)**. To provide a more detailed structural characterization of the polysaccharide/polyphenol complexes, the Et80Sn of Fr7, containing a purified arabinan, was submitted to NMR spectroscopic analysis.

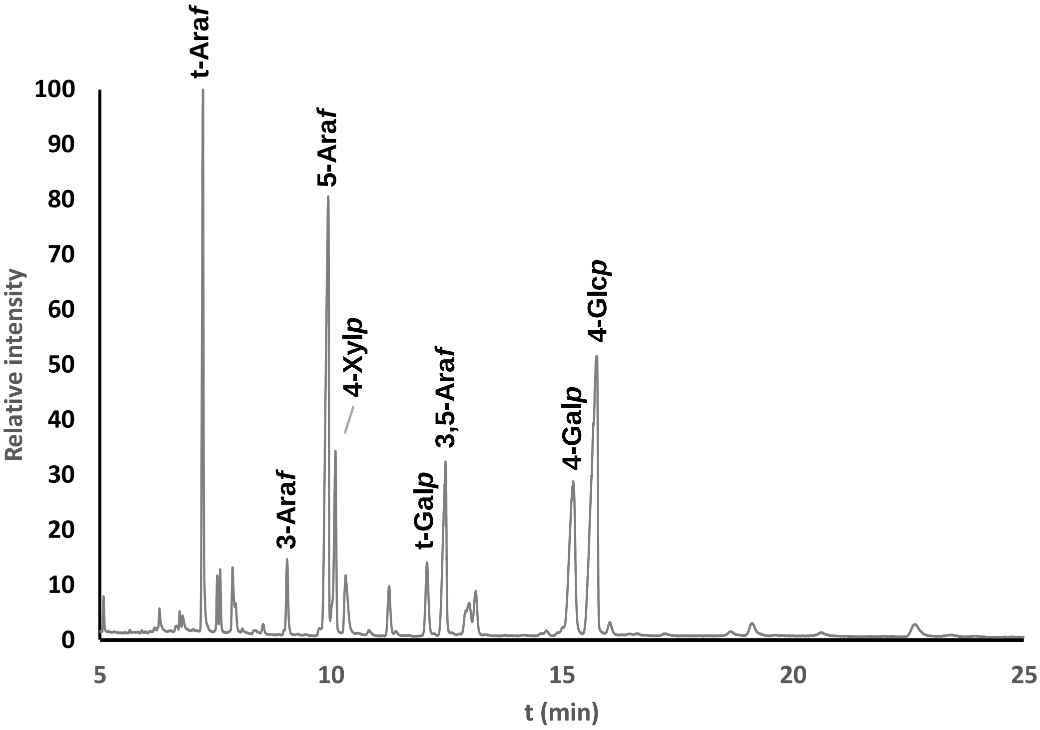

Figure 3.7 - Total ion chromatogram of the partially methylated alditol acetates (PMAA) from the Et50Pp resultant from the NrFr extraction.

Table 3.10- Main glycosidic linkages observed for the non-retained fraction insoluble in 50% (v/v) ethanol (NrFr Et50Pp).

Rt (min)	**PMAAs**	**Fragments** (diagnostic ions, m/z)	**Linkage**	**Average Mol %**
7.94	1,5-Di-O-acetyl-1-deuterio-6-deoxy-2,3,4-tri-O-methyl-L-mannitol	59, 72, 89, 102, 115, 118, 131, 162	t-Rha*p*	0.4
10.10	1,2,5-Tri-O-acetyl-1-deuterio-6-deoxy-3,4-di-O-methyl-L-mannitol	72, 88, 89, 100, 115, 130, 131, 190	2-Rha*p*	1.9
13.13	1,2,4,5-Tetra-O-acetyl-1-deuterio-6-deoxy-3-O-methyl-L-mannitol	88, 101, 130, 143, 190, 203	2,4-Rha*p*	1.1
			Total Rha*p*	3.4
8.53	1,5-Di-O-acetyl-1-deuterio-6-deoxy-2,3,4-tri-O-methyl-L-galactitol	59, 72, 88, 89, 101, 102, 115, 118, 131, 175	t-Fuc*p*	0.2
			Total Fuc*p*	0.2
7.23	1,4-Di-O-acetyl-1-deuterio-2,3,5-tri-O-methyl-L-arabinitol	59, 71, 87, 101, 102, 118, 129, 161	t-Ara*f*	14.0
8.99	1,2,4-Tri-O-acetyl-1-deuterio-3,5-di-O-methyl-L-arabinitol	71, 87, 88, 100, 101, 129, 130, 161, 190	2-Ara*f*	0.1
9.05	1,3,4-Tri-O-acetyl-1-deuterio-2,5-di-O-methyl-L-arabinitol	59, 87, 99, 113, 118, 129, 160, 233	3-Ara*f*	2.1
9.94	1,4,5-Tri-O-acetyl-1-deuterio-2,3-di-O-methyl-L-arabinitol	87, 99, 102, 118, 129, 189	1,5-Ara*f*	21.5
12.48	1,3,4,5-Tetra-O-acetyl-1-deuterio-2-O-methyl-L-arabinitol	59, 85, 99, 118, 127, 261	3,5-Ara*f*	10.7
12.95	1,2,4,5-Tetra-O-acetyl-1-deuterio-3-O-methyl-L-arabinitol	87, 88, 129, 131, 189, 190	2,5-Araf	1.9
			Total Ara	50.3
7.87	1,5-Di-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-xylitol	58, 59, 87, 88, 101, 102, 117, 118, 161, 162	t-Xyl*p*	2.2
10.06	1,4,5-Tri-O-acetyl-1-deuterio-2,3-di-O-methyl-D-xylitol	87, 99, 102, 118, 129, 189	4-Xyl*p*	1.5
			Total Xyl*p*	3.7
11.41	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-mannitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	t-Man*p*	0.2
			Total Man	0.2
12.08	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-galactitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	t-*Galp*	1.9
15.25	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-galactitol	87, 99, 102, 113, 118, 129, 131, 162, 173, 233	4-Gal*p*	9.5
16.04	1,3,5-Tri-O-acetyl-1-deuterio-2,4,6-tri-O-methyl-D-galactitol	74, 87, 101, 118, 128, 129, 143, 160, 161, 174, 234	3-Gal*p*	0.5
18.66	1,5,6-Tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-galactitol	87, 99, 102, 118, 129, 159, 162, 173, 189, 233	6-Gal*p*	0.2
19.11	1,3,4,5-Tetra-O-acetyl-1-deuterio-2,6-di-O-methyl-D-galactitol	59, 87, 118, 129, 143	3,4-Gal*p*	0.9
23.41	1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-D-galactitol	85, 99, 102, 117, 118, 127, 261	4,6-Gal*p*	0.3
25.70	1,3,5,6-Tetra-O-acetyl-1-deuterio-2,4-di-O-methyl-D-galactitol	87, 101, 118, 129, 139, 160, 174	3,6-Gal*p*	0.4
			Total Gal	14.8
11.26	1,5-Di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methyl-D-glucitol	71, 87, 101, 102, 118, 129, 145, 161, 162, 205	t-Glc*p*	1.7
15.75	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-glucitol	87, 99, 102, 113, 118, 129, 131, 162, 173, 233	4-Glc*p*	24.7
22.63	1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-D-glucitol	85, 99, 102, 118, 127, 261	4,6-Glc*p*	1.6
			Total Glc	25.2

3.2.2.2 NMR spectroscopic analysis

The ^{13}C NMR spectrum of the Fr7 high molecular weight material soluble in 80% ethanol is represented in **Figure 3.8 a** (DEPT-135 in **Figure 3.8 b**). Based on the NMR data from arabinans **(Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Xia, Liang, Yang, Wang, & Kuang, 2015)** and arabinan rich-pectins **(Renard & Jarvis, 1999; Shakhmatov et al., 2018; Shakhmatov, Toukach, Michailowa, & Makarova, 2014)**, it was possible to identify →5)-Ara*f*-(α1→ (**A**), →3,5)-Ara*f*-(α1→ (**B**), →3)- Ara*f*-(α1→ (**C**), →2,5)-Ara*f*-(α1→ (**D**) and Ara*f*-(α1→ (**E**) residues in the characteristic chemical shifts of anomeric carbons C-1 (δ_C 107.5–106.5) from arabinofuranosyl residues.

The signal at δ_C 101.3 could be attributed to Ara*f*-(β1→ (**F**), identified to occur in olive pomace pectic polysaccharides **(Cardoso et al., 2007; Cardoso et al., 2002)**. Also, the well-defined carbon signals in the DEPT-135 at δ_C 67.0, 66.8, 62.9, and 61.1 could be attributed to the C-5 of the residues **A**, **B/D**, **F**, and **C/E** respectively (**Table 3.11**).

Table 3.11 - ^{1}H and ^{13}C chemical shifts (δ) of Fr7 >12-14 kDa Et80Sn in D2O at 500.13 MHz and 125.77 MHz

Residue	*Sugar linkage*	*H1-C1*	*H2-C2*	*H3-C3*	*H4-C4*	*H5-C5*
A	→5)-Ara*f*-(α1→	4.97 [a,b,c]	4.02 [a,b,c]	3.88 [a,b,c]	4.11 [a,b,c]	3.65-3.70/3.76-3.78 [a,b,c]
		107.5 [b,c]	80.8 [b,c]	76.6 [b,c]	82.3 [b,c]	67.0 [b,c,d]
B	→3,5)-Ara*f*-(α1→	5.00 [a,b,c]	4.17 [a,b]	3.97 [a,b]	3.92 [a,b,c]	3.65-3.70/3.76-3.78 [a,b,c]
		107.5 [b,c]	79.2 [b]	83.9 [b,c]	82.1 [b,c]	66.8 [b,c,d]
C	→3)-Ara*f*-(α1→	5.06 [a,b,c]	4.03 [a,b]	3.82 [b,c]	3.99 [a,b]	3.57-3.61/3.67-3.72 [a,b]
		107.1[b]	80.8 [b]	83.9 [b,c]	81.4 [b]	61.0 [b,c,d]
D	→2,5)-Ara*f*-(α1→	5.08 [a,c]	4.05[a]	4.10[b]	4.18[a]	3.65-3.70/3.75-3.79 [a,b]
		106.5 [b,c]	87.0 [b,c]	79.5 [b,c]	81.4 [b,c]	66.8 [b,c,d]
E	Ara*f*-(α1→	5.03 [a,b,c]	4.02 [a,c]	3.85 [a,c]	3.95 [a,c]	3.57-3.61/3.67-3.72 [a,b,c]
		107.4 [b,c]	81.8 [b,c]	76.5 [b,c]	83.9 [b,c]	61.1 [b,c,d]
F	Ara*f*-(β1→	4.96 [a,b,c]	4.01[a,c]	3.80 [a,c]	3.79 [a,b]	3.53-3.60/3.65-3.70 [a,b]
		101.4 [b]	76.3 [b]	74.2 [b]	82.0 [b,c]	62.9 [b,d]

[a] COSY [b]HSQC, [c]HMBC, [d]DEPT-135

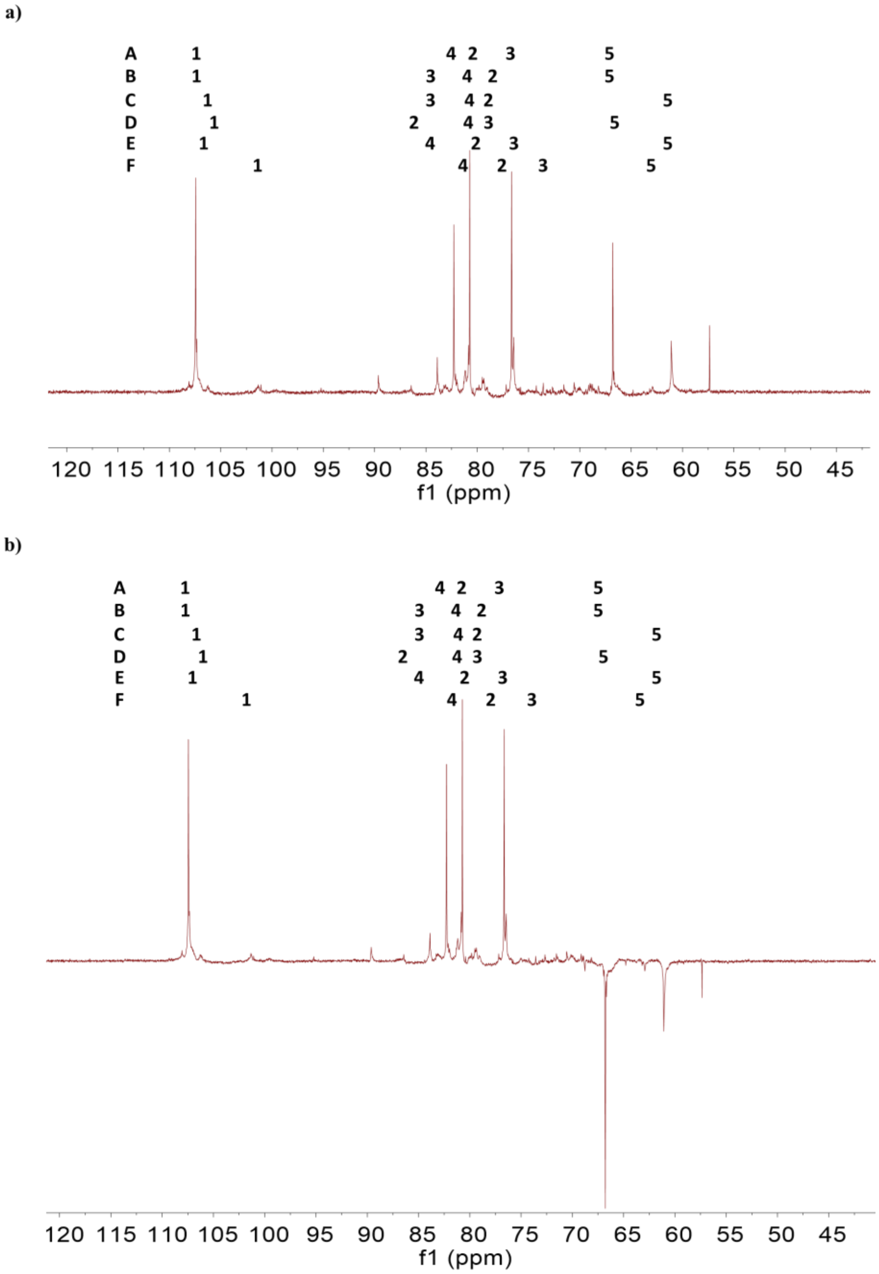

Figure 3.8 - Representation of the key a) ^{13}C NMR spectrum and b) DEPT-135 spectrum of Fr7 >12 kDa Et80Sn. Letters represent the type of arabinofuranosyl unit: A - →5)-Ara*f*-(α1→; B - →3,5)-Ara*f*-(α1→; C - →3)-Ara*f*-(α1→; D - →2,5)-Ara*f*-(α1→; E - Ara*f*-(α1→; F - Ara*f*-(β1→. Numbers represent the carbon number starting from the anomeric carbon

Minor signals of *O*-acetyl and carboxyl groups were also observed in the ^{13}C NMR at δ_C 20.3 and 173.9, respectively. HSQC and HMBC analysis revealed that *O*-acetyl moiety at δ_C 20.3/δ_H 2.06 was correlated with δ_C 173.9 (not shown). Although no direct evidences of acetylation were determined for this fraction, Gal*p*A in apple pectin can be acetylated at O-2 and/or O-3 positions, depending on the botanical species **(Perrone et al., 2002)**. In the

NMR analysis, a peak characteristic of CH_3O- groups (δ_C 57.4) was also observed suggesting that some of the GalA could be methyl esterified. No additional assignments were possible for GalA, possibly due to their lower degree of freedom when compared to the arabinose units **(Dourado et al., 2006; Schols et al., 1990)**.

