UNIVERSIDADE FEDERAL DO PARANÁ

CRISTIANE COLODEL

POTENCIALIDADES DO REAPROVEITAMENTO DE CASCA DE PONKAN E DE BAGAÇO DE UVA PARA OBTENÇÃO DE PECTINAS VISANDO APLICAÇÕES INDUSTRIAIS, BIOLÓGICAS E AMBIENTAIS

CURITIBA

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Tese apresentada ao curso de Pós-Graduação em Ciências - Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Ciências - Bioquímica.

Orientador(a): Profa. Dra. Carmen Lúcia de Oliveira Petkowicz
Co-orientadora: Dra. Lúcia Cristina Vriesmann

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **CRISTIANE COLODEL**, intitulada: **POTENCIALIDADES DO REAPROVEITAMENTO DE CASCA DE PONKAN E DE BAGAÇO DE UVA PARA OBTENÇÃO DE PECTINAS VISANDO APLICAÇÕES INDUSTRIAIS, BIOLÓGICAS E AMBIENTAIS**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de Doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 12 de Abril de 0019.

CARMEN LUCIA DE OLIVEIRA PETKOWICZ
Presidente da Banca Examinadora

VICELMA LUIZ CARDOSO
Avaliador Externo

MARCO TADEU GRASSI
Avaliador Externo

SELMA FARIA ZAWADZKI BAGGIO
Avaliador Externo

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“Não é sobre chegar no topo do mundo e saber que venceu
É sobre escalar e sentir que o caminho te fortaleceu”
(“Trem Bala”, Ana Vilela)

RESUMO

Pectinas são polissacarídeos amplamente utilizados na indústria alimentícia. São obtidas da casca de laranja e de limão e do bagaço de maçã. Entretanto, outros resíduos agroindustriais podem ser promissoras fontes de pectinas. Neste trabalho, foram investigadas a casca de ponkan e o bagaço de uva Chardonnay como fontes alternativas de pectina. Os materiais foram submetidos a extrações ácidas fervente usando soluções de HNO_3 empregando-se um planejamento experimental do tipo composto central (CCD) no qual foi avaliado o efeito do pH, do tempo de extração e da razão líquido:sólido no rendimento e no teor de ácido urônico (AU) das pectinas extraídas. Para a casca da ponkan, o rendimento e o teor de AU da pectina foram afetados negativamente pelo pH e positivamente pelo tempo de extração e razão líquido:sólido. A condição ótima determinada foi pH 1,6, 100 min e 36 mL/g, resultando em uma pectina, denominada PPOP, com rendimento de 25,6% e 84,5% de AU, composta principalmente por homogalacturonana com grau de metil-esterificação (DM) de 85,7% e massa molar média (Mw) de 80.650 g/mol. PPOP apresentou comportamento de fluxo pseudoplástico e formou gel em meio ácido na presença de sacarose, sendo o gel mais forte obtido na concentração de 2,5% (m/m) em pH 2,5 com 60% de sacarose. Este gel apresentou alta estabilidade térmica e comportamento semelhante a uma pectina cítrica comercial testada nas mesmas condições. Para o bagaço de uva, o rendimento foi diretamente proporcional ao tempo de extração e inversamente proporcional ao pH, e o teor de AU foi inversamente proporcional ao pH e diretamente proporcional ao tempo de extração e à razão líquido:sólido. A condição ótima foi definida como pH 2,08, 135 min e 35,11 mL/g e a pectina obtida nestas condições (GPOP) teve 11,1% de rendimento e 56,8% de AU. GPOP apresentou contaminação por amido, que foi removido enzimaticamente, resultando na fração α-GPOP, composta por 63,5% de AU e uma alta proporção de ramnogalacturonana I (35,2%) altamente ramificada por cadeias curtas de arabinose e galactose, com DM = 18,1% e Mw = 154.100 g/mol. Nas concentrações de 50 a 500 μg/mL, α-GPOP estimulou a produção de NO por macrófagos, não apresentando citotoxicidade. PPOP e α-GPOP (2 g/L) foram avaliadas quanto a capacidade de remoção de Pb^{2+} (5 mg/L) e Cu^{2+} (10 mg/L) em solução, mas não foram eficientes nas condições testadas. A casca de ponkan também foi submetida a extrações sequenciais usando água fria e fervente, oxalato de amônio (0,5%), ácido cítrico (pH 2.5), Na_2CO_3 0,05 M, NaOH 2 M e 4 M, dando origem a frações pécticas e hemicelulósicas. Uma pectina com 57,7% de AU, 3,1% de Rha, 14,1% de Ara e 15,5% de Gal, com DM = 39,4% e Mw = 161.500 g/mol (α-WSP) foi purificada a partir da fração extraída com água fria por tratamento com α-amilase e amiloglucosidase, e uma homogalacturonana (HWSP-Cu) com 93.5% UA, DM = 76,2% e Mw = 135.200 g/mol foi purificada da fração extraída com água fervente por precipitação com $CuSO_4$. Quando testadas quanto à capacidade de estimulação de produção de NO por macrófagos, α-WSP teve efeito estimulador estatisticamente mais alto que o controle positivo enquanto HWSP-Cu não estimulou a produção de NO. O bagaço de uva também foi testado quanto à capacidade de sorção dos corantes azul de metileno, cristal violeta, safranina, auramina O e verde malaquita, e teve capacidade máxima de sorção de 177,6, 160,5, 82,3, 76,0 e 307,0 mg/g, respectivamente. Os resultados sugerem que o bagaço de uva pode ser uma fonte de pectinas com propriedade imunoestimuladora e pode ser um promissor bioadsorvente para a remoção de corantes em meio aquoso. Por sua vez, a casca de ponkan destaca-se por fornecer pectina com alto rendimento e características estruturais e reológicas comparáveis a pectinas cítricas já utilizadas comercialmente.

Palavras-chave: Caracterização química e estrutural. Propriedades reológicas. Imunomodulação. Remoção de espécies metálicas. Remoção de corantes catiônicos.

ABSTRACT

Pectins are polysaccharides widely used in the food industry. They are obtained from orange and lemon peels and from apple pomace. However, other agroindustrial residues can be promising sources of pectins. In this work, the ponkan peel and Chardonnay grape pomace were investigated as alternative sources of pectin. The materials were subjected to boiling acid extraction by HNO_3 solutions using a central composite experimental design (CCD) in which the effect of pH, extraction time and liquid:solid ratio on the yield and the content of uronic acid (UA) of the extracted pectins. For the ponkan peel, the yield and the UA content of the pectin were negatively affected by the pH and positively by the time of extraction and liquid:solid ratio. The optimum condition was pH 1.6, 100 min and 36 mL/ g, resulting in a pectin, denominated PPOP, with yield of 25.6% and 84.5% of UA, composed mainly of homogalacturonan with degree of methyl esterification (DM) of 85.7% and average molar mass (Mw) of 80,650 g / mol. PPOP presented a pseudoplastic flow behavior and formed gel in acid medium in the presence of sucrose, the strongest gel being obtained in the concentration of 2.5% (w/w) at pH 2.5 with 60% of sucrose. This gel showed high thermal stability and similar behavior to commercial citrus pectin tested under the same conditions. For grape pomace, the yield was directly proportional to the extraction time and inversely proportional to the pH, and the UA content was inversely proportional to the pH and directly proportional to the extraction time and the liquid:solid ratio. The optimum condition was defined as pH 2.08, 135 min and 35.11 mL/g and pectin obtained under these conditions (GPOP) had 11.1% yield and 56.8% UA. GPOP contained starch contamination, which was enzymatically removed, resulting in the α-GPOP fraction, composed of 63.5% of UA and a high proportion of ramnogalacturonan I (35.2%) highly branched by short chains of arabinose and galactose, with DM = 18.1% and Mw = 154,100 g/mol. At concentrations of 50 to 500 µg/mL, α-GPOP stimulated the production of NO by macrophages, showing no cytotoxicity. PPOP and α-GPOP (2 g/L) were evaluated for the ability to remove Pb^{2+} (5 mg/L) and Cu^{2+} (10 mg/L) in solution but were not efficient under the conditions tested. The ponkan peel was also subjected to sequential extraction using cold and boiling water, ammonium oxalate (0.5%), citric acid (pH 2.5), 0.05 M Na_2CO_3, 2 M and 4 M NaOH, giving rise to pectic and hemicellulosic fractions. A pectin with 57.7% UA, 3.1% Rha, 14.1% Ara and 15.5% Gal, with DM = 39.4% and Mw = 161,500 g / mol (α-WSP) was purified from the fraction extracted with cold water by treatment with α-amylase and amyloglucosidase, and a homogalacturonan (HWSP-Cu) with 93.5% UA, DM = 76.2% and Mw = 135,200 g / mol was purified from the fraction extracted with boiling water by precipitation with $CuSO_4$. When tested for the ability to stimulate NO production by macrophages, α-WSP had a statistically higher stimulatory effect than the positive control whereas HWSP-Cu did not stimulate NO production. Grape pomace was also tested for the adsorption capacity of methylene blue, violet crystal, safranin, auramine O and malachite green cationic dyes, and had adsorption capacity of 177.6, 160.5, 82.3, 76, 0 and 307.0 mg/g, respectively. The results suggest that grape pomace may be a source of pectins with immunostimulatory properties and may be a promising biosorbent for the removal of dyes in aqueous medium. In turn, the ponkan peel stands out for providing pectin with high yield and structural and rheological characteristics comparable to commercially used citrus pectins.

Key words: Chemical and structural characterization. Rheological properties. Immunomodulation. Removal of metallic species. Removal of cationic dyes.

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LISTA DE ABREVIATURAS OU SIGLAS

α-GPOP - pectina otimizada do bagaço de uva livre de amido

α-WSP - pectina purificada da fração WSP por remoção enzimática de amido

AIR - resíduo insolúvel em álcool *(alcool insoluble residue)*

ANOVA - análise de variância

ASPI - parte insolúvel após diálise da pectina solúvel em álcali diluído *(alkali-soluble pectin, insoluble)*

ASPS - parte solúvel após diálise da pectina solúvel em álcali diluído *(alkali-soluble pectin, soluble)*

AU - ácido urônico

BSA - soroalbumina bovina

CCD - planejamento composto central

CCP - pectina cítrica comercial *(commercial citrus pectin)*

CGP - bagaço de uva Chardonnay *(Chardonnay grape pomace)*

CI - *color index*

CSP - pectina solúvel em quelante *(chelant-soluble pectin)*

DA - grau de acetilação *(degree of acetylation)*

DM - grau de metil-esterificação *(degree of methyl-esterification)*

DMSO - dimetilsulfóxido

Et - tempo de extração *(extraction time)*

FBS - soro fetal bovino *(fetal bovine serum)*

FT-IR - espectroscopia no infravermelho com transformada de Fourier *(Fourier transformed-infrared)*

Gal-3 - galectina-3

GC - cromatografia gasosa *(gas chromatography)*

GC-MS - cromatografia gasosa acoplada à espectrometria de massas *(gas chromatography-mass spectrometry)*

GLC - cromatografia líquido-gasosa *(gas-liquid chromatography)*

GPOP - pectina otimizada do bagaço de uva *(grape pomace optimized pectin)*

GPP - bagaço de uva moído *(grape pomace powder)*

HA2 - hemicelulose A solúvel em NaOH 2 M

HA4 - hemicelulose A solúvel em NaOH 4 M

HAc - ácido acético

HB4 - hemicelulose B solúvel em NaOH 4 M

HBSS - solução salina balanceada de Hank

HG - homogalacturonana

HM - pectina de alto grau de metil-esterificação *(high methoxyl)*

HPAEC-PAD - cromatografia de troca iônica de alta performance com deteção de pulso amperométrico (*high performance anion exchange chromatography with pulsed amperometric detection)*

HPSEC - cromatografia de exclusão estérica de alta pressão (*high pressure size exclusion chromatography)*

HSQC - *heteronuclear single quantum coherence*

HSP - pectina solúvel em ácido diluído *(H^+-soluble pectin)*

HWSP - pectina solúvel em água quente *(hot water-soluble pectin)*

HWSP-Cu - pectina purificada da fração HWSP por precipitação com $CuSO_4$

ICP-OES - espectrometria de emissão óptica por plasma acoplado indutivamente *(inductively coupled plasma optical emission spectrometry)*

KDa - quilodalton

LM - pectina de alto grau de metil-esterificação *(low methoxyl)*

LPS - lipopolissacarídeo

LS - razão líquido:sólido

MALLS - espalhamento de luz laser multiângulos *(multi angle laser light scattering)*

MeOH - metanol

MLR - regressão linear múltipla *(multiple linear regression)*

MTT - brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio

NaOAc - acetato de sódio

NMR - *nuclear magnetic ressonance*

NO - óxido nítrico

PP - casca de ponkan *(ponkan peel)*

PPOP - pectin otimizada da casca de ponkan *(ponkan peel optimized pectin*)

RG-I - ramnogalacturonana-I

RG-II - ramnogalacturonana-II

Rha - ramnose

RI - índice de refração *(refraction index)*

RMN - ressonância magnética nuclear

RSM - método de superfície de resposta *(response surface method)*

SD - desvio padrão *(standard deviation)*

SEM - erro padrão da média *(standard error of mean)*

TFA - ácido trifluoroacético

UA - *uronic acid* (ácido urônico)

WSP - pectina solúvel em água a 25ºC *(water-soluble pectin)*

LISTA DE SÍMBOLOS

δ	- deslocamento químico
ω	- frequência angular
C_f	- concentração final
C_i	- concentração inicial
dn/dc	- variação no índice de refração com relação à variação na concentração do soluto
G'	- módulo elástico
G"	- módulo viscoso
m	- massa
M_w	- massa molar média
M_w/M_n	- grau de polidispersão
q_e	- capacidade de sorção
V	- volume

SUMÁRIO

1 INTRODUÇÃO

A pectina é um polissacarídeo de origem vegetal amplamente empregado por suas propriedades espessantes, estabilizantes e, mais notadamente, gelificantes. Seu principal campo de aplicação é na área alimentícia, usado como agente modificador de textura, mas sua capacidade de atuar como um alimento funcional vem ampliando suas aplicações nesta área e, consequentemente, sua demanda. Além disso, a pectina também encontra usos na área farmacêutica e cosmética (CIRIMINNA et al., 2016; CHAN et al., 2017).

As pectinas comerciais são produzidas a partir da casca de cítricos (cerca de 85% do total), mais especificamente, laranja, limão Thaiti e limão siciliano, e de bagaço de maçã (cerca de 14%) (CIRIMINNA et al., 2016). No entanto, o mercado de pectinas encontra-se em crescente demanda, com projeção de taxa de crescimento anual no período de 2018 a 2023 de 7,3%, de acordo com dados apresentados pela Industry Research (THE CW SAN DIEGO, 2019). Por este motivo, tem-se buscado novas fontes de pectina, que possam fornecer pectinas com a produtividade e qualidade exigida pelo mercado. Procurando suprir a demanda de pectinas, diversas pesquisas vêm sendo realizadas, principalmente utilizando resíduos agroindustriais com possíveis fontes comerciais deste importante polímero (DRANCA, OROIAN, 2018).

É considerando este cenário que o presente trabalho apresenta resultados a respeito da extração e da caracterização estrutural das pectinas de dois resíduos agroindustriais, a casca de ponkan e o bagaço de uva, bem como a investigação de propriedades relacionadas a potenciais aplicações industriais, biológicas e ambientais destas pectinas.

Os resultados desta pesquisa estão apresentados em 7 capítulos. Os capítulos 1, 2 , 3 e 4 apresentam os dados a respeito das pectinas da ponkan. O primeiro expõe os resultados acerca da extração sequencial dos polissacarídeos de parede celular, caracterização parcial das frações obtidas e caracterização química estrutural de uma pectina purificada a partir da fração obtida com água fria, α-WSP. Estes dados foram publicados na forma de artigo no periódico *Carbohydrate Polymers*. O segundo capítulo apresenta o processo de purificação da pectina obtida por extração com água quente, HWSP-Cu, e traz uma comparação preliminar entre a propriedade imunomoduladora das pectinas α-WSP e HWSP-Cu. O terceiro trata da otimização do processo de extração ácida e da caracterização estrutural da pectina obtida nas condições otimizadas, e o artigo resultante foi publicado no periódico *International Journal of Biological Macromolecules.* O quarto capítulo apresenta a caracterização reológica da pectina obtida nas condições otimizadas, cujos resultados foram compilados em um artigo que foi

publicado no periódico *Food Hydrocolloids*. O capítulo 5 trata do bagaço de uva Chardonnay, e relata o processo de otimização da extração ácida, a caracterização estrutural da pectina extraída sob as condições otimizadas e os resultados do teste de imunoestimulação, e o artigo com estes resultados será submetido ao periódico *Industrial Crops and Products*. O capítulo 6 apresenta os resultados dos testes de remoção de Pb^{2+} e Cu^{2+} em solução pelas pectinas otimizadas da casca da ponkan e do bagaço de uva. E, por fim, o capítulo 7 traz os resultados da aplicação do bagaço de uva Chardonnay como biosorvente para a remoção de corantes em meio aquoso. Uma breve revisão bibliográfica será apresentada previamente, a fim de contextualizar a proposta da pesquisa e dos resultados obtidos.

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2 REVISÃO DE LITERATURA

2.1 RESÍDUOS VEGETAIS AGROALIMENTARES: UM RISCO AMBIENTAL OU UMA FONTE DE PRODUTOS DE ALTO VALOR AGREGADO?

A agricultura e a agroindústria geram uma grande quantidade de resíduos de material vegetal. Estes resíduos são produzidos ao longo de toda a cadeia produtiva, desde a produção na lavoura, durante o transporte até as indústrias de beneficiamento ou diretamente para venda, durante o processo de industrialização e também após o consumo (SAGAR et al., 2018).

De acordo com a Organização das Nações Unidas para Alimentação e Agricultura (FAO) cerca de 1,3 bilhão de toneladas de resíduos alimentares são produzidos anualmente em todo o mundo, sendo que aproximadamente 60% é constituído por materiais de origem vegetal (SAGAR et al., 2018).

Quando não manejados de maneira adequada, os resíduos agroalimentares podem acarretar grandes prejuízos ambientais. Em geral, estes resíduos são descartados em aterros, e como contêm grande quantidade de matéria orgânica, geram alta demanda química e bioquímica de oxigênio no ambiente onde são descartados, alteram o pH do meio, além de representar alto risco de contaminação pela proliferação de microrganismos. O fato de muitas culturas estarem concentradas nas épocas de safra agrava ainda mais este problema, devido à alta taxa de acumulação (PING et al., 2011; PFALTZGRAFF et al., 2013; RAVINDRAN, JAISWAL, 2016).

Os destinos mais comuns dos resíduos agroalimentares são a utilização como fertilizante nas lavouras, o descarte em aterros e principalmente o uso na alimentação do gado (VAN DYK et al., 2013; CHRISTIAENS et al., 2015;), porém todas estas opções apresentam desvantagens. O descarte em aterros, além de causar prejuízos ambientais, pode também acarretar custos de transporte e tratamento em alguns países (VAN DYK et al., 2013). Quando utilizados como fertilizantes, alguns resíduos podem modificar as propriedades físicas, químicas e biológicas do solo, devido à alta carga de matéria orgânica, a alterações no pH, e à presença de compostos nocivos, como espécies metálicas potencialmente tóxicas, ou que afetam a microbiota do solo, como metabólitos com ação antimicrobiana (BUSTAMANTE et al., 2005; DOMÍNGUEZ, SANCHEZ-HERNANDEZ, LORES, 2017). Por sua vez, a limitação do uso na alimentação animal encontra-se no fato de que a maioria dos resíduos vegetais possui baixo teor de proteínas e aqueles com alto conteúdo de lignina podem ser de

difícil digestão. Há também a desvantagem econômica, pois o resíduo precisa ser seco e peletizado para ser comercializado, gerando custos, e geralmente o valor de mercado é muito baixo e não cobre os gastos gerados (VAN DYK et al., 2013).

A busca por soluções para um melhor aproveitamento dos resíduos agroalimentares é importante tanto para reduzir o impacto ambiental, como para agregar valor comercial a um material que geralmente é descartado ou ainda acarreta custos para sua disposição correta (PING et al., 2011; PFALTZGRAFF et al., 2013). Neste contexto, o uso dos resíduos vegetais agroalimentares como matéria-prima para a obtenção de produtos com alto valor de mercado é uma alternativa atraente para reduzir tanto os prejuízos econômicos quanto os ambientais.

Resíduos vegetais são materiais particularmente ricos em metabólitos primários e secundários de grande interesse industrial. Compostos fenólicos, alcaloides, glicosídeos, óleos essenciais, flavorizantes, ácidos orgânicos e resinas são exemplos de metabólitos secundários que podem ser obtidos de resíduos vegetais e que possuem amplas aplicações. Entre os compostos primários de interesse, encontram-se enzimas, lignina e polissacarídeos, estes últimos compreendendo as hemiceluloses, pectinas e celulose. Os compostos obtidos a partir de resíduos vegetais e seus derivados possuem variadas aplicações na indústria farmacêutica, alimentícia, química e na área da saúde (BANERJEE et al., 2017). Considerando a possibilidade do uso de resíduos vegetais para a obtenção de produtos com valor agregado, esta opção apresenta inquestionável vantagem sobre o seu descarte ou seu uso para fins menos nobres.

A presença de altos conteúdos de polissacarídeos nos resíduos vegetais também permite aplicações que dispensam processos de extração, necessários para a obtenção dos metabólitos citados anteriormente. Um exemplo deste tipo de aplicação seria o uso como biosorventes de baixo custo para remoção de espécies tóxicas em meio aquoso. Algumas revisões já foram publicadas no esforço de compilar os dados obtidos em diversos estudos utilizando resíduos na remoção de corantes e de espécies metálicas dissolvidos (SALLEH et al., 2011; NGUYEN et al., 2013; ADEGOKE, BELO, 2015; BHATNAGAR, SILLANPÄÄ, WITEK-KROWIAK, 2015; PATHAK, MANDAVGANE, KULKARNI, 2015).

Salleh et al. (2011) compararam a eficiência de diversos resíduos agrícolas na remoção de corantes catiônicos e aniônicos. Os autores concluem que os resíduos agrícolas tendem a ser mais eficientes na remoção de corantes catiônicos quando usados em seu estado natural, enquanto que para melhorar a eficiência de remoção de corantes aniônicos são necessários processos de tratamento prévio. Este comportamento é justificado pela presença de grupos carboxílicos como principais grupos funcionais na composição dos resíduos de

origem vegetal, que possuem carga negativa e assim interagem mais facilmente com os corantes catiônicos, positivamente carregados. Adegoke e Belo (2015) reuniram os dados a respeito da sorção de corantes de diferentes classes por resíduos provenientes da agricultura, abrangendo aspectos cinéticos, possíveis mecanismos e a capacidade máxima de sorção de diferentes resíduos. Estes resíduos incluem folhas, bagaços, serragens e cascas de frutas e cereais, por vezes ativados física ou quimicamente. Os autores apontam que estes resíduos são ricos em lignina e polissacarídeos, e que estes compostos são capazes de adsorver as moléculas de corantes através de mecanismos que envolvem complexação, troca iônica e ligações de hidrogênio. Os dados compilados por Bhatnagar, Sillanpää e Witek-Krowiak (2015) também apontam as interações entre as cargas opostas dos corantes catiônicos dos corantes e os grupos carboxílicos presentes nos resíduos vegetais oriundos de cascas como as principais responsáveis pelo mecanismo de sorção, porém não o único, uma vez que os grupos hidroxila também interagem com os pares de elétrons livres presentes na estrutura do corante.

Os grupos carboxílicos também parecem ser os principais responsáveis pela capacidade de interação dos resíduos com íons metálicos. Como observado por Nguyen et al. (2013), os resíduos vegetais possuem em sua composição uma vasta gama de grupos funcionais capazes de estabelecer interações com espécies metálicas, mas diversos estudos elencados pelos autores apontam para os grupos carboxílicos e hidroxílicos como os mais importantes para o mecanismo de sorção. A importância dos grupos carboxílicos para o mecanismo de sorção por resíduos vegetais e consequentemente sua eficiência como adsorventes também foi destacada por Pathak, Mandavgane, e Kulkarni (2015), que reuniram os resultados obtidos por diversos estudos realizados a respeito do uso de resíduos de cascas de frutas para remoção de metais pesados e corantes.

2.2 A PAREDE CELULAR VEGETAL

Os polissacarídeos, já mencionados como um dos principais componentes dos resíduos vegetais, encontram-se localizados em uma estrutura bem organizada: a parede celular.

A parede celular vegetal é uma estrutura que circunda a membrana plasmática e possui importantes funções. Ela é responsável pelo controle da forma e tamanho da célula, pelo suporte mecânico da planta, pela regulação osmótica da célula, pela adesão e comunicação intercelular, protege a membrana plasmática e atua no reconhecimento e defesa

contra patógenos (KEEGSTRA, 2010; PALIN, GEITMAN, 2012; HÖFTE, VOXEUR, 2018). É classificada em dois tipos, a parede celular primária e a parede celular secundária, além da região de adesão intercelular denominada lamela média (KEEGSTRA, 2010), conforme mostra a Fig. 1.

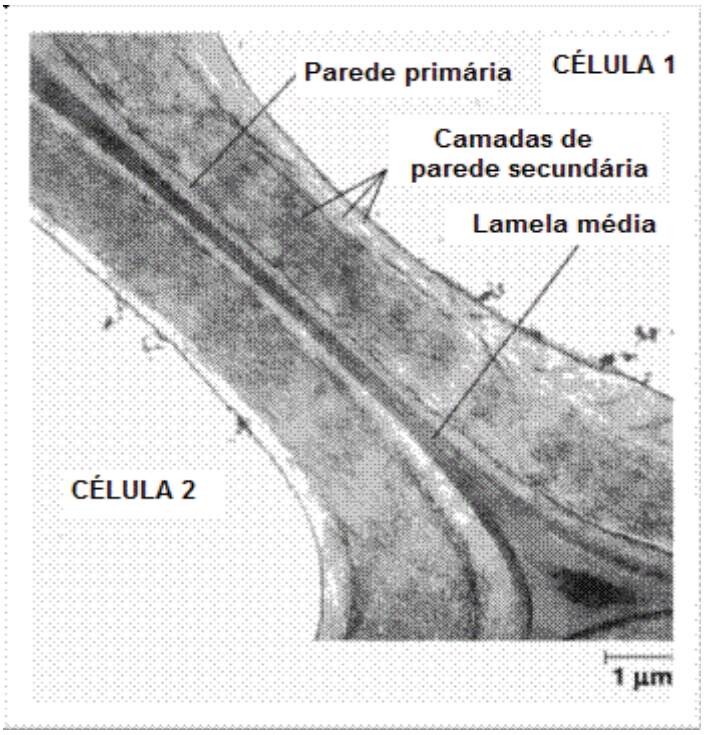

Fig. 1 – Micrografia eletrônica da região de junção entre duas células vegetais, mostrando a localização da lamela média, da parede celular primária e da parede celular secundária. FONTE: adaptado de http://preuniversity.grkraj.org/html/1_CELL_STRUCTURE.htm.

De maneira geral, cerca de 90% da massa da parede celular das plantas superiores é composta por três famílias de polissacarídeos - a celulose, as hemiceluloses e as pectinas - e compostos fenólicos (CARPITA; McCANN, 2000; PALIN, GEITMAN, 2012; DAHER, BRAYBOOK, 2015; HÖFTE, VOXEUR, 2018). Os 10% restantes são formados por proteínas e compostos inorgânicos (PALIN, GEITMAN, 2012). Porém, são as diferenças na composição química e estrutural da parede celular primária e da parede celular secundária que refletem diretamente as distintas propriedades mecânicas destas duas regiões.

