Universidade de Aveiro Departamento de Química
Ano 2020

Sónia dos Santos Ferreira

Estabilização e valorização de subprodutos de brócolo como ingredientes, fonte de compostos bioativos e biomateriais

Stabilization and valorisation of broccoli by-products as ingredients, source of bioactive compounds, and biomaterials

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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 do Doutor Manuel António Coimbra Rodrigues da Silva, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro, da Doutora Susana Cardoso, Investigadora Doutorada do Departamento de Química da Universidade de Aveiro, e da Doutora Dulcineia Ferreira Wessel, Professora Coordenadora da Escola Superior Agrária do Instituto Politécnico de Viseu

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Dedico este trabalho aos meus pais, aos meus irmãos e ao Pedro Oliveira.

"A vida é uma aprendizagem diária.
Afasto-me do caos e sigo um simples pensamento:
Quanto mais simples melhor!"
José Saramago

o júri

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palavras-chave

Subprodutos de brócolo, valorização, glucosinolatos, compostos fenólicos, polissacarídeos, atividade imunoestimuladora, filmes com brócolo.

resumo

Na indústria de congelação de brócolo, as perdas podem ultrapassar mais de 45% da matéria-prima, resultando numa grande quantidade de subprodutos com baixo valor económico e impacto ambiental considerável. Os principais subprodutos são caules, folhas e restos de inflorescências, partilhando o valor nutricional e os compostos bioativos dos brócolos, como glucosinolatos, fenólicos, pigmentos e polissacarídeos (como fibra alimentar). No entanto, a elevada quantidade de água (cerca de 90%) e consequente perecibilidade limitam a valorização destes subprodutos. Neste sentido, os subprodutos de brócolo, quando adequadamente estabilizados para preservar os compostos bioativos, podem ser uma fonte de ingredientes alimentares e biomateriais para serem utilizados em embalagens de base biológica.

Para estabilizar os subprodutos de brócolo, a tecnologia de microondas por hidrodifusão e gravidade (MHG) foi usada para desidratar e recuperar simultaneamente os compostos solúveis em água para incorporação como ingredientes alimentares. A hidrodifusão permitiu obter um material seco com 12% de humidade em 43 min, quando foram utilizados 550 g de subprodutos de brócolo, preservando os polissacarídeos e a proteína. A água recuperada por hidrodifusão continha até 317 µg/mL de compostos fenólicos como equivalentes de ácido gálico, 11 mg/mL de açúcares livres, 9 mg/mL de aminoácidos e 356 µg/mL de glucosinolatos, dependendo do tipo de subproduto utilizado. A estabilização por desidratação e simultânea extração de compostos bioativos mostrou o potencial da tecnologia MHG para valorização de subprodutos de brócolo.

O branqueamento como pré-tratamento tem sido usado para melhorar a taxa de secagem dos brócolos, promovendo simultaneamente a inativação das enzimas. Atendendo a esta informação, o impacto do branqueamento antes da desidratação por liofilização, por convecção de ar e assistida por MHG nos níveis de pigmentos, glucosinolatos e fenólicos foi estudada por UHPLC-DAD-ESI/MS^n. Quando comparada à liofilização, uma técnica conhecida por preservar compostos, o branqueamento aumentou a capacidade de extração de pigmentos e fenólicos, enquanto a secagem por convecção de ar reteve apenas 49% dos pigmentos e 70% de fenólicos, ambos os tratamentos sem afetar os glucosinolatos. No entanto, quando a secagem por convecção de ar foi precedida de branqueamento, menos de 50% dos compostos foram retidos. Por outro lado, a desidratação por MHG aumentou a capacidade de extração de fenólicos em 26%, particularmente a dos derivados de kaempferol, além de reter a quantidade do glucosinolato glucorafanina, quando comparada à liofilização. No entanto, apenas 23% dos glucosinolatos com um grupo indole foram recuperados e os pigmentos foram severamente reduzidos, com a luteína representando apenas 32% e apenas a clorofila *b* foi observada em pequenas quantidades após a desidratação por MHG. Portanto, para valorizar os subprodutos de brócolo, diferentes tecnologias de secagem podem ser usadas para se obter ingredientes com composição variada:

a liofilização é adequada para pigmentos e glucosinolatos, a secagem por convecção de ar é adequada para glucosinolatos, enquanto a tecnologia de MHG promove a extração de compostos fenólicos.

Como existem poucas informações sobre a bioatividade dos polissacarídeos de brócolo, os polissacarídeos pécticos foram extraídos, fracionados e caracterizados, e a sua atividade imunoestimuladora foi avaliada por incubação *in vitro* com BALB/c splenócitos e citometria de fluxo. Os polissacarídeos foram posteriormente fracionados por tamanho e carga, tratados enzimaticamente e sulfatados quimicamente para estabelecer relações estrutura-função dos polissacarídeos pécticos com ativação dos linfócitos B. Polissacarídeos pécticos obtidos após a preparação de resíduo insolúvel em álcool (AIR, estabilizado por secagem com solvente), extração com água quente e precipitação com etanol a 80% (Et80) estimularam os linfócitos B. Por outro lado, os polissacarídeos pécticos extraídos com soluções de imidazol, Na_2CO_3 e KOH não estimularam os linfócitos B. A fração Et80 possuía polissacarídeos pécticos esterificados com grupos metilo e acetilo, galactanas em ligação 1-4, arabinanas e arabinogalactanas tipo II com resíduos de 4-O-Me-GlcA. O fracionamento dos polissacarídeos Et80 por peso molecular (de 12 a 400 kDa) mostrou que todas as frações apresentavam atividade imunoestimuladora. A remoção da Ara terminal ou pequenas cadeias de Ara também não alterou a atividade imunoestimuladora. No entanto, a desesterificação dos grupos metilo e remoção das regiões de homogalacturonanas dos polissacarídeos pécticos reduziram a atividade imunoestimuladora. O fracionamento desses polissacarídeos permitiu obter uma fração acídica com atividade imunoestimuladora, indicando que a atividade destes polissacarídeos acídicos era ocultada pela presença de polissacarídeos neutros. A sulfatação química também reduziu a atividade, provavelmente por obstruir as características estruturais importantes para a ativação das células B. Estes resultados mostram que os subprodutos de brócolo podem ser valorizados como fonte de polissacarídeos pécticos para ingredientes funcionais que melhorem a função imunológica e promovam a saúde.

Com a preocupação de reduzir as questões ambientais dos plásticos à base de petróleo, desenvolveram-se materiais renováveis e biodegradáveis à base de amido com inclusão de subprodutos de brócolo e foi avaliado o impacto da sua adição nas propriedades mecânicas e na hidrofilicidade da superfície dos filmes. A adição de subprodutos antes das etapas de gelatinização e filtração do amido permitiu obter filmes com maior resistência, rigidez e elasticidade do que o filme de amido controlo. Além disso, superfícies menos hidrofílicas foram obtidas com a adição de subprodutos de brócolo. A atividade antioxidante, o teor de água e a solubilidade em água foram mantidos com a adição destes subprodutos. Os compostos extractáveis com água foram os principais contribuintes para as alterações observadas nas propriedades mecânicas. Uma elasticidade ainda maior foi obtida quando a genipina, um reticulante natural, foi adicionada. Após um ano de armazenamento, os filmes tenderam a ser mais resistentes, menos flexíveis, mais rígidos e com superfícies mais hidrofóbicas, indicando que a adição de subprodutos de brócolo promoveu rearranjos da matriz de amido ao longo do armazenamento. Estes resultados promissores da incorporação de subprodutos de brócolo nos filmes de amido mostram o potencial de valorização dos subprodutos de brócolo para aplicação em bioplásticos para embalagens.

Em conclusão, os subprodutos de brócolo podem ser estabilizados após desidratação e extração simultâneas de compostos bioativos que podem ser valorizados como ingredientes alimentares e como aditivos na área de embalagens, contribuindo para a redução de resíduos industriais e uma economia circular.

keywords

Broccoli by-products, valorisation, glucosinolates, phenolic compounds, polisaccharides, immunostimulatory activity, films with broccoli.

abstract

Losses in the frozen-food industry of broccoli can account for more than 45% of the raw material, resulting in a large amount of by-products with low economic value and of considerable environmental impact. The main by-products are stalks, inflorescence remains and leaves, sharing broccoli nutritional value and bioactive compounds such as glucosinolates, phenolics, pigments, and polysaccharides (as dietary fibre). However, their high amount of water (around 90%) and consequent perishability limit by-products valorisation. In this regard, broccoli by-products, if adequately stabilized to preserve the characteristics of the bioactive compounds, can be a source of food ingredients and biomaterials to be used in biobased packaging.

To stabilize broccoli by-products, microwave hydrodiffusion and gravity (MHG) was used to dehydrate and simultaneously recover the water-soluble diffused compounds for incorporation as food ingredients. The hydrodiffusion allowed to obtain a dried material with 12% moisture in 43 min when 550 g of broccoli by-products were used, preserving polysaccharides and proteins. Diffused water contained up to 317 µg/mL gallic acid equivalents of phenolic compounds, 11 mg/mL free sugars, 9 mg/mL amino acids, and 356 µg/mL glucosinolates, depending on the type of by-product used. The simultaneous stabilization by dehydration and extraction of bioactive compounds showed the potential of MHG technology for valorisation of broccoli by-products.

Blanching pre-treatment has been reported to improve the drying rate of broccoli, promoting simultaneous enzyme inactivation. Therefore, the impact of pre-dehydration blanching step, freeze-drying, air-drying, and MHG dehydration on the levels of pigments, glucosinolates, and phenolics, was studied by UHPLC-DAD-ESI/MS^n. When compared to freeze-drying, a technique known to preserve compounds, a pre-blanching step increased the extractability of both pigments and phenolics, while air-drying only retained 49% of the pigments and 70% of phenolics, both without affecting glucosinolates. However, when air-drying was preceded by blanching, less than 50% of compounds were retained. On the other hand, MHG dehydration increased the phenolics extractability by 26%, particularly that of kaempferol derivatives while also retaining the amount of the glucosinolate glucoraphanin, when compared to freeze-drying. Nevertheless, only 23% of indole glucosinolates were recovered and pigments were severely reduced, with lutein accounting only for 32% and only chlorophyll *b* was observed in trace amounts after MHG dehydration. Therefore, to valorise broccoli by-products as ingredients, different drying technologies may be used when targeting different composition richness: freeze-drying is suitable for pigments and glucosinolates, air-drying is suitable for glucosinolates, while MHG promotes the extractability of phenolic compounds.

As there is scarce information about broccoli polysaccharide bioactivities, pectic polysaccharides were extracted, fractionated, and characterized and their immunostimulatory activity was evaluated by *in vitro* incubation with BALB/c

splenocytes and flow cytometry. Polysaccharides were further fractionated by size and charge, enzymatically treated, and chemically sulfated to establish structure-function relationships of pectic polysaccharides with B lymphocytes activation. Pectic polysaccharides obtained after preparation of alcohol insoluble residue (AIR, stabilized by solvent drying), hot water extraction, and 80% ethanol precipitation (Et80) stimulated B lymphocytes. In contrast, pectic polysaccharides extracted with imidazole, Na_2CO_3, and KOH solutions did not stimulate B lymphocytes. Et80 fraction had methyl- and acetyl-esterified pectic polysaccharides, 4-linked galactans, arabinans, and type II arabinogalactans with 4-O-Me-GlcA residues, as well as α-glucans. Fractionation of Et80 polysaccharides by molecular weight (from 12 to 400 kDa) showed that all fractions had immunostimulatory activity. Removal of terminal Ara or small chains of Ara also did not change immunostimulatory activity. However, the methyl deesterification of pectic polysaccharides and removal of galacturonan regions reduced polysaccharides immunostimulatory activity. Fractionation of these polysaccharides allowed to obtain an acidic fraction with immunostimulatory activity, indicating that neutral polysaccharides were masking the immunostimulatory activity of the acidic ones. Chemical sulfation also reduced activity, probably hiding structural features important for B cells activation. These results show that broccoli by-products can be valorised as a source of pectic polysaccharides to serve as functional food ingredients to improve immune function and promote health.

Taking the opportunity to contribute to reduce the environmental issues of petroleum-based plastics by developing starch-based renewable and biodegradable materials, broccoli by-products were used to evaluate their impact on mechanical properties and surface hydrophilicity of the films. Addition of by-products before starch gelatinization and filtration steps allowed to obtain films with higher strength, stiffness, and elasticity than starch control film. Moreover, less hydrophilic surfaces were obtained with addition of broccoli by-products. Antioxidant activity, water content, and water solubility were maintained with addition of broccoli by-products. Water extractable compounds were the main contributors to the observed mechanical properties changes. Even higher elasticity was obtained when genipin was added. After one-year storage, films tended to be more strength, less flexible, stiffer, and with increased hydrophobicity of surfaces, indicating that broccoli by-products addition promoted starch matrix rearrangements along storage. These promising results of incorporation of broccoli by-products in starch films show the valorisation potential of broccoli by-products by application as bioplastics on the packaging field.

In conclusion, broccoli by-products can be stabilized after simultaneous dehydration and extraction of bioactive compounds that can be valorised as food ingredients and as additives in the packaging field, contributing to a circular economy and reduction of industrial wastes.

Publications and communications

The results presented in this thesis originated several publications in international scientific journals with Referee, as well as oral and poster communications (as first author and presenter) in national and international meetings.

Publications in international scientific journals with Referee

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia F. Wessel, Manuel A. Coimbra (**2018**). Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds. *Food Chemistry*, 246, 386-393. IF 5.399.

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Sónia S. Ferreira, Alexandra Correia, Manuel Vilanova, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra (**2020**). Pectic polysaccharides from broccoli by-products with *in vitro* B lymphocytes stimulatory activity. (In preparation)

Sónia S. Ferreira, Idalina Gonçalves, Paula Ferreira, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra (**2020**). Hydrophobization of starch films surfaces with broccoli by-products. (In preparation)

Oral communications

Sónia S. Ferreira, Alexandra Correia, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel Vilanova, Manuel A. Coimbra. *In vitro* immunostimulatory activity of pectic polysaccharides from broccoli by-products. *6th EPNOE International Polysaccharide Conference*, **21-25 October 2019**, Aveiro, Portugal.

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Broccoli by-products as ingredients rich in bioactive compounds after microwave assisted dehydration. *XX EuroFoodChem Conference*, **17-19 June 2019**, Porto, Portugal.

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Simultaneous dehydration and extraction of broccoli by-products by microwave hydrodiffusion and gravity. *XXIV Encontro Luso Galego de Química*, **21-23 November 2018**, Porto, Portugal.

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Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Identification of glucosinolates from broccoli extracts obtained by microwave hydrodiffusion and gravity. *7th Workshop of Mass spectrometry*, **7 March 2018**, Aveiro, Portugal.

Sónia S. Ferreira, Ana M. Costa, Manuel A. Coimbra, Susana M. Cardoso, Dulcineia Ferreira Wessel. Characterization of dietary fibre polysaccharides from broccoli by-products. *11th International Meeting of the Portuguese Carbohydrate Group and 6th Iberian Carbohydrate Meeting*, **6-10 September 2015**, Viseu, Portugal.

Poster communications

Sónia S. Ferreira, Filipa Monteiro, Dulcineia Ferreira Wessel, Artur M. S. Silva, Manuel A. Coimbra, Susana M. Cardoso. Effect of blanching and drying in pigments of broccoli by-products flours, *2nd Conference on Food Bioactives & Health*, **26-28 September 2018**, Lisbon, Portugal. Award for 2nd best poster presentation on carotenoids.

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds. *7th joint congress of Microbiology and Biotechnology: Microbiotec17*, **7-9 December 2017**, Porto, Portugal.

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Extraction of carbohydrates from broccoli by-products dehydrated by microwave hydrodiffusion and gravity. *12th National Meeting of the Portuguese Carbohydrate Group – Glupor12*, **11-13 September 2017**, Aveiro, Portugal. Award for best poster presentation.

Sónia S. Ferreira, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. Broccoli by-products as a source of glucosinolates, phenolic compounds, protein, and polysaccharides. *XIII Encontro de Química dos Alimentos*, **14-16 September 2016**, Porto, Portugal.

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra. NEOS-GR assisted extraction of glucosinolates from broccoli by-products. *6th joint congress of Microbiology and Biotechnology: Microbiotec15*, **10-12 December 2015**, Évora, Portugal.

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Abbreviations

2D COSYPR	Homonuclear shift correlation with presaturation during relaxation delay
ABTS	2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
AIR	Alcohol insoluble residue
Ara	Arabinose
CDTA	Cyclohexane diamine tetraacetic acid
DAD	Photodiode-array detector
DEAE	Dietilaminoetil cellulose
DEPT-135	Distortionless enhancement by polarization transfer
ESI	Electrospray ionization
dw	Dry weight
***-f* (suffix)**	Furanose
FACS	Fluorescence-activated cell sorting
FAME	3-*O*-acetyl fatty acid methyl esters
FID	Flame ionization detector
Fru	Fructose
FTIR	Fourier transform infrared
Fuc	Fucose
GAE	Gallic acid equivalents
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography
GCqMS	Gas chromatography quadrupole mass spectrometry
HMBC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence
Glc	Glucose
GlcA	Glucuronic acid
GLS	Glucosinolates
HBSS	Hanks' balanced salt solution
ITC	Isothiocyanates
LPS	Lipopolysaccharides
Man	Mannose
MHG	Microwave hydrodiffusion and gravity
MS	Mass spectrometry
MS^n	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
***-p* (suffix)**	Piranose

PBS	Phosphate buffered saline
PCA	Principal component analysis
Rha	Rhamnose
RPMI	RPMI-1640 medium supplemented with foetal calf serum (10%), penicillin (100 IU/mL), streptomycin (50 mg/L), and 2-mercaptoethanol (0.05 mol/L)
RPMI-1640	Medium Roswell Park Memorial Institute
SEM	Scanning electron microscopy
TFA	Trifluoroacetic acid
TPC	Total phenolic compounds
UA	Uronic acids
UHPLC	Ultra-high-performance liquid chromatography
WCA	Water contact angle
XRD	X-Ray diffractometry
Xyl	Xylose

CHAPTER 1 - INTRODUCTION

1.1. Bioactive compounds of broccoli by-products

1.1.1. Glucosinolates and derivatives

1.1.2. Phenolic compounds

1.1.3. Pigments

1.1.4. Polysaccharides

1.2. Dehydration of broccoli by-products

1.3. Hypothesis, aims, and strategy of the work

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Sónia S. Ferreira, Filipa Monteiro, Cláudia P. Passos, Artur M. S. Silva, Dulcineia Ferreira Wessel, Manuel A. Coimbra, Susana M. Cardoso (**2020**). Blanching impact on pigments, glucosinolates and phenolics of dehydrated broccoli by-products. *Food Research International*, 132, 109055. IF 4.972.

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Sónia S. Ferreira, Idalina Gonçalves, Paula Ferreira, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra (**2020**). Hydrophobization of starch films surfaces with broccoli by-products. (In preparation).

1. Introduction

Broccoli belongs to genus *Brassica* that includes cabbage, cauliflower, kale, brussels sprouts, and turnip and are the world's most cultivated and consumed vegetables, due to their flavour and health promoting composition (Ares, Nozal, & Bernal, 2013; Fahey, 2003). Worldwide production of broccoli and other brassicas accounted for more than 97,436 tonnes in 2017, from which 0.2% were produced in Portugal (FAO, 2017). These vegetables are delivered to the final consumer mostly as fresh products, although they can be packed in modified atmospheres, held at reduced temperatures, or be frozen. The broccoli head usually used by frozen-food industry accounts only for 55% of the commercial product. The remaining 45% are broccoli by-products comprising stalks, leaves, and inflorescences remains (Monliz, 2013). This high percentage of by-products is usually managed as refuse or used to produce animal feed creating a high economic and environmental impact. Nonetheless, such by-products share the same composition of the commercially available broccoli heads representing a source of nutritional and bioactive compounds that can be valorised through different applications after stabilization and extraction, which is being encouraged by the European Union legislation (Heeres, 2009).

1.1. Broccoli by-products bioactive compounds

Broccoli by-products are a source of protein, amino acids, lipids, sugars, vitamins (vitamin C, E, A, K) and essential minerals (N, K, Ca, Fe) (Ares et al., 2013). Additionally, they are rich in bioactive compounds such as glucosinolates (and their degradation products isothiocyanates), phenolic acids, flavonoids, pigments, and polysaccharides as dietary fibre (Berndtsson, Andersson, Johansson, & Olsson, 2020; Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Femenia, Bestard, Sanjuan, Rosselló, & Mulet, 2000; Schaefer et al., 2006), which have health-beneficial effects (Jeffery & Araya, 2009; Juge, Mithen, & Traka, 2007; Paturi et al., 2010; L. Xu, Cao, & Chen, 2015). In the next sections these bioactive compounds will be introduced, elucidating their structure, bioactivities, and presence in broccoli.

1.1.1. Glucosinolates and derivatives

Glucosinolates (GLS) are *β*-thioglucoside *N*-hydroxysulfates with a variable side chain (R) derived from amino acids (**Figure 1.1**). Based on the diversity of amino acid precursors and further modifications, more than 120 glucosinolates have already been identified and classified as **1.** glucosinolates derived from leucine, isoleucine, methionine or valine, which are aliphatic; **2.** glucosinolates derived from tyrosine and phenylalanine, which are aromatic, and **3.** glucosinolates derived from tryptophan, which contain an indole group (Sønderby, Geu-Flores, & Halkier, 2010; Thomas, Badr, Desjardins, Gosselin, & Angers, 2018). These hydrophilic anionic molecules are plant secondary metabolites sequestered in the vacuoles as potassium salts. Upon cellular disruption, glucosinolates may interact with myrosinase (EC 3.2.3.1 thioglucosidase), an endogenous enzyme that hydrolyses the glucose (Glc) moiety (Shirakawa & Hara-Nishimura, 2018). The release of Glc from glucosinolates forms an unstable intermediate, which upon spontaneous rearrangement leads to a variety of degradation products, such as isothiocyanates (ITC), thiocyanates, nitriles and epithionitriles. The way this degradation products are formed depends on several factors such as structure of the parent glucosinolate, pH, temperature, and presence of cofactors, as Fe^{2+} ions and epithiospecifier proteins (Latté, Appel, & Lampen, 2011; Witzel, Abu Risha, Albers, Börnke, & Hanschen, 2019).

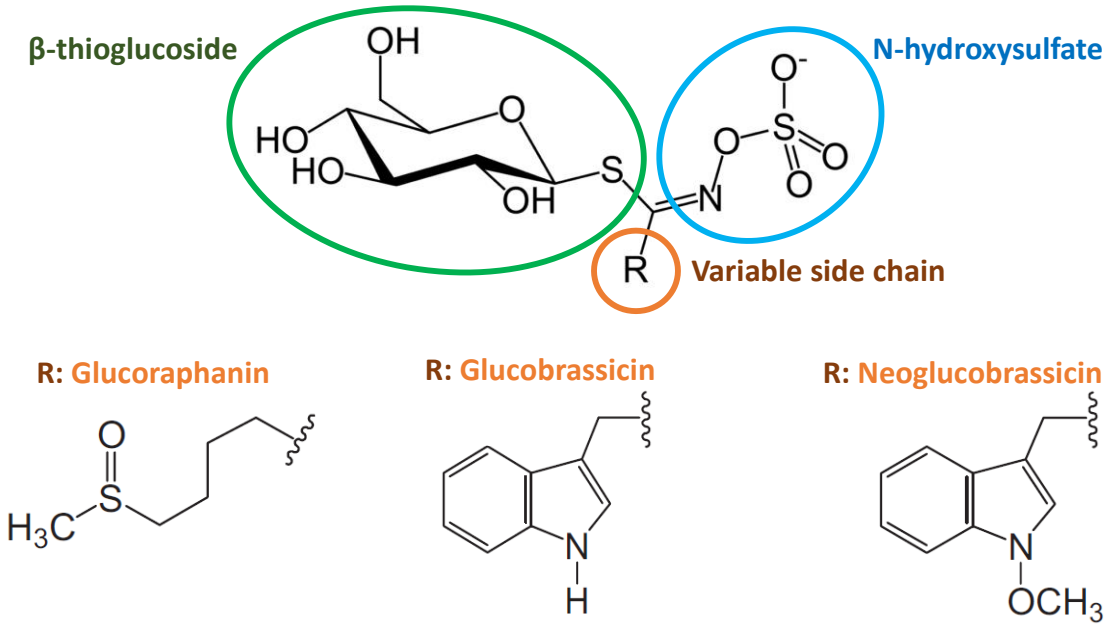


Figure 1.1. General chemical structure of glucosinolates and R side chains of major glucosinolates in broccoli: glucoraphanin, glucobrassicin and neoglucobrassicin.

Glucosinolates and/or their degradation products have long been known for their importance in agriculture through potential fungicidal, bactericidal, nematocidal and allelopathic properties by grinded plant incorporation into soils (Björkman et al., 2011; Neugart et al., 2018; Rosa & Rodrigues, 1999; Zasada & Ferris, 2004). More recently, they have gained research interest because of their health-related activities. *In vitro* and *in vivo* studies indicate GLS and ITC, particularly sulforaphane, affect many steps of cancer development, including modulation of phases I and II detoxification enzymes, and have beneficial health effects in models of cardiovascular and neurological diseases (Ares et al., 2013; Jeffery & Araya, 2009; Juge et al., 2007; Latté et al., 2011; Mandrich & Caputo, 2020). Studies of bioaccessibility and bioavailability have shown that ITC can reach the absorption sites in the small intestine and reach the blood circulation, in contrast with their precursors GLS. Notwithstanding, GLS are more stable than ITC, and can be absorbed in the colon after microbiota metabolization of GLS into ITC (Liou et al., 2020). These evidences for health benefits fomented the characterization of these compounds in *Brassica* plants, including several broccoli cultivars (Latté et al., 2011) and the development of food products with high concentration of glucosinolates, such as soups and tea (Alvarez-Jubete et al., 2014; Dominguez-Perles, Moreno, Carvajal, & Garcia-Viguera, 2011; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

The major glucosinolates in broccoli are glucoraphanin and the indole glucosinolates glucobrassicin and neoglucobrassicin (**Figure 1.1**) (Gliszczyńska-Świgło et al., 2006; Meyer & Adam, 2008). However, there are large variations regarding the total amount of glucosinolates and the individual components. Differences are found between cultivars, plant age, cultivation practices, and also environmental factors like temperature, soil characteristics, pathogen stress, wounding, and plant growth regulators (Björkman et al., 2011; Del Carmen Martínez-Ballesta, Moreno, & Carvajal, 2013; Meyer & Adam, 2008). Storage and processing conditions also affects glucosinolates content (Gliszczyńska-Świgło et al., 2006; Sikorska-Zimny & Beneduce, 2020). Therefore, broccoli by-products should be stabilized to save these compounds.

1.1.2. Phenolic compounds

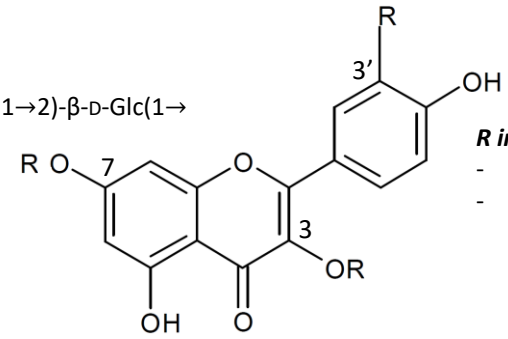
Phenolic compounds are a group of more than 8,000 secondary metabolites characterized by having at least one aromatic ring with one or more hydroxyl substituents. They range from single aromatic-ringed compounds to large derived polyphenols and are classified in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (hydroxycinnamic acids, stilbenes and others), commonly found conjugated to sugars and organic acids (Cartea, Francisco, Soengas, & Velasco, 2011). Standing by this concept, some glucosinolates derived from tyrosine are also phenolic compounds.

The dietary intake of phenolic compounds has been related to beneficial health effects, especially by epidemiological studies about fruit and vegetable intake. Their activity is mainly associated with their potent antioxidant properties and inhibition of oxidative damage diseases such as cardiovascular, neurodegenerative and cancer (Björkman et al., 2011; Del Rio et al., 2013; Rashmi & Negi, 2020; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). However, the exact nature of the antioxidant or protective effects *in vivo* is not well established and only the metabolic fates of few compounds have been explored (Traka & Mithen, 2011).

The phenolic composition of *Brassica* vegetables is well established. It has been investigated for broccoli, cabbage, white cabbage, Italian kale, and even for some by-products (Cartea et al., 2011; Francisco et al., 2009; Velasco et al., 2011). In broccoli, a high number of chlorogenic acids, flavonoids (prevailing hydroxycinnamic acid esters of kaempferol and quercetin glycosides), and hydroxycinnamic acyl glycosides (with predominance of *p*-coumaric, sinapic and ferulic acids in conjugation with sugar or other hydroxycinnamic acids) have been identified (**Figure 1.2**). This kind of compounds are usually correlated with high antioxidant activity, despite the polyglycosylated flavonols. As for GLS, phenolics are also affected by cultivation, storage, and processing conditions (Cai et al., 2016; Cartea et al., 2011; Rybarczyk-Plonska et al., 2016).

Flavonols glucosides derivatives

R in position 7
- H
- β-D-Glc(1→
- Sophoroside = β-D-Glc(1→2)-β-D-Glc(1→

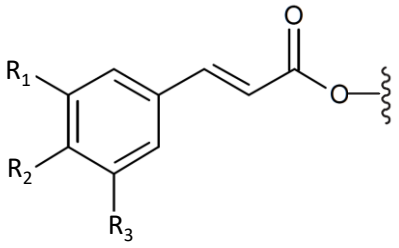


R in position 3'
- H = Kaempferol derivatives
- OH = Quercetin derivatives

R in position 3
- β-D-Glc(1→
- Sophoroside
- Sophorotrioside = β-D-Glc(1→2)-β-D-Glc(1→2)-β-D-Glc(1→
- Sophoroside or Sophorotrioside monoacylated with hydroxycinnamic acids
- Sophorotrioside diacylated with hydroxycinnamic acids

Hydroxycinnamic acids derivatives

R_1: OCH_3, **R_2**: OH, **R_3**: OCH_3 = sinapoyl group
R_1: OCH_3, **R_2**: OH, **R_3**: H = feruloyl group
R_1: OH, **R_2**: OH, **R_3**: H = caffeoyl group
R_1: OCH_3, **R_2**: OH, **R_3**: OH = methoxycaffeoyl group
R_1: H, **R_2**: OH, **R_3**: H = p-coumaroyl group



Esterified with
- Sophoroside or Sophorotrioside of kaempferol or quercetin derivatives
- Gentiobiose = β-D-Glc(1→6)-β-D-Glc(1→
- Quinic acid

Figure 1.2. Chemical structures of flavonols and hydroxycinnamic acids derivatives found in broccoli.

1.1.3. Pigments

The two main classes of vegetable pigments are the carotenoids and the chlorophylls, which are involved in the photosynthesis conferring a broad range of light-harvesting. More than 700 carotenoid pigments have been identified in foods (Traka & Mithen, 2011). These compounds have a 40-carbon isoprenoid structure, which confers a nonpolar nature and low water solubility. They can be classified as carotenes or, when hydroxyl or carbonyl are present, as xanthophylls (**Figure 1.3**). Some carotenoids are acyclic whereas others contain terminal ring structures, but all have in common a system of conjugated single and double bonds. These structural combinations give rise to differences in the absorption spectrum, with maximum wavelengths around 440–489 nm, which results in different color, from yellow to orange-red.

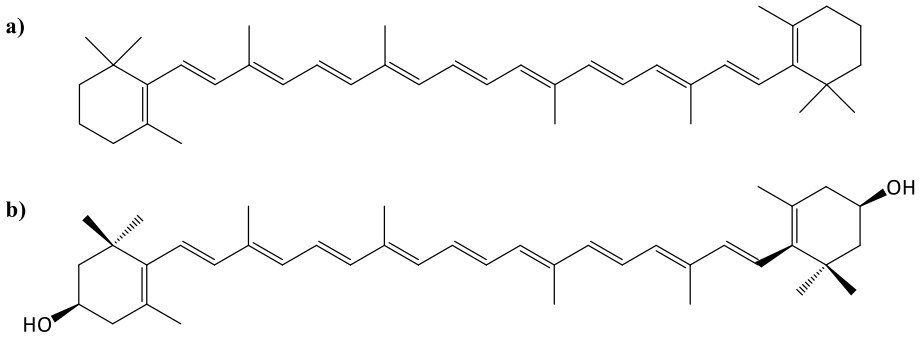


Figure 1.3. Chemical structure of the main carotenoids found in broccoli: **a)** β-carotene and **b)** lutein.

Chlorophylls have a tetrapyrrole moiety with one reduced pyrrole ring and a long chain esterified terpenoid alcohol phytol, which is esterified to a carboxylic acid functional group. In plants, the most abundant chlorophylls are the green chlorophylls *a* and *b*, both featuring a magnesium ion at the centre of the tetrapyrrole ring (**Figure 1.4**). Due to their high lability, these natural chlorophylls can be susceptible to chemical alterations along processing and plant senescence namely demetallation, saponification, and epimerization (**Figure 4.1**), resulting in compounds with different color (Ryan & Senge, 2015).

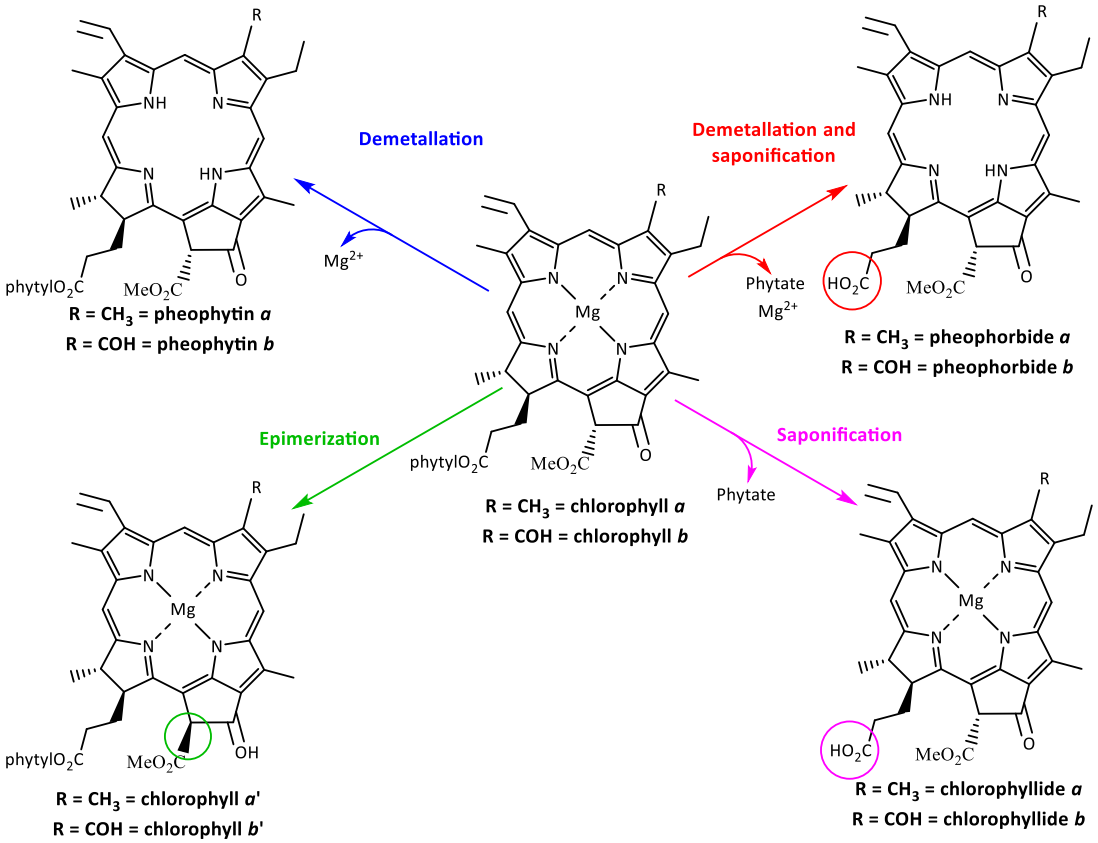


Figure 1.4. Chemical structure of chlorophylls *a* and *b* and resulting products after processing.

Carotenoid bioactivities are mainly related to the fact that they are precursors of vitamin A and should be taken in the diet, as they are not synthesised by humans. Moreover, their varying degrees of antioxidant activity may also contribute to human health (Fernández-García et al., 2012). Chlorophylls health related activities were also associated with their antioxidant activity and, more recently, to cancer prevention due to antimutagenic activity, mutagen trapping, modulation of xenobiotic metabolism, and induction of apoptosis, especially at gastrointestinal level (Ferruzzi & Blakeslee, 2007).

In general, green vegetables are a main source of carotenoids and chlorophylls. The xanthophyll lutein and the carotene β-carotene were the main carotenoids in broccoli, found in high amounts in different broccoli parts such as florets, stalks, and especially in the leaves (Guzman, Yousef, & Brown, 2012; Murcia, López-Ayerra, Martínez-Tomé, & García-Carmona, 2001). Chlorophylls *a* and *b* were also found in broccoli (Ares et al., 2013). The amount of carotenoids and chlorophylls is higher in leaves than in inflorescences and stalks. These compounds decrease along storage and processing (Cai et al., 2016). For this reason, the pigments of broccoli by-products vary greatly, reaching 10-fold differences (Neugart et al., 2018).

1.1.4. Polysaccharides

Polysaccharides are carbohydrate polymers composed of more than 10 monosaccharide units connected by glycosidic bonds. They are classified depending on their monosaccharide composition, and they are named with the suffix “an” after the name of the residue present in higher amount. For example, a polysaccharide composed by Glc residues is named glucan, and a polysaccharide composed by xylose (Xyl) but with higher amount Glc is named xyloglucan. Notwithstanding, some have common names, as starch and cellulose, two kinds of glucans. In addition to monosaccharide composition, polysaccharides complexity can increase due to different glycosidic linkages, degree of polymerization, and functional groups (McNaught, 1996; Perez & Aoki-Kinoshita, 2017).

Polysaccharides are a central component of food. Starch, composed by amylose and amylopectin, both homopolysaccharides of Glc, is ingested and, after enzymatic hydrolysis in the small intestine, Glc is absorbed to the bloodstream. Glc can then reach every cell of the organism and then be oxidized to produce energy or be stored in humans, as glycogen. However, not all ingested polysaccharides can be digested in the small intestine. This type

of compounds that are resistant to hydrolysis (digestion) by the alimentary enzymes are called dietary fibre (EFSA, 2010; EU, 2011).

Most of dietary fibre polysaccharides are part of plant cell walls, a complex organelle that plays an essential role in plant physiology and consists on cellulose (15-30%), hemicellulose (20-40%), and pectic polysaccharides (~35%) (Sticklen, 2008). Cellulose is a linear homoglucan consisting of (β1→4)-D-Glucopyranosyl (Glc*p*) residues, attached to each other at 180° orientation. In the plant cell wall, cellulose chains, of 2000-8000 Glc*p* residues each, are associated through hydrogen bonding into a tightly packed structure known as a microfibril (Waldron & Faulds, 2007). Microfibrils are generally crystalline structures but also contain some amorphous regions, in which the molecules are less compact. These structures are impermeable to water, and their high tensile strength provides strong resistance to chemical and enzymatic attack.

The plant cell wall polysaccharides with a main skeleton like cellulose (β1→4 linkages) and usually branched are called hemicelluloses. Overall, the hemicelluloses comprise glucans such as xyloglucan, xylans (including glucuronoxylan, arabinoxylan and glucuronoarabinoxylan), and mannans (including glucomannan, galactomannan, arabinogalactomannan, and galactoglucomannan) (Waldron & Faulds, 2007).

Pectic polysaccharides are complex heteropolysaccharides with a high proportion of galactopyranosyluronic acid (Gal*p*A) (Waldron & Faulds, 2007). They include various fragments of linear and ramified regions covalently connected (**Figure 1.5**). The linear region consists of units of (α1→4)-D-Gal*p*A residues (homogalacturonan region) that can carry methyl ester groups and can also be acetylated at the galacturonan backbone. A backbone of alternating (α1→4)-D-Gal*p*A and (α1→2)-L-Rhamnopyranosyl (L-Rha*p*) residues, ramified in the Rha by galactans, arabinogalactans, and arabinans of varying structure is named type I rhamnogalacturonans (Pérez, Rodríguez-Carvajal, & Doco, 2003; Vincken et al., 2003; Yapo, 2011).

Plant cell wall polysaccharides have been associated to health effects, from anticancer to immunostimulatory activity (Ramberg, Nelson, & Sinnott, 2010; Schepetkin & Quinn, 2006). Immunostimulatory polysaccharides are able to interact with several cells and humoral components of the immune system, strengthening the innate and adaptive immunity responses, either by exhibiting the effect themselves or by inducing effects via complex reaction cascades (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015).

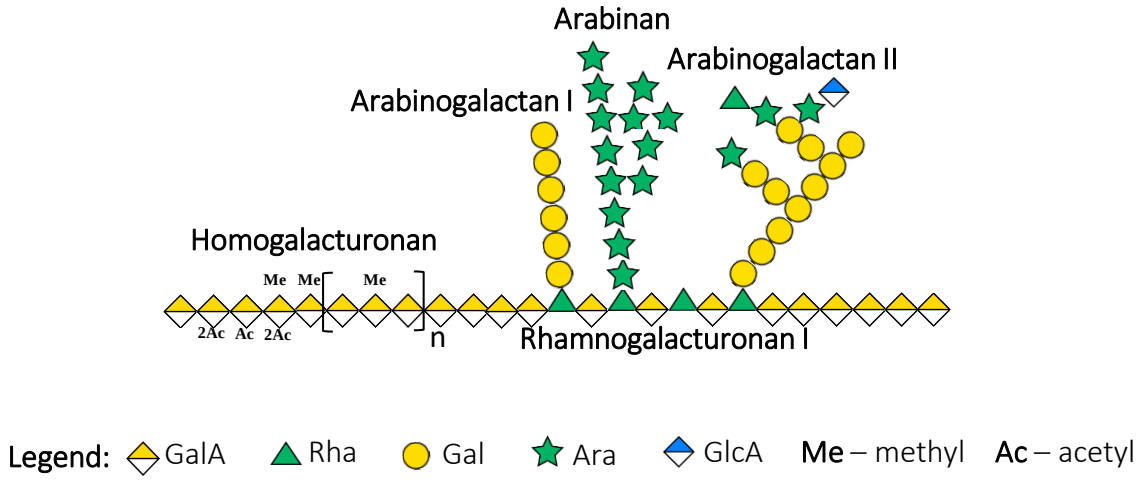


Figure 1.5. Illustration of chemical structure of pectic polysaccharides, using nomenclature proposed by Neelamegham et al. (2019).