From the ^{1}H–^{1}H COSY spectrum (**Figure 3.9**) it was possible to correlate protons for each one of the identified arabinofuranosyl units (**Table 3.11**), while by one-bond ^{13}C-^{1}H correlation of NMR resonances, as HSQC spectrum (**Figure 3.10 a** and **Table 3.11**), it was possible to assign the carbon signals of the residues **A-F**. Due to the overlapping nature of the spectra, some assignments might be interchangeable but agreeing with those of literature. Cross peaks of both anomeric protons and carbons of each sugar residue were observed both at intra and inter-residual level by HMBC analysis (**Figure 3.10 b** and **Table 3.11**). Besides the intra-residual correlations, long-range correlations were observed, allowing to infer about 15 different sequences (**Table 3.12**) present along the arabinan structure.

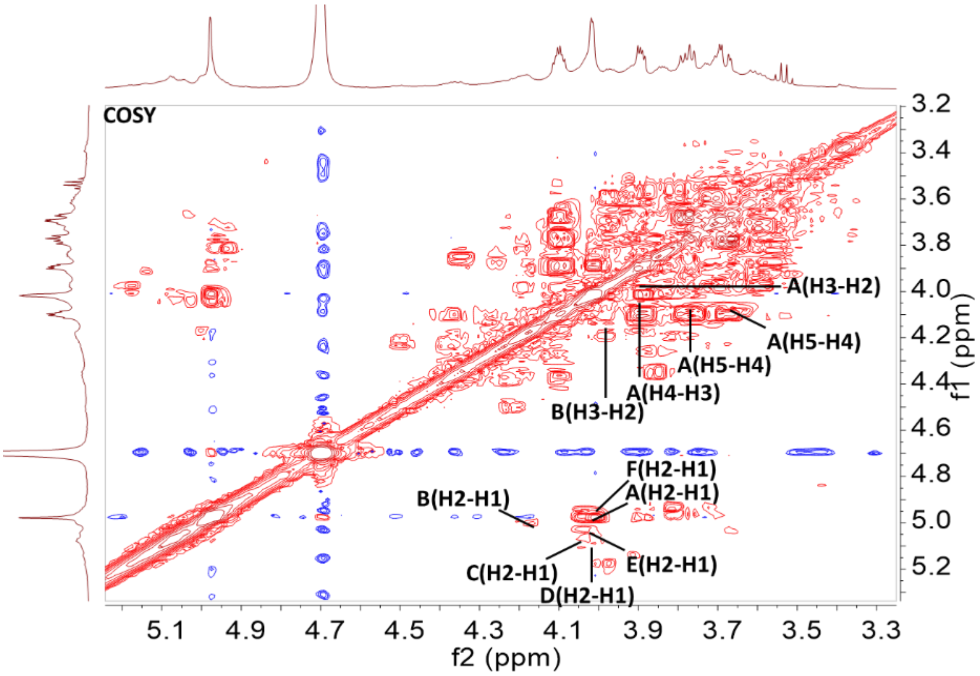

Figure 3.9 - ^{1}H-^{1}H COSY spectrum of Fr7 >12 kDa Et80Sn.

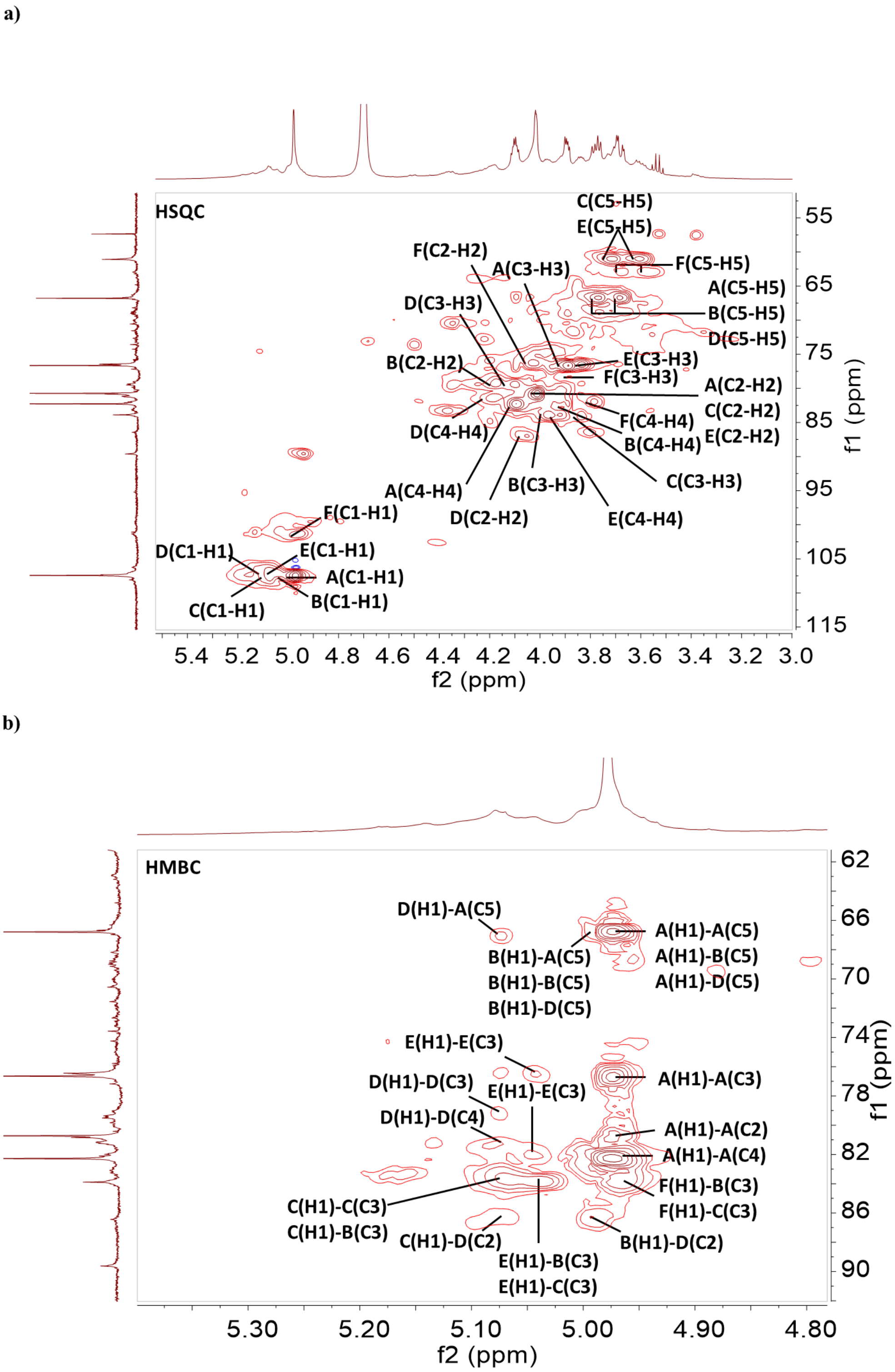

Figure 3.10 - HSQC spectrum and HMBC spectrum of Fr7 >12-14 kDa Et80Sn fraction. Letters represent the type of arabinofuranosyl unit : A - →5)-Ara*f*-(α1→; B - →3,5)-Ara*f*-(α1→; C - →3)-Ara*f*-(α1→; D - →2,5)-Ara*f*-(α1→; E - Ara*f*-(α1→; F - Ara*f*-(β1→. Numbers represent the carbon and proton number starting from the anomeric carbon.

Table 3.12 - Key $J_{H,C}$ connectivities observed in the HMBC spectrum of Fr7 >12 kDa Et80Sn.

Residue	**Sugar linkage**	**H (δ_H)**	**Observed connectivities**			**Sequence**
			δ_H /δ_C	Residue	Atom	
A	→5)-Ara*f*-(α1→	4.97	67.0	A	C5	1
		4.97	66.9	B	C5	2
		4.97	66.7	D	C5	3
		4.97	76.7	A	C3	-
		4.97	81.0	A	C2	-
		4.97	82.3	A	C4	-
		3.65-3.70 3.76-3.78	107.5	A	C1	-
		3.65-3.70 3.76-3.78	107.5	B	C1	-
B	→3,5)-Ara*f*-(α1→	5.00	67.0	A	C5	4
		5.00	66.7	B	C5	5
		5.00	66.7	D	C5	6
		5.00	87.0	D	C2	7
		3.65-3.70 3.76-3.78	107.5	A	C5	-
		3.65-3.70 3.76-3.78	107.5	B	C5	-
		3.65-3.70 3.76-3.78	106.5	D	C5	-
C	→3)-Ara*f*-(α1→	5.06	83.9	B	C3	8
		5.06	83.9	C	C3	9
		5.06	87.0	D	C2	10
D	→2,5)-Ara*f*-(α1→	5.08	67.0	A	C5	11
		5.08	79.5	D	C3	-
		5.08	81.4	D	C4	-
E	Ara*f*-(α1→	5.03	83.9	B	C3	12
		5.03	83.9	C	C3	13
		5.03	76.5	E	C3	-
		5.03	81.9	E	C2	-
F	Ara*f*-(β1→	4.96	83.9	B	C3	14
		4.96	83.9	C	C3	15

Based on the data obtained, a putative model structure was suggested (**Figure 3.11**) which includes the →5)-Ara*f*-(α1→ backbone substituted at O-2 or O-3 mainly with →3)-Ara*f*-(α1→ similarly to arabinans isolated from other sources **(Dourado et al., 2006; Shakhmatov et al., 2014; Xia et al., 2015).** In addition to the occurrence of Ara*f*-(α1→, unusual Ara*f*-(β1→, a rare structural feature in arabinans **(Cardoso et al., 2007; Cardoso et al., 2002; Shakhmatov et al., 2018)** was also shown to occur. In this model structure are also included the bonded polyphenols whose linkages to carbohydrates remain to be elucidated.

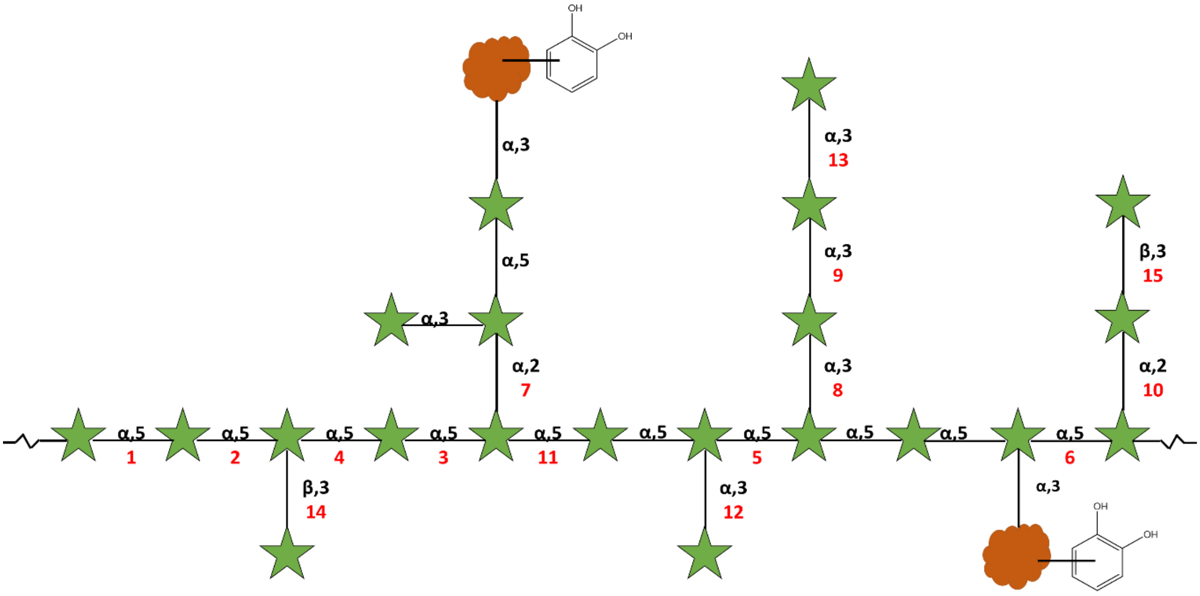

Figure 3.11 – Model structure representative of the isolated arabinan/polyphenol complex as provided by the complementarity between GC-MS, ^{13}C NMR and alkaline fusion analysis. Orange/brownish clouds are representative of the unknown phenolic structures with a catechol group that are attached to the arabinan. In red are shown the glycosidic sequences observed by ^{13}C NMR.