A parede celular primária é depositada sobre a membrana plasmática durante o crescimento celular. Costuma ser uma estrutura relativamente fina, flexível e altamente hidratada, enquanto a parede celular secundária é uma estrutura mais forte e rígida, que se deposita sob a parte interna da parede celular primária nos estádios mais avançados do desenvolvimento, após a interrupção do crescimento celular (COSGROVE, JARVIS, 2012).

A parede celular secundária é composta primariamente por celulose, hemiceluloses (tipicamente xilanas e glucomananas) e lignina. A proporção de cada um destes componentes pode variar de espécie para espécie, mas o teor aproximado de celulose é de cerca de 41-48%, o de hemiceluloses cerca de 27% e o de lignina cerca de 21-29% (ZHONG, YE, 2015; KUMAR, CAMPBEL, TURNER, 2016). O teor de água é reduzido em comparação à parede primária, sendo cerca de 30% (COSGROVE, JARVIS, 2012). A rigidez típica desta estrutura é conferida principalmente pelo alto teor de celulose, organizada em microfibrilas altamente orientadas, com as quais as cadeias hemicelulósicas formam ligações cruzadas para formar a estrutura básica da parede secundária. Esta estrutura encontra-se impregnada pela lignina, que promove resistência adicional e hidrofobicidade à parede celular secundária (ZHONG, YE, 2015).

Por sua vez, a parede celular primária possui menor conteúdo de celulose, entre 15% e 40%, que se encontra mais dispersa, em contraste com as microfibrilas altamente orientadas da parede secundária. Contém também de 20 a 30% de hemiceluloses, associadas às microfibrilas de celulose. Destaca-se a presença de pectinas, que compreendem de 30% a 50% e que são de fundamental importância para a flexibilidade da parede celular primária (COSGROVE, JARVIS, 2012).

Além das paredes primária e secundária, existe outra região importante na morfologia do tecido vegetal: a lamela média, que compreende a zona de junção entre células adjacentes, composta majoritariamente por pectinas e que desempenha um importante papel na adesão celular (PALIN, GEITMAN, 2012; DAHER, BRAYBOOK, 2015).

2.2.1 POLISSACARÍDEOS DE PAREDE CELULAR VEGETAL

2.2.1.1 CELULOSE E HEMICELULOSES

A celulose é o polissacarídeo de parede celular que apresenta a estrutura mais simples. Ela é composta por cadeias lineares formadas por unidades de β-D-glucose unidas por ligações glicosídicas do tipo (1→4). O grau de polimerização pode variar bastante, mas gira em torno de 10.000 (BURTON, GIDLEY, FINCHER, 2010; ZHONG, YE, 2015).

As hemiceluloses possuem cadeia principal formada por unidades monossacarídicas unidas por ligações β(1→4) e com configuração equatorial nas posições C-1 e C-4, de forma muito semelhante à celulose, embora a composição monossacarídica seja variável

(SCHELLER, ULVSKOV, 2010). As hemiceluloses são um grupo de heteropolissacarídeos, e podem ser classificadas em xilanas, xiloglucanas, mananas e β-glucanas mistas, sendo estas últimas restritas à família das Poaceas (EBRINGEROVÁ, 2006; SCHELLER, ULVSKOV, 2010). A predominância de um ou de outro grupo de hemiceluloses, bem como a estrutura destes grupos podem variar conforme a classe ou família botânica à qual o vegetal pertence (SCHELLER, ULVSKOV, 2010; FRY, 2017).

A Figura 2 apresenta a estrutura química esquemática de algumas hemiceluloses mais comuns.

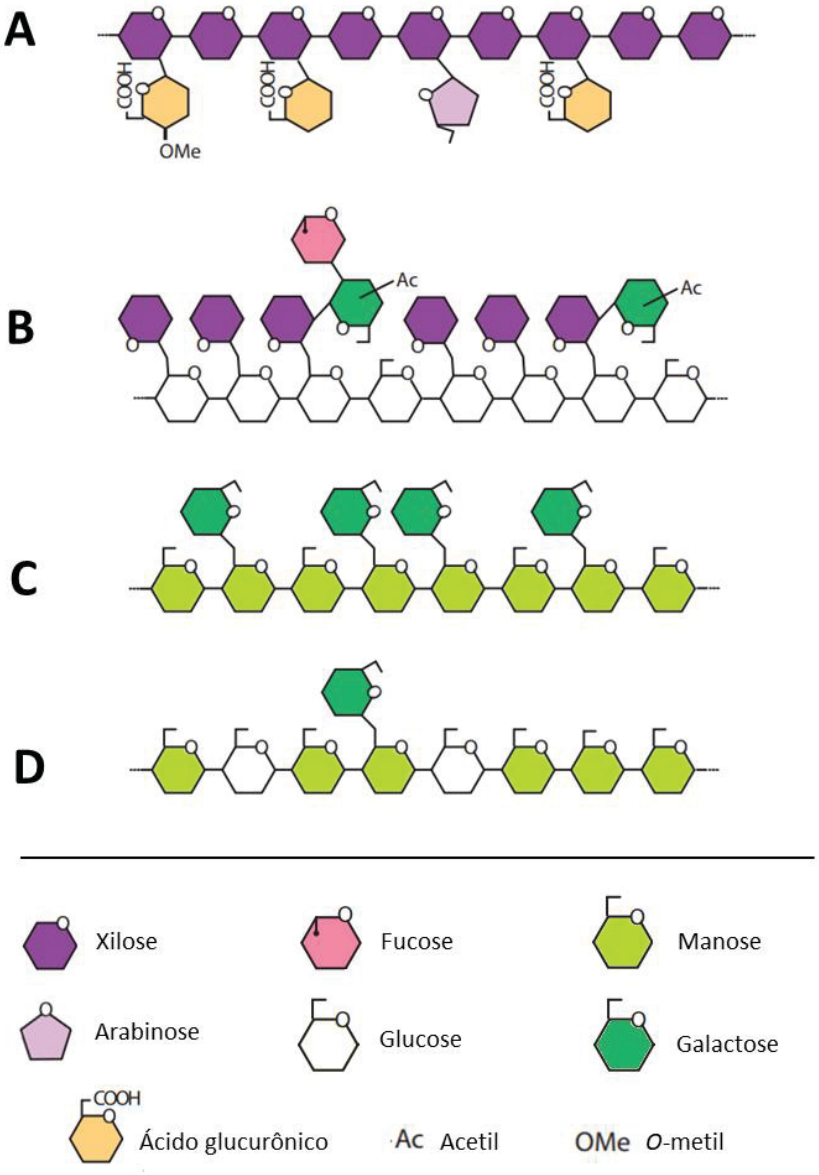

Fig. 2 – Estrutura esquemática de algumas hemiceluloses comuns. (A) glucuronoxilana; (B) xiloglucana; (C) galactomanana e (D) galactoglucomanana. Fonte: adaptado de SCHELLER, ULVSKOV (2010).

2.2.1.3 PECTINAS

O termo pectina abrange um complexo grupo de polissacarídeos ácidos, compostos primariamente por α-D-GalA. Conforme sua estrutura química, as pectinas são classificadas em três principais grupos: as homogalacturonanas (HG), as ramnogalaturonanas I (RG-I) e as ramnogalacturonanas II (RG-II) (CHAN et al., 2017).

As HG são o grupo estruturalmente mais simples entre as pectinas e correspondem ao polímero linear composto unicamente por unidades de α-D-GalA unidas por ligações (1→4), com grau de polimerização comumente entre 100 e 200 unidades, embora cadeias ainda maiores sejam possíveis. O grupo carboxílico na posição C-6 pode ser metil-esterificado e grupamentos acetil podem estar presentes como substituintes nas posições *O*-2 e/ou *O*-3 (CHAN et al., 2017).

Por sua vez, as RG-I são estruturas nas quais a cadeia principal é formada por unidades de α-D-GalA intercaladas por unidades de α-L-Rha, em uma estrutura repetitiva →4)α-D-GalA(1→2)α-L-Rha(1→ de até 100 unidades dissacarídicas. Entre 20% e 80% das unidades de Rha podem ser substituídas na posição C-4 por cadeias laterais neutras contendo arabinose e/ou galactose, denominadas arabinanas, galactanas e arabinogalactanas (VINCKEN et al., 2003; CHAN et al., 2017). As galactanas são constituídas por cadeias lineares de β-D-Gal ligadas (1→4), enquanto as arabinanas costumam exibir estruturas altamente ramificadas, com uma cadeia principal formada por unidades de α-L-Ara(1→5) substituídas em *O*-2 ou *O*-3 por unidades individuais de α-L-Ara(1→ (HWANG, PYUN, KOKINI, 1993). Já as arabinogalactanas podem ser do tipo I (AG-I), compostas por uma cadeia principal de β-D-Gal(1→4) que podem ser substituídas em *O*-3 por α-L-Ara(1→, ou do tipo II (AG-II) onde a cadeia principal é formada por β-D-Gal(1→3) contendo cadeias laterais curtas de α-L-Ara(1→6)β-D-Gal(1→6), nas quais as unidades de β-D-Gal podem ser substituídas por α-L-Ara(1→3) (VINCKEN et al., 2003). O tamanho, o tipo e o grau de ramificação das cadeias laterais nas RG-I são bastante variáveis dependendo da fonte vegetal, resultando em uma vasta diversidade estrutural (CHAN et al., 2017).

Por outro lado, as RG-II são estruturas bastante complexas, porém altamente conservadas. As RG-II são compostas por uma cadeia principal de HG com cerca de 9 unidades de →4)α-D-GalA(1→ ramificadas por quatro oligossacarídeos de composição bem definida. Estes oligossacarídeos contém monossacarídeos raros, tais como a apiose, 2-*O*-metil-L-Fuc, 2-*O*-metil-D-Xyl, ácido acérico, ácido 3-deoxi-D-mano-octulosônico (Kdo) e ácido 3-deoxi-D-lixoheptulosárico (Dha). Seus monossacarídeos ligam-se através de variados

tipos de ligações glicosídicas, e destaca-se a presença de grupos borato, que permitem a ligação cruzada entre duas cadeias de RG-II (CHAN et al., 2017).

Além das HG, RG-I e RG-II, também são descritas na literatura outras classes de pectinas como as xilogalacturonanas e as apiogalacturonanas. A Fig. 3 apresenta a estrutura esquemática das diferentes classes de pectinas.

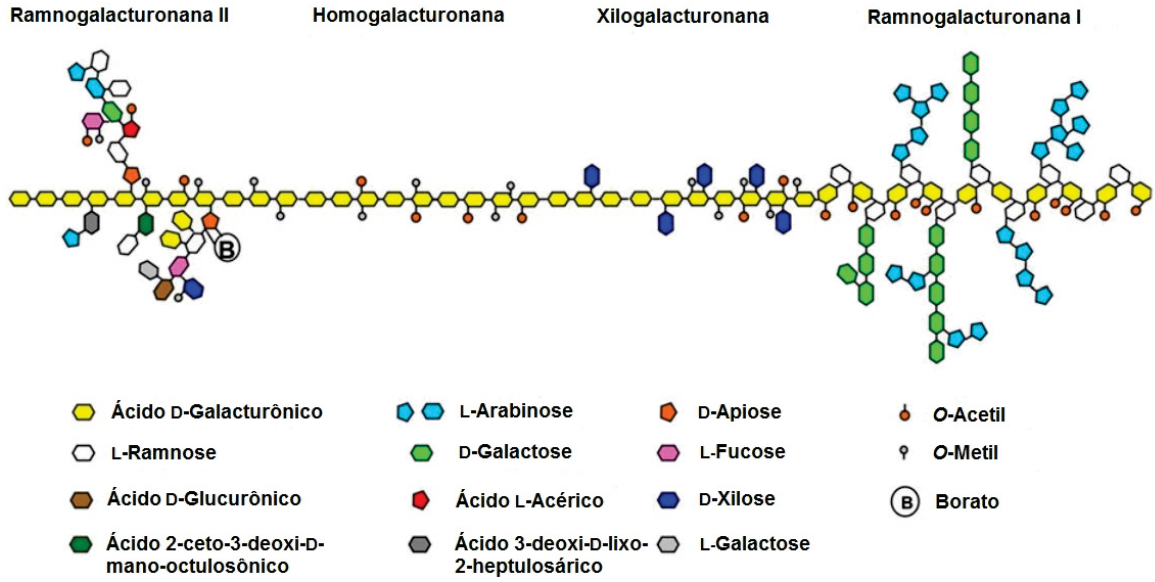

Fig. 3 – Estrutura esquemática das diferentes classes de pectinas. FONTE: adaptado de HARHOLT, SUTTANGKAKUL, SCHELLER (2010).

As pectinas encontram vasta aplicação como modificadores de textura, particularmente no campo alimentício, sendo reconhecidas como um aditivo alimentar sob o registro 440 do Codex Alimentarius (CHAN et al., 2017). A capacidade de formar géis sob condições específicas é a propriedade mais notável das pectinas e a principal razão para suas aplicações industriais e tecnológicas (CHRISTIAENS et al., 2016).

As condições sob as quais as pectinas gelificam dependem, primariamente, do grau de metil-esterificação. Quando mais de 50% das unidades de GalA encontram-se na forma metil-esterificada, a pectina é classificada como HM (do inglês, *high methoxyl*, de alto grau de metoxilação) e forma gel na presença de altas concentrações de co-solutos em meio ácido, geralmente em pH < 3,5. A presença do co-soluto, habitualmente sacarose, diminui a atividade da água e promove interações hidrofóbicas entre os grupos metil. Por sua vez, o pH < 3,5, que é o pKa médio das pectinas, protona os grupos carboxílicos não-esterificados, evitando, assim, a repulsão eletrostática entre as cargas negativas destes grupos e tornando-os aptos a estabelecer ligações de hidrogênio, o que também contribui para a interação entre as cadeias e consequentemente a estabilização da rede polimérica que forma o gel (KASTNER,

EINHORN-STOLL, SENGE, 2012; CHRISTIAENS et al., 2016; CHAN et al., 2017). Por outro lado, quando menos de 50% dos grupos carboxílicos são metil-esterificados, a pectina é classificada como LM (do inglês, *low methoxyl*, de baixo grau de metoxilação) e forma géis na presença de cátions bivalentes, sendo o Ca^{2+} o mais comum. Os íons, positivamente carregados, interagem eletrostaticamente com os grupos carboxílicos, com carga negativa, formando zonas de junção entre as cadeias de pectina e permitindo a formação da rede tridimensional. A gelificação das pectinas LM não requer uma faixa específica de pH, mas é favorecida quando pH > pKa, pois as cadeias precisam estar desprotonadas para que a interação pectina-Ca^{2+} aconteça (KASTNER, EINHORN-STOLL, SENGE, 2012; CHRISTIAENS et al., 2016; CHAN et al., 2017). No entanto, para que ocorra a formação de zonas de junção suficientemente estáveis para levar à gelificação, é necessário que a distribuição dos grupos carboxílicos livres permita a existência de um número mínimo de unidades sucessivas não metil-esterificadas, que estima-se que seja entre 6 e 20 (MUNARIN, TANZI, PETRINI, 2012).

Na indústria alimentícia, área em que as pectinas são mais amplamente empregadas, sua principal aplicação é na confecção de doces e geleias de frutas, de géis para confeitaria, e no preparo de sobremesas lácteas, destacando-se aquelas de baixo teor de açúcares e de gordura (ENDREβ, CHRISTENSEN, 2009). No entanto, a capacidade de gelificação das pectinas também permite promissoras aplicações em outras áreas, particularmente na área biomédica, tais como o desenvolvimento de veículos para liberação controlada de medicamentos de uso oral, nasal ou ocular, como biomaterial para engenharia de tecidos e como adesivos cicatrizantes (MUNARIN, TANZI, PETRINI, 2012; NOREEN et al., 2017).

Além do uso biomédico na forma de hidrogéis, as pectinas também apresentam possibilidades farmacológicas devido às suas interessantes atividades biológicas, com destaque aos efeitos antitumorais (ZONG, CAO, WANG, 2012; ZHANG, XU, ZHANG, 2015) e imunomoduladores (RAMBERG, NELSON, SINNOTT, 2010; POPOV, OVODOV, 2013). Porém, em contraste às suas propriedades gelificantes, para a qual o domínio mais importante da estrutura é a região linear de HG, para os efeitos biológicos as cadeias laterais do domínio RG-I são de grande relevância. É sugerido que a interação entre a proteína pró-metastática galectina-3 (Gal-3) e as unidades terminais de galactose, presentes nas cadeias laterais da RG-I, seja um dos mecanismos chave para o efeito antitumoral de algumas pectinas. Esta interação inibe a proteína, bloqueando a adesão e migração de células tumorais e induzindo estas células à apoptose (MAXWELL et al., 2012; ZHANG, XU, ZHANG, 2015). Quanto à atividade imunomoduladora, a relação entre a estrutura e o efeito parece ser

mais complexa, mas de maneira geral regiões ramificadas de RG-I contendo AG-I tendem a apresentar efeito imunoestimulador enquanto a região HG tende a induzir efeito imunossupressor, especialmente quando possuem grande número de carboxilas livres e grupos acetil (POPOV, OVODOV, 2013; FERREIRA et al., 2015).

Embora as pectinas sejam biopolímeros tão versáteis e com vasta aplicação, as pectinas comerciais disponíveis no mercado são obtidas de fontes ainda restritas, sendo as cascas de cítricos e o bagaço de maçã remanescentes da indústria de sucos as principais matérias-primas para a sua obtenção. Cerca de 85% de toda a pectina produzida mundialmente é obtida de fontes cítricas, 14% do bagaço de maçã e 0,5% é obtido da polpa de beterraba (CIRIMINNA et al., 2015; CHAN et al., 2017). A matéria-prima utilizada depende do material mais disponível na região onde está localizada a empresa produtora. A produção de pectina concentra-se na Europa, mas também existem fábricas na China, onde o bagaço de maçã é a fonte mais utilizada, e no Brasil e no México, onde os cítricos são a principal matéria-prima (CIRIMINNA et al., 2015).

Embora atualmente os resíduos de cítricos e de maçã ainda sejam as principais fontes de obtenção das pectinas comerciais, muitas pesquisas vêm sendo desenvolvidas no esforço de estabelecer fontes alternativas de pectinas, com destaque para a utilização de resíduos agroindustriais como matéria-prima. A busca por novas fontes de pectinas entre os resíduos agroindustriais pode valorizar estes resíduos e evitar seu descarte e, ao mesmo tempo, suprir a demanda da produção industrial de pectina, que devido ao amplo uso em diferentes áreas pode não ser suficiente utilizando apenas as fontes convencionais (DRANCA, OROIAN, 2018). No entanto, embora virtualmente todo tecido vegetal contenha pectinas, para que possa ser considerada como uma fonte alternativa para a produção comercial o material precisa proporcionar uma pectina de alto rendimento e também de boa qualidade. Uma vez que o domínio HG é a estrutura que mais influencia as propriedades físico-químicas das pectinas, um dos principais parâmetros que determinam a qualidade das pectinas comerciais é o teor de ácido galacturônico, que deve ser maior que 65% (CIRIMINNA et al., 2015). Recentemente, Marenda et al. (2019) publicaram uma ampla revisão que compilou diversos resíduos vegetais já investigados quanto à sua empregabilidade para obtenção de pectinas. Dos 28 resíduos citados, 18 forneceram pectinas com teor de ácido urônico acima de 65% (casca de banana, casca de cacau, bagaço de cenoura, casca de cubiu, casca de pitaia, semente de frutos de unha-de-gato, bagaço de uva vermelha, casca de grapefruit, bagaço de kiwi, casca de manga, casca de maracujá, casca de pistache, bagaço de ameixa, casca de romã, bagaço de abóbora, bagaço de beterraba, bagaço de tomate e casca de melancia) e alguns deles tiveram baixo rendimento.

Assim, mais pesquisas são necessárias para buscar novas fontes que possam proporcionar pectinas com alto rendimento e alta qualidade.

2.3 CASCA DE TANGERINA PONKAN: UM RESÍDUO CÍTRICO AINDA NÃO EXPLORADO

O gênero *Citrus* (família Rutaceae) compreende um número não bem definido de espécies, uma vez que existem controvérsias entre seus taxonomistas, mas que varia entre 16 e 162 entre espécies não-híbridas e híbridas (KRUEGER, NAVARRO, 2007). Há quase um século a casca de cítricos vem sendo utilizada para a extração industrial de pectina (CANTERI et al., 2012), sendo atualmente a matéria-prima que dá origem a mais de 85% da pectina de uso comercial produzida mundialmente (CIRIMINNA et al., 2015). No entanto, o número de espécies usualmente utilizadas para este fim é bastante limitado, abrangendo apenas a laranja, a toranja e os limões das variedades Taiti e siciliano (YAPO, 2009), dependendo da disponibilidade do material na região onde a pectina é produzida (CIRIMINNA et al., 2015).

A ponkan (*Citrus reticulata* Blanco *cv. Ponkan*) é a mais importante variedade de mandarina do ponto de vista comercial (DUTT et al., 2010). É extensivamente produzida no Brasil (principalmente nas regiões Sul e Sudeste) e principalmente na China (DUTT et al., 2010; EMBRAPA, 2018), onde foram produzidos mais de 52% das cerca de 33 milhões de toneladas de tangerinas, mandarinas, clementinas e satsumas produzidas em todo o mundo em 2016 (FAOSTAT, 2018). Considerando que as regiões de grande produção de ponkan são, coincidentemente, regiões onde estão localizadas importantes indústrias de fabricação de pectina de uso comercial (CIRIMINNA et al., 2015), a casca de ponkan apresenta-se como uma interessante fonte alternativa deste polissacarídeo, que pode ser inclusa entre o rol de cítricos utilizados para este fim.

Embora as cascas de espécies cítricas sejam amplamente estudadas quanto às suas pectinas (TAMAKI, KONISHI, TAKO, 2008; KANMANI et al., 2014; XU et al., 2014; KAYA et al., 2014; HOSSEINI, KHODAIYAN, YARMAND, 2016; PASANDIDE et al., 2017; LIEW et al., 2018), poucas investigações a respeito das pectinas da casca de tangerinas e mandarinas (*C. reticulata*) são encontradas na literatura. Liu et al. (2001) quantificaram o teor de pectinas na casca de limão, grapefruit, laranja e tangerina da variedade Dancy e observaram que a tangerina teve o teor mais elevado entre os frutos, de 5,29% em relação ao peso fresco da casca. Chen et al. (2016) realizaram um estudo buscando otimizar o

rendimento da extração de pectina da casca de tangerina usando a técnica de extração por microondas, porém não especificaram a variedade de tangerina utilizada. Os autores obtiveram 19,9% de rendimento nas condições otimizadas (704 W, 52,2º C e 41,8 min). Precisamente a respeito da casca de ponkan, o único estudo encontrado na literatura foi o de Wang, Chuang e Hsu (2008), que realizaram apenas a quantificação, encontrando 37,7 mg/g de pectina na casca seca. A presença de altos teores de pectina na casca de ponkan é sugerida em alguns estudos utilizando este resíduo como bioadsorvente. Pavan et al. (2006) investigaram o uso da casca de ponkan como bioadsorvente para remoção de Ni^{2+}, Co^{2+} e Cu^{2+}, e verificaram que este material é bastante eficiente na remoção destes metais tóxicos em meio aquoso. Os autores apontaram a presença de altos teores de pectinas como responsáveis pelo bom desempenho do material em interagir com espécies positivamente carregadas, embora não tenham realizado quantificação ou caracterização das pectinas.

2.4 BAGAÇO DE UVA: UM RESÍDUO DA VINIFICAÇÃO

As uvas pertencem ao gênero *Vitis*, da família das Vitáceas (JACKSON, 2014) e estão entre as frutas mais cultivadas em todo o mundo (GARCÍA-LOMILLO, GONZÁLEZ-SANJOSÉ, 2017), somando uma produção mundial de quase 70,5 milhões de toneladas no ano de 2016 (FAOSTAT, 2018). O Brasil ocupa a 16ª posição entre os produtores de uva, com uma produção de 984.481 toneladas em 2016, e a região Sul é a região que mais produz, correspondendo a 76% do total nacional no mesmo ano (IBGE, 2016).

As uvas podem ser consumidas *in natura* ou para a produção de doces, geleias, sucos e uvas passas, mas o mais importante produto obtido da uva é o vinho. Cerca de 80% de toda a produção de uvas é direcionada para a produção de vinhos, dando origem a aproximadamente 27 bilhões de litros de vinho produzidos em todo o mundo anualmente (MAROUN et al., 2017; BERES et al., 2017). No entanto, a fabricação de vinhos gera como resíduo o bagaço, que corresponde a cerca de 20% da massa dos frutos (GARCÍA-LOMILLO, GONZÁLEZ-SANJOSÉ, 2017) o que gira em torno de 11 milhões de toneladas de bagaço produzidas mundialmente por ano, e é composto por aproximadamente 40% de cascas, 30% de sementes e 30% de ráquis (DOMÍNGUEZ, SANCHEZ-HERNANDEZ, LORES, 2017).

O bagaço de uva é geralmente descartado como lixo orgânico, usado na alimentação do gado ou devolvido à lavoura como fertilizante (MENDES *et al*., 2013). No entanto, este material é bastante rico em compostos de alto valor comercial e pode ser utilizado como

matéria-prima para a obtenção de óleos, fibras alimentares e compostos fenólicos (SCHIEBER, STINTZING, CARLE, 2001; BERES et al., 2017).

Diversos estudos já foram publicados a respeito do isolamento e aplicações de fibras alimentares obtidas do bagaço de uva (VALIENTE et al., 1995; BRAVO, SAURA-CALIXTO, 1998; DENG, PENNER, ZHAO, 2011; ZHU et al., 2015; ZHANG et al., 2017). No entanto, embora as pectinas façam parte do grupo de biomoléculas classificadas como fibras alimentares, há poucas informações a respeito das pectinas do bagaço de uva na literatura. Deng, Penner e Zhao (2011) fracionaram as pectinas do bagaço das variedades brancas Muller Thurgau e Morio Muscat e vermelhas Cabernet Suavignon, Merlot e Pinot Noir de acordo com a sua solubilidade em água, quelante e álcali diluído. Os autores não indicaram valores de rendimento para cada fração, mas observaram teores de ácido galacturônico consideravelmente mais elevados na fração obtida com quelante e geralmente mais altos para as variedades vermelhas. Müller-Maatsch et al. (2016) quantificaram o teor de pectinas em diversos resíduos alimentares, incluindo o bagaço de uva (variedade não especificada), extraindo as pectinas sequencialmente usando quelante e álcali diluído. De maneira semelhante aos resultados de Deng, Penner e Zhao (2011), a fração solúvel em quelante teve maior rendimento (11%) e maior teor de ácidos urônicos (89,4%), enquanto a fração solúvel em álcali diluído teve 8% de rendimento e menos de 40% de ácidos urônicos. O grau de metil-esterificação foi determinado somente para a fração extraída com quelante, sendo de apenas 5%, o mais baixo DM entre todos os 24 materiais avaliados. González-Centeno et al. (2010) não realizaram a extração das pectinas, mas apresentaram algumas predições a respeito das características estruturais das pectinas presentes no bagaço de dez variedades de uvas, incluindo vermelhas e brancas, a partir da composição monossacarídica e DM do AIR (*alcohol insoluble residue*). Com base na proporção dos monossacarídeos, os autores deduziram uma proporção média de 74% de HG, 22% de RG-I e 4% de RG-II nos bagaços de uva, e todos os materiais apresentaram baixo grau de metil-esterificação (<50%). O trabalho com maior enfoque na obtenção de pectinas usando o bagaço de uva como fonte, visando parâmetros de qualidade para a utilização comercial da pectina foi o realizado por Minjares-Fuentes et al. (2014). Os autores utilizaram a técnica de ultrassom para extrair pectina com maior rendimento, massa molar e grau de metil-esterificação a partir do bagaço de uva da variedade Cabernet Sauvignon, usando ácido cítrico como solvente e aplicando um planejamento experimental do tipo Box-Behnken variando a temperatura, o pH e o tempo de extração. As condições ótimas encontradas foram 75ºC, pH 2,0 e 60 minutos, resultando em uma pectina composta quase totalmente por HG, contendo cerca de 97% de ácido

galacturônico, com massa molar média de 163.900 g/mol e grau de metil-esterificação de 55,2%, corroborando os demais autores que apontam o bagaço de uva como fonte de pectinas de baixo grau de metil-esterificação. O baixo DM das pectinas do bagaço de uva é interessante do ponto de vista comercial, visto que a maior parte das pectinas LM disponíveis no mercado são obtidas através de tratamentos químicos ou enzimáticos realizados com as pectinas de alto DM geralmente obtidas pela extração convencional (CHAN et al., 2017).