Immunostimulatory polysaccharides may be isolated from primary plant cell walls (Leung, Liu, Koon, & Fung, 2006). Contrarily to cellulose, hemicelluloses (e.g. acetylated galactomannans (Simões et al., 2009; Simões, Nunes, Domingues, & Coimbra, 2010; Simões, Nunes, Domingues, Coimbra, & Domingues, 2012) and xylans (Zhou et al., 2010)) can show immunostimulatory activity. The immunostimulatory activity of pectic polysaccharides has been associated to the branched regions (e.g. arabinans (Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Dourado et al., 2004; Popov & Ovodov, 2013)). In fact, the removal of the linear regions of pectic polysaccharides by enzymatic treatments with endo-polygalacturonase has been shown to improve their immunostimulatory activity (Togola et al., 2008) whereas the opposite effect was observed by the removal of the branching regions with exo-α-L-arabinofuranosidase and exo-β-D-galactosidase (Nergard et al., 2005). Decrement of activity was also observed by removal of arabinofuranosyl (Ara*f*) residues with weak acid hydrolysis (Diallo, Paulsen, Liljebäck, & Michaelsen, 2001, 2003; Duan, Dong, Ding, & Fang, 2010; Inngjerdingen et al., 2007; Wang, Liu, & Fang, 2005). However, the removal of homogalacturonan after endo-polygalacturonase treatment of *Pterospartum tridentatum* (L.) Willk. inflorescence pectic polysaccharides was shown to decrease the nitric oxide production by macrophages (Martins et al., 2017), showing that different structural features of pectic polysaccharides are involved in the immune response.

Moreover, chemical modification of polysaccharides can add/increase their biological activity, e.g. sulfated cellulose has been patented with anti-inflammatory and anti-viral properties (Grassauer & Meier, 2009; Grassauer & Pretsch, 2010). The presence of sulfate groups has been closely associated with immunostimulatory activity, highlighted by an increase of activity after chemical sulfation (Wang, Zhang, Yu, & Cheung, 2009) and a decrease of this activity after desulfation (Khil'chenko et al., 2011).

Therefore, monosaccharide and glycosidic-linkage composition, molecular weight, branching characteristics, and functional groups are structural features important for immunostimulatory activity (Ferreira et al., 2015). Still, information about the relevance of each structural features when combined with each other is scarce and it should be noted that studies relating immunostimulatory activity to polysaccharides generally lack structural characterization (Schepetkin & Quinn, 2006). Hence, polysaccharide structure-function relationships are still far from being understood.

Broccoli polysaccharides found in cell wall material can account for more than 30% of dried broccoli (Bestard, Sanjuan, Rosselló, Mulet, & Femenia, 2001; Femenia et al., 2000; Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011; Monro & Mishra, 2010). The available studies about these polysaccharides mainly focus on their monosaccharide composition, but it can be inferred the presence of mainly pectic polysaccharides and cellulose (Femenia et al., 2000; Villanueva-Suárez, et al., 2003), taking into account the high amount of Glc (25-35 %mol), uronic acids (UA, 21-43 %mol), Ara (12-19 %mol), and Gal (6-12 %mol, **Table 1.1**), and vestigial amounts of Xyl, Rha, mannose (Man), and fucose (Fuc) (Bestard et al., 2001; Müller et al., 2003; Villanueva-Suárez et al., 2003; L. Xu et al., 2015). Ara can occur in arabinans, arabinogalactans, and arabinogalactan proteins associated with pectic polysaccharides, as found in cabbage (Beldman, Schols, & Pitson, 1997). At present, it is not known if pectic polysaccharides and hemicelluloses from broccoli might exert immunostimulatory activity, though this ability has been observed for polysaccharides of similar structures from other plants (Dourado et al., 2006, 2004; Ferreira et al., 2018; Popov & Ovodov, 2013; Schepetkin & Quinn, 2006; Simões, Nunes, Domingues, & Coimbra, 2011).

Table 1.1. Monosaccharide composition of cell wall polysaccharides from broccoli.

Broccoli organ type	CW isolation and fractionation		Sugar composition (%mol)								Total Sugars (mg/g)[*1]	Ref
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
Inflorescences	Amylases and protease		3	2	18	8	3	11	35	21	218	a
Inflorescences	AIR		1	1	14	4	2	10	25	43	563	b
Rehydrated Inflorescences	AIR	η (%)	3	1	17	5	2	10	29	33	469	c
	Water	11.2	1	1	39	1	3	21	7	27	221	c
	CDTA	14.4	4	0	16	2	0	7	2	70	690	c
	Na_2CO_3	22.1	6	0	37	1	0	9	3	43	598	c
	KOH	21.8	1	1	18	24	2	11	28	14	410	c
	Residue	15.6	5	0	13	2	1	8	69	3	768	c
Rehydrated Stalks	AIR		2	1	19	5	3	12	29	30	546	c
Stalks	Secondary xylem		2	1	15	7	3	6	28	39	868	d
Stalks	Parenchyma		2	1	12	7	3	7	30	38	764	d

CW: cell wall; AIR: alcohol insoluble residue; CDTA: cyclohexane diamine tetraacetic acid; η: yield; *[1] mg of sugars per g of sample dry matter. Ref.: a. Villanueva-Suárez, et al., (2003); b. Bestard, et al. (2001); c. Femenia, et al., (2000); d. Müller, et al. (2003).

1.2. Dehydration of broccoli by-products

Given the composition of broccoli by-products and potential beneficial effects, they can be used in the development of several food ingredients or products, namely soups (Alvarez-Jubete et al., 2014), fermented products (Bekhit, Lingming, Mason, Zhou, & Sedcole, 2013), flours (Campas-Baypoli et al., 2009), and enriched tea (Dominguez-Perles, Moreno, Carvajal, & Garcia-Viguera, 2011) and pasta (Oliviero & Fogliano, 2016). However, due to their high moisture content, techniques to extend postharvest life are required. Broccoli by-products can be blanched to reduce deterioration (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2008; Gonçalves et al., 2009; Morales-Blancas, Chandia, & Cisneros-Zevallos, 2002), but without a frozen chain (-20 ºC), blanching does not prevent broccoli microbial growth and perishability. The stabilization of broccoli by-products by dehydration, decreasing the moisture content, overcome these drawbacks, allowing longer storage periods and reducing shipping weights (Doymaz, 2014).

For high quality products, freeze-drying is the dehydration technique of choice because it combines low temperature and pressure to sublimate water (Duan, 2017), preserving cell structure when fast freezing methods are employed (Jha, Xanthakis, Chevallier, Jury, & Le-Bail, 2019). Still, due to the high energy demand of freeze-drying

(Duan, 2017), air-drying has been the most applied technique for drying broccoli (Doymaz, 2014; Femenia et al., 2000; Jin, Sman, & van Boxtel, 2011; Karaaslan, 2016; Mrkic, Redovnikovic, Jolic, Delonga, & Dragovic-Uzelac, 2010; Oliviero, Verkerk, & Dekker, 2013; Salim, Gariépy, & Raghavan, 2017; Xu, Jin, Zhang, & Chen, 2017). Nevertheless, regardless its convenience, depending on the temperature (20ºC to 100ºC), air-drying may promote the degradation of some broccoli bioactive compounds, namely indole glucosinolates (Mrkic, Redovnikovic, Jolic, Delonga, & Dragovic-Uzelac, 2010), ascorbic acid (Jin et al., 2011; Mrkic, Cocci, Rosa, & Sacchetti, 2006), and pigments (Zhang et al., 2018). Reduction of dehydration temperature and/or time were shown to partially preserve the contents of phenolic compounds and antioxidant activity of broccoli. These conditions can be achieved by increasing air flow-rate (Mrkic et al., 2006), by combining air-drying with zeolites to absorb the released water (Oliviero et al., 2013), and air-drying with microwaves (Salim et al., 2017; Vadivambal & Jayas, 2007). Microwave assisted drying uses microwave energy to heat the water present in the plant material without the need to heat the surroundings of the sample, providing time and energy savings (Guo, Sun, Cheng, & Han, 2017). The purpose of this methodology has been to achieve the reduction of moisture of the products by water vaporization, promoting simultaneously a blanching effect (Vadivambal & Jayas, 2007). Using the same principle, but avoiding water vaporization, a recent technology, the microwave hydrodiffusion and gravity (MHG) enabled the simultaneous drying and collection of an extract by hydrodiffusion of the internal water of the samples by gravity (Cendres, Chemat, Maingonnat, & Renard, 2011; Périno, Pierson, Ruiz, Cravotto, & Chemat, 2016). This technique has already been used for extraction of broccoli by-products for development of hydrogels with antioxidant activity (López-Hortas, Conde, Falqué, Domínguez, & Torres, 2019).

The negative impact of dehydration on broccoli active compounds can also be minimized by a blanching pre-treatment, which is reported to improve the drying rate (Doymaz & Sahin, 2016), and inactivate lipoxygenase (Morales-Blancas et al., 2002), myrosinase (Oliviero, Verkerk, & Dekker, 2013; Rungapamestry et al., 2008), and peroxidase (Gonçalves et al., 2009), i.e, the enzymes related with the deterioration of carotenoids, glucosinolates, and phenolic compounds, respectively.

1.3. Hypothesis, aims, and strategy of the work

It is hypothesized that broccoli by-products are a source of compounds that can be valorised as food ingredients with bioactive compounds and as biobased materials for food packaging.

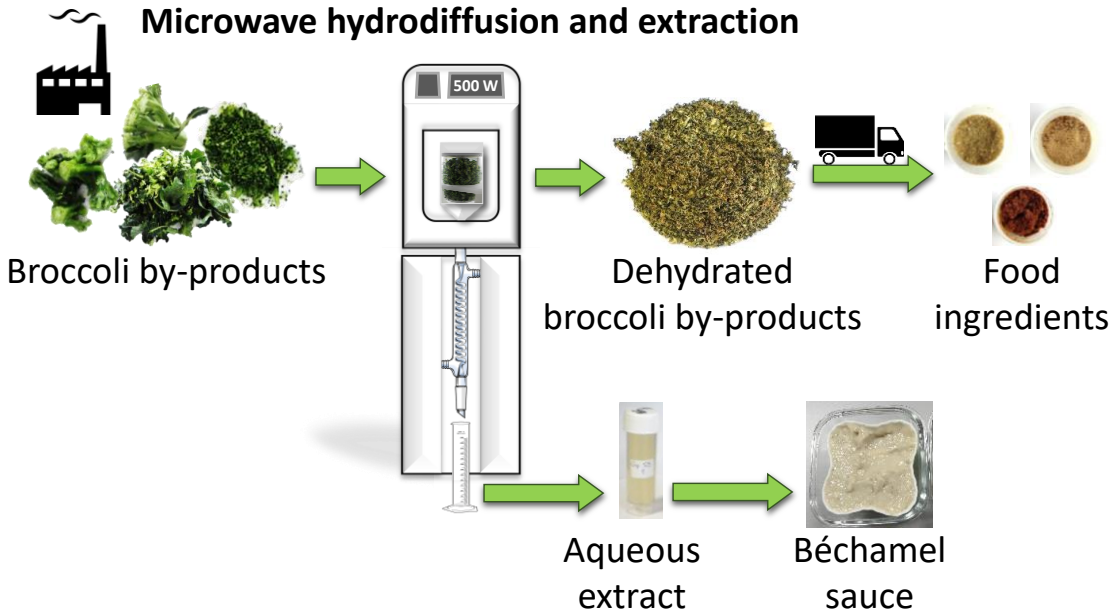
As broccoli by-products high amount of water (around 90%) limits its valorisation, the first objective to fulfil the hypothesis was to study a recent technology, MHG, allowing for dehydration and simultaneously recovery of diffused water with bioactive compounds, stabilizing the broccoli by-products. The products recovered by MHG were characterized and used to develop ingredients for innovative food products (**Chapter 2**). Freeze-drying and air-drying were also used to evaluate the impact of other dehydration processes, with an early blanching step, on broccoli by-products pigments, glucosinolates, and phenolic compounds (**Chapter 3**). As there is scarce information about broccoli polysaccharides bioactivities, pectic polysaccharides were extracted, fractionated, and characterized to stablish structure-function relationships of pectic polysaccharides *in vitro* B lymphocytes activation (**Chapter 4**).

Taking advantage of the opportunity to contribute to reducing the environmental issues of petroleum-based plastics, by developing renewable and biodegradable starch-based materials with improved features, broccoli by-products were used to assess their impact on surface hydrophilicity and mechanical properties of starch films (**Chapter 5**).

CHAPTER 2

Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds

Graphical abstract:



CHAPTER 2

Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds

Parts of the text of this chapter were published in the following publications:

Sónia S. Ferreira, Cláudia P. Passos, Susana M. Cardoso, Dulcineia F. Wessel, Manuel A. Coimbra (**2018**). Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds. *Food Chemistry*, 246, 386-393. IF 5.399.

Sónia S. Ferreira, Filipa Monteiro, Cláudia P. Passos, Artur M. S. Silva, Dulcineia Ferreira Wessel, Manuel A. Coimbra, Susana M. Cardoso (**2020**). Blanching impact on pigments, glucosinolates and phenolics of dehydrated broccoli by-products. *Food Research International*, 132, 109055. IF 4.972.

2.1. Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds - approach

Broccoli by-products from frozen-food industry account for 45% of the initial broccoli heads. They consist on stalks, inflorescences, and leaves, blanched and non-blanched, sharing the nutritional value and bioactive compounds of commercial broccoli heads. However, their high perishability prevents further valorisation. Therefore, in this study microwave hydrodiffusion and gravity (MHG) technology was used to dehydrate broccoli by-products and simultaneously recover the water-soluble diffused compounds for food ingredients use. In order to evaluate the feasibility of MHG for dehydration and simultaneous extraction of compounds from broccoli by-products, in the present study several broccoli by-products from the frozen food industry were placed in the MHG reactor, without addition of any solvent or water. Moisture, protein and sugar contents of broccoli by-products and carbohydrate extraction were evaluated before and after the experiments. The extracts collected during MHG experiments were characterized for total phenolic compounds, sugars, free amino acids and glucosinolates, and used as food ingredient.

2.2. Materials and methods

2.2.1. Broccoli samples and chemicals

Broccoli (*Brassica oleracea* var. Parthenon) by-products from the frozen food industry were provided by Monliz SA, Alpiarça, Portugal, in January 2015. The six initial by-products (iBB) were obtained and numbered according to the order of flow in frozen broccoli production line (**Figure 2.1.a**): 1. large stalks (iBB 1); 2. and 3. mixtures of small inflorescences and leaves (iBB 2 and iBB 3); 4. blanched inflorescences remains (iBB 4); 5. blanched small inflorescences (iBB 5); and 6. blanched stalks, smaller than iBB 1 (iBB 6). Broccoli by-products recovered in the cutting and cooler rooms and during calibration accounted for 45% of broccoli head (Monliz, 2013). The iBB samples were frozen and maintained under -20 ºC until use.

All reagents used were of analytical grade or higher available purity.

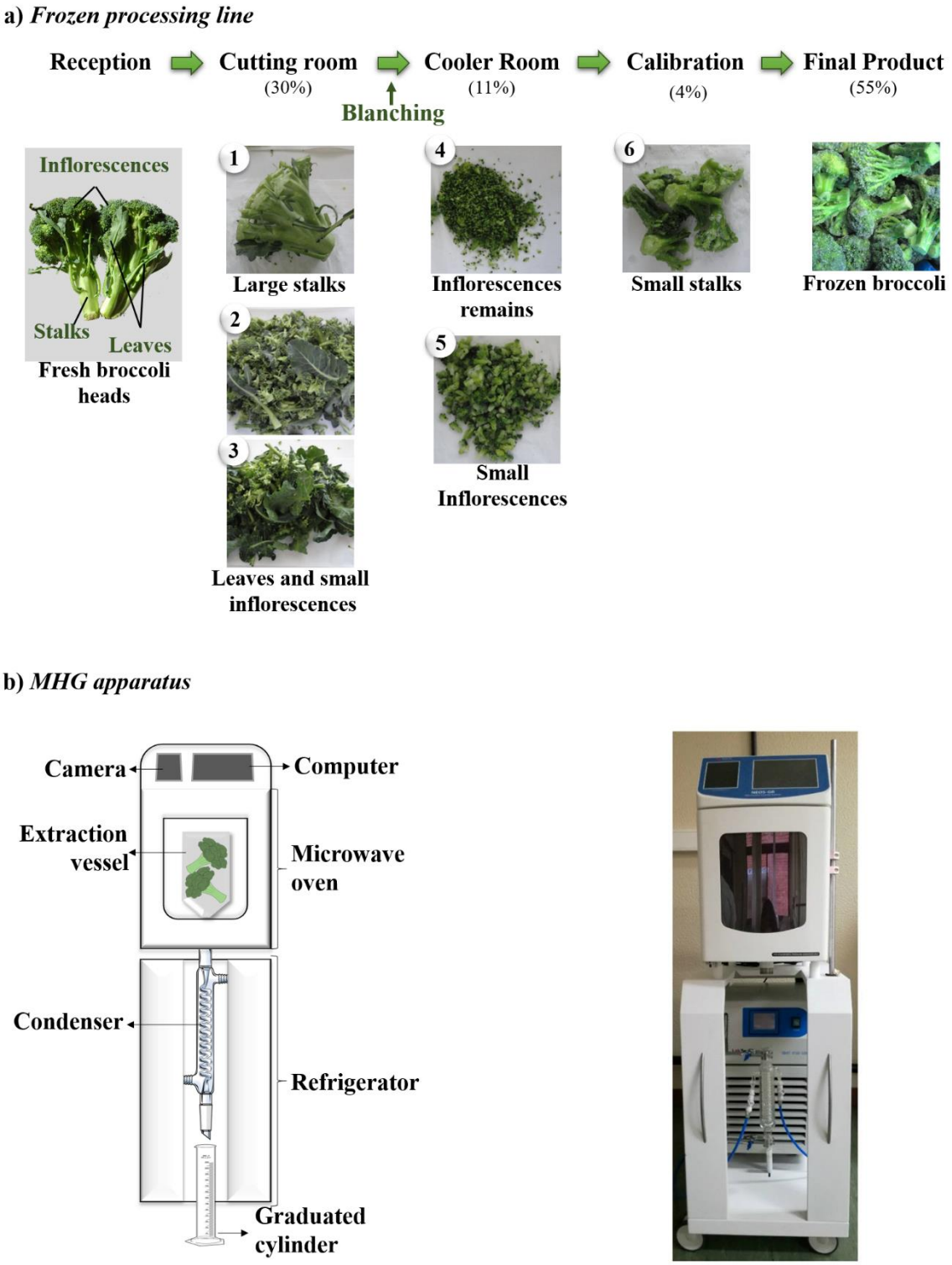


Figure 2.1. Representation of **a)** frozen processing line of broccoli fresh heads and resulting products and by-products used in this study, **b)** scheme and photography of MHG apparatus.

2.2.2. Microwave hydrodiffusion and gravity assisted extraction and dehydration

A NEOS-GR apparatus (**Figure 2.1.b**), from Milestone Srl, Italy, was used for MHG assisted extraction and dehydration of broccoli by-products. Samples (550 g, fresh weight) were placed in the Pyrex extraction vessel (1 L capacity) and irradiated with 500 W under atmospheric pressure. The extraction vessel was rotated from time to time (each 5 min, approximately) to prevent the burning out of the plant material. The condensed water solutes were continuously collected in 50 mL fractions. The procedure was stopped when the flow of the water diffusion by gravity through the bottom of the MHG device decreased, avoiding reaching the burning point. The temperature was monitored during the experiments by optic fibres sensors, and time and temperature were registered for each collected fraction. The aqueous extracts were frozen at -20 ºC until analysis. The dry weight material was determined after freeze-drying aliquots of each sample. The dehydrated broccoli by-products (BB_MHG) obtained after the microwave treatment were also frozen, freeze-dried, and crushed into powder for storage, giving origin to samples BB_MHG 1 to 6.

2.2.3. Broccoli by-products hot water extraction

For comparison of the extraction yield obtained by MHG and the conventional hot-water extraction, sample iBB 6 (blanched stalks, 55 g) was boiled in 200 mL of water during 20 min. The mixture was cooled in a cold-water bath, filtered, and freeze-dried.

2.2.4. Dehydrated broccoli by-products extraction and fractionation

A mix of 39.5 g dehydrated broccoli by-products (equal parts of BB_MHG 1 to 6) was hydrated with water (1 L) during 5 h under agitation, using a solid liquid ratio of 1:25, and then boiled for 1 h and cooled at 4 ºC. The material was filtered with a nylon cloth and then with a fritted funnel (N1). The hot water extraction was repeated for the residue left five more times to obtain a total of six aqueous extracts (1^{st}-6^{th} BB_MHG) and a final residue (BB_Residue). For comparison, the same sequential extractions were performed on a mix of freeze-dried initial broccoli by-products, giving fractions (1^{st}-6^{th} iBB) and a final residue (iBB_Residue).

Additionally, the water-soluble material obtained from the 1st extraction of BB_MHG was further purified by ethanol precipitation. The addition of 50% absolute ethanol, kept for 16 h at 4 ºC before centrifugation (24,652 g, 10 min, 4 ºC), allowed to obtain the 50% ethanol soluble material, which was separated from the precipitate (Et50_BB_MHG). Absolute ethanol was then added to the solution containing the material soluble in 50% ethanol until reaching a concentration of 80% ethanol. After centrifugation, the precipitate (Et80_BB_MHG) was separated from the supernatant (EtSn_BB_MHG). The three fractions obtained by ethanol fractionation were evaporated under reduced pressure and freeze-dried for yield determination and carbohydrate analysis.

2.2.5. Broccoli by-products chemical characterization

2.2.5.1. Preparation of alcohol insoluble residue (AIR)

To quantify the amount of cell wall polysaccharides in iBB 6 and BB_MHG 6, the AIR were prepared. Ethanol enrich the insoluble residues in cell wall polysaccharides by removing low molecular weight compounds (Knee, 1973). Briefly, 115 mg of material was hydrated with 1 mL of distilled water for 30 min. Afterwards, ethanol was added to the hydrated samples, in order to obtain a final concentration of 85% (v/v). After boiling for 10 min, the mixture was cooled in a cold-water bath and filtered. The filtration residue was dispersed again in 80% (v/v) ethanol, boiled for 10 min, filtered, washed with ethanol and acetone, and allowed to dry at room temperature. The AIR was characterized for their neutral sugars and UA contents.

2.2.5.2. Carbohydrates analyses

Samples were analysed for their sugar content after acid hydrolysis, derivatisation to alditol acetates, and analysis of individual neutral sugars by gas chromatography with flame ionization detector (GC-FID) and quantification of UA by the 3-phenylphenol colorimetric method, as described below. The total sugars were determined by the sum of the amount of the individual sugars. Free sugars were analysed after reduction, derivatisation to alditol acetates, and analysis of individual neutral sugars by GC-FID. Fructose (Fru) was quantified as a ratio of mannitol and sorbitol (Brunton, Gormley, & Murray, 2007). The total polymeric sugars were determined by the difference between total sugars and free sugars.

Hydrolysis with 1M sulfuric acid

To carry out hydrolysis 2-3 mg of each sample was weighted in 10 mL tubes, and 200 µL of 72% H_2SO_4 were added (Bastos, Coelho, & Coimbra, 2015; Selvendran, March, & Ring, 1979). After incubation for 3 h at room temperature with occasional stirring, 2.2 mL of distilled water was added to reach 1 M and incubated for 1 h at 100 ºC. The tubes were cooled down in a cold-water bath, and 0.5 mL were removed for later UA analysis. The hydrolysis continued for another 1.5 h at 100ºC.

Reduction and acetylation

After adding 200 µL of internal standard (2-deoxy-Glc 1 g/L), 0.5 mL of sample were transferred to other tube and neutralized with 200 µL 25% NH_3. The reduction was performed adding 100 µL of 15% (m/v) $NaBH_4$ in 3 M NH_3 to samples and incubating for 1 h at 30 °C. After cooling down the tubes in a cold-water bath and adding 50 µL of acetic acid twice, 300 µL of sample were transferred to sovirel tubes. The acetylation was performed by adding 450 µL of 1-methylimidazole and 3 mL of acetic anhydride (Blakeney, Harris, Henry, & Stone, 1983). After mixing on vortex, samples were incubated for 30 min at 30 °C. The resulting alditol acetates were extracted to an organic phase by adding 3 mL of distilled water and 2.5 mL of dichloromethane, followed by vigorous stirring, and separation by centrifugation (30 s, 1400 g). Afterwards, the aqueous phase was removed by suction with vacuum. Addition of distilled water and dichloromethane, stirring, centrifuging, and removing the aqueous phase were repeated. Later the organic phase was washed twice with 3 mL of distilled water, mixed, and centrifuged, after which the aqueous phase was completely removed. The organic phase was transferred to specific speedvac tubes and the dichloromethane was evaporated. Afterwards, 1 mL of anhydrous acetone was added and evaporated, twice.

GC-FID analysis

The alditol acetates were dissolved in 10-50 µL of anhydrous acetone and analysed by GC-FID (Perkin Elmer–Clarus 400). The GC was equipped with a 30 m column DB-225 (J&W Scientific, Fol-som, CA, USA) with i.d. and film thickness of 0.25 mm and 0.15 µm, respectively. The oven temperature program used was initial temperature 220 ºC, standing for 7 min and a rise in temperature at a rate of 5 ºC/min until 240 ºC. The injector and detector

temperatures were, respectively, 220 and 240 ºC. The flow rate of the carrier gas (H_2) was set at 1.7 mL/min.

Uronic acid analysis

Uronic acids (UA) were determined by the *m*-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973), using GalA (20 to 160 µg/mL) as standard. To 0.5 mL of diluted hydrolysed sample (1:4 or 1:5) were added 3 mL of 200 mM boric acid in 96%(w/w) H_2SO_4. After shaken, the test tubes were heated at 100 ºC, during 10 min. After cooling, 100 µL of m-phenylphenol were added, the test tubes were shaken and let to react 20 min in dark, and the absorbance was read at 520 nm.

2.2.5.3. Protein quantification

Protein was quantified by the nitrogen content (N) determined by elemental analysis using Truspec 630-200-200 elemental analyser. Standard nitrogen-to-protein conversion factor of 6.25 was used (Campas-Baypoli et al., 2009).

2.2.5.4. Statistical analysis

Differences between iBB and BB_MHG were analysed through unpaired Student's t-test with GraphPad Prism 7.02 (OriginLab Corporation, Northampton, MA, trial version). P values of less than 10% and 5% ($p < 0.1$ and $p < 0.05$, respectively), were considered to be significant.

2.2.6. MHG extracts chemical characterization

2.2.6.1. Determination of the total phenolic compounds

Total phenolic compounds (TPC) of fractions 1 to 4 (first 200 mL hydrodiffused) were estimated using the Folin-Ciocalteu method (Ferreira et al., 2002). Briefly, a mixture of 500 µL of distilled water, 125 µL of Folin-Ciocalteu reagent and 125 µL of each fraction or standard was prepared. After 5 min in the dark, 1.25 mL of 75 g/L Na_2CO_3 was added and the mixture was homogenized and incubated for 1h at 25 °C. The absorbance was measured at 760 nm and total phenolic compounds were expressed as gallic acid equivalents, using a calibration curve of gallic acid as standard (0.2–19.6 µg/ml gallic acid in the tube). The data

were expressed as mean ± standard error (SD) of three independent assays performed in different days.

2.2.6.2. Phenolic compounds and glucosinolates characterization

Phenolic compounds and intact glucosinolates present in the first collected extract from iBB 6 (fraction 1) were simultaneously analysed by ultra-high-performance liquid chromatography (UHPLC), using a photodiode-array detector (DAD) coupled with electrospray ionization/tandem mass spectrometry detection (ESI/MS^n). Phenolic compounds were identified at 320 nm using DAD and MS data. Glucosinolates were identified using MS data and quantified using sinigrin (Sigma-aldrich, Germany) as standard at 227 nm and response factors of 1 for aliphatic glucosinolates and of 0.25 for indole glucosinolates according Buchner, R. (1987), as described by Dominguez-Perles et al. (2011). Briefly, the extract was filtered through a 0.2 µm filter disk (cellulose acetate, 30 mm syringe filters, Whatman, UK) and injected into the UHPLC-DAD-ESI/MS^n system. UHPLC system consisted on an Ultimate 3000 (Dionex Co., USA) apparatus equipped with a quaternary pump, an autosampler, an ultimate 3000 Diode Array Detector (Dionex Co., USA), and an automatic thermostatic column compartment (30ºC).

The compounds were separated in a Hypersil GOLD C18 column (100 mm length; 2.1 mm i.d.; 1.9 µm particle diameter, end-capped from Thermo Scientific, USA) and its temperature was maintained at 30 ºC. The mobile phase used for separation was a mixture of (A) formic acid in water (0.5%, v/v) and (B) formic acid in acetonitrile (0.5%, v/v). The flow rate was 0.10 mL/min in a linear gradient, for 6 min, with 1% B, reaching 0.15 mL/min gradient and 17% B at 12.5 min. The flow rate was 0.15 mL/min in a linear gradient until the end of the separation. The eluent B was maintained for 1.67 min, before reaching 25% at 18.34 min, 35% at 25.01 min, 50% at 29.18, and 99% at 33.35 min. UV-Vis spectral data for all peaks were accumulated in the range 190–700 nm, while the chromatographic profiles were recorded at 227 nm for glucosinolates and 280 and 320 nm for phenolic compounds.

The analyses were performed by using a mass spectrometer 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 simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units.

2.2.6.3. Carbohydrates and alditol analyses

Fractions 1 to 4 (first 200 mL hydrodiffused) were analysed for their carbohydrate content as described in **Section 2.2.5.2**. Free alditols were determined after derivatisation to alditol acetates and analysis by GC-FID.

2.2.6.4. Identification and determination of free amino acids

Free amino acids present in the first collected extracts (fraction 1) were analysed according to the method described by Ribeiro et al. (2015). Briefly, to 600 µL of extract were added 400 µL of anhydrous ethanol, 100 µL of the internal standard, L-norleucine (0.1 mg/mL), 100 µL of pyridine, and 50 µL of ethyl chloroformate. The mixture was sonicated for 60 s at room temperature (25 °C) in an ultrasonic bath (Ultrasons-H, Selecta). Subsequently, 300 µL of chloroform were added followed by addition of 100 µL of 7 mol/L NaOH and 50 µL of ethyl chloroformate. The layers formed were mixed by vortex for 30 s and centrifuged for 3 min at 1400 g. The aqueous layer was removed, while the remaining chloroform layer containing derivatives were dried with 100 mg of anhydrous sodium sulfate. After derivatisation with ethyl chloroformate, amino acids were analysed by gas chromatography quadrupole mass spectrometry (GCqMS) (GC-2010 Plus, Shimadzu, Japan) using a non-polar column DB1 (30 m length, 0.25 mm internal diameter and 0.10 µm stationary phase, Agilent, USA), by injection of 1 µL in split mode (split ratio 2.0) with an injection temperature of 260 ºC. The initial column temperature was 70 ºC, and the temperature increased to 260 ºC at 10 ºC/min and then to 300 ºC at a rate of 20 ºC/min. The carrier gas (helium) was maintained at a constant flow rate of 1.51 mL/min. The transfer line temperature was 300 ºC and the temperature of the ionization source was 250 ºC. Mass spectra were acquired in the full-scan mode (50-700 m/z) after ionization by electron impact with 70 eV.

2.2.7. Food applications of MHG extracts

A béchamel sauce was prepared with the combined MHG extracts from broccoli by-products 1 to 6 in order to evaluate their potential as food ingredients. Butter with salt (20 g, Mimosa, Lactogal, Portugal) and wheat flour T55 without ferment (10 g, Auchan Portugal) were continuously mixed in the stove under low temperature until the mixture was homogeneous and started to thicken. Then, milk powder (20 g, Molico, Nestlé Portugal) dissolved in 180 mL of tap water (control sauce) or MHG extract were slowly added to the first mixture. When the final mix thickened, it was flavoured with ground black pepper (1 g, Auchan Portugal) and marine refined salt (1 g, Auchan Portugal). The mixture was kept under 4 ºC until consumption.

2.3. Results and discussion

2.3.1. Broccoli by-products characterization

The six initial by-products (iBB) used (iBB 1: large stalks; iBB 2 and iBB 3: mixtures of small inflorescences and leaves; iBB 4: blanched inflorescences remains; iBB 5: blanched small inflorescences; and iBB 6: blanched stalks, smaller than iBB 1, **Figure 2.1.a**) were characterized for their moisture, total protein, and sugars content. The moisture ranged from 87 to 91% per product weight (**Figure 2.2**), showing similar values despite their origin (stalks, leaves, and/or inflorescences remains) and size, obtained before or after blanching step in the frozen processing line (**Figure 2.1.a**). These values are comparable with literature (Campas-Baypoli et al., 2009) and are associated with the high perishability of broccoli.

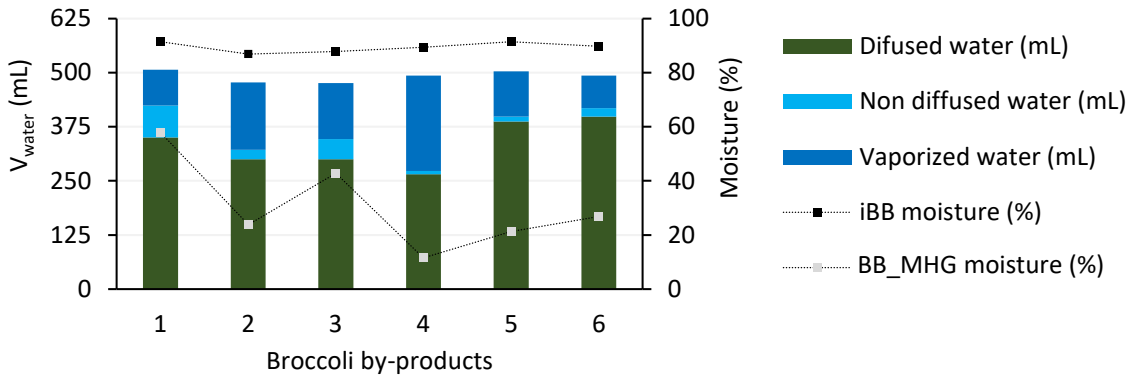


Figure 2.2. Estimated water volume for each broccoli by-product as the sum of diffused water, non diffused water, and vaporized water. Moistures of initial (iBB) and dried broccoli by-products (BB_MHG) are represented in the secondary axis.

The samples with higher content in protein were those rich in inflorescences and leaves (15-17% dry weight, dw), whereas the two stalk-rich samples (iBB 1 and iBB 6) only accounted for 6-11% dw (**Table 2.1**), which is in accordance with literature (Campas-Baypoli et al., 2009). On the contrary, stalks were richer in free sugars, Fru and Glc, accounting for 12-15% dw, whereas the samples rich in inflorescences and leaves had only 3-7% (**Table 2.1**), in accordance with literature (Bhandari & Kwak, 2015). The polysaccharides of all samples ranged from 28 to 44% dw. The main polymeric sugars were UA (49.2%), Glc (36.0%), Ara (6.2%) and Gal (4.7%), with lower amounts of Man, Xyl, Fuc and Rha (**Table 2.1**). This sugars pattern is characteristic of the presence of pectic polysaccharides and cellulose as major components, with low amounts of hemicelluloses, as already reported for *Brassica* cell walls (Femenia, Bestard, Sanjuan, Rosselló, & Mulet, 2000; Femenia, Waldron, Robertson, & Selvendran, 1999).

Table 2.1. Sugar composition, polymeric sugars, free sugars, and protein of initial (iBB) and dried (BB_MHG) broccoli by-products. Some results are expressed as mean ± standard deviation. Values of polymeric sugars, free sugars, and protein of BB_MHG with a sign are significantly different from iBB after a student's t-test: * $p < 0.1$, ** $p < 0.05$.

Samples	Sugar Composition (%mol)										Polymeric sugars (mg/g)	Free sugars (mg/g)	Protein (mg/g)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Glc Free	Fru Free			
iBB 1	[tr]	[tr]	4	2	1	3	29	35	16	10	349 ± 17	119 ± 5	106 ± 10
BB_MHG 1	1	[tr]	3	2	3	5	33	38	10	6	391 ± 27	**72 ± 15	92 ± 12
iBB 2	1	[tr]	4	1	2	4	27	50	7	4	275 ± 18	34 ± 4	158 ± 19
BB_MHG 2	1	[tr]	6	2	3	4	29	45	7	5	286 ± 8	37 ± 2	159 ± 16
iBB 3	1	[tr]	7	2	2	4	26	47	5	6	298 ± 10	37 ± 1	172 ± 12
BB_MHG 3	1	[tr]	5	2	2	6	32	42	6	3	344 ± 31	*32 ± 1	172 ± 11
iBB 4	[tr]	[tr]	8	2	2	5	32	40	7	3	370 ± 25	44 ± 3	164 ± 18
BB_MHG 4	1	[tr]	6	2	2	7	32	41	7	2	313 ± 15	**31 ± 1	162 ± 8
iBB 5	1	[tr]	5	1	1	4	31	41	12	5	317 ± 41	65 ± 2	147 ± 15
BB_MHG 5	[tr]	[tr]	4	2	2	6	33	40	8	5	282 ± 1	*42 ± 9	147 ± 8
iBB 6	[tr]	[tr]	3	1	1	3	32	34	17	9	435 ± 13	149 ± 17	63 ± 2
BB_MHG 6	[tr]	[tr]	4	1	1	4	30	37	14	8	**397 ± 1	*111 ± 7	70 ± 6

[tr] traces.

2.3.2. Microwave hydrodiffusion and gravity experiments

In order to evaluate the feasibility of MHG for dehydration and simultaneous extraction, the broccoli by-products were irradiated directly using the MHG reactor without addition of any solvent or water (**Figure 2.3.a**).

Under the same MHG conditions (37 to 43 min), the broccoli by-products that presented the lowest and the highest moisture content were samples iBB 4 (blanched inflorescences remains, 12%) and iBB 1 (non blanched larger stalks, 58%), respectively (**Figure 2.2**). The different moistures observed between samples were probably the effect of higher water diffusion in the blanched samples as compared to non-blanched ones (Doymaz, 2014) and the different size of broccoli (Jin, Sman, & van Boxtel, 2011) as well as different tissue structures (Xu et al., 2017). After MHG experiments, the volume of the broccoli by-products in the extraction vessel was reduced to about one-third (**Figure 2.3.a**). This shrinkage was also observed by (Salim, Gariépy, & Raghavan, 2017) when combining microwave and hot air-drying techniques. These MHG dehydrated broccoli by-products (BB_MHG) were then freeze-dried and grinded, resulting in powders with a browner colour than the green iBB (**Figure 2.3.b**). To evaluate the impact of the dehydration by MHG on broccoli by-products and water extracts, the dehydrated samples (BB_MHG) and the water-soluble compounds that diffused by gravity during this process were characterized.

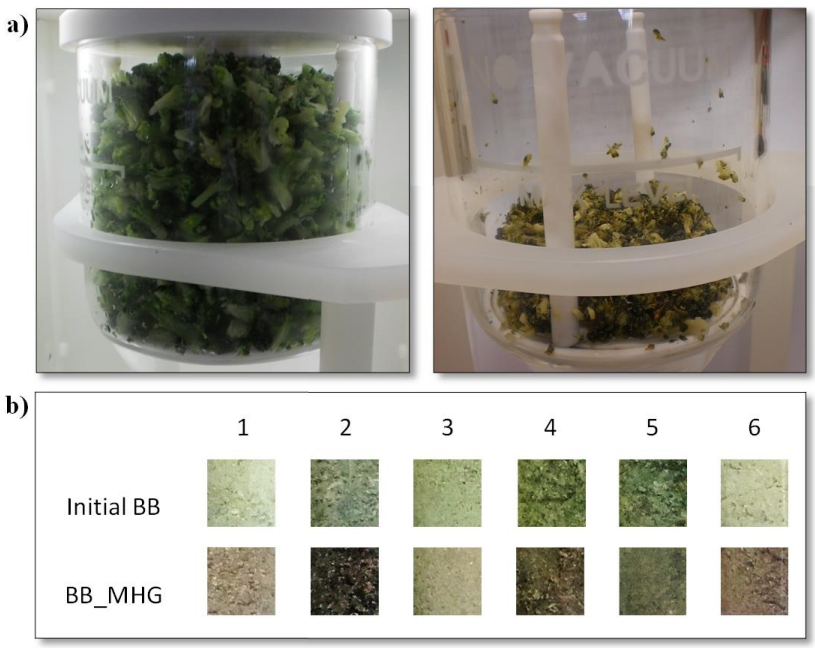


Figure 2.3. Detail of **a)** extraction vessel with broccoli by-products before and after MHG experiment and of **b)** colours of iBB and BB_MHG materials.

2.3.2.1. Protein and carbohydrate content of dehydrated broccoli by-products

Overall, the protein content and the polymeric sugars of BB_MHG samples were not significantly different ($p > 0.05$) from iBB ones (**Table 2.1**), suggesting the general preservation of protein and polysaccharides after MHG dehydration. In contrast, significant differences ($p < 0.1$) in total free sugars were observed after MHG experiments (**Table 2.1**), showing a loss of up to 39% of free sugars in MHG dehydrated broccoli by-products, possibly by hydrodiffusion. To evaluate the impact of MHG on further extraction of carbohydrates, BB_MHG and iBB were submitted to sequential hot water extractions and their carbohydrate composition was compared.

2.3.2.2. Impact of MHG on aqueous extraction of carbohydrates from dehydrated broccoli by-products

The hot water extractions allowed to recover 56% of BB_MHG dw, from which 72% were recovered in the first extraction (1^{st} BB_MHG). The total soluble solids in iBB extractions was similar to that of BB_MHG whereas the total sugar content was higher in 1^{st} BB_MHG (37%) than in 1^{st} iBB (27%) (**Table 2.2**) but with a similar sugar composition, showing that the MHG drying process promoted a higher carbohydrate extraction. Glc (49 and 54 mol%, respectively) may exist in free form and may also arise from starch. UA (27 and 23 mol%), Ara (9 and 7 mol%), Gal (5 mol%), and the vestigial amounts of Xyl, Rha, and Fuc may be components of pectic polysaccharides, whereas Man (9 mol%) may derive from Fru or from mannitol.

To separate the free sugars from polysaccharides, the 1^{st} BB_MHG extract was submitted to an ethanol fractionation, allowing to obtain two fractions that precipitate with 50% (Et50_BB_MHG) and 80% (Et80_BB_MHG) ethanol, accounting for 18% and 15% dw, respectively. Their sugar composition show enrichment in total sugars, UA, Ara and Gal, which are characteristics of the presence of pectic polysaccharides. The small amount of Glc may be due to the presence of starch. The carbohydrates present in the ethanol soluble fraction (EtSn_BB_MHG) were mainly Fru (46 mol%) and Glc (29 mol%) in free form.

The sequential extractions of both BB_MHG and iBB with hot water (2^{nd}-6^{th}) allowed to obtain extracts rich in pectic polysaccharides (41% to 61% total sugars per extract). The sum of all hot water fractions showed a total of 60% of iBB pectic polysaccharides extracted, whereas the application of MHG dehydration allowed an increase

up to 76% of pectic polysaccharides. These results show that MHG had a pre-treatment function on broccoli by-products. The remaining pectic polysaccharides were still retained in the unextracted residues, together with cellulose. Therefore, MHG has the potential to increase pectic polysaccharides extraction with hot water from the dehydrate broccoli by-products, while promoting by-products preservation and easy transport, as well as recovering the solutes washed out with condensed water by gravity. In order to evaluate the composition of the washed out solutes, the extracts obtained by MHG from broccoli by-products were collected and analysed.

Table 2.2. Sugar composition of dehydrated broccoli by-products mixture (equal parts of BB_MHG 1 to 6 and iBB 1 to 6) and samples from hot water extraction and ethanol precipitation. Total sugars are expressed as mean ± standard deviation.