The →5)-Ara*f*-(α1→ and →3,5)-Ara*f*-(α1→ linkages of arabinans result in an helicoidal backbone that allow the orientation of the side chain outside the helix **(Pérez, Mazeau, & Hervé du Penhoat, 2000)**. It is feasible also that polyphenols bonded to the arabinan structure can additionally result in the formation of hydrophobic domains, especially considering the high flexibility of the arabinofuranose ring **(Cros, Imberty, Bouchemal, Du Penhoat, & Perez, 1994),** explaining the isolation by retention in C_{18} cartridges of the hydrophobic polysaccharides herein detected. Acetyl and methyl groups associated to the GalA residues adjacent to the arabinan side chains of pectins may also contribute to the observed hydrophobicity. In fact, as shown by apple enzymatic liquefaction, apple arabinans from pectins present high degrees of acetylation and methyl-esterification of GalA **(Schols et al., 1990)**. Nevertheless, its extent should be very limited as apple pomace usually results from ripe and technologically treated fruits, where enzymes such as

PME, PG, and acetylesterase are very active **(Kennedy et al., 1999)**. Therefore, although methyl esterified and acetyl groups might contribute to pectic polysaccharides hydrophobicity, it is very unlikely that their contribution surpassed the one provided by the covalently linked polyphenols, which differed in more that 10-fold between the retained and non-retained fractions. To consolidate the relevance of the polyphenols bonded to the arabinan towards C_{18} cartridges, solid-phase extraction was performed on a sugar beet arabinan (SB Ara), which is a polysaccharide with covalently linked ferulic acid derivatives to the O-5 and O-2 positions of arabinose residues **(Levigne et al., 2004)**. The experiment was performed at pH 3 to potentiate the protonation of GalA carboxylic groups and, in this way, promoting the retention of the hydrophobic compounds. The SB Ara used presented 790 g/kg of carbohydrates, mainly Ara (74 mol%), Gal (13 mol%), and GalA (10 mol%). Polyphenols represented 7 g PLZE/kg (**Table 3.13**).

Table 3.13 – Yield (%), carbohydrate composition (mol %), total carbohydrates (g/kg) and total polyphenols (g phloridzin equivalents (PLZE)/kg) of the sugar beet arabinan (SB Ara) and its retained (RFr) and non-retained (NrFr) fraction obtained by solid-phase extraction. In parenthesis are presented that data in gallic acid equiv (g GAE/kg) for total polyphenols.

Fraction	**Yield (%)**	**Carbohydrate composition (molar%)**								**Total Carbohydrates (g/kg)**	**Total PC (g PLZE/kg)**
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA		
SB Ara	100	3	tr	74	tr	tr	13	-	10	785	6 (2)
NrFr	81	2	tr	77	tr	1	10	1	9	816	5 (2)
RFr	19	5	tr	52	tr	1	23	2	17	777	15 (5)

3.2.3 Contribution of polyphenols on arabinan retention in C18 cartridges

As observed for apple pomace, solid-phase extraction of SB Ara gave rise to a non-retained fraction (SB Ara NrFr) and a retained fraction (SB Ara RFr) that represented 81 and 19% of the SB Ara (**Table 3.13**), respectively. These two fractions presented similar sugars content (780-820 g/kg), rich in Ara (52-77 mol%), Gal (10-23 mol%) and GalA (9-17 mol%). SB Ara RFr contained 15 g PLZE/kg while for SB Ara NrFr, polyphenols accounted 5 g PLZE/kg. This agreed with the retention of apple pomace polysaccharides with higher amounts of bonded polyphenols. However, the amount of polyphenols present in the SB Ara RFr was of the same order of magnitude as that observed for the non-retained hydrophilic material of apple pomace, thus suggesting that other structural features, in addition to polyphenols, contribute to hydrophobicity of the polysaccharides. The GalA content was 3-fold lower in SB Ara RFr than in apple pomace hydrophilic fraction suggesting that, in addition to polyphenols, the low prevalence of negative charges contribute to polysaccharides hydrophobicity. Still, as suggested by the isolation of an apple pomace hydrophobic fraction at pH 3, highly rich in GalA and polyphenols, there appears to exist a threshold limit where the effect of negative charges is negligible when compared to the hydrophobicity provided by polyphenols. It must be noted, however, that the type of phenolic structures present in SB Ara and arabinan fractions derived from apple pomace are of a completely different nature. In the case of SB Ara, the phenolic structure covalently linked to the arabinose unit result from a biosynthetic process **(Levigne et al., 2004)**. As far as we are aware, despite the extensive studies already performed for apple cell wall composition, no covalently linked polyphenols to polysaccharides, clearly associated to biosynthetic processes were identified for apple. Therefore, in the case of apple pomace derived fractions the covalently linked phenolic structures outcomes from polyphenol oxidation. The understanding of the type of linkages involved and their effect on the three-dimensional structure of the polymer is essential to unveil the hydrophobicity observed in this study, as well as in the nutritional, functional and biological properties generally attributed to polysaccharides. In order to understand how polysaccharide/polyphenol covalent complexes are formed, the capability of carbohydrates to non-covalently interact with polyphenols was assessed. This was achieved in Chapter 3.3 by the determination of polyphenol diffusion performance along a dialysis membrane, the thermodynamic parameters involved in the interactions by isothermal titration calorimetry and the formation of aggregates by phase-dyagram. For this purpose, carbohydrates structurally related to those involved on the covalent complexes and three of the most relevant polyphenols found in apple (5-*O*-caffeoylquinic acid, phloridzin and procyanidins) were used.

3.3 Interaction of arabinan rich pectic polysaccharides with polyphenols

This chapter accounted with the contributions of:

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This chapter includes information of the publication:

Fernandes, P. A. R., Le Bourvellec, C., Renard, C. M. G.C., Wessel, D. F., Cardoso, S. M., Coimbra, M. A. "*Interactions of arabinan-rich pectic polysaccharides with polyphenols*". Carbohydrate Polymers, **2019**, DOI: 10.1016/j.carbpol.2019.115644.

3.3.1 Arabinan interaction with polyphenols

Pectic polysaccharide-polyphenols interactions result from the contribution of their specific domains, which include the arabinans commonly found in apple pectic polysaccharides **(Renard et al., 1991)**. To assess the possible contribution of arabinans, as well as the impact of side chains of these branched structures, this work took advantage of sugar beet arabinan (SB Ara) and sugar beet debranched arabinan (SB dAra) obtained by enzymatic treatment with arabinofuranosidase. In SB Ara and SB dAra, carbohydrates accounted for >900 g/kg, mainly composed by Ara (62-78 mol %), Gal (12-18 mol %) and GalA (9-14 mol %) (**Table 3.14**). Protein accounted for 1-2 g/kg while polyphenols (presumably ferulic acid **(Rombouts & Thibault, 1986)**) accounted for 6-8 g PLZE/kg of sample.

Table 3.14 – Carbohydrate composition (mol %), total polysaccharide (g/kg), protein (g/kg) and total polyphenols (g phloridzin equivalents (PLZE)/kg) of the different polysaccharide fractions. For total polyphenols, data in gallic acid equivalents (g/GAE/kg) are also presented in parenthesis. Data is expressed as Mean and Pooled Standard Deviation (Polled std) of three replicates.

Fraction	**Carbohydrates (molar %)**								**Total Polysacc. (g/kg)**	**Protein (g/kg)**	**Total PC (g PLZE/kg)**
	Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**GalA**			
SB Ara	2	tr	78	tr	tr	12	tr	9	928	2	6 (2)
SB dAra	3	tr	62	tr	tr	18	4	14	944	1	8 (3)
AP Ara	3	tr	60	5	tr	7	11	13	620	0.9	62 (26)
AP Pect	2	tr	25	5	tr	8	7	52	914	-	5 (2)
Pooled std	*0*	*0*	*1*	*0*	*0*	*0*	*0*	*1*	*22*	*0*	*1 (0)*

SB Ara – sugar beet arabinan; SB dAra – sugar beet debranched arabinan; AP Ara - apple pomace arabinan; AP Pect - apple pomace pectic polysaccharides; tr – traces; PLZE – phloridzin equivalents.

SB Ara and SB dAra presented typical arabinan structures with (1→5)-Ara*f* (32-68%), (1→3,5)-Ara*f* (2-33%) and (1→2,5)-Ara*f* (2%) (**Figure 3.12 a** and **b** and **Table 3.15**), as shown by methylation analysis. The average branching degree, estimated by the ratio between the branching units and total arabinose, was higher for SB Ara (0.41) than for SB dAra (0.05), which is in accordance with the enzymatic treatment with arabinofuranosidase. The difference between t-Ara*f* and the branching units, alongside with the detection of polyphenols in these fractions (6-8 g PLZE/kg of sample) allows to infer that at about 21%

and 1% of the arabinofuranosyl units were O-5 or O-2 substituted with ferulic acid **(Levigne et al., 2004)**.

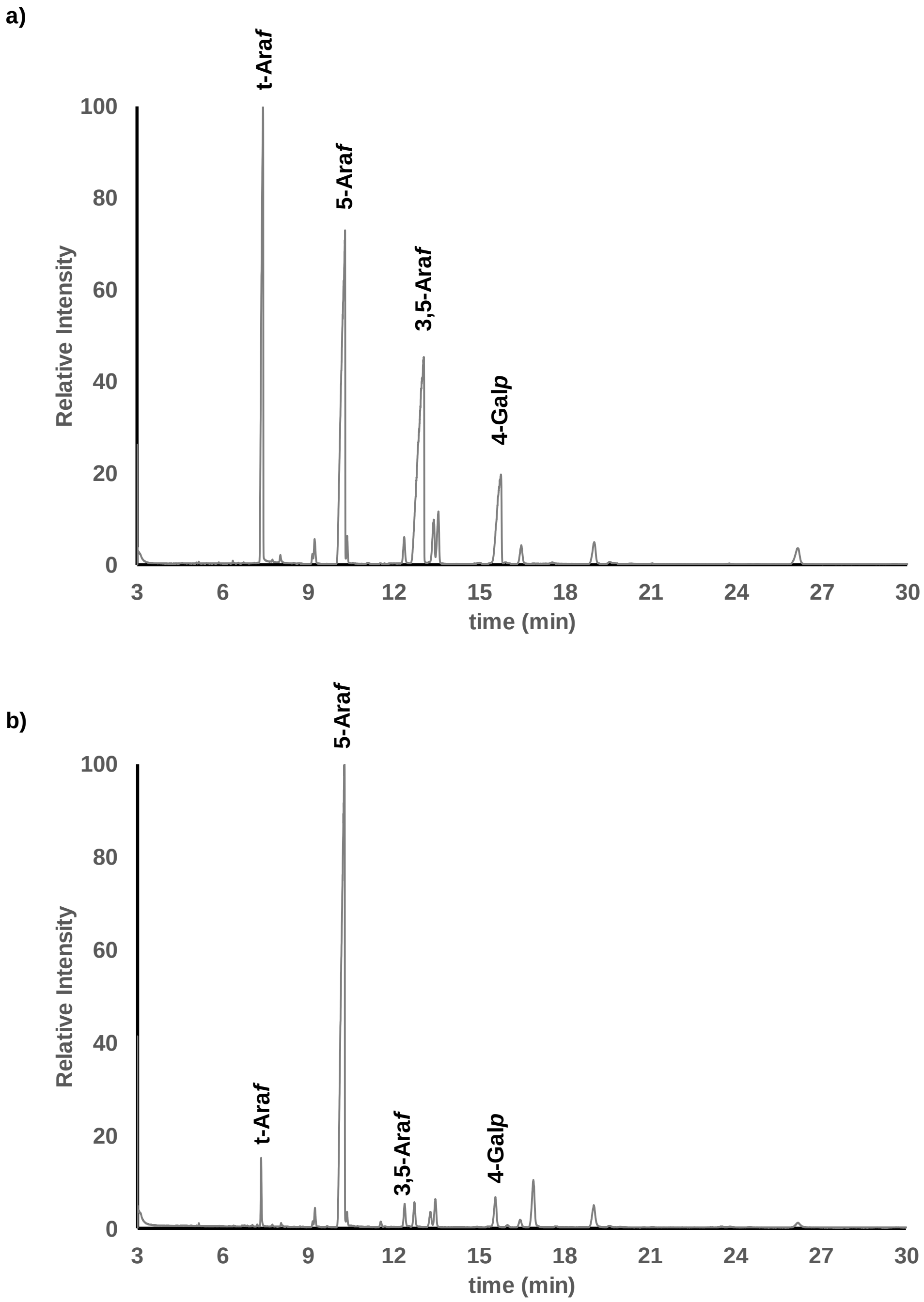

Figure 3.12 - Total ion chromatogram of the partially methylated alditol acetates (PMAA) from a) the SB Ara and b) SB dAra fractions used in the interactions.

Table 3.15 - Partially methylated alditol acetates (molar %) of sugar beet arabinan (SB Ara), sugar beet debranched arabinan (SB dAra), apple pomace arabinan (AP Ara) and apple pomace pectic polysaccharides (AP Pec). Data for the precipitates obtained after interaction with procyanidins are also present for SB Ara, SB dAra and AP Ara.