Os vinhos são, em sua maioria, fabricados a partir de duas espécies de uvas: a *V. labrusca*, com as quais são produzidos os vinhos de mesa, e a *V. vinífera*, das quais se obtém os vinhos finos (ARCANJO et al., 2017). De acordo com a coloração da casca, resultante da acumulação de antocianinas, as uvas podem ser classificadas como brancas ou vermelhas (ou pretas) (MASSONET et al. 2017). Os vinhos também são classificados de acordo com a coloração, como vinhos brancos, tintos ou rosados, porém isso não obrigatoriamente se relaciona com a coloração da uva utilizada para sua fabricação, sendo possível obter vinhos brancos a partir de uvas vermelhas (BRUCH, 2012). O que diferencia os vinhos brancos dos vinhos tintos e rosados é o processo de fabricação, particularmente a etapa de fermentação, como apresentado na Fig. 4. Os vinhos tintos são obtidos pela fermentação alcoólica do mosto na presença das partes sólidas do fruto, como cascas e sementes, e só após a fermentação é que ocorre a prensagem, etapa em que o bagaço é gerado. Já na fabricação dos vinhos brancos, os frutos são prensados e apenas o suco é fermentado e dessa forma o bagaço não sofre o processo de fermentação (BRUCH, 2012; IANNONE et al., 2016).

Fig. 4 – Etapas do processo de fabricação dos vinhos brancos e tinto/rosado. FONTE: www.tudodevinho.wordpress.com, 2018.

3 JUSTIFICATIVA

Considerando que a ponkan ainda não é uma espécie cítrica empregada para a produção comercial de pectina, que ainda não existem estudos a respeito da extração e caracterização de pectinas presentes na casca dos frutos desta espécie, que é amplamente produzida no Brasil e na China, onde estão localizadas fábricas de pectina (CIRIMINNA et al., 2015), é neste contexto que se justifica a proposta do uso da casca de ponkan para a extração de pectina apresentada neste trabalho.

A grande produção mundial de vinhos e a escassez de estudos sobre o uso do bagaço de uva como fonte de pectinas embasa a proposta de investigação a respeito do uso deste resíduo como uma possível fonte comercial de pectinas. Quanto à variedade escolhida, por não passar pelo processo de fermentação, é possível supor que as pectinas contidas no bagaço de uvas utilizadas para a fabricação de vinhos brancos sejam provavelmente menos degradadas e possuam maior massa molar, o que é importante para suas propriedades gelificantes e aplicações industriais (CHAN et al., 2017). Considerando que, entre as uvas produzidas para fabricação de vinhos brancos, a Chardonnay (*Vitis vinífera cv. Chardonnay*) é

a variedade mais amplamente produzida e distribuída mundialmente entre as variedades de uvas brancas (OIV, 2017), esta variedade foi selecionada para ser investigada neste trabalho.

Com este trabalho, espera-se contribuir para o estabelecimento de novas fontes alternativas de pectina, além de ampliar as possibilidades de aplicação destes resíduos, agregando valor e direcionando-os a destinos mais nobres.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Investigar a potencialidade da casca de ponkan e do bagaço de uva Chardonnay como fonte de pectinas e suas aplicações industriais, biológicas e ambientais.

4.2 OBJETIVOS ESPECÍFICOS

- Extrair os polissacarídeos de parede celular da casca de ponkan através de extrações sequenciais empregando diferentes solventes e caracterizar as frações obtidas;
- Extrair pectinas a partir da casca de ponkan e do bagaço de uva por meio de extrações ácidas, empregando um planejamento experimental com o objetivo de definir condições ótimas para extração de pectinas com máximo rendimento e máximo teor de ácidos urônicos;
- Caracterizar as pectinas obtidas da casca de ponkan e do bagaço de uva nas condições otimizadas quanto à sua estrutura química;
- Avaliar o comportamento reológico da pectina otimizada da casca da ponkan;
- Avaliar a citotoxicidade e o potencial de estimulação da produção de NO por macrófagos das pectinas obtidas da casca de ponkan e do bagaço de uva;
- Avaliar a capacidade das pectinas obtidas do bagaço de uva de remover espécies metálicas tóxicas (metais pesados) em solução;
- Avaliar a capacidade do bagaço de uva de remover corantes em solução.

CAPÍTULO 1

Artigo: “Cell wall polysaccharides from Ponkan mandarin (*Citrus reticulata* Blanco *cv. Ponkan*) peel”

Cell wall polysaccharides from Ponkan mandarin (*Citrus reticulata* Blanco *cv. Ponkan*) peel

Cristiane Colodel, Lúcia Cristina Vriesmann, Carmen Lúcia de Oliveira Petkowicz

Department of Biochemistry and Molecular Biology, Federal University of Parana, PO Box 19046, 81531-980 Curitiba, Parana, Brazil

Abstract

Cell wall polysaccharides from ponkan peel were investigated with the aim of gain knowledge about their potential for different applications and the use of ponkan peel as raw material for pectin extraction. The plant material was defatted using MeOH:$CHCl_3$, pretreated with DMSO and then subjected to sequential extractions with cold and hot water, ammonium oxalate, HCl, Na_2CO_3, 2 M and 4 M NaOH in order to obtain polysaccharides. The polysaccharide fractions were analyzed by chemical, chromatographic and spectroscopic methods. Cold and hot water-soluble pectins contained higher amounts of GalA and higher degrees of methyl-esterification (DM) than ammonium oxalate and HCl fractions. Na_2CO_3 extraction provided non-esterified arabinose-rich pectins which formed gel in a dialysis step. NaOH solubilized hemicelluloses, composed mainly of xyloglucans, galactomannans and galactoglucomannans. The water-soluble fraction (WSP) was purified using α-amylase and amyloglucosidase and gave rise to the subfraction named α-WSP. The α-WSP was a pectin composed of HG and RG-I domains containing side chains of arabinans and short-chains of galactans, with low DM (39.4%) and M_w of 1.615×10^5 g/mol.

Keywords: hemicelluloses; pectins; sequential extractions; structural characterization.

1. Introduction

Cell wall polysaccharides are important and promising raw materials for biomedical (Liu, Willför, & Xu, 2015) and food applications (Chapple, & Carpita, 1998; Harris, & Smith, 2006). Since the composition of the polysaccharides differs depending on the plant source (Nevins, English, & Albersheim, 1967) and even the cell type (Keegstra, 2010), it is

important to increase the knowledge about the polysaccharides present in different species. Cellulose is the most characteristic plant cell wall polysaccharide and forms the structure of the cell wall by association with other two groups of polysaccharides: pectins and hemicelluloses (Keegstra, 2010).

Pectins are acidic heteropolysaccharides which are classified into three main groups: homogalacturonans (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Willats, Knox, & Mikkelsen, 2006). HG consists of a linear chain of covalently linked →4)-α-D-GalA*p*-(1→ units, which can be partially methyl-esterified at C-6 and acetylated at *O*-2 and/or *O*-3 (Yapo, 2011; Voragen, Coenen, Verhoef, & Schols, 2009). In RG-I, the backbone is composed of [→4)-α-D-GalA*p*-(1→2)-α-L-Rha*p*(1→] repeated disaccharide and side chains of arabinans, galactans and/or arabinogalactans are attached to the backbone at *O*-4 of some rhamnosyl residues (Yapo, 2011; Voragen *et al*., 2009). In its turn, RG-II consists of HG main chain containing quite complex side chains. (Voragen *et al.*, 2009; Mohnen, 2008). Pectins are widely used due to their gelling properties (Willats, Knox, & Mikkelsen, 2006), which are strongly affected by their structure, especially on the degree of methyl-esterification (Thakur, Singh, & Handa, 1997).

Hemicelluloses are described as the polysaccharides that are solubilized from the cell wall by aqueous alkaline solutions and have β-(1→4)-linked pyranosyl residues with the O-4 in the equatorial position. They comprise the groups of xylans, mannans and xyloglucans (Caffall, & Mohnen, 2009).

Citrus peel are the major sources of commercial pectins (Willats, Knox, & Mikkelsen, 2006; Ciriminna, Chavarría-Hernández, Hernández, & Pagliaro, 2015). Often, the botanical source is not precisely identified in citrus commercial pectins, but orange, lime, grapefruit and lemon are commonly used (Yapo, 2009).

Ponkan *(Citrus reticulata* Blanco *cv. Ponkan*) is a non-climateric citrus fruit originated from Asia which belongs to a cultivar of mandarin (Lee, Zhong, & Chang, 2015; Bao, Yuan, Zhao, Liu, & Gao, 2015). It is one of the most appreciate mandarins for consumption, being the main mandarin cultivated in Brazil (Ramos *et al.*, 2009; Mendonça, Ramos, Rufini, Araújo Neto, & Rossi, 2006). The fruits are flattened, with few seeds and the peel is rough and loose (Coelho, 1996), which makes it easy for consumption *in natura*. The fruits are also processed for the production of concentrated juice (Pavan, Mazzocato, Jacques, & Dias, 2008).

Previous studies on the ponkan peel composition investigated mainly secondary metabolites, such as flavonoids, carotenoids (Wang, Chuang & Hsu, 2008),

polymethoxyflavones (Du & Chen, 2010), and the essential oil (Lota, Serra, Tomi & Casanova, 2000). Ascorbic acid and mineral contents were also evaluated (Barros, Ferreira & Genovese, 2012). Concerning the polysaccharides, the pectin content in ponkan peel was reported to be around 37% (Wang, Chuang and Hsu, 2008). However, only the amount of pectin was evaluated, and no studies were found in the literature concerning the structure and properties. Thus, the present work describes the fractionation and characterization of polysaccharides from cell wall of ponkan peel.

2. Materials and methods

2.1. Preparation of plant material

Fresh ripe fruits were collected in Ponta Grossa, Paraná, in southern Brazil (25°04'18.7"S; 50°07'44.3"W). Fruits were washed and peeled, and the peels were freeze-dried and ground into powder using an analytical mill IKA A-11 basic. The peel powder was defatted in a Soxhlet apparatus using chloroform/methanol, in a ratio of 2:1. Then, a pretreatment with DMSO 90% for 12 h was carried out for removal of starch.

2.2. Sequential extraction of cell wall polysaccharides from ponkan peel

After starch removal, sequential extractions of ponkan peel (PP) were performed using water, chelating agents, dilute acid and alkali in order to obtain pectins (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995) and increasing concentrations of aqueous NaOH for extraction of hemicelluloses (Vriesmann, & Petkowicz, 2009), as shown in Fig. 1. All the extractions were carried out using a solid/liquid ratio of 1:25 under mechanical stirring. NaOH extractions were performed in the presence of 20 mM $NaBH_4$ to avoid alkaline degradation. After each extraction, the material was centrifuged at 5,000 rpm for 20 min at 10°C and the supernatant was filtered with a synthetic fabric. For salt removal, ammonium oxalate and sodium carbonate extracts were dialyzed (6-8 KDa membrane) against tap water for three days. The extracts obtained using water (at 25ºC and 100°C), ammonium oxalate and citric acid were precipitated with 2 volumes of ethanol, kept at 4°C overnight, and the polysaccharides were recovered by filtration, washed three times with ethanol and dried under vacuum, resulting in WSP (water-soluble pectin), HWSP (hot water-soluble pectin), CSP

(chelator-soluble pectin) and HSP (diluted acid-soluble pectin) fractions, respectively. The sodium carbonate extract formed a gel within the membrane during dialysis which was then separated in soluble and insoluble parts, giving rise to fractions ASPS (soluble part from diluted alkali-soluble pectin after dialysis) and ASPI (insoluble part from diluted alkali-soluble pectin after dialysis). For NaOH extractions, the extracts were neutralized with 50% acetic acid and the precipitate formed in 2 M NaOH extract resulted in the polysaccharide fraction HA2 (hemicellulose A soluble in 2M NaOH), whereas no precipitate was found for 4 M extract after neutralization. After the neutralization and removal of the precipitate by centrifugation, the supernatants were dialyzed for three days, precipitated with 2 volumes of ethanol and the polysaccharides were recovered and dried as previously described, giving rise to fractions HB2 (hemicellulose B soluble in 2M NaOH) and HB4 (hemicellulose B soluble in 4M NaOH).

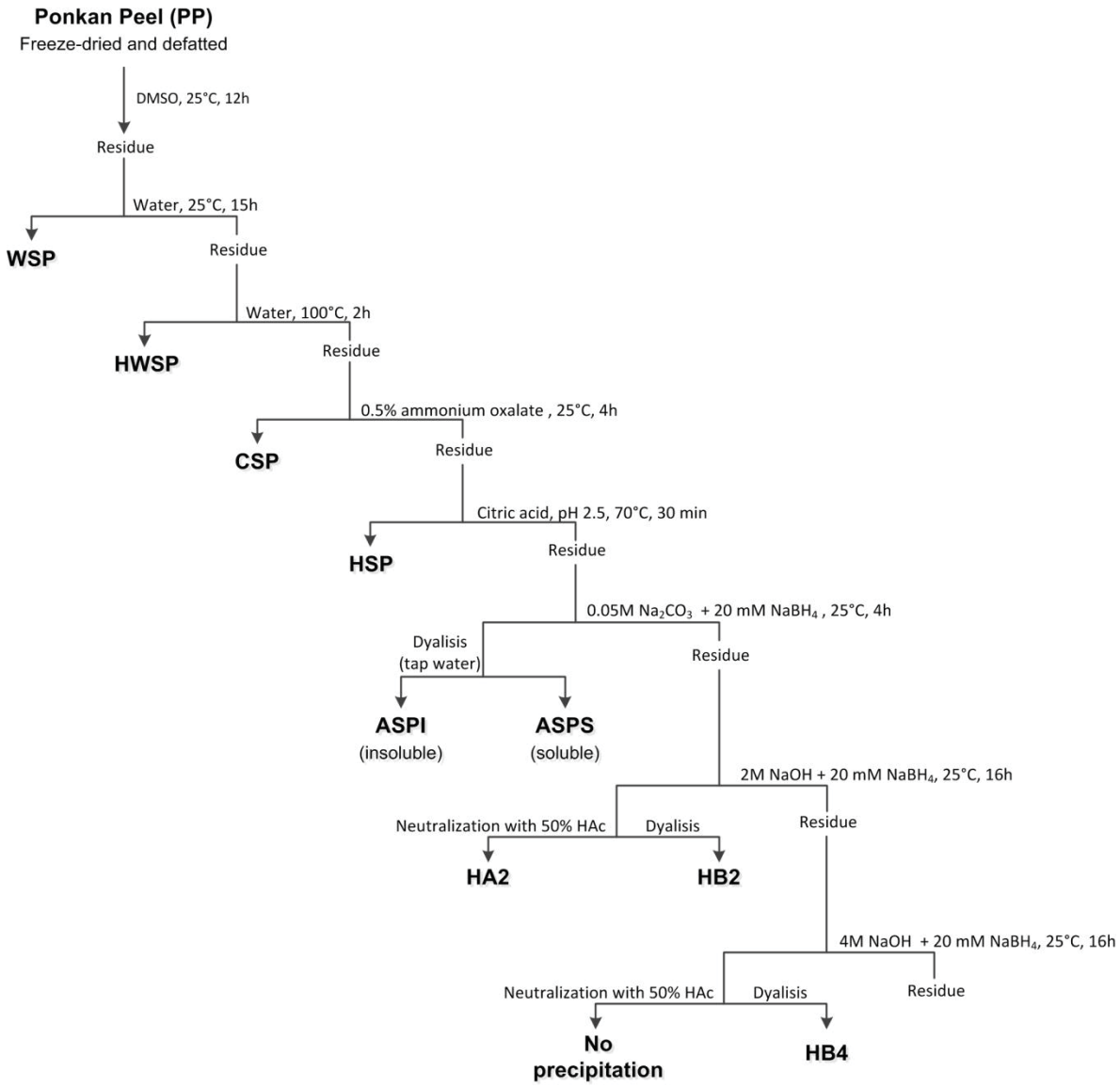

Fig. 1. Scheme of sequential extraction of polysaccharides from ponkan peel.
WSP = water-soluble pectin; HWSP = hot water-soluble pectin; CSP = chelator-soluble pectin; HSP = diluted acid-soluble pectin; ASPI = insoluble part from diluted alkali-soluble pectin after dialysis; ASPS = soluble part from diluted alkali-soluble pectin after dialysis; HA2 = hemicellulose A from 2M NaOH extraction; HB2 = hemicellulose B from 2M NaOH extraction; HB4 = hemicellulose B from 4M NaOH extraction.

2.3. Purification of WSP

WSP was solubilized (1 mg/mL) and kept at 55°C in a water bath. 30 μL α-amylase (Sigma, Germany) and 30 μL amyloglucosidase (Sigma, Germany) were added and the system was incubated for 1 h, followed by 15 minutes boiling for inactivation and precipitation of the enzymes. The solution was filtered, dialyzed for 2 days against deionized

water in 6-8 kDa membrane and then lyophilized, giving rise to the fraction α-WSP which was then characterized.

2.4. Monosaccharide composition

For determination of neutral monosaccharides, fractions were hydrolyzed with 2 M TFA at 120°C for 2 h. The hydrolyzate was evaporated to dryness, solubilized in water, reduced with $NaBH_4$ (Wolfrom, & Thompson, 1963a) and acetylated with pyridine–acetic anhydride (1:1 v/v, 16h, 25°) (Wolfrom, & Thompson, 1963b). The resulting alditol acetates from fractions obtained by sequential extractions were extracted with $CHCl_3$ and examined by a Varian Saturn 2000R − 3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer using a DB-225-MS column (0.32 mm internal diameter × 30 m × film thickness 0.25 μm) programmed from 50 to 220 ºC at a heating rate of 40 ºC/min, using He as carrier gas at 1 mL/min.

The purified polysaccharide was hydrolyzed and derivatized as described above and analyzed by a Thermo Scientific Trace GC Ultra gas chromatograph using a DB-225-MS column (0.32 mm internal diameter × 30 m × film thickness 0.25 μm) programmed from 60ºC to 200ºC at a heating rate of 40 ºC/min, using a mixture of He, N_2 and compressed air as carrier gas at 1 mL/min.

Uronic acids were quantified according to Blumenkrantz and Asboe-Hansen (1973) method, using galacturonic acid as standard, and were identified by anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). After hydrolysis with 2 M TFA (8 h, 100 ºC), drying and repeated washing until total removal of the acid, the sample (1 mg/mL) was filtered through a membrane of 0.22 μm, injected in a Thermo Scientific Dionex ICS-5000 chromatograph (Thermo Fisher Scientific, USA) with CarboPac PA20 column (3 × 150 mm) using gradient of 1 M NaOH and 1 M NaOAc as eluent (Nagel, Sirisakulwat, Carle, & Neidhart, 2014) in N_2 atmosphere in a flow of 0.2 mL/min at 30 ºC. Analyses were carried out in triplicate. Data were collected and analyzed using the ChromeleonTM 7.2 Chromatography Data System software.

2.5. Colorimetric methods

The acetyl content was determined by Hestrin (1949) method, using erythritol tetraacetate as standard, and the degree of acetylation (DA) was calculated as described by

Colodel, Bagatin, Tavares, & Petkowicz (2017). Protein was measured by the Bradford (1976) method using BSA as standard. Total phenolics were estimated by the Singleton and Rossi (1965) method, using galic acid as standard.

2.6. High Pressure Size Exclusion Chromatography (HPSEC)

HPSEC analyses were performed using a Waters unit coupled to a refractive index (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS). Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled to the multidetection instrument. A solution of 0.1 M $NaNO_2$ and 0.02% NaN_3 was used as eluent at a flux of 0.6 mL/min. Prior to the analyses, the samples (1.0 mg/mL) were filtered through a 0.22 μm cellulose acetate membrane. All the analyses were carried out at 25 °C. The refractive index increment of the solvent-solute solution with respect to a change in solute concentration (dn/dc) was determined using concentrations 0.2 - 1.0 mg/mL, and the average molar mass (Mw) was calculated using a Wyatt Technology ASTRA software.

2.7. Spectroscopic methods

The heteronuclear single quantum coherence nuclear magnetic resonance (HSQC NMR) spectrum was obtained for α-WSP in D_2O (40 mg/mL) at 70°C using a Bruker DRX 400 Avance spectrometer. Acetone was used as internal standard (δ30.2 for ^{13}C and δ2.22 for ^{1}H). The data was collected and analyzed by Bruker TopSpin software.

The Fourier-transform infrared (FT-IR) spectra of crude and purified pectic fractions were collected at the absorbance mode in the frequency range of 4000–400 cm^{-1} using a Vertex 70 spectrophotometer (Bruker, Germany), at 4 cm^{-1} resolution. Spectroscopic grade KBr powder was used and discs were prepared using a 99:1 salt/sample proportion. The spectra were obtained using OriginPro 8 software.

3. Results and discussion

The freeze-dried and defatted ponkan peel (PP) was extracted with DMSO to remove starch (yield 1.6%; Lugol test positive). The residue was submitted to sequential extractions with different solvents in order to obtain cell wall polysaccharides. Pectins were fractionated

into water-soluble pectins (WSP), hot water-soluble pectins (HWSP), chelator-soluble pectins (CSP), acid-soluble pectin (HSP) and alkali-soluble pectins, which were fractionated into soluble (ASPS) and insoluble (ASPI) subfractions after dialysis. Hemicelluloses were extracted with 2M and 4M NaOH, yielding fractions HA2 and HB2, precipitated by neutralization with acetic acid and by ethanol from 2M extract, respectively, and HB4 fraction from 4M extract precipitated by ethanol since there was no precipitation after its neutralization.

The yield and the composition of polysaccharide fractions extracted from ponkan peel are shown in Table 1.

Table 1. Yield and composition of polysaccharide fractions obtained from ponkan peel by sequential extractions.

Fraction	Yield[a] (%)	Monosaccharides[b] (%)								Protein[c] (%)	Phenolics[c] (%)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[c]		
WSP	2.9	2.7	0.9	11.5	1.3	1.9	19.5	11.8	50.5	4.5	0.5
HWSP	12.4	2.7	tr	17.7	tr	1.8	17.6	2.9	57.3	4.1	0.2
CSP	7.2	4.2	tr	26.7	tr	1.0	18.7	1.5	47.9	12.7	0.2
HSP	0.6	3.9	tr	29.4	0.8	0.9	34.8	3.4	26.8	8.0	0.3
ASPI	2.4	1.5	tr	55.7	tr	tr	21.8	tr	21.0	nd	nd
ASPS	0.1	4.8	0.7	48.4	0.7	1.4	39.4	2.7	1.9	nd	nd
HA2	0.7	3.6	tr	28.8	18.3	8.3	28.0	8.3	TR	53.6	1.7
HB2	2.3	0.8	4.0	6.6	14.7	18.6	19.7	34.0	1.5	7.0	0.3
HB4	0.6	1.5	2.6	10.4	8.7	23.8	26.5	24.1	2.3	4.3	0.3

[a] Yields are based on freeze-dried and defatted PP after starch removal using 90% DMSO.
[b] Neutral monosaccharides were determined by GC-MS after hydrolysis and derivatization.
[c] Colorimetric methods were used for estimation of uronic acids (UA), protein and phenolics.
nd = not-determined; tr = trace.
WSP = water-soluble pectin; HWSP = hot water-soluble pectin; CSP = chelator-soluble pectin; HSP = diluted acid-soluble pectin; ASPI = insoluble part from diluted alkali-soluble pectin after dialysis; ASPS = soluble part from diluted alkali-soluble pectin after dialysis; HA2 = hemicellulose A from 2M NaOH extraction; HB2 = hemicellulose B from 2M NaOH extraction; HB4 = hemicellulose B from 4M NaOH extraction.

The peels of citrus species are known for their high content of pectin (Ciriminna *et al.*, 2015). In this study, the total content of pectin was 25.6%, which is in agreement with the 25-35% of pectin content reported for citrus species (Ciriminna *et al*., 2015). The amount of hemicellulose extracted was low, 3.6%. Pectin contents about 3-6 times higher than the hemicellulose content were reported for orange and grapefruit (Ting, & Deszyck, 1961).

3.1. Pectin fractions

The yield of pectin fractions varied greatly, from 0.1% to 12.4%. Thehighest yield was found for HWSP fraction, followed by CSP and WSP. Acid and alkali-soluble pectins (HSP and ASPI/ASPS) had lower yields, ≤ 2.5%. Water and chelator-soluble pectins also had the highest yields among the fractions obtained by sequential extractions from purple pitaya pericarp (Montoya-Arroyo *et al*., 2014) and Styrian oil-pumpkin biomass (Košťálová, Hromádková, & Ebringerová, 2013).

As shown in Table 2, the proportion of neutral sugars, mainly arabinose and galactose, increased with the strength of extraction. The side chains of arabinans and galactans present in the pectic domain RG-I are described as capable of binding to other components of the cell wall. Zykwinska, Ralet, Garnier, & Thibault (2005) observed that arabinans and galactans from side chains of pectins from sugar beet and potato bound to cellulose microfibrils under *in vitro* conditions and the linear arabinans had the highest binding capacity. Arabinans and galactans present in RG I may also be covalently linked to xyloglucans, as Popper and Fry (2008) observed in cultured *Arabidopsis* cells. The authors suggest that the bonds formed between the pectic side chains and other polysaccharides may play a key role in the cell wall architecture. Thus, under these circumstances, this class of polysaccharides would require more drastic conditions to be extracted, such as dilute acids and alkalis that can hydrolyze covalent bonds (Sila *et al*., 2009).

All pectic fractions had low degree of acetylation (DA 0.2 - 0.5%) (Table 2). Pectins from orange albedo (Prabasari, Pettolino, Liao, & Bacic, 2011), obtained by sequential extraction, also showed low DA (2%).

Table 2. Degree of methyl-esterification, degree of acetylation and proportions of HG and RG-I domains for pectin fractions from ponkan peel.