Samples	Yield (%)	Sugar composition (mol%)									Total sugars (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Fru	
BB_MHG		1	1	15	4	4	9	40	26	[e]	375 ± 28
1st BB_MHG	41 [a]	0	0	9	0	9	5	49	27	[e]	374
Et50_BB_MHG	18 [b]	1	0	13	1	2	8	20	56	[e]	512
Et80_BB_MHG	15 [b]	1	0	16	0	2	13	10	56	[e]	587
EtSn_BB_MHG	67 [b]	0	0	6	0	0	2	34 (29)[d]	12	46	315 (234)[d]
2nd BB_MHG	7 [a]	1	0	28	1	3	12	22	32	[e]	460
3rd BB_MHG	3 [a]	1	0	38	2	2	15	13	29	[e]	509
4th BB_MHG	2 [a]	1	0	41	2	1	17	10	27	[e]	559
5th BB_MHG	2 [a]	1	0	40	2	1	18	9	29	[e]	611
6th BB_MHG	1 [a]	1	0	38	3	1	18	15	25	[e]	547
Res. BB_MHG	40 [a]	0	1	9	8	3	9	52	18	[e]	413
iBB		0	0	15	4	4	9	39	28	[e]	375 ± 46
1st iBB	40 [c]	1	0	7	1	9	5	54	23	[e]	274
2nd iBB	8 [c]	1	0	24	1	3	9	23	39	[e]	432
3rd iBB	3 [c]	1	0	32	1	1	13	10	41	[e]	634
4th iBB	5 [c]	1	0	36	1	1	14	9	37	[e]	633
5th iBB	2 [c]	1	0	38	2	1	16	11	32	[e]	590
6th iBB	1 [c]	1	0	41	2	1	17	10	29	[e]	596
Res. iBB	36 [c]	1	1	10	8	3	9	49	20	[e]	416

[a] Yield is expressed as a percentage of dry weight dehydrated broccoli by-products (BB_MHG). [b] Yield is expressed as a percentage of dry weight 1st BB_MHG. [c] Yield is expressed as a percentage of dry weight of initial broccoli by-products (iBB). [d] Amount of free sugars are in brackets. [e] Not determined. Res. – Residue.

2.3.2.3. Characterization of MHG extracts

Different amount of solutes were collected in MHG extracts (265 to 398 mL) from the different samples of broccoli by-products. Combining the amount of water retained in by-products after MHG dehydration (non diffused water in **Figure 2.2**) with the amount of water recovered in the extracts (diffused water in **Figure 2.2**), it was possible to infer that an amount of water ranging from 19% to 46%, with 32% on average, was lost due to vapour loss retained by condensation in the microwave reactor (vaporized water in **Figure 2.2**) (Cendres, Chemat, Maingonnat, & Renard, 2011).

Along MHG experiments, 92 to 100% of the collected material was in the three first fractions (150 mL) (**Table 2.3**), with exception for the experiment with blanched mixture of leaves and inflorescences remains (iBB 5), where the material was collected up to 200 mL. Overall, the dry weight material recovered ranged from 1.6 to 4.2 g, with a mean value of 2.6 ± 1.1 g, which corresponds to a mean yield of extraction of 4.4 ± 1.9% dw. This yield was 2 to 4 times higher than the observed for blanched and non-blanched broccoli, respectively, after MHG experiments performed by López-Hortas, et al. (2019). The same recovery of 2.6 g dw was achieved by conventional hot water extraction (150 mL in 55 g) of fresh iBB 6 (that showed the highest MHG yield), which corresponds to a yield of 45.3% dw of iBB 6. This result shows that the yield of extraction by MHG methodology for iBB 6 (6.3% dw) was about 7 times lower as compared to that of hot water one, as already stated for lettuce (Périno, Pierson, Ruiz, Cravotto, & Chemat, 2016). Nonetheless, MHG takes advantage on the concentration of the broccoli by-product extracts (7.8 to 28 mg/mL) when related with the hot water extraction (17 mg/mL). When considering drying purposes, this can be an advantage of obtaining a concentrated extract as a co-product of the drying process.

In general, the first 3 fractions obtained during the MHG experiments were coloured green to yellow (**Figure 2.4**), indicating the extraction of pigments, mainly chlorophylls, known to be present in broccoli (Guzman, Yousef, & Brown, 2012). The concentration of total phenolic compounds (TPC) estimated in first 150 or 200 mL of MHG fractions was 131 to 317 µg/mL of gallic acid equivalents (**Table 2.4**), in accordance with other MGH experiments (López-Hortas, et al., 2019) and with the concentration in TPC of infusions prepared from broccoli by-products (Dominguez-Perles, Moreno, Carvajal, & Garcia-Viguera, 2011) , pointing out the fact that these MHG extracts could be used without any concentration step to prepare broccoli-based beverages. Identification of these phenolics by

UHPLC-DAD-ESI/MS^n **(Figure 2.5)** showed the presence of hydroxycinnamic acids (sinapic, ferulic, caffeic, and coumaric acids) esterified with quinic acid, kaempferol glucosides, and gentiobiose **(Table 2.5)**, in accordance with the phenolic profile of broccoli and other *Brassicas* (Cartea, Francisco, Soengas, & Velasco, 2011).

Table 2.3. Percentage of dry weight (dw) material recovered in the collected fractions (50 mL each) and total dw collected along the MHG extraction of the distinct iBB samples.

iBB samples	% dw in each fraction								Total dw (g)
	1	2	3	4	5	6	7	8	
1	42	35	17	3	2	0	1	[a]	4.45
2	81	14	3	3	0	0	[a]	[a]	1.75
3	58	40	2	0	0	0	[a]	[a]	2.70
4	40	49	9	1	1	1	[a]	[a]	1.73
5	24	22	27	24	1	1	1	1	1.69
6	39	40	13	1	2	3	2	1	3.91

[a] the diffusion stopped before.

Figure 2.4. Detail of colour change of extracts along fractions collected during MHG experiment of iBB 6.

Table 2.4. Sugar composition, total sugars, mannitol, total phenolic compounds (TPC) as gallic acid equivalents (GAE), and free amino acids from the extracts (sum of fractions 1 to 4) obtained in the MHG experiments with broccoli by-products 1 to 6. Results are expressed as mean of total compounds ± standard deviation.

Ext N.º	Sugar composition (%mol)										Total sugars (mg/mL)	Mannitol (mg/mL)	TPC (µg/mL)	Amino acids (mg/mL)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Free Glc	Free Fru				
1	[tr]	[tr]	1	[tr]	[tr]	1	[tr]	15	41	41	13.1 ± 1.0	1.94 ± 0.09	232 ± 21	7.83 ± 0.79
2	[tr]	3	2	4	[tr]	1	[tr]	6	40	45	3.7 ± 0.1	0.02 ± 0.00	137 ± 7	9.30 ± 0.52
3	[tr]	2	7	2	[tr]	3	7	8	25	46	5.2 ± 0.0	0.05 ± 0.01	261 ± 15	9.30 ± 1.36
4	[tr]	3	4	1	[tr]	4	2	23	5	57	2.1 ± 0.2	0.65 ± 0.03	194 ± 13	4.24 ± 1.59
5	[tr]	[tr]	1	[tr]	[tr]	1	[tr]	17	28	52	2.3 ± 0.5	0.79 ± 0.03	131 ± 9	1.98 ± 0.18
6	[tr]	[tr]	1	[tr]	[tr]	1	25	11	8	53	11.4 ± 0.7	2.56 ± 0.07	317 ± 34	0.86 ± 0.14

Ext N.º - extraction number. [tr] - traces.

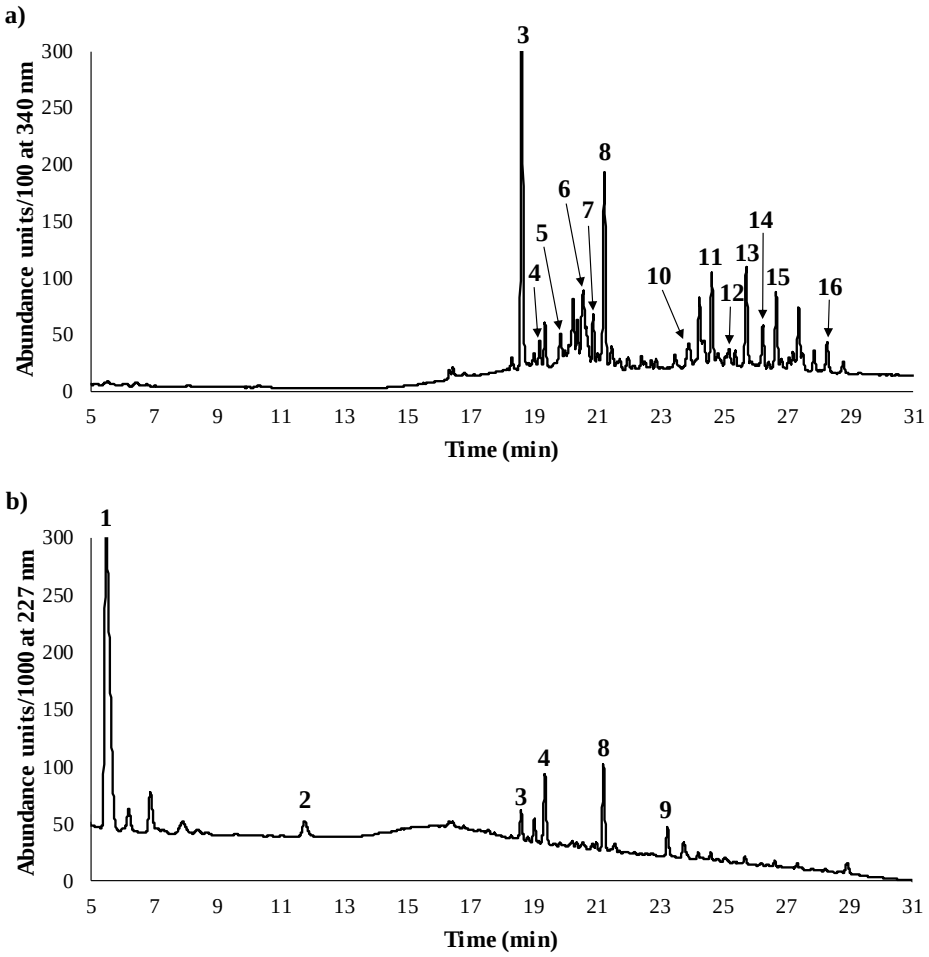


Figure 2.5. Chromatographic profile at **a)** 340 nm and **b)** 227 nm of the first fraction collected in the experiment with broccoli by-product 6. The numbers in the chromatograms correspond to the UHPLC-DAD-ESI-MS^n peaks described in **Table 2.5**.

Total sugars in the first 150 to 200 mL of extracts ranged from 2.1 to 13.1 mg/mL (**Table 2.4**). Fru, accounting for 41 to 56% of total sugars, and Glc (5-41%) were the only free sugars present, as no sucrose was detected. The amount of collected free sugars was higher in extracts rich in stalks, iBB 1 and 6, in accordance with the higher amount of free sugars in these by-products (**Table 2.1**). It was quantified also mannitol, in the amount of 18 µg/mL to 2.6 mg/mL (**Table 2.4**), which is a metabolite already reported to occur in broccoli (Pierre et al., 2012). The extracts richer in mannitol were those containing the stalks (iBB 1 and 6), as was observed for the free sugars, in tissues with higher content of phloem structures, where mannitol is present (Patel & Williamson, 2016). The presence of both free sugars and mannitol, with sweet character (Grembecka, 2015), should confer a sweetener character to MHG extracts.

Table 2.5. Glucosinolates and phenolic compounds identified in the hydrodiffused extracts collected in the MHG experiment, analysed by UHPLC-DAD-ESI-MS^n. Quantification of glucosinolates identified in the first fraction collected in the experiment with broccoli by-product 6.

P	RT	$[M-H]^-$	MS^2 $[M-H]^-$, m/z (%)	λ_{max}	C (µg/mL)	Compound
1	4.87	436	421 (1); 372 (100); 356 (1); 291 (2); 275 (1); 259 (4); 258 (1)	201, 288	318.2	Glucoraphanin
2	11.47	463	383 (2); 285 (100); 267 (25); 263 (5); 240 (20); 187 (8); 160 (16)	224, 275	3.6	4-hydroxyglucobrassicin
3	18.63	420	340 (34); 359 (8); 291 (7); 275 (44); 259 (100); 242 (5); 224 (21); 195 (24); 178 (41)	221sh, 238, 298sh, 324, 368sh #	12.7	Glucoerucin
3	18.63	353		221sh, 238, 298sh, 324, 368sh #		5-caffeoylquinic acid
4	19.36	447	367 (6); 291 (40); 285 (11); 275 (31); 269 (18); 259 (100); 251 (28); 241 (11); 227 (10); 224 (8); 205 (44)	221, 270	8.5	Glucobrassicin
5	19.86	337		241, 310, 368sh		4-p-coumaroylquinic acid
6	20.57	353		244, 270, 329, 368sh #		Chlorogenic acid
6	20.66	1139		246, 269, 333, 368sh #		Kaempferol-3-sinapoylsophorotrioside-7-glucoside
7	20.88	977		244, 268, 334, 368sh		Kaempferol-3-sinapoylsophoroside-7-glucoside
8	21.23	477	397 (2); 315 (7); 299 (17); 291 (100); 284 (8); 281 (20); 275 (53); 259 (91); 235 (9); 227 (5); 204 (12); 201 (7); 195 (5)	222, 268, 292, 331	9.8	4-methoxyglucobrassicin
9	23.24	477	447 (82); 446 (100); 367 (1); 301 (2); 284 (2); 275 (1); 259 (2); 229 (1); 170 (1)	232, 275, 289	3.4	Neoglucobrassicin
10	23.92	367		244, 328, 369sh		5-feruloylquinic acid
11	24.64	1345		244, 271, 329, 368sh#		Kaempferol-3-disinapoylsophorotrioside-7-glucoside
12	25.18	1315		246, 278, 329, 368sh		Kaempferol-3-feruloylsinapoylsophorotrioside-7-glucoside
13	25.73	915		243, 329, 367sh		disinapoylcaffeoylgentiobiose
14	26.25	885		245, 329, 367sh		sinapoylcaffeoylfereloylgentiobiose
15	26.67	753		244, 329, 368sh		1,2-disinapoylgentiobiose
16	28.28	959		244, 326, 368sh		1,2,2′-Trisynapoylgentiobioside
				Total Glucosinolates	356.2	Total Glucosinolates

P – peak number; RT – retention time (min); [M-H]- : molecular ion; λmax - maximum wavelengths (nm); #λmax of co-eluting compounds; sh –indicate a shoulder in λmax.RT-retention time.

Although not having sucrose, the first 150 to 200 mL of extracts were also composed by glycosidic-linked sugars. The stalks (iBB 1 and 6) have high content, ranging from 2.4 to 4.5 mg/mL then the other samples, which ranged from 0.5 to 1.5 mg/mL **(Table 2.4)**. UA accounted for 29 to 91% of glycosidic-linked sugars **(Table 2.4)**. These acid sugars should have origin in intercellular space pectic polysaccharides (Christiaens, Van Buggenhout, Houben, et al., 2011; Christiaens, Van Buggenhout, Vandevenne, et al., 2011), which may be more easily diffused by heated water than other plant cell wall polysaccharides. The diffusion of pectic polysaccharides may also explain the traces of Rha, Ara and Gal.

Amino acids accounted for 0.86 to 4.24 mg/mL in the first 50 mL extracted from blanched iBB (4, 5, and 6, **Table 2.4**) and 7.83 to 9.32 mg/mL in the extracts from non-blanched iBB (1, 2, and 3, **Table 2.4**). On average, there was a reduction of 3.7 times for extracts from blanched iBB in comparison with extracts from non-blanched, an effect also observed in broccoli stalks and florets after a blanching treatment(Murcia, López-Ayerra, Martínez-Tomé, & García-Carmona, 2001). Furthermore, the amount of free amino acids observed in the stalk-rich extracts (iBB 1 and iBB 6) was lower than the other blanched and non-blanched extracts, as broccoli florets are known to have more free amino acids than the stalks (Murcia et al., 2001). Glutamine was the major amino acid (**Table 2.6**) , representing 38-61% of the total amino acids, followed by aspartic acid, proline, asparagine and glutamic acid, in accordance with the literature (Gomes & Rosa, 2001; Murcia et al., 2001; Zaghdoud, Carvajal, Moreno, Ferchichi, & del Carmen Martínez-Ballesta, 2016). As the amino acids are present in the conducting tissues of the broccoli vascular system (xylem and phloem) (Shelp, 1987) they can be easily extracted by diffusion of xylem and phloem contents.

As intact glucosinolates are known to be preserved after blanching processes due to the inactivation of myrosinase (Alanís-Garza, Becerra-Moreno, Mora-Nieves, Mora-Mora, & Jacobo-Velázquez, 2015; Oliviero, Verkerk, & Dekker, 2013), only in blanched samples were observed the presence of glucosinolates. A detailed analysis of the first 50 mL extract from iBB 6 allowed the quantification of six glucosinolates (GLS), accounting for a total of 379 µg/mL (**Table 2.5**). Glucoraphanin represented 74% of the GLS amount, followed by 4-hydroxyglucobrassicin, glucoerucin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin (**Figure 2.5**), which was in accordance with the GLS identified by Dominguez-Perles, Moreno, Carvajal, & Garcia-Viguera (2011). Glucoraphanin, as

precursor of isothyocyanate sulforaphane, is a valuable compound leading to several bioactivities, in particular as chemopreventive (Juge, Mithen, & Traka, 2007).

Table 2.6. Amino acids composition of extracts obtained from broccoli by-products 1 to 6 after MHG experiments. Total amino acids are expressed as mean of total compounds ± standard deviation.

Amino acids (mg/mL)	Extracts					
	1	2	3	4	5	6
Ala & Gly[a]	0.14	0.36	0.39	0.04	0.01	0.01
Val	0.21	0.40	0.54	0.03	0.00	0.00
Leu	0.10	0.15	0.21	0.01	0.01	0.01
Ser & Thr[a]	0.36	0.65	0.35	0.16	0.06	0.05
Ile	0.04	0.12	0.16	0.01	0.00	0.00
Pro	1.01	1.18	1.03	0.46	0.27	0.22
Asn	0.33	0.34	0.21	0.09	0.22	0.00
Asp	0.37	0.51	0.59	0.87	0.36	0.17
Met	0.02	0.02	0.02	0.01	0.00	0.00
Glu	0.15	0.55	0.64	0.50	0.00	0.02
Phe	0.00	0.05	0.08	0.00	0.00	0.00
Gln	4.78	4.41	4.03	1.86	0.96	0.33
Orn	0.00	0.00	0.18	0.04	0.00	0.00
Lys	0.10	0.28	0.40	0.00	0.00	0.00
His	0.10	0.15	0.30	0.13	0.06	0.02
Tyr	0.03	0.06	0.08	0.00	0.00	0.00
Trp	0.08	0.06	0.12	0.02	0.01	0.02
Total	7.83 ± 0.79	9.30 ± 0.52	9.32 ± 1.36	4.24 ± 1.59	1.98 ± 0.18	0.86 ± 0.14

[a]Amino acids co-eluted.

2.3.2.4. Food applications of MHG extracts

The combined MHG extracts were used to develop a béchamel sauce where the added water is substituted by the broccoli aqueous extract. In comparison with the béchamel sauce made with water no changes in taste are noticed by the addition of the MHG extracts. However, an improving on its flavour is observed, adding a cruciferous pleasant flavour to the sauce. Bioactive compounds present in the extracts, namely glucosinolates, are expected to be preserved during the sauce preparation, as myrosinase is deactivated under microwave heating (Vadivambal & Jayas, 2007) and glucosinolates have a good thermal stability (Franco et al., 2016). Therefore, the incorporation of MHG extracts enriched the sauce with bioactive compounds, representing a healthier choice and maintaining the main characteristics of a béchamel sauce, showing the potential of these extracts to be used directly by the food industry.

2.4. Concluding remarks

MHG technology allows the simultaneous dehydration of the different perishable parts of broccoli by-products, including inflorescences, leaves and stalks in a short period of time, and the extraction of a small fraction as an aqueous solution rich in phenolic compounds, Fru, Glc, mannitol, pectic polysaccharides, free amino acids, and glucosinolates.

For dehydration purposes, MHG allowed to achieve broccoli by-products until 12% of moisture without affecting polysaccharides and protein content. The stabilised dehydrated product can be used for further processing as a source of ingredients rich in carbohydrates. For this, MHG technology behaves as pre-treatment for extraction of pectic polysaccharides. On the other hand, the washed out solutes obtained by MHG represented only 4.4% per dry weight of broccoli by-products, indicating that most bioactive compounds, characteristic of broccoli matrix, are still present in dried by-products.

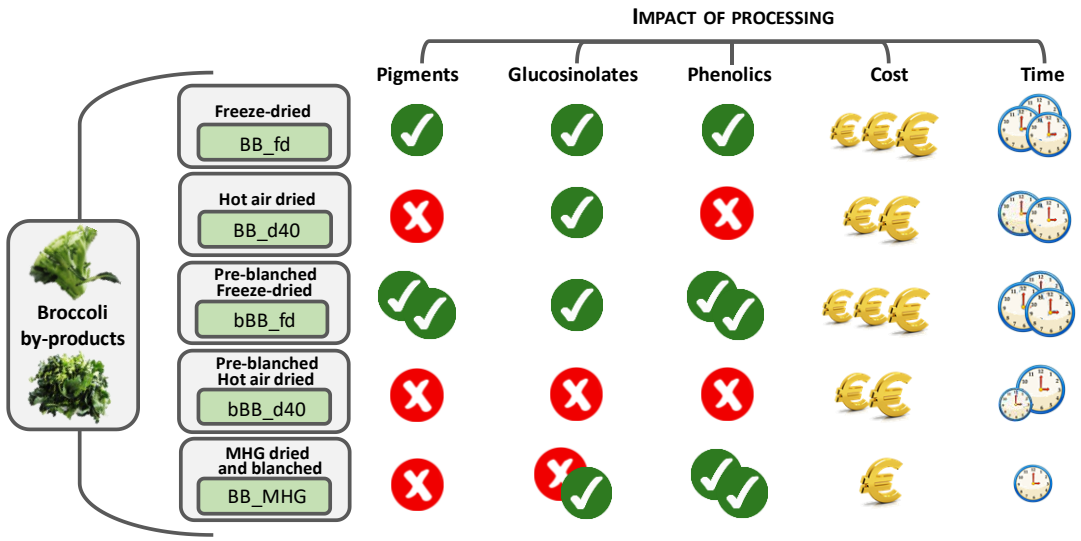
MHG aqueous washed out solutes had up to 10.8 mg/mL of free sugars, 9.32 mg/mL of amino acids, 2.5 mg/mL of mannitol, 2.2 mg/mL of pectic polysaccharides, 379 µg/mL of glucosinolates, and 317 µg/mL of phenolic compounds, showing that these extracts are also a source of bioactive compounds without the need of a filtration and concentration steps usually performed when aqueous extractions are used.

The simultaneous dehydration of the material and extraction of bioactive compounds indicates that MHG technology has potential to be used for valorisation of industrial by-products.

CHAPTER 3

Blanching impact on pigments, glucosinolates, and phenolics of dehydrated broccoli by-products

Graphical abstract:



CHAPTER 3

Blanching impact on pigments, glucosinolates, and phenolics of dehydrated broccoli by-products

3.1. Blanching impact on pigments, glucosinolates, and phenolics of dehydrated broccoli by-products - approach

3.2. Material and methods

3.2.1. Broccoli by-products and chemicals

3.2.2. Dehydration and blanching of broccoli by-products

3.2.3. Pigments characterization

3.2.4. Glucosinolates and phenolic compounds characterization

3.2.5. Estimation of energy consumption

3.2.6. Statistical analysis and principal component analysis

3.3. Results and discussion

3.3.1. Impact of blanching and dehydration on pigments

3.3.2. Impact of blanching and dehydration on glucosinolates

3.3.3. Impact of blanching and dehydration on phenolic compounds

3.3.4. Impact of blanching and dehydration techniques on broccoli by-products valorisation

3.4. Concluding remarks

Parts of the text of this chapter were published/submitted in the following publications:

Sónia S. Ferreira, Filipa Monteiro, Cláudia P. Passos, Artur M. S. Silva, Dulcineia Ferreira Wessel, Manuel A. Coimbra, Susana M. Cardoso (**2020**). Blanching impact on pigments, glucosinolates and phenolics of dehydrated broccoli by-products. Food Research International, 132, 109055. IF 4.972.

3.1. Blanching impact on pigments, glucosinolates, and phenolics of dehydrated broccoli by-products - approach

Blanching pre-treatment has been reported to improve the drying rate of broccoli. As a thermal treatment, blanching also promotes enzyme inactivation. Therefore, this work aimed to evaluate the impact of blanching and dehydration on bioactive compounds, such as pigments, glucosinolates and phenolics of broccoli by-products making use of MHG technology studied in **Chapter 2**, oven-drying and freeze-drying.

3.2. Material and methods

3.2.1. Broccoli by-products and chemicals

Two batches of broccoli by-products were used. The first batch consisted of non-blanched stalks and leaves from fresh broccoli heads (harvested in March 2018). These by-products represented 31% of broccoli head. The second batch of broccoli by-products comprised a mixture of stalks, leaves, and inflorescence remains provided by Monliz SA, Portugal, in January 2015 (**Chapter 2**, **Section 2.2.1**). These by-products represented 45% of broccoli head and were obtained along the freezing line in the industrial process and after the thermal blanching step (33% of by-product).

All reagents used were of analytical grade or higher available purity.

3.2.2. Blanching and dehydration of broccoli by-products

To evaluate the effect of blanching and dehydration, broccoli by-products (BB) from the first batch were divided in two groups (**Figure 3.1**). One group (300 g) was not submitted to blanching, while other was blanched in a domestic microwave, which consisted of three treatments at 800 W for 2 min each, with intercalated mixing, according to Silva et al. (2013). A part of the non-blanched group (BB) and of the blanched broccoli by-products group (bBB) was air-dried at 40 ºC (VENTI-Line, VL-115, VWR) until constant weight (64 h for BB_d40 and 40 h for bBB_d40, respectively). Alternatively, other part of BB and bBB was frozen with liquid nitrogen and freeze-dried at -45 to -50ºC and less than 150 mTorr (benchtop K, VirTis with Vacuumbrand pump) for one week (samples BB_fd1 and bBB_fd, respectively).

To evaluate the effect of simultaneous blanching and dehydration, the second batch of broccoli by-products was dehydrated by MHG (BB_MHG in **Chapter 2**, 10 min bellow 100ºC, and 35 min at 100ºC,) or alternatively, it was freeze-dried (BB_fd2, iBB in **Chapter 2**, **Figure 3.1**).

All dehydrated broccoli by-products (BB_d40, bBB_d40, BB_fd1, BB_MHG, BB_fd2, and bBB_fd) were placed in the dark. Before analysis, the samples were milled in a cooled analytical grinder (Yellowline, A10 IKA).

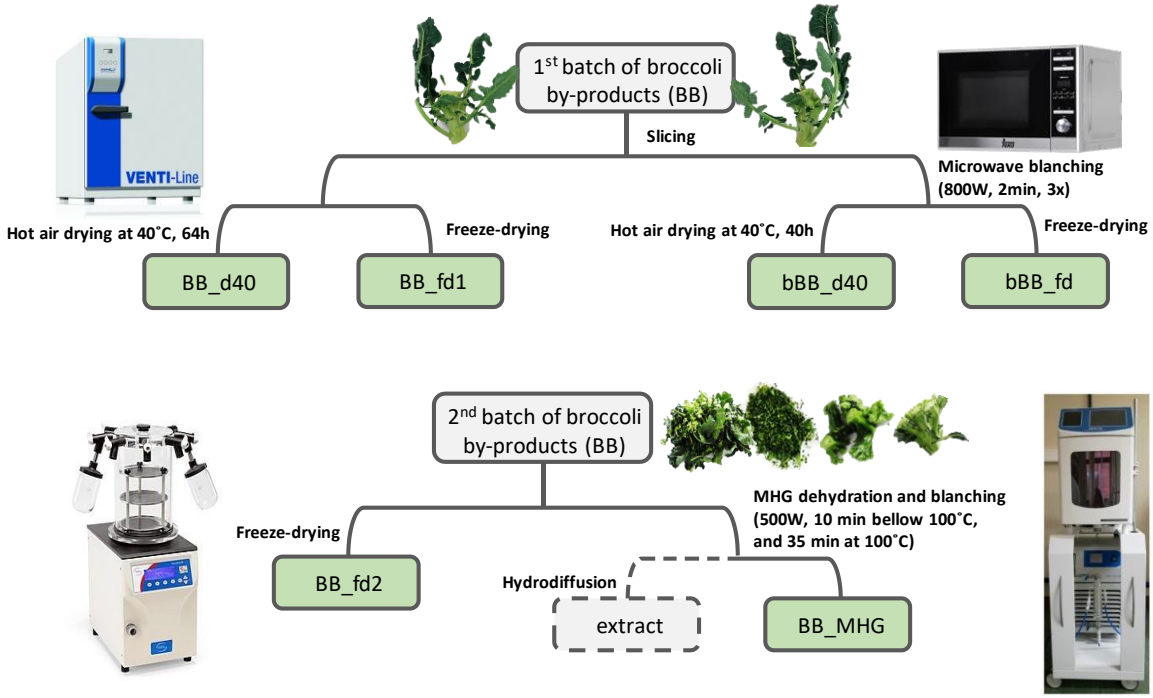


Figure 3.1. Scheme of broccoli by-product samples processed by oven-drying, freeze-drying and MHG.

3.2.3. Pigments characterization

Pigments were analysed by UHPLC-DAD-ESI/MS^n after extraction according to Guzman, Yousef, & Brown (2012), with some modifications. Dehydrated broccoli by-products (0.2 g) were extracted with 5 mL of 0.1% butylated hydroxytoluene in pure ethanol, for 24 h under agitation, at room temperature, in the dark. After each extraction, the supernatants were separated by gravity settling, filtered through a 0.2 µm filter (cellulose acetate, 30 mm syringe filters, imChem, France) into an amber vial, and stored at −20°C until injection. Triplicate extractions were performed for each sample.

The chromatographic apparatus used was the same as described in **Chapter 2**, **Section 2.2.6.2** for glucosinolates and phenolic compounds characterization. The instrument was operated in positive-ion mode and the full scan covered the mass range from *m/z* 100 to 2000. ESI needle voltage was set at 4.80 kV and an ESI capillary temperature of 275ºC.

Gradient elution was carried out with a mixture of 0.1% (v/v) formic acid in water (solvent A) and 30% (v/v) methanol in acetonitrile (solvent B), with a flow rate of 0.200 mL/min in a linear gradient. The solvent gradient started with 5% of solvent B, reaching 40% at 14 min, 100% at 16 min, and being maintained for two minutes before returning to the initial conditions at 20 min. UV-Vis spectral data for all peaks were accumulated in the range 200–700 nm, while the chromatographic profiles were recorded at 450 and 655 nm for carotenoids (xanthophylls and carotenes) and chlorophylls analysis, respectively. The identification of pigments was performed by comparison of retention times, absorption spectra, and MS data with standards and literature. Quantification was performed by peak integration, using the external standard method, with the exact (chlorophyll a, chlorophyll b, lutein, β-carotene and fucoxanthin) or structurally-related standard compounds (chlorophyll b in the case of other chlorophylls and lutein in the case of other xanthophylls (**Table 3.1**).

Table 3.1. Concentration range, calibration curve, and regression coefficients of standards used to quantify pigments.

Compound	λ (nm)	Concentration range (µg/ml)	Calibration curve	Regression coefficient (R^2)
Lutein	450	1 - 18	y = 36221x – 5139	0.9990
Chlorophyll b	655	2 - 25	y = 20166x + 5011	0.9962
Chlorophyll a	655	5 - 70	y = 14135x + 11077	0.9946
β-carotene	450	2 - 42	y = 15423x + 374	0.9983

3.2.4. Glucosinolates and phenolic compounds characterization

Phenolic compounds and intact glucosinolates were simultaneously analysed by UHPLC-DAD-ESI/MS^n after extraction of 100 mg of dehydrated broccoli by-products in 3 mL of 70% methanol for 30 min under stirring at 70 ºC, in the dark, according to Domínguez-Perles, et al. (2010). After each extraction, the supernatants were cooled down in a cool water bath, separated by gravity, filtered through a 0.2 µm filter into an amber vial, and stored at −20°C until injection. Triplicate extractions were performed for each sample.

Glucosinolates and phenolic compounds were characterized as described in **Chapter 2**, **Section 2.2.6.2** with some modifications of gradient elutions and mobile phases. Gradient

elution was carried out with a mixture of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B), with a flow rate of 0.200 mL/min in a linear gradient. The solvent gradient started with 5% of solvent B, reaching 40% at 14 min, 100% at 16 min, maintained for two minutes before returning to the initial conditions. UV-Vis spectral data for all peaks were accumulated in the range 190–700 nm, while the chromatographic profiles were recorded at 227 nm for glucosinolates and 280 and 320 nm for phenolic compounds. The identification of glucosinolates and phenolic compounds was performed by comparison of retention times, absorption spectra, and MS data with standards and literature. Glucosinolates were quantified using sinigrin (Sigma-Aldrich, Germany) and response factors according Buchner, R. (1987) **(Table 3.2)**.

Table 3.2. Response factors of glucosinolates and concentration range, calibration curve, and regression coefficients of external standard sinigrin used to quantify glucosinolates according their response factor.

Compound	λ (nm)	Response factor	External standard	Concentration range (µmol/ml)	Calibration curve	Regression coefficient (R^2)
Glucoraphanin	227	1	Sinigrin	0.1 - 14	y = 4.827x	0.9852
Glucobrassicin	227	0.25				
4-methoxyglucobrassicin	227	0.25				
Neoglucobrassicin	227	0.25				

Quantification of phenolic compounds was performed by peak integration, using the external standard method, with structurally related standard compounds **(Table 3.3)**. Esters of hydroxycinnamic acids with gentiobiose and esters of hydroxycinnamic acids with quinic acid were quantified as 5-O-caffeoylquinic acid; quercetin derivatives were quantified as quercetin-7-O-galactoside; and kaempferol glucosides, monoacylated kaempferol glucosides and diacylated kaempferol glucosides were quantified as kaempferol.

Table 3.3. Concentration range, calibration curve, and regression coefficients of standards used to quantify phenolic compounds.

Compound	λ (nm)	Concentration range (µg/ml)	Calibration curve	Regression coefficient (R^2)
Quercetin-7-*O*-galactoside	340	13 - 215	y=10139x-12806	0.990
Kaempferol	340	10 - 310	y=17655x-35885	0.998
5-*O*-caffeoylquinic acid	320	5 - 90	y=14621x-13860	0.996

3.2.5. Estimation of energy consumption

Energy consumption was measured or estimated for all processing techniques to compare their efficiencies. Energy consumption of oven was quantified with power meter (DANIU, Intertek). Energy consumption during microwave blanching was calculated by the input of microwave power with time and energy consumption during MHG dehydration estimated according López-Hortas, et al. (2019) data. For estimation of freeze-drying energy consumption, steps of freezing, sublimation and vacuum pump were considered, summing the energetic consumption of all equipment.

3.2.6. Statistical analysis and principal component analysis

Differences among dehydrated products from the first batch (BB_fd1, BB_d40, bBB_fd, and bBB_d40) and from the second batch (BB_fd2 and BB_MHG) were analysed through One-Way ANOVA followed by Tukey's multiple comparisons tests and unpaired Student's t-test, respectively, in GraphPad prism, Trial Version 6.01 (GraphPad Software, Inc. La Jolla, CA, USA). Differences were deemed significant at p-value<0.001.

Principal component analysis (PCA) was applied to all compounds identified (pigments, glucosinolates, and phenolic compounds) using the values of µg/g of dry weight. The data matrix comprised 6 samples of dehydrated broccoli by-products (3 repetitions) and 64 compounds, thus given a matrix with 18 columns and 64 rows. The data matrix was analysed using MetaboAnalyst (https://www.metaboanalyst.ca, Chong, Wishart, & Xia, (2019). Data were normalized after auto scaling: mean-centring and division by the standard deviation of each variable.

3.3. Results and discussion

To evaluate the impact of blanching and dehydration on pigments, glucosinolates, and phenolic compounds, broccoli by-products from batch 1 were freeze-dried (BB_fd1 and bBB_fd) and air-dried at 40 ºC (BB_d40 and bBB_d40), in the absence or presence of a blanching pre-treatment (bBB_fd and bBB_d40). In addition, to assess the effect of simultaneous blanching and dehydration, broccoli by-products (batch 2) were dehydrated by the technology MHG (BB_MHG) and freeze-dried (BB_fd2). Note that freeze-drying was used for comparison purposed as this is the elected dehydration technique to preserve compounds and the cells structure (Duan, 2017; Jha et al., 2018). The potential of each

processing technique to dehydrate and valorise broccoli by-products was studied after estimation of energy consumption and by means of PCA.

3.3.1. Impact of blanching and dehydration on pigments

The first batch of broccoli by-products (BB_fd1) was much richer in pigments (8.26 mg/g dw) than the second (BB_fd2, 0.206 mg/g dw) (**Table 3.4**), and differences are probably attributed to a multitude of factors, including the different agronomic operations, cultivars, edaphoclimatic conditions, and previous processing (Björkman et al., 2011; Neugart et al., 2018). In this regard, one must highlight that the first batch was obtained in March as fresh broccoli and promptly cut, while the second batch was provided by the industry in December, after cutting, washing, thermal processing, and/or cooling, which could affect this kind of compounds (Cai et al., 2016). Nevertheless, in general, the same pigment derivatives were found in the two batches (**Table 3.4**), as depicted in bellow for bBB_fd sample (**Figure 3.2**).

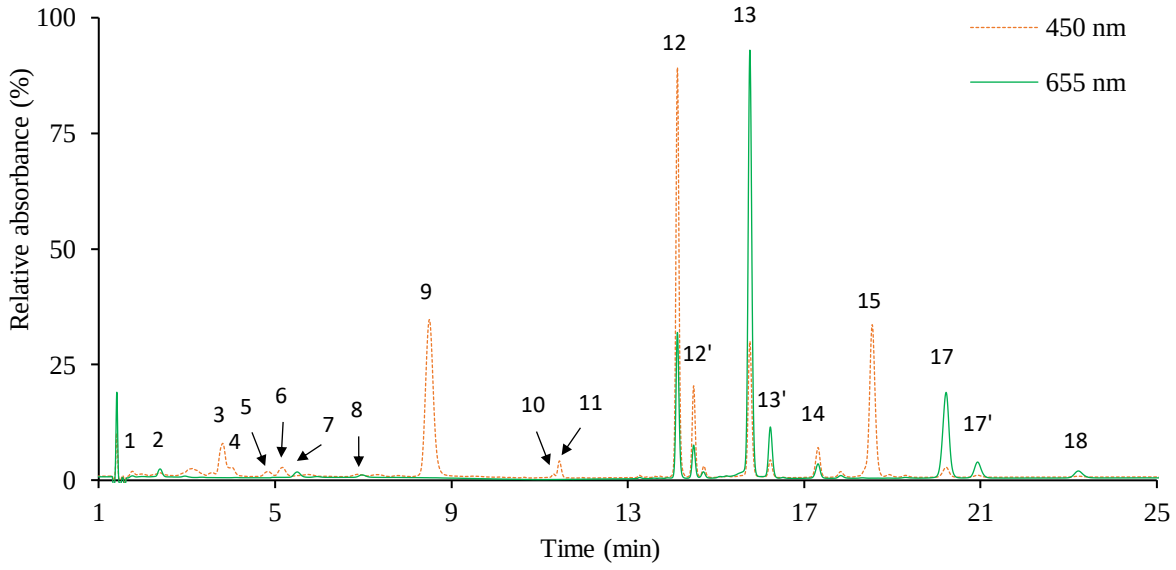


Figure 3.2. Representative chromatograms from 1-25 min at 450 nm and 655 nm, from the first batch of broccoli by-products after freeze-drying with a pre-blanching step (bBB_fd). The numbers in the chromatograms correspond to the UHPLC-DAD-ESI-MS peaks described in **Table 3.4**.

3.3.1.1. Carotenoids

The main carotenoid found in the first batch of broccoli by-products was β-carotene (up to 1.54 mg/g dw). This amount is comparable to the values reported for *Brassica* leaves (Lefsrud, Kopsell, Wenzel, & Sheehan, 2007; Müller, 1997), but much higher than previously reported values for broccoli inflorescences (Reis et al., 2015; Guzman et al., 2012; Zhang et al., 2018) and broccoli sprouts (Luo et al., 2019). Lutein was the main xanthophyll (up to 904 µg/g dw), but neoxanthin and violaxanthin, luteoxanthin- and lutein-like structures were also found in moderate amounts (105 µg/g dw, 12.1 µg/g dw, 69.4 µg/g dw, 62.8 µg/g dw, respectively). As for β-carotene, the content of lutein herein found is comparable to literature values reported for *Brassica* leaves (Lefsrud et al., 2007) and higher than those described for processed broccoli by-products (Liu et al., 2018). In the second batch, carotenoids were about 50 folds lower than in the first batch and opposing to the last, lutein was its major carotenoid, representing about 65% of the total carotenoids.

Air-drying at 40ºC reduced by 43% the amount of carotenoids recovered from broccoli by-products, when compared to freeze-drying (BB_d40 vs BB_fd1, **Table 3.4**). These results are consistent with literature data. In fact, air-drying of broccoli inflorescences was previously shown to reduce about 62% the contents of the main carotenoids (lutein and β-carotene), independently of dehydration temperature (60-80ºC), due to cell wall disruption and further degradation of these compounds along dehydration (Zhang et al., 2018). On the other hand, our results showed that the application of a blanching pre-treatment before air-drying, which reduced the time of dehydration of the by-products by about 40%, had no significant effect on the amounts of carotenoids (bBB_d40 vs BB_d40). Nevertheless, in the freeze-dried products, this blanching step promoted the increase of carotenoids extraction (67%), suggesting that plant cell disruption promoted by the blanching step only improves extractability (Reis et al., 2015; Murador, da Cunha, & de Rosso, 2014; Oliveira, Brandão, & Silva, 2016; Podsędek, 2007) when freeze-drying is used (Duan, 2017; Jha et al., 2018). MHG dehydration, which was used in the second batch, reduced the carotenoids from 31 µg/g dw to 16 µg/g dw (BB_fd2 vs BB_MHG). Regardless of whether the total quantities were lower than those observed in the first batch, the carotenoid loss caused by this treatment was in the same range as that observed in lower temperature (40 ºC) air-dried samples.

3.3.1.2. Chlorophylls

The main chlorophylls identified in broccoli by-products were chlorophylls a (up to 3.57 mg/g dw) and *b* (up to 0.77 mg/g dw). Chlorophyll derived compounds were also found, namely pheophytins *a* and *b*, pyropheophytin *a*, pheophorbide *a*, pyropheophorbide *a*, chlorophyllides *a* and *b*, and pheophytin *a* allomer. These compounds were naturally present (Luo et al., 2019) or could have been produced by the acidic conditions promoted by cellular lysis, by the temperature of blanching and/or dehydration used (Gonçalves et al., 2009), or by the light exposure (Yasuda, Oda, Ueda, & Tabata, 2019). The ratio of chlorophylls *a* to *b* was 4.8, which is in accordance with broccoli head values (Guzman et al., 2012). Total chlorophylls *a* and *b* were also in accordance with the data observed for broccoli by-products: 4.62 mg/g dw and 0.80 mg/g dw, respectively (Liu et al., 2018).