Linkage	**SB Ara**		**SB dAra**		**AP Ara**		**AP Pec***
	Initial	Precipitate	Initial	Precipitate	Initial	Precipitate	Initial
t-Rha*p*					0.8	0.5	0.5
2-Rha*p*	0.6		0.6		0.9		1.8
2,4-Rha*p*	1.8		2.8		0.5	0.4	1.7
Total Rha*p*	2.4		3.4		2.2	0.9	4.0
t-Fuc*p*					0.9	1.4	0.3
Total Fuc*p*					0.9	1.4	0.3
t-Ara*f*	17.6	23.5	3.0	2.9	10	9.3	14.6
2-Ara*f*	0.1	0.1	0.2	0.3	1.5	1.1	0.1
3-Ara*f*	0.7	0.8	1.0		5.2	4.1	2.7
5-Ara*f*	31.6	39.1	68.0	91.3	38.2	23.6	26.0
3,5-Ara*f*	32.7	29.7	2.3	1.7	10.8	8.1	11.1
2,3-Ara*f*					0.5	0.3	
2,5-Ara*f*	2.3	1.1	1.7	2.0	4.5	1.7	2.4
2,3,5-Ara*f*	0.2				2.3	0.0	
Total Ara*f*	85.3	95.7	76.2	98.2	73.0	48.2	56.9
t-Xyl*p*	0.1		0.2		2.9	6.9	1.9
2-Xyl*p*					2.0	6.0	
4-Xyl*p*							3.1
2,4-Xyl*p*							0.7
Total Xyl*p*	0.1		0.2		4.9	12.9	5.7
t-Man*p*					0.8		1.6
Total Man*p*					0.8		1.6
t-Gal*p*	0.9	0.5	2.2	0.6	1.3	1.9	2.9
2-Gal*p*					1.1	2.1	
3-Gal*p*	0.7		1.2				0.6
4-Gal*p*	7.9	3.6	4.8	1.0	3.4	5.4	10.7
6-Gal*p*	1.6		6.5			0.9	0.3
3,4-Gal*p*	0.1		0.1			0.3	0.6
3,6-Gal*p*	1.1		1.3			0.5	0.7
4,6-Gal*p*						0.4	1.0
Total Gal*p*	12.2	4.0	16.2	1.6	5.8	11.5	16.8
t-Glc*p*						1.8	0.2
4-Glc*p*		0.3		0.1	7.5	12.0	14.5
2,4-Glc*p*						0.3	
4,6-Glc*p*				0.1	5.0	12.5	
Total Glc*p*		0.3		0.2	12.5	26.8	14.7

*Et80Pp of the non-retained material obtained after solid-phase extraction using C_{18} cartridges (NrFr Et80Pp)

To determine the interactions of the two-sugar beet arabinans with low molecular weight polyphenols, 5-*O*-caffeoylquinic acid and phloridzin were dialysed in the presence and absence of SB Ara and SB dAra. Five dialysate solutions were obtained for each polysaccharide, in accordance with the number of water changes performed (**Table 3.16)**. From the percentage of polyphenols that diffused in the first 3 h of dialysis in the presence and absence of arabinans, a retention coefficient was determined, allowing to infer the interactions of each arabinan structure with 5-*O*-caffeoylquinic acid and phloridzin. SB Ara presented a RC of 0.10 and 0.22 towards 5-*O*-caffeoylquinic acid and phloridzin, respectively (**Table 3.17**), while those of SB dAra were 0.32 and 0.39. These differences allowed to infer higher interactions of SB dAra than SB Ara.

Table 3.16 – Amount (µg) of 5-*O*-caffeolyuinic acid and phloridzin that diffused along the dialysis membranes in the absence (control) and presence of sugar beet arabinan (SB Ara) or sugar beet debranched arabinan (SB dAra). In parentheses are represented the amounts retained inside the dialysis membrane.

Polyphenol	**Experience**	**Dialysis number**				
		1	2	3	4	5
5-*O*-caffeoylquinic acid	Control	41.8 (58.2)	16.6 (41.7)	9.8 (31.8)	6.8 (25.0)	5.1 (20.0)
	SB Ara	37.6 (61.1)	18.6 (42.6)	12.7 (29.9)	6.8 (23.0)	2.7 (21.9)
	SB dAra	28.1 (71.9)	18.7 (53.1)	8.1 (45.0)	5.2 (39.8)	3.8 (36.0)
	Pooled Std	*1.5* *(1.5)*	*0.7* *(1.1)*	*0.7* *(0.5)*	*0.3* *(0.7)*	*0.1* *(0.6)*
Phloridzin	Control	50.7 (49.3)	20.0 (29.3)	7.3 (22.0)	3.4 (18.6)	1.6 (17.0)
	SB Ara	39.7 (60.4)	14.5 (45.9)	7.0 (38.8)	4.1 (34.7)	3.5 (33.0)
	SB dAra	30.8 (69.2)	15.3 (53.8)	6.6 (47.2)	2.0 (45.2)	0.6 (44.6)
	Pooled Std	*1.5* *(1.5)*	*0.2* *(1.7)*	*0.5* *(1.6)*	*0.1* *(1.7)*	*0.1* *(1.6)*

Following the analysis of the first dialysate, the analysis to the subsequent dialysates in the presence or absence of polysaccharides were also performed to monitor 5-*O*-caffeoylquinic acid and phloridzin diffusion from the dialysis membrane (**Table 3.16**). The amount of polyphenols retained inside the dialysis membrane (**Figure 3.13 a** and **b** and **Table 3.16**) decreased sharply in the first two dialysates and tended to a plateau phase (3rd-5th dialysates).

Table 3.17 – Retention coefficient (RC) and normalized retention capacity (NRC) (µg/mg of polysaccharide) of sugar beet arabinan (SB Ara) and sugar beet debranched arabinan (SB dAra) towards 5-*O*-caffeoylquinic acid and phloridzin.

Sample	**RC**		**NRC** ***(µg/mg of polysaccharide)***	
	5-*O*-caffeoylquinic acid	Phloridzin	5-*O*-caffeoylquinic acid	Phloridzin
SB Ara	0.10	0.22	0.95	8.01
SB dAra	0.32	0.39	8.04	13.81
Pooled Std	*0.02*	*0.02*	*0.13*	*0.38*

The measurement of the difference of the plateau stage in the presence of polysaccharides and in their absence, allowed to determine a normalized retention capacity (NRC). For SB Ara the NRC was 1 and 8 µg/mg of polysaccharide for 5-*O*-caffeoylquinic acid and phloridzin, respectively, while for SB dAra the values were 8 and 14 µg/mg of polysaccharide. These results confirm the higher interactions of SB dAra with polyphenols, when compared to SB Ara.

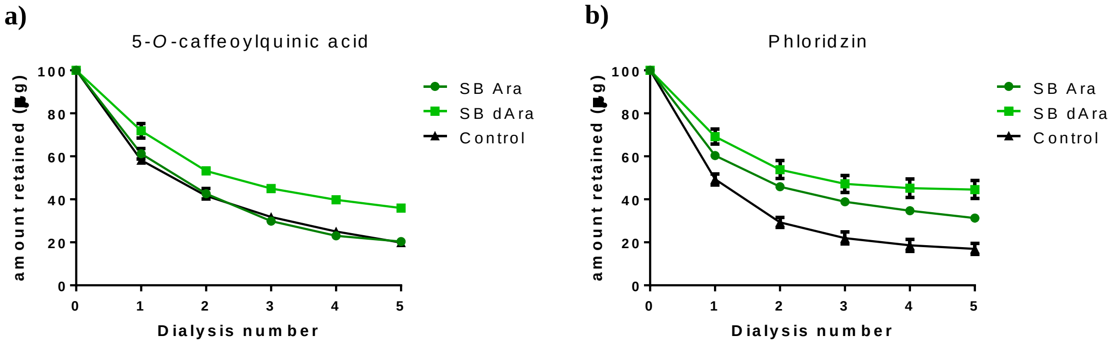

Figure 3.13 – Amount (µg) of retained a) 5-*O*-caffeoylquinic acid and b) phloridzin along the different dialysis experiments for the Control, sugar beet arabinan (SB Ara) and sugar beet debranched arabinan (SB dAra).

Linear arabinans are generally associated to an helicoidal structure **(Pérez et al., 2000)** in which the arabinofuranose units present high flexibility **(Cros et al., 1994)**. These features are probably favourable for the formation of hydrophobic domains by polymer entanglement in which both 5-*O*-caffeoylquinic acid and phloridzin might be encapsulated in a similar mechanism, as reported for dextrins **(Saura-Calixto, 2011).** However, in the case of SB Ara, lower interactions were observed as these hydrophobic cavities might be less prone to be formed or even, when existing, too small to allow the encapsulation of the polyphenol due to steric hindrance caused by the polysaccharide branches. The lower interaction of both polysaccharides towards 5-*O*-caffeoylquinic acid as compared to phloridzin can be explained by the overall negative charge of 5-*O*-caffeoylquinic acid (pKa=3.3) at pH 4.0 (86% ionization), which might impair further association of other molecules, as well as its encapsulation by the arabinans.

To determine the interactions of the two-sugar beet arabinans with high molecular weight polyphenols, procyanidins with a DP 9, isolated from apple, were tested by Isothermal Titration Calorimetry (ITC) and aggregate formation in the presence and absence of SB Ara and SB dAra.

3.3.2 Interactions of arabinans with procyanidins

3.3.2.1 Effect of arabinan branching degree

The isothermal titration calorimetry (ITC) assay of the SB Ara and SB dAra with procyanidins showed strong exothermic peaks (**Figure 3.14**). After subtraction of the controls (titration only with procyanidins), the peak areas were plotted against the molar ratio of (-)-epicatechin/GalA for a direct comparison with previous experiments using other pectic polysaccharide structures **(Watrelot et al., 2013, 2014)**. The resulting curve was adjusted to an independent model. This allowed to estimate the thermodynamic parameters associated to arabinan-procyanidin interactions, which included molarity (*n*), affinity constant (K_a), enthalpy (ΔH), Gibbs energy (ΔG), and entropy (ΔS).

According with the thermodynamic parameters of the interactions (**Table 3.18**), stoichiometry (defined as the ratio of (-)-epicatechin/GalA) was 0.04 and 0.2 for SB Ara and SB dAra, showing that 1 molecule of (-)-epicatechin binds with 25 and 5 molecules of GalA, respectively. The affinity constant for SB Ara was 391 M^{-1} while for SB dAra was 540 M^{-1}, lower than the 1955 M^{-1} determined for apple arabinan interaction with procyanidins of similar DP **(Watrelot et al., 2014).** Such difference may result from the occurrence of ferulic acid in sugar beet arabinan side chains that can restrain arabinan interaction with procyanidins.

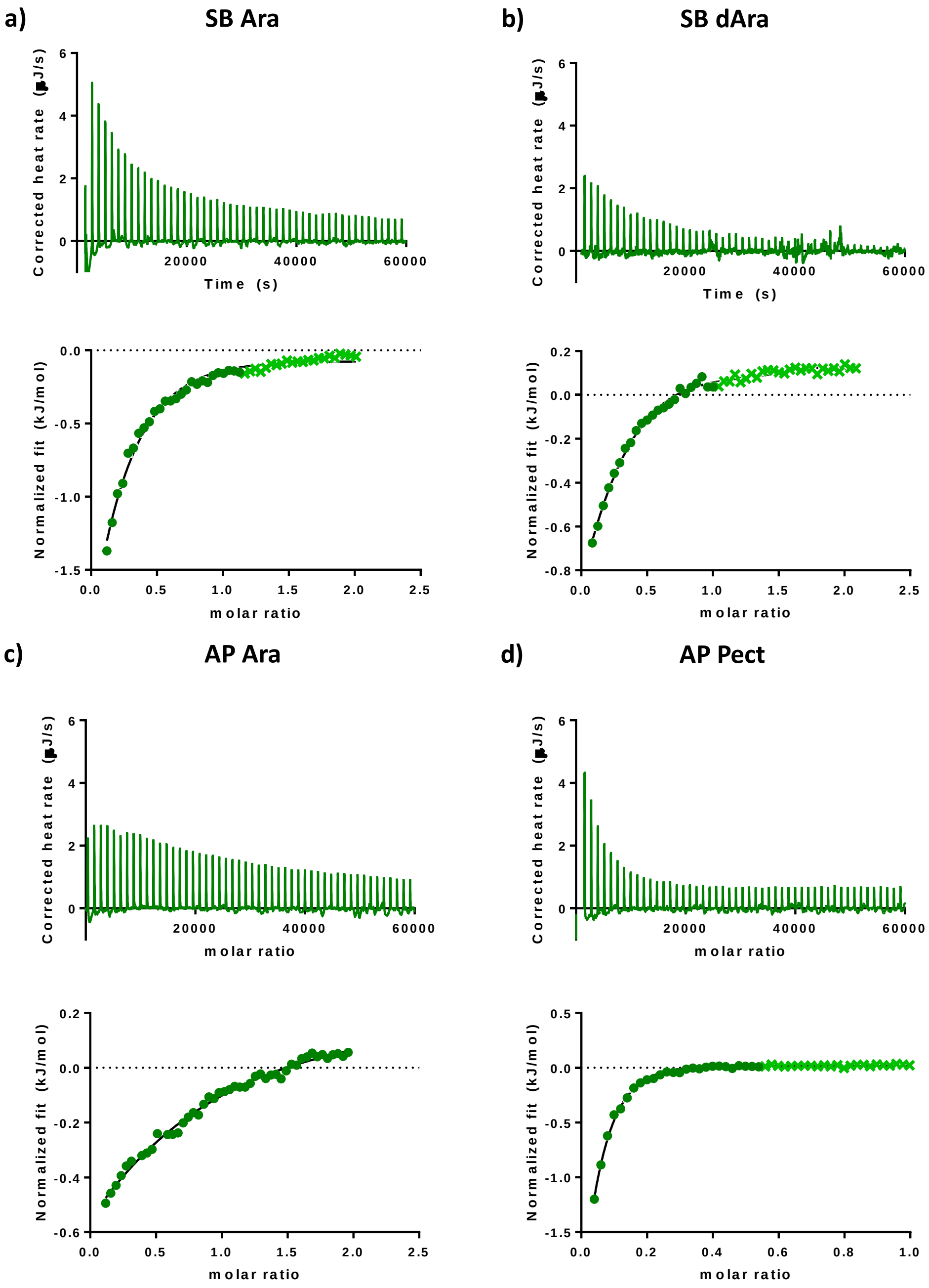

Figure 3.14 – Representative thermograms of titration of a) sugar beet arabinan (SB Ara), b) sugar beet debranched arabinan (SB dAra), c) apple pomace arabinan (AP Ara) and d) apple pomace pectin (AP Pect) with apple procyanidins DP 9: (top) measurement of heat release; (bottom) molar enthalpy change against (-)-epicatechin/GalA ratio after peak integration. The lighter green crosses highlight the plateau phase of the titration.