Pectin fraction	DM [a] (%)	DA [b] (%)	HG [c] (%)	RG-I [c] (%)	HG/RG-I [c]
WSP	51.2 ± 0.4	0.03 ± 0.005	47.8	36.4	1.3
HWSP	52.6 ± 1.3	0.4 ± 0.002	54.6	40.7	1.3
CSP	34.1 ± 0.6	0.2 ± 0.01	43.7	53.8	0.8
HSP	38.4 ± 3.4	0.2 ± 0.003	22.9	72.0	0.3
ASPI	0	nd	19.5	80.5	0.2
ASPS	nd	nd	-	94.5	-

nd = not determined.
[a] DM, degree of methyl-esterification, determined by FT-IR.
[b] DA, degree of acetylation, calculated as described by Colodel *et al.*, 2017.
[c] Calculated as described by M'sakni *et al*., 2006.
WSP = water-soluble pectin; HWSP = hot water-soluble pectin; CSP = chelator-soluble pectin; HSP = diluted acid-soluble pectin; ASPI = insoluble part from diluted alkali-soluble pectin after dialysis; ASPS = soluble part from diluted alkali-soluble pectin after dialysis.

Water-soluble pectins include pectins and colloidal pectinic acids of high methyl ester (Majumder, 2012). They are commonly involved in weak interactions, as Van der Waals bonds, such as cold-water soluble pectins and hydrogen bonds such as hot-water soluble pectins (Sila *et al*., 2009). Jansen, Jang, Albersheim, & Bonner (1960) classified as classical pectin fraction only the polysaccharides extracted with hot water, which would be associated with the cell wall, while the cold water-soluble would be composed of pectins not associated with the cell wall. In the present study, cold and hot water-soluble pectins were sequentially extracted, resulting in WSP and HWSP, which were composed mainly of uronic acids, 50.5% and 57.3%, respectively (Table 1). The values were lower than that reported by Yapo, Lerouge, Thibault, & Ralet (2007) for a water-soluble pectin obtained from industrial citrus peels (61.5%). WSP and HWSP had similar degrees of methyl-esterification, 51.2% and 52.6%, respectively (Table 2). Homogalacturonan domains were predominant in WSP and HWSP, as demonstrated by the HG/RG-I ratio >1 (Table 2). They were probably extracted from the middle lamella, where HGs are deposited in highly methyl-esterified form and are subsequently modified through the action of enzymes to regulate mechanical properties and cell adhesion (Willats *et al*., 2001).

A great amount of pectin (7.2%) was extracted from ponkan peel using ammonium oxalate. The chelator-soluble pectin (CSP) was composed of 47.9% of uronic acids, differing strongly from the content of uronic acids found in pectins extracted with chelants from other

citric sources, such as orange albedo (85% of GalA, Prabasari *et al*., 2011) and citrus peel from industrial waste (70.4%, Yapo *et al*., 2007). Chelating agents solubilize those pectins that are bound together by means of junctions formed by Ca^{2+} ions in the cell wall by loosening the egg box structures formed by homogalacturonan and calcium complex (Babbar *et al.*, 2016). The low DM (34.1%) of CSP corroborates this hypothesis.

The acid extracted pectin (HSP) had the lowest yield among the pectin fractions, only 0.6% of the PP. Uronic acids were 26.8% (DM = 38.4%), and a high proportion of galactose (34.8%), arabinose (29.4%) and rhamnose (3.9%) was found. A similar composition and DM was reported for an acid-soluble pectin obtained by sequential extraction from purple pitaya skin, which also displayed the lowest yield (Montoya-Arroyo *et al*., 2014).

The extract obtained using diluted alkali (0.05 M Na_2CO_3) formed a gel (Supplementary data - Fig. S1) within the membrane during dialysis. The gel was separated from the liquid phase by filtration, yielding the fractions ASPI and ASPS, respectively. The formation of a gel during the dialysis was described by Bochichio, Petkowicz, Alquini, Busato, & Reicher (2006) for 0.1 M Na_2CO_3 extracts obtained from *A. angustifolia* plantlets. The authors suggested that gelation took place due to the formation of Ca^{2+} cross-links between homogalacturonans chains. However, ASPI had mainly arabinose (55.7%), followed by galactose (21.8%) and only 21.0% of uronic acids. It was insoluble in water, 1 M HNO_3, 1 M NaOH and DMSO. ASPI showed to be totally non-esterified, probably as a result of saponification due to the alkaline conditions of extraction (Wang, Pagán, & Shi, 2002). ASP-S, which corresponds to the liquid phase subfraction, had a very low yield and was composed predominantly of arabinose (48.4%), galactose (39.4%) and rhamnose (4.8%), being probably a highly branched fragment of RG-I.

The presence of a high content of pectins with DM up to 53% in ponkan peel is in agreement with the efficiency demonstrated by this biomaterial when it was used for biosorption of heavy metals in aqueous medium, since it was suggested that the uptake of ions occurred through the ionic interaction with the carboxylic groups from pectins (Pavan, Lima, Lima, Airoldi, & Gushikem, 2006; Pavan *et al*., 2008).

3.2. Hemicellulose fractions

As depicted in Table 1, HA2 fraction was mainly composed of protein (53.6%). Among the monosaccharides, the predominance of arabinose (28.8%) and galactose (28.0%)

suggests the presence of arabinans, galactans and/or arabinogalactans. Xylose (18.3%), probably from xylans, was also found.

The monosaccharide composition of fraction HB2 suggests the presence of xyloglucans and galactomannans. Xyloglucans were the main polysaccharides from 1 M and 4 M KOH extracts obtained from orange albedo (Prabasari *et al*., 2011).

Glucose, galactose and mannose were the main monosaccharides in HB4 fraction, suggesting the presence of galactoglucomannans. Galactoglucomannans were found in a fraction obtained from apple flesh using 24% KOH (Nara, Ito, Kato, & Kato, 2004) and in a fraction extracted from kiwi pericarp using 4 M KOH (Schröder *et al*., 2001). A minor amount of fucogalactoxyloglucans might be also present in HB4 fraction, considering the amounts of xylose (8.7%) and fucose (2.6%). The predominance of fucogalactoxyloglucans in 1 M KOH extract and galactoglucomannans in 4 KOH extract was reported by Missang, Renard, Baron, & Drilleau (2001) for bush butter fruit pulp, similar to the results found in the present study.

3.3. Purification of the water-soluble pectin (WSP)

As discussed previously, monosaccharide analysis of fraction WSP revealed that this fraction was composed of uronic acids, galactose, arabinose and rhamnose, which are typical components of pectic polysaccharides (Yapo, 2011; Voragen *et al*., 2009). A small amount of glucose (11.8%) was detected, suggesting the presence of starch as contaminant. When analyzed by HPSEC-RI, WSP showed a bimodal elution profile (Fig. 2), confirming that this fraction was composed of two families of polysaccharides. Therefore, WSP was subjected to a treatment using α-amylase and amyloglucosidase, in order to remove starch, resulting in a subfraction named α-WSP (76.5% of WSP). The efficient removal of starch through the enzymatic process was demonstrated by the reduction in the glucose content by 80% (Table 3). After the enzymatic treatment, α-WSP had a monomodal elution profile by HPSEC-RI (Fig. 2).

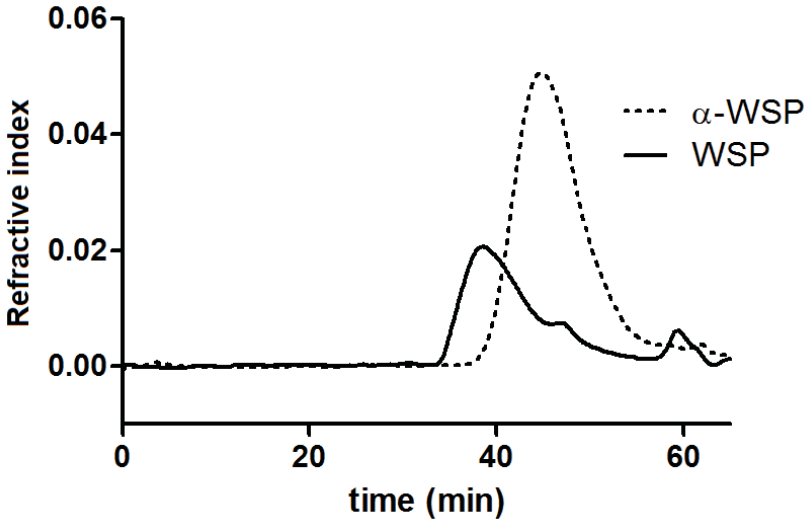

Fig. 2. HPSEC elution profile of water-soluble pectin (WSP) and enzyme-treated and reduced in starch content fraction (α-WSP).

Table 3. Comparison between monosaccharide composition and degree of methyl-esterification of water-soluble pectin (WSP) and enzyme-treated and reduced in starch content fraction (α-WSP).

Fraction	Monosaccharides[a] (%)								DM[c] (%)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA[b]	
WSP	2.7	0.9	11.5	1.3	1.9	19.5	11.8	50.5	51.2 ± 0.4
α-WSP	3.1	0.7	14.1	1.5	5.0	15.6	2.4	57.7	39.4 ± 0.8

[a] Neutral monosaccharides were determined by GC-MS after hydrolysis and derivatization.
[b] Quantified by colorimetric method and identified by HPAEC-PAD.
[c] DM, degree of methyl-esterification, determined by FT-IR.
WSP = water-soluble pectin; α-WSP = enzymatically purified pectin from WSP.

Compared to WSP, α-WSP showed a slight increase in the content of galacturonic acid, rhamnose, galactose and arabinose. A reduction in the degree of methyl esterification also occurred, probably due to the synergistic action of temperature (55 ºC) and pH of the medium, which was naturally close to 4. High temperatures increase the rate of demethoxylation of pectins at the same time that lead to depolymerization (El-Nawawi, & Heikal, 1995; Constenla, & Lozano, 2003). This is consistent with the decrease of ~23% in the DM, which was found to be 39.4%, as determined by FT-IR (Fig. 3) and with the increase in elution time of α-WSP (Fig. 2) compared with WSP.

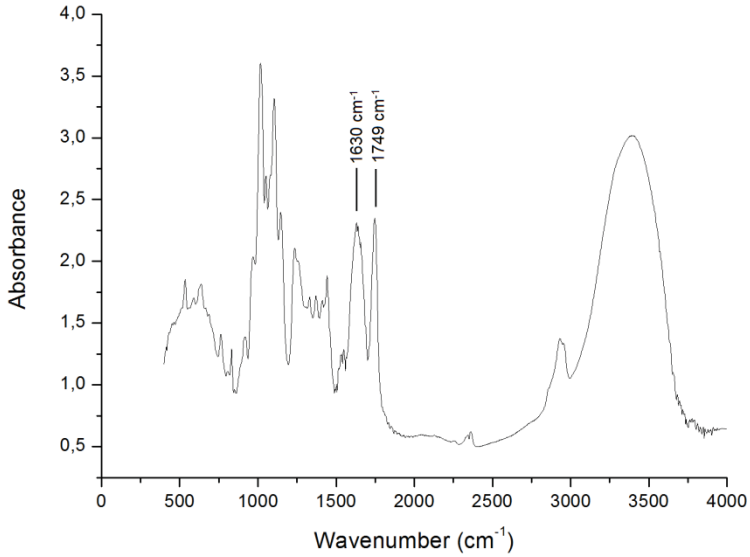

Fig. 3. FT-IR spectrum of α-WSP showing esterified (1749 cm^{-1}) and non-esterified (1630 cm^{-1}) carboxyl bands.

The average molar mass of α-WSP was calculated by HPSEC/MALLS-RI (Supplementary data, Fig. S2) as 1.615×10^5 g/mol. Berth (1988) obtained a purified fraction from a citrus pectin by ion exchange chromatography with average molar mass of 1.65×10^5 g/mol. Morris *et al.* (2008) determined the molar mass of a series of commercial citrus pectins and found values in the range of $1.45 – 1.95\times10^5$ g/mol.

The structural features of α-WSP were investigated by bidimensional HSQC NMR (Table 4 and Fig. 4).

Table 4. Chemical shifts of the glycosyl residues of α-WSP from $^{1}H–^{13}C$ HSQC NMR spectra.

Glycosil residues	Chemical shifts, δ (ppm)							
	H1/C1	*H2/C2*	*H3/C3*	*H4/C4*	*H5/C5*	*H6/C6*	*-COCH₃*	*-OCH₃*
→4)α-D-GalA(1→	5.14/99.5	3.76/70.5	4.14/68.5	4.46/78.9	5.06/70.6	-	2.09/20.2 (*O*-3)	
→4)α-D-6Me-GalA*p*(1→	4.97/100.2	3.74/68.3	3.99/68.5	4.46/78.9	5.24/70.5	-	2.18/20.4 (*O*-2)	3.82/52.8
→2)α-L-Rha*p*(1→	5.26/99.9	4.02/76.9	3.88/73.9	-	3.82/71.0	1.27/16.9		
→2,4)α-L-Rha*p*(1→	5.26/99.9	4.14/77.3	3.88/73.9	3.72/80.6	3.54/70.7	1.27/17.2		
→4)β-D-Gal*p*(1→	4.62/104.4	3.68/72.4	3.77/72.1	4.16/77.6	-	3.82/61.4		
t-β-D-Gal*p*(1→	4.50/103.4	3.59/70.6	-	-	-	3.75/61.5		
→5)α-L-Ara(1→	5.08/107.6	4.09/83.4	4.02/77.2	4.22/81.5	3.82/67.3			
→3,5)α-L-Ara(1→	5.15/107.5	4.28/79.6	-	4.31/81.2	-			
t- α-L-Ara(1→	5.26/109.3	4.13/81.2	3.96/76.9	-	3.85/61.5			

Acetone was used as internal standard (δ2.22/30.2), and the analysis was carried out at 70ºC.

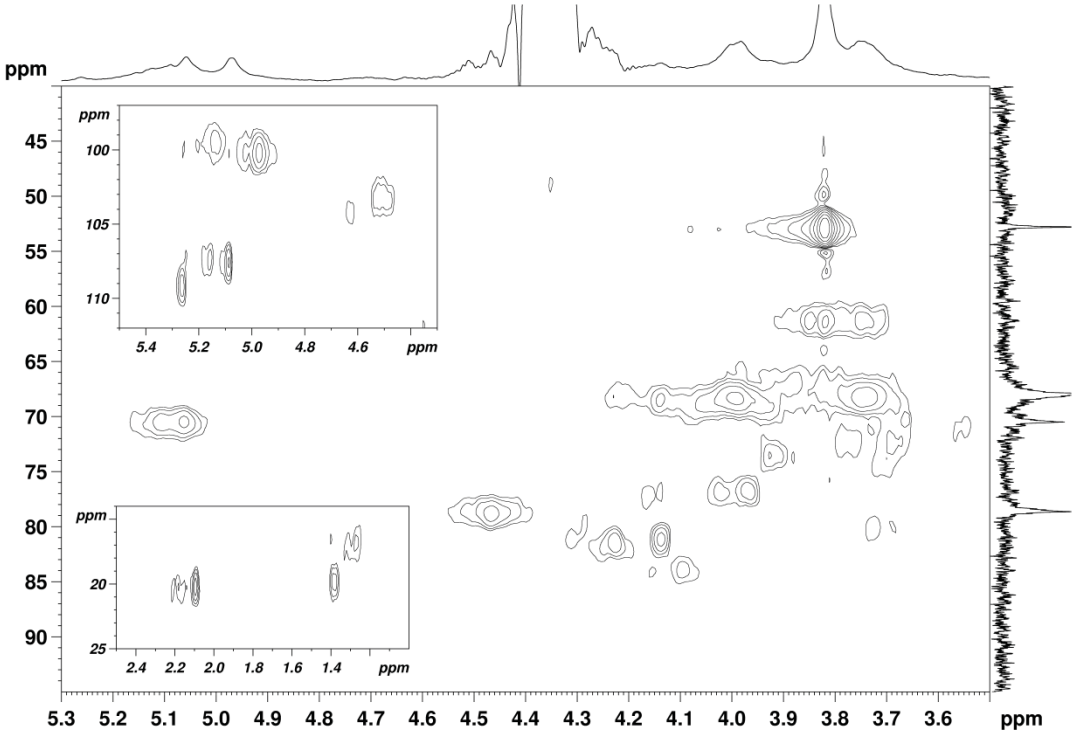

Fig. 4. ^{1}H-^{13}C HSQC NMR spectrum of α-WSP in D_2O at 70ºC.

The ^{1}H-^{13}C HSQC spectrum (Fig. 4, Table 4) displayed signals of non-esterified and methyl-esterified GalA*p* residues from homogalacturonans (Ovodova, Bushneva, Shashkov, Chizhov, & Ovodov, 2005; Golovchenko *et al.,* 2007; Kost'álová, Hromádková, & Ebringerová, 2014). Although the crude WSP fraction contained a very low degree of acetylation (DA 0.31%), correlations found at δ2.09/20.2 and at δ2.18/20.4 were attributed to the presence of acetyl groups attached at the O-3 and O-2 positions of the GalA units, respectively. No correlation from simultaneous acetylation at *O*-2 and *O*-3 was found (Renard & Jarvis, 1999).

Correlations assigned to unbranched →2)α-L-Rha*p*(1→ and to branched →2,4)α-L-Rha*p*(1→ units from RG-I backbone were observed (Habibi, Heyraud, Mahrouz, & Vignon, 2004; Ovodova *et al.*, 2005; Golovchenko *et al.*, 2007; Kost'álová, Hromádková, & Ebringerová, 2014). The higher signal intensity related to H6/C6 from →2,4)α-L-Rha*p*(1→ compared to →2)α-L-Rha*p*(1→ is an evidence that the RG-I domain of this pectin is highly substituted.

According to the HSQC correlations, the side chains of α-WSP seem to be composed of branched arabinans and by short chains of galactans. As shown in Table 4, signals from the linear backbone of →5)α-L-Ara(1→ residues (Habibi *et al*., 2004; Kost'álová, Hromádková, & Ebringerová, 2014; Shakhmatov, Toukach, Michailowa, & Makarova, 2014) were found, as well as from the branched →3,5)α-L-Ara(1→ units (Shakhmatov *et al*., 2014). Correlations assigned to *t*-α-L-Ara(1→ were also identified (Habibi *et al*., 2004; Kost'álová, Hromádková, & Ebringerová, 2014), and the similarity in the intensity of the anomeric signal of terminal and substituted units suggests that the chains are numerous and short.

Correlations from →4)β-D-Gal*p*(1→ (Habibi *et al*., 2004; Ovodova *et al.*, 2005; Kost'álová, Hromádková, & Ebringerová, 2014) and from *t*-β-D-Gal*p*(1→ (Golovchenko *et al.*, 2007) from galactans were found, being the anomeric signal of terminal units more intense than the anomeric signal of substituted units.

The results obtained by the HSQC analysis suggest that the purified polysaccharide consists of a pectin formed predominantly by HG regions, but contains fairly branched RG-I inserts with numerous and short chains of galactans, branched arabinans and possibly arabinogalactans.

Conclusions

Ponkan peel is an agroindustrial waste that can provide a high content of pectins with DM up to 53% and low DA. The results obtained in this study indicate that ponkan peel could be used for industrial production of pectin in the regions where the fruit is produced in large amounts. Apart from pectins, ponkan peel contains xyloglucans, galactomannans and galactoglucomannan, identified among the hemicelluloses. The fractionation and characterization of polysaccharides from the cell wall of ponkan peel open up possibilities for applications of both pectins and hemicelluloses present in this waste.

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Supplementary data

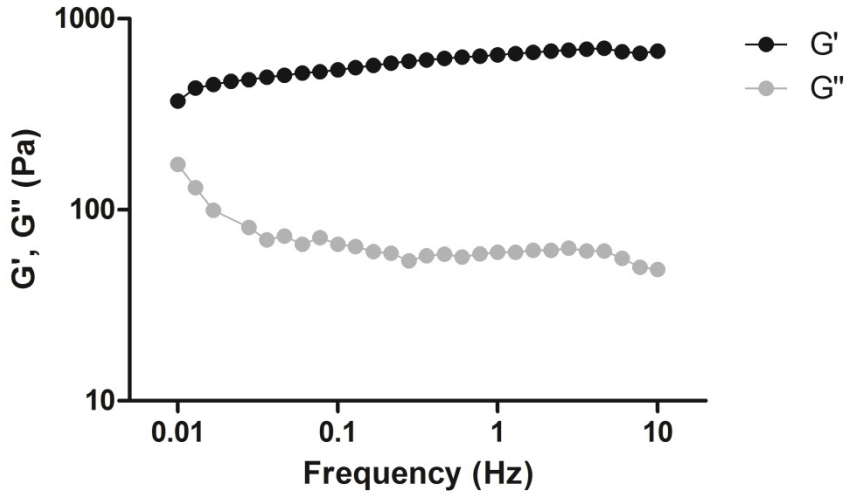

Figure S1. Frequency sweep of ASP-I at 25ºC.

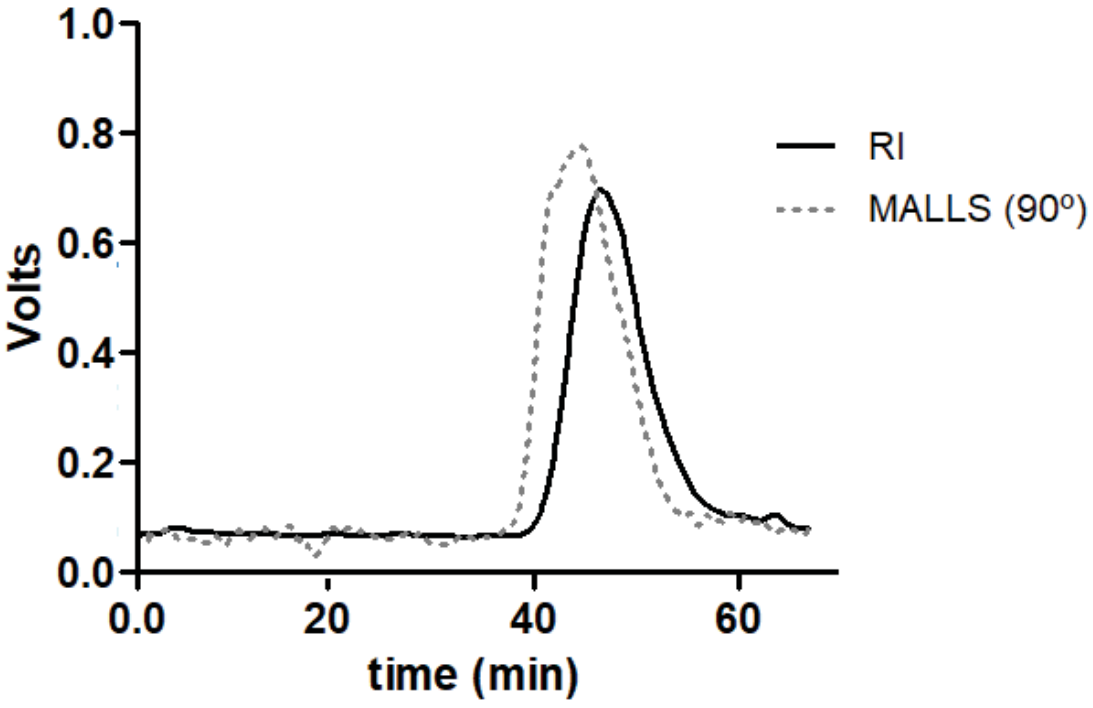

Figure S2. Elution profile of α-WSP by HPSEC/MALLS-RI.

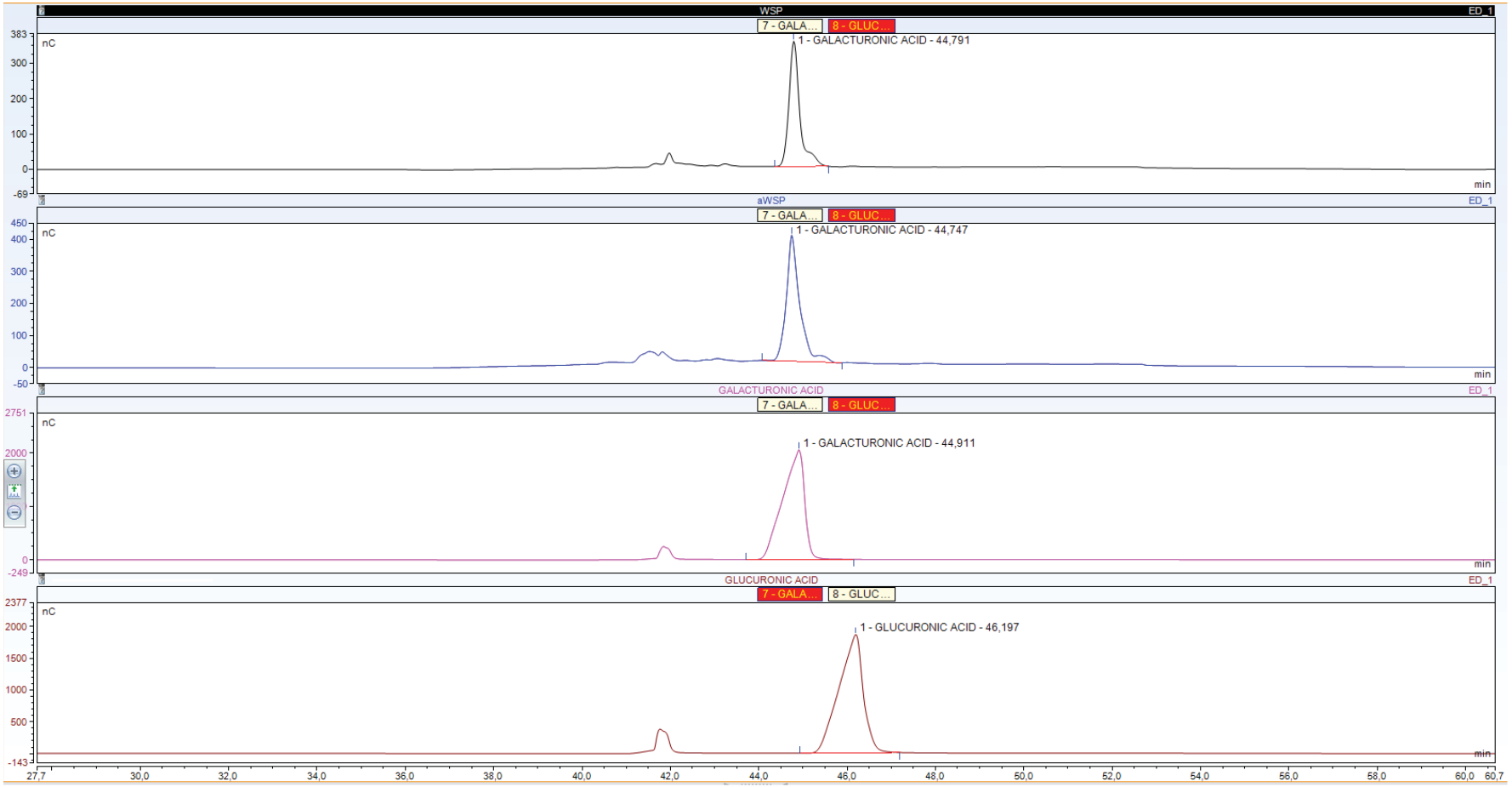

Figure S3. HPAEC-PAD chromatogram of WSP (black) and α-WSP (blue), compared to galacturonic (pink) and glucuronic (red) acid standards.