Air-drying reduced the amount of chlorophylls by 54% when comparing to freeze-drying but kept the same chlorophylls constituents. The sequential application of blanching and air-drying reduced even further the total chlorophylls contents (by 60%). Nevertheless, in the freeze-dried products, this blanching step promoted the increase of chlorophylls *a* and *b* extraction (8%), as observed by other authors (Cai et al., 2016). Moreover, the blanching step promoted the formation of chlorophylls derived compounds, mainly by the loss of Mg^{2+} (demetallization) of chlorophyll *a* into pheophytin *a* and, in lower extend, of chlorophyll *b* into pheophytin *b*, as observed for broccoli florets blanched before frozen (Murcia, López-Ayerra, Martínez-Tomé, & García-Carmona, 2000). Other chlorophylls derived compounds were observed after blanching, namely pyropheophytin *a*, pheophorbide *a*, chlorophyllides *a* and *b*, and pyrophaeophorbide *a*, which formation has been associated to thermal processing (Ferruzzi & Blakeslee, 2007; Schelbert et al., 2009). In particular, the formation of pheophorbide occurred after breadmaking of doughs with incorporated fresh plant material from *Brassica* species (Klopsch et al., 2019).

After MHG dehydration, total chlorophylls amount was not significantly different from freeze-dried by-product. Nevertheless, chlorophyll *b* was reduced to traces and chlorophyll *a* was not detected, in accordance with favourable degradation of chlorophyll *a* into pheophytin *a* and other derived compounds (Canjura, Schwartz, & Nunes, 1991; Murcia, et al., 2000). Pheophytins *a* and *b* were the main chlorophylls, followed by pyropheophytin *a* and pheophorbide *a*, which have been associated to light brown color (Gauthier-Jaques, Bortlik, Hau, & Fay, 2001), in accordance with the color of the samples

Table 3.4. Identification and quantification of pigments from dehydrated broccoli by-products analysed by UHPLC-DAD-ESI-MS.

P	RT (min)	$[M+H]^+$	λmax	Probable Compound	Batch 1				Batch 2	
					BB_fd1	BB_d40	bBB_fd	bBB_d40	BB_fd2	BB_MHG
1	1.8	630	461, 601, 648	Chlorophyllide b	-	-	0.3 ± 0.0	tr	tr	-
2	2.3	616	415sh, 430, 590, 619, 663	Chlorophyllide a	tr	7.0 ± 0.1^{d}	42.4 ± 0.1^{a}	9.35 ± 0.24^{c}	tr	tr
3	3.8	602	414, 437, 465	Neoxanthin	98.2 ± 0.1^{b}	55.3 ± 0.0^{d}	104.6 ± 0.9^{a}	39.3 ± 0.2^{e}	2.7 ± 0.0′	1.8 ± 0.0″
4	4.1	602	417,439,469	Violaxanthin	146.0 ± 0.0^{a}	76.1 ± 0.0^{b}	12.1 ± 0.2^{c}	4.7 ± 0.0^{d}	2.5 ± 0.0′	2.3 ± 0.0″
5	4.9	602	399, 422, 448	Luteoxanthin alike	11.0 ± 0.0^{c}	7.7 ± 0.0^{e}	26.1 ± 0.1^{a}	8.2 ± 0.0^{d}	-	-
6	5.2	602	399, 422, 448	Luteoxanthin alike	14.9 ± 0.0^{c}	11.3 ± 0.0^{d}	43.3 ± 0.7^{a}	11.0 ± 0.1^{d}	-	-
7*	5.5 & 5.9	594	407, 506, 536, 608, 664	Pheophorbide a	-	tr	49.9 ± 0.1^{c}	38.5 ± 0.1^{c}	3.0 ± 0.5′	5.8 ± 0.6′
8	6.9	536	405, 507, 538, 606, 665	Pyrophaeophorbide a	-	tr	20.7 ± 0.1^{b}	10.7 ± 0.0^{c}	4.0 ± 0.1′	1.9 ± 0.3″
9	8.5	569	423sh, 446, 473	Lutein	503.3 ± 1.1^{c}	308.2 ± 0.3^{d}	903.7 ± 2.1^{a}	298.8 ± 1.0^{d}	19.8 ± 0.3′	6.3 ± 1.6″
10	11.3	569	418sh, 442, 469	Lutein alike	13.5 ± 0.0^{b}	6.5 ± 0.1^{d}	13.1 ± 0.1^{b}	7.9 ± 0.2^{c}	1.9 ± 0.0′	2.1 ± 0.1′
11	11.5	569	417sh, 439, 467	Lutein alike	34.0 ± 0.1^{c}	17.6 ± 0.2^{d}	49.7 ± 0.0^{b}	17.7 ± 0.2^{d}	2.6 ± 0.0′	2.0 ± 0.0″
12*	14.1 & 14.5	908	462, 599, 648	Chlorophyll b	530.5 ± 0.9^{c}	262.7 ± 0.1^{d}	766.3 ± 0.7^{a}	234.6 ± 0.3^{e}	33.1 ± 0.1	tr
13*	15.8 & 16.2	894	412sh, 430, 537, 580, 617, 663	Chlorophyll a	3492.3 ± 1.5^{ab}	1609.8 ± 0.0^{c}	3573.4 ± 1.5^{a}	835.0 ± 2.6^{d}	111.0 ± 0.3	-
14*	17.3 & 17.9	886	434, 526, 598, 652	Pheophytin b	tr	-	103.0 ± 0.1^{b}	48.2 ± 0.1^{c}	tr	15.0 ± 1.2
15	18.6	537	427sh, 452, 476	β-carotene	788.2 ± 4.5^{c}	428.9 ± 1.0^{e}	1536.1 ± 1.9^{a}	540.4 ± 4.5^{d}	1.1 ± 0.1′	1.1 ± 0.3′
16	19.5	888	402, 500, 530, 607, 665	Pheophytin a allomer	-	-	-	-	tr	tr
17*	20.2 & 20.9	872	408, 505, 535, 608, 664	Pheophytin a	80.9 ± 1.0^{d}	26.7 ± 0.0^{e}	917.6 ± 0.9^{a}	416.1 ± 0.1^{c}	23.2 ± 0.0″	90.1 ± 6.5′
18	23.3	814	409, 507, 537, 608, 665	Pyropheophytin a	-	-	95.9 ± 0.1^{a}	35.7 ± 0.0^{c}	1.0 ± 0.0″	26.1 ± 1.9′
				Total Carotenoids	1609.2 ± 5.3^{c}	911.7 ± 1.3^{d}	2688.7 ± 4.1^{a}	963.0 ± 5.3^{d}	30.6 ± 0.4′	15.6 ± 1.5″
				Total Chlorophylls	4103.7 ± 0.4^{c}	1906.1 ± 0.1^{d}	5569.4 ± 0.9^{a}	1628.2 ± 3.0^{e}	175.3 ± 0.9′	138.9 ± 10.5′
				Total Pigments	**5712.9 ± 5.7^{c}**	**2817.8 ± 1.4^{d}**	**8258.1 ± 3.2^{a}**	**2556.2 ± 3.7^{e}**	**205.9 ± 1.3′**	**154.5 ± 12.0′**

P – peak number; RT – retention time (min); $[M+H]^+$ – positive molecular ion (*m/z*); λ_{max} - maximum wavelengths (nm); BB_fd1 – batch 1 broccoli by-products submitted to freeze-drying; BB_d40 – batch 1 broccoli by-products submitted to air-drying at 40ºC; bBB_fd – batch 1 broccoli by-products submitted to blanching, followed by freeze-drying; bBB_d40 - batch 1 broccoli by-products submitted to blanching, followed by air-drying at 40ºC; BB_fd2 - batch 2 broccoli by-products submitted to freeze-drying; BB_MHG - batch 2 broccoli by-products submitted to microwave hydrodiffusion and gravity (MHG); *epimers eluting in different RT; sh –indicate a shoulder in λ_{max}; - not detected; tr – traces were detected. Values are expressed as µg/g of broccoli by-product dry weight. In each line different letters (a,b,c, and d) or different symbols (′ and ″) mean significant differences ($p < 0.001$).

obtained. On the other hand, chlorophylls reduction was also due to hydrodiffusion of these compounds, as collected extracts were green coloured (**Figure 2.4**).

3.3.2. Impact of blanching and dehydration on glucosinolates

Glucosinolates were analysed after 70% methanol extraction and UHPLC-DAD-ESI/MS^n analysis using an elution program that allowed to identify both glucosinolates (**Figure 3.3**) and phenolic compounds (**Figure 3.4**). In opposition to pigments, the first batch of broccoli by-products presented lower amounts of total glucosinolates than the second (5.00 mg/g dw and 7.85 mg/g dw, respectively). Differences between glucosinolates and pigments could be attributed agronomic operations, cultivars, edaphoclimatic conditions, and previous processing (Alanís-Garza, Becerra-Moreno, Mora-Nieves, Mora-Mora, & Jacobo-Velázquez, 2015; Bell & Wagstaff, 2017).

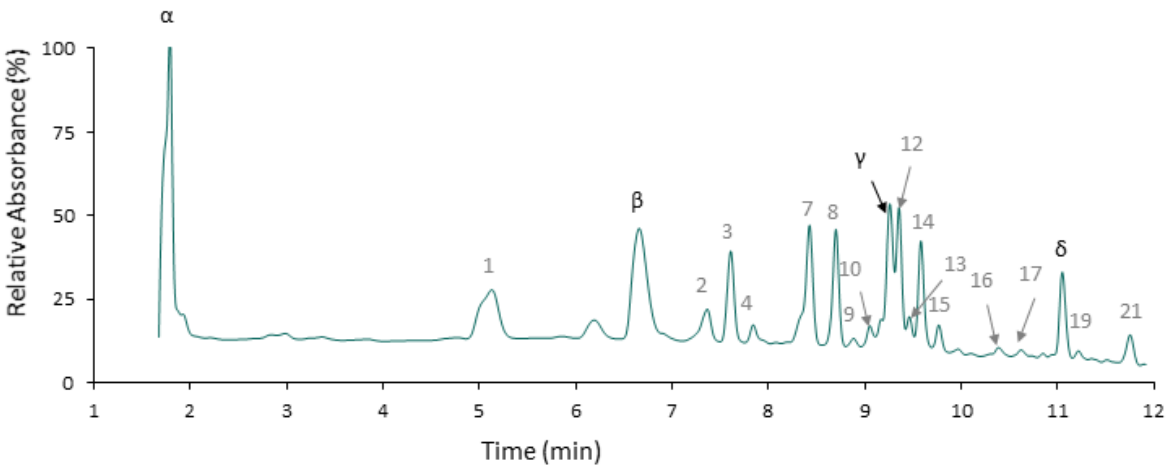


Figure 3.3. Representative chromatogram from 1.5-12 min at 227 nm (for glucosinolates detection), from the first batch of broccoli by-products after freeze-drying with a pre-blanching step (bBB_fd). Greek letters (glucosinolates) and numbers (phenolic compounds) in the chromatogram correspond to the UHPLC-DAD-ESI-MSn peaks described in **Table 3.5** and **3.6**, respectively.

Four glucosinolates were identified: glucoraphanin, an aliphatic compound, and 3 indoles (glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin) (**Table 3.5**). Glucoraphanin was the main glucosinolate, representing 74% and 48% of total glucosinolates in the first and second batches, respectively. The profile of glucosinolates in these broccoli by-products was similar to that observed for broccoli stalks, leaves, and inflorescences by-products (3 to 15 mg/g dw) (Dominguez-Perles, Moreno, Carvajal, &

Garcia-Viguera, 2011; Liu et al., 2018) and broccoli heads (Bell & Wagstaff, 2017; Latté, Appel, & Lampen, 2011).

Table 3.5. Identification and quantification of glucosinolates from dehydrated broccoli by-products analysed by UHPLC-DAD-ESI-MS^n.

P	RT	Probable compound $[M-H]^-$: Main MS^2 $[M-H]^-$	Batch 1				Batch 2	
			BB_fd1	BB_d40	bBB_fd	bBB_d40	BB_fd2	BB_MHG
α	1.8	**Glucoraphanin** **436:** 372(100); 259(2)	2.41 ± 0.13^a	2.24 ± 0.12^a	2.34 ± 0.08^a	1.42 ± 0.24^b	5.80 ± 0.18′	5.64 ± 0.14′
β	6.7	**Glucobrassicin** **447:** 259(100)	1.02 ± 0.03^b	1.21 ± 0.08^{ab}	1.36 ± 0.04^a	0.52 ± 0.05^c	1.28 ± 0.03′	0.20 ± 0.00″
γ	9.3	**4-methoxyglucobrassicin** **477:** 259(100); 274(60)	0.65 ± 0.04^a	0.66 ± 0.00^a	0.77 ± 0.00^a	0.28 ± 0.03^b	0.42 ± 0.00′	0.11 ± 0.00″
δ	11.1	**Neoglucobrassicin** **477:** 446 (100); 259(2)	0.92 ± 0.06^a	1.05 ± 0.04^a	0.47 ± 0.01^b	0.28 ± 0.01^b	0.34 ± 0.00′	0.12 ± 0.00″
		Total (μg/g)	**5.00 ± 0.23^a**	**5.15 ± 0.05^a**	**4.93 ± 0.04^a**	**2.50 ± 0.33^b**	**7.85 ± 0.15′**	**6.06 ± 0.14″**

P – peak number; RT – retention time (min); $[M-H]^-$: Main MS^2 $[M-H]^-$ – molecular ion and main product ions observed with percentage in brackets (m/z), other ions were found although not discriminated; BB_fd1 – batch 1 broccoli by-products submitted to freeze-dried; BB_d40 – batch 1 broccoli by-products submitted to air-drying at 40ºC; bBB_fd – Batch 1 broccoli by-products submitted to blanching, followed by freeze-drying; bBB_d40 - Batch 1 broccoli by-products submitted to blanching, followed by air-drying at 40ºC; BB_fd2 - Batch 2 broccoli by-products submitted to freeze-drying; BB_MHG - Batch 2 broccoli by-products submitted to microwave hydrodiffusion and gravity (MHG);Values are expressed as mg/g of dry weight. In each line different letters (a,b,c, and d) or different symbols (′ and ″) mean significant differences ($p < 0.001$).

Air-drying at 40 ºC did not impact significantly the total amount of glucosinolates. However, when broccoli by-products were blanched before air-drying, levels of total glucosinolates decreased by 49%. Glucobrassicin and 4-methoxyglucobrassicin were the most affected ones, decreasing by 61 and 64%, respectively. As observed for pigments, promotion of cell disruption by blanching (Alanís-Garza et al., 2015) led to glucosinolates degradation at 40 ºC, owing to non-enzymatic degradation (Hanschen et al., 2012), and/or residual myrosinase activity due to partial blanching inactivation (Oliviero et al., 2013), responsible for the degradation of glucosinolates into isothiocyanates.

MHG dehydration reduced to 23% the amount of total glucosinolates. Glucoraphanin amount was not affected by this dehydration, but indole glucosinolates glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicina were reduced by 85, 75, and 65%, respectively, in accordance with the observed in other dehydration processes at high temperatures (Lafarga, Bobo, Viñas, Collazo, & Aguiló-Aguayo, 2018; Mrkic et al., 2010; Tabart, Pincemail, Kevers, Defraigne, & Dommes, 2018). From the total glucosinolates initially accounted in freeze-dried by-products, only 50% were lost due to degradation along

MHG dehydration, as the remaining were hydrodiffused and recovered in the aqueous extracts (**Chapter 2**, **Section 2.3.2.4**).

3.3.3. Impact of blanching and dehydration on phenolic compounds

The first batch of broccoli by-products presented lower amounts of total phenolic compounds than the second batch (2.20 mg/g dw and 10.6 mg/g dw, respectively), thus following a similar tendency to glucosinolates and contrasting with that observed for pigments. The main phenolic compounds found in broccoli by-products comprised glycosylated flavonoids (quercetin and kaempferol), either non-esterified or esterified with one or two hydroxycinnamic acids (caffeic, methoxycaffeic, sinapic, ferulic, and coumaric acids), as well as hydroxycinnamic acids esterified with carbohydrates (gentiobiose) or with quinic acid, which overall could be divided in six groups (**Figure 3.4**, **Table 4.6**). Notably, the 42 compounds identified were previously reported to occur in broccoli heads (Cartea, Francisco, Soengas, & Velasco, 2011; Vallejo, Tomás-Barberán, & Ferreres, 2004), broccoli sprouts (Sun et al., 2013), and other *Brassica* (Fiol et al., 2012; Francisco et al., 2009; Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003). However, the exception is for the isomers with parent ions at *m/z* 807 and 817 and the compound with parent ion at *m/z* 563, which were herein tentatively assigned to kaempferol derivatives, according to their elution order, absorbance spectra and MS-MS data. As observed in **Table 4.6**, albeit both batches of broccoli contained the same phenolic compounds, their specific concentrations were variable between them, a fact that has been reported before and accepted to depend on plant part and environmental factors (Cartea et al., 2011).

Air-drying reduced the amount of total phenolics by 30%. Individually, 19 phenolic compounds had a reduction in their abundance (17% to 79%), 19 were not significantly affected, while 4 of them increased their availability with air-drying, namely kaempferol-3-caffeoylsinapoylsophorotrioside-7-sophoroside (47%), kaempferol-3-methoxycaffeoylsinapoylsophorotrioside-7-glucoside (47%), 1,2,2′-trisynapoylgentiobioside (27%), and sinapoyldiferuloylgentiobiose (23%). To our knowledge, this is the first report of air-drying impact on each phenolic compound of *Brassica*. It was only reported the impact of air-drying (50-100ºC) on kaempferol, when analysed by HPLC as aglycon after hydrolysis of the parent glycosides, showing contrasting results as kaempferol increased by 12% to 22% after air-drying (Mrkic et al., 2006).

However, these authors also observed that total polyphenols decreased by 13% to 56% when analysed after reaction with Folin–Ciocalteu reagent, a colorimetric assay, indicating the reduction of antioxidant compounds in general, including the most reactive phenolic compounds.

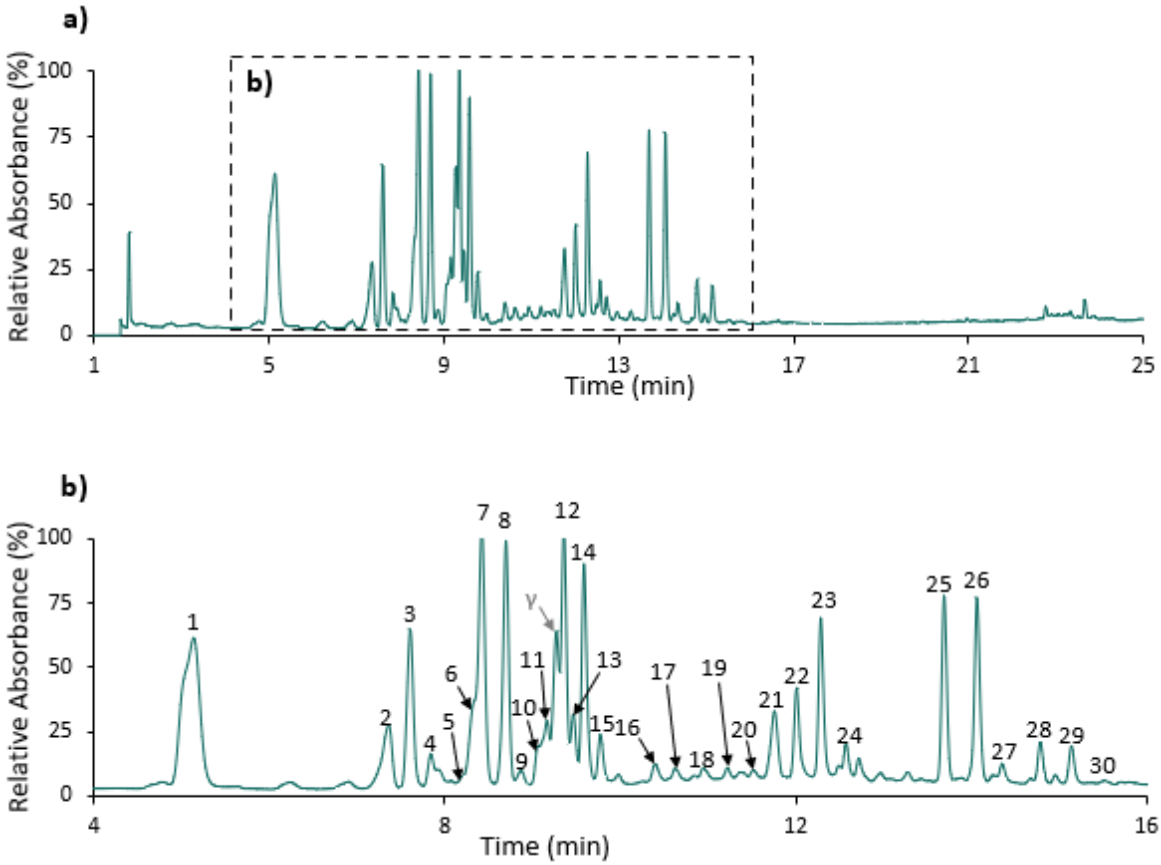


Figure 3.4. Representative chromatogram from a) 1–25 min of phenolic compounds (at 340 nm), from the first batch of broccoli by-products after freeze-drying with a pre-blanching step (bBB_fd). b) Inset shows expanded region of the chromatogram with retention time of 4–16 min. The numbers in the chromatogram correspond to the UHPLC-DAD-ESI-MS^n peaks described in **Table 3.6**.

The sequential blanching and freeze-drying of broccoli by-products resulted in an incremented recovery of phenolic compounds (51%), compared to freeze-dried by-products. Despite that, two monoacylated kaempferol glucosides, namely kaempferol-3-sinapoylsophorotrioside-7-sophoroside (33%) and kaempferol-3-sinapoylsophorotrioside-7-glucoside (72%), were significantly reduced. The increase of total phenolic content was already observed for broccoli subjected to blanching (Alanís-Garza et al., 2015; Cai et al., 2016), although also without individual identification of phenolic compounds. Moreover,

Table 3.6. Identification and quantification of phenolic compounds from dehydrated broccoli by-products analysed by UHPLC-DAD-ESI-MS^n.

P	RT	[M-H]$^-$: Main MS^2 [M-H]$^-$	λ_{max}	Probable compound	Batch 1				Batch 2	
					BB_fd1	BB_d40	bBB_fd	bBB_d40	BB_fd2	BB_MHG
Monoacylated quercetin glucosides										
2	**7.4**	**949:** 787 (100)	228, 266, 316, 343sh, 368sh$^{\#}$	Q-3-C-dG-7-G	66.9 ± 1.2^{b}	60.1 ± 0.9^{b}	80.3 ± 0.6^{a}	50.8 ± 0.9^{c}	46.3 ± 0.2′	47.4 ± 0.5′
9	**8.9**	**993:** 831 (100); 787 (40)	243, 275sh, 317, 368sh	Q-3-S-dG-7-G	263.5 ± 6.3^{a}	193.0 ± 5.1^{b}	304.7 ± 13.4^{a}	176.1 ± 3.3^{b}	128.1 ± 2.4′	133.9 ± 2.1′
Kaempferol glucosides										
2	**7.4**	**817:** 655 (100)	228, 266, 316, 343sh, 368sh$^{\#}$	K derivative	14.6 ± 0.7^{b}	10.6 ± 0.5^{b}	22.7 ± 0.4^{a}	5.0 ± 0.5^{c}	2.4 ± 0.1′	3.0 ± 0.3′
2	**7.4**	**807:** 609 (100)	228, 266, 316, 343sh, 368sh$^{\#}$	K derivative	10.0 ± 0.5^{b}	7.2 ± 0.4^{b}	15.5 ± 0.3^{a}	3.4 ± 0.4^{c}	1.6 ± 0.1′	2.0 ± 0.2′
3	**7.6**	**771:** 609 (100)	228sh, 265, 320sh, 349, 368sh$^{\#}$	K-3-dG-7-G	476.9 ± 5.0^{b}	222.9 ± 3.2^{c}	855.5 ± 23.6^{a}	162.0 ± 17.6^{c}	17.1 ± 0.9″	36.1 ± 0.6′
3	**7.6**	**817:** 655 (100)	228sh, 265, 320sh, 349, 368sh$^{\#}$	K derivative	15.8 ± 0.2^{b}	7.4 ± 0.1^{c}	28.4 ± 0.8^{a}	5.4 ± 0.6^{c}	0.6 ± 0.0″	1.2 ± 0.0′
3	**7.6**	**807:** 609 (100)	228sh, 265, 320sh, 349, 368sh$^{\#}$	K derivative	55.5 ± 0.6^{b}	26.0 ± 0.4^{c}	99.6 ± 2.8^{a}	18.9 ± 2.1^{c}	2.0 ± 0.1″	4.2 ± 0.1′
4	**7.9**	**933:** 771 (100)	229sh, 265, 355, 368sh	K-3-dG-7-dG	227.5 ± 6.5^{a}	73.0 ± 5.7^{b}	221.6 ± 15.5^{a}	42.5 ± 4.5^{b}	13.6 ± 1.7″	47.1 ± 2.8′
5	**8.2**	**563**	222sh, 239, 331	K derivative	tr	tr	tr	tr	50.1 ± 0.6″	64.0 ± 0.7′
17	**10.6**	**609:** 447 (100)	244, 269, 344, 368sh$^{\#}$	K-3-G-7-G	1.9 ± 0.2^{a}	1.8 ± 0.1^{a}	2.8 ± 0.1^{a}	0.5 ± 0.0^{b}	1.0 ± 0.1′	0.9 ± 0.0′
19	**11.4**	**609:** 429 (100)	245, 267, 355, 368sh	K-3-dG	13.0 ± 0.9^{ab}	17.2 ± 1.5^{ab}	27.9 ± 1.7^{a}	8.0 ± 0.2^{b}	2.8 ± 0.5′	0.6 ± 0.3′
Monoacylated kaempferol glucosides										
7	**8.4**	**963:** 801 (100); 771 (20)	238, 269, 332, 368sh	K-3-MC-dG-7-G	797.3 ± 23.8^{b}	575.7 ± 35.2^{b}	1875.1 ± 64.2^{a}	475.0 ± 47.0^{b}	38.5 ± 0.8″	112.1 ± 2.9′
8	**8.7**	**933:** 771 (100); 609 (5)	243, 268, 335$^{\#}$	K-3-C-dG-7-G	731.5 ± 3.3^{b}	412.8 ± 30.2^{c}	1324.6 ± 54.9^{a}	335.0 ± 35.6^{c}	29.4 ± 1.6″	66.5 ± 3.7′
8	**8.7**	**1095:** 771 (100); 609 (37)	243, 268, 335$^{\#}$	K-3-C-tG-7-G	151.9 ± 0.7^{b}	85.7 ± 6.3^{c}	275.2 ± 11.4^{a}	69.6 ± 7.4^{c}	6.1 ± 0.3″	13.8 ± 0.8′
10	**9.1**	**1301**	241, 270, 332, 368sh	K-3-S-tG-7-dG	197.0 ± 6.8^{a}	161.8 ± 14.6^{ab}	132.8 ± 12.5^{b}	63.2 ± 3.0^{c}	9.9 ± 1.7′	17.6 ± 0.8′
11	**9.2**	**1139:** 815 (100); 609 (29)	240, 269, 333, 368sh	K-3-S-tG-7-G	387.1 ± 14.5^{a}	81.8 ± 8.0^{b}	108.2 ± 8.1^{b}	116.4 ± 9.7^{b}	18.7 ± 5.2″	110.6 ± 1.9′
12	**9.3**	**977:** 815 (100); 609 (15)	239, 268, 334, 368sh	K-3-S-dG-7-G	823.0 ± 23.5^{a}	390.7 ± 20.8^{b}	851.8 ± 26.5^{a}	199.1 ± 11.5^{c}	tr	23.9 ± 4.4
13	**9.5**	**1109**	243, 268, 338, 368sh	K-3-F-tG-7-G	257.2 ± 10.0^{a}	84.0 ± 5.1^{b}	222.8 ± 14.1^{a}	46.6 ± 2.4^{b}	8.4 ± 1.3′	15.5 ± 1.2′
14	**9.6**	**947:** 785 (100); 771 (75)	242, 268, 333, 368sh	K-3-F-dG-7-G 1	687.2 ± 11.5^{b}	353.7 ± 20.2^{c}	1175.7 ± 17.0^{a}	242.0 ± 16.7^{d}	3.4 ± 0.4″	10.0 ± 1.1′
15	**9.8**	**917:** 755 (100);771 (60)	239, 367, 320, 348sh, 368sh$^{\#}$	K-3-Co-dG-7-G 1	107.9 ± 8.7^{b}	64.9 ± 3.0^{c}	153.9 ± 4.3^{a}	31.2 ± 3.1^{d}	2.4 ± 0.7″	17.6 ± 1.1′
17	**10.6**	**917:** 755 (100)	244, 269, 344, 368sh$^{\#}$	K-3-Co-dG-7-G 2	30.8 ± 2.6^{a}	29.9 ± 2.3^{a}	46.1 ± 2.2^{a}	8.1 ± 0.6^{b}	16.3 ± 2.1′	15.0 ± 0.6′
17	**10.6**	**947:** 785 (100); 609 (2)	244, 269, 344, 368sh$^{\#}$	K-3-F-dG-7-G 2	21.5 ± 1.9^{a}	21.0 ± 1.6^{a}	32.3 ± 1.5^{a}	5.7 ± 0.4^{b}	11.4 ± 1.5′	10.5 ± 0.4′
Diacylated kaempferol glucosides										
20	**11.5**	**1493:** 977 (100); 1169 (71)	244, 270, 340, 368sh	K-3-MC/S-tG-7-dG	41.7 ± 2.9^{b}	33.7 ± 3.1^{bc}	90.7 ± 3.7^{a}	11.2 ± 0.0^{c}	tr	1.5 ± 0.3
21	**11.8**	**1463:** 1139 (100)	242, 270, 328, 367sh$^{\#}$	K-3-C/S-tG-7-dG	87.8 ± 1.4^{c}	128.7 ± 4.7^{b}	191.2 ± 6.2^{a}	41.7 ± 3.6^{d}	16.7 ± 0.6″	91.2 ± 1.9′
21	**11.8**	**1331:** 1169 (100)	242, 270, 328, 367sh$^{\#}$	K-3-MC/S-tG-7-G	275.9 ± 4.5^{c}	404.6 ± 14.6^{b}	601.1 ± 19.4^{a}	131.1 ± 11.2^{d}	52.6 ± 2.0″	286.6 ± 6.0′
22	**12.0**	**1301:** 1139 (100)	242, 269, 330, 368sh$^{\#}$	K-3-C/S-tG-7-G	296.7 ± 2.8^{b}	246.4 ± 8.0^{b}	556.8 ± 35.3^{a}	105.1 ± 10.8^{c}	40.5 ± 0.7″	58.8 ± 0.5′

22	**12.0**	**1507**	242, 269, 330, 368sh[#]	K-3-dS-tG-7-dG	147.2 ± 1.4[b]	122.2 ± 4.0[b]	276.1 ± 17.5[a]	52.1 ± 5.4[c]	20.1 ± 0.3″	29.2 ± 0.2′
23	**12.3**	**1345:** 977 (100)	241, 269, 330, 368sh[#]	K-3-dS-tG-7-G	668.7 ± 23.1[b]	466.6 ± 22.5[c]	1164.3 ± 21.2[a]	244.7 ± 29.3[d]	88.8 ± 4.9″	148.0 ± 1.2′
23	**12.3**	**1477:** 1153 (100)	241, 269, 330, 368sh[#]	K-3-F/S-tG-7-dG	79.2 ± 2.7[b]	55.3 ± 2.7[c]	137.9 ± 2.5[a]	29.0 ± 3.5[d]	10.5 ± 0.6″	17.5 ± 0.1′
24	**12.6**	**1315:** 1153 (100)	243, 269, 330, 368sh	K-3-F/S-tG-7-G	142.5 ± 8.6[b]	83.0 ± 5.4[c]	231.1 ± 4.8[a]	46.1 ± 3.2[c]	3.1 ± 0.3″	23.8 ± 3.9′
Esters of hydroxycinnamic acids with gentiobiose										
15	**9.8**	**431:** 385 (100)	239, 367, 320, 348sh, 368sh[#]	Sinp-Glc + formic acid	70.6 ± 2.9[b]	56.3 ± 1.0[c]	85.9 ± 1.4[a]	45.1 ± 1.0[d]	35.5 ± 0.2″	40.5 ± 0.4′
18	**10.9**	**885:** 867 (100)	243, 275sh, 317, 368sh	SinpCafFer-Gentb	118.8 ± 4.2[b]	116.4 ± 4.3[b]	140.2 ± 2.6[a]	90.1 ± 0.8[c]	78.5 ± 0.4′	73.8 ± 1.0′
25	**13.7**	**753:** 529 (100)	241, 330, 368sh	diSinp-Gentb	649.9 ± 5.5[b]	563.5 ± 48.7[b]	886.2 ± 24.8[a]	298.8 ± 21.6[c]	292.7 ± 8.9′	197.2 ± 1.7″
26	**14.1**	**723:** 499 (100)	241, 273sh, 329	SinpFer-Gentb	588.6 ± 3.6[b]	451.7 ± 18.4[c]	904.3 ± 25.4[a]	250.1 ± 21.1[d]	291.1 ± 12.7′	245.8 ± 3.8′
27	**14.4**	**693:** 469 (100)	246, 326	SinpCoum-Gentb	101.4 ± 4.7[b]	104.3 ± 4.0[ba]	133.8 ± 4.2[a]	68.8 ± 0.8[c]	80.7 ± 4.7′	74.0 ± 0.6′
28	**14.8**	**959:** 735 (100)	244, 323	triSinp-Gentb	315.6 ± 13.7[a]	399.8 ± 24.2[a]	325.9 ± 18.6[a]	210.7 ± 15.8[b]	233.5 ± 11.1′	176.8 ± 6.9′
29	**15.2**	**929:** 705 (100)	245, 320	diSinpFer-Gentb	252.2 ± 4.2[b]	245.8 ± 7.5[b]	306.2 ± 2.8[a]	137.8 ± 8.1[c]	201.5 ± 3.6′	174.5 ± 3.0″
30	**15.5**	**899:** 705 (100)	239, 327	SinpdiFer-Gentb	90.0 ± 1.5[b]	111.0 ± 2.5[a]	98.3 ± 1.5[ab]	tr	89.5 ± 1.1′	98.8 ± 1.6′
Esters of hydroxycinnamic acids with quinic acid										
1	**5.2**	**353:** 191 (100); 179 (45)	200, 263sh, 289	5-CQA	721.6 ± 10.2[b]	456.2 ± 41.3[c]	1059.8 ± 3.5[a]	336.4 ± 22.5[c]	74.1 ± 2.6′	64.0 ± 2.1′
2	**7.4**	**337:** 163 (100); 191 (6);	228, 266, 316, 343sh, 368sh[#]	4-p-CoQA	405.3 ± 16.2[b]	311.1 ± 12.3[b]	592.3 ± 8.8[a]	182.7 ± 12.5[c]	120.3 ± 2.5′	134.9 ± 6.8′
6	**8.3**	**367:** 193 (100); 134 (4)	240, 327	4-FQA	111.7 ± 2.5[b]	88.9 ± 3.6[b]	222.8 ± 6.6[a]	78.5 ± 4.8[b]	33.5 ± 0.2″	41.1 ± 0.3′
16	**10.4**	**367:** 191 (100); 173 (64)	241, 277sh, 332, 369sh	5-FQA	56.5 ± 2.0[b]	47.8 ± 1.5[bc]	78.8 ± 0.6[a]	41.3 ± 1.5[c]	tr	34.1 ± 0.4
				Total	**10605.5 ± 148.6[b]**	**7419.7 ± 378.0[c]**	**15986.7 ± 270.3[a]**	**4588.7 ± 319.8[d]**	**2203.0 ± 50.9″**	**2765.5 ± 2.0′**

P – peak number; RT – retention time (min); $[M-H]^-$: Main MS^2 $[M-H]^-$ – molecular ion and main product ions observed with percentage in brackets (*m/z*), other ions were found although not discriminated; λ_{max} - maximum wavelengths (nm); $^{\#}\lambda_{max}$ of co-eluting compounds; sh –indicate a shoulder in λ_{max}; tr – traces were detected. BB_fd1 – batch 1 broccoli by-products submitted to freeze-dried; BB_d40 – batch 1 broccoli by-products submitted to air-drying at 40ºC; bBB_fd – Batch 1 broccoli by-products submitted to blanching, followed by freeze-drying; bBB_d40 - Batch 1 broccoli by-products submitted to blanching, followed by air-drying at 40ºC; BB_fd2 - Batch 2 broccoli by-products submitted to freeze-drying; BB_MHG - Batch 2 broccoli by-products submitted to microwave hydrodiffusion and gravity (MHG);CQA: caffeoylquinic acid; *p*-CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acid. Q: quercetin; dG: sophoroside; G: Glc; K: kaempferol; MC: methoxycaffeoyl; C:Caf: caffeoyl; tG: sophorotrioside; S: Sinp: sinapoyl; F: Fer: Feruloyl; Co:Coum: Coumaroyl; Gentb: gentiobiose. Values are expressed as µg/g of by-products dry weight. In each line different letters (a,b,c, and d) or different symbols (′ and ″) mean significant differences ($p < 0.001$).

this increase was hypothesized as a result of cell lysis (Cai et al., 2016) with release of bound phenolics and consequent promotion of their extraction (Gonzales et al., 2015; Harbaum, Hubbermann, Zhu, & Schwarz, 2008). However, when blanching preceded air-drying, the amount of recovered phenolic compounds decreased by 71%. In this case, only kaempferol-3-sinapoylsophorotrioside-7-glucoside was not affected by the dehydration process. These results showed that, for phenolics preservation, air-drying did not benefit from blanching pre-treatment, possibly due to the promotion of oxidation processes.

Similarly to what was observed when blanching preceded freeze-drying, MHG dehydration increased the amount of recovered phenolic compounds (26%). However, the impact of MHG dehydration was different for each group of compounds. Among esters of hydroxycinnamic acids and quinic acid, feruloylquinic acid isomers increased 2.2 folds by increase of isomer 5-feruloylquinic acid from trace amounts. Regarding kaempferol derivatives, kaempferol glucosides increased 74% and kaempferol glucosides esterified with hydroxycinnamic acids increased 3 folds. These results can be explained by the higher stability of kaempferol derivatives to thermal treatments (Wu, Zhao, Haytowitz, Chen, & Pehrsson, 2019). On the other hand, esters of hydroxycinnamic acids and gentiobiose, which represent 59% of total phenolics, decreased by about 17%. It should be noted that the decrease of these compounds after drying cannot be associated only to degradation, but also to their hydrodiffusion to the aqueous extracts recovered during MHG drying, where these compounds were detected (**Chapter 2**, **Section 2.3.2.4**).

3.3.4. Impact of blanching and dehydration techniques on broccoli by-products valorisation

The potential of each processing technique to dehydrate and valorise broccoli by-products was studied after estimation of energy consumption and by means of principal component analysis (PCA). As observed in **Table 3.7**, MHG dehydration was the faster processing technique and the one that spent less energy (1350 J) to dehydrate broccoli by-products. Oven air-drying needed 53 to 83 more time and 32 to 51 more energy than MHG dehydration, depending on the application or the absence of a blanching pre-treatment, respectively. Freeze-drying was the most time and energy consuming technique. The scale up of these technologies to industrial level can be adopted according the composition needed, this is, the amount of pigments, glucosinolates, and phenolics. However, it should be

reminded that presently the biggest MHG apparatus only allow to process a volume of broccoli by-products of 70 L (MAC70 Milestone Srl, Italy) (Périno et al., 2016).

Table 3.7. Energy consumption for each processing technique used to dehydrate broccoli by-products.

Processing technique	Equipments	Time of operation (h)	Energy consumption (kJ)
Freeze-drying	Freezer, freeze-drier, and vacuum pump	168	131 328
Air-drying	Oven	64	69 120
Freeze-drying with a pre-blanching step	Microwave, freezer, freeze-drier, and vacuum pump	168	131 616
Air-drying with a pre-blanching step	Microwave and oven	40	43 560
MHG dehydration	MHG apparatus	0.75	1 350

PCA was applied to all dehydrated samples to determine the main sources of variability based on samples pigments, glucosinolates, and phenolic compounds. **Figure 3.5.a** shows the scores plot (PC1 x PC2) of the data provided where six groups were obtained. These two axes contain 88.8% of the total variance (PC1 =77.4% and PC2 = 11.4%). According to the loadings plot (**Figure 3.5.b**) samples with lower amounts of compounds were separated from samples with high amounts of compounds by PC1. Blanched samples (bBB_fd; bBB_d40; and BB_MHG) were separated from non-blanched samples (BB_fd1; BB_d40; and BB_fd2) by PC2, mainly due to chlorophyll derivatives contribution. These results corroborate that the different processing techniques allows to obtain dehydrated broccoli by-products with different characteristics and the processing techniques should be chosen according both final composition required and environmental concern (energy and time expenditures).

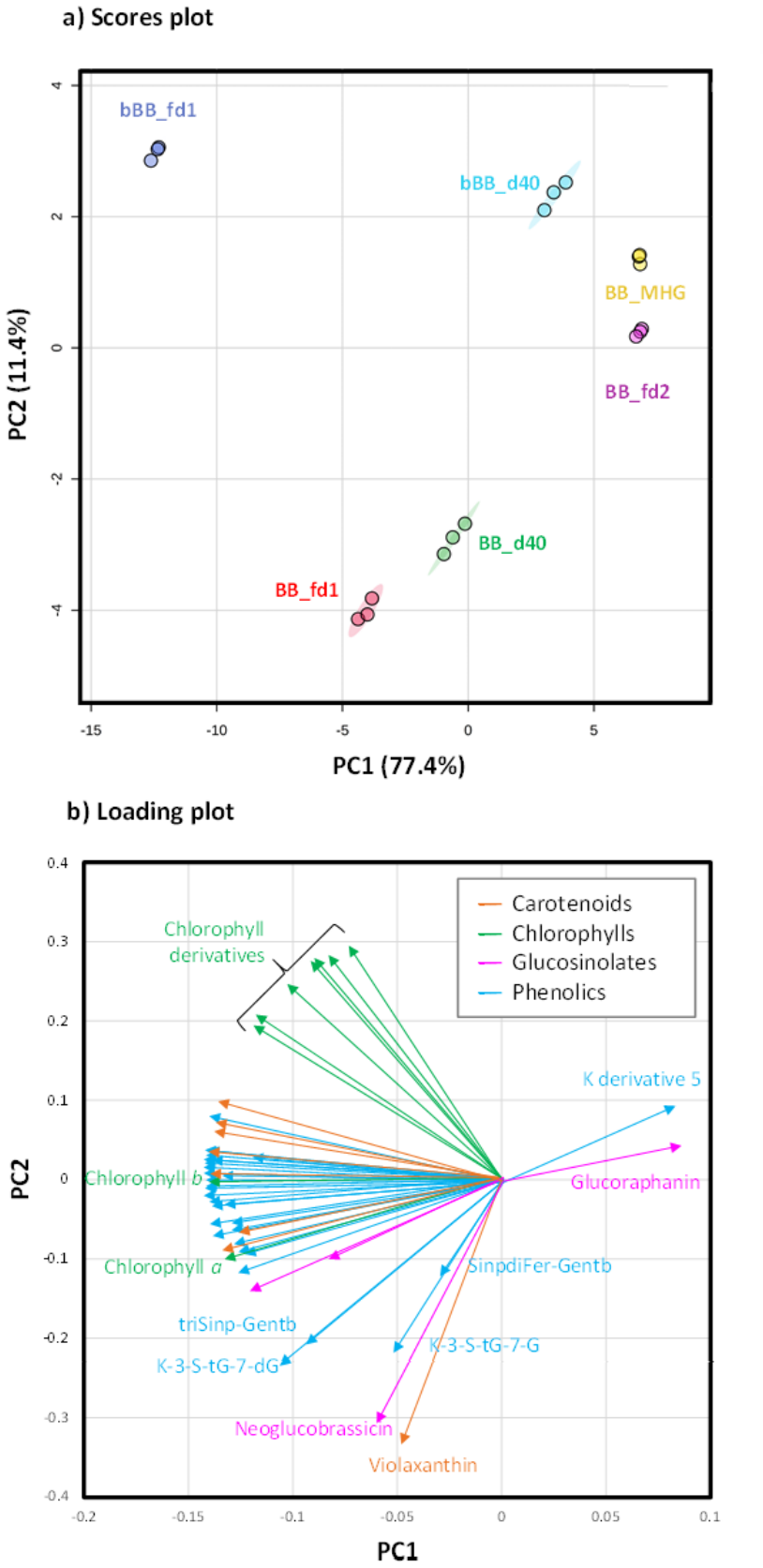


Figure 3.5. PCA of the pigments, glucosinolates and phenolic compounds from dehydrated broccoli by-products: **a)** scores scatter plot (PC1 vs PC2) and **b)** PC1 vs PC2 loadings.