The analysis of the thermodynamic contributions showed that the interactions with procyanidins had a negative enthalpy contribution (ΔH= -24.6 and -2.1 kJ/mol for SB Ara and SB dAra, respectively), related to exothermic interactions. Moreover, the entropy contribution for SB Ara was negative (-TΔS=9.8 kJ/mol), indicating that the interactions were mostly driven by enthalpy and therefore, mostly involving hydrogen bonding **(Frazier et al., 2010; Poncet-Legrand et al., 2006).** In contrast, for SB dAra, a favourable contribution of entropy was observed (-TΔS = -14.8 kJ/mol), thus suggesting the that interactions of this polysaccharide and procyanidins mostly involve hydrophobic interactions. In fact, in a ligand/design approach, entropy contribution may result from conformational changes of the polymer and desolvation, generally attributed to the establishment of hydrophobic interactions between the interacting molecules **(Frazier et al., 2010; Whitesides & Krishnamurthy, 2005)**.

Table 3.18 – Thermodynamic binding parameters of the interactions between the different polysaccharide structures with procyanidin DP 9. Results are expressed as average of duplicate measurements. Stoichiometry is represented as the molar ratio value of (-)-epicatechin/GalA.

PCA DP8	SB Ara	SB dAra	AP Ara	AP Pect
n	0.04±0.00	0.20±0.01	0.70±0.1	0.04±0.01
K_a (M^{-1})	391±11	540±10	85±8	1719±65
ΔH ($kJ.mol^{-1}$)	-24.6±0.8	-2.1±0.2	-2.5±0.1	-3.5±0.3
ΔG ($kJ.mol^{-1}$)	-15±0	-16±0	-11±0	-19±0
ΔS ($J.mol^{-1}.K^{-1}$)	-33±3	45±1	29±0	50±1
ΔDP	-1.0	-0.8	-0.7	-1.4
ΔBD	-0.11	-0.01	-0.07	n.d

The capacity of each arabinan structure to form insoluble complexes with procyanidins was measured by the optical density at 650 nm, a wavelength at which neither pectic polysaccharides nor procyanidins absorb. The absorbance of polysaccharides solution with procyanidins DP 9 at 30 mM equivalents (-)-epicatechin increased with the concentration of polysaccharides **(Figure 3.15 a** and **b)**. This increase was more evident for SB dAra than for SB Ara, reaching a maximum of about 0.1 and 0.07, respectively, at 30 mM GalA equivalents. **Figure 3.15 c** and **d** shows the absorbance of procyanidins solution with the different polysaccharide structures at 30 mM GalA equivalents. As the concentration of procyanidins increased, the initial turbidity observed for SB dAra increased until an absorbance of 0.1 at 30 mM of (-)-epicatechin equivalents. In contrast, for SB Ara the turbidity values were less affected, only increasing from the initial 0.06 to 0.07 at the higher concentration of procyanidins. This behaviour showed that the interactions between arabinans and procyanidins include the formation of insoluble aggregates, being higher for SB dAra that for SB Ara as observed by ITC.

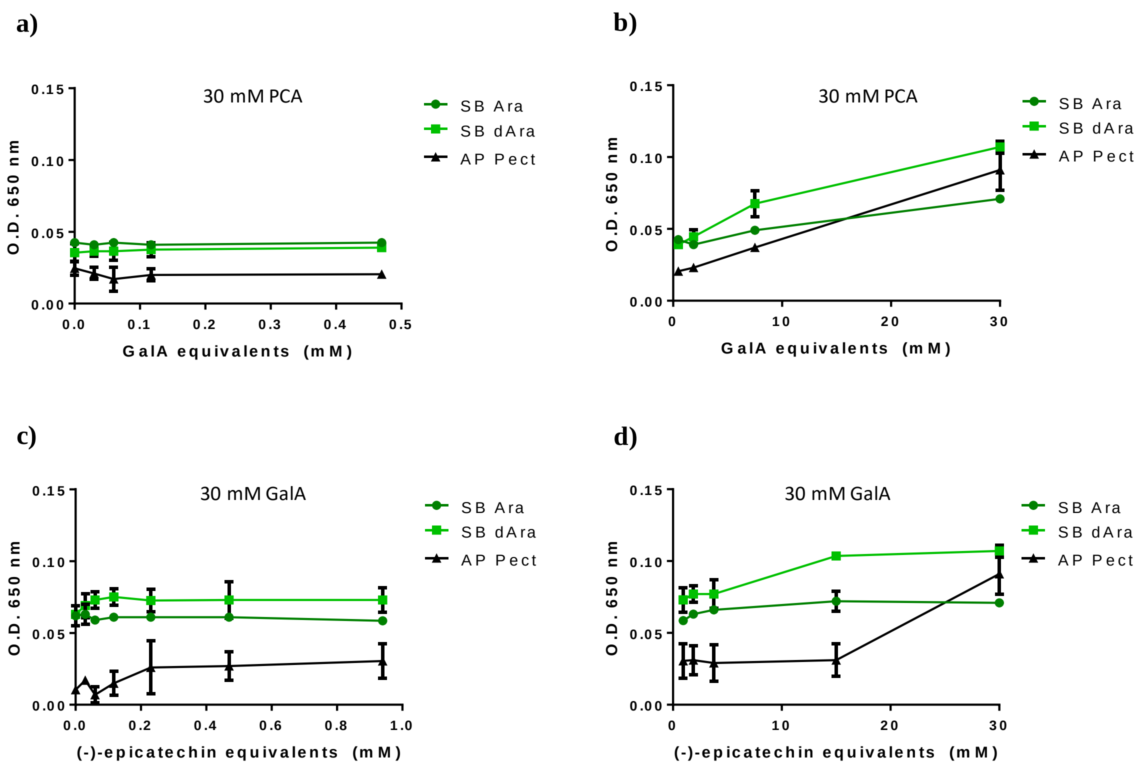

Figure 3.15 – Optical density at 650 nm obtained in the phase diagram of the arabinans and apple pomace pectin fractions and procyanidins DP 9. Variation in the optical density of procyanidins DP9 solutions (30 mM (-)-epicatechin equivalents) in the presence a) of low (0-0.47 mM GalA equivalents) and b) high concentrations (0.47-30 mM GalA equivalents) of polysaccharides and of the optical density of polysaccharide solutions (30 mM GalA) in the presence c) of low (0-0.94 mM (-)-epicatechin equivalents) and d) high concentrations (0.94-30 mM (-)-epicatechin equivalents) of procyanidins DP9.

The observed differences in the molarity, constant affinity and optical density at 650 nm between the SB Ara and SB dAra demonstrated that the branches at the arabinans backbone limit their interaction with procyanidins, as observed for low molecular weight polyphenols. This behaviour can be explained by the capability of a less branched structure to allow the accommodation of many procyanidins by stacking, given the capability of these molecules to self-associate **(Poncet-Legrand et al., 2006).** Additionally, the encapsulation of procyanidins by polysaccharides, also mediated by hydrophobic interactions, may occur. However, this mechanism is more likely to be expected for procyanidins of lower degree of polymerization as the cavity dimensions may condition the size of the molecule able to penetrate **(Bindon, Smith, & Kennedy, 2010; Gonçalves et al., 2011)**. In the case of more branched polysaccharides, due to steric hindrance caused by the side chains, interactions are decreased and mostly established via multiple hydrogen bonding between the phenolic structure and the carbohydrate residues. These complexes are generally soluble **(Riou et al., 2002)** and present lower density than those formed by proteins **(Carn et al., 2012)**. However, ultracentrifugation of the arabinan/procyanidin solutions obtained after ITC experiments allowed to separate the insoluble material from the soluble one. To obtain information about the influence of the degree of polymerization of the procyanidins on the interaction with arabinans having different branching degree, analyses were performed on both the supernatant and precipitate.

The ΔDP corresponds to the difference in the degree of polymerization between the procyanidins that had not formed aggregates with arabinans (at 7.5 mM GalA equivalents), and the initial degree of polymerization in buffer before the ITC experiment. After centrifugation, for both SB Ara and SB dAra supernatants, the ΔDP was -1.0 (-11%) and -0.8 (-9%), respectively (**Table 3.18**). As the variation was, on average, 1 (-)-epicatechin unit, it suggests that the sugar beet arabinans formed insoluble aggregates preferentially with procyanidins of higher degree of polymerization. The selective binding of larger procyanidin molecules has already been observed for apple and grape cell wall material **(Bindon, Smith, & Kennedy, 2010; Le Bourvellec et al., 2005)** and agreed with the relationship between the length of procyanidins with the type of complexes formed **(Carn et al., 2012)**. Glycosidic linkage analysis of the material that precipitated alongside with procyanidins allowed to determine the ΔBD, i.e., the difference in the degree of branching between the polysaccharides that formed insoluble aggregates with procyanidins, and the initial branching degree in buffer. The ΔBD was -0.11 (-27%) and -0.01 (-20%) for SB Ara and SB dAra (**Table 3.18**), indicating that the precipitated arabinan structures were less branched. For SB Ara, the precipitate presented, on average, 1 branching unit per 3 arabinofuranosyl units, lower than the initial 1 branching unit per 2 arabinofuranosyl units. For SB dAra, this resulted in a decrease to 1 branching unit per 26 arabinofuranosyl residues from the initial 1 to 19.

3.3.2.2 Effect of covalently bonded polyphenols to arabinan backbone

As arabinans may include different amount of polyphenolic structures attached to their backbone, the apple pomace arabinan rich fraction (AP Ara) isolated from the HWE was used. This arabinan presented 10-fold the amount of covalently linked polyphenols (62 PLZE g/kg) of sugar beet arabinans (**Table 3.14**), allowing to infer the effect of covalently bonded polyphenols to arabinans on arabinan-procyanidins interaction. In AP Ara, carbohydrates represented 620 g/kg, composed of Ara (60 mol%), GalA (13 mol%), Glc (11 mol%), and Gal (7 mol%). Glycosidic linkage analysis demonstrated the occurrence of (1→5)-Ara*f* (40%) and (1→3,5)-Ara*f* (10%) (**Table 3.15**). A branching degree of 0.28 was estimated, between that observed for SB Ara and SB dAra. The difference between t-Ara*f* and branching units demonstrated that about 14% of the arabinofuranosyl units should be branched with phenolic structures. Protein content (1 g/kg) was also within the values for the other arabinans herein studied.

AP Ara-procyanidins interaction presented a molar ratio of 0.7 between the (-)-epicatechin units and GalA units, suggesting an interaction of approximately 1:1. As the entropy value (-TΔS= -8.5 kJ/mol) was higher than that observed for enthalpy (ΔH= -2.5 kJ/mol) (**Table 3.18**), the interactions should be hydrophobic. The affinity constant of 85 M^{-1} was much lower than those observed for sugar beet arabinans with higher and lower branching degrees than AP Ara. Hence, it is feasible to hypothesize that the lower affinity of AP Ara towards Procyanidins DP9 might be due to steric hindrances caused by covalently linked polyphenols that limit further association of procyanidins, as suggested to occur with grape cell walls **(Bindon, Smith, & Kennedy, 2010).** Alternatively, it may result from the three-dimensional structure of the polysaccharides less prone to interact with procyanidins, despite its hydrophobicity. In fact, arabinans are highly mobile polymers in the cell walls **(Ha, Viëtor, Jardine, Apperley, & Jarvis, 2005)**, but when having covalently linked polyphenols such mobility might be restricted in a similar way as for arabinans crosslinked with ferulic acid in sugar beet cell walls **(Waldron, Ng, Parker, & Parr, 1997)**.

The decrease of procyanidins DP (-0.7) for those which remained in solution after ultracentrifugation, which was lower than that observed for sugar beet arabinans (-0.8 to -1.0), suggested a lower selectivity towards highly polymerized procyanidins than sugar beet arabinans. The branching degree of the polysaccharides recovered as insoluble material after interaction with procyanidins was -0.07 (-25%) lower than that of the initial AP Ara, suggesting that less branched arabinans were more prone to form insoluble aggregates. The occurrence of (1→4)-Glc*p* (12.0 mol %), (1→4,6)-Glc*p* (12.5%), (1→2)-Xyl*p* (6.0%), t-Gal*p* (1.9%), (1→2)-Gal*p* (2.1%), t-Fuc*p* (1.4%) and t-Xyl*p* (6.9%) (**Table 3.15**), diagnostic linkages of xyloglucans **(Renard et al., 1995)** also suggested that these polysaccharides formed insoluble aggregates with procyanidins. This fact is consistent with the previously described capability of xyloglucans to interact with polyphenols **(Ruiz-Garcia et al., 2014)**. However, given the high prevalence of polyphenols in the apple pomace arabinan in comparison with those obtained from sugar beet, it is feasible to suggest that procyanidins may stack with the phenolic structures covalently bonded to the apple pomace arabinan, inducing the polysaccharide precipitation. This mechanism has been attributed to procyanidins aggregation and precipitation in wine **(Riou et al., 2002)** and apple ciders **(Millet et al., 2019)**.