CAPÍTULO 2

Pectinas isoladas das frações aquosas extraídas da casca de ponkan: caracterização estrutural de uma homogalacturonana e comparação da capacidade de estimulação da produção de óxido nítrico por macrófagos

PECTINAS ISOLADAS DAS FRAÇÕES AQUOSAS EXTRAÍDAS DA CASCA DE PONKAN: CARACTERIZAÇÃO ESTRUTURAL DE UMA HOMOGALACTURONANA E COMPARAÇÃO DA CAPACIDADE DE ESTIMULAÇÃO DA PRODUÇÃO DE ÓXIDO NÍTRICO POR MACRÓFAGOS

INTRODUÇÃO

Além de suas funções estruturais como componentes da parede celular vegetal, quando isolados, os polissacarídeos são capazes de exercer importantes funções biológicas com efeitos terapêuticos. Ao mesmo tempo em que costumam apresentar baixa toxicidade e ausência de efeitos colaterais, diversos polissacarídeos de origem vegetal apresentam atividade antitumoral, antiviral, antioxidante e imunomoduladora (SHI, 2016; YU et al., 2018).

A ação imunomoduladora dos polissacarídeos ocorre através da capacidade destes biopolímeros de ativar células que fazem parte do sistema imunológico, como as células T, linfócitos B, células *natural killers* e, principalmente, macrófagos. Os macrófagos compõem, juntamente com os neutrófilos, a primeira linha de defesa do organismo. Estas células possuem em sua superfície receptores que reconhecem certos polissacarídeos, que podem estimular a atividade fagocítica, a proliferação celular e a produção e secreção de citocinas, espécies reativas de oxigênio e óxido nítrico pelos macrófagos (SCHEPETKIN; QUINN, 2006; YIN; ZHANG; LI, 2019).

Pectinas são polissacarídeos estruturais presentes na parede celular vegetal primária e são o principal componente da lamela média. São classificadas, de acordo com a sua estrutura química, em três grupos principais: as homogalacturonanas (HG), compostas unicamente por unidades de →4)α-D-GalA(1→ organizadas em uma estrutura linear, que podem encontra-se acetiladas nas posições *O-2* e/ou *O-3* e metil-esterificadas na posição C-6; as ramnogalacturonanas-I (RG-I), formadas por uma cadeia principal composta pela repetição de dímeros →4)α-D-GalA(1→2)α-L-Rha(1→, onde as unidades de α-L-Rha podem ser decoradas por cadeias laterais de arabinanas, galactanas ou arabinogalactanas ligadas na posição 4; e as ramnogalacturonanas-II, nas quais a cadeia principal de HG pode conter quatro oligossacarídeos bastante complexos e conservados (CHAN et al., 2017).

A atividade imunomoduladora das pectinas está relacionada às suas características estrturais. A presença de grupos metil, acetil e, particularmente, de regiões ramificadas

contendo arabinose e galactose parecem ser fundamentais para as propriedades imunomoduladoras destas moléculas (AMORIM et al., 2016). Popov e Ovodov (2013) reuniram resultados obtidos em testes de imunomodulação usando pectinas isoladas de plantas do norte da Europa e da Rússia e a partir destes resultados, propuseram um modelo de relação entre estrutura e efeito das pectinas no sistema imune. De acordo com este modelo, as regiões ramificadas tais como as RG-I possuem efeito imunoestimulador, enquanto as pectinas contendo mais de 75% de GalA, predominantemente HG, possuem efeito imunossupressor.

Neste capítulo, serão apresentados os resultados a respeito da purificação e caracterização química e estrutural da HWSP-Cu, uma subfração obtida da fração HWSP, isolada da casca de ponkan por extração com água fervente, conforme descrito no capítulo 1 (COLODEL; VRIESMANN; PETKOWICZ, 2018). Em seguida, serão demonstrados os resultados referentes aos testes de citotoxicidade e de capacidade de estimulação da produção de óxido nítrico (NO) por macrófagos usando a HWSP-Cu e a α-WSP, purificada da fração WSP, extraída da casca de ponkan por extração com água fria conforme descrito anteriormente (COLODEL; VRIESMANN; PETKOWICZ, 2018), efetuando uma avaliação preliminar da propriedade imunomoduladora das pectinas.

MATERIAIS E MÉTODOS

ISOLAMENTO DA HOMOGALACTURONANA

O isolamento da homogalacturonana foi realizado usando-se o método descrito por Hwang et al. (1992) como algumas modificações. A fração HWSP, obtida da casca de ponkan através da extração com água fervente conforme descrito previamente (COLODEL, VRIESMANN, PETKOWICZ, 2018), foi solubilizada em 100 mL de água destilada (10 mg/mL) e o pH foi ajustado para ~7 usando NaOH 0,1 M. Em seguida, foram adicionados 10 mL de $CuSO_4$ 5% (m/v) lentamente sob agitação. O material foi então filtrado e o precipitado foi tratado com etanol acidificado (contendo 1% de HCl) por 30 minutos, recuperado por filtração e lavado repetidamente com etanol até completa remoção do HCl, que foi monitorada através de testes no etanol recolhido após a lavagem adicionando-se algumas gotas de solução de $AgNO_3$ 0,1M, que forma um precipitado branco na presença de íons Cl^-. O tratamento com etanol acidificado foi repetido até que fosse observada a ausência de

coloração azulada ou esverdeada característica da presença de Cu^{2+}. Todo o procedimento de solubilização, precipitação com $CuSO_4$ e tratamento com etanol acidificado foi repetido por 5 vezes, e então o material resultante foi dissolvido em 50 mL de água destilada, deixado por 2 dias em diálise fechada e por fim liofilizado, sendo nomeado HWSP-Cu.

CARACTERIZAÇÃO QUÍMICA E ESTRUTURAL DA FRAÇÃO HWSP-Cu

Toda a caracterização química e estrutural da fração HWSP-Cu, incluindo a composição monossacarídica, dosagem colorimétrica de ácido urônico e de acetil, determinação do grau de acetilação, determinação do grau de metil-esterificação, cálculo de proporção de HG e RG-I, análise de homogeneidade, determinação da massa molar e análises de RMN foi realizada como descrito anteriormente (COLODEL, VRIESMANN, PETKOWICZ, 2018). A identificação do ácido urônico foi realizada para a fração bruta HWSP por cromatografia de troca iônica de alta performance acoplada a detector de pulso amperométrico (HPAEC-PAD), conforme a metodologia apresentada previamente (COLODEL, VRIESMANN, PETKOWICZ, 2018).

VIABILIDADE CELULAR E PRODUÇÃO DE ÓXIDO NÍTRICO

A viabilidade celular foi determinada pelo método do brometo de 3-[4,5-dimetiltiazol-2-il] -2,5-difeniltetrazólio (MTT) como descrito por Mosmann (1983) e Noleto et al. (2002). Os macrófagos aderentes foram incubados na ausência (controle) ou na presença das pectinas nas concentrações de 100, 250 e 500 μg / mL diluídos em meio por 48 horas, nas mesmas condições. Em seguida, o meio foi substituído por MTT 500 μg/mL em HBSS e a placa incubada no escuro por 10 minutos. Após a adição de DMSO para a diluição de cristais de formazan, determinou-se a absorbância em 550 nm. O controle (macrófagos incubados apenas no meio) foi considerado como 100% de viabilidade.

A produção de óxido nítrico foi quantificada indiretamente na forma de nitrito no meio de cultura, utilizando a reação de Griess com algumas modificações, conforme descrito anteriormente por Keller et al. (1990). Os macrófagos aderentes foram incubados por 48h na ausência (controle) ou presença de lipopolissacarídeo de *Escherichia coli* (LPS) (controle positivo) ou pectinas nas concentrações de 100, 250 e 500 μg / mL diluídos em meio por 48h,

nas mesmas condições. Em seguida, 100 µL de sobrenadante foram misturados com igual volume de reagente de Griess e incubados a 25 ºC por 10 minutos. A absorbância foi medida a 550 nm em leitor de microplacas. A concentração de nitrito foi calculada a partir de uma curva padrão de $NaNO_2$ e os resultados foram expressos em µmol.

RESULTADOS E DISCUSSÃO

Como pode ser observado na Fig. 1, a fração HWSP apresentou perfil de eluição bimodal, com um pico de menor intensidade detectado em 39 minutos, e outro pico mais intenso eluindo em 45,7 min, quando analisada por HPSEC-RI. Após a precipitação com $CuSO_4$, a fração resultante HWSP-Cu, que teve rendimento de 30,2% em relação à massa inicial de HWSP, apresentou perfil de eluição monomodal, com um único pico eluindo em 46,2 min, indicando que o tratamento foi eficiente para a purificação da amostra.

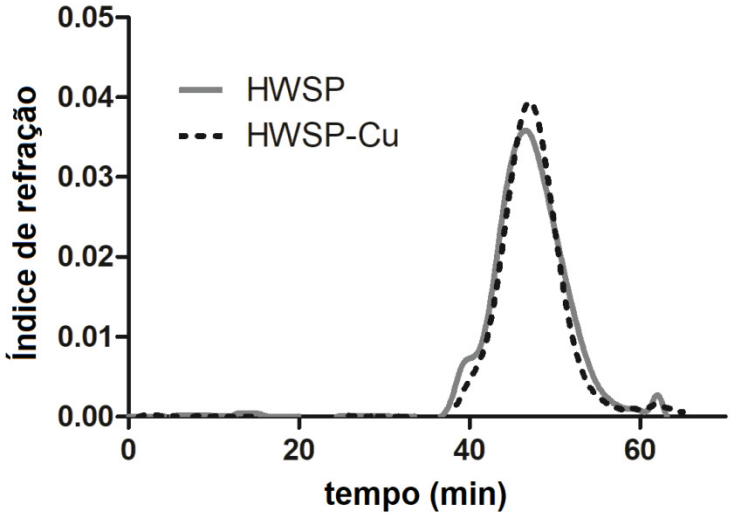

Fig. 1 – Comparação entre os perfis de eluição por HPSEC da fração bruta HWSP (linha contínua em cinza) e da sub-fração purificada HWSP-Cu (linha tracejada em preto). FONTE: A AUTORA.

A composição monossacarídica da amostra evidenciou que o polissacarídeo isolado corresponde a uma homogalacturonana, uma vez que a amostra é composta por 93,5% de ácido galacturônico, único monossacarídeo ácido identificado por HPAEC-PAD na fração bruta HWSP (Tabela 1). Ramnose, arabinose e galactose, que são monossacarídeos típicos de RG-I, estão presentes em teores baixos, resultando em uma proporção de apenas 6,2% de RG-I, 15 vezes menor que a proporção de HG, que foi de 93% (Tabela 1). Comparando-se a

composição monossacarídica e as proporções de HG e RG-I da fração HWSP e da subfração purificada HWSP-Cu, pode-se sugerir que o processo de precipitação seletiva com $CuSO_4$ removeu uma família de RG-I bastante ramificada por arabinose e galactose. Esta observação corrobora o resultado obtido pelas análises de HPSEC, considerando que as RG-I são a classe de pectinas mais volumosa e de maior massa molar, conforme aponta Yapo (2011).

Tabela 1 – Comparação entre a composição química e aspectos estruturais da fração bruta HWSP, da sua sub-fração purificada HWSP-Cu e da sub-fração α-WSP, purificada da fração WSP obtida por extração com água fria.

	HWSP	**HWSP-Cu**	**α-WSP**
Rha	2,7	0,5	3,1
Fuc	Tr	0,1	0,7
Ara	17,7	2,6	14,1
Xyl	Tr	Tr	1,5
Man	1,8	0,2	5,0
Gal	17,6	2,6	15,6
Glc	2,9	0,5	2,4
UA	57,3	93,5	57,7
DM	52,6 ± 1,3	76,2 ± 3,5	39,4 ± 0,8
DA	0,4 ± 0,002	0,3 ± 0,02	nd
HG	54,6	93,0	54,6
RG-I	40,7	6,2	35,9
HG/RG-I	1,3	15	1,5

nd = não determinado.

De acordo com Hwang et al. (1992), a adição de íons Cu^{2+} leva à formação de um complexo com a pectina através da interação eletrostática do cátion com os grupos carboxílicos negativamente carregados das unidades de ácido galacturônico, justificando assim a purificação de uma homogalacturonana por meio deste método. No entanto, a fração HWSP-Cu revelou ser altamente metil-esterificada, com DM = 76,2%, determinado por FT-IR (Fig. 2). Embora o mecanismo não seja elucidado, May (1990) aponta que pectinas de alto grau de metil-esterificação podem precipitar pela adição de Al^{3+} e Cu^{2+} em certas condições, o que parece ter ocorrido neste processo.

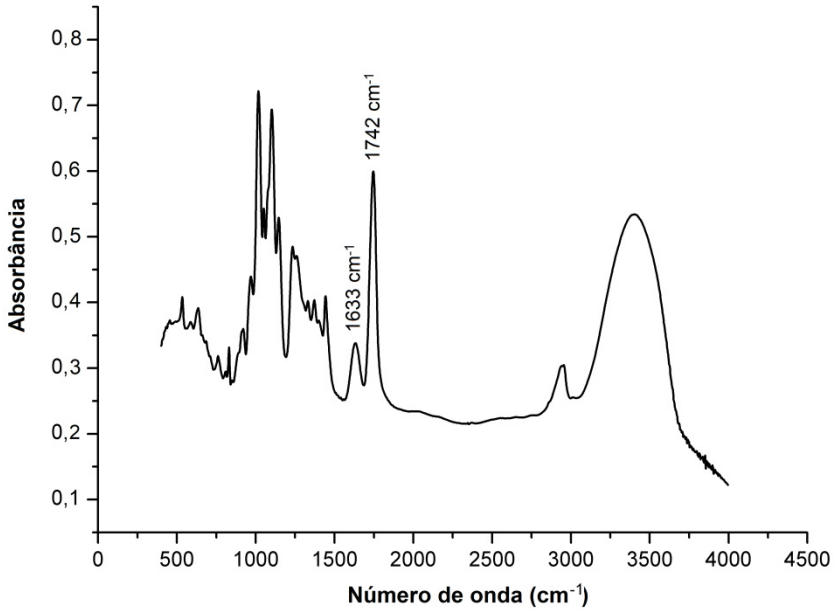

Fig. 2 – Espectro de FT-IR da sub-fração HWSP-Cu, indicando as bandas correspondentes às carboxilas livres (1633 cm^{-1}) e metil-esterificadas (1742 cm^{-1}). FONTE: A AUTORA.

A massa molar média (M_w), determinada por HPSEC-MALLS e calculada conforme mostra a curva de distribuição de massa ilustrada na Fig. 3, foi de $1{,}352\times10^5$ g/mol, ligeiramente menor que a massa molar da pectina purificada α-WSP ($1{,}615\times10^5$ g/mol), obtida a partir da fração extraída com água fria (COLODEL, VRIESMANN, PETKOWICZ, 2018) e maior do que a massa da pectina otimizada obtida da casca da ponkan por meio de extração HNO_3 fervente em pH 1.6 ($8{,}065\times10^4$ g/mol) (COLODEL et al., 2018). A diferença entre as massas molares das pectinas extraídas da casca da ponkan é coerente com cada processo de extração empregado, uma vez que as pectinas são suscetíveis a processos de hidrólise conforme aumenta a drasticidade do processo de extração, ou seja, o aumento na acidez do meio e na temperatura podem degradar as cadeias de pectina (CHAN, CHOO, 2013). O grau de polidispersão (M_w/M_n) da HWSP-Cu calculado foi de 1,6, comparável aos valores descritos por Corredig e Wicker (2001) para pectinas comerciais.

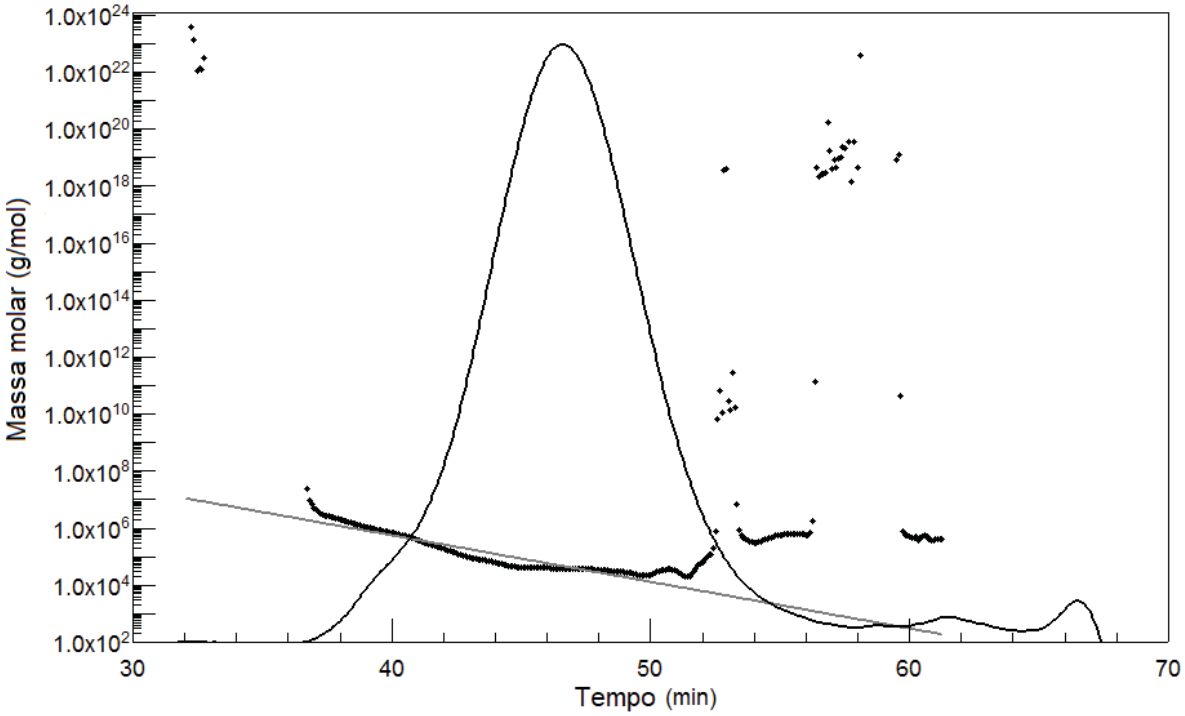

Fig. 3 – Curva de distribuição de massa molar da sub-fração HWSP-Cu. FONTE: A AUTORA.

O mapa de correlação bidimensional de $^{1}H/^{13}C$-HSQC RMN da sub-fração HWSP-Cu (Fig. 4) apresentou sinais intensos característicos de homogalacturonana altamente metil-esterificada, concordando com a composição monossacarídica que evidenciou a presença de GalA como componente majoritário. Para o H-1/C-1 das unidades de →4)α-D-(6Me)GalA(1→, dois sinais foram identificados: em δ5,99/99,7, correspondente às unidades não-esterificadas, e em δ4,96/100,1, atribuído às unidades metil-esterificadas e detectado em maior intensidade, evidenciando o alto grau de metil-esterificação. O intenso sinal detectado em δ3,81/52,9, relacionado ao grupo $\underline{CH_3}O$- ligado à carboxila, e o sinal detectado em δ170,5 no espectro de ^{13}C (inserção na Fig. 4), relacionado ao C-6 das unidades metil-esterificadas, proporcionalmente bem mais intenso que o sinal em δ172,6, que resulta das unidades não-esterificadas, também corroboram o alto DM da HWSP-Cu. Os sinais relacionados ao C-2, C-3, C-4 e C-5 das unidades de →4)α-D-(6Me)GalA(1→ foram identificados, respectivamente, em δ3,74/68,1, δ4,00/68,2, δ4,46/78,6 e δ5,04/70,6. Embora a HWSP-Cu tenha apresentado baixo grau de acetilação, de 0,3% (Tabela 1), a presença de grupos acetil foi evidenciada pelo sinal detectado em δ2,08/20,0. De acordo com Renard e Jarvis (1999), este sinal estaria correlacionado à acetilação na posição C-3 das unidades de GalA. Ainda que a proporção de arabinose e de galactose presentes na HWSP-Cu tenham sido baixas (2,6% para ambos os monossacarídeos, Tabela 1), foram detectados sinais de arabinanas e galactanas. Em

δ4,61/104,5 e em δ4,13/81,3, foram identificados os sinais de C-1 e C-5 das unidades de →4)β-D-Gal(1→. Já em δ5,08/107,6 e em δ3,80/61,1 foram encontrados sinais correspondentes a unidades de →5)α-L-Ara(1→, respectivamente. Apenas o sinal anomérico dos pontos de ramificação das arabinanas, →3,5)α-L-Ara(1→, foi observado em δ5,15/107,4.

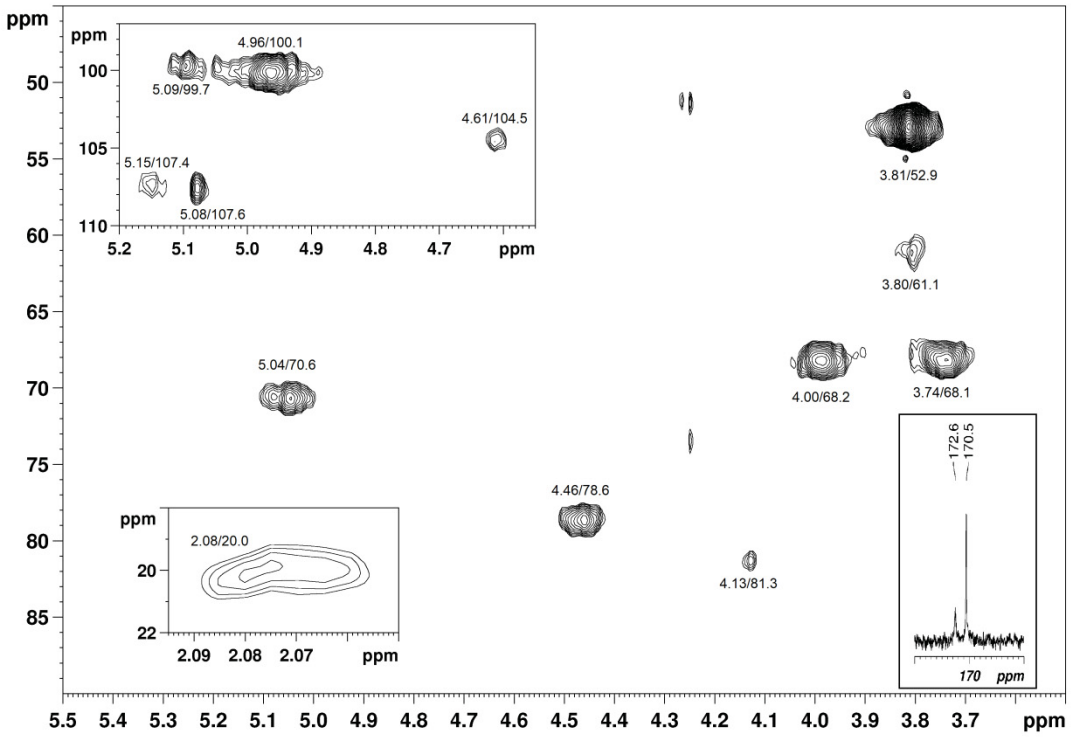

Fig. 4 – Mapa de correlação de $^{1}H/^{13}C$-HSQC RMN da sub-fração HWSP-Cu. FONTE: A AUTORA.

Os resultados da caracterização estrutural da HWSP-Cu e a caracterização estrutural da fração α-WSP, já descrita anteriormente (COLODEL, VRIESMANN, PETKOWICZ, 2018) demonstraram que, embora as duas sub-frações tenham sido obtidas de frações aquosas e que ambas as frações brutas extraídas da casca da ponkan com água fria (WSP) e com água fervente (HWSP) tenham apresentado composição monossacarídica e DM médio muito semelhantes (COLODEL, VRIESMANN, PETKOWICZ, 2018), elas são compostas por famílias de polissacarídeos pécticos muito distintos entre si, como mostra a Tabela 1.

Considerando as diferenças estruturais encontradas nas sub-frações purificadas α-WSP e HWSP-Cu, o fato de que pectinas são polissacarídeos com propriedades imunomodulatórios e que o efeito das pectinas no sistema imune está intimamente ligado à sua estrutura (POPOV, OVODOV, 2013; FERREIRA et al., 2015; ZHANG et al., 2016), ambas foram testadas quanto à sua citotoxicidade e sua capacidade de estimular a produção de NO por macrófagos.

Como pode ser observado na Fig. 5, nenhuma das amostras apresentou citotoxicidade nas concentrações testadas (100 – 500 μg/mL). Quanto à capacidade de indução da produção de NO por macrófagos, na concentração de 250 μg/mL a sub-fração α-WSP, que contém alta proporção de RG-I ramificadas por cadeias laterais de galactanas, arabinanas e arabinogalactanas, aumentou a produção de NO em 272% em relação ao controle. Nas concentrações testadas, α-WSP apresentou um efeito 31% a 43% superior ao LPS (controle positivo). Contrariamente, a sub-fração HWSP-Cu, rica em HG, não foi capaz de estimular os macrófagos a produzirem NO, apresentando resposta igual ao controle. Estes resultados estão de acordo com as conclusões apresentadas por Popov e Ovodov (2013), sugerindo que as pectinas mais ramificadas, tais como apiogalacturonanas, xilogalacturonanas e RG-I têm efeito imunoestimulador.

A α-WSP apresentou melhor desempenho na estimulação da produção de NO por macrófagos comparada a frações pécticas ricas em RG-I bastante ramificadas isoladas de outras fontes, tais como os frutos de goji berry preto, que resultou em um aumento de cerca de 120% na produção de NO por macrófagos tratados com a pectina em concentração de 200 μg/mL (PENG et al., 2014); a raíz de ginseng, de onde foi isolada uma RG-I altamente ramificada (5,3% GalA, 4,1% Rha, 40,9% Ara e 44,4% Gal) que promoveu um aumento de até 78,2% na produção de NO por macrófagos na concentração de 200 μg/mL (ZHANG et al., 2012); a casca de *Parkia biglobosa*, de onde foram isoladas quatro frações ricas em RG-I que foram usadas no tratamento de macrófagos em concentrações de 1, 10 e 100 μg/mL. Três das quatro amostras apresentaram efeito imunoestimulatório na concentração de 100 μg/mL, com aumento na produção de NO entre 100% e 170%, aproximadamente, em relação ao controle (ZOU et al., 2014). O efeito estimulatório de produção de NO da α-WSP foi similar ao de uma RG-I (10% GalA, 10% Rha, 50% Ara e 30% Gal) obtida das flores de oleandra, que promoveu um aumento de 245% na produção de NO por macrófagos na concentração de 500 μg/mL (DONG et al., 2010).

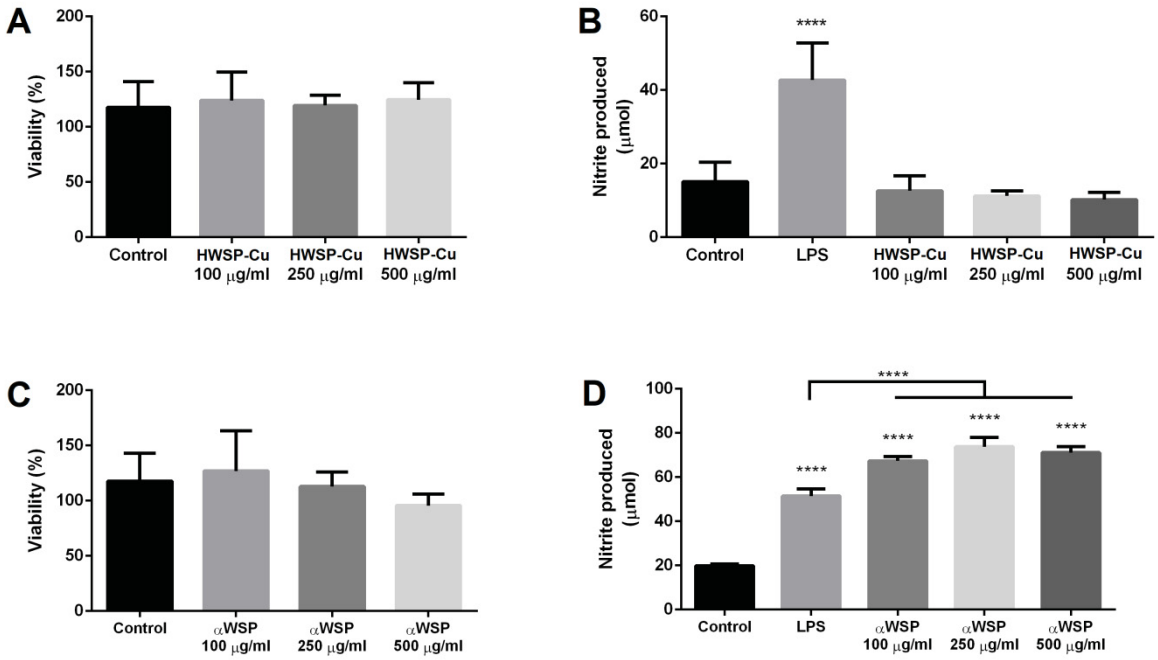

Fig. 5 – Viabilidade celular (A e C) e produção de NO (B e D) pelos macrófagos tratados com diferentes concentrações de HWSP-Cu e de α-WSP.