3.4. Concluding remarks

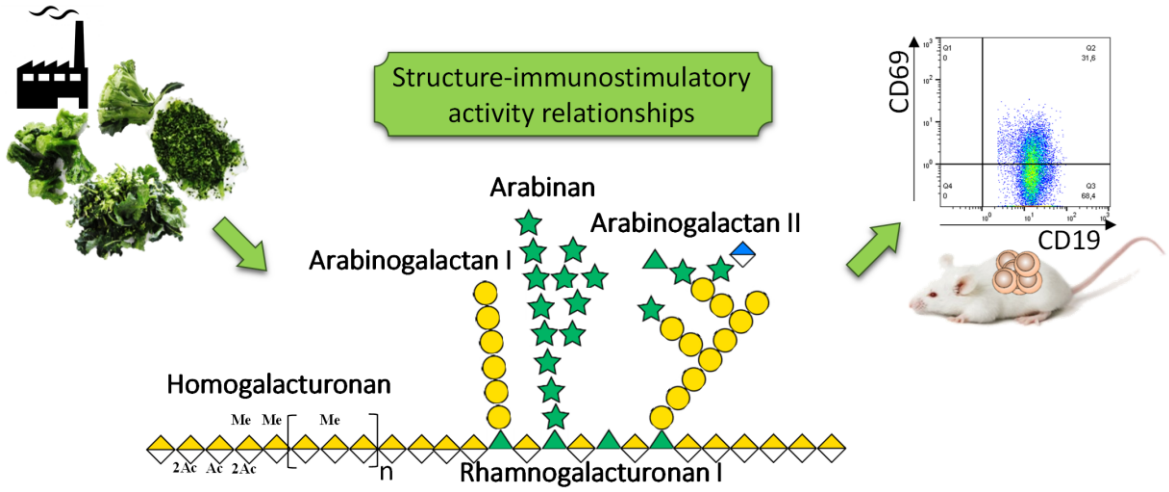
Dehydrated broccoli by-products were shown to represent a source of pigments, including carotenoids (16 to 2689 µg/g dw) and chlorophylls (139 to 5569 µg/g dw), glucosinolates (2502 to 7846 µg/g dw), and phenolic compounds (2203 to 15987 µg/g dw). Depending on the blanching pre-treatment and dehydration process applied, products with different profiles were obtained.

Air-drying at 40 ºC did not impact glucosinolates but only retained 49% of the pigments and 70% of phenolics. Air-drying benefited from a previously blanching step, by reduction of dehydration time. However, this step also promoted higher degradation of compounds along air-drying. MHG dehydration preserved the glucosinolate glucoraphanin, increased the total amount of phenolic compounds by 26%, with especial contribution of kaempferol glucosides, but had a negative impact on pigments by reducing the content of lutein and promoting the degradation of chlorophylls *a* and *b* into their derivatives. In comparison with freeze-drying, both air-drying and MHG dehydration technologies impacted pigments, glucosinolates, and phenolics at different extents. Nonetheless, these technologies offer benefits in stabilizing broccoli by-products by dehydration, in a more energetically friendly way, making them ready to be milled into flours and used as potential food ingredients, promoting broccoli by-products valorisation.

CHAPTER 4

Pectic polysaccharides from broccoli by-products with *in vitro* B lymphocytes stimulatory activity

Graphical abstract:



CHAPTER 4

Pectic polysaccharides from broccoli by-products with *in vitro* B lymphocytes stimulatory activity

4.1. Pectic polysaccharides from broccoli by-products with in vitro B lymphocytes stimulatory activity – approach

4.2. Material and methods

4.2.1. Broccoli by-products samples and materials

4.2.2. Preparation of AIR

4.2.3. Sequential extraction of pectic polysaccharides from AIR

4.2.4. Extraction of hot water (HW) soluble polysaccharides from AIR

4.2.5. Ethanol precipitation of HW

4.2.6. Fractionation and enzymatic treatments of HW and fraction Et80

4.2.7. Chemical sulfation of fraction Et80

4.2.8. Protein and free amino acid analysis

4.2.9. Characterization of carbohydrates

4.2.10. *In vitro* immunostimulatory activity assays

4.2.11. Detection of lipopolysaccharides

4.3. Results and discussion

4.3.1. Extraction and characterization of carbohydrates from broccoli by-products AIR

4.3.2. Evaluation of immunostimulatory activity of broccoli by-products pectic polysaccharides

4.3.3. Detailing of structural features from broccoli by-product pectic polysaccharides

4.4. Concluding remarks

Parts of the text of this chapter will be submitted in the following publication:

Sónia S. Ferreira, Alexandra Correia, Manuel Vilanova, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra (**2020**). Preparation of pectic polysaccharides from broccoli by-products with *in vitro* B lymphocytes stimulatory activity. (In preparation).

4.1. Pectic polysaccharides from broccoli by-products with *in vitro* B lymphocytes stimulatory activity – approach

Broccoli by-products are a source of bioactive compounds that have been exhaustively studied in literature, namely pigments, glucosinolates, and phenolics. Notably, broccoli by-products are also a source of pectic polysaccharides, which information about their structure and potential bioactivities is lacking. These polysaccharides comprise a diversity of structures dependent of plant source, stages of maturity, plant part, or previous processing. Pectic polysaccharides found in several plants have been related with potential immunostimulatory activity. Although main structural features of pectic polysaccharides from plant cell walls are well known for many years, their complex structure is far from being fully revealed, which puzzle the establishment of structure-function relationships. Following the current state of knowledge about polysaccharides immunostimulatory activity, the pectic polysaccharides from broccoli by-products were extracted, characterized, fractionated, and derivatized to study the *in vitro* potential immunostimulatory activity and to establish structure-function relationships. The data gathered in this study will also contribute to an integrated valorisation of broccoli by-products.

4.2. Materials and methods

4.2.1. Broccoli by-products samples and materials

Broccoli (*Brassica oleracea* var. Parthenon) by-products from the frozen food industry were provided by Monliz SA, Alpiarça, Portugal, in December 2015. The by-products obtained in frozen broccoli production line (**Chapter 2**, **Section 2.2.1**) were analysed separately as stalks, leaves, and inflorescences remains, or mixed. They were frozen and maintained under -20 ˚C until use. All reagents used were of analytical grade or higher available purity.

4.2.2. Preparation of AIR

To isolate polysaccharides from broccoli by-products cell walls and to remove low molecular weight compounds (Knee, 1973), AIR was prepared. Briefly, ethanol was added to 150 g of frozen material, previously blended, to obtain a final concentration of 80% (v/v). After boiling for 10 min, the mixture was cooled in a cold-water bath and filtered in fritted

funnel (G-1). The filtration residue was dispersed again in 80% (v/v) ethanol, boiled for 10 min, filtered, washed with 500 mL of ethanol and 500 mL of acetone, and allowed to dry at room temperature. The AIR obtained was further milled in a coffee grinder and the moisture content was determined by freeze-drying. This procedure was repeated, if necessary, to obtain more AIR material. Ethanol and acetone solutions were concentrated to remove the solvents and freeze-dried for further characterization of alcohol soluble material. Part of alcohol soluble material was suspended in water and dialysed using membranes with 12-14 kDa cut-off.

4.2.3. Sequential extraction of pectic polysaccharides from AIR

To extract pectic polysaccharides from broccoli by-products cell walls, AIR was submitted to sequential extractions with different solvents (water, imidazole, sodium carbonate, and alkaline solutions) usually used to disassemble and characterize plant cell wall polysaccharides (Nunes, Saraiva, & Coimbra, 2008) (**Figure 4.1**). The AIR (10,5 g) was sequentially extracted with: (1) 750 mL of water for 16 h at 4 ˚C; (2) 700 mL of water for 6 h at room temperature (RT, 25 ˚C); (3) 750 ml of 0.5 M imidazole/HCl pH 7.0 for 16 h at RT; (4) 700 mL of 0.5 M imidazole/HCl pH 6.5 for 2 h at RT; (5) 750 mL of 50 mM Na_2CO_3, 600 mL, for 16 h at 4 ˚C; (6) 700 mL of 50 mM Na_2CO_3, for 2 h at RT; (7) 500 mL of 0.5 M KOH for 30 min at RT; (8) 500 mL of 0.5 M KOH for 2 h at RT; (9) 600 mL 1 M KOH for 2 h at RT; (10) 500 mL of 4 M KOH for 2 h at RT; (11) 500 mL of 4 M KOH and 3.5% H_3BO_3 for 2 h at RT; and (12) 500 mL of 8 M KOH for 2 h at RT.

To prevent β-elimination, peeling reactions, and alkaline oxidation of the polysaccharides, Na_2CO_3 and KOH extractions, steps (5) to (12), were carried out with solutions containing 20 mM $NaBH_4$. Furthermore, extractions with KOH solutions, steps (7) to (12), were prepared with O_2-free solutions and under N_2.

After extraction steps (1) to (6), the solubilised polymers were separated from the insoluble residue by centrifugation (24,652 g for 30 min at 4 ˚C) followed by filtration of the supernatant through a fritted funnel (G-3). After extraction steps (3) to (6) and first centrifugation, the residue was resuspended in distilled water, stirred for 30 min at RT, and centrifuged (24,652 g for 30 min at 4 ˚C). The supernatant was filtrated through a fritted funnel (G-3) and combined with the respective extract. These extracts were concentrated and dialysed using membranes with 12-14 kDa cut-off.

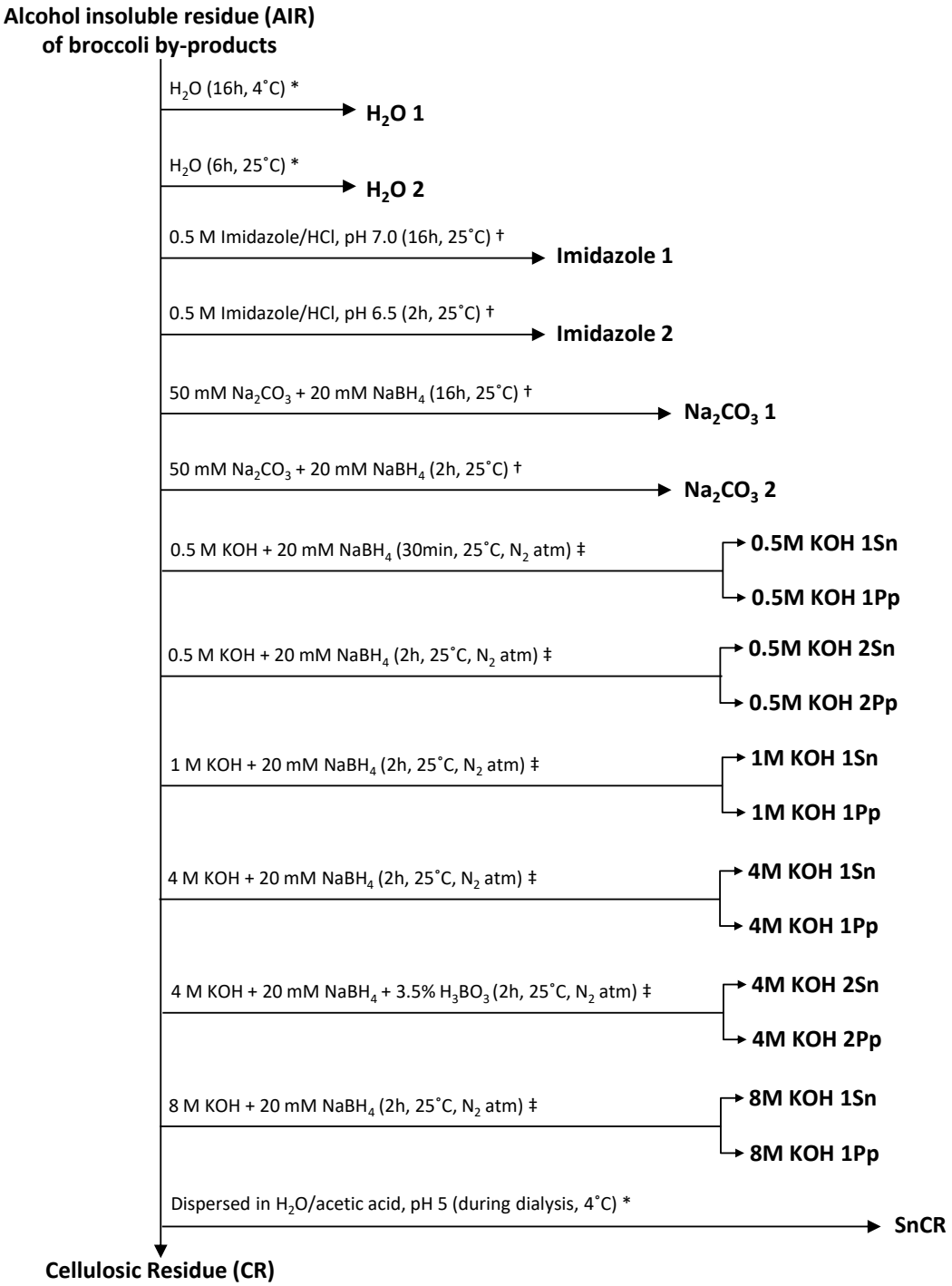


Figure 4.1. Scheme of polysaccharide sequential extractions from AIR. *Centrifuged (24,652 g, 4 ˚C, 30 min) to separate the supernatant, which was filtered (G-3), concentrated, and freeze-dried. †Centrifuged (24,652 g, 4 ˚C, 30 min) to separate the supernatant from the residue. The residue was dispersed in H_2O (30 min) and centrifuged. The supernatants were combined, filtered (G-3), concentrated, and freeze-dried. ‡Filtered in a fritted funnel (G-1) to separate the filtrate, which was acidified to pH 5 with acetic acid, dialysed, concentrated, centrifuged (24,652 g, 4 ˚C, 30 min), and freeze-dried to give a supernatant (Sn) and a precipitate (Pp).

After extraction steps (7) to (12), the solubilised polymers were separated from the insoluble residue by filtration through a fritted funnel (G-1), after centrifugation at 24,652 g for 30 min at 4 ˚C not being successful. The extracts were neutralised to pH 5–6, in a cold-water bath, with glacial acetic acid, prior to dialysis. The cellulosic residue (CR) obtained after the alkali extractions was suspended in water, neutralised (pH 5–6) and dialysed. After dialysis, extracts were concentrated under reduced pressure and precipitates were collected separately by centrifugation. The supernatant from the dialysis of the CR was collected separately from the residue by centrifugation and filtration. All extracts were frozen and freeze-dried.

4.2.4. Extraction of hot water (HW) soluble polysaccharides from AIR

To extract pectic polysaccharides with green solvents, AIR was submitted to a hot water extraction (Xu et al., 2015). Briefly, 1 g of AIR was hydrated with 100 mL of distilled water for 2 h, under constant stirring. Afterwards, it was boiled for 1 h, using a condenser to avoid liquid loss. The mixture was cooled in a cold-water bath and the soluble material was separated from un-extracted residue by centrifugation (24,652 g, 4ºC, for 30 min) followed by filtration in a fritted funnel (G-3). The soluble material was concentrated in a rotary evaporator and dialysed using membranes with 12-14 kDa cut-off (HW). HW and residue were freeze-dried for yield determination. HW carbohydrates were analysed and further purified, fractionated, and derivatized (**Figure 4.2**).

4.2.5. Ethanol precipitation of HW

Polysaccharides from HW were fractionated according their solubility in ethanol solutions. HW (100 mg) was hydrated by the addition of 20 mL of water, heated at 40 ºC, for 5 min, and vortexed. Some insoluble particles, less than 1% of Sol_AIR dry weight, were removed by centrifugation at 2,500 g, for 10 min. absolute ethanol was added to the soluble material to obtain a solution of 50% ethanol. After 2 h, at 4 ºC, no precipitate was observed. More absolute ethanol was added until reaching a concentration of 80% ethanol, that was kept for 2 h at 4 ºC before centrifugation (24,652 g, 10 min, 4 ºC), allowing to obtain the 80% ethanol soluble material (SnEt), which was separated from the precipitate (Et80) (**Figure 4.2**). Both fractions were evaporated under reduced pressure and freeze-dried for yield determination and carbohydrate analysis.

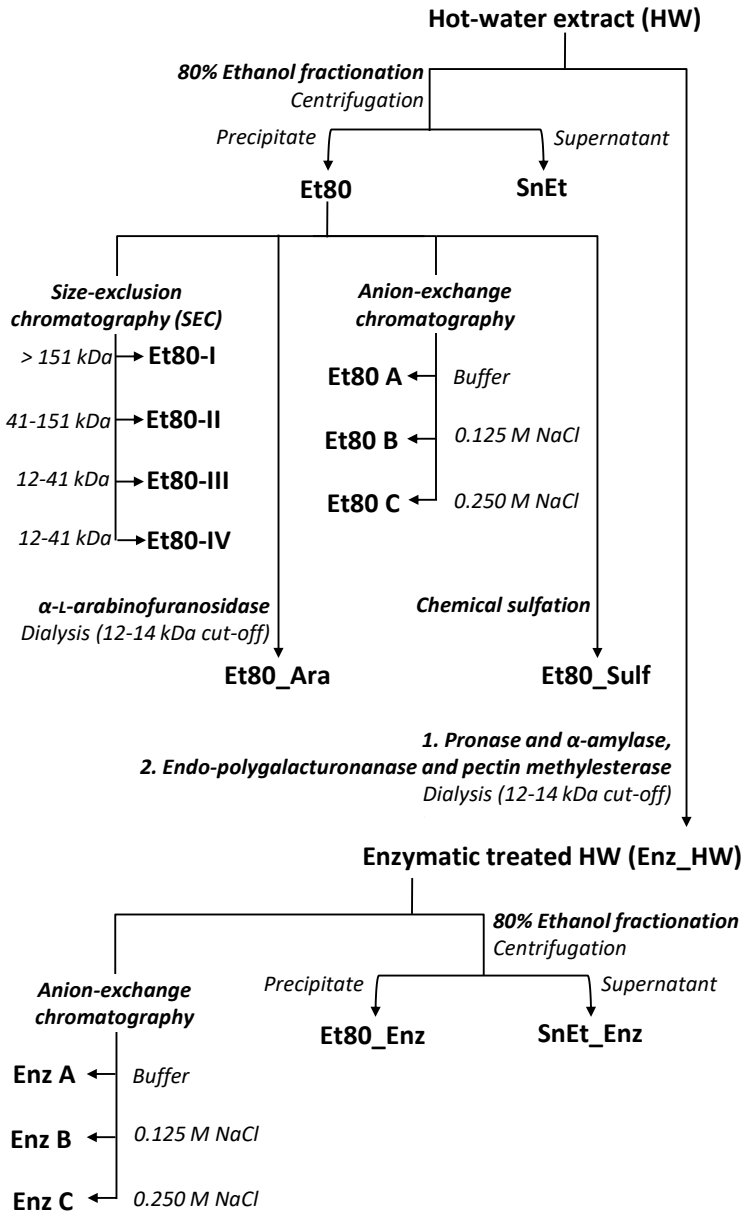


Figure 4.2. Scheme of hot water extraction and following fractionations from broccoli by-products.

4.2.6. Fractionation and enzymatic treatments of HW and fraction Et80

4.2.6.1. Size-exclusion chromatography of fraction Et80

To unveil the molecular weight of polysaccharides found in fraction Et80, this was fractionated in a size-exclusion chromatography (SEC) column (XK 16/100, Pharmacia) of Sephacryl S400 HR (10–2000 kDa fractionation range for dextrans, Pharmacia–Biotech). The fractionation was run using 200 mM phosphate buffer, pH 7, 200 mM NaCl, and 0.02% sodium azide as eluent, with a flow rate of 1.0 mL/min, at room temperature. The sample

(10 mg) was dissolved in 1 mL of the same elution buffer and then passed through the column. Fractions of 2 mL were collected and assayed for total sugars according to phenol-concentrated sulfuric acid method (**Section 4.2.12.2**) and at 280 nm for phenolics. The appropriate fractions were combined, dialysed and freeze-dried. The average molecular weight of each fraction was established after a calibration of the column with dextrans of average molecular weights 12 kDa (2.8 mg, 31418-100 mg sigma), 80 kDa (2.45 mg, 31421-100 mg sigma), and 270 kDa (2.84 mg, 31423 sigma). The void volume (V_0) was determined with blue dextran (2000 kDa) and the inner volume (V_t) of the column was determined with Glc.

4.2.6.2. α-L-Arabinofuranosidase treatment of fraction Et80

To study structures with terminal Ara in the Et80 fraction (30 mg), this was hydrolysed with 2 U of *Clostridium thermocellum* α-L-arabinofuranosidase 51 A (EC 3.2.1.55, Nzytech), purified from a recombinant *Escherichia coli* strain, for 48 h at 37 °C with continuous gentle stirring in 5 mL of 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide (Ferreira et al., 2018). After enzymatic treatment and enzyme denaturation by placing in boiling water for 5 min, the hydrolysed material was dialysed and freeze-dried to get sample Et80_Ara.

4.2.6.3. Enzymatic treatment of HW

Ramified regions of pectic polysaccharides from HW were purified by enzymatic treatment to remove protein, starch, and pectin. First, HW (20 mg) was solubilized in 200 mL of 50 mM phosphate buffer, pH 7, with 0.1% sodium azide. Pronase, a mixture of proteases (2 mg, from *Streptomyces griseus*, ROCHE), was added to this solution and incubated for 24 h, at 37 ºC, under constant stirring. Enzymes were denatured by placing in boiling water for 5 min. After cooling down, 10 µL of α-amylase (1210U/mg protein, 27 mg protein/mL, A-2643, Sigma), 10 µL of endo-polygalacturonase (5000 U/mL, M2, Megazyme), and 1 mL of pectin methylesterase (75.2 U/mL, from *Streptomyces avermitilis*, PROZOMIX) were added and incubated for 24 h, at 37 ºC, also under constant stirring. Enzymes were denatured as previous described and the solution was dialysed, using membranes with 12-14 kDa cut-off, before freeze-drying to obtain Enz_HW. Sample Enz_HW was fractionated according its solubility in ethanol solutions, as performed for

sample HW in **Section 4.2.5**. Fractionation allowed to obtain 80% ethanol soluble material (SnEt_Enz), which was separated from the precipitate (Et80_Enz).

4.2.6.4. Anion-exchange chromatography of fraction Et80 and Enz_HW

Anion-exchange chromatography on dietilaminoetil cellulose (DEAE)-Trisacryl M (1 mL of resin/7.5 µmol of UA, Sigma) was performed on a 40 × 16 mm column (XK 16/20, Pharmacia), at a flow rate of 0.4 mL/min. Et80 (20 mg) and Enz_AIR (10 mg) were dissolved in 1 mL of 50 mM Tris-HCl buffer, pH 7.4, 0.02% sodium azide without formation of insoluble material after centrifugation (2490 g, 15 min). Each sample was eluted with the same volume of Tris-HCl buffer, and buffer containing 0.125, 0.250, 0.500, and 1.00 M NaCl. Fractions of 1.9 mL were collected and assayed for total sugars according to phenol-concentrated sulfuric acid method (**Section 4.2.12.2**) and at 280 nm for phenolics. The fractions of interest were combined, dialysed, and freeze-dried.

4.2.7. Chemical sulfation of fraction Et80

Fraction Et80, rich in pectic polysaccharides, was sulfated to study the presence of this functional group in pectic polysaccharides immunostimulatory activity. Sulfation was performed using a methodology with chlorosulfonic acid and pyridine, according (Du et al., 2010). Et80 (100 mg) was suspended in formamide (10 mL) under agitation at room temperature for 15 min, followed by dropwise addition of sulfating reagent (286 µL chlorosulfonic acid and 1714 µL of pyridine). The reaction was stirred at room temperature for 2 h and maintained at 30 °C for 5 h with continuous stirring. After cooling to room temperature, the solution was neutralized with 15 % (w/v) NaOH solution, dialyzed (Mw cut-off 12-14 kDa) against distilled water, concentrated, and freeze-dried to obtain the sulfated polysaccharide (Sulf_Et80).

4.2.8. Protein and free amino acid analysis

Protein was quantified by the nitrogen content (%N×6.25) determined by elemental analysis (**Chapter 2, Section 2.2.5.3**). The amino acids from alcohol soluble material were analysed after derivatization with ethyl chloroformate according to the method described in **Chapter 2**, **Section 2.2.6.4**.

4.2.9. Characterization of carbohydrates

4.2.9.1. Neutral sugar and uronic acids analysis

Samples were analysed for their sugar content after acid hydrolysis, derivatisation to alditol acetates, and analysis of individual neutral sugars by GC-FID and quantification of uronic acids (UA) by the 3-phenylphenol colorimetric method, as described in **Chapter 2**, **Section 2.2.5.2**.

Sugars were mostly analysed after hydrolysis with 1 M of sulfuric acid (**Chapter 2**, **Section 2.2.5.2**). To analyse smaller amounts of water-soluble samples hydrolysis was performed with 2 M trifluoroacetic acid (TFA). Briefly, to ~0.2 mg of samples dissolved in 500 µL of distilled water were added 500 µL of 4 M TFA and incubated for 1 h at 121 ºC (Albersheim, Nevins, English, & Karr, 1967). After incubation, TFA was evaporated and 20-50 µL of internal standard (2-deoxy-Glc 0.1-1 g/L) were added. The reduction and acetylation were performed in the same tube as described for hydrolysis with 1 M of sulfuric acid.

4.2.9.2. Total sugars analysis

Fractions of anion exchange chromatography and SEC-S400 were assayed for total sugars by the phenol-sulfuric acid method, measuring the absorbance at 490 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Briefly, to 80 µL of samples or blank solution (water), 150 µL of phenol (5 %) and 1 mL of concentrated sulfuric acid were added to the test tubes. Test tubes were manually shaken and kept in a boiling water bath for 5 min. After cooling to room temperature in a water bath, 300 µL of each test tube were transferred to two wells of 96 wells microplate and the absorbance was measured at 490 nm.

4.2.9.3. Methylation analysis

Glycosidic linkage composition of broccoli by-products was analysed by methylation analysis (Passos & Coimbra, 2013). Polysaccharides (1–2 mg) were dissolved in 1 mL of anhydrous dimethylsulfoxide and then powdered NaOH (40 mg) was added under an argon atmosphere. The samples were methylated by addition of 80 µL of CH_3I during 20 min with stirring, following by a second addition of CH_3I (80 µL) and stirring for another 20 min. $CHCl_3$/MeOH (1:1, v/v, 3 mL) was added, and the solution was dialyzed using

membranes with 12-14 kDa cut-off against 3 lots of 50% EtOH. The dialysate was evaporated to dryness and the material was remethylated using the same procedure. The remethylated material was hydrolysed with 2 M TFA (1 mL) at 120 ºC for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis (using $NaBD_4$ instead of $NaBH_4$).

The partially methylated alditol acetates were separated and analysed in the GC-MS apparatus used for amino acids analysis (**Chapter 2**, **Section 2.2.6.4**). The samples were injected in a split mode with the injector operating at 250 ºC. The GC oven temperature program was set to an initial temperature of 80 ºC, raised to 140 ºC at 10 ºC/min, holding for 5 min, raised to 150 ºC at 0.20 ºC/min, then raised to 250 ºC at 60 ºC/min, holding for 2 min. The flow rate of the carrier gas (He) was set at 1.84 mL/min. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV scanning the range 50–700 m/z, in a full scan acquisition mode. Chromatogram peaks were identified comparing all mass spectra with a laboratory made database of partially methylated alditol acetates.

4.2.9.4. Carboxyl reduction of methylated polysaccharides.

Carboxyl reduction of methylated polysaccharides was performed to analyse UA linkages (Dourado et al., 2004). To the freeze-dried methylated material, 20 mg of $LiAlD_4$ and 1 mL anhydrous tetrahydrofuran were added, and the mixture was left to react for 4 h, at 65 ºC. The reagent in excess was eliminated by adding 2–3 drops of EtOH and 2–3 drops of distilled water. The pH of the mixture was reduced to neutrality by adding 2 M H_3PO_4. Two mL of 2:1 $CHCl_3$–MeOH mixture were then added. The reduced polymers were removed from the white precipitate by centrifugation and washed thoroughly with 2:1 $CHCl_3$–MeOH. The supernatant collected was evaporated, and the carboxyl-reduced material was submitted to hydrolysis, reduction, and acetylation, and analysed by GC–MS as described above.

4.2.9.5. Nuclear magnetic resonance (NMR) studies

^{1}H and ^{13}C NMR spectra were recorded in D_2O on a Bruker DRX 500 spectrometer operating at 500.13 and 125.77 MHz, respectively; the chemical shifts are expressed in δ (ppm) values relative to TSS as external reference (Fernandes et al., 2019). 2D COSYPR (homonuclear shift correlation with presaturation during relaxation delay) spectrum was

recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 1200 Hz. The repetition time was 1.8 s. These data were processed in the absolute-value mode. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) HSQC (heteronuclear single quantum coherence) using gradient pulses for selection spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 1280 Hz in F2 and 7000 Hz in F1. The repetition time was 2.3 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant 1J(CH) of 149 Hz. The HMBC (heteronuclear multiple quantum coherence) using gradient pulses for selection spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 1280 Hz in F2 and 7000 Hz in F1. The repetition time was 2.3 s. A sine multiplication was applied in both dimensions. The low-pass J-filter of the experiment was adjusted for an average coupling constant 1J (CH) of 149 Hz and the long-range delay utilised to excite the heteronuclear multiple quantum coherence was optimised for 7 Hz.

4.2.9.6. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum BX FTIR spectrometer, using a horizontal one single reflection ATR Golden Gate (Specac, Germany). Between determinations, the crystal was carefully cleaned with water. The spectra were registered between 4000 and 600 cm−1, collected at a resolution of 8 cm−1, with 64 scans co-added before Fourier transformation. All spectra are the average of ten independent measurements after baseline-correction and smooth correction; moreover, background spectrum was subtracted to aid clarity (Ferreira et al., 2018).

4.2.10. *In vitro* immunostimulatory activity assays

4.2.10.1. Mice

BALB/c mice were purchased from Charles River (Barcelona, Spain) and were kept at the animal facilities of the Institute for Biomedical Sciences Abel Salazar. Experiments were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and directive

2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of the animals used for scientific purposes, and Portuguese rules (DL 113/2013).

4.2.10.2. *In vitro* lymphocyte stimulating effect by flow cytometry analysis

The preparation of spleen lymphocytes for stimulation tests and flow cytometry analysis was performed according to Ferreira et al. (2020). Spleens were aseptically removed from BALB/c mice and splenocyte suspensions were obtained by gently teasing the organ in Hanks' balanced salt solution (HBSS, Sigma) and filtered through 100 µm cell strainers. Splenocytes were resuspended in ammonium-chloride-potassium lysing buffer (ACK) for 3 min, for lysing erythrocytes, washed with HBSS, and resuspended in Roswell Park Memorial Institute medium (RPMI-1640, Sigma, St. Louis, USA) supplemented with 10% foetal calf serum (Biowest, Nuaillé, France), 10 mM HEPES solution (Sigma), 100 IU/mL penicillin (Sigma), 50 mg/L streptomycin (Sigma), and 50 nM 2-mercaptoethanol (Merk, Darmstadt, Germany) (RPMI). Spleen cell suspensions were distributed on 96-well plates (10^6 cells/well) and cultured for 6 h at 37 °C, in 95% humidified atmosphere containing 5% CO_2. Plated cells were stimulated with RPMI medium alone (negative control), 2.5 mg/L of bacterial lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma, St. Louis) (B cells positive control), 2.5 mg/L of concanavalin A (Sigma, St. Louis) (T cells positive control), or with 25, 100, and 250 mg/L of samples. Co-incubation with 100 mg/L polymyxin B (PB; Sigma) was done in parallel to evaluate possible endotoxin contamination.

After incubation, the cells supernatant was removed by centrifugation at 500 g for 5 min and cells were washed by centrifugation with 150 µL of phosphate buffered saline (PBS). The pelleted spleen cells were suspended in 25 µL of fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin and 10 mM sodium azide in PBS) containing the following mAbs diluted 1:100: anti-CD19 (PE-conjugate; clone 1D3; Biolegend), anti-CD3 (PE/Cy7-conjugate; clone 145-2C11; BD Bioscience) and anti-CD69 (FITC-conjugate; clone H1.2F3; Biolegend). After incubation for 30 min at 4 °C in the dark, cells were washed by centrifugation with 150 µL of FACS buffer to remove unbound antibodies.

The washed cells were then suspended in FACS buffer and analysed in an BD FACSCanto II flow cytometer using BD FACSDiva Software (Biosciences BD). Dead cells

were excluded by propidium iodide (Sigma) incorporation. The collected data files were analysed using the FlowJo v10.3. software (Tree Star inc., Ashland, OR, USA).

4.2.10.3. Statistical analysis

Statistical analysis of the B cell stimulatory activity was done using Two-Way ANOVA in GraphPad prism, Version 6.0 (GraphPad Software, Inc. La Jolla, CA, USA) and Tukey's multiple comparisons test. Differences were deemed significant at p-value<0.05.

4.2.11. Detection of lipopolysaccharides

Lipopolysaccharides (LPS) presence was evaluated after methanolysis, hexane extractions, and acetylation of fatty acids for GC-MS analysis as 3-*O*-acetyl fatty acid methyl esters (FAME), according Santana-Filho et al. (2012). Fraction Et80 (5 mg) was dissolved in 400 µL methanol and subjected to methanolysis by the addition of 100 µL of 3 M HCl in methanol, vortexing (1 min), and incubation at 80 °C for 20 h. The solution was then partitioned between hexane (1 mL) and ultrapure water (0.5 mL). The hexane phase was evaporated under a N_2 stream and acetylated, with a mixture of pyridine (100 µL) and acetic anhydride (100 µL), at 100 ºC for 1 h. After removal of acetylation solvents under a N_2 stream, samples were dissolved in acetone for GC-MS analysis, in the apparatus used for amino acids analysis (**Chapter 2**, **Section 2.2.6.4**). FAME were analysed by injection of 1 µL in split mode (split ratio 2.0) with an injection temperature of 250 ºC. The initial column temperature was 80 ºC, and the temperature increased to 140 ºC at 10 ºC/min, held for 5 min, heated to 150 ºC at 0.2 ºC/min, heated to 250 ºC at 60 ºC/min, and held for 2 min. The carrier gas (helium) was maintained at a constant flow rate of 1.51 mL/min. The transfer line temperature was 300 ºC and the temperature of the ionization source was 250 ºC. Mass spectra were acquired in the full-scan mode (50-700 m/z) after ionization by electron impact with 70 eV. LPS from *Escherichia coli* serotype O111:B4 (0.3 mg, Sigma-Aldrich, USA) was used as standard.

4.3. Results and discussion

4.3.1. Extraction and characterization of carbohydrates from broccoli by-products AIR

To evaluate the *in vitro* potential immunostimulatory activity of pectic polysaccharides from broccoli by-products, they were extracted following two approaches after preparation of AIR. In the first approach pectic polysaccharides were obtained by sequential extraction with water and aqueous solutions of imidazole, Na_2CO_3, and KOH (**Figure 4.1**), while in the second approach theywere obtained by a simple hot-water extraction, mimicking the boiling performed to cook vegetables, followed by an ethanol fractionation to purify polysaccharides (**Figure 4.2**).

4.3.1.1. Characterization of broccoli by-products before and after AIR preparation

Before AIR preparation, total carbohydrates of broccoli by-products components (stalks, leaves, and inflorescence remains) accounted for 37% to 45% (**Table 4.1**). Stalks had more carbohydrates followed by leaves and inflorescence remains as observed for the batch of broccoli by-products analysed in **Chapter 2** and in accordance with literature (Campas-Baypoli et al., 2009). Protein was inversely correlated with total carbohydrates, ranging from 8.2% to 17%. As at industrial level it is not feasible to separate components these by-products were mixed, yielding 38% and 11% of the total carbohydrates and protein, respectively.

The preparation of AIR promoted the stabilization of broccoli by-products by enzymes thermal inhibition and dehydration along solvent evaporation. The AIR from isolated stalks, leaves, inflorescence remains, and mixed by-products contained 68%, 54%, 43%, and 55% of total carbohydrates, respectively, representing 79% to 87% of broccoli by-products initial carbohydrates content (**Table 4.1**). Carbohydrates recovered in soluble fraction were free sugars, pectic polysaccharides loosely linked to cell walls, including Ara containing polysaccharides (**Table 4.1**), and glycosides from glucosinolates and phenolic compounds (characterized in **Chapter 5**).

Stalks, leaves, and inflorescence remains had a similar carbohydrate composition (**Table 4.1**) despite having different amounts of total carbohydrates, in accordance with cauliflower components (Femenia et al.,1998; 1999). Both broccoli by-products and AIR were composed mainly by Glc (46 and 33 mol%), UA (23 and 34 mol%), Ara (13 and 16

mol%), and Gal (9 mol%), and small amounts of Xyl (4 and 5 mol%), Man (4 and 2 mol%), Fuc (less than 1 mol%), and Rha (1 mol%), indicating the presence of cellulose, pectic polysaccharides, and small amount of xyloglucans, known to be present in the cell walls of *Brassica* and contributing to dietary fibre present in broccoli (Bestard, et al., 2001; Campas-Baypoli et al., 2009; Schäfer, et al., 2017; Villanueva-Suárez, et al., 2003).

Table 4.1. Yield, carbohydrate composition, total carbohydrates, and total protein of broccoli by-products components and mixture and their resulting AIR and alcohol soluble material.

Sample	η (%)	Carbohydrate composition (mol%)								Total carbohydrates (mg/g)	Total protein (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
Stalks	100	tr	tr	9	3	3	7	57	21	450 ± 40	82 ± 8
AIR of stalks	57	1	tr	12	5	3	11	39	29	682 ± 60	82 ± 12
Leaves	100	1	tr	8	3	3	5	53	27	408 ± 28	117 ± 10
AIR of leaves	60	1	tr	10	5	3	6	38	37	538 ± 23	121 ± 10
Inflorescences	100	1	tr	15	4	3	10	41	26	372 ± 22	166 ± 20
AIR of Inflorescences	69	1	tr	18	4	3	10	34	30	438 ± 9	224 ± 3
Mixture	100	1	tr	13	4	4	9	46	23	384 ± 24	111 ± 12
AIR of Mixture	60	1	tr	16	5	2	9	33	34	555 ± 4	127 ± 9
Alcohol soluble material	31	1	tr	5	1	3	14	65	11	196 ± 5	106 ± 0*
(>12-14 kDa)		(2)	(1)	(37)	(6)	(12)	(11)	(12)	(19)	(317 ± 0.7)	

[tr] traces; * only free amino acids. Data are expressed as mean ± standard deviation of three replicates.

4.3.1.2. Sequential extraction of broccoli by-product polysaccharides with water and aqueous solutions of imidazole, Na_2CO_3, and KOH

In the first approach to extract pectic polysaccharides from broccoli by-products, AIR was submitted to sequential extractions with water and aqueous solutions of imidazole, Na_2CO_3, and KOH. The combination of these sequential extraction procedures is usually used to break the complex organization of cell wall polysaccharides, allowing simultaneous solubilization of polysaccharides and characterization of cell walls. The extracts obtained were characterized for both carbohydrates and protein content (**Table 4.2**). Along the extractions with water and aqueous solutions of imidazole the obtained extracts had

increasing amount of total carbohydrates (from 18% to 34%). The main polysaccharides extracted were pectic polysaccharides (sum of Rha, Fuc, Ara, Gal, and UA in the range of 55 to 73%) and hemicelluloses, namely xylans and xyloglucans (sum of Glc and Xyl in the range of 23 to 42%). The following extractions with Na_2CO_3 allowed to obtain extracts richer in polysaccharides (55 to 58%), composed mainly by homogalacturonans regions or pectin, by the presence of 63 mol% to 78 mol% of UA.

Extractions using lower concentration of KOH (0.5M and 1M) followed by neutralization originated precipitates rich in protein (31 to 51%) and soluble fractions enriched in hemicelluloses (40 to 65%) (**Table 4.2**). Increasing KOH concentration to 4 M without or with borate originated also precipitates rich in protein (24 and 18%, respectively) and soluble fractions richer in pectic polysaccharides (47 and 76%, respectively) than hemicelluloses (43 and 21%, respectively). Using 8 M KOH solution was also possible to extract pectic polysaccharides, although yield of 1.1% was considerably lower than previous extractions (1.3 to 8.5%). A last water-soluble extract was obtained after dialysis of cellulose enriched residue (CR), which was rich in pectic polysaccharides (snCR, 94%). The remaining residue, in addition to cellulose (59% of Glc), also contained pectic polysaccharides resistant to the extraction conditions, as observed for other *Brassica* along cell walls extractions (Femenia et al., 2000).

To elucidate the type of polysaccharides extracted, the glycosidic linkages of neutral sugars were determined by methylation and analysed as partially methylated alditol acetates by GC-MS (**Table 4.3**). The mol% were pondered with the amount of UA determined by the colorimetric method, as they were one of the main sugar residues. (1→5)-Ara*f* residues, ramified in position 2 and/or 3, and terminal-Ara*f* (*t*-Ara*f*) indicated the presence of arabinans or Ara side chains of arabinogalactans. Type II arabinogalactans were observed by the presence of (1→3,6)-Gal*p* with *t*-Gal*p*, (1→3)-Gal*p*, and (1→6)-Gal*p*, usually found associated to proteins, or, less commonly, associated to pectic polysaccharides. (1→4)-Gal*p* indicated the presence of type I galactans in ramified regions of pectic polysaccharides. The occurrence of (1→4)-Glc*p* units, ramified in position 6, (1→2)-Xyl*p* and *t*-Xyl*p* units allowed to infer the presence of xyloglucans. The occurrence of (1→4)-Xyl*p* indicated the presence xylans. These kinds of structures that form broccoli by-products cell walls were previously observed in other *Brassica* cell walls, namely cauliflower (Femenia, Waldron, Robertson, & Selvendran, 1999; Schäfer et al., 2017).

Table 4.2. Yield, carbohydrate composition, total carbohydrates, estimated amounts of pectic polysaccharides (sum of Rha, Fuc, Ara, Gal, and UA mol%) and of xyloglucans (sum of Glc and Xyl mol%), and protein for samples obtained by sequential extraction with water and aqueous solutions of imidazole, Na_2CO_3, and KOH from AIR of broccoli by-products.