3.3.3 Contribution of the arabinans to pectic polysaccharides interactions with procyanidins

In order to relate the interaction of the arabinans as constituents of pectic polysaccharides side chains, a representative apple pomace pectic fraction was used. This pectic polysaccharide material, defined as AP Pect, presented 914 g/kg of carbohydrates, composed of GalA (52 mol%), Ara (25 mol%) and Gal (8 mol%). The molar ratio of GalA:Rha was 26, suggesting the prevalence of homogalacturonans (**Table 3.14**). Glycosidic linkage analysis (**Table 3.15**) showed that the arabinans were the main neutral sugar side chains, as shown by the presence of (1→5)-Ara*f* (26%), (1→3,5)-Ara*f* (11%), (1→2,5)-Ara*f* (2%) and t-Ara*f* (15%), and some residual galactan chains represented by (1→4)-Gal*p* (11%), (1→4,6)-Gal*p* (1%), (1→3,6)-Gal*p* (1%) and t-Gal*p* (3%) **(Renard et al., 1991; Selvendran, 1985)**.

The stoichiometry (ratio of (-)-epicatechin/GalA) was 0.04 for AP Pect (**Table 3.18**), showing that 1 molecule of (-)-epicatechin bound with 25 molecules of GalA, respectively. The affinity constant was of 1719 M^{-1}, in agreement with the values previously estimated for apple pectin (1240 M^{-1}) **(Watrelot et al., 2013)**. The observed ΔH (-3.5 kJ/mol) and -TΔS (-14.9 kJ/mol) suggested that the interactions were mostly driven by entropy, with the prevalence of hydrophobic interactions. The optical density of procyanidin solutions (30 mM

(-)-epicatechin equivalents) increased with the concentration of pectic polysaccharides, reaching a value of 0.09 at 30 mM GalA equivalents (**Figure 3.15 a** and **b)**. The same behaviour (**Figure 3.15 c** and **d)** was observed when pectic polysaccharides concentration was fixed at 30 mM GalA equivalents and increasing concentrations of procyanidins were tested, thus suggesting that apple pomace pectic polysaccharides presented the capability to form insoluble aggregates with procyanidins, agreeing with the interactions observed by ITC.

Considering the relevant fraction that Ara (25% mol) represents, and supposing that their share in the affinity constant is directly proportional to their relative abundance in the pectic polysaccharides fraction, it could be estimated that 1-28% of the affinity observed for AP Pect can be explained by the arabinan fractions herein studied and those tested by **(Watrelot et al., 2014)**. Type I galactans, which presented a K_a of 2225 M^{-1} for procyanidins of similar degree of polymerization **(Watrelot et al., 2014)**, with a relative 7 mol% of Gal, explained an additional 10%, totalling 38% of the observed affinity for apple pomace pectic polysaccharides. The remaining 80% could be explained by the contribution of homogalacturonan chains of the AP Pect. As these pectic polysaccharides were obtained from apple pomace attained from ripe and enzymatically treated fruits (PME, PG, and acetylesterase) **(Kennedy et al., 1999)**, AP Pec might present a low methyl esterification and/or even acetylation. ITC and optical density at 650 nm showed that low methyl esterified homogalacturonans did not interact with procyanidins of DP9 **(Watrelot et al., 2013)**. Therefore, it can be hypothesized that other structural features are relevant for polysaccharide-polyphenol interactions. In fact, there might exist a synergetic contribution between chain flexibility and hydrophobicity provided by the occurrence of methyl esterified groups in pectin **(Morris, Foster, & Harding, 2000)**, increased flexibility provided by the increasing content in Rha between GalA residues **(Cros, Garnier, Axelos, Imberty, & Pérez, 1996)**, mobility of pectic polysaccharide side chains **(Ha et al., 2005)**, molecular weight **(McManus et al., 1985)**, and branching degree herein tested. All contribute to the acquisition of a three-dimensional structure and interactions that might differ from those observed with the isolated polysaccharides. Nevertheless, it is generally observed that all these polysaccharides present the capability to interact with polyphenols. This close spatial relationship between the two different molecules is very probably essential and the first step for polyphenol covalent bonding to polysaccharides upon oxidation. However, to take advantage of these chimeric polysaccharide/polyphenol complexes, it is essential to provide drying techniques that surpass the limitation imposed by the conventional hot-air drying by meeting the industry demands, as well as assuring apple pomace preservation. For this purpose, in Chapter 3.4, microwave hydrodiffusion and gravity (MHG) was assessed as a technology for apple pomace drying by comparing the drying rates to the conventional hot-air drying.

3.4 Microwave assisted drying of apple pomace

3.4.1 Conventional apple pomace drying

Apple pomace (APIII), on a dry weight basis, is characterized by having a high carbohydrate content (649 g/kg of the apple pomace), as show in **Table 3.19**. These mostly include polysaccharides (469 g/kg), of which about 25% correspond to pectic ones, as shown by the high prevalence of GalA (10 mol%), Ara (5 mol%) and Gal (9 mol%). However, the high-water content of apple pomace (810 g/kg) renders this byproduct highly perishable impairing its use for the recovery of these components. To overcome this perishability, apple pomace water content must be reduced **(Lavelli & Corti, 2011)**. Hot-air drying is one, if not the most used technic for drying several vegetable and other food matrixes **(Motevali et al., 2011)**. For this reason, apple pomace was dried at different temperatures and relative air humidity. As described by **Lavelli and Corti (2011)**, the drying process followed a falling rate period (**Figure 3.16 a** and **b**) characteristic of a progressive water removal differently bounded to the apple pomace components.

At 40 ºC and a relative air humidity of 60%, an average drying rate of 1.6 g/min was observed, resulting in a drying time of almost 10 h and a final pomace with a moisture content of 14% (**Table 3.20**). By decreasing the relative air humidity to 30%, the time required for apple pomace drying at 40 ºC was reduced to about 8 h, yielding a product with a moisture content of 12%. As these values were higher than the maximal moisture content 10% required to assure apple pomace stability **(Lavelli & Corti, 2011)**, higher temperatures of drying were assayed. The increase of temperature to 60-100 ºC, resulted in drying rates that could be increased up to 2-fold when comparing to 40 ºC (**Table 3.20**). Furthermore, the apple pomace final moisture was reduced to 0.1-1.3%, i.e. below the 10% threshold to assure the stability of the dried apple pomace. However, hot-air drying represents time-consuming process that requires a constant airflow, temperature and moisture control, whose high investment and energetic inputs may impair a profitable apple pomace valuation. Therefore, to improve the drying rate of apple pomace, as well as energetic drying efficiency, microwave-hydrodiffusion and gravity (MHG) was tested as an alternative approach.

Table 3.19 – Carbohydrate composition (mol %), total carbohydrates (g/kg) and total polyphenols (phloridzin equivalents (PLZE)/kg) of the different apple pomace fractions. In parentheses are also presented the total polyphenols in g Galic acid equivalents (GAE)/kg. Data is expressed as Mean ± standard deviation of three replicates. n.d means for not determined.

Type of Extraction	Sample	Fraction		Carbohydrates (mol%)									Total Carbohydrates (g/kg)	Total Polyphenols (g PLZE/kg)
				Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Fru**	**Gal**	**Glc**	**GalA**		
Microwave-assisted drying	Apple Pomace III		Polysac.	1	-	6	16	-	-	10	56	11	439	n.d.
			Free Sugars	-	-	25	1	-	44	-	30	-	180	
	300 W	1	Polysac.	3	tr	31	7	tr	-	10	8	41	728	14.4
			Free Sugars	-	-	8	12	-	24	8	48	-	111	(6.1)
	400 W	1	Polysac.	2	tr	32	9	tr	-	9	3	45	578	16.5
			Free Sugars	-	-	10	1	-	26	11	51	-	84	(7.0)
	500 W	1	Polysac.	2	tr	32	7	tr	-	10	4	49	592	12.7
			Free Sugars	-	-	8	12	-	24	8	51	-	95	(6.3)
	600 W	1	Polysac.	2	tr	30	7	tr	-	9	4	50	695	14.7
			Free Sugars	-	-	9	13	-	17	9	52	-	90	(6.5)
	900 W	1	Polysac.	2	tr	29	6	tr	-	8	1	54	652	14.3
			Free Sugars	-	-	7	12	-	19	8	53	-	111	(6.1)
	Apple pomace II		Polysac.	1	1	12	10	5	-	9	41	19	534	n.d.
			Free Sugars	-	-	2	1	-	76	-	22	-	183	
	400 W	1	Polysac.	1	tr	11	2	tr	-	3	8	75	265	5.0
			Free Sugars	-	-	-	1	-	61	-	38	-	665	(2.3)
	400 W + EtOH	12	Polysac.	4	tr	59	tr	tr	-	6	4	27	161	12.2
			Free Sugars	-	-	-	1	-	68	-	31	-	787	(3.0)
		13	Polysac.	4	tr	60	1	tr	-	5	2	28	152	12.6
			Free Sugars	-	-	-	2	-	67	-	31	-	663	(6.9)
	Pooled std			*0*	*0*	*0*	*0*	*0*	*1*	*0*	*0*	*1*	*8*	*0.2* *(0.1)*

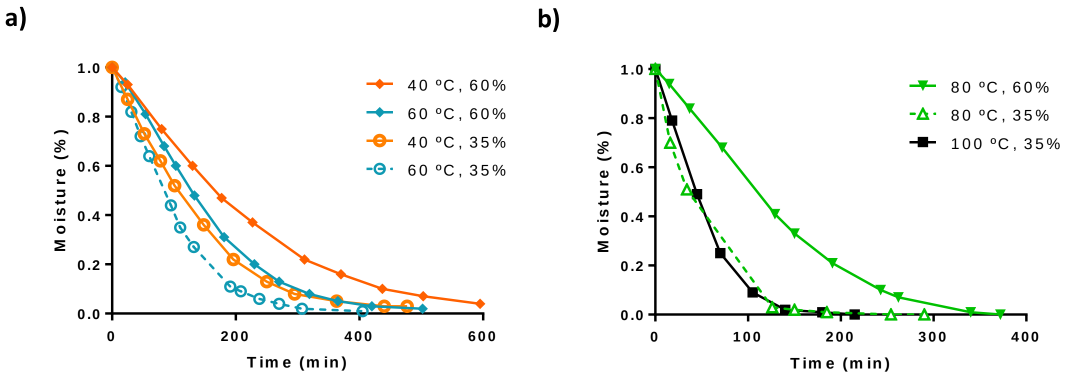

Figure 3.16 -Evolution of the moisture content of apple pomace dried at a) low (40 and 60 ºC) and b) high temperatures (80 and 100 ºC) and relative air humidity of 35 and 60%.

Table 3.20 – Hot-air drying average drying rate, total drying time and final moisture content of apple pomace at different relative air humidity (35 and 60%) and temperatures (40, 60, 80 and 100 ºC).

Relative Humidity (%)	Temperature (ºC)	Average drying rate (Δ g/min)	Total Drying time (h)	Final moisture (%)
35	40	1.6	8.0	11.8
	60	2.2	6.8	5.1
	80	2.1	4.8	0.1
	100	3.5	3.6	1.3
60	40	1.0	9.9	13.9
	60	1.4	8.4	7.6
	80	1.6	6.2	1.3

3.4.2 Microwave-hydrodiffusion and gravity drying

For apple pomace MHG assisted drying, powers of 300 to 900 W were tested. This drying procedure is characterized by a three-stage process as represented in **Figure 3.17 a** to **Figure 3.17 e**. In a first step, heating of the *in situ* water of apple pomace occurs until reaching 100 ºC (boiling point of water), then diffusing from the plant material and eluting downward under the influence of earths' gravity **(Zill, Abert-Vian, Elmaataoui, & Chemat, 2011)**. This heating time took 29 min at 300 W and decreased linearly (y=-0.03+38, R^2 =0.97) to 10 min at 900 W, ending approximately when the first 50 mL of water were collected. This heating time was in the same order of magnitude to that described for algae **(Pérez, Conde, & Domínguez, 2014)** and broccoli byproducts drying **(Ferreira et al., 2018)**. The second phase of the MHG drying process corresponded to a steady state plateau (at 100 ºC) with a continuous flow of *in situ* (non-bounded) water of 5 mL/min at 300 W, and increasing linearly (y=-0.02+0.61, R^2 =0.97) to 14 mL/min at 900 W (**Table 3.21)**. This continuous flow is a typical behavior for high water content matrices **(Zill et al., 2011)** and represents a faster water removal from apple pomace than that observed at the optimal conditions for oven-drying (1.6 mL/min at 100 ºC, relative air humidity 35%). In the third part of MHG drying, which was delimited by the last fractions collected, temperature increased very quickly to about 103ºC and signals of burning started to appear. This phase is called burning point phase. In open vessel equipment's, as the NEOS-GR, the maximum temperature is determined by the boiling point of the solvent at atmospheric pressure. Therefore, this burning stage signalizes the low water content of the apple pomace at this stage.