CONCLUSÕES

O uso da precipitação seletiva usando $CuSO_4$ permitiu o isolamento de uma homogalacturonana altamente metil-esterificada e de alta massa molar, denominada HWSP-Cu, a partir da fração extraída da casca de ponkan usando água fervente. Quando testadas quanto à capacidade de estimulação da produção de NO por macrófagos, a HWSP-Cu, rica em HG, não induziu a produção de NO enquanto a fração α-WSP, previamente isolada da fração extraída usando água fria e que apresentou estrutura rica em RG-I, induziu a produção de NO, sugerindo efeito imunomodulador.

CAPÍTULO 3

Artigo: “Extraction of pectin from ponkan (*Citrus reticulata* Blanco *cv. Ponkan*) peel: Optimization and structural characterization”

Extraction of pectin from ponkan (*Citrus reticulata* Blanco *cv. Ponkan*) peel: optimization and structural characterization

Cristiane Colodel [a], Lúcia Cristina Vriesmann [a], Reinaldo Francisco Teófilo [b], Carmen Lúcia de Oliveira Petkowicz [a*].

[a] Federal University of Parana, Department of Biochemistry and Molecular Biology, PO Box 19046, 81531-980 Curitiba, Parana, Brazil.

[b] Federal University of Viçosa, Department of Chemistry, 36570-000 Viçosa, Minas Gerais, Brazil

Abstract

A central composite experimental design was used to evaluate the influence of pH, extraction time and liquid:solid ratio on the yield and uronic acid content of the pectin from ponkan peel. The response surface methodology showed that the yield is positively influenced by lower pHs, longer extraction times and higher liquid:solid ratio, whereas the uronic acid content decreases with increasing extraction time. The conditions that resulted in the highest yield and highest uronic acid content were defined as pH 1.6, extraction time of 100 min and liquid:solid ratio of 36 mL/g. The pectin obtained under these conditions (PPOP) had an experimental yield of 25.6%, below the predicted theoretical value despite the good fit of the model ($R^2 = 0.96$) and the galacturonic acid content was 84.5%, in close agreement with the predicted theoretical value. PPOP was composed mainly of a homogalacturonan with degree of methyl esterification of 85.7% and a rhamnogalacturonan I region mainly branched by galactans. In addition, PPOP had a very low degree of acetylation (0.1%) and average molar mass of 80,650 g/mol, determined by light scattering. The results showed that ponkan peel may be used as a source of citrus pectin in the regions where this species is cultivated.

Keywords: pectin; response surface methodology; structural characterization

1. Introduction

Pectin is a widely used food additive (INS 440), being used as stabilizer, emulsifier, thickener and mainly as gelling agent [1]. Its properties are derived from its chemical structure, which consists of a backbone of D-galacturonic acid residues α(1→4) linked. The

α-D-galacturonic acid units can be interspersed by (1→2) α-L-rhamnose which in turn may be substituted with side chains of arabinans, galactans and arabinogalactans giving rise to rhamnogalacturonan I; or the polygalacturonic acid chain may be decorated with more complex side chains, being known as rhamnogalacturonan II [1,2,3]. Nevertheless, only pectins having 65% or more of galacturonic acid meet the regulations for commercial purposes [1].

The most important structural feature that affects the gelling ability of pectins is the methyl esterification of carboxylic group in the C-6 of the galacturonic acid units. Depending on the degree of methyl-esterification, pectins form gel under different conditions, which determine their applications. Pectins with more than 50% of methyl esterified galacturonic acid units are classified as "high methoxyl" and form gel in acidic medium in the presence of high soluble solids, generally sucrose, being used mainly for production of jellies and jams. On the other hand, pectins with less than 50% of methyl esterified galacturonic acid units are classified as "low methoxyl" and form gel in the presence of Ca^{2+}, being suitable for application mainly in dairy products [1]. Acetyl groups at *O*-2 and/or *O*-3 also interfer on gelling ability of pectins and degree of acetylation higher than 5% is considered critical for gelation [4], although there is evidence in the literature that pectins with degree of acetylation >5% can form gels under specific conditions [5,6].

Commercial pectins are produced from apple pomace and citrus peel by extraction using hydrochloric, nitric or sulfuric acid at pH 1.0-3.0 at high temperature and extraction times of 1 to 12h, depending on the desired properties of the pectin [7,8]. Although both raw materials produce chemically equivalent pectins, citrus contains twice pectin than apple (20-30% and 10-15%, respectively) [9], and it is preferable for application in clear products once apple pectins are often darker due to the presence of phenolic compounds [10,11].

Despite the identity of the citrus species used for extraction of commercial "citrus pectin" is not usually informed, the most common citrus used are orange, lime, grapefruit and lemon [12]. In Brazil and Mexico, citrus pectin is obtained from orange and lime peels [13].

Tangerines also belong to citrus group, but they are not commercially exploited for pectin extraction. In fact, there is a lack of studies about pectins from tangerines and most of them are only quantitative. Pectin contents of 3.69% [14] and 3.73% [15] on dry basis were reported for tangerine peel. Liu *et al*. [16] found 5.29% of pectin on wet basis for Dancy tangerine. Chen *et al.* [17] investigated the extraction conditions to achieve the highest yield of pectin from tangerine peel. The optimal conditions were: microwave power 704 W,

temperature 52.2°C, and extraction time 41.8 min. Under these conditions, the yield of pectin was 19.9% based on alcohol-insoluble residue.

Ponkan (*Citrus reticulata* Blanco *cv. Ponkan)* is one of the most important tangerine cultivars, being China and Brazil the major producers [18]. It is a sweet and easy to peel fruit [18] mainly consumed freshly but also processed as juice [19].

The use of the ponkan peel for pectin extraction may represent an additional source of citrus pectin in the regions where this species is cultivated, as well as add value to the waste resulting of the industrial processing or of the consuming of the fruit. Recently, Colodel, Vriesmann and Petkowicz [20] investigated the cell wall polysaccharides from ponkan peel employing sequential extractions using various solvents and found 25.6% of pectins besides 3.6% of hemicelluloses. Considering the high content of pectin, the present study investigated the optimization of extraction of pectin from ponkan peel using response surface methodology, aiming simultaneously the highest yield and the highest uronic acid content.

2. Materials and methods

2.1. Plant material

Fresh ripe fruits were collected in Ponta Grossa, Parana, in Southern Brazil (25°04'18.7"S; 50°07'44.3"W) in July, 2015. The fruits were washed, peeled and the peel was freeze-dried and ground into powder using an analytical mill IKA A-11 basic (Staufen, Germany). This material was defatted in a Soxhlet apparatus using chloroform/methanol at a ratio of 2:1 (v/v) to give rise to the ponkan peel powder which was used for pectin extraction.

2.2. Experimental design

Response surface methodology was used to determine the optimum condition for pectin extraction from ponkan peel powder. A central composite design 2^3 was used to determine the effect of three extraction variables – pH, extraction time and liquid:solid ratio – on yield and uronic acid content of pectin, chosen as responses. The central composite design consisted of 19 experiments including: eight factorial experiments (+1, -1 levels), six axial experiments (+α, -α levels), and five replicates of center point (0 levels) to make the estimation of possible pure error, using the independent variables as described in Table 1. All experiments were carried randomly to minimize the effect of possible systematic errors.

Table 1 – Independent variables and their levels used in central composite design 2^3 for pectin extraction from ponkan peel.

Factor	Levels				
	-α	*1*	*0*	*1*	*+α*
pH	1.66	2	2.5	3	3.34
Extraction time (Et, min)	10.77	36	73	10	135.23
Liquid-solid ratio (LS, mL/g)	9.89	15	22.5	30	35.11

The regression coefficients for the linear, quadratic and interaction terms were determined using multiple linear regression analysis. The significance of each effect and the regression coefficient was judged statistically by computing the t-value from associated errors. The regression coefficients were then used to generate the response surfaces, and the model was validated using the plot of observed vs. predicted values and the plot of observed vs. raw residuals [21]. All calculations and graphics in this work were performed using Statistica 13.0 software. A difference was considered statistically significant when $p < 0.065$.

2.3. Pectin extraction

Pectin was extracted from ponkan peel powder using boiling aqueous HNO_3 at different conditions in a heating mantle under a reflux system according to the experimental design (Table 1). After centrifugation (4695.6 × *g*, 20 min, 4°C), the supernatant was filtered on a synthetic fabric, precipitated with absolute ethanol 1:2 (v/v) and then left to rest at 4°C overnight. The precipitate was recovered by vacuum filtration, rinsed with absolute ethanol and dried under vacuum.

2.4. Yield and uronic acid content

The pectin yield was determined as follows [22]:

$$yield\ (\%) = \frac{mass\ of\ dried\ pectin\ (g)}{mass\ of\ ponkan\ peel\ powder\ used\ for\ extraction\ (g)} \times 100$$

The uronic acid content was measured by the *m*-hydroxybiphenyl method [23], using galacturonic acid (5-100 μg/mL) as standard.

2.5. Obtention and characterization of the ponkan peel optimized pectin (PPOP)

Once the optimal extraction conditions were identified in which the highest yield and the highest uronic acid content were reached, extractions were performed to obtain a higher amount of pectin (PPOP) for chemical characterization.

Neutral monosaccharide composition was determined by gas-liquid chromatography after hydrolysis and derivatization. PPOP was hydrolyzed using 2 mol/L trifluoroacetic acid (8 h, 100 °C) and evaporated to dryness. The monosaccharides were reduced with $NaBH_4$ (16 h, 4°C) [24] and then acetylated with pyridine-acetic anhydride (1:1 v/v, 16 h, 25°C) [25]. The resulting alditol acetates were extracted with $CHCl_3$ and analyzed using a 5890 S II Hewlett–Packard gas chromatograph at 220°C (flame ionization detector and injector temperature, 250°C) with a DB-210 capillary column (0.25 mm internal diameter × 30 m), film thickness 0.25 μm, using nitrogen as the carrier gas (2.0 mL/min).

Uronic acid content was measured as described in section 2.4 and the identity of uronic acid was determined by anion exchange chromatography with pulse amperometric detection. After hydrolysis with 2 mol/L trifluoroacetic acid (8 h, 100°C) and acid removal, the sample (1 mg/mL) was filtered through a membrane of 0.22 μm and analyzed in a Thermo Scientific Dionex ICS-5000 chromatograph (Thermo Fisher Scientific, USA) with CarboPac PA20 column (3 ×150 mm) using gradient of 1 mol/L NaOH and 1 mol/L NaOAc as eluent in N_2 atmosphere in a flow of 0.2 mL/min at 30 °C [26]. Data were collected and analyzed using the Chromeleon™ 7.2 Chromatography Data System software.

The molar mass distribution of the pectin was investigated by high pressure size exclusion chromatography. The sample (1.0 mg/mL) was solubilized in 0.1 mol/L $NaNO_2$ containing 0.5 g/L NaN_3, filtered (Millipore, Canada, 0.22 μm) and analyzed using a Waters unit coupled to a refractive index, a Wyatt Technology Dawn-F multi-angle laser light scattering detector. Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled to the multidetection instrument. A solution of 0.1 mol/L $NaNO_2$ and 0.5 g/L NaN_3 was used as eluent at a flow of 0.6 mL/min.

The degree of methyl esterification was obtained by ^1H NMR [27]. For ^1H and HSQC NMR analysis, 20 mg of the pectin was dissolved in D_2O (10 mg/mL) and analyzed in a Bruker Avance DRX400 spectrometer (Bruker, Germany) at 70°C. Chemical shifts were expressed in δ (ppm), using acetone (δ 30.2/2.22) as reference.

Colorimetric methods were used to determine the content of acetyl [28], using erythritol acetate as standard; phenolics [29], expressed as gallic acid equivalents; and protein [30], using bovine serum albumin as standard. The degree of acetylation was calculated as described by Colodel, Bagatin and Petkowicz [31].

3. Results and discussion

3.1. Preparation of the plant material

The average mass of ponkan fruits was 212 g, greater than 178g previously reported [32]. The percentage of peel relative to the fresh fruit weight was 35.6%, which represents a great amount of waste and a plentiful source to obtain pectins. The value is higher than those described for other citrus fruits such as Lima orange (16.7%), Pera orange (27.8%), Tahiti lime (19.3%) and Sweet lime (18.2%) [32].

The moisture of the ponkan peel was 77.1%, calculated as the difference between the mass of the fresh and lyophilized material. It may be considered low compared to the peel of other fruits previously investigated for pectin extraction, such as passion fruit (81.5%) [33] and cacao (~90%) [34], resulting in a higher proportion of raw material to obtain pectins.

The $CHCl_3$/MeOH soluble material was found to be 26.3% on dry basis. Citrus species contain nonpolar components of industrial interest, such as essential oil, flavonoids, polyphenols and carotenoids [35]. They can be extracted prior to the use of raw material for pectin extraction. Thus, the ponkan peel can be a source of several compounds of high commercial value.

3.2. Central Composite Design

A three variables central composite design was employed to determine the optimum condition of pH, extraction time and liquid-solid ratio for extraction of pectin from ponkan peel to obtain maximum yield and maximum content of uronic acid. These characteristics are desirable for industrial applications. Experimental values obtained for yield and uronic acid content at each designed point are shown in Table 2, and the regression coefficients for yield and uronic acid content, obtained from the experimental results, are given in Table 3.

Table 2 – Decoded and coded levels of variables and responses for central composite design for pectin extractions from ponkan peel.

Assay	pH	Et (min)	LS (mL/g)	Yield (%)	Uronic acid (%)
5	2 (-)	36 (-)	30 (+)	22.0	53.4
15	2.5 (0)	73 (0)	22.5 (0)	14.6	52.3
7	2 (-)	110 (+)	30 (+)	25.8	60.8
3	2 (-)	110 (+)	15 (-)	14.6	47.2
18	2.5 (0)	73 (0)	22.5 (0)	14.8	55.8
11	2.5 (0)	10.77 (-α)	22.5 (0)	7.6	56.9
6	3 (+)	36 (-)	30 (+)	10.6	52.3
4	3 (+)	110 (+)	15 (-)	19.5	51.1
8	3 (+)	110 (+)	30 (+)	17.2	55.7
17	2.5 (0)	73 (0)	22.5 (0)	15.4	55.5
10	3.34 (+α)	73 (0)	22.5 (0)	18.6	67.4
14	2.5 (0)	73 (0)	35.11 (+α)	14.0	61.8
12	2.5 (0)	135.23 (+α)	22.5 (0)	18.3	44.8
1	2 (-)	36 (-)	15 (-)	9.4	62.6
19	2.5 (0)	73 (0)	22.5 (0)	15.1	51.7
13	2.5 (0)	73 (0)	9.89 (-α)	11.7	55.4
2	3 (+)	36 (-)	15 (-)	15.3	57.2
9	1.66 (-α)	73 (0)	22.5 (0)	25.2	70.9
16	2.5 (0)	73 (0)	22.5 (0)	15.4	51.8

Et = Extraction time; LS = liquid:solid ratio.

Table 3 - Coefficient estimates for central composite design and statistical parameters for models of pectin yield and uronic acid content for pectins extracted from ponkan peel.

	Yield				Uronic acid			
	Coeff.	Std.Err.	t(9)	p	Coeff.	Std.Err.	t(9)	p
Mean	***15.04***	0.57	26.27	8.11×10^{-10}	***53.60***	1.74	30.73	2.0×10^{-10}
pH	***-1.50***	0.35	-4.31	0.002	-0.98	1.06	-0.93	0.377
pH^2	***2.60***	0.35	7.48	3.80×10^{-5}	***4.54***	1.06	4.29	0.002
Et	**2.76**	0.35	7.96	2.30×10^{-5}	***-2.27***	1.06	-2.15	0.060
Et^2	-0.56	0.35	-1.60	0.143	-1.94	1.06	-1.83	0.100
LS	***1.51***	0.35	4.36	0.002	1.08	1.06	1.02	0.335
LS^2	-0.61	0.35	-1.76	0.112	0.80	1.06	0.75	0.471
pH × Et	0.22	0.45	0.47	0.647	0.66	1.38	0.48	0.644
pH × LS	***-3.87***	0.45	-8.53	1.30×10^{-5}	-0.58	1.38	-0.42	0.684
Et × LS	0.13	0.45	0.29	0.781	***4.03***	1.38	2.92	0.017

Values in bold and italics are significant at $\alpha = 0.065$ with nine degrees of freedom.
Et = Extraction time; LS = liquid:solid ratio.

3.2.1. Effect of the variables on the yield of pectins

The yield of pectin ranged from 7.6% to 25.8%, higher to those obtained for pectins from orange peel using a factorial experiment 2^4 evaluating the influence of pH, temperature, time and reduction of the extract volume before precipitation of pectin with ethanol [36]. Similar yields were described for pectins from peel of three *Citrus* species (*C. sinensis, C. limetta* and *C. limon*) using different conditions of pH and temperature [37] and from *Citrus medica* peel using a Box-Behnken design where the effect of the temperature, time and liquid:solid ratio was investigated [22].

As can be seen in Table 3, the yield was affected by the three variables, especially by the linear effect. The yield was increased within the studied levels with the decrease of the pH. At lower pHs, insoluble pectin constituents are more prone to undergo hydrolysis into soluble pectin, thus increasing the pectin recovery from the plant material [38]. Many authors have described pH as one of the most significant variables that influence the yield of pectin extraction, commonly obtaining higher yields at lower pH values [22, 38, 39, 40, 41].

It was observed that increase of the extraction time (Et) resulted in increase in the yield. Longer contact times between the extractor and the plant material provide greater mass transfer from the solid particles into the solution [22]. For the extraction of pectin from lemon

by-products, an increase in the yield was also observed as a result of the decrease in pH and increase in extraction time [42]. A similar trend was observed for pectins from sugar beet pulp [43].

The increase of the liquid:solid (LS) ratio was directly proportional to the yield. A higher liquid:solid ratio favors the yield by increasing the contact surface of the plant material with the solvent. In addition, it enhances the swelling of the material, leading to cell disruption and thereby facilitates the solubilization of pectin [22, 44].

It was also observed an interaction between pH and liquid:solid ratio. This interaction indicates that when pH decreases and liquid:solid ratio increases simultaneously within the levels studied, the yield increases.

3.2.2. Effect of the variables on the uronic acid content of pectins

The uronic acid content varied between 44.8% and 70.9%. Saberian, Hamidi-Esfahani, Gavlighi, and Barzegar [45] reported slightly higher and less variable values for the uronic acid content (66.74 - 79.58%) for pectins from orange juice waste. The authors used ohmic heating process and carried out a central composite design in which the effects of voltage gradient, temperature and time of extraction on yield, uronic acid content and degree of esterification of pectins were investigated.

For the uronic acid content, the significant variables were the extraction time and the interaction extraction time x liquid:solid ratio (Et×LS). Unlike the yield, when the extraction time was increased, within the studied levels, the uronic acid content decreased. However, the interaction extraction time x liquid:solid ratio indicated that the simultaneous increase in the extraction time and the liquid:solid ratio, within the studied levels, increased the uronic acid content. Different conclusions have been depicted for uronic acid content as a function of extraction conditions depending on the raw material and the experimental protocol. Vriesmann, Teófilo, & Petkowicz [46, 47] observed that the uronic acid content of pectins extracted from cacao pod husk using both nitric acid and citric acid was not significantly (linearly or quadratically) affected by any of the studied variables (pH and extraction time). On the other hand, the uronic acid content of pectins extracted from banana peel was reported to be significantly affected by all the variables studied (temperature, extraction time and pH) [40]. However, in the study carried out by Emaga *et al*. [48], only pH had a significant influence and it was inversely related to the uronic acid content of the pectins. Liu, Cao, Huang, Cai, & Yao [49] performed a single-factor design in order to optimize the quality of

pectins extracted from mulberry branch bark and observed that lower pH decreased the uronic acid content whereas longer extraction times and higher liquid:solid ratios increased it.

3.2.3. Optimization of the extraction conditions

From the results observed in Table 3, the response surfaces (Fig. 1) were built applying the models.

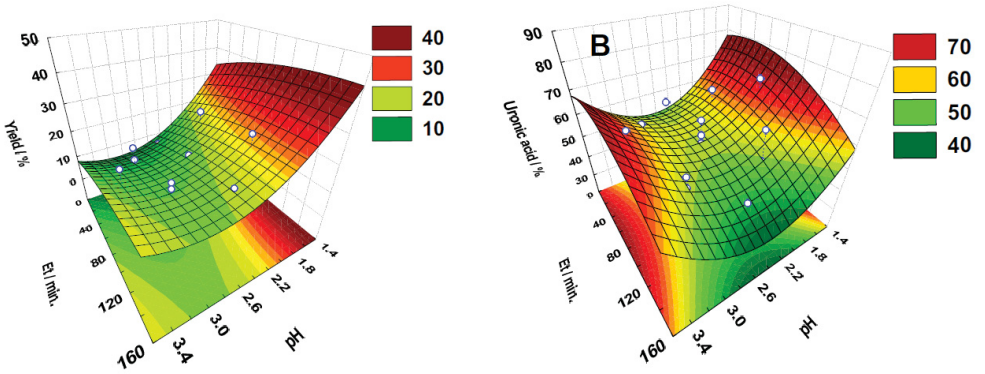

Fig. 1 – Response surfaces showing the effect of the variables on the yield (A) and uronic acid content (B) for pectins extracted from ponkan peel. The level of liquid:solid ratio was fixed in central point. Et = Extraction time.

The results of observed versus predicted values for both responses are depicted in Fig. 2 and show that built models were fitted and had a good prediction capacity. The values of determination coefficient (R^2) for uronic acid and yield were 0.82 and 0.96, respectively.

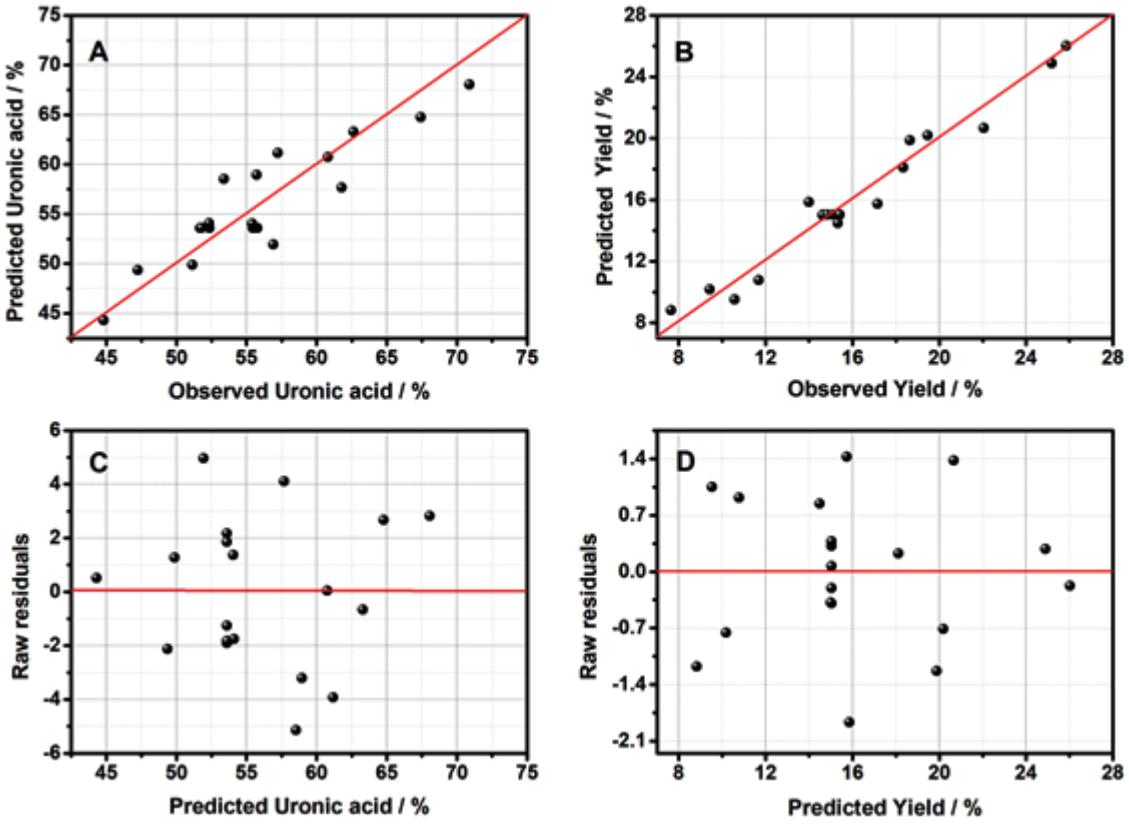

Fig. 2 – Observed versus predicted values for uronic acid (A) and yield (B) and predicted versus residues values for uronic acid (C) and yield (D) for pectins extracted from ponkan peel.

Simultaneous analysis of the responses using the desirability function of Derringer and Suich [50] were applied to obtain the highest yield and the highest uronic acid content. The results indicated that the variables levels to obtain the optimized pectin with the desired characteristics were pH 1.6, extraction time of 100 minutes and liquid:solid ratio of 36 mL/g. These conditions were satisfactory since more extreme conditions become unsuitable because they can lead to the undesirable degradation or modification of the pectin. According to the models, the theoretical yield and uronic acid content for the pectin extracted at these conditions were 41.0% and 78.2%, respectively.

3.3. Characterization of ponkan peel optimized pectin (PPOP)

The pectin obtained under the optimized conditions (pH 1.6, 100 min, 36 mL/g) was named PPOP and it was characterized.

The experimental yield was 25.6%, much lower than the value predicted by the mathematical model (41.0%). On the other hand, the experimental uronic acid content was

84.5%, slightly higher than the theoretical value, 78.2%. The considerable difference between the theoretical and the experimental yield may be due to the fact that low pH together with long extraction times and high temperatures can result in degradation of the pectin, which may not have been predicted by the model.