		η	Carbohydrate composition (mol%)								Total Carb.	Pectic PS	XG	Total protein
		(%)	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*UA*	(mg/g)	(%)*[1]	(%)*[2]	(mg/g)
H_2O														
	1	**10.3**	*3*	*0*	*18*	*2*	*4*	*25*	*22*	*26*	**179**	72	24	**111**
	2	**2.5**	*2*	*0*	*16*	*2*	*5*	*23*	*31*	*21*	**191**	62	33	**115**
Imidazole														
	1	**1.9**	*1*	*0*	*21*	*1*	*3*	*21*	*22*	*30*	**325**	74	23	**248**
	2	**0.4**	*0*	*0*	*11*	*2*	*2*	*9*	*41*	*35*	**340**	56	42	**278**
Na_2CO_3														
	1	**1.0**	*1*	*0*	*12*	*4*	*1*	*10*	*9*	*63*	**550**	86	13	**140**
	2	**5.4**	*1*	*0*	*13*	*0*	*0*	*6*	*2*	*78*	**575**	97	2	**46**
0.5M KOH														
	1Sn	**1.1**	*0*	*1*	*20*	*15*	*2*	*21*	*25*	*17*	**281**	58	40	**190**
	1Pp	**7.4**	*1*	*0*	*15*	*1*	*2*	*17*	*14*	*50*	**133**	82	15	**505**
	2Sn	**0.6**	*1*	*0*	*13*	*19*	*2*	*18*	*38*	*8*	**375**	40	57	**198**
	2Pp	**3.1**	*0*	*0*	*16*	*2*	*3*	*12*	*30*	*36*	**87**	65	32	**422**
1M KOH														
	Sn	**0.7**	*0*	*2*	*5*	*21*	*8*	*13*	*44*	*7*	**472**	27	65	**90**
	Pp	**3.0**	*0*	*0*	*15*	*7*	*2*	*11*	*45*	*19*	**99**	46	52	**311**
4M KOH														
	1Sn	**0.9**	*1*	*2*	*13*	*13*	*7*	*14*	*30*	*20*	**780**	50	43	**49**
	1Pp	**0.4**	*1*	*0*	*21*	*2*	*1*	*8*	*20*	*47*	**272**	77	22	**242**
	2Sn	**1.0**	*1*	*1*	*27*	*6*	*2*	*13*	*15*	*35*	**765**	77	21	**52**
	2Pp	**0.5**	*1*	*0*	*22*	*1*	*2*	*9*	*26*	*40*	**210**	72	27	**181**
8M KOH														
	Sn	**1.0**	*1*	*0*	*32*	*3*	*1*	*13*	*9*	*41*	**800**	88	11	**31**
	Pp	**0.1**	*1*	*0*	*15*	*1*	*2*	*6*	*54*	*21*	**292**	42	55	**133**
Residue														
	CR_Sn	**1.5**	*2*	*0*	*34*	*1*	*0*	*15*	*5*	*44*	**690**	94	5	**20**
	CR	**12.2**	*1*	*0*	*14*	*1*	*1*	*6*	*59*	*19*	**850**	40	*	**28**

*[1]Pectic polysaccharides quantified as the sum of the content of Rha, Fuc, Ara, Gal, and UA; *[2]hemicelluloses, namely xylans and xyloglucans quantified as the sum of the content of Glc and Xyl.

Table 4.3. Heatmap of glycosidic linkage analysis of samples obtained by sequential extraction with water and aqueous solutions of imidazole, Na_2CO_3, and KOH from AIR of broccoli by-products. Mol% of glycosidic linkages were pondered with the amount of UA determined by the colorimetric method.

Deduced linkages (mol%)	H_2O		Imidazole		Na_2CO_3		0.5M KOH		1M KOH	4M KOH		8M KOH	Residue	
	1	2	1	2	1	2	1Sn	2Sn	Sn	1Sn	2Sn	Sn	CR_Sn	CR
t-Rha*p*	-	-	-	-	-	-	-	0.7	0.6	2.0	-	-	-	-
1,2-Rha*p*	-	-	-	-	-	-	0.7	-	-	0.5	1.1	1.7	1.0	0.6
1,2,4 Rha*p*	-	-	-	-	-	-	0.5	0.6	-	-	0.8	0.8	0.9	0.5
t-Ara*f*	5.6	4.1	3.5	2.1	2.0	3.4	5.7	1.9	1.0	2.6	7.0	7.7	10.3	4.2
1,5-Ara*f*	6.9	4.8	6.3	3.6	3.4	4.0	7.7	2.8	1.1	5.2	12.3	14.8	17.1	7.4
1,2,5-Ara*f*	5.0	3.4	2.7	4.4	1.7	3.7	4.1	-	1.2	0.9	1.3	2.3	2.8	1.1
1,3,5-Ara*f*	1.1	0.8	1.2	0.7	0.9	2.0	2.0	-	-	2.0	5.3	7.0	8.6	4.1
1,2,3,5-Ara*f*	4.8	3.1	2.1	2.6	2.6	3.1	0.6	-	-	2.9	2.1	1.0	2.0	4.5
t-Xyl*p*	0.4	0.4	-	0.5	0.6	-	3.4	3.5	9.6	9.1	4.0	2.2	0.6	0.6
1,2-Xyl*p*	0.5	-	-	-	-	-	2.7	3.2	5.2	5.7	2.3	1.3	-	0.5
1,4-Xyl*p*	2.1	2.5	1.9	1.9	3.5	-	19.3	17.4	15.5	3.7	2.1	0.8	0.6	1.7
1,4,6-Man*p*	-	-	-	-	-	-	-	0.6	2.7	2.4	0.5	-	-	-
t-Gal*p*	1.3	1.1	1.5	0.8	0.6	-	1.7	1.4	4.3	4.4	2.2	2.0	1.4	1.0
1,3-Gal*p*	4.6	3.4	2.7	1.2	0.6	-	1.8	2.3	1.8	4.3	1.7	0.5	-	-
1,4-Gal*p*	2.2	2.6	2.7	2.0	6.6	0.9	2.1	2.7	4.7	7.4	3.5	6.3	5.2	-
1,6-Gal*p*	4.3	2.2	3.6	1.0	1.0	-	3.1	2.0	0.6	-	-	-	-	-
1,3,6-Gal*p*	11.8	7.8	9.6	4.6	2.3	1.9	6.9	6.6	1.2	0.4	0.5	-	-	-
t-Glc*p*	1.4	2.1	1.3	2.0	0.5	-	1.2	2.0	1.0	0.5	0.5	-	-	1.1
1,4-Glc*p*	21.6	37.9	28.8	36.1	9.6	3.1	16.6	36.4	24.6	13.3	9.9	7.3	5.4	53.0
1,4,6-Glc*p*	0.5	2.8	1.9	1.6	1.2	-	3.1	8.3	18.3	12.5	7.9	3.5	0.3	1.1
UA	25.9	21.0	30.2	34.9	62.9	77.9	16.8	7.6	6.6	20.2	35.0	40.8	43.8	18.6

Giving the carbohydrate and linkage composition observed, the following extracts were shown to represent the variety of polysaccharide structures found in broccoli by-products: Imidazole 2 represents pectic polysaccharides with type II arabinogalactans, Na_2CO_3 2 represents pectate (high amount of deesterified UA), 1M KOH sn represents xyloglucans and xylans, 4M KOH 2 sn represents a mixture of arabinans with high amount of xyloglucans and xylans, and snCR represents a mixture of arabinans with low amount of xyloglucans and xylans.

4.3.1.3. Hot water extraction and ethanol fractionation of broccoli by-product polysaccharides

Hot water (HW) extraction yielded 12% of high molecular weight material, dialysed against membrane with a molecular weight cut-off between 12 and 14 kDa, and 73% of insoluble material (residue). HW had 69% of polysaccharides and was composed by 64 mol% of UA, 18 mol% of Ara, 10 mol% of Gal, 5 mol% of Glc, and small amounts of Xyl,Man, Rha, and Fuc (**Table 4.4**), indicating that pectic polysaccharides were the main extracted polysaccharides. The residue left had 54% of polysaccharides and was composed by 38 mol% of Glc, 27 mol% of UA, 16 mol% of Ara, 9 mol% of Gal, 6 mol% of Xyl, and small amounts of Man, Rha and Fuc, allowing to infer that as observed in previous sequential extractions, broccoli by-products have a high amount of pectic polysaccharides that need harsh conditions to be extracted. Hemicelluloses, namely xyloglucans and xylans, together with cellulose, remained in the hot water extraction residue, shown by the Xyl and Glc enrichment.

The HW extract was dissolved in cold water, centrifuged, and submitted to ethanol precipitation using 50% and 80% ethanol solutions. All material was soluble in 50% ethanol and fractionation was only observed using 80% ethanol yielding a fraction insoluble in 80% ethanol (Et80) that was separated from the 80% ethanol soluble material (SnEt).

The Et80 fraction accounted for 85% of HW and contained 64% of polysaccharides (**Table 4.4**). This fraction contained as major sugars UA (65 mol%), Ara (17 mol%), Gal (10 mol%), and Glc (6 mol%), and in lower amounts Man, Xyl, Rha, and Fuc, allowing to infer the presence of pectic polysaccharides, resembling HW extract. The SnEt fraction accounted for 14% of HW and had only 34% of carbohydrates, mainly Ara (43 mol%) and UA (36 mol%), inferring the presence of pectic polysaccharides rich in Ara and a high

amount of non-carbohydrate material, probably high molecular weight phenolic compounds or phenolic compounds adsorbed to high molecular weight material (Fernandes et al., 2020; Gonçalves et al., 2018).

Table 4.4. Yield, carbohydrate composition, and total carbohydrates of samples obtained by hot water extractions, fractionations, enzymatic treatments, and chemical sulfation. Data are expressed as mean ± standard deviation of three replicates.

Sample	η (%)	Carbohydrate composition (mol%)								Total Carbohydrates (mg/g)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
Hot water extraction										
HW	12	1	1	18	1	tr	10	5	64	685 ± 5
Residue	73	1	tr	16	6	3	9	38	27	539 ± 20
80% ethanol fractionation of HW										
Et80	85	1	tr	17	1	1	10	6	65	642 ± 25
Sn80	14	2	tr	43	3	3	7	6	36	336 ± 10
Size-exclusion chomatography of Et80										
Et80-I $^{>151\ kDa}$	20	2	1	26	1	1	10	7	52	366 ± 22
Et80-II $^{41\text{-}151\ kDa}$	34	1	tr	22	1	1	13	4	58	729 ± 22
Et80-III $^{12\text{-}41\ kDa}$	25	1	1	12	2	3	11	10	60	591 ± 16
Et80-IV $^{<12\ kDa}$	11	2	1	9	4	6	20	42	15.6	23 ± 2
α-L-Arabinofuranosidase treatment of Et80										
Et80-Ara	79	1	tr	10	1	1	14	9	64	609 ± 43
Anion-exchange chomatography of Et80										
Et80 A	34	1	1	12	1	2	12	10	61	533 ± 3
Et80 B	45	2	tr	20	1	tr	10	2	65	587 ± 4
Et80 C	8	2	3	11	7	7	16	12	*42*	71 ± 2
Chemical sulfation of Et80										
Et80-Sulf	104	1	tr	17	1	2	11	9	59	515 ± 12
Protease and glicosidase treatments of HW*										
Enz_HW	32	2	tr	33	2	3	19	4	37	601 ± 14
80% ethanol fractionation of Enz_HW										
Enz-Et80	8	2	tr	28	5	4	16	6	39	363 ± 21
Enz-Sn80	92	3	tr	32	1	3	17	3	41	660 ± 34
Anion-exchange chomatography of Enz_HW										
Enz_HW A	36	1	tr	31	2	4	25	3	34	677 ± 0
Enz_HW B	50	1	tr	38	1	tr	19	1	40	628 ± 12
Enz_HW C	14	1	1	43	1	1	*11*	4	*38*	17 ± 2

^{tr} traces; *treatments with pronase, α-amylase, endo-polygalacturonase, and pectin methylesterase.

Table 4.5. Heatmap of glycosidic linkage analysis of samples obtained by hot water extraction (HW) from AIR of broccoli by-products and following purification, fractionation, and chemical derivatization steps. Mol% of glycosidic linkages were pondered with the amount of UA determined by the colorimetric method.

Deduced linkages (mol%)	HW	HW - 80% ethanol fractionation		Et80 - Size-exclusion chromatography			Et80 - Anion-exchange chromatography		Et80 Ara removal	Et80 sulfation	HW enzymatic treated	Enz - 80% ethanol fractionation		Enz - Anion-exchange chromatography	
		Et80	Sn80	Et80-I	Et80-II	Et80-III	Et80A	Et80B	Et80-Ara	Et80-Sulf	Enz_HW	Enz-Et80	Enz-Sn80	Enz_HWA	Enz_HWB
1,2-Rha*p*	0.3	0.3	0.1	1.2	0.3	0.3	0.2	0.8	0.4	0.2	0.6	0.5	0.2	0.1	0.5
1,2,4 Rha*p*	0.3	0.5	0.2	0.8	0.4	0.2	0.5	1.1	0.8	0.7	1.1	1.0	0.5	0.4	2.1
t*-Fuc*p	0.1	0.1	-	0.4	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.1	0.2	-	0.2
t*-Ara*f	6.0	5.0	11.4	8.0	7.9	3.6	4.1	3.9	1.8	1.4	10.3	11.6	11.6	9.3	10.7
1,3-Ara*f*	0.1	0.1	1.2	0.2	0.1	0.3	0.3	0.2	0.1	0.7	0.7	0.3	0.4	1.0	0.2
1,5-Ara*f*	7.1	6.4	17.8	13.0	8.4	5.4	5.3	7.6	6.2	3.0	14.1	17.1	12.3	13.7	15.2
1,2,5-Ara*f*	0.8	0.8	2.2	0.5	1.6	0.3	0.8	1.8	0.2	1.8	1.8	1.3	1.4	1.1	0.8
1,3,5-Ara*f*	1.6	1.4	7.0	2.5	2.1	0.7	1.5	3.1	0.1	1.7	3.7	3.1	3.1	2.9	5.2
1,2,3,5-Ara*f*	1.4	0.9	1.8	1.2	1.2	0.2	0.8	4.1	0.8	1.9	0.4	0.1	2.0	1.5	2.8
t*-Xyl*p	0.5	0.6	0.5	0.6	0.2	0.3	0.6	0.3	1.3	1.2	1.2	1.7	1.0	0.2	0.9
1,4-Xyl*p*	0.6	0.7	3.3	0.8	1.0	3.1	2.1	0.8	0.9	1.0	2.2	1.7	2.2	5.1	2.1
t*-Gal*p	0.8	1.1	0.7	0.5	0.6	1.0	1.2	0.8	1.4	1.1	2.8	1.0	2.3	2.1	1.8
1,3-Gal*p*	2.1	1.6	1.0	2.6	1.7	5.4	4.1	1.0	1.7	1.4	1.8	1.2	1.6	3.0	4.9
1,4-Gal*p*	0.6	1.0	1.9	0.1	0.3	1.5	1.4	0.2	0.6	0.7	2.3	2.0	2.3	5.0	0.5
1,6-Gal*p*	1.7	2.2	1.5	0.5	2.6	1.8	1.5	1.3	4.5	3.7	4.4	4.5	4.3	4.6	3.5
1,3,6-Gal*p*	3.4	4.2	5.8	2.2	4.3	3.1	3.0	2.1	2.2	4.4	9.0	5.4	7.5	9.4	4.7
1,4,6-Gal*p*	-	-	-	-	-	-	-	-	-	1.4	-	-	-	-	-
1,2,4,6-Gal*p*	-	-	-	-	-	-	-	-	-	1.4	-	-	-	-	-
1,3,4,6-Gal*p*	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-
t*-Glc*p	0.5	0.4	0.9	0.7	0.5	1.1	0.5	0.3	0.9	0.4	0.3	0.3	0.3	0.1	0.1
1,4-Glc*p*	6.5	6.6	4.7	7.9	7.2	6.7	8.6	3.8	11.0	4.4	3.8	7.0	3.4	3.4	1.4
1,4,6-Glc*p*	0.7	0.6	0.9	0.5	0.3	2.7	0.6	0.1	0.4	3.7	0.3	0.2	0.8	0.2	0.1
UA	64.2	65.0	35.5	51.8	58.2	60.3	61.0	65.1	63.5	59.1	37.1	38.9	40.8	33.6	40.3

Glycosidic linkage analysis after carboxyl reduction with $LiAlD_4$ of methylated polysaccharides confirmed the presence of (1→4)-Gal*p*A residues indicating the presence of pectic polysaccharides in both HW and Et80 and SnEt fractions (**Table 4.5**). The presence of (1→3)- and (1→3,6)-Gal*p* residues, as well as *t*-Gal*p* and (1→6)-Gal*p* residues were diagnostic of type II arabinogalactans. *t*-Galactopyranosyluronic acid (*t*-Glc*p*A) residues were also detected which could indicate the presence of arabinogalactans with these residues. The presence of (1→2,4)-Rha*p* residues in these fractions supports the presence of rhamnogalacturonan-I, the ramified region of pectic polysaccharides. The presence of (1→4)-Gal*p* was diagnostic of type I arabinogalactans, which are often found attached to the rhamnogalacturonan moiety. The presence of high amount of (1→5)-Ara*f* could indicate that in addition of its presence in arabinogalactans, arabinans can be found in the ramified domain of pectic polysaccharides, as found in other *Brassica*, namely cabbage (Stevens et al.,1980). Part of water-soluble arabinans and arabinogalactans in HW material were recovered in EtSn, in agreement with their high solubility in ethanol (Fernandes et al 2019).

In addition to pectic polysaccharides, a small amount of Glc containing polysaccharides were also identified by the presence of *t*-Glc*p*, (1→4)-Glc*p*, and (1→4,6)-Glc*p* (**Table 4.5**), which could indicate the presence of starch-like polysaccharides or water-soluble hemicelluloses, namely xyloglucans and xylans, by the presence of *t*-Xyl*p* and (1→4)-Xyl*p*.

4.3.2. Evaluation of immunostimulatory activity of broccoli by-products pectic polysaccharides

To evaluate broccoli by-products polysaccharides immunostimulatory activity, samples obtained from sequential extraction were studied (Imidazole 2, Na_2CO_3 2, 1M KOH Sn, 4M KOH 2Sn, and SnCR) in addition with fractions obtained after hot water extraction and 80% ethanol fractionation (HW and Et80). These extracts were incubated with BALB/c splenocytes to evaluate the activation of B and T lymphocytes by the expression of an early activation marker (CD69) after 6 h of cells stimulation and flow cytometry analysis. Cells viability was maintained when cell were stimulated with broccoli by-products polysaccharides, in the range of concentrations tested, from 25 to 250 mg/L. $CD3^+$ cells expressed insignificant amounts of CD69 on the surface, showing that the extracts did not stimulate T cells. In contrast, $CD19^+$ cells (B cells) were stimulated from 11% to 37% by

fraction Et80, in a dose-response relationship from 25 to 250 mg/L, respectively (**Figure 4.3**). However, HW only activated 16% of B cells and samples obtained by sequential extraction (Imidazole 2; Na_2CO_3 2; 1M KOH sn; 4M KOH sn2) only activated 8.3% to 11% of B cells, which were values of B cells activation not statistically different from non-stimulated cells (RPMI, 7.7%). Immunostimulation due to lipopolysaccharides contamination in fraction Et80 was excluded by non-detection of lipid A structures.

These results show that pectic polysaccharides from broccoli by-products have potential immunostimulatory activity. However, only the combination of structures found in Et80 fraction were capable to stimulate B cells. As these polysaccharides were purified from HW material, it could be inferred that the lower purification or the presence of antagonist structural features contributed to HW material reduced activity. Furthermore, from the methylation analysis, extracts obtained from sequential extraction also shared some linkages that were not enough to stimulate B cells, possibly by the presence of other non-carbohydrate material (Imidazole 2 extract had only 34% of polysaccharides), by the presence of high contribution of UA in Na_2CO_3 2 extract, that was shown to be immunosuppressive in other studies (Yamada & Kiyohara, 2007); by the presence of high amount of hemicelluloses in 1M KOH sn and 4MKOH sn, which diluted pectic polysaccharides; by deesterification of functional groups from polysaccharides after extraction with alkaline solutions (Na_2CO_3 and KOH), as acetyl groups, which have been related with immunostimulatory activity (Martins et al., 2017; Simões et al., 2009; Simões, Nunes, Domingues, & Coimbra, 2010; Simões, Nunes, Domingues, Coimbra, & Domingues, 2012); and by different combinations of arabinogalactans or arabinans observed in the ratio of Ara:Gal and the amount of (1→5)-Ara*f* linkage as observed for pectic polysaccharides from other sources (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Martins et al., 2017; Westereng, Michaelsen, Samuelsen, & Knutsen, 2008; Bjorge Westereng et al., 2009).

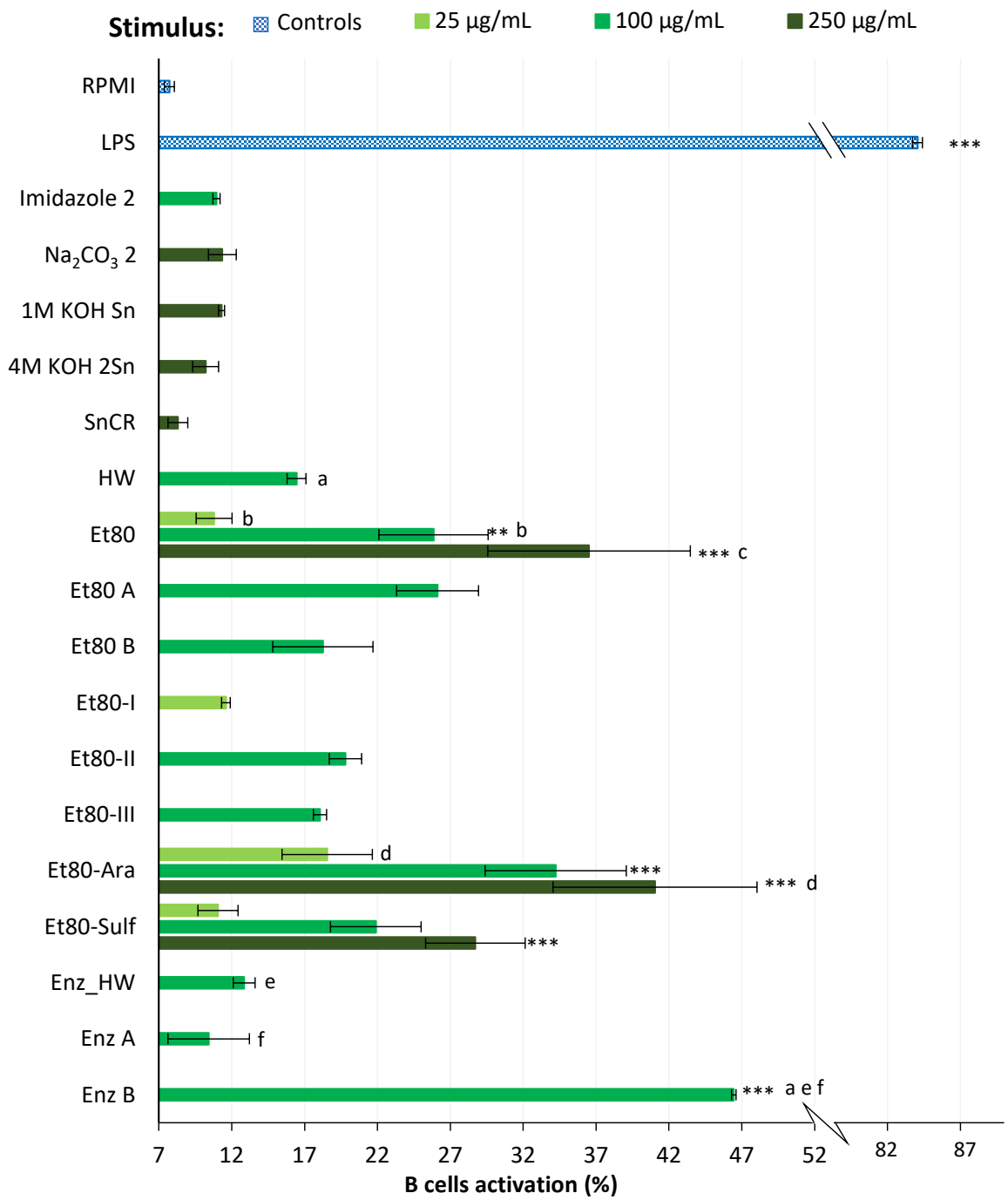


Figure 4.3. Percentage of B cells activated ($CD69^+$) by treatment with polysaccharides from broccoli by-products at the concentrations of 25, 100, and 250 mg/L. Culture medium alone (RPMI) was used as negative control and lipopolysaccharides (LPS) were used as positive control. Statistical differences between RPMI (negative control) and different stimulus are indicated above bars (* $p < 0.01$; ** $p < 0.001$; *** $p<0.0001$). Statistical differences among samples are highlighted with letters: similar letters above bars indicate significant differences between compared groups (a, b, e: $p < 0.05$; d, f: $p < 0.01$; c: $p < 0.0001$ two-Way ANOVA and Tukey's multiple comparisons test).

To detail the structural features responsible for broccoli by-product polysaccharides immunostimulatory potential, Et80 fraction was further characterized by NMR spectroscopy, its molecular weight was characterized by size-exclusion chromatography, the ramified regions of pectic polysaccharides were characterized after enzymatic treatments and charge was characterized by anion-exchange chromatography. Fractions obtained from these characterization procedures were incubated with spleen cells to evaluate their potential immunostimulatory activity and stablish structure-function relationships. Furthermore, Et80 fraction was chemically sulfated to evaluate the presence of this functional group in immunostimulatory activity of pectic polysaccharides from broccoli by-products.

4.3.3. Detailing of structural features from broccoli by-product pectic polysaccharides

4.3.3.1. NMR spectroscopy

The ^{13}C NMR, HSQC, and HMBC spectra of polysaccharides from fraction Et80 are represented in **Figures 4.4.a**, **4.5**, and **4.6**, respectively. According to these 1D and 2D NMR spectra, methylation analysis (**Table 4.5**), and literature about pectic polysaccharides rich in UA, arabinans, and arabinogalactans (Cardoso, Silva, & Coimbra, 2002; Dourado et al., 2006; Fernandes, et al., 2019; Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013; Makarova, Shakhmatov, & Belyy, 2016; Schols, Posthumus, & Voragen, 1990; Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Makarova, & Belyy, 2019; Shakhmatov, Toukach, Michailowa, & Makarova, 2014), intense signals of the anomeric carbon of the ^{13}C NMR spectrum were attributed to C-1 of α-Ara*f* (106.3-109.2 ppm), α-Gal*p*A (100.2 ppm), and β-Gal*p* (104.1 ppm). Based on literature about α-glucans (Chen et al., 2017; Guo, et al., 2015), the signal at 99.5 ppm was attributed to C-1 of α-Glc*p*. Low intense signals of C-1 from β-Gal*p* (102.9 ppm), α-Rha*p* (99.2 and 100.1 ppm), and β-Glc*p*A (102.9 ppm) were also observed.

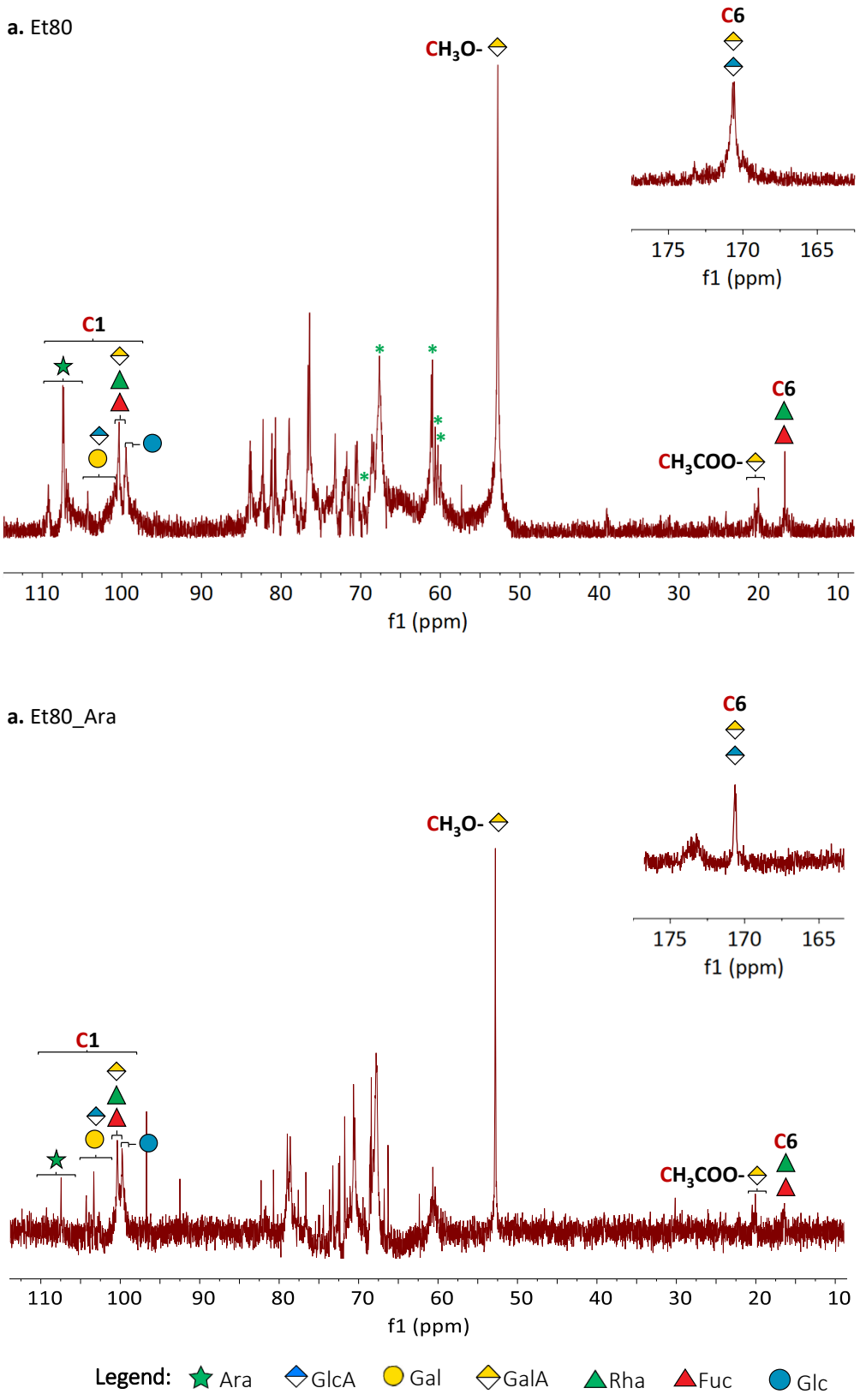


Figure 4.4. Representation of the ^{13}C NMR spectra from **a.** Et80 and **b.** Et80_Ara. Illustration of sugar residues using the nomenclature proposed by Neelamegham et al. (2019).*Identification of negative signals of DEPT-135 NMR spectrum from Et80.

Proeminent signals in the region of carboxyl groups were observed in the ^{13}C NMR at δC range of 163.4-172.3, indicating the existence of UA with different vicinities. Methyl and acetyl esterification were suggested by both one-bond ^{13}C-^{1}H correlation of NMR resonances, in the HSQC spectrum, and by three-bond ^{13}C-^{1}H correlation of NMR resonances, in the HMBC spectrum. The signals of CH_3O-groups at 52.6/3.69 ppm and of methyl groups of acetate CH_3CO- at 20.3/2.04 ppm were observed in the HSQC spectrum. The signals of the carbon of carboxyl correlation with methyl groups of CH_3CO- at 173.6/2.06 ppm and 172.9/2.05 ppm (176.0/2.09) ppm were observed in the HMBC spectrum (Makarova, Patova, Shakhmatov, Kuznetsov, & Ovodov, 2013; Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Makarova, & Belyy, 2019). Some assignments were not possible for GalA due to overlapping of signals and to its lower degree of freedom when compared to other sugar residues (Dourado et al., 2006; Schols, Posthumus, & Voragen, 1990). Overall, the data indicate the presence of a methyl and acetyl esterified galacturonan in Et80 fraction. Methyl esterification and acetylation, specifically at *O*-2 and/or *O*-3 positions, were previously observed in pectins from *Brassica* (Westereng et al., 2006).

In ^{13}C NMR spectrum, C6 of deoxy-sugar residues Rha were observed at 16.1-16.8/1.11-1.18 ppm (Shakhmatov, Belyy, & Makarova, 2017). These results indicated the presence of *t*-Rha*p*, usually found in the non-reducing terminal of arabinogalactans, and the presence of (α1→2)-Rha*p* and (α1→2,4)-Rha*p*, typical of in RG-I regions, according with what was observed in methylation analysis.

The negative carbon signals in the ^{13}C Distortionless Enhancement by Polarization Transfer (DEPT-135) found at δ_C 69.4, 66.3-66.8, 60.9, 60.7, and 60.5 can be attributed to the -CH_2- of sugar residues, namely the C-5 of Ara and the C-6 of Gal and Glc. Other assignments of the proton and carbon signals of Ara, Gal, and Glc were reported in **Table 4.6**. The identification of signals belonging to (α1→5)-Ara*f*, (α1→3,5)-Ara*f*, (α1→3)-Ara*f*, (α1→2,5)-Ara*f*, (α1→2,3,5)-Ara*f*, and *t*-α-Araf indicated the presence of arabinans (Fernandes, et al., 2019; Dourado, et al., 2006; Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Makarova, & Belyy, 2019; Shakhmatov, Toukach, Michailowa, & Makarova, 2014), which were previously isolated from cabbage. However, downfielded chemical shifts of Ara residues were found in HSQC spectrum, confirming linkages between Ara-Gal residues, as usually found in arabinogalactans (Makarova, Shakhmatov, & Belyy, 2016;

Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Toukach, Michailowa, & Makarova, 2014). Therefore, it cannot be excluded that the Ara attributed to arabinans are part of side chains of arabinogalactans.

Table 4.6. ^{1}H and ^{13}C chemical shifts (δ) of fraction Et80 in D2O at 500.13 MHz and 125.77 MHz.

Residue		**C-1** **H-1**	**C-2** **H-2**	**C-3** **H-3**	**C-4** **H-4**	**C-5** **H-5;5'**	**C-6** **H-6;6'**	***CH₃O-***	**CH₃COO**
→4)-α-Gal*p*A-(1→	GalA	100.2 4.84	- -	- 3.97	79.0 4.33	- -	170.3		20.3 2.04
→4)-α-Gal*p*A-(1→	GalA Me	100.2 4.84	- -	- -	- -	70.5 4.98	164.1	52.6 3.67	
α-Rha*p*	Rha	100.1	-	-	-	-	16.1-16.8		
		4.63	-	-	-	-	1.04-1.11		
→5)-α-Ara*f*-(1→	A_5	107.3 4.96	81.0 4.01	76.6 3.88	82.3 4.08	66.5 3.67;3.75			
→3,5)-α-Ara*f*-(1→	A_{35}	107.1 5.00	79.1 4.16	83.9 3.95	81.1 3.89	66.8 3.67;3.75			
→3)-α-Ara*f*-(1→	A_3	107.1 5.04	81.0 4.01	83.7 3.83	81.3 3.99	60.9 3.60;3.69			
→2,5)-α-Ara*f*-(1→	A_{25}	107.0 5.07	86.7 4.04	79.4 4.09	81.5 4.17	66.8 3.67;3.75			
α-Ara*f*-(1→	A_t	107.0 5.02	81.7 3.99	76.4 3.83	83.9 3.92	60.9 3.60;3.69			
→2,3,5)-α-Ara*f*-(1→	A_{235}	106.7 5.12	84.5 4.18	80.9 4.22	81.9 3.99	66.5 3.67;3.75			
α-Ara*f*-(1→ Gal	A_t^*	109.2 5.12	- -	- -	- -	- -			
β-Gal*p*-(1→	G_t	103.1 4.36	70.5 3.4	72.5 3.53	- 3.84	- 3.53	60.4 3.60;3.69		
→6)-β-Gal*p*-(1→	G_6	103.1 4.36	70.5 3.42	72.5 3.53	- 3.80	- 3.84	69.2 3.89;3.80		
→3,6)-β-Gal*p*-(1→	G_{36}	103.1 4.36	69.5 3.53	79.6 3.55	- 3.97	- 3.84	69.2 3.89;3.80		
→3)-β-Gal*p*-(1→	G_3	104.1 4.51	70.0 3.56	80.0 3.61	- 4.04	- 3.53	60.4 3.60;3.69		
→4)-β-Gal*p*-(1→	G_4	104.1 4.51	71.7 3.54	73.2 3.56	77.5 4.05	- 3.54	60.4 3.60;3.69		
4-*O*Me-α-Glc*p*A-(1→	GA	102.9 4.36	72.9 3.22	73.9 -	82.1 3.23	- 4.17	- -	60 3.35	
α-Glc*p*	Glc	99.7 5.28	- 3.53	- -	- -	- -	- -		

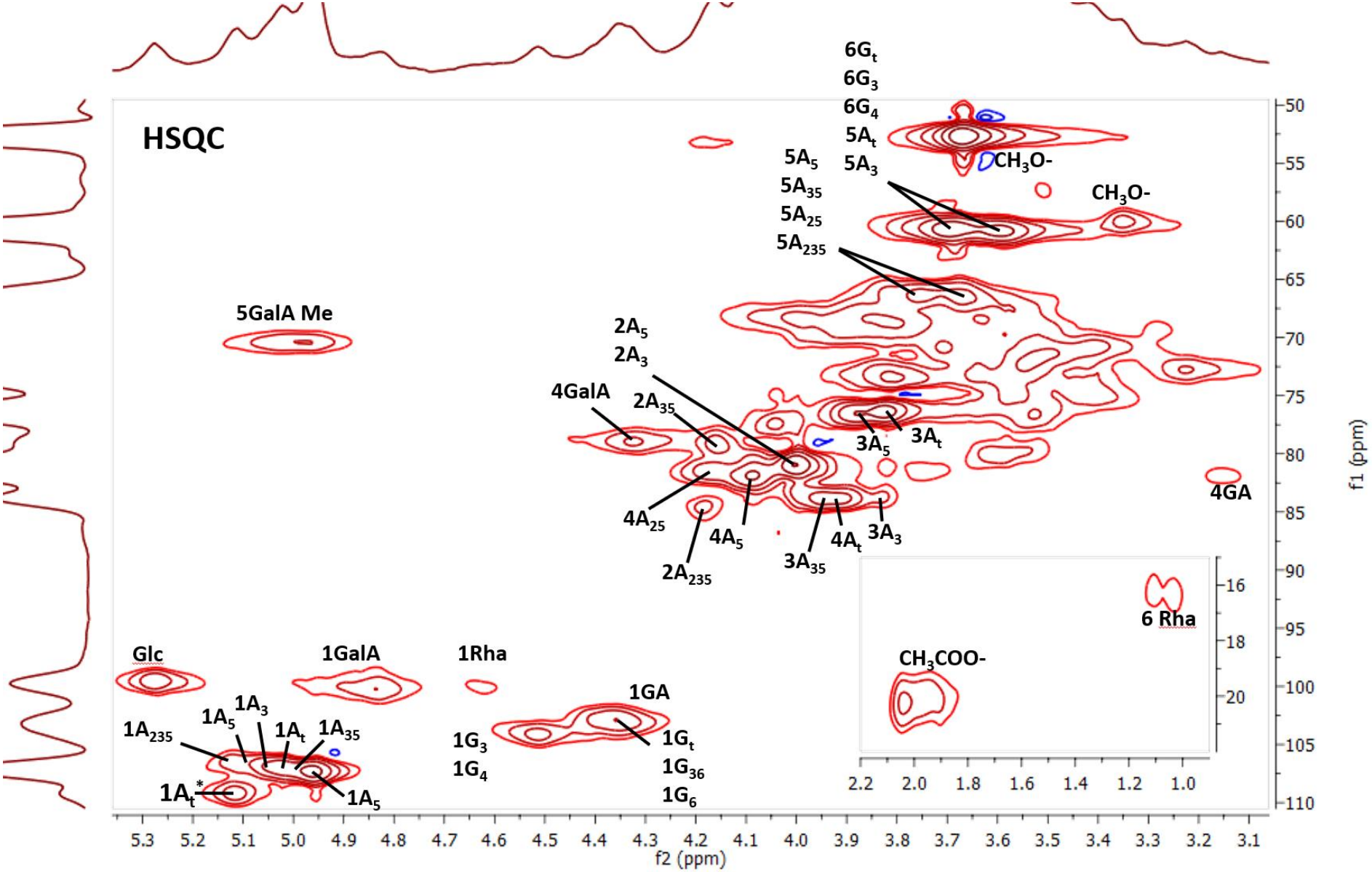


Figure 4.5. Representation of HSQC spectrum of fraction Et80. The notations used are given in **Table 4.6.**

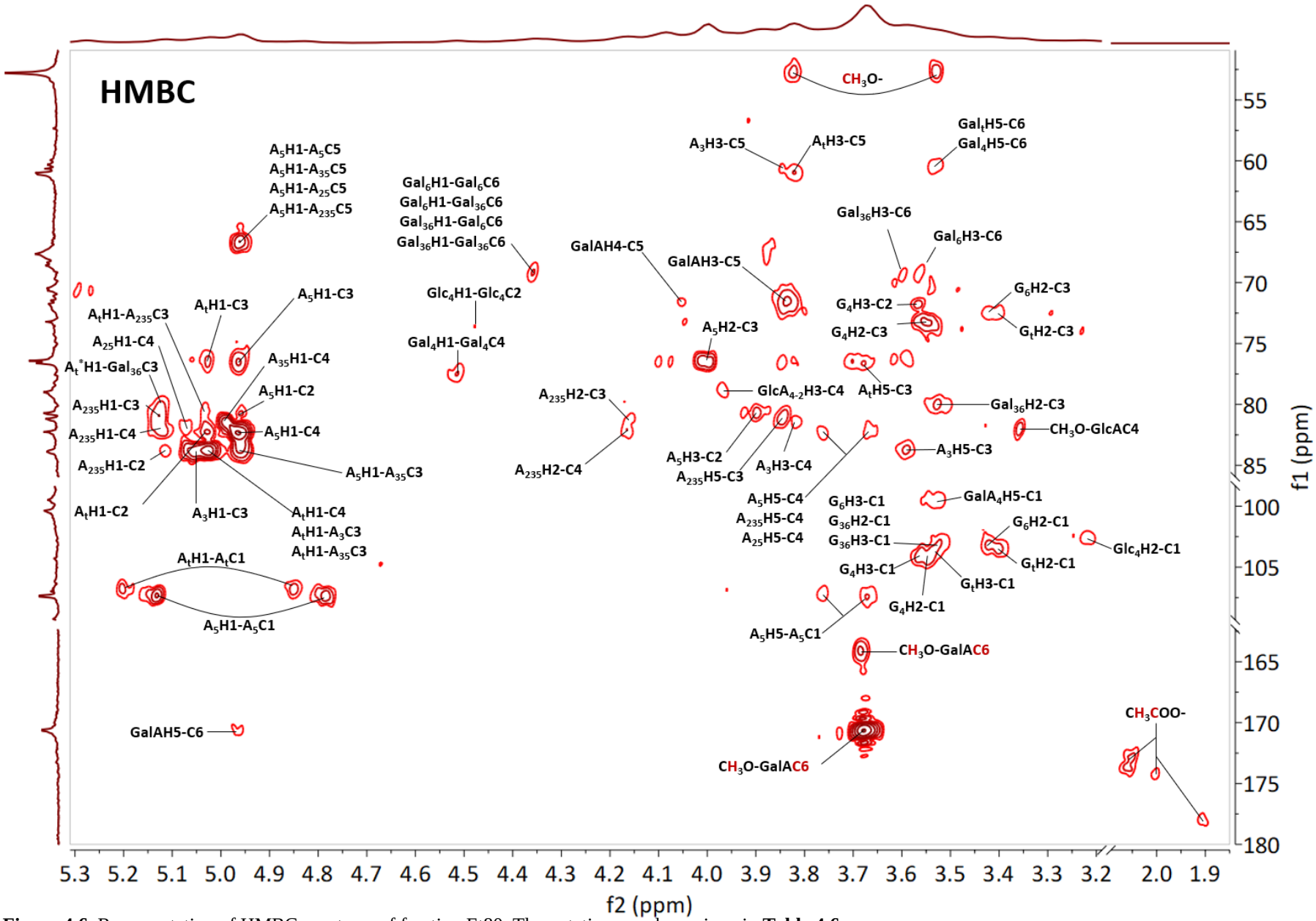


Figure 4.6. Representation of HMBC spectrum of fraction Et80. The notations used are given in **Table 4.6.**

The identification of signals belonging to *t*-βGal*p*, (β1→4)-Gal*p*, (β1→3)-Gal*p*, (β1→6)-Gal*p*, and (β1→3,6)-Gal*p* were in agreement with the presence of both galactans, 1→4 linked, like the ones found in type I arabinogalactans, and 1→6 linked Gal ramified at 3 like the ones found in type II arabinogalactans. Galactans are usually found in ramified regions of pectic polysaccharides and type II arabinogalactans can be found both as free polysaccharides and as side chains of rhamnogalacturonan (Makarova, Shakhmatov, & Belyy, 2016; Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Makarova, & Belyy, 2019). In this work, no correlation was observed between these two polysaccharides and RG-I, which can be due to low intensity of these possible interactions.