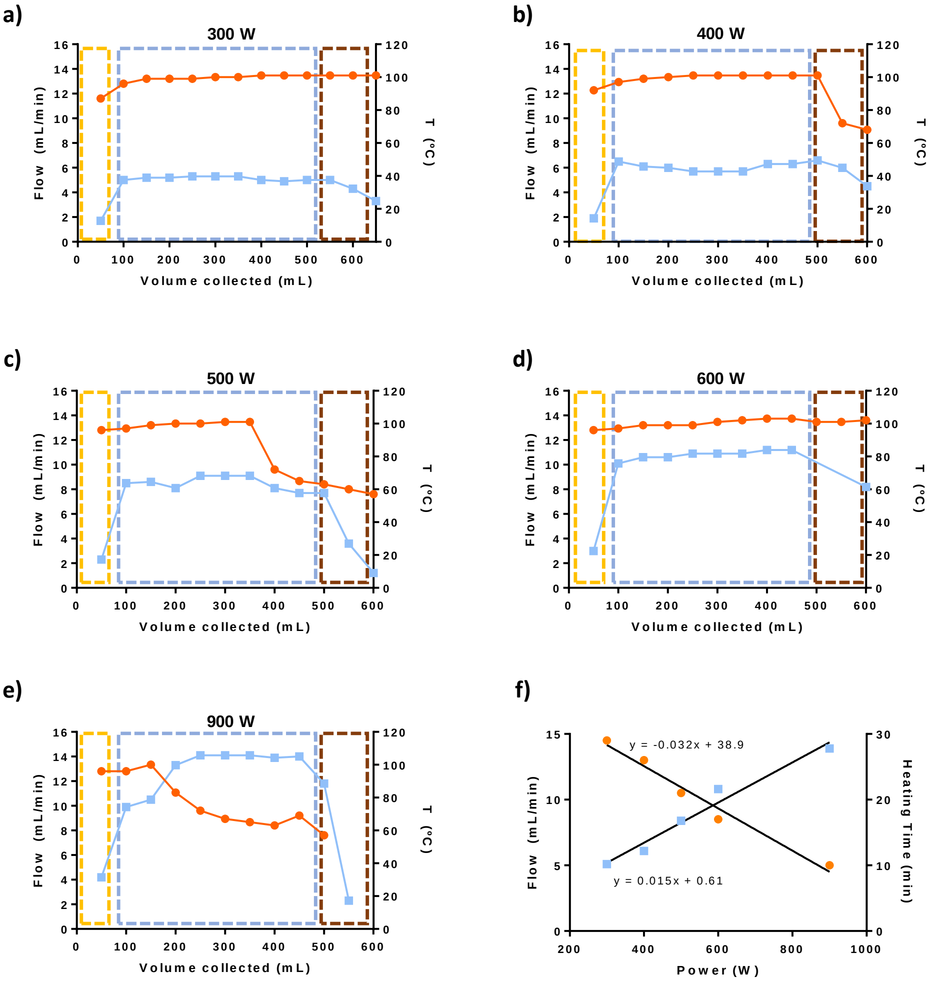

Figure 3.17 – Representation of the temperature (circles) and flow (squares) during apple pomace MHG at a) 300 W, b) 400 W, c) 500 W, d) 600 W and e) 900 W. In f) is represented the linear regression obtained by the relationship between heating time and flow with the maximum delivery power used during drying.

Table 3.21 – Microwave-hydrodiffusion and gravity (MHG) drying powers and processing parameters (volume of water recovered as liquid, heating time, water flow, total drying time and theorical energy expended). In parentheses are provided the energy efficiency (%) when compared to the energy required for water heating and evaporation.

Power (W)	Power density (W/g)	Total recovered Volume (mL)	Heating time (h)	Flow (mL/min)	Drying time (h)	Theorical energy (kJ/ kg fresh)
300	0.25	650	0.48	5.1	2.6	2310 (90%)
400	0.33	595	0.43	6.1	2.0	2369 (88%)
500	0.42	595	0.35	8.4	2.1	3142 (66%)
600	0.50	600	0.25	10.8	1.1	2073 (100%)
900	0.75	530	0.17	13.9	1.0	2631 (80%)

Moisture analysis showed that at the burning point, the average water content of the pomace is around 5%, reaching values between those obtained by conventional oven-drying. This, together with the fact that 1.2 kg of apple pomace presents 932 mL of water, allowed to estimate a drying kinetics associated to MHG by normalizing the apple pomace water content to the total obtained as liquid water during the experiments (530-650 mL) and assuming that the amount of water lost by evaporations remained constant along the drying process. As represented in **Figure 3.18**, apple pomace drying by MHG took between 1 to 2.6 h. This shows that MHG dries at least 2-fold faster and twice the amount of apple pomace that can be dried by oven-drying. Furthermore, based on the power used and the operation time, it was estimated an energy input of about 2500 kJ/kg of fresh weight apple pomace by MHG drying (**Table 3.21**), while the theorical energy required to heat apple pomace water to 100 ºC and promote its evaporation was 2084 kJ/kg. As in MHG heating is only based on the capability of water to absorb energy, inducing its heating to 100 ºC and transition from liquid to gaseous phase, it was estimated a theoretically energetic efficiency of about 83%. This efficiency is expected to be much higher than that of oven-drying as it required air heating, flow and moisture control. Although not directly comparable, **Motevali et al. (2011)** estimated a minimum energy requirement of 51 (kW/h/kg) for hot-air drying of sour pomegranate aril (initial moisture content of 71%), 500-fold the minimum required for drying apple pomace by MHG (0.1 kW/h/kg).

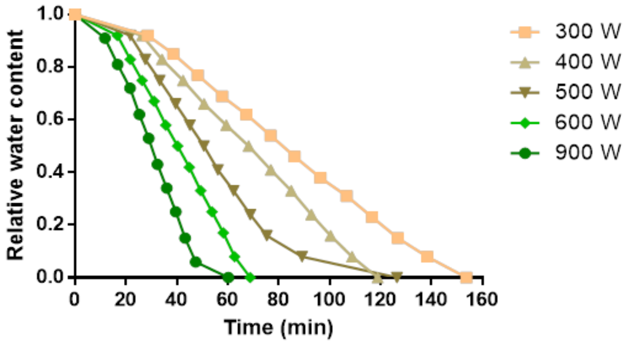

Figure 3.18 – Relative water content during apple pomace MHG drying at 300 to 900 W.

In contrast to oven-drying, MHG led to a partial burning of the apple pomace attributed to the uneven spatial distribution of the electromagnetic field inside the drying cavity. However, this can be overcome by rotation or stirring of the byproduct, thus allowing to standardize the average of electromagnetic field intensity in the whole pomace **(Vadivambal & Jayas, 2007)**. Furthermore, the final pomace obtained by hot-air drying presented a grain-like appearance, while for that obtained by MHG presented a highly compact cylindrical shape (**Figure 3.19**). This type of shrinkage effect has been observed for microwave dehydration, being attributed to the accelerated removal of water from tissues at high powers **(Krokida & Maroulis, 2001)**.

Hot-air dried pomace

MHG dried pomace

Figure 3.19 – Apple pomace obtained after hot-air drying (granular appearance) and after MHG drying (highly compact pomace with cylindric shape).

3.4.3 Microwave-hydrodiffusion and gravity extraction

In MHG, in addition to a drying approach, an extraction kinetics (mass yield recovery) can also be considered given that from the 932 mL of water in the pomace (determined as the moisture content), about 550-650 mL at 300-900 W was obtained as liquid form. This is possible because the extraction vessel is maintained inside the NEOS-GR oven during the experiment, and the gravitational selection allows the recovery of the liquefied fraction. In this case, and as observed for the recovery of essential oils **(Santos et al., 2019)**, the extraction kinetics can be divided into two phases, correspondent to the recovery of material from the surface of the vegetable matrix and a second one corresponding to the removal of water. In the first phase **(Figure 3.20 a)**, the *in-situ* water drags most of the solid material (0.4-0.7 g), as the recovery of the two extracts correspond to more than 99% of the extracted material. The amount of soluble material extracted at this stage appeared to be inversely proportional to the power applied (**Figure 3.20 b**), reaching a minimum of 0.4 g in the first 50 mL recovered at 900 W, in contrast to what was previously reported for algae **(Pérez et al., 2014)**. This is possibly attributed to the shortened heating stage, extraction duration, increased flow rate and more water vapor formed at higher powers thus limiting the extraction of soluble material **(Cendres, Chemat, Maingonnat, & Renard, 2011)**. In the second phase, only water is recovered, associated to the drying effect of MHG. Similar observations were also taken in broccoli byproduct **(Ferreira et al., 2018)**, onions **(Zill et al., 2011)** and algae **(Pérez et al., 2014)**.

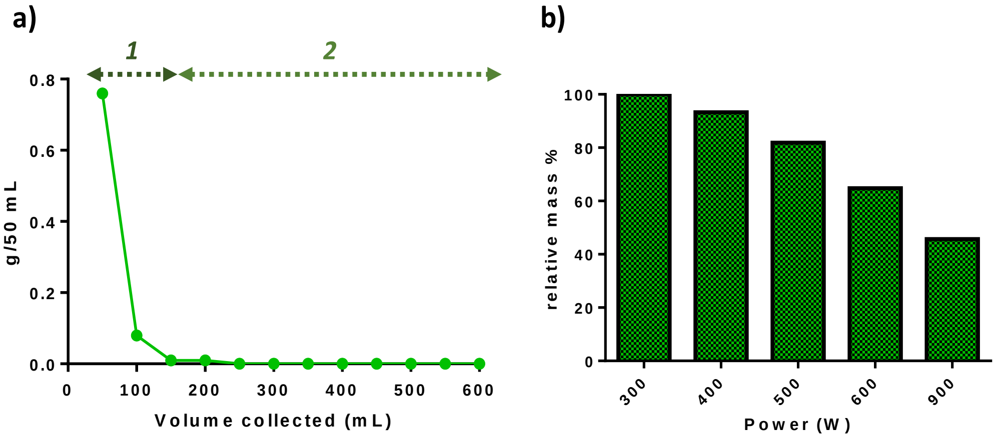

Figure 3.20 – Amount of soluble material recovered in the 50 mL fraction during apple pomace MGH drying a) and relative mass of soluble material recovered while increasing the applied power from 300 to 900 W b).

Sugar analysis of the soluble material (**Table 3.19)** revealed that this was mainly represented by polysaccharides (578-728 g/kg). The occurrence of Ara (29-32 mol%) and GalA (41-54 mol%) suggested the prevalence of pectic polysaccharides. Free sugars represented 111 g/kg, comprising mostly Glc (48-53 mol%), Fru (17-26 mol%), and some Xyl (1-13 mol%), Gal (8-11 mol%) and Ara (8-10 mol%). Free Fru and Glc are commonly found in apples while the remaining sugars are not. As these free sugars were already present in the initial pomace, it is very likely that they result from the juice extraction process during apple processing **(Kennedy et al., 1999)**. Analysis of this fraction by the Folin-Ciocalteu method additionally showed the occurrence of polyphenols, 13-17 g PLZE/kg. These data thus showed that, in addition to a drying technology, MHG simultaneously works as an extraction technique for apple pomace. In this context, a new pomace was subjected to a tailored drying process by MHG, taking advantage of the internal water of the byproduct, as well as the intermittent addition of hydroethanolic solutions. This process was performed with the principle that, by adding any additional solvent, more soluble material could be obtained by heating and recovered at the bottom phase of the apparatus by gravity.

3.4.4 MHG tailored extractions

The apple pomace material (AP II) to be subjected to MHG tailored drying by adding hydroethanolic solutions presented a general composition like the one previously used in the drying experiments. However, the free sugars content in this pomace was higher reaching 183 g/kg (**Table 3.19**). Additionally, the GalA (19 mol %) and Ara (12 mol %) molar ratio was higher than those observed for APIII. These observations suggest differences in juice production processing between AP II and AP III **(Kennedy et al., 1999)**. 400 W of power were selected for this study, as at this condition for APIII, the amount of material recovered in the first 50 mL fraction was almost the same as the amount recovered at 300 W (93% as represented in **Figure 3.20 b**) but with a lower dehydration time (-23%) as observed in **Table 3.21**.

MHG drying of apple pomace at 400 W behaved similarly to what was reported in the initial experiments, presenting a heating stage of about 26 min (time at which the first 50 mL of water were recovered). This process was performed until 200 mL (50 mL fractions) of water were collected (**Figure 3.21 a**). Freeze-drying showed that the soluble material ranged from 2.4 g/50 mL in the first fraction to 0.2 g/50 mL in the 4th fraction. The higher recovery of soluble material in this batch than in the one used in previous experiments, alongside with the higher amount of sugars in APII, suggested that the total soluble solids in AP II was higher than those in APIII. Sugar analysis revealed that these fractions were mainly composed of free sugars (>650 g/kg), mainly free Fru (61 mol%) and Glc (38 mol%), containing also some polysaccharides (>260 g/kg).

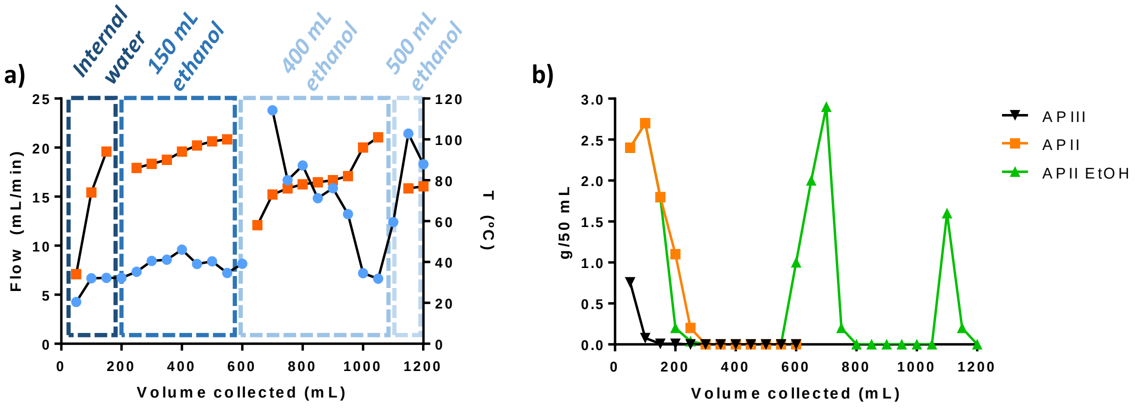

Figure 3.21 - Representation of the a) temperature (circles) and flow (squares) during apple pomace MHG at 400 W in the different stages of ethanol addition and b) extraction yields when compared to the process for APII without ethanol addition.