It has been reported that pH<2 can lead to breakdown of glycosidic linkages between acidic and neutral sugars, causing loss of branched regions [51], and, as consequence, the polysaccharide yield is diminished. At the same time, the cleavage of neutral sugars rises the proportion of galacturonic acid [7], as observed in the present study. Another limiting factor for the yield is the actual pectin content in the ponkan peel, which cannot be predicted by the mathematical model and may be lower than the theoretically predicted, considering that the pectin content of citrus peels ranges from 20 to 30% on dry basis [9, 52]. Interestingly, the yield obtained with the acid extraction under the optimized conditions was the same as the total yield of the pectic fractions obtained by sequential extraction with different solvents [20]. However, all pectins obtained by sequential extractions presented lower content of uronic acid (21.0 – 57.3%) and higher content of neutral sugars than PPOP. The structural characteristics of PPOP, compared to the pectins obtained by sequential extraction, suggest that although the yield was the same, the acid extraction was more efficient. The high degree of methyl esterification suggests that the pectin obtained might be originated from protopectins early deposited on the primary cell wall [53].

Although the yield obtained was lower than expected, it was higher than that reported by Chen *et al.* [17] using microwave-assisted extraction evaluating the effect of potency, temperature and extraction time on the yield of tangerine peel pectin using a Box-Behnken Design (19.9%). Methacanon, Kronsing and Gamonpilas [54] used central composite design for the optimization of pectin extraction from pomelo testing the effect of temperature, time and calcium concentration and found a yield of 23.2% at the optimal conditions. However, in a preliminary experiment using HNO_3 at pH 2 at 85°C for 120 min they found a yield of 24.6% and galacturonic acid content of 86.3%, which are very similar to the values obtained in the present study for ponkan peel. Hosseini, Khodaiyan and Yarmand [38], employed a Box-Behnken design for the optimization of pectin extraction from sour orange peel. They studied the effect of irradiation time, microwave power and pH on the yield and degree of esterification of pectin. They obtained a yield of 29.1% under optimized conditions, slightly higher than that obtained in the present work, and 71.0% of uronic acid, lower than PPOP. Others citrus species, such as *Citrus depressa* and *Citrus tankan*, had pectins with uronic acid content 79.2% and 80.0%, respectively [55, 56]. These pectins were

extracted using HCl at pH 1.3, which is close to the pH used for PPOP extraction and had yields similar to that obtained in the present study. Compared to optimized pectins from other fruit by-products, the ponkan peel provided higher yield and uronic acid content than banana peel (14.23% and 75.91%, respectively) [40] and cacao pod husk (9.0% and 66.0%, respectively) [46].

The presence of galactose, rhamnose and arabinose in PPOP (Table 4) suggests the occurence of rhamnogalacturonan I (RG-I) regions. It had 6.1% of protein and although citrus peel is rich in phenolic compounds [57, 58], low amount of phenolics was found (0.9%).

Table 4 – Chemical composition of PPOP.

Monosaccharide composition [a] *(%)*	
Rha	2.8
Ara	1.4
Xyl	0.4
Man	0.4
Gal	9.2
Glc	2.0
GalA	84.5
DM [b] (%)	85.7
Acetyl content [c] / DA [d] (%)	2.1 / 0.1
Protein [c] (%)	6.1
Phenolics [c] (%)	0.9

[a] Neutral monosaccharides were determined by GLC after hydrolysis and derivatization; uronic acid was estimated by colorimetric method and identified by anion exchange chromatography with pulse amperometric detection.
[b] DM = degree of methyl-esterification. Determined by ^{1}H NMR.
[c] Determined by colorimetric methods.
[d] DA = degree of acetylation. Calculated as described by Colodel, Bagatin and Petkowicz [27].

The degree of methyl-esterification was determined by ^{1}H NMR (Fig. 3) and it was found to be 85.7% (Table 4), which characterizes PPOP as a high methoxyl pectin. High methoxyl pectins are widely used in the food industry as gelling agents in the preparation of acidic products containing high solids content such as jams and jellies [1] and as stabilizers in dairy products, avoiding casein aggregation and precipitation at low pH [59].

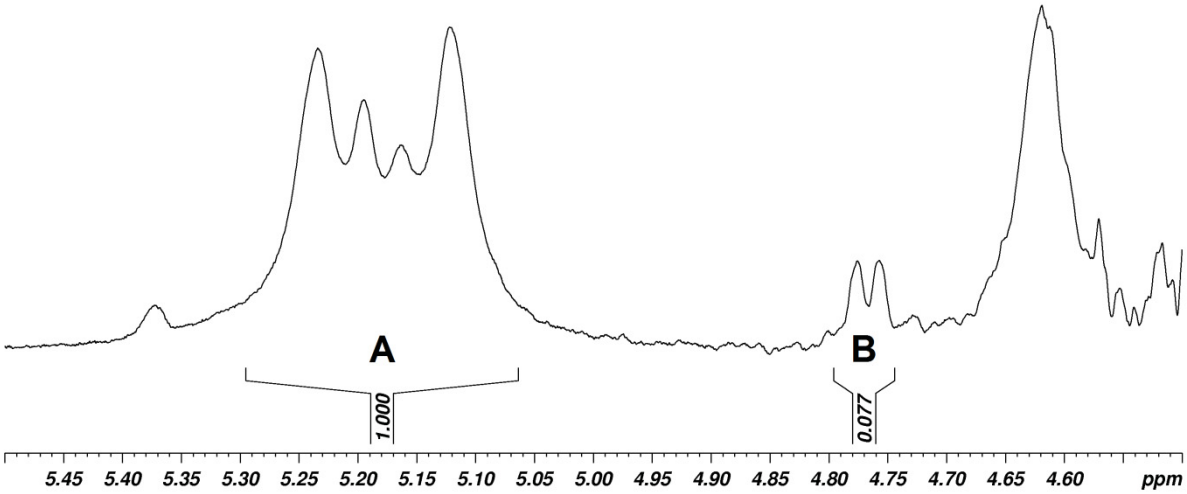

Fig. 3 – ^{1}H NMR spectrum of PPOP, showing the region A, which denotes signals from H-1 and H-5 of esterified galacturonic acid units, and region B, where appear the signals of H-5 of non-esterified galacturonic acid units. The degree of methyl esterification (DM) is given by the ratio between the areas, DM=(∫A-∫B)/(∫A+∫B), as described by Grasdalen, Bakøy and Larsen [23].

The degree of acetylation was low (0.1%, Table 4), a desirable feature since degrees of acetylation above 4% usually hinder the gelation of pectins [4].

The elution profile of PPOP by high pressure size exclusion chromatography is depicted in Fig. 4. A main peak eluting at ~46 min was detected by refractive index with high intensity. The simultaneous detection by light scattering enabled the calculation of the average molar mass of PPOP as 80,650 g/mol. The value is close to that reported for acid-extracted pectins from orange peel after microwave treatment (82,000 – 87,000 g/mol) [60] and for citric acid-extracted pectin from orange pomace (83,486 g/mol) [61].

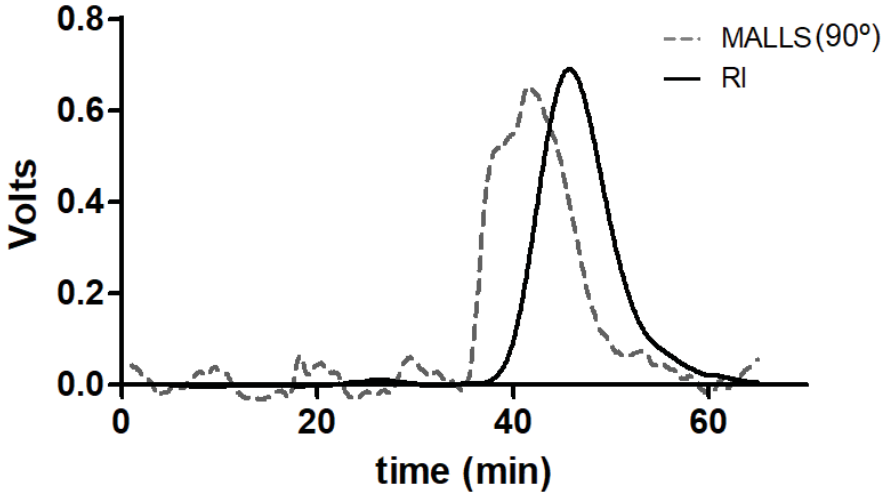

Fig. 4 – Elution profile of PPOP by high pressure size exclusion chromatography using refractive index (RI) and multi-angle laser light scattering (MALLS; 90° is shown) detectors.

The HSQC NMR spectrum (Fig. 5) revealed correlations assigned to the anomeric carbon of α-D-6Me-GalA*p*(1→4) at δ100.1/4.97 and of α-D-GalA*p*(1→4) at δ99.8/5.09. C-2 and C-4 of the units of α-D-(6Me)-GalA*p*(1→4) was detected at δ68.0/3.76 and δ78.3/4.47, respectively and the C-3 was detected at δ68.6/3.93 and that from non-esterified was identified at δ68.1/4.05. The signal of C-5 from esterified units of GalA*p*(1→4) was found at δ70.3/5.02 [62,63], whereas no signal of C-5 from non-esterified α-D-GalA*p*(1→4) was observed, which is probably due to the high degree of methyl esterification of PPOP. The signal of $\underline{C}H_3$-C6 appeared at δ52.9/3.81 [62]. Although PPOP had a fairly low DA, signals at δ20.2/2.09 and δ19.9/2.07 indicated acetylation in *O*-3 and both *O*-2/*O*-3 of some units of GalA*p* [64,65]. In addition to the typical signals of homogalacturonan, signals at δ16.3/1.26 and at δ16.6/1.32 were found and assigned to α-L-Rha*p*(1→2) and substituted α-L-Rha*p*(1→2,4) [62,66], another evidence of the presence of regions of rhamnogalacturonan I, in agreement with the monosaccharide composition (Table 4). According to Table 4, the side chains of rhamnogalacturonan I are composed predominantly of galactans, which is confirmed by the signals of β-D-Gal*p*(1→4) found at δ104.3/4.63 (C-1), δ74.5/3.70 (C-2), δ73.4/3.75 (C-3), δ73.3/3.78 (C-4) and δ60.8/3.81 (C-6) [62,66]. No Ara*f* signals were identified on HSQC spectrum, and only a minor amount of arabinose (1.4%) was present in the monosaccharide composition, as showed in Table 4. Nevertheless, the signals at δ104.4/4.61 (C-1), δ72.1/3.68 (C-2) and δ77.6/4.15 (C-4) evidenced the presence of β-D-Gal*p*(1→3,6) [31] suggesting the presence of type-II arabinogalactans in PPOP. It is possible that the native pectin contained higher arabinose content, but strongly acidic extraction

conditions may cause the hydrolysis of this monosaccharide, since arabinose is the most labile among the pectin sugars [59].

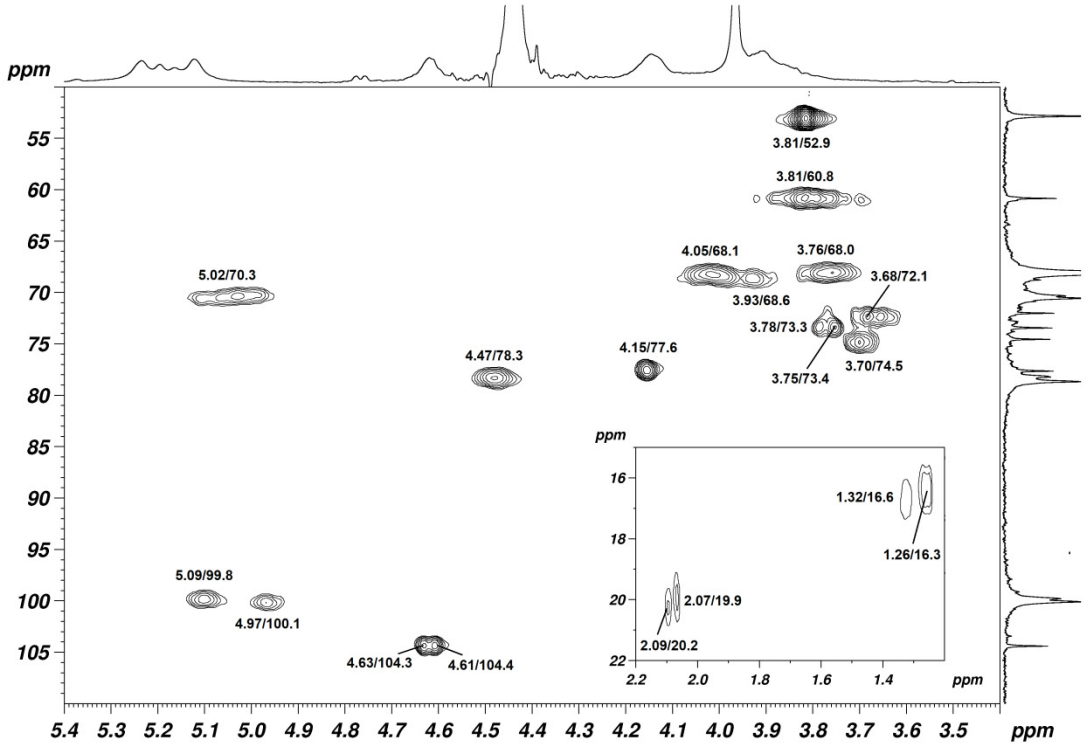

Fig. 5 – HSQC NMR spectrum of PPOP in D_2O at 70ºC. Acetone (δ30.2/2.22) was used as internal standard.

Conclusions

The highest yield of the pectin from ponkan peel was obtained with the decrease of the pH, increase of the extraction time and increase of the ratio LS, whereas the content of uronic acid diminished with greater times of extraction, within the levels studied. Applying the desirability function, the optimal condition to obtain the highest yield and highest uronic acid content simultaneously was defined as pH 1.6, extraction time of 100 minutes and liquid:solid ratio of 36 mL/g. The pectin obtained under these conditions (PPOP) had an experimental yield of 25.6% and the galacturonic acid content was 84.5%. PPOP had high degree of methyl-esterification, low degree of acetylation and average molar mass of 80,650 g/mol.

Acknowledgements

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CAPÍTULO 4

Artigo “Rheological characterization of a pectin extracted from ponkan (*Citrus reticulata* Blanco *cv. Ponkan)* peel”

Rheological characterization of a pectin extracted from Ponkan (*Citrus reticulata* Blanco *cv. Ponkan)* peel

Cristiane Colodel, Lucia Cristina Vriesmann, Carmen Lucia de Oliveira Petkowicz*.

Federal University of Parana, Department of Biochemistry and Molecular Biology, PO Box 19046, 81531-980 Curitiba, Parana, Brazil.

Abstract

The rheological properties of the optimized high-methoxyl pectin (PPOP), obtained from ponkan peel using nitric acid extraction (pH 1.6, 100 min, 36 mL/g liquid:solid ratio) were characterized. The flow behavior of aqueous and 0.1 M NaCl solutions at concentrations of 1%, 3% and 5% (w/w) was evaluated. The apparent viscosity of the solutions was directly related to the concentration. At the same concentrations and low shear rates the apparent viscosity values were close to those of a commercial citrus pectin. However, at higher shear rates, PPOP had a more shear thinning behavior. For both pectins, the presence of 0.1 M NaCl did not affect the apparent viscosity. The influence of pH, pectin and sucrose concentration on the gelling behavior of PPOP was investigated. Gelation occurred at pH <3.5 and the strongest gel was obtained with 2.5% (w/w) pectin and 60% (w/w) sucrose at pH 2.5. The gel prepared under these conditions had viscoelastic behavior comparable to the gel prepared with a high-methoxyl commercial citrus pectin under the same conditions and displayed high thermal stability.

Keywords: gel; heating-cooling cycle; HM pectin; homogalacturonan; rhamnogalacturonan I; rheology.

1. Introduction

Polysaccharides are widely used to improve the viscosity and texture of food preparations (Saha & Bhattacharya, 2010). Pectin is one of the polysaccharides used as a rheology modifier, displaying stabilizing and thickening properties, in addition to its main application as gelling agent due to its ability of trapping water and forming gels at low concentration (Chan, Choo, Young, & Loh, 2017; Einhorn-Stoll, 2018). The physicochemical

properties of pectins, which include their rheological behavior, are influenced by intrinsic and extrinsic factors. The intrinsic factors refer to its fine structure and molar mass. The extrinsic factors include the polymer concentration, temperature, pH, ionic strength and the presence of co-solutes and specific ions (Lopes da Silva, & Rao, 2006; Einhorn-Stoll, 2018).

Pectins are classified into three main groups, according to its chemical structure: homogalacturonanan (HG), composed of α-D-GalA(1→4) linear chains, which may contain the carboxylic group at the C-6 position in both forms, free or methyl esterified, and may also be acetylated at the *O*-2 position and/or *O*-3; rhamnogalacturonan I (RG-I), in which the backbone is formed by repeating units of →4)α-D-GalA(1→2)α-L-Rha(1→, and the rhamnosyl residues may be substituted at *O*-4 by arabinans, galactans and/or arabinogalactans; and rhamnogalacturonan II (RG-II), which has side chains formed by four complex oligosaccharides attached to a HG backbone (Mohnen, 2008).

Low-methoxyl pectins (LM), which have the degree of methyl-esterification (DM) lower than 50%, form gels in the presence of bivalent ions, due to the formation of junction zones through electrostatic interactions between the ions and the negative charges of the carboxyl groups of GalA. On the other hand, high-methoxyl pectins (HM, DM> 50%) form gels in acidic medium with reduced water activity by the junction and stabilization of the chain network by hydrophobic interactions between methyl groups and hydrogen bonds between the protonated carboxylates and hydroxyl groups (Chan et al., 2017). Thus, the homogalacturonan domain contributes the most to the gel formation. However, the side chains, depending on the size and structure, can also contribute to the formation and stabilization of the three-dimensional network leading to gelation (Hwang, Pyun & Kokini, 1993; Sousa, Nielsen, Armagan, Larsen & Sørensen, 2015).

The main sources of commercial pectins are citrus peel and apple pomace, depending on the availability and abundance of the raw material in the region where they are produced (Ciriminna et al., 2015). The citrus species which are used for pectin extraction are orange, lime, grapefruit and lemon (Yapo, 2009). Recently, we reported a study on the optimization of pectin extraction from ponkan (*Citrus reticulata* Blanco *cv. Ponkan*) peel (Colodel, Vriesmann, Teófilo & Petkowicz, 2018). Ponkan is a citrus species belonging to the group of mandarins, being the most cultivated mandarin in the world, mainly in Brazil, China and India (Kahn & Vidalakis, 2014). Ponkan peel is commonly discarded, but proved to be a good source of pectins, with yields and structural features comparable to commercial citrus pectins. The pectin obtained using the optimized conditions (HNO_3, pH 1.6, 100 min and liquid/solid ratio 36 mL/g), named PPOP, had yield of 25.6%, 84.5% GalA, DM 85.7% and Mw 80.650

g/mol. It was composed mainly of HG with a minor region of RG-I branched by galactans and type II arabinogalactans. In the present study the rheological behavior of PPOP was investigated in order to provide evidences that it could be used as gelling agent and to define the best conditions for gel formation.

2. Materials and methods

2.1 Pectin

PPOP was obtained as previously described (Colodel et al., 2018), and had 84.5% GalA, DM 85.7% and Mw 80,650 g/mol. Commercial citrus pectin (CCP, GENU® pectin type B rapid set-Z, not standardized with sucrose, DM = 71.4%) was kindly provided by CP Kelco (Brazil). The galacturonic acid content of CCP was quantified by a colorimetric method (Blumenkrantz & Asboe-Hansen, 1973) and its molar mass was determined by light scattering (Colodel et al., 2018).

2.2. Pectin solutions and gels

For preparation of the solutions, the pectins PPOP and CCP were dispersed in distilled water or 0.1 M NaCl at concentrations of 1%, 3% and 5% (w/w). The pH was maintained as per pectin solutions, close to 3.

The conditions for gel formation were tested by varying the pH (1.5-3.5), the pectin concentration (1.0-3.0% w/w) and the sucrose content (55-65% w/w). To prepare the gels, the pectins were solubilized in 5 mL of distilled water or 0.1 M NaCl, and the solution was then added to and appropriate amount of 80% sucrose, according to the desired final sucrose concentration. After homogenization for 30 min, the system was heated in a boiling water bath for 15 min, the pH was adjusted using 1 M HNO_3 and the final weight was adjusted using distilled water or 0.1 M NaCl. Then, the samples were kept under refrigeration at 4°C overnight and allowed to stand at 20°C for at least 1 h prior to the analyses.

2.3. Rheological measurements

The rheological measurements were carried out using a Thermo Scientific HAAKE MARS II rheometer (Haake GmbH, Germany) coupled to a HAAKE K15 thermostatic water bath, a HAAKE DC5 heating control and HAAKE UTMC unit. All data were collected and analyzed using the software RheoWin 4.

Flow behavior analyses of the pectin solutions were performed at 25°C, in the range of 0.001-500 s^{-1}, using a C60/2°TiL cone-plate geometry.

Oscillatory experiments were performed using a P35TiL parallel plate geometry. Stress sweeps were performed under a range of 0.01–10 Pa at 6.28 rad/s (25ºC) to determine the linear viscoelastic region, where the storage and loss moduli were independent of the shear stress. Thereafter, frequency sweeps were carried out at 25°C in a frequency range of 0.0628-628 rad/s under shear stress within the linear viscoelastic region.

For thermal stability evaluation, the experiments were performed at oscillatory mode at frequency of 6.28 rad/s and shear stress of 2 Pa with heating (5°C-90°C) and subsequent cooling (90°C-5°C) at a rate of 1°C/min. Mineral oil was used around in the exposed sample edge to prevent water loss.

3. Results and discussion

3.1. Flow behavior of aqueous solutions

Polysaccharides influence the viscosity of aqueous solutions because of the large volume that the molecules occupy when hydrated (Kontogiorgos, Margelou, Giorgiadis & Ritzoulis, 2012).

Figures 1-A and 1-C show the results of the rotational flow tests carried out using aqueous and 0.1 M NaCl solutions for PPOP at concentrations of 1, 3 and 5% (w/w). At low shear rates, the apparent viscosities were similar at all concentrations for both solvents water and 0.1 M NaCl. However, at high shear rates, the increase in pectin concentration resulted in an increase of the apparent viscosity values. A directly proportional relationship between the concentration and the viscosity of pectins in solution had been previously demonstrated by others authors (Owens, Lotzkar, Merrill & Peterson,1944; Kar & Arslan, 1999; Kjøniksen, Hiorth & Nyström, 2005; Guimarães, Coelho Junior & Rojas, 2009; Kontogiorgos et al., 2012; Sousa et al., 2015). The increase in the viscosity caused by the increase in concentration can be explained by the higher number of hydrogen bonds formed by the increased availability in the hydroxyl groups that lead to more entangled polymer coils. (Kar & Arslan, 1999; Guimarães, Coelho Junior & Rojas, 2009). Thus, the increase in polymer concentration forces the mutual interpenetration between the molecules and limits their movement, increasing the viscosity (Morris, 1990).

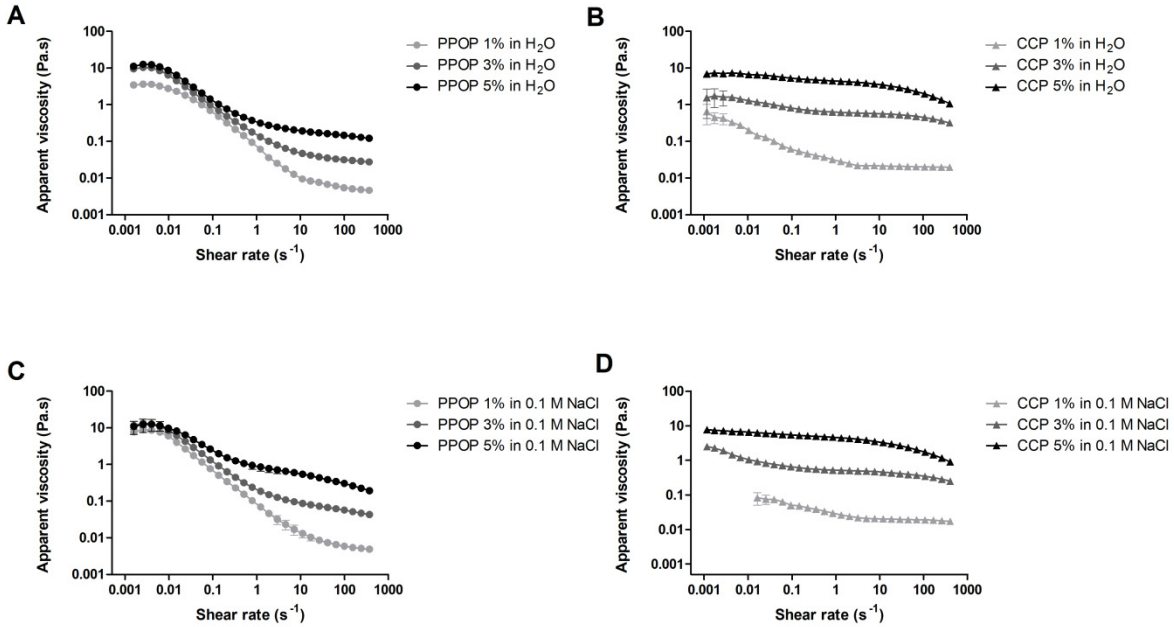

Fig. 1. Viscosity curves of PPOP and CCP solutions at concentration of 1%, 3% and 5% (w/w) in water (A and B) and in 0.1 M NaCl (C and D). The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

For comparison purposes, the flow behavior of a commercial citrus pectin (CCP) at concentrations of 1, 3 and 5% (w/w) is shown in Fig. 1-B and 1-D. CCP was composed of 84.4% of galacturonic acid, similar to PPOP (84.5% GalA), and was also highly methyl-esterified (71.4%) although it had a DM smaller than PPOP (85.7%). The average molar mass of CCP was determined as 1.407×10^5 g/mol, about 1.75 times higher than that of PPOP. Although the same trend between concentration and viscosity was also observed for CCP, the viscosity of the CCP showed to be less dependent on the shear rate, whereas the PPOP had a more pseudoplastic behavior. This difference might be due to the higher molecular mass of CCP compared to PPOP. The shear induced molecular orientation might be easier for short chains than for longer ones.

Despite the lower molecular mass PPOP reached values of viscosity comparable to those of the CCP. It has been reported that in the absence of metal interactions, the viscosity increase with the increase in the degree of esterification (Rolin & de Vries, 1990) and PPOP had a higher degree of methyl esterification than CCP. Due to its higher shear thinning behavior, at higher shear rates, the apparent viscosity of PPOP was markedly lower than CCP. This might be a positive aspect for food applications as it has been pointed out that foods with a rapid decrease of viscosity with shear rate are easier to swallow (Szczesniak, & Farkas, 1962).

The presence of salt did not affect the flow behavior of either PPOP or CCP, since the results obtained using water and 0.1 M NaCl were very similar (Fig 1). A test was performed using a higher concentration of salt (1 M NaCl) and the results were similar (data not shown). Usually in diluted pectin solutions, the presence of salts of monovalent cations reduces the viscosity as a result of the decrease of the effects of negative charges (Rolin & de Vries, 1990; Pals & Hermans, 1952; Lima, Soldi & Borsali, 2009). On the other hand, in concentrated solutions as used in the present study, the presence of monovalent cations may even increase the viscosity due to the aggregation of the pectin chains without the repulsive effect of the charges (Lopes da Silva & Rao, 2006). As PPOP and CCP had a high DM, with few ionizable groups, it was expected a lower effect of the presence of monovalent cations. Recently, Alba et al. (2018) investigated the conformation of pectins with diferent DM (10, 30, 50 and 70%). They found that at high DM the influence of counterion is minimized due the charge density decrease. The authors observed by small angle X-ray scattering that the presence of salt did not significantly affect the results for the pectin with DM 70%, differently from the pectins with lower DM.