A methyl group signal not related with carboxyl group of UA was found in the HSQC spectrum at 60.0/3.35 ppm and the chemical shift of C4/H4 at 82.1/3.23 ppm confirmed the existence of 4-*O*-Me-GlcA. A cross-peak at 82.1/3.35 ppm in the HMBC spectrum confirmed an O-methyl substituent at position 4 of β-D-Glc*p*A. According to literature, these residues can occur as terminals of side chains of (β1→6)-Gal*p* chains from type II arabinogalactans (Shakhmatov, Belyy, & Makarova, 2017; Shakhmatov, Toukach, Michailowa, & Makarova, 2014).

NMR spectra interpretation corroborated glycosidic linkage data obtained by methylation analysis. Furthermore, the data revealed that Et80 fraction had methyl- and acetyl-esterified pectic polysaccharides, 4-linked galactans, arabinans, and type II arabinogalactans with 4-*O*-Me-GlcA residues, as well as α-glucans.

4.3.3.2. Characterization of molecular weight by size-exclusion chromatography

The Et80 fraction was submitted to size-exclusion chromatography on a Sephacryl 400-HR to characterize its molecular weight distribution. Elution of compounds was evaluated by phenol-sulfuric acid method and absorbance at 280 nm to monitor polysaccharides and phenolic groups, respectively (**Figure 4.7**). Polysaccharides were eluted first followed by co-elution with phenolic containing compounds, which could indicate the elution of protein and/or polyphenolic compounds adsorbed. Although it was not possible to resolve polysaccharides by size, eluted compounds were separated in four fractions: Et80-I consisted on the material with molecular weight higher than 151 kDa, yielded 20% of the eluted material, and had 37% of polysaccharides; Et80-II consisted on the material with molecular weight between 41 and 151 kDa, yielded 34% of the eluted material, and was the

fraction richest in polysaccharides (73%); Et80-III consisted on the material with molecular weight between 12 and 41 kDa, yielded 25% of the eluted material, had 59% of sugars and represented the peak of material that absorbs at 280 nm; Et80-IV consisted on the material with molecular weight lower than 12 kDa, yielded 11%, and had vestigial amounts of carbohydrates, only 2%.

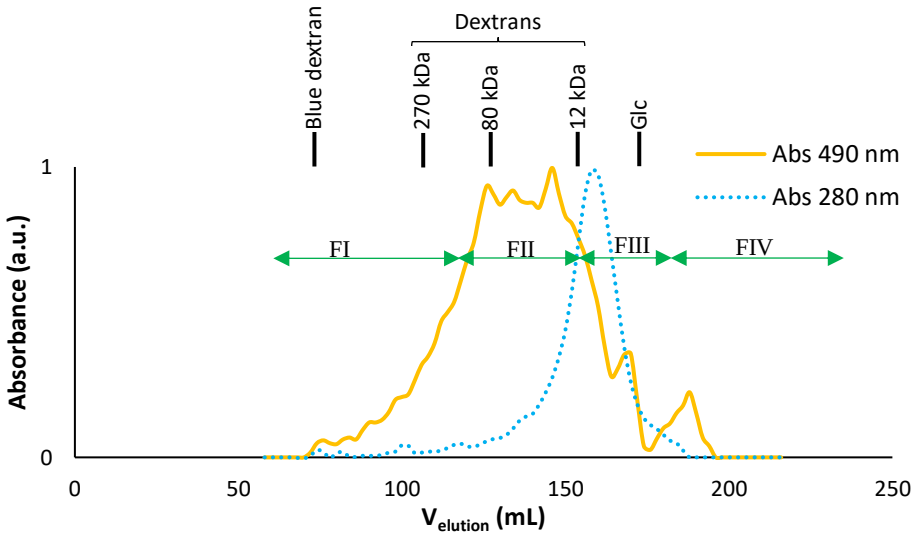


Figure 4.7. Size exclusion chromatography in Sephacryl 400 HR XK 16/100 of Et80 and indication of recovered fractions (FI, FII, FIII, and FIV), blue dextran (void volume), dextrans (12 kDa, 80 kDa, and 270 kDa), and Glc (inner volume of the column) elution volumes.

Carbohydrate composition and linkage analysis of fractions Et80-I, Et80-II, and Et80-III (**Tables 4.4** and **4.5**, respectively) revealed their resemblance with initial Et80, as all consisted in pectic polysaccharides, rich in UA, arabinans, arabinogalactans, and glucans. At the level of detail, it was found that bigger polysaccharides (Et80-I) had higher amount of Ara and smaller polysaccharides (Et80-III) had higher amount of UA, Glc and Gal. Linkage analysis showed that the average branching points of arabinans, estimated by the ratio between the branching units and total Ara, were lower for fractions Et80-I and Et80-III (0.17 and 0.11, respectively) than for fraction Et80-II and initial Et80 (0.23 and 0.21, respectively). The amount of Gal from type II arabinogalactans represented more than 88% of its overall amount and was higher in fractions with high molecular weight (from 0.88 to 0.98). Inversely, the amount of Gal from type I galactans was higher in fractions with lower molecular weight. The branching degree of type II galactans, estimated by the ratio between the branching units ((1→3,6)-Gal*p*) and total Gal from type II galactans (sum of (1→3,6)-,

(1→3)-, (1→6)-, and *t*-Gal*p* linkages), was lower for fractions Et80-I and Et80-III (0.38 and 0.27, respectively) than for fraction Et80-II and initial Et80 (0.47 and 0.46, respectively). Therefore, branching degree from arabinans and type II galactans was correlated, possibly indicating that branched Ara residues are part of type II galactans branching sites.

To evaluate the contribution of molecular weight to fraction Et80 immunostimulatory activity, fractions Et80 I, Et80 II, and Et80 III were incubated with spleen cells. All fractions presented immunostimulatory activity comparable to Et80 (**Figure 4.5**), showing a lack of molecular weight-immunostimulatory activity relationship in Et80, despite the variation of arabinans and type II galactans branching degrees.

4.3.3.3. Characterization of ramified regions of pectic polysaccharides after enzymatic treatments

To detail ramified regions of pectic polysaccharides found in broccoli by-products polysaccharides, two approaches of enzymatic treatments were followed. In the first approach, Et80 fraction was treated with α-L-arabinofuranosidase to characterize arabinans and arabinogalactans domains. The second approach consisted of two steps applied to HW: a purification with pronase and α-amylase, to remove proteins and starch-like polysaccharides, respectively, and the treatment with pectin methylesterase and endo-polygalacturonase to deesterify methyl groups and remove homogalacturonan domains from pectic polysaccharides.

First approach: α-L-arabinofuranosidase treatment

After α-L-arabinofuranosidase treatment, 79% of Et80 fraction was recovered in Et80_Ara. Et80_Ara had 61% of total sugars and was mainly composed by UA, Gal and Ara. Comparing to Et80 fraction, 42% of Ara was lost. Linkage analysis revealed that both arabinans and type II galactans branching degrees were reduced to 56% (0.12) and 49% (0.22), respectively. The decrease of 3,6-linked Gal and the increase of 6-linked Gal indicated that terminal Ara residues or small chains of Ara were mainly linked to the position 3 of 6-linked Gal. The remaining 58% Ara residues in Et80_Ara should be part of longer arabinan chains, as α-L-arabinofuranosidase preferably act in single Ara residues and not on the longer arabinan chains (Taylor et al., 2006).

The reduction of Ara residues linked to Gal residues was also observed by NMR spectroscopy, by the reduction of signals with higher chemical shifts from C-1 of Ara residues linked Gal (**Figure 4.4**). This effect was described in literature for type II arabinogalactans submitted to arabinofuranosidase treatment (Leboeuf, et al., 2004) and corroborate the information obtained by methylation linkage analysis.

The sample Et80_Ara was incubated with spleen cells, resulting in stimulation of B cells in the same extension of the observed for Et80 fraction. These results show that removal of terminal Ara or small Ara chains did not interfere with immunostimulatory activity of broccoli by-products pectic polysaccharides, which was previously observed with other arabinogalactans anticomplementary activity, indicating that the core (1→3,6)- and (1→6)-β-D-galactan might be essential for the expression of the activity (Yamada & Kiyohara, 2007; Zou et al., 2019).

Second approach – purification with pronase and α-amylase, and treatment with pectin methylesterase and endo-polygalacturonase

To disclose more structural details about pectic polysaccharides, a second approach for purification of branched regions was performed by removing protein, starch-like material, and homogalacturonan regions of sample HW, before preparation of fraction Et80. After these enzymatic treatments, 32% of HW material was recovered in Enz_HW, that had 60% of sugars, mainly UA (37 mol%), Ara (33 mol%), and Gal (19 mol%) (**Table 4.4**). Only 28% of HW polysaccharides were recovered in Enz_HW: 16% of UA, 51% of Ara, 53% of Gal, and 22% of Glc, indicating that the reduction of sugars was mainly due to the degradation of homogalacturonans and starch-like polysaccharides. Moreover, homogalacturonans degradation may have promoted the reduction of molecular weight of ramified regions with Ara and Gal, leading to their loss by wash out through the dialysis membranes (cut off 12-14 kDa).

The remaining 37% of UA in Enz_HW is expected to belong to homogalacturonans regions with acetyl groups observed by NMR spectroscopy in Et80 fraction, as it is known that endo-polygalacturonase activity is impaired by the presence of acetyl groups (Searle-van Leeuwen,Vincken, Schipper, Voragen, & Beldman, 1996). Moreover, rhamnogalacturonan regions, Glc*p*A and 4-methyl-Glc*p*A, also observed by NMR spectroscopy in Et80 fraction, would contribute to the amount of UA quantified.

The glycosidic linkage analysis confirmed the isolation of pectic polysaccharides with arabinans and arabinogalactans from Enz_HW (**Table 4.5**). The estimation of branching degree of arabinans from Enz_HW (0.19) was lower than both HW and fraction Et80 (0.22 and 0.21, respectively) and the branching degree of galactan moiety from type II arabinogalactans from Enz_HW (0.50) was higher than that observed in both HW and fraction Et80 (0.43 and 0.46, respectively). These results indicate that ramified Ara moieties were more prone to be localized in accessible sites for enzyme hydrolysis and be washed out. On the other hand, more ramified type II galactans were associated to higher molecular weight and remained in Enz_HW fraction.

The structural changes observed in Enz_HW favoured the solubility of broccoli by-products polysaccharides in 80% ethanol (92 % of the Enz_HW material), in contrast with the solubilization of only 14% of HW in 80% Ethanol (fraction Sn80), probably due to the removal of homogalacturonan know to be insoluble in ethanol and the reduction of pectic polysaccharides molecular weight (Guo, Zhang, Meng, & Yu, 2017). Et80_Enz and SnEt_Enz sugar composition resembled Enz_HW. Notwithstanding, Et80_Enz had lower sugar content than SnEt_Enz_HW, 36% and 66%, respectively.

Enz_HW was incubated with spleen cells and stimulated less B cells than HW and fraction Et80. This result questions the importance of homogalacturonans to the core structure of polysaccharides immunostimulatory sites or the existence of masking elements in Enz_HW. To reveal the impact of homogalacturonan removal and charge in pectic polysaccharides immunostimulatory activity, both Et80 and Enz_HW fractions were submitted to anion-exchange chromatography.

4.3.3.4. Characterization of charge by anion-exchange chromatography

To evaluate the contribution of charge of both Et80 and Enz_HW fractions, they were fractionated on a DEAE-Trisacryl M column eluted with buffer without NaCl and with increasing concentrations of NaCl (**Figure 4.8**). Et80 and Enz_HW were mainly eluted with buffer without NaCl (34% for Et80A and 36% for Enz_HWA, respectively) and with 0.125 M of NaCl (45% for Et80B and 50% for Enz_HWB, respectively). With 0.250 M NaCl were eluted the compounds that absorbed more at 280 nm, representing low yields of Et80 (8%) and of Enz_HW (14%), and having low amount of sugars (7.1% and 1.7% respectively). Only traces were eluted with higher concentrations of NaCl and were not considered.

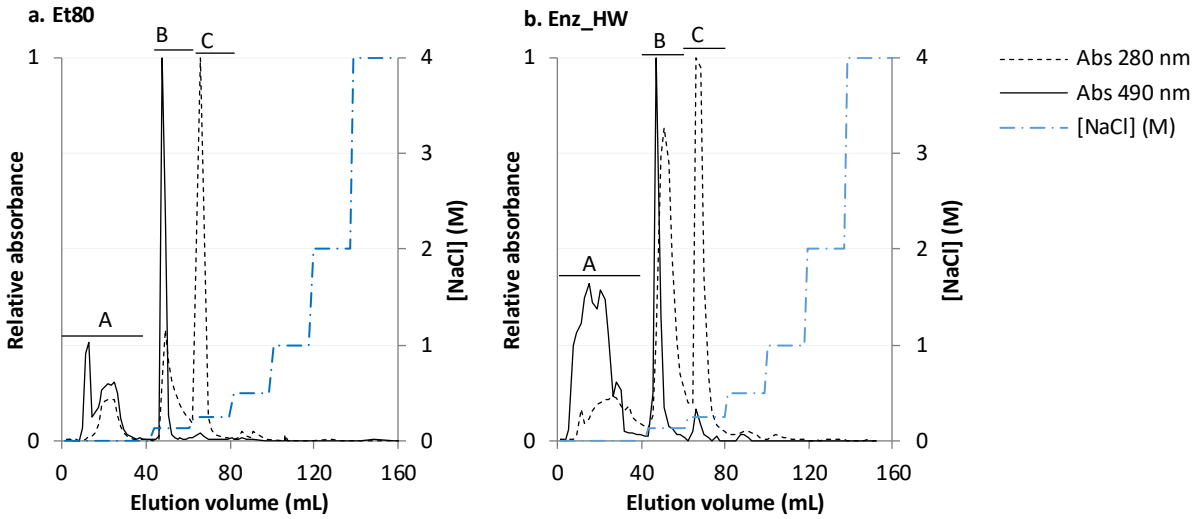


Figure 4.8. Anion-exchange chromatograms of samples **a.** Et80 and **b.** Enz_HW

Both fractions Et80 A and Et80 B, eluted without and with NaCl had similar amount of UA (63-65 mol%) than initial Et80 (**Table 4.4**). As Et80A fraction was the neutral fraction eluted without ionic strength, its UA residues must be the ones protected by methyl and acetyl esterification, observed by NMR spectroscopy, and the ones protected by hindrance, providing neutral global charge. Differences between Et80A and Et80B were found in Ara:Gal ratio: fraction Et80A had a ratio of 1 whereas fraction Et80B had a ratio of 2. Linkage analysis showed that fraction Et80A had arabinans and type II galactans branching degrees (0.24 and 0.31, respectively) lower than Et80B (0.43 and 0.40, respectively). Moreover, the amount of 4-linked Gal revealed that type I galactans were mainly recovered in Et80A.

Fraction Enz_HWA had lower amount of UA than Enz_HWB (34 vs 40 mol%), but values were near the amount found in the initial Enz_HW (37 mol%). As justified for Et80 fractions, UA found in Enz_HWA must be protected providing neutral global charge. Ara:Gal ratios of Enz_HWA and Enz_HWB were the same observed for Et80A and Et80B, respectively. Linkage analysis showed that fraction Enz_HWA had arabinans and type II galactans branching degrees (0.19 and 0.49, respectively) equal to initial Enz_HW, whereas Enz_HWB had higher arabinans branching degree (0.25) and lower type II galactans branching degree (0.32).

The fractions Et80A, Et80B, Enz_HWA, and Enz_HWB were incubated with spleen cells to evaluate their potential immunostimulatory activity. Et80A was as active as Et80, but Et80B was less active. Enz_HWA had lower activity as Enz_HW, while Enz_HWB had much higher activity (46%). Therefore, Enz_HW had structural features with immunostimulatory activity, which were recovered in Enz_HWB. The biological potential of the latter was comparable to that of Et80 fraction, despite Enz_HWB not having the long homogalacturonans and lower type II galactans branching degree (0.32). Arabinans branching degree from Enz_HWB (0.25) was slightly higher than the one from Et80 (0.21). These results indicate that the acidic fraction from Enz_HW has immunostimulatory activity, albeit this was masked by the polysaccharides recoverd in Enz_HWA.

4.3.3.5. Effect of chemical sulfation on pectic polysaccharides

To study the effect of chemical modification with sulfate groups, known to contribute to immunostimulatory activity, Et80 fraction was incubated with chlorosulfonic acid and pyridine to produce sulfated polysaccharides (Et80_Sulf). Et80_Sulf had 52% of total sugars and a sugar composition resembling Et80 fraction. Linkage analysis showed an increase of ramifications points in Ara, Gal and Glc, indicating the sulfation of these residues. Moreover, the sulfation was observed by FTIR analysis (**Figure 4.9**) by the observation of a strong S=O absorption band at 1260 cm^{-1} (Castro et al., 2006).

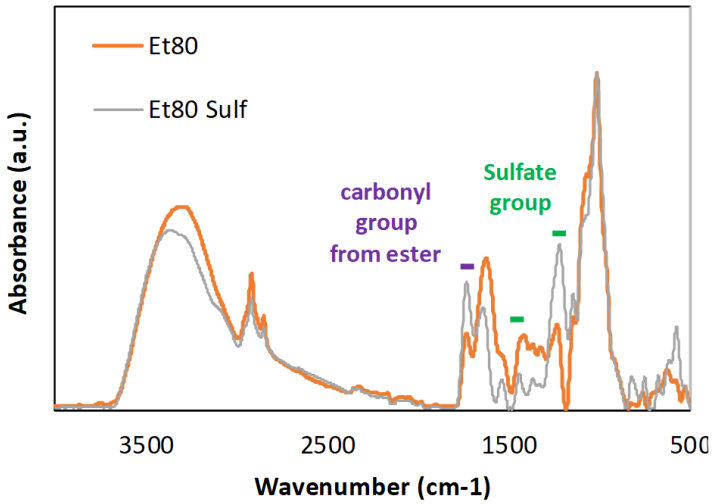


Figure 4.9. FTIR spectra of samples Et80 and Et80_Sulf acquired by ATR sampling technique (shown after baseline correction and smooth correction; background spectrum subtracted to aid clarity).

When incubated with spleen cells, Et80_Sulf had lower immunostimulatory activity than Et80, hence it is feasible to conclude that the derivatization may hidden structural features more important for polysaccharides bioactivity than the presence of sulfate groups itself.

4.4. Concluding remarks

The results herein collected showed that broccoli by-products were a source of pectic polysaccharides with *in vitro* B lymphocytes stimulatory activity. Immunostimulatory pectic polysaccharides were obtained after preparation of AIR (stabilized by solvent drying), hot water extraction, and 80% ethanol precipitation (Et80). In contrast, pectic polysaccharides, xyloglucans, and xylans obtained after sequential extractions with imidazole, Na_2CO_3, and KOH solutions did not stimulate B lymphocytes.

Pectic polysaccharides from Et80 fraction were methyl- and acetyl-esterified and had ramified regions with 4-linked galactans, arabinans, and type II arabinogalactans with 4-O-Me-GlcA residues. Fractionation of Et80 polysaccharides by molecular weight (from 12 to 400 kDa) showed that all fractions had immunostimulatory activity. Removal of terminal Ara or small chains of Ara also did not change immunostimulatory activity. However, the methyl deesterification of pectic polysaccharides and removal of galacturonan regions reduced polysaccharides immunostimulatory activity. Fractionation of these polysaccharides allowed to obtain an acidic fraction with immunostimulatory activity, indicating that neutral polysaccharides were masking the immunostimulatory activity of the acidic ones. Chemical sulfation also reduced activity, probably by hiding the structural features for B cells activation.

The presence of pectic polysaccharides with potential immunostimulatory activity in broccoli by-products showed that these by-products can be valorised as functional food ingredients that improve immune function and promote health.

CHAPTER 5

Potential of broccoli by-products for hydrophobization of starch films surfaces

Graphical abstract:

CHAPTER 5

Potential of broccoli by-products for hydrophobization of starch films surfaces

5.1. Potential of broccoli by-products for hydrophobization of starch film surfaces - approach

5.2. Material and methods

5.2.1. Broccoli by-products, starch and chemicals

5.2.2. Characterization of broccoli by-products

5.2.3. Preparation of films

5.2.4. Films characterization

5.2.5. Statistical analysis

5.3. Results and discussion

5.3.1. Characterization of broccoli by-products incorporated in the films

5.3.2. Impact of broccoli by-products addition on starch films

5.3.3. Effect of one-year storage in starch films with broccoli by-products

5.4. Concluding remarks

Parts of the text of this chapter will be submitted in the following publication:

Sónia S. Ferreira, Idalina Gonçalves, Paula Ferreira, Susana M. Cardoso, Dulcineia Ferreira Wessel, Manuel A. Coimbra (**2020**). Hydrophobization of starch films surfaces with broccoli by-products. (In preparation)

5.1. Potential of broccoli by-products for hydrophobization of starch film surfaces - approach

Broccoli by-products represent a range of compounds that could be used as biomaterials to improve starch films properties. For that, broccoli by-products were characterized concerning the glucosinolates, phenolic compounds, free amino acids, protein, and carbohydrates and were applied in the formulation of starch-based films for food packaging. Three approaches were followed in the preparation of starch films: addition of by-products after gelatinization; addition of by-products before gelatinization; and addition of by-products with a natural crosslinker, genipin. Impact of broccoli by-products incorporation in starch films properties was evaluated by analysis of surface wettability and mechanical properties. Properties of the most promising films with broccoli by-products were re-evaluated after one year of storage. These films were further characterized according to their antioxidant activities, their optical properties measured by CIELAB and FTIR spectroscopy, crystallinity measured by X-Ray Diffractometry (XRD), and surface analysis shown by Scanning Electron Microscopy (SEM).

5.2. Material and methods

5.2.1. Broccoli by-products, starch and chemicals

Broccoli by-products, provided by the frozen food industry Monliz SA, Portugal, were used dehydrated by microwave hydrodiffusion and gravity technology (BB_MHG, **Chapters 2 and 3**), after dehydration and water extraction (BB_Residue, **Chapter 2**), and unprocessed (BB, **Chapter 4**). BB_MHG and BB_Residue were milled and sieved to obtain a powder with particle size lower than 63 µm and BB was milled before films preparation.

The starch used in this work was obtained from the wastewater of potato industry, A Saloinha, Portugal. The starch was isolated after wastewater decanting, washing, and dehydration. Before use, the starch was milled and sieved to obtain a particle size lower than 150 µm, dried at 105 ºC overnight, and placed in a desiccator in order to control the water content, as it also functions as a plasticizer.

All reagents used were of analytical grade or higher available purity.

5.2.2. Characterization of broccoli by-products

To explain the properties of starch films when broccoli by-products were added, broccoli by-products were characterized concerning glucosinolates, phenolic compounds, free amino acids, protein, and carbohydrates, as shown in previous chapters. Intact glucosinolates and phenolic compounds were extracted and analysed by UHPLC-DAD-ESI/MS^n, as previously described (**Chapter 3, Section 0 and Chapter 4, Sections 4.22 and 4.2.3**). Total phenolic compounds (TPC) were also estimated using the Folin-Ciocalteu colorimetric method (**Chapter 3, Section 2.2.6.1**). Free amino acid composition of extracts hydrodiffused by MHG experiments or obtained with 80% boiling ethanol (**Chapter 2, Section 2.2.2** and **Chapter 4, Section 4.2.2**, respectively) were derivatized with ethyl chloroformate according to the method described in **Chapter 2**, **Section 2.2.6.4**. Total protein content of broccoli by-products was estimated by the determination of total nitrogen (N) using elemental analysis (**Chapter 2, Section 2.2.5.8**). Carbohydrates were analysed after acid hydrolysis, derivatisation to alditol acetates, and analysis of individual neutral sugars by GC-FID and quantification of UA by the 3-phenylphenol colorimetric method, as described in **Chapter 2**, **Section 2.2.5.2**.

5.2.3. Preparation of films

To study the effect of broccoli by-products addition to starch films, three film preparation approaches were used: **1st**: addition of by-products after gelatinization; **2nd**: addition of by-products before gelatinization; and **3rd**: addition of by-products before gelatinization, with addition of genipin as crosslinker halfway at the gelatinization process. In the **1st approach**, starch (4 g) and 30% w/w of glycerol (1.2 g in 4 g of starch) were dispersed in 100 mL of distilled water at room temperature (RT, 20 ºC) for 5-10 min with stirring. The resulting starch suspension was gelatinized at 95 ºC, for 30 min with stirring. Afterwards, it was filtered and placed again at 95 ºC with addition of BB_MHG or Res_MHG powders in the proportions of 0.5%, 1%, or 2% w/w of starch or 0% as control, for 15 min with stirring. The solution was degassed, 21 g were transferred into plexiglass plates (144 cm^2 of area and 3 mm of deep) and placed in a convective oven at 25 ºC overnight for film formation by solvent casting.

In the **2nd approach**, starch (4 g), 30% w/w of glycerol (1.2 g), and broccoli by-products (BB_MHG, Res_MHG, or BB on a dry weight basis) in the proportions of 0.5%,

1%, or 2% w/w of starch were dispersed in 100 mL of distilled water or less to account BB own water (90% water content), at room temperature (RT, 20 ºC) for 5-10 min with stirring. A control without addition of broccoli by-products was also prepared. The resulting suspension was gelatinized at 95 ºC, for 30 min with stirring. Afterwards, it was filtered, degassed, and 21 g were transferred into plexiglass plates (144 cm^2 of area and 3 mm of deep) and placed in a convective oven at 25 ºC overnight for film formation by solvent casting.

In the **3rd approach**, 0.05% w/v of genipin (50 mg in 500 µL of ethanol in final 100 mL of water) was added halfway through the gelatinization process, this is, after 15 min of gelatinization, following the conditions of the **2nd approach** with addition of BB unprocessed.

All films were removed from plexiglass plates and stored under controlled equilibrium relative humidity (54%) for five days before characterization. The water contact angle (wettability) and the mechanical properties were determined for all films. Films with better properties were further characterized for their water solubility and antioxidant activity and reanalysed after 1-year storage in the dark. Furthermore, films were also characterized by means of FTIR spectroscopy, XRD, and SEM analysis to relate the molecular interactions between film materials.

5.2.4. Films characterization

5.2.4.1. FTIR spectroscopy

FTIR spectra of the films were obtained using a Golden Gate single reflection diamond ATR system in a Perkin Elmer Spectrum BX spectrometer according **Chapter 4**, **section 4.2.12.6**.

5.2.4.2. Optical properties

The evaluation of color change was assessed by tristimulus colorimetry (CIELab). CIELab parameters, namely *a** (red/green), *b** (yellow/blue), and *L** (luminosity) components were determined using a CR400 Chroma Meter (Oliveira et al., 2020).

5.2.4.3. Wettability

The contact angle of ultrapure water drops with film surfaces was calculated by an image analysis software (SCA20M4 Dataphysics) using the Laplace–Young method (Nunes et al., 2013). The up surface was the one in contact with air and the down surface was the one in contact with the plate during casting. At least nine droplet images were obtained for each film surface.

5.2.4.4. Mechanical properties

The mechanical properties of films were evaluated as described by Nunes et al. (2013). The tensile test was performed at room temperature using a texture analyser equipment (model TA.Hdi, Stable Micro Systems) equipped with fixed grips lined with thin rubber on the ends. All films were cut in strips with 90 mm length and 10 mm wide for the determination of tensile properties. The film ends were assembled on the grips and the initial grip separation was set at 50 mm. The crosshead speed was set at a constant rate of 0.5 mm/s. Young's modulus, percentage elongation or strain at break, and tensile strength or stress at break were determined from stress–strain curves obtained from uniaxial tensile tests to film failure. These parameters were calculated based on ASTM D 882-83 standard method. At least five samples of each film type were tested. Film thickness was measured using a hand-held micrometer (Mitutoyo Corporation), along the length of the rectangular strip.

5.2.4.5. Antioxidant activity - ABTS assay

The antioxidant activity of the films produced was determined by an adaptation of the method of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, ABTS, as described by Nunes et al. (2013). Briefly, a solution of 7 mM ABTS was prepared in 2.45 mM potassium persulfate. This solution was left in the dark, at room temperature, for 12–16 h for $ABTS^{\bullet+}$ formation and its absorbance adjusted to 0.7-0.8 absorbance values measured at 734 nm (EON, BioTek) when transferring 300 µL to 96 wells plate, by diluting 1:80 in ethanol. One square (1 cm^2) of film was placed in 1.5 mL of $ABTS^{\bullet+}$ solution and left to react in the dark with orbital stirring at 80 rpm (Lab-Line 4626-1, USA), over 7 days. The absorbance at 734 nm of the solution was measured after 0.5 h, 1 h, 2 h, 4 h, 6 h, 24 h, 48 h, 72 h, and 7 days. All measurements were performed in triplicate.

5.2.4.6. Moisture and water solubility

Moisture of the films was determined by measuring their loss of weight, upon drying in a convective oven at 105˚C until reaching a constant weight (dry film weight). Solubility of the films was determined in distilled water. One square (4 cm^2) of film was placed in 30 mL of water at room temperature with orbital agitation (80 rpm, Lab-Line 4626-1, USA) for 7 days. Then, films were placed in an oven at 105 ˚C for 16 h, allowed to cool at room temperature, and weighed. The solubility was determined as follows: $Solubility = (w_b - w_a)/w_b \times 100$, where w_b and w_a were the weight of dry film before and after being immersed in water, respectively. These determinations were performed in triplicate.

5.2.4.7. XRD

X-ray diffractograms were conducted using a PANalytical Empyrean X-Ray diffractometer. The radiation used was Cu-Kα (λ = 0.15406 nm). The scan was carried out at 45 kV and 40 mA for 2θ range of 5-60º with a step size of 0.04º and a collection time of 196 seconds at each step.

5.2.4.8. SEM

SEM of films surface was performed using a SU-70 (Hitachi) SEM microscope at an accelerating voltage of 4 kV and a working distance of about 7 mm. Film samples were fixed at the holder using carbon tape and covered with a thin carbon layer.

5.2.5. Statistical analysis

Differences among starch control films and starch films with broccoli by-products were analysed through One-Way ANOVA followed by Tukey's multiple comparisons tests and paired Student's t-test, respectively, in GraphPad prism, Trial Version 6.01 (GraphPad Software, Inc. La Jolla, CA, USA). Differences were deemed significant at p-value<0.05.

5.3. Results and discussion

5.3.1. Characterization of broccoli by-products incorporated in the films

Carbohydrates accounted for 38% to 41% of broccoli by-products incorporated in the starch films (**Table 5.1**). BB_MHG and BB unprocessed had similar sugar composition and amount of pectic polysaccharides (52% and 46% as sum of Rha, Fuc, Ara, Gal, and UA, respectively), which are the main hot water extractable polysaccharides (**Chapters 2** and **4**). In contrast with BB, BB_MHG contained less free sugars, due to their hydrodiffusion along MHG experiments, and BB_MHG pectic polysaccharides were more prone to extraction (**Chapter 2**, **Section 2.3.2.2)**. BB_Residue presented lower amount of pectic polysaccharides (37%), intrinsically connected to the cellulosic matrix of plant cell wall, in accordance with the 6 exhaustive hot water extractions submitted, where part of pectic polysaccharides and small molecular weight compounds were removed (**Chapter 2**, **Section 2.3.2.2)**.

Total protein accounted for 13% and 11% of BB_MHG and BB unprocessed, respectively (**Table 5.1**). Free amino acids comprised 3.2% of BB unprocessed and are expected to be present in BB_MHG, as only part of them where hydrodiffused after MHG experiments. Both free amino acids in BB unprocessed and in MHG extracts were rich in glutamine (**Chapter 2, Section 2.3.2.3**).

Table 5.1. Yield, carbohydrate composition, total carbohydrates, total protein and extractable material from broccoli by-products added to starch films.

Sample	η (%)	Carbohydrate composition (mol%)								Total carbohydrates (mg/g)	Total protein (mg/g)	Extractable material (%)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA			
BB_MHG	100	1	1	15	4	4	9	40	26	375 ± 28	134 ± 10	41[a]
BB_Residue	40[b]	[tr]	1	9	8	3	9	52	18	413 ± 4	[n.d.]	[tr]
BB unprocessed	100	1	[tr]	13	4	4	9	46	23	384 ± 24	111 ± 12	38[c]

[a] Material extracted after 1 hot water extraction (**Table 2.2**); [b] yield of residue from BB_MHG after 6 sequential hot water extractions; [c] material extracted with 80% boiling ethanol (31%, **Table 4.1**) and hot water (12% of AIR, this is 7%, **Table 4.4**); [tr] traces; [n.d.] not determined. Some data are expressed as mean ± standard deviation of three replicates.

The glucosinolates identified by UHPLC-DAD-ESI-MS^n in unprocessed broccoli by-products were glucoraphanin, glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin (**Figure 5.1**). Quantification of these glucosinolates showed that they accounted for 6.0 mg/g dw of by-products, which is in the range of values found in BB_MHG and in the other broccoli by-products studied (**Chapter**

3, Section 3.3.2). Although only four glucosinolates (glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin) were identified in BB_MHG, glucoraphanin was the main glucosinolate in both broccoli by-products (**Table 5.2**).

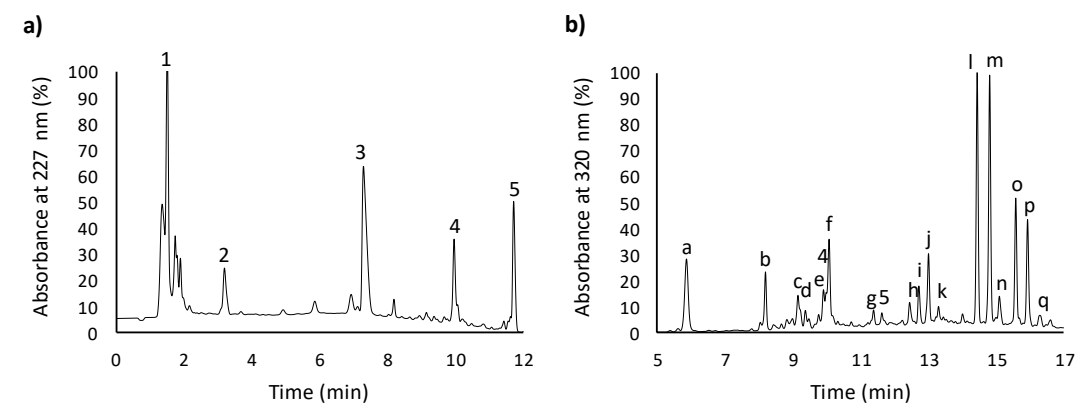


Figure 5.1. Chromatograms obtained at **a)** 227 nm and **b)** 320 nm for glucosinolates and phenolic compounds detection, respectively, from unprocessed broccoli by-products. Numbers (glucosinolates) and letters (phenolic compounds) in the chromatograms correspond to the UHPLC-DAD-ESI-MS^n peaks described in **Tables 5.2** and **5.3**, respectively.

Table 5.2. Identification and quantification of glucosinolates from BB_MHG and BB unprocessed by UHPLC-DAD-ESI-MS^n.

P	RT	$[M-H]^-$: Main MS^2 $[M-H]^-$	Probable Compound	C (mg/g)	
				BB_MHG	BB
1	1.5	**436:** 372 (100); 259 (4)	Glucoraphanin	5.7	4.0
2	3.2	**463:** 259 (100); 275 (44)	4-Hydroxyglucobrassicin	-	0.2
3	7.3	**447:** 259 (100); 205 (44); 291 (40); 275 (31)	Glucobrassicin	0.2	1.0
4	10.0	**477:** 259 (100); 275 (53); 281 (20); 299 (17)	4-Methoxyglucobrassicin	0.1	0.4
5	11.7	**477:** 446 (100); 259 (2)	Neoglucobrassicin	0.1	0.4
			Total	**6.1**	**6.0**

P – peak number in **Figure 5.1**; RT – retention time (min); $[M-H]^-$: Main MS^2 $[M-H]^-$ – molecular ion and main product ions observed with percentage in brackets (m/z), other ions were found although not discriminated; C (mg/g) – Concentration of glucosinolates expressed as mg/g of material dry weight.

The analysis of phenolic compounds from unprocessed broccoli by-products showed the presence of glucosylated flavonoids (quercetin and kaempferol) esterified with one or two hydroxycinnamic acids (caffeic, sinapic, and ferulic) and hydroxycinnamic acids (sinapic, methoxycaffeic, ferulic, and coumaric) esterified with Glc or gentiobiose (Glc disaccharide). Only esters of hydroxycinnamic acids with quinic acid were found as non-glycosylated phenolic compounds (**Table 5.3**). Overall, these compounds were also observed in BB_MHG (**Chapter 3**). However, diversity of compounds was lower in BB than in BB_MHG, which may be due to the different solvents used to extract phenolic compounds

Table 5.3. Identification and quantification of phenolic compounds from BB_MHG and BB analysed by UHPLC-DAD-ESI-MS^n.

P	RT	$[M-H]^-$: Main MS^2 $[M-H]^-$	λ_{max}	Probable compound	C (mg/g)	
					BB_MHG	BB
Quercetin glucosides						
2	**8.1**	**949:** 787 (100)	228, 266, 316, 343sh, 368sh#	Q-3-C-dG-7-G	0.05	-
9	**9.6**	**993:** 831 (100); 787 (40)	243, 275sh, 317, 368sh	Q-3-S-dG-7-G	0.13	-
j	**13.0**	**1523:** 599 (100)	243, 270, 330, 367sh#	Q-3-dS-tG-7-dG	-	0.59
Kaempferol glucosides						
3	**8.3**	**771:** 609 (100)	228sh, 265, 320sh, 349, 368sh#	K-3-dG-7-G	0.04	-
4	**8.6**	**933:** 771 (100)	229sh, 265, 355, 368sh	K-3-dG-7-dG	0.05	-
5	**8.9**	**563**	222sh, 239, 331	K derivative	0.06	-
Monoacylated kaempferol glucosides						
7	**9.1**	**963:** 801 (100); 771 (20)	238, 269, 332, 368sh	K-3-MC-dG-7-G	0.11	-
8 **d**	**9.4**	**933:** 771 (100); 609 (5)	243, 268, 335#	K-3-C-dG-7-G	0.07	0.18
8	**9.4**	**1095:** 771 (100); 609 (37)	243, 268, 335#	K-3-C-tG-7-G	0.01	-
10	**9.8**	**1301**	241, 270, 332, 368sh	K-3-S-tG-7-dG	0.02	-
11 **e**	**9.9**	**1139:** 815 (100); 609 (29)	240, 269, 333, 368sh	K-3-S-tG-7-G	0.11	0.15
12	**10.0**	**977:** 815 (100); 609 (15)	239, 268, 334, 368sh	K-3-S-dG-7-G 1	0.02	-
13	**10.2**	**1109**	243, 268, 338, 368sh	K-3-F-tG-7-G	0.02	-
14	**10.3**	**947:** 785 (100); 771 (75)	242, 268, 333, 368sh	K-3-F-dG-7-G 1	0.01	-
15	**10.5**	**917:** 755 (100);771 (60)	239, 367, 320, 348sh, 368sh#	K-3-Co-dG-7-G 1	0.02	-
17	**11.3**	**917:** 755 (100)	244, 269, 344, 368sh#	K-3-Co-dG-7-G 2	0.02	-
17	**11.3**	**947:** 785 (100); 609 (2)	244, 269, 344, 368sh#	K-3-F-dG-7-G 2	0.01	-
h	**12.4**	**977:** 815 (100); 609 (16)	244, 268, 330, 368sh	K-3-S-dG-7-G 2	-	0.22
i	**12.7**	**947:** 785 (100); 609 (6)	243, 270, 330, 367sh#	K-3-F-dG-7-G 3	-	0.12
Diacylated kaempferol glucosides						
21	**12.5**	**1463:** 1139 (100)	242, 270, 328, 367sh#	K-3-C/S-tG-7-dG	0.09	-
21	**12.5**	**1331:** 1169 (100)	242, 270, 328, 367sh#	K-3-MC/S-tG-7-G	0.29	-
22	**12.7**	**1301:** 1139 (100)	242, 269, 330, 368sh#	K-3-C/S-tG-7-G	0.06	-
22 **i**	**12.7**	**1507**	242, 269, 330, 368sh#	K-3-dS-tG-7-dG	0.03	0.18
23 **j**	**13.0**	**1345:** 977 (100)	241, 269, 330, 368sh#	K-3-dS-tG-7-G	0.15	0.22
23 **j**	**13.0**	**1477:** 1153 (100)	241, 269, 330, 368sh#	K-3-F/S-tG-7-dG	0.02	0.22
24	**13.3**	**1315:** 1153 (100)	243, 269, 330, 368sh	K-3-F/S-tG-7-G	0.02	-
Esters of hydroxycinnamic acids with gentiobiose						
15 **f**	**10.1**	**385:** 223 (100); 247 (56); 205 (40)	240, 330	Sinp-Glc	0.04	0.25
g	**11.4**	**753:** 529 (100)	243, 325	diSinp-Gentb 1	-	0.12
18	**11.6**	**885:** 867 (100)	243, 275sh, 317, 368sh	SinpCafFer-Gentb	0.07	-
k	**13.3**	**739:** 515 (100)	244, 280sh, 328	SinpMeCafGentb	-	0.12
25 **l**	**14.4**	**753:** 529 (100)	241, 330, 368sh	diSinp-Gentb	0.20	1.26
26 **m**	**14.8**	**723:** 499 (100)	241, 273sh, 329	SinpFer-Gentb	0.25	1.20
27 **n**	**15.1**	**693:** 469 (100)	246, 326	SinpCoum-Gentb	0.07	0.22
28 **o**	**15.5**	**959:** 735 (100)	244, 323	triSinp-Gentb	0.18	0.83
29 **p**	**15.9**	**929:** 705 (100)	245, 320	diSinpFer-Gentb	0.17	0.68
30 **q**	**16.2**	**899:** 705 (100)	239, 327	SinpdiFer-Gentb	0.10	0.15
Esters of hydroxycinnamic acids with quinic acid						
1 **a**	**5.9**	**353:** 191 (100); 179 (45)	200, 263sh, 289	5-CQA	0.06	0.25
2 **b**	**8.2**	**337:** 163 (100); 191 (6);	228, 266, 316, 343sh, 368sh#	4-p-CoQA	0.13	0.15
6	**9.0**	**367:** 193 (100); 134 (4)	240, 327	4-FQA	0.04	-
c	**9.2**	**353:** 173 (100); 179 (51); 191 (13)	241, 330, 368sh	4-CQA	-	0.15
16	**11.1**	**367:** 191 (100); 173 (64)	241, 277sh, 332, 369sh	5-FQA	0.03	-
				Total	**2.75**	**7.27**

P – peak number and letter in **Figure 3.4** and **5.1**, respectively; RT – retention time (min); $[M-H]^-$: Main MS^2 $[M-H]^-$ – molecular ion and main product ions observed with percentage in brackets (m/z), other ions were found although not discriminated; λmax - maximum wavelengths (nm); BB_MHG - Broccoli by-products dehydrated by microwave hydrodiffusion and gravity (MHG, **Chapter 3**); BB – Broccoli by-products unprocessed (**Chapter 4**); C (mg/g) – Concentration of glucosinolates expressed as mg/g of material dry weight; Q: quercetin; dG: sophoroside; G: Glc; K: kaempferol; MC: MeCaf: methoxycaffeoyl; C: Caf: caffeoyl; tG: sophorotrioside; S: Sinp: sinapoyl; F: Fer: Feruloyl; Co: Coum: Coumaroyl; Gentb: gentiobiose; CQA: caffeoylquinic acid; p-CoQA: pcoumaroylquinic acid; FQA: feruloylquinic acid.

before analysis (80% ethanol and 70% methanol). Total phenolics accounted for 2.8 and 7.3 mg/g dw for BB_MHG samples and BB, respectively.