To assess if additional material could be recovered from the apple pomace during the drying procedure, 150, 400 and 500 mL of ethanol were added when the cumulative amount of liquid fraction reached 200 mL, 600 mL and 1050 mL, respectively (**Figure 3.21 b**). The addition of 150 mL allowed to recover low amounts of soluble material (0.04 g/50 mL). However, when 400 mL of ethanol were added, more than 6 g of material were recovered, increasing to more 1.5 g when adding more 500 mL of ethanol (**Figure 3.21 b** and **Table 3.19**). Sugar analysis revealed that the obtained extracts were mainly composed of free sugars (657-776 g/kg), mostly Fru (67-70 mol%) and Glc (29-32 mol%) (**Table 3.19**). Polysaccharides accounted for 132-149 g/kg, presenting Ara (59-60 mol%) and GalA (27-28 mol%), i.e., sugars characteristic of pectic polysaccharides. Polyphenols were also present, accounting for 12-13 g PLZE/kg of extract. These results thus show that the drying procedure by MHG can be tailored in order to allow the recovery of value-added compounds with the simultaneous drying of apple pomace. At the end of the MHG drying procedure, apple pomace presented a moisture content of about 5%.

3.4.5 Apple pomace stability and valuation

The dried APIII by MHG, after a storage period of 2 years at variable room temperature and atmospheric pressure appeared to remain stable without visual signs of spoilage (**Figure 3.19**), attributed to its very low moisture content that decreased from its initial 5% after MGH-assisted drying to 3% after the 2-year storage period. To assess the potential reuse of this dried material as a source of pectic polysaccharide and polyphenols, sequential extractions were performed to the APIII dried at 900 W. This pomace was selected to assess if, even when applying high delivery powers, valuable components such as polyphenols could be recovered. Acidified hot water extractions were performed to promote β-elimination reactions **(Voragen et al., 2009)** and preventing oxidation reactions **(Ferreira et al., 2002)** thus allowing to obtain higher polysaccharide and polyphenol extractions yields.

In the first extraction, about 9% (w/w) of the dried apple pomace was obtained, with 8% potentially recoverable by performing a second extraction (**Table 3.22**). A third hot water extraction was performed, but only 1.4% of material was recovered showing that most of the extractable material is recoverable in the first two hot water extractions. Sugar analysis of the extracts showed that they were mainly composed of polysaccharides (517-683 g/kg) with GalA (38-59 mol%) Ara (7-33 mol%) and Gal (9-14 mol%) as major monosaccharides. As GalA, Ara and Gal are characteristic of pectic polysaccharides **(Renard et al., 1991; Schols et al., 1990)**, it can be inferred that more than 70% of the polysaccharides extracted from apple pomace were of pectic nature, with an additional presence of Glc (8-12 mol%) and Xyl (6-9 mol%), typical of xyloglucans **(Renard et al., 1995)**. Total polyphenol analysis of the obtained extracts suggested that about 38 and 50 g PLZE/kg of extract were present. Of these, an average of 0.55 g/kg could be explained by UHPLC-DAD-ESI-MS^n (**Table 3.23**), comprising hydroxycinnamic acids (5-O-Caffeoylquinic acid), flavonols (Hyperoside, Isoquercetin, Reynoutrin, Avicularin, Quercitrin and Quercetin), and the dihydrochalcones (phloretin-2-*O*-glucoside), which are all polyphenols characteristic of apple **(Guyot, Marnet, Sanoner, & Drilleau, 2001)**. The difference between the total polyphenolic content determined by the Folin-Ciocalteu and UHPLC can be attributed to apple native procyanidins, only quantifiable after thioacidolysis **(Guyot, Marnet, & Drilleau, 2001)**, but also to polyphenol oxidation products derived from dihydrochalcones **(Guyot et al., 2007)** and hydroxycinnamic acids oxidation by PPO **(Castillo-Fraire et al., 2019)** and procyanidins oxidation by coupled-oxidation reactions.

Table 3.22 – Yield (%), carbohydrate composition (mol %), total carbohydrates (g/kg) and total polyphenols (g phloridzin equivalents (PLZE)/kg) of the HWE from APIII dried by MHG at 900 W. For total polyphenols, values are also provided as Gallic acid equivalents (g GAE/kg) between parentheses. Data are expressed as pooled standard deviation (*Pooled Std*) of three replicates. n.d means for not determined.

Sample	**Yield (%)**		**Carbohydrates (mol%)**									**Total Carboh. (g/kg)**	**Total Polyphenol (g PLZE/kg)**
			Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Fru**	**Gal**	**Glc**	**GalA**		
APIII 900 W	100	Polysac.	1	-	12	14	-	-	6	50	17	445	n.d.
		Free Sugars	-	-	tr	-	-	tr	-	100	-	8	
HWE I	8.9	Polysac.	3	-	28	8	-	-	11	5	46	629	38 (13)
		Free Sugars	-	-	13	-	-	21	-	66	-	69	
HWE II	8.1	Polysac.	2	-	29	8	-	-	10	6	45	709	50 (17)
		Free Sugars	-	-	14	-	-	25	-	61	-	64	
HWE III	1.4	Polysac.	2	-	34	7	-	-	10	5	42	625	50 (17)
		Free Sugars	-	-	15	-	-	24	-	61	-	78	
Residue	80	Polysac.	1	1	4	16	3	-	5	53	9	599	n.d.
Pooled std			*0*	*1*	*1*	*0*	*0*	*3*	*0*	*1*	*1*	*13*	*2 (1)*

Mass balance showed that 117 g of polysaccharides and 8 g PLZE per kg of apple pomace could be potentially recovered. These yields were lower than those observed for AP I, as well as to those reported in the literature **(Çam & Aaby, 2010; Fernandes et al., 2019)** possibly due to differences in the processing conditions while performing apple juice extraction. The analysis of the residue obtained after hot water extraction showed that 80% of the initial pomace had a high prevalence of Glc (53 mol%), Xyl (16 mol%), GalA (9 mol %) and some Gal (5 mol%), Ara (4 mol%) and Man (3 mol%). These observations suggest, as reported for AP I, the occurrence of cellulose, xyloglucans and pectic polysaccharides **(Mehrländer et al., 2002; Renard et al., 1991; Stevens & Selvendran, 1984)**. Moreover, the residue still contained polyphenols, which remained in the water insoluble cell wall material due to their capability to establish interactions with polysaccharides. These could be obtained by methanol or water/acetone extractions given the chaotropic nature of these solvents **(Renard et al., 2001)**. In the case of those covalently bonded to the water insoluble cell wall material due to oxidation reactions, further processing by microwave superheated water extraction can be additionally performed thus allowing to recover a still relevant phenolic fraction**.** These correspond to valuable compounds with potential to tailor the overall organoleptic properties of foods (e.g bitterness and astringency) **(Symoneaux, Baron, Marnet, Bauduin, & Chollet, 2014),** colour **(Nicolas et al., 1994)** but most importantly because these compounds have been shown to possess several biological properties with implications on Human health **(Del Rio et al., 2012)**. However, the apparatus used in this work presented a maximum processing of 1.2 kg per batch. Therefore, for this technology to be industrially implementable, larger scale apparatus must be developed.

Table 3.23 - Individual polyphenolic composition and total polyphenols (mg/kg) of the sequential hot water extracts (HWE), as determined by UHPLC-DAD-ESI-MS^n. Data are expressed as Pooled standard deviation (Pooled std) of three replicates.

N°	**RT**	**Compound**	**HWE I**	**HWE II**	**HWE III**	***Pooled std***
1	7.9	5-*O*-Caffeoylquinic Acid	57	57	65	*1*
2	12.6	Hyperoside[b]	86	99	117	*2*
3	12.7	Isoquercetin [a]	24	28	35	*1*
4	13.2	Reynoutrin [b]	23	24	27	*1*
5	13.5	Avicularin [b]	18	10	14	*1*
6	13.8	Quercitrin [b]	72	77	99	*1*
8	14.9	Phloridzin [a]	192	192	243	*3*
7	15.9	Quercetin	24	35	19	*0*
		Total	496	521	619	*1*

Chapter 4- Conclusions and Future Perspectives

During apple juice extraction, polyphenol oxidation reactions are known to occur thus yielding polyphenol oxidation products and affecting the structure and properties of the polysaccharides in the pomace. This and the lack of an efficient drying procedure capable to meet industry demands, rend apple pomace a disposable. In this context, this work was conducted aiming to obtain an overview of the structural modifications of polyphenols and polysaccharides derived from polyphenol oxidation reactions, their properties, and their potential recovery upon drying for apple pomace stabilization.

Apple pomace was composed, on a general basis, mostly by polysaccharides (470-620 g/kg), constituted by Glc (44-58 mol %) and Xyl (7-15 mol %), characteristic of cellulose and xyloglucans. GalA (9-25 mol %), Ara (5-14 mol %), and Gal (7-9 mol %), characteristic of pectic polysaccharides, were also found. Alongside with these, polyphenols were also observed. They accounted for about 5 g/kg of apple pomace dry weight, mostly flavan-3-ols with an average degree of polymerization of 5 (59%), flavonols (39%), dihydrochalcones (3%), and traces of hydroxycinnamic acids. Polyphenol oxidation products derived from dihydrochalcones and hydroxycinnamic acids were also found to occur as extractable components. On the other hand, a significant amount of flavan-3-ols, about 4-fold of the total apple native polyphenols, were found to be non-extractable, attributed to flavan-3-ol oxidation. Microwave superheated water extractions, aiming cell wall degradation, followed by ethanol precipitation of the obtained extracts, showed that these compounds were covalently attached to pectic polysaccharides, xyloglucans and cellulose, accounting for 40% of the potentially extractable polyphenols from apple pomace. Such observations show the relevance of phenolic structures derived from oxidation reactions when considering the biological properties of these extracts, either for food or pharmaceutical purposes. The higher polyphenol yields obtained from apple pomace when applying cell wall degrading technologies showed also the presence of covalent polysaccharide/polyphenol complexes in the pomace.

Fractionation of the hot water-soluble material by solid-phase extraction using C_{18} cartridges demonstrated that in apple pomace about 17% of the polymeric material is mostly composed of polysaccharides that behaved hydrophobically at pH 7 and an additional 1% behaved hydrophobically at pH 3. Glycosidic linkage analysis, their sequence by NMR, and the use of sugar beet arabinans as standard polysaccharides with covalently attached ferulic acids suggested that the hydrophobic polymeric material corresponded to xyloglucan-polyphenol-pectic polysaccharides and arabinan-polyphenol complexes, where polyphenols dictated their hydrophobicity. However, as this hydrophobic behaviour was observed by performing solid-phase extraction using C_{18} cartridges, it remains to be elucidated if this hydrophobicity derives from the interaction between the polysaccharide-polyphenol complexes and the stationary phase of the cartridge or instead, from an intrinsic conformational hydrophobicity of the polysaccharides.

Studies of the interactions between pectic polysaccharides and polyphenols demonstrated that pectic polysaccharides can interact both with low molecular weight polyphenols (5-*O*-caffeoylquinic acid and phloridzin) and procyanidins. The use of branched and unbranched arabinans allowed to observe that branching of the pectic backbone negatively impact the interactions with polyphenols. Moreover, interactions are also hampered in the case of polysaccharides with covalently attached polyphenols. In this context, the suppression of polysaccharide debranching enzymes during juice extraction would result in a higher transfer of polyphenols to the juice fraction together with the polysaccharides. On the other hand, these observations demonstrate that the polysaccharides found to occur both as soluble and insoluble covalent polysaccharide-polyphenols complexes can interact with polyphenols through non-covalently linkages. This probably represents the phenomenon that preceedes to the formation of the covalent linkages due to oxidation. In addition, arabinans, despite being highly abundant in apple pectic polysaccharides, only explained 8% of the interaction between pectic-polysaccharides and polyphenols. Such observation suggests that for polyphenol interactions, the whole polysaccharide structure is more relevant than each part. In the case of pectic polysaccharides, this means that the interaction capacity of isolated arabinans towards polyphenols is lower than that of the arabinans when present as part of the pectic polysaccharides structures, probably due to a concerted action of the different structures, providing differences on their structural conformation.

The dehydration processes of apple pomace by MHG presents a high energetic efficiency (80% on average), with at least 4-fold higher drying rates than those observed for conventional hot-air drying. MHG drying process is composed of three different stages: the heating phase, a drying phase, and a burning point phase. The latter, although not of industrial interest, signalizes the complete drying of the byproduct, composed of about 5% of water. This same drying technology also allows to recover some of the water that is removed from the pomace and flows downwards by the action of gravity. Part of this water presents a high concentration of soluble solids, mostly carbohydrates, that can be reused by apple processing industries. On the other hand, by adding other solvents such as ethanol, higher amounts of soluble material can be recovered, including both polysaccharides and polyphenols. The generated pomace presents a highly compact structure, ideal for long term storage and visually stable for at least 2 years after which polysaccharides and polyphenols are still recoverable. This approach has, however, a limited sample processing (maximum 1.2 kg) that although partially surpassed by the pilot-scale MAC-75, may still not fully meet the amounts that need to be industrially processed.

From a general point of view, the results obtained provide information about the structure and properties of polyphenols and polysaccharides modified by polyphenol oxidation reactions that, so far, have been neglected and perceived as negative by both food chemists and industries. Furthermore, the results show the potential of MHG technology as a drying approach to be considered by apple processing industries to assure apple pomace stability and its valuation. Some new features on apple pomace research were also opened, including:

- Influence of the level of oxidation of apple pomace in the type of phenolic structures and their occurrence as covalent complexes with polysaccharides;

- Fine structure of the polysaccharide-polyphenol covalent complexes, considering structural changes on carbohydrates and polyphenols, particularly the type of linkages;

- Biological and functional properties of the polysaccharide-polyphenol complexes, namelly in relation to their hydrophobic behaviour;

- Relationship between the polysaccharides structure and conformation, when isolated or as part of a more complex structure, towards the interactions with polyphenols;

- Design of an MHG drying process capable of reaching a dried pomace without the burning stage;

- Transposition to larger scale apparatus for the drying of apple pomace;

- Microbial and toxicological stability of the dehydrated pomace.

The elucidation of these aspects might culminate in the design of more diversified solutions for agro-food waste valuation, apple pomace mitigation and the implementation of a circular economy model in the apple processing sector.

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