3.2. Gelling properties

Gel formation is the most important property of pectins. It has already been well established that the gelation of pectins depends on certain factors, such as the degree of esterification, pH, pectin concentration and the presence of salts or co-solutes (Wang & Cui, 2005; Lopes da Silva & Rao, 2006). Since the structure and properties of pectins strongly depend on the source and method of extraction (Petkowicz, Vriesmann, & Williams, 2017), the optimal gelation may occur at different conditions for different pectins. As PPOP had a degree of methyl-esterification, the viscoelastic properties were evaluated at different concentrations of sucrose and acid in order to identify the best conditions of gelling within the tested ranges.

3.2.1. Influence of pH on PPOP gelation

Considering that HM pectins usually form gels at pH bellow 3.5, which is approximately the pKa of pectins (Bulone, Martorana, Xiao & San Biagio, 2002; Einhorn-Stoll, 2018), a pH range between 1.5 and 3.5 was selected for the tests. The influence of pH on the viscoelastic properties of PPOP is shown in Fig. 2 (A and B).

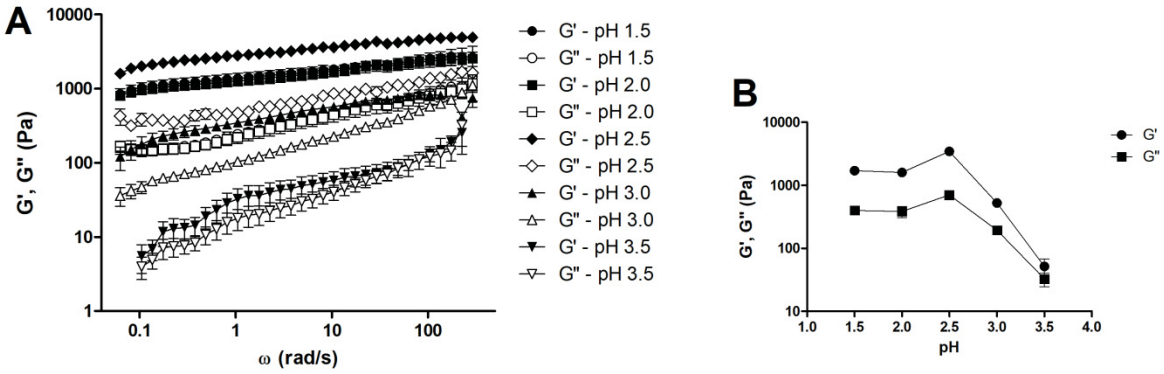

Fig. 2. Viscoelastic properties of PPOP at 2% (w/w) concentration and 60% (w/w) sucrose at different pHs. (A) Frequency sweeps for gels prepared at different pHs. (B) Elastic (G') and viscous (G'') moduli at ω = 6.283 rad/s as a function of pH. The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

Gelation of PPOP did not take place properly at pH 3.5, as evidenced by the overlapping between G' and G''. However, at pH < 3.5 PPOP showed typical gel behavior, with G'>G'' throughout over the applied frequency range.

As the pH decreases from 3.0 to 2.5, the gels became less frequency dependent and an increase in the values and in the difference between the moduli was observed, indicating that the increase in H^+ concentration increased the gel strength. The literature describes pH as an important factor for the gelation of HM pectins due to the role that H^+ ions exert by protonating the free carboxylic groups. H^+ ions neutralize the electrostatic repulsions and make these groups able to form hydrogen bonds, reinforcing the three-dimensional structure (Chan et al., 2017). An inverse correlation between pH and gel strength was also reported by several authors (El-Nawawi & Heikel, 1997; Sharma, Liptay & Le Maguer, 1998; Evageliou, Richardson & Morris, 2000; Löfgren, Guillotin, Evenbratt, Schols, & Hermansson, 2005; Colodel, Petkowicz, 2018).

The strongest gel was found at pH 2.5 as shown in Fig. 2-B, which shows the values of G' and G'' at the angular frequency of 6.283 rad/s as a function of pH. However, the progressive decrease of pH from 2.5 to 1.5 resulted in decrease in the moduli values. This was possibly due to pre-gelation, that results in non-homogeneous and weaker gels (Endreβ & Christensen, 2009), as previously observed by Kastner *et al*. (2014) for gels prepared with commercial HM citrus pectin at pH 2.03 to 2.52.

When G' is plotted as a function of ω (angular frequency), the degree of dependence of G' can be determined by the power-law parameters, given by the relation $G' = A\omega^B$. From

this equation, A is a constant and B is the slope in a log–log plot of G' versus ω. The constant A is directly related to the magnitude of G', but the parameter B can provide information about the structure of the gel. In covalent gels, B=0, whereas in physical gels B>0. Thus, B is related to the strength and nature of the gel (Khondkar, Tester, Hudson, Karkalas & Morrow, 2007). The table 1 gives the parameters A and B determined for each condition tested for gel formation. At all pH values, B>0, which was expected since HM pectins form physical gels (Munarin, Tanzi & Petrini, 2012). Comparing the parameters A and B at the different pHs, the highest A-value and the lowest B-value was found at pH 2.5 which was the strongest gel. The gels prepared at pH ≤2.5 showed similar B-values.

Based on the results, the pH 2.5 was selected for further tests.

3.2.2. Influence of pectin concentration on PPOP gelation

The gelation of polysaccharides occurs when the concentration of the polymer exceeds the critical concentration, where the amount of chains is sufficient for the formation of junction zones and the tridimensional network and the gel strength typically increases with increasing concentration (Wang & Cui, 2005). An ideal pectin concentration is not well established, but HM pectins are generally used for gelation at concentrations from 2% up to 4% (Burey, Bhandari, Howes & Gidley, 2002). Although a minimum concentration of 1% had been recommended (May, 1990), it was demonstrated that gelation also takes place at lower concentrations (Guo et al., 2012). Concentrations of PPOP from 1% to 3% (w/w) were used to evaluate the influence of the pectin concentration on gel properties. The sucrose concentration was fixed at 60% (w/w) and the pH at 2.5, as previously defined. The mechanical spectra are depicted in Fig. 3-A. A typical gel behavior (G'>G'') was observed at all concentrations. A positive correlation was found between the pectin concentration and the gel strength, since the values of the elastic modulus G' increased progressively as the concentration increased. The same trend was observed for gels prepared using pectins from tomato (Sharma et al., 1998), orange peel (Guo *et al*., 2012), cacao pod husks (Vriesmann & Petkowicz, 2013), cupuassu (Vriesmann, Silveira & Petkowicz, 2010) and pomelo albedo (Gamonpilas, Krongsin, Methacanon & Goh, 2015).

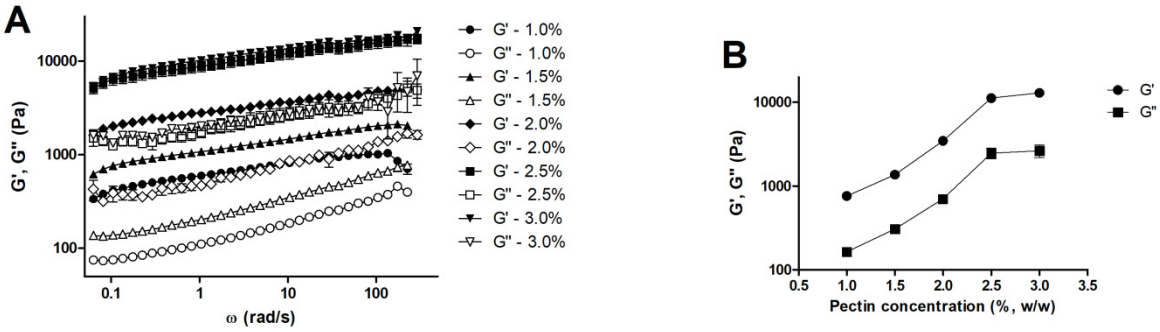

Fig. 3. Viscoelastic properties of PPOP at different concentrations and 60% (w/w) sucrose at pH 2.5. (A) Frequency sweeps for gels prepared with different pectin concentrations. (B) Elastic (G') and viscous (G'') moduli at ω = 6.283 rad/s as a function of pectin concentration. The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

As can be seen in Fig. 3-B, the correlation between the gel strength and pectin concentration was not linear, being more pronounced at concentrations 1% to 2.5%. However, the raising in the concentration from 2.5% to 3% did not result in a considerable difference in the gel strength.

The increase in amount of polymer when the other parameters are kept constant allows the formation of more junction zones between the chains (BeMiller, 1986; Guo *et al*., 2012). However, it appears there is an upper limit, from which the increase in the number of pectin chains do not contribute significantly to the network, since no changes in the gel strength were found when the concentration of PPOP increased from 2.5% to 3%. Regarding the A and B parameters from the relation $G' = A\omega^B$, the B-value was very similar at all concentrations, between 0.122 and 0.140 (Table 1). Although in the frequency sweeps, G' values for concentrations 2.5% and 3.0% were almost superimposed (Fig. 3-A), the A-value was slightly higher for 3% concentration. Considering the results, the pectin concentration of 2.5% was chosen as the best concentration for PPOP gelation.

3.2.3. Influence of the sucrose concentration on PPOP gelation

Gels of HM pectins are stabilized by hydrophobic interactions between the ester methyl groups and hydrogen bonds occurring between the protonated carboxyl and hydroxyl groups of the pectin chains (Oakenfull & Scott, 1984; Chan et al., 2017; Einhorn-Stoll, 2018). The gel formation takes place under conditions of low water activity, which is often achieved by the addition of co-solutes, favoring the inter chain interactions (Lopes da Silva & Rao, 2006; Sousa et al., 2015; Chan et al., 2017; Einhorn-Stoll, 2018).

A minimum of 55% (w/w) of co-solute is required to induce the gelation of pectin, while 60-65% (w/w) is considered the ideal concentration (BeMiller, 1986; Rolin & de Vries, 1990; May, 1990). Some studies have been carried out using glucose and fructose as co-solutes (Evageliou, Richardson & Morris, 2000; Yoo, Yoo, Kim & Lim, 2003; Tsoga, Richardson & Morris, 2004). In order to seek healthier formulations, sorbitol (Yoo et al., 2003; Tsoga, Richardson & Morris, 2004), glycerol, xylitol and ethan-1,2-diol (Tsoga, Richardson & Morris, 2004) have also been used to prepare gels of HM pectins. However, the most widely used co-solute is sucrose (Chan et al., 2017). In the present study, sucrose at concentrations 55, 60 and 65% was used to induce PPOP gelation at pH 2.5.

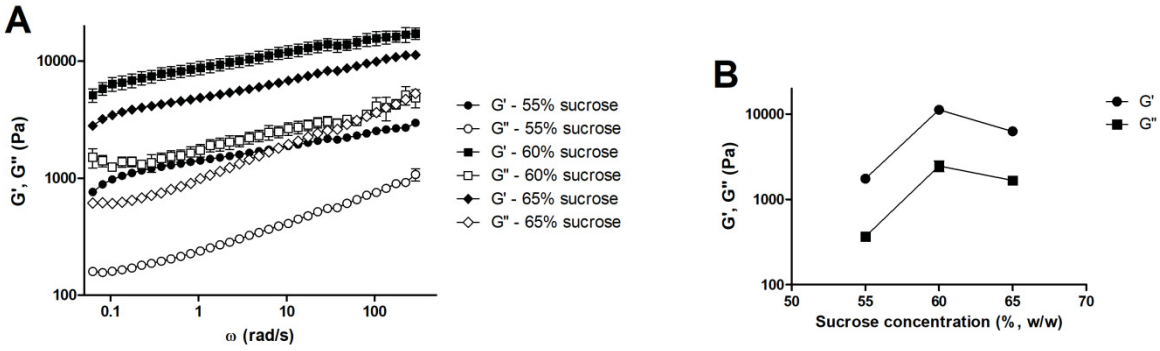

Fig. 4. Viscoelastic properties of PPOP at 2.5% (w/w) concentration and pH 2.5 at different sucrose concentrations. (A) Frequency sweeps for gels prepared with different sucrose concentrations. (B) Elastic (G') and viscous (G") moduli at ω = 6.283 rad/s as a function of sucrose concentration. The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

As can be seen in Fig. 4-A, gelation took place at the three sucrose concentrations tested. A noticeable increase in the magnitude of the moduli was found when the sucrose concentration was increased from 55% to 60%. However, the addition of 65% sucrose decreased the gel strength compared to 60%, as shown in Fig. 4-B. A direct correlation between the gel strength and the sucrose content was observed by Vriesmann, Silveira & Petkowicz (2010), using the same amounts of sucrose (55, 60 and 65%). Giacomazza, Bulone, Biagio, Marino & Lapasin (2018) also reported increasing values of G' and G" with the increment of the sucrose concentration (56-65%) for gels prepared using 0.2% (w/w) commercial HM citrus pectin. Similarly to the results found in the present study, Sharma, Liptay & Le Maguer (1998) achieved the most satisfactory gel formation of HM pectins from tomato (DM ~88%) using 60% sucrose. The parameters A and B also confirmed the best gelation of PPOP in the presence of 60% of sucrose (Table 1).

Table 1. Power–law parameters for the relationship between the storage modulus (G') and the angular frequency (ω) for the gels prepared with PPOP.

Variable	Variable level	A (Pa.s)	B (dimensionless)
pH	1.5	1363.4	0.129
	2.0	1275.5	0.130
	2.5	2706.9	0.122
	3.0	323.0	0.210
	3.5	23.3	0.392
PPOP concentration (%, w/w)	1.0	578.7	0.137
	1.5	1055.8	0.140
	2.0	2706.9	0.122
	2.5	8618.2	0.132
	3.0	9707.4	0.133
Sucrose concentration (%, w/w)	55	1360.5	0.135
	60	8618.2	0.132
	65	4826.7	0.153

3.2.4. Comparison between the viscoelastic properties of PPOP and CCP gels

After defining the best gelation conditions for PPOP - pH 2.5, pectin concentration of 2.5% (w/w) and sucrose concentration of 60% (w/w) - gels were prepared using PPOP and a commercial HM pectin (CCP; DM 71.4%) in order to compare their viscoelastic behavior at the same conditions. The mechanical spectra of the gels are depicted in Fig. 5.

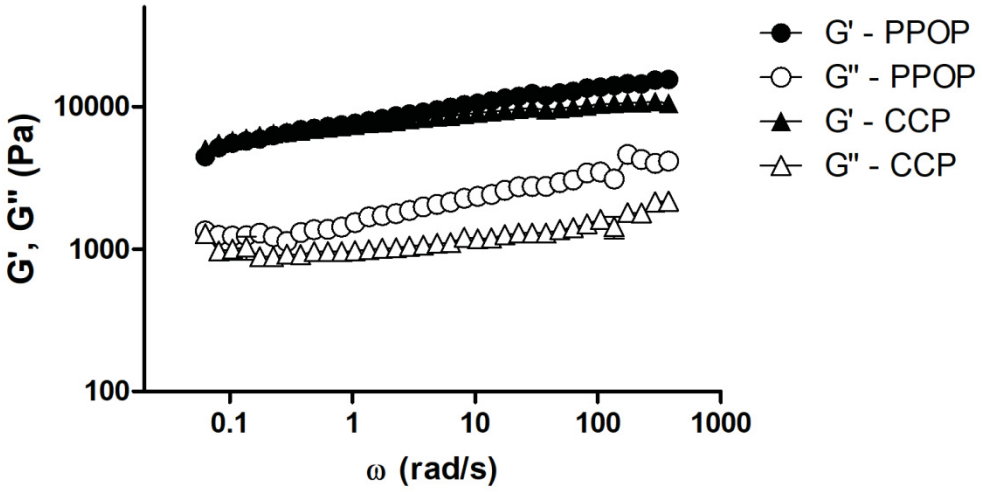

Fig. 5. Comparison between the viscoelastic behavior of the gels of PPOP and CCP prepared with 2.5% (w/w) pectin, 60% (w/w) sucrose at pH 2.5. The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

Comparing the mechanical spectra of the gels of PPOP and CCP prepared and analyzed at the same conditions, it can be seen that G' and G" are less frequency dependent for the CCP. This was confirmed by the B-value (0.081) from the power-law relationship $G' = A\omega^B$, which was lower than the B-value found for PPOP gel (0.132).

On the other hand, the magnitude of the moduli was similar for CCP and PPOP, confirming the good gelling properties of PPOP.

3.3. Thermal stability of PPOP gel

Heating and cooling cycles is one of the approaches used to study the thermal behavior of biopolymer gels (Lopes da Silva & Rao, 2007). Although HM pectins gels are considered thermo-irreversible (Lopes da Silva & Rao, 2006), the heating affects the intermolecular interactions. However, it does not cause rupture to the gel-sol transition point as occurs in thermo-reversible LM pectins (Axelos & Thibault, 1991).

Fig. 6 shows the behavior of the gel prepared with 2.5% (w/w) PPOP, 60% (w/w) sucrose at pH 2.5 subjected to four cycles of heating (5-90°C) and cooling (90-5°C). A temperature dependence of G' and G" was observed throughout the heating-cooling cycle of PPOP gel. The values of G' and G" were inversely proportional to the temperature, which plays a crucial role in the formation and disruption of the junction zones and cross-links (Lopes da Silva & Rao, 2007). Heating produces opposite effects on the interacting forces that

stabilize the HM pectin gel network. At the same time that hydrogen bonds become weaker by increasing temperature, hydrophobic interactions are known to be strengthened when the temperature increases up to 85°C (Ben-Naim, 1980; Oakenfull & Scott, 1984). These opposite effects resulted in decrease in the moduli on heating of PPOP gel in the range 5-90°C and were reported to cause a non-linear decrease in the rupture strength of an HM pectin gel with increase in the temperature from 0 to 50°C (Oakenfull & Scott, 1984).

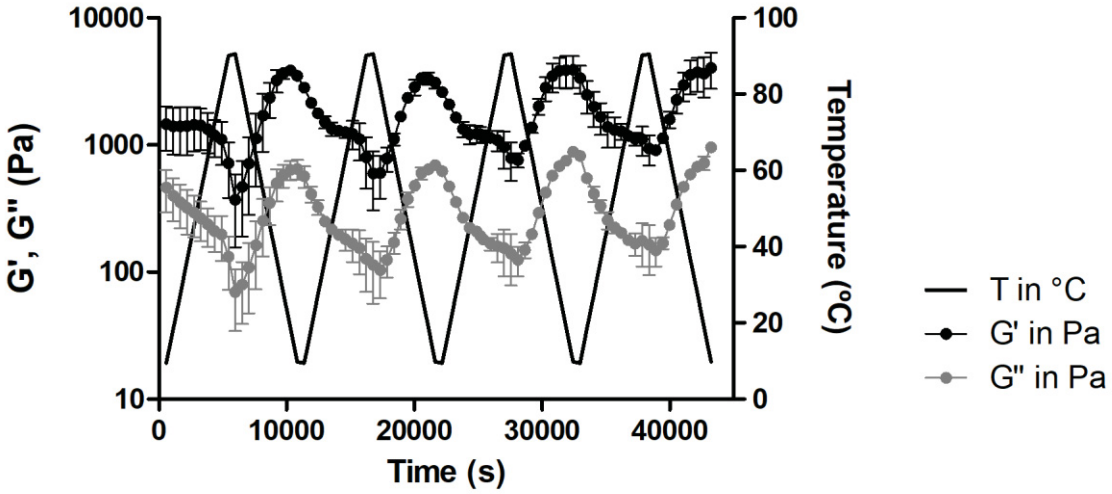

Fig. 6. Heating and cooling cycles (5-95°C and 95-5°C) of 2.5% (w/w) PPOP gel prepared at pH 2.5 and 60% (w/w) sucrose. Four heating and cooling cycles were performed at a rate of 1°C/min. The results are expressed as mean of triplicate experiments and the error bars represent the standard deviation.

PPOP gel displayed high thermal stability as G' > G" throughout the set temperature range and at the end of each heating and cooling cycle the values of G' and G" were only slightly higher the initial ones. The water loss may have contributed to the differences between the values of the moduli observed comparing the beginning and the end of the experiment.

Conclusion

At low shear rates, aqueous solutions (1%-5% w/w) of optimized pectin from ponkan peel (PPOP) showed apparent viscosities similar to a HM commercial citrus pectin (CCP). However, PPOP had a more pseudoplastic flow behavior. The apparent viscosity was directly related to the concentration. PPOP formed gels at pH <3.5 in the presence of sucrose, and the

strongest gel was obtained using 2.5% (w/w) pectin, 60% (w/w) sucrose at pH 2.5. The viscoelastic behavior of PPOP gel was close to that observed for the gel prepared with CCP under the same conditions. In addition, the gel showed to be thermally stable after four heating and cooling cycles. The results suggest that PPOP has properties comparable to commercial citrus pectin. PPOP could be used as a thickener in viscous foods where mouth-feel depends on the rapid decrease of viscosity, such as nectars and yoghurts or as a gelling agent in acidic products containing high soluble solids content and can be prepared or packaged under wide temperature range.

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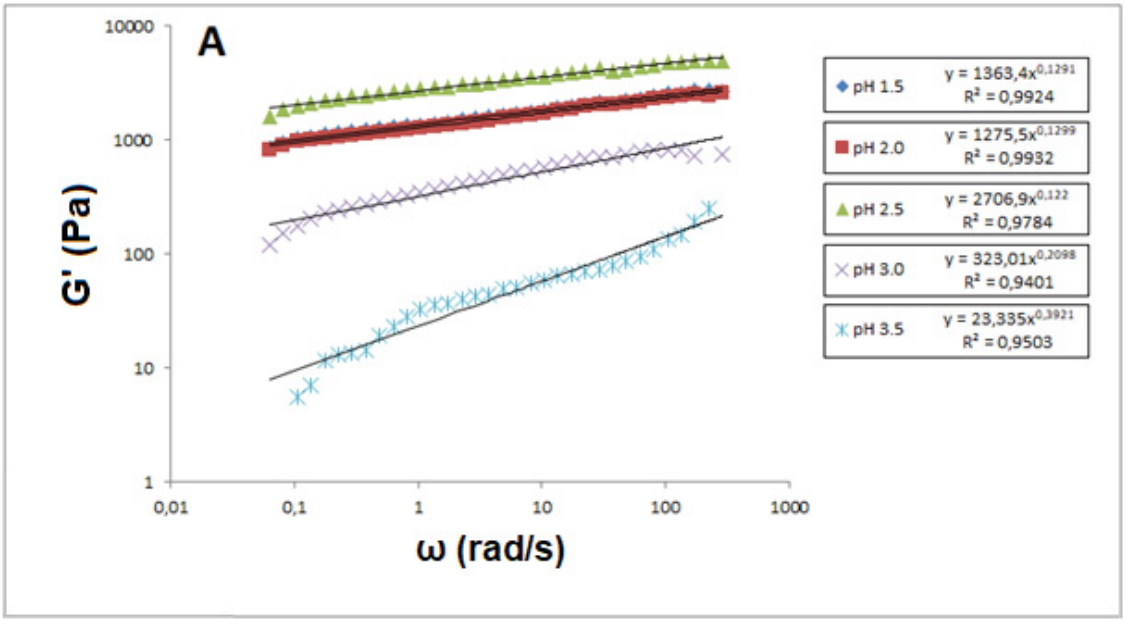
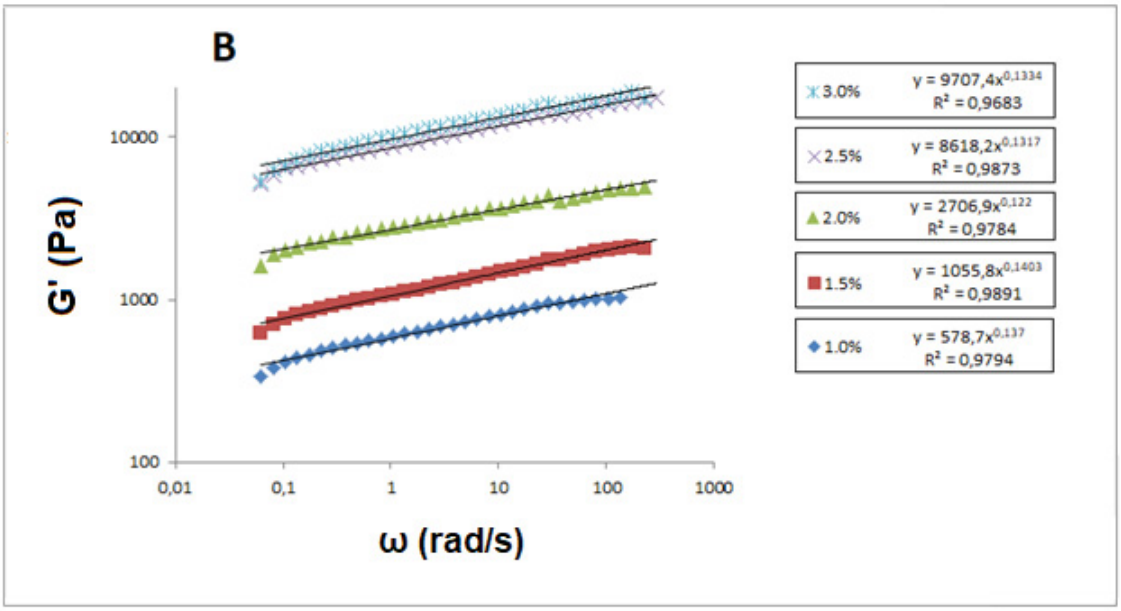
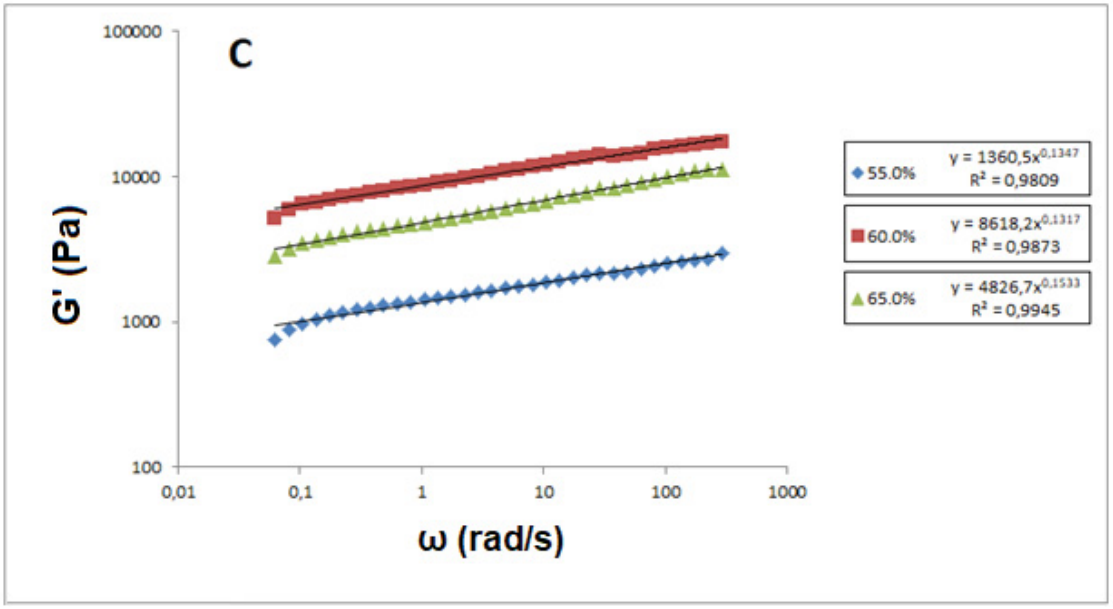
Supplementary Data