Given the composition diversity and the potential of broccoli by-products stabilized by MHG, the potential to valorise the residue obtained after hot water extraction (BB_residue), and the use of unprocessed BB as biomaterials, they were used in the preparation of starch films. Impact of broccoli by-products on films wettability, evaluated by the water contact angle, and mechanical properties were studied.

5.3.2. Impact of broccoli by-products addition on starch films

The addition of broccoli by-products (0.5%, 1%, and 2% on dry weight basis of starch) allowed to obtain starch films by solvent casting. In the **1st approach**, when BB_MHG and BB_Residue were added after gelatinization, spots were observed in the films (**Figure 5.2.a**). In fact, although broccoli by-products powders had a particle size lower than 63 µm, they tended to agglomerate when added to hot gelatinized starch. These spots were avoided in the **2nd approach** by adding of broccoli by-products before gelatinization and removal of agglomerates by filtration (**Figures 5.2 and 5.3**). This approach was also used to add blended unprocessed BB. In this approach starch films with broccoli were uniform and transparent as the control at macroscopic level (**Figure 5.2.b**). Films with broccoli were more yellow and less green with the increase of broccoli by-products (**Table 5.2**) The transparency of the films indicates that the semicrystalline structure of starch granules has been destroyed during gelatinization and that broccoli addition did not affected it. Moreover, FTIR analysis showed that spectra of films with broccoli by-products resembled the major components in the film (starch and glycerol), as the control film (**Figure 5.4**).

To further improve the properties of starch films with BB, the crosslinker genipin was added halfway through gelatinization process (**3rd approach**). Genipin crosslinking was observed by the blue color of starch films (**Table 5.4**).

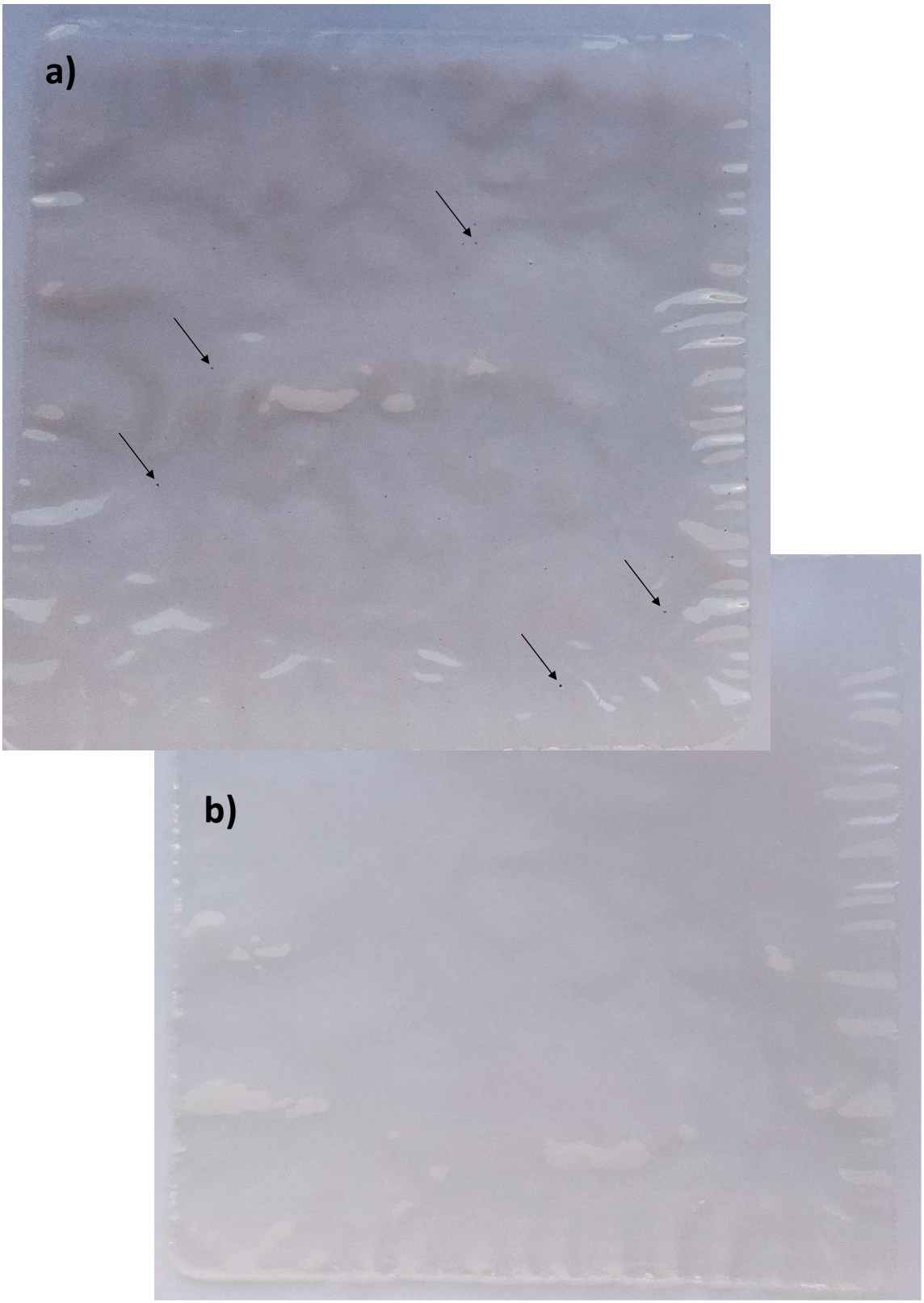


Figure 5.2. Visual appearance of starch films with BB_MHG added **a)** after (**1st approach**) or **b)** before gelatinization and filtration steps (**2nd approach**). Spots are highlighted with arrows ↑.

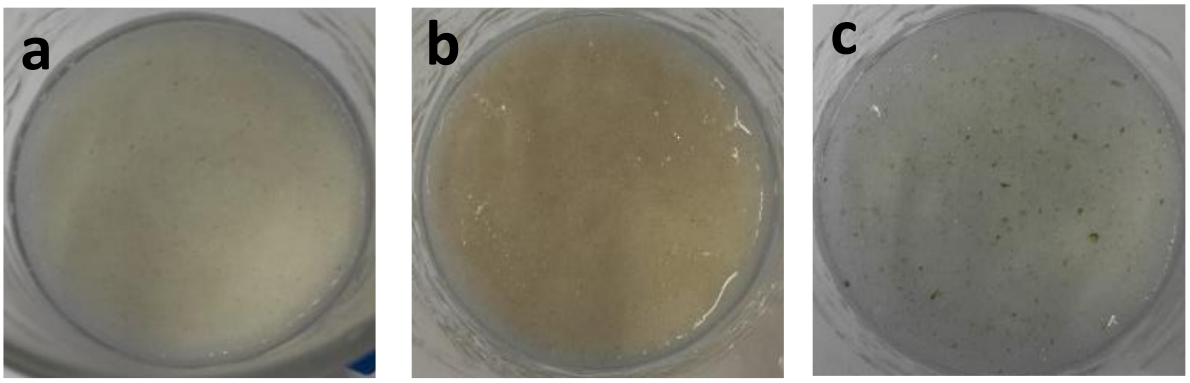


Figure 5.3. Photographs of fritted funnels after filtration of gelatinized starch with **a)** BB_MHG, and **b)** BB_Residue, and **c)** BB.

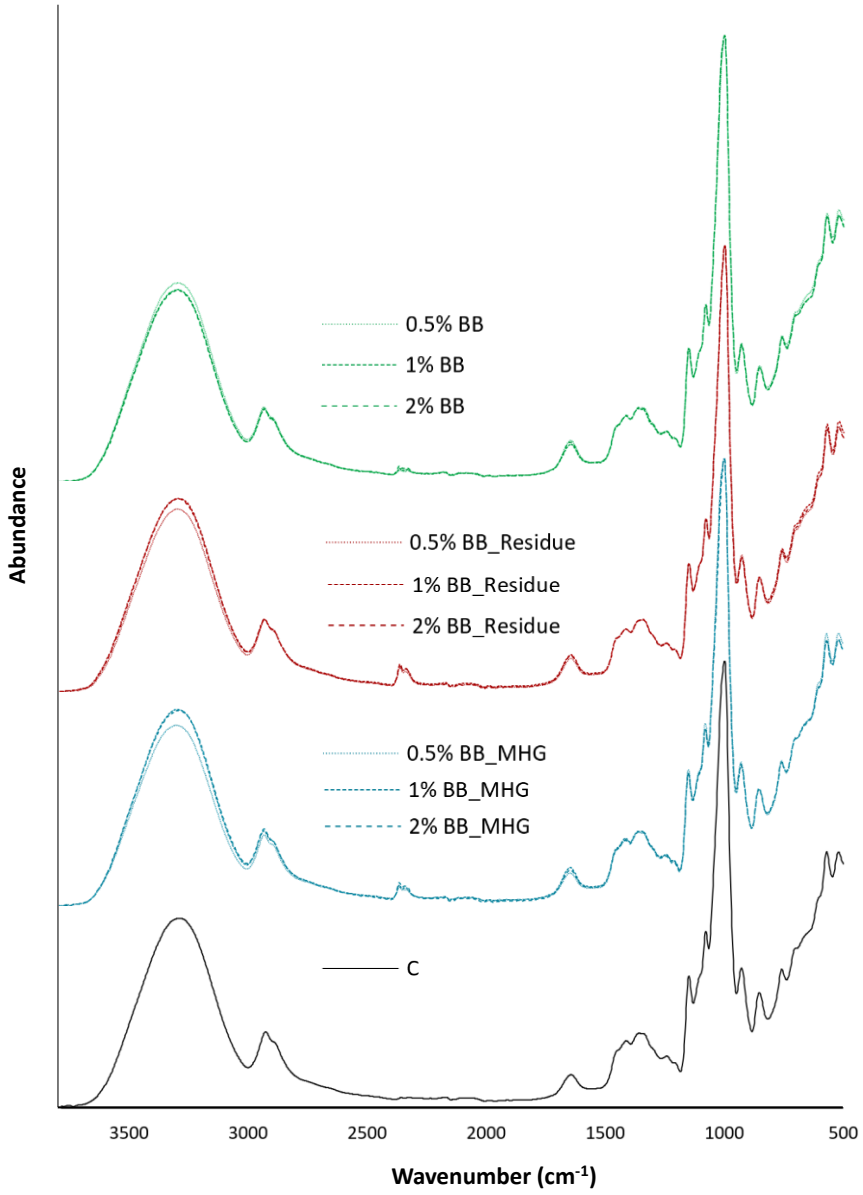


Figure 5.4 FTIR spectra of starch films with broccoli by-products from **2nd approach**.

Table 5.4. CIELab values and visual appearance of starch films with broccoli by-products (**2nd approach**).

		L	*a*	*b*	Visual appearance
Control		92.71 ± 0.22^{a}	1.34 ± 0.02^{a}	-1.79 ± 0.02^{e}	
BB_MHG	**0.5%**	93.28 ± 0.35^{a}	1.34 ± 0.03^{a}	-1.50 ± 0.06^{cde}	
	1%	93.02 ± 0.41^{a}	1.20 ± 0.06^{ab}	-0.85 ± 0.35^{bc}	
	2%	92.73 ± 0.83^{a}	0.92 ± 0.10^{c}	0.34 ± 0.62^{a}	
BB_Residue	**0.5%**	93.01 ± 0.39^{a}	1.25 ± 0.11^{ab}	-1.43 ± 0.15^{bcde}	
	1%	92.61 ± 0.65^{a}	1.16 ± 0.09^{b}	-0.73 ± 0.79^{b}	
	2%	92.43 ± 0.65^{a}	0.90 ± 0.12^{c}	0.61 ± 0.55^{a}	
BB	**0.5%**	92.89 ± 0.31^{a}	1.22 ± 0.03^{ab}	-1.81 ± 0.04^{e}	
	1%	92.81 ± 0.19^{a}	1.14 ± 0.01^{b}	-1.75 ± 0.06^{de}	
	2%	93.07 ± 0.11^{a}	0.87 ± 0.07^{c}	-0.93 ± 0.24^{bcd}	
BB with genipin	**0%**	-	-	-	
	0.5%	-	-	-	
	1%	-	-	-	
	2%	-	-	-	

Data are expressed as mean ± standard deviation of 6 replicates. Different letters between each line in a column means values significantly different $p<0.05$.

5.3.2.1. Mechanical properties

Films with incorporation of BB_MHG and BB_Residue had lower values of tensile strength (10-24 MPa) and Young's modulus (0.8-1.3 GPa) than control film (30 MPa and 1.5 GPa, respectively, **Table 5.5**). These values were inversely correlated with increasing amount of broccoli by-products from 0.5 to 2%. As Young's modulus is a measure of the stiffness of the film and the tensile strength is related to the mechanical resistance of the films, addition of broccoli by-products created films with less stiffness and with lower mechanical resistance. The decrease of tensile strength can be explained by the presence of spots due to agglomeration of broccoli by-products powder, promoting the discontinuity of starch matrix around them and consequent loss of strength (Versino & García, 2014). This hypothesis was confirmed by the results where these agglomerates were absent by addition of BB_MHG, BB_Residue and BB before gelatinization and filtration steps, showing an increase of the mechanical resistance and stiffness. The films prepared with BB, containing all the extractable compounds, showed the higher values of tensile strength (34 to 36 MPa) and Young's modulus (1.8 to 1.9 GPa), independently of the concentration used (0.5 to 2%). The films prepared with BB_MHG and BB_Residue, containing less amount of extractable compounds than BB, showed lower tensile strength (24-33 MPa and 25-26 MPa, respectively) and Young's modulus (1.3-1.5 GPa and 1.4-1.6 GPa, respectively), indicating the importance of these extractable compounds for films strength and stiffness.

Addition of genipin to starch films increased their tensile strength (31 MPa) and Young's modulus (1.7 GPa, **Table 5.5**). However, when broccoli by-products were added with genipin, tensile strength (25-33 MPa) and Young's modulus (1.2-1.5 GPa) decreased in comparison with starch films with broccoli by-products. These results indicate that addition of genipin promoted the crosslinking of protein material in starch enough to rearrange polymers organization and improve mechanical resistance and stiffness, but the addition of broccoli by-products, which were able to react with genipin, decreased the amount of genipin available.

Table 5.5. Mechanical parameters of starch films with broccoli by-products.

		1st approach – addition after filtration			2nd approach – addition before filtration			2nd approach - 1 year storage			3rd approach – addition of genipin		
		TS (MPa)	*YM (GPa)*	*E (%)*	*TS (MPa)*	*YM (GPa)*	*E (%)*	*TS (MPa)*	*YM (GPa)*	*E (%)*	*TS (MPa)*	*YM (GPa)*	*E (%)*
C	**0%**	29.5 ± 1.8^{f}	1.51 ± 0.05^{c}	2.40 ± 0.21^{b}	21.9 ± 1.0^{a}	1.28 ± 0.03^{a}	2.42 ± 0.24^{a}	$33.3 \pm 1.0^{ab'}$	1.44 ± 0.07^{a}	2.80 ± 0.26^{ab}	$31.3 \pm 0.8^{b*}$	$1.67 \pm 0.07^{b*}$	2.88 ± 0.27^{a}
BB_MHG	**0.5%**	24.3 ± 1.4^{ef}	1.20 ± 0.03^{b}	3.46 ± 0.4^{c}	30.4 ± 0.9^{b}	1.60 ± 0.06^{cde}	3.33 ± 0.24ac	$39.9 \pm 1.0^{cd'}$	1.53 ± 0.03^{ab}	3.46 ± 0.33^{abc}	-	-	-
	1%	22.2 ± 1.5^{ed}	1.21 ± 0.03^{b}	2.50 ± 0.24^{b}	23.8 ± 0.9a	1.31 ± 0.05^{a}	4.86 ± 0.64^{d}	$36.5 \pm 1.4^{abc'}$	$1.51 \pm 0.04^{ab'}$	$3.13 \pm 0.31^{abc'}$	-	-	-
	2%	14.5 ± 0.7^{abc}	0.88 ± 0.03^{a}	2.65 ± 0.38^{b}	33.2 ± 0.9^{bc}	1.49 ± 0.02^{ac}	3.31 ± 0.13^{ac}	-	-	-	-	-	-
BB_Residue	**0.5%**	20.9 ± 1.5^{cd}	1.27 ± 0.04^{b}	2.43 ± 0.33^{b}	26.5 ± 1.1^{a}	1.38 ± 0.10^{ab}	2.71 ± 0.28^{ab}	$32.0 \pm 2.8^{a'}$	1.45 ± 0.04^{a}	2.69 ± 0.19^{ab}	-	-	-
	1%	10.1 ± 2.1^{a}	0.76 ± 0.13^{a}	2.65 ± 0.78^{b}	24.9 ± 1.3^{a}	1.56 ± 0.10^{bcd}	2.38 ± 0.24^{a}	$39.4 \pm 1.2^{c'}$	1.53 ± 0.05^{ab}	$3.95 \pm 0.39^{c'}$	-	-	-
	2%	15.9 ± 1.7^{abcd}	1.20 ± 0.05^{b}	1.82 ± 0.12^{a}	25.5 ± 1.0^{a}	1.46 ± 0.04^{abc}	2.65 ± 0.13^{ab}	$40.8 \pm 1.8^{cd'}$	1.65 ± 0.04^{ab}	$3.69 \pm 0.37^{bc'}$	-	-	-
BB	**0.5%**	-	-	-	36.0 ± 1.4^{c}	1.80 ± 0.05^{ef}	3.68 ± 0.47^{bcd}	$44.6 \pm 3.4^{d'}$	1.71 ± 0.10^{b}	4.19 ± 0.40^{c}	33.0 ± 1.0^{b}	$1.50 \pm 0.01^{b*}$	3.54 ± 0.33^{ab}
	1%	-	-	-	36.1 ± 1.3^{c}	1.88 ± 0.04f	3.86 ± 0.32^{cd}	32.7 ± 2.8^{a}	$1.44 \pm 0.04^{a'}$	$2.56 \pm 0.40^{a'}$	$25.0 \pm 0.8^{a*}$	$1.18 \pm 0.04^{a*}$	5.13 ± 0.79^{b}
	2%	-	-	-	33.7 ± 1.4^{bc}	1.76 ± 0.05^{def}	3.08 ± 0.13^{ac}	37.3 ± 1.7^{bc}	$1.44 \pm 0.04^{a'}$	3.35 ± 0.30^{abc}	$25.4 \pm 0.3^{a*}$	$1.28 \pm 0.05^{a*}$	$4.05 \pm 0.52^{b*}$

TS: tensile strength; YM: Young's modulus; E: Elongation at break; C: control film. Data are expressed as mean ± standard error of mean. Different letters (a,b,c, and d) between each line of a column mean significant differences ($p<0.05$). Symbol (') in 2nd approach - 1 year storage columns indicate significant differences between initial and 1 year storage values ($p < 0.05$). Symbol (*) in 3rd approach – addition of genipin indicate significant differences between films without and with genipin ($p < 0.05$).

Elongation at break increased with addition of 0.5% of BB_MHG (3.5%) and decreased with addition of 2% of BB_Residue (1.8%), whereas it remained equal to control (2.4%) with addition of higher amounts of BB_MHG (2.5 to 2.7%) or lower amounts of BB_Residue (2.4 to 2.7%, **Table 5.5**). As addition of lower amount of broccoli by-products reduced the amount of agglomeration spots, a lower amount of BB_MHG was enough to add plasticizing compounds present naturally in broccoli, including free sugars, amino acids, and other small molecular weight compounds (Vieira, da Silva, dos Santos, & Beppu, 2011). On the other hand, a higher amount of BB_Residue, where these plasticizing compounds were absent, contributed to the formation of more agglomerates and to restrictions on the film molecular mobility (Gilfillan, Moghaddam, & Doherty, 2014; Nasri-Nasrabadi, Behzad, & Bagheri, 2014). The importance of extractable compounds to elongation at break was observed when agglomerates were removed by adding broccoli by-products before gelatinization and filtration steps: BB and BB_MHG with high amount of extractable compounds promoted higher elongation at break (3.1 to 4.9%) than BB_residue (2.4-2.7%), which had values comparable to control film. The crosslinking of these compounds with genipin also increased the elongation at break of starch films with broccoli and genipin (3.1-3.9%). Therefore, addition of genipin with broccoli by-products can be used when more elastic and less strength films are needed and broccoli by-products alone can be used when elastic and more strength films are preferable.

5.3.2.2. Wettability

Wettability of starch films with broccoli was evaluated by analysis of water contact angle (WCA) of both down and up surfaces of the films. Films with incorporation of lower amounts of BB_MHG (0.5% and 1%) and BB_Residue (0.5%) had higher values of WCA of down surface (68º, 59, and 69º, respectively) than control film (52%), whereas addition of 2% of BB_MHG and 1% and 2% of BB_Residue did not change WCA of down surface (**Table 5.6**). On the other hand, films with 0.5% and 2% of BB_MHG had higher WCA of up surface (61º and 57º) than control film (56º), whereas addition of 1% of BB_MHG and 0.5% to 2% of BB_Residue had lower WCA of up surface (50º and 47º to 38º, respectively). These results indicate that small amounts of extractable compounds from BB_MHG were enough to impact starch polymers organization and get films with less hydrophilic surfaces and less wettability, although not hydrophobic, as WCA were lower than 90˚ (Law, 2014).

Moreover, decreasing WCA of up surfaces could be linked with surface imperfections, promoted by the evaporation of water during solvent casting due to the presence of agglomerates. After removal of agglomerates from films by addition of broccoli by-products before gelatinization and filtration (**2nd approach**), WCA of both down and up surfaces (49º-94º and 49º-87º, respectively) were higher than control film (44º and 43º, respectively), excepting WCA of down surface when 1% of BB_Residue was added. Hydrophobic down surfaces (WCA higher than 90º) were observed when lower amount of BB_MHG and BB were added. On the other hand, hydrophobization of both surfaces was accomplished when genipin was added, allowing to obtain WCA of down and up surfaces of 124º and 121º, independently of addition of BB. Overall, a small amount of broccoli (20 mg as dry weight in 100 mL) was enough to hydrophobicize starch films down surface, although not so effective has the addition of genipin (50 mg in 100 mL).

Table 5.6. Water contact angle with starch films surfaces.

Starch films		Initial WCA (˚)		WCA after 1 year (˚)	
		up	*down*	*up*	*down*
Control		43.1 ± 2.1^{e}	43.7 ± 0.4^{d}	$54.9 \pm 1.3^{c'}$	$70.1 \pm 1.7^{d'}$
BB	**0.5%**	74.5 ± 0.4^{b}	94.0 ± 1.5^{a}	72.7 ± 2.2^{b}	$128.6 \pm 0.4^{a'}$
	1%	61.2 ± 7.6^{cd}	61.5 ± 6.2^{bc}	63.3 ± 1.1^{bc}	$113.2 \pm 2.5^{bc'}$
	2%	86.7 ± 0.8^{a}	48.8 ± 1.2^{cd}	$109.7 \pm 2.0^{a'}$	$125.1 \pm 2.6^{ab'}$
BB_MHG	**0.5%**	63.0 ± 3.5^{bcd}	94.4 ± 1.6^{a}	72.9 ± 2.8^{b}	$111.7 \pm 7.3^{bc'}$
	1%	56.5 ± 2.0^{c}	51.2 ± 3.3^{cd}	$69.1 \pm 2.0^{b'}$	$106.0 \pm 2.2^{c'}$
	2%	59.9 ± 6.9^{c}	63.3 ± 4.3^{bc}	71.2 ± 2.3^{b}	$120.6 \pm 3.0^{ab'}$
BB_Residue	**0.5%**	59.3 ± 0.4^{c}	67.8 ± 5.5^{b}	$70.7 \pm 2.2^{b'}$	$124.5 \pm 0.4^{ab'}$
	1%	49.0 ± 1.2^{cde}	38.2 ± 1.7^{d}	57.4 ± 1.1^{c}	$119.8 \pm 2.9^{ab'}$
	2%	57.5 ± 1.5^{c}	60.1 ± 5.6^{b}	$74.1 \pm 0.9^{b'}$	$113.2 \pm 2.2^{bc'}$

WCA water contact angle; Different letters between each line in a column means WCA values significantly different $p<0.05$; Symbols ' and " mean WCA values after 1 year are significantly different ($p<0.05$), ($n = 6$).

5.3.2.3. Antioxidant activity

The antioxidant activity of starch films with broccoli by-products was evaluated by the capacity to inhibit ABTS$^{\bullet+}$ along 7 days. This antioxidant activity was evaluated for films with incorporation of broccoli by-products before gelatinization and filtration steps (**2nd Approach**) and films with incorporation of genipin (**3rd Approach**). Addition of broccoli maintained or decreased the radical ABTS inhibition observed for the control film after 7 days of incubation (45%, **Figure 5.5**). Lower antioxidant potential was observed when BB_Residue was added (11 to 28%), as this sample was obtained as the remaining material after six hot water extractions. On the other hand, although BB_MHG and BB had phenolic compounds with known antioxidant activity, the low amount added to the films did not impact films antioxidant activity (22-51% and 31-52%, respectively). In contrast, 100% inhibition of radical ABTS was observed after 4 days of incubation when the crosslinker genipin, which is known to have high antioxidant activity (Lee, Lee, & Jeong, 2009), was used independently of addition of BB.

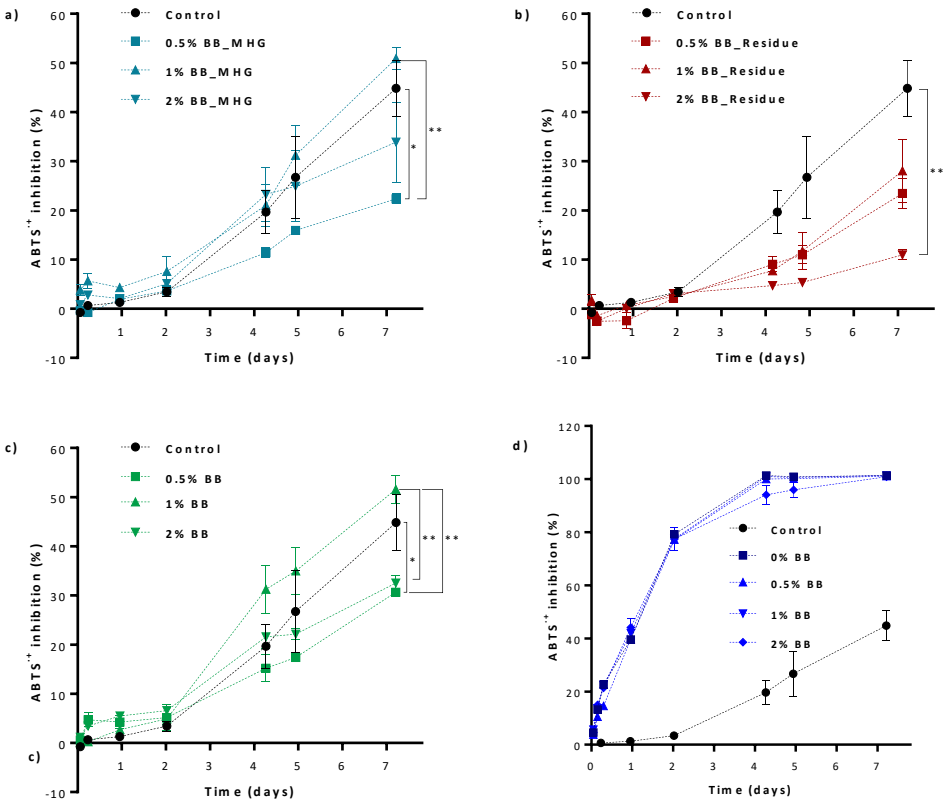


Figure 5.5. Antioxidant activity expressed in ABTS$^{\bullet+}$ inhibition (%) over 7 days of incubation of starch control film and starch film with the addition of 0.5%, 1%, and 2% of **a)** BB, **b)** BB_MHG, **c)** BB_Residue, and **d)** BB with genipin following **2nd** and **3rd approaches** to prepare films. Statistical differences of ABTS•+ inhibition (%) after 7 days between films are indicated near symbols (* $p < 0.05$; **$p < 0.01$).

5.3.2.4. Water content and solubility

Incorporation of broccoli by-products in starch films did not show statistically significant differences in water content (8.3 to 9.1%) when compared with control films (9.3%, **Figure 5.6.a**). No statistically significant differences were also observed with addition of genipin alone (9.1%). However, when 1% and 2% of BB were incorporated together with genipin, water content increased to 9.8%. These results indicate that broccoli by-products influenced the rearrangement of starch chains by promoting the retention of water when combined with genipin. The higher water content of the films may explain their higher elongation at break values (**Table 5.5**).

When water solubility was evaluated, films curled up in water. After 7 days in water, some films also loss their integrity, which could be responsible for the high standard deviation values, especially observed in films with higher amount of broccoli by-products (**Figure 5.6.b**). Due to this variability, no statistically significant differences in water solubility were observed when broccoli by-products were incorporated in starch films, with or without genipin (9.1 to 16%).

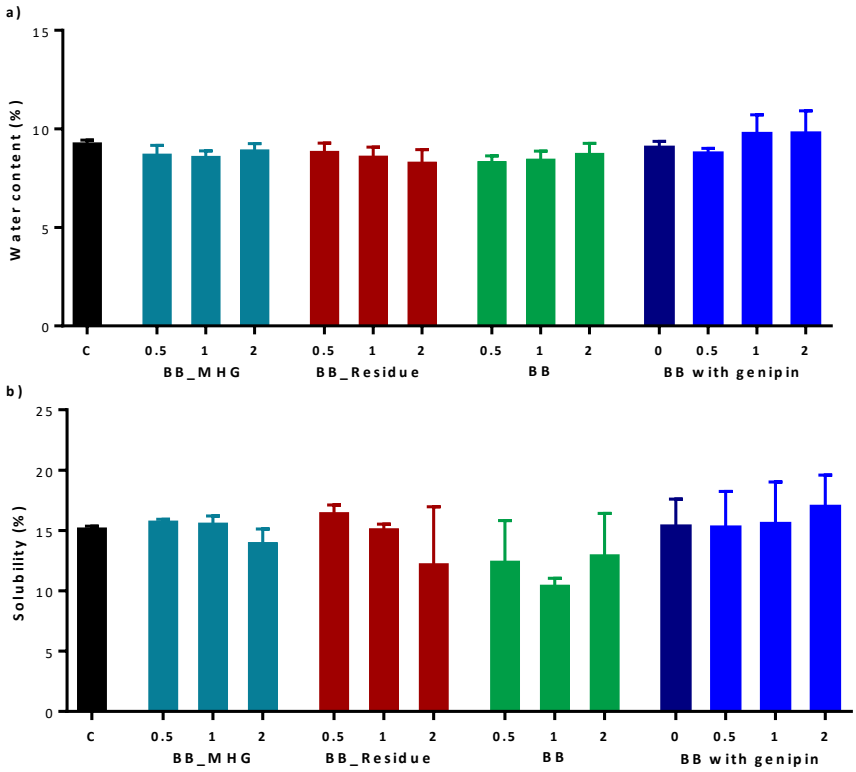


Figure 5.6 a) Water content and **b)** solubility in distilled water of starch films with broccoli after storage under controlled conditions of moisture (45% humidity) and temperature (25 ˚C). Columns values represent mean ± standard deviation of n=3 assays.

5.3.3. Effect of one-year storage in starch films with broccoli by-products

Mechanical properties and wettability of films with broccoli by-products were re-evaluated after one-year of storage (**Table 5.5**). Tensile strength of films increased with storage (from 22-36 to 32-45 MPa) and Young's modulus of films with 1 and 2% of BB decreased (from 1.8-1.9 to 1.4 GPa), indicating that films were strengthen with storage independently of broccoli by-products addition, and only the films with BB decreased their stiffness after one year. Elongation at break of films with 1% BB_MHG and 1% BB decreased with storage (from 3.9-4.9% to 2.6-3.1%), showing that stored films with 1% BB_MHG and 1% BB were less flexible. In contrast, elongation at break of films with 1 and 2% BB_Residue increased with storage (from 2.4-2.7 to 4.0-3.7). These results indicate that extractable compounds were able to promote rearrangement of starch matrix, decreasing their mobility, as observed for starch films stored for 45 days (Zhang et al., 2019). The films with BB_Residue showed higher starch mobility after storage, possibly by preventing or changing starch matrix rearrangements.

Wettability of films decreased with storage, as observed by increasing of WCA of films surfaces, in particular WCA of down surface (**Table 5.6**). Down surfaces of films with broccoli were all hydrophobic (WCA higher than 90º). As this phenomenon was not described before, films were further characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD) analysis. The analysis of films surfaces by SEM showed the accumulation of irregular shapes (**Figure 5.7**). In particular, films with BB_MHG and BB_Residue had higher deposition of these structures. XRD patterns of films were analysed in the interval from 11° to 29° (2θ). The diffraction peaks of starch films were observed at 2θ of 17.4º, 19.6º and 21.4º (**Figure 5.8**), which were assigned to B-type crystallinity, characteristic of retrograded gelatinized starch after storage (Domene-López, García-Quesada, Martin-Gullon, & Montalbán, 2019; Fu, Wang, Li, Zhou, & Adhikari, 2013). Films with broccoli by-products maintained the same XRD pattern, with minor differences in the intensity and width of the peaks observed.

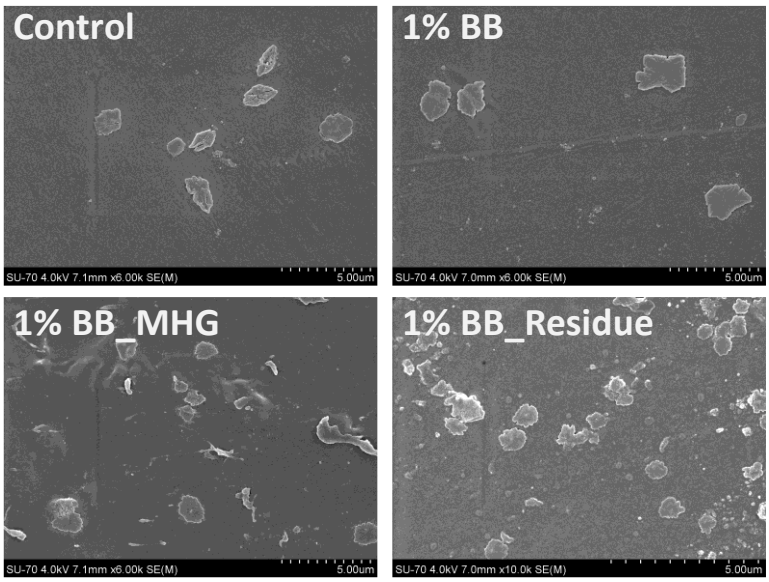


Figure 5.7. Scanning electron images of starch film control and starch films with broccoli by-products.

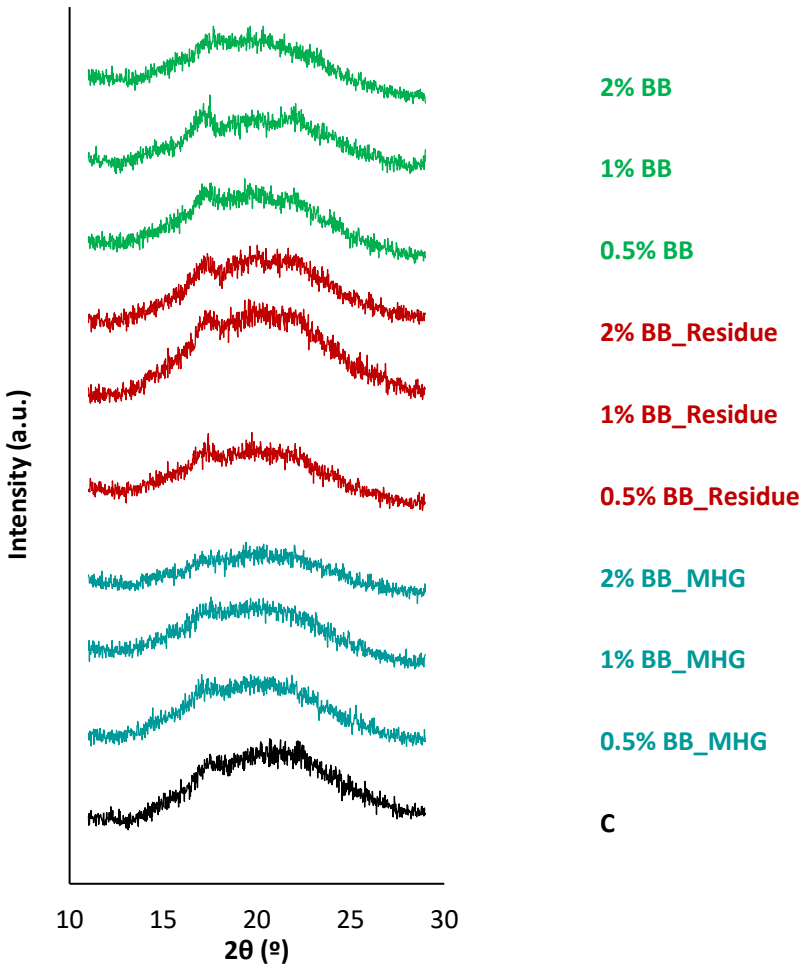


Figure 5.8. X-ray diffraction pattern of starch films with broccoli by-products.

5.4. Concluding remarks

Incorporation of broccoli by-products allowed to obtain starch films by solvent casting. Addition of by-products after gelatinization of starch promoted the formation of agglomeration spots that impaired starch films properties. On the other hand, the addition of by-products before starch gelatinization and filtration steps promoted films with higher strength, stiffness, and elasticity than starch control film. Moreover, less hydrophilic surfaces were obtained with addition of broccoli by-products. Antioxidant activity, water content, and water solubility were maintained with the addition of broccoli by-products. The differences between incorporation of broccoli by-products unprocessed and dehydrated (BB and BB_MHG, respectively) and incorporation of BB_Residue, showed that water extractable compounds were the main contributors to the observed changes of mechanical properties and wettability. The use of genipin promoted starch films with broccoli by-products with higher elasticity. After one-year storage, films tended to be more strength, less flexible, stiffer, and with increased hydrophobicity of surfaces, indicating that broccoli by-products propelled starch matrix rearrangements along storage. These promising results of incorporation of broccoli by-products in starch films highlight the valorisation potential of broccoli by-products by application on the packaging field.

CHAPTER 6

Conclusions and Future Perspectives

This thesis contributed to develop a methodology to dehydrate and stabilize broccoli by-products using microwave hydrodiffusion and gravity (MHG) technology, which allows to minimize the time and energy of dehydration. With exception of pigments, this technology did not compromise the amount of other compounds, such as carbohydrates, protein, glucoraphanin, and phenolic compounds with antioxidant activity. Simultaneously, MHG allowed to recover concentrated extracts with free sugars, amino acids, mannitol, pectic polysaccharides, glucosinolates, and phenolic compounds, ready to be used as ingredients, demonstrated by the bechamel sauce that was produced in the aim of this thesis.

Broccoli by-products were shown to be a source of polysaccharides with *in vitro* B lymphocytes stimulatory activity, namely pectic polysaccharides methyl- and acetyl-esterified with ramified regions with 4-linked galactans, arabinans, and type II arabinogalactans with 4-O-Me-GlcA residues. This activity was observed in pectic polysaccharides soluble in 50% ethanol aqueous solutions and insoluble in 80%, independently of the molecular weight of polysaccharides (12 to 400 kDa). Removal of terminal Ara or small chains of Ara also did not change the bioactive potential. However, the methyl deesterification of pectic polysaccharides and removal of galacturonan regions reduced polysaccharides immunostimulatory activity, showing that the acidic regions contribute to the activity observed. Moreover, the chemical sulfation of pectic polysaccharides also reduced their activity. In contrast, other pectic polysaccharides, xyloglucans, and xylans did not show immunostimulatory activity. Therefore, besides the known bioactive compounds of broccoli, pectic polysaccharides with immunostimulatory activity should also be considered for their valorisation.

The incorporation of broccoli by-products in biobased materials was exploited in this work, using wastewater starch from potato industry as thermoplastic matrix and glycerol as plasticizer. Broccoli by-products increased strength, stiffness, and elasticity of the films and decreased the hydrophilicity of their surfaces, while maintaining their antioxidant activity, water content, and water solubility. Addition of the crosslinker genipin to the formulation allowed to obtain starch films with broccoli by-products with higher elasticity, diversifying the properties and applications of the films. These results showed that incorporation of broccoli by-products promoted starch matrix rearrangements, which were more pronounced after one-year storage, providing films with higher strength, stiffness, and hydrophobicity and lower flexibility, able to be used in the biobased packaging field.

The results obtained have created the scientific basis for a dehydration broccoli by-product approach fostering a set of applications in the area of healthy food ingredients and the development of improved biobased materials for food packaging, contributing to more sustainable food systems and a more circular economy. Some of the dehydration approaches can now be tested in large pilot processes to boost the scale-up and allow the validation of applications, including the development of functional ingredients after disclosure of polysaccharides immunostimulatory activity mechanisms, both *in vitro* and *in vivo*, and development of biodegradable food packages.

CHAPTER 7

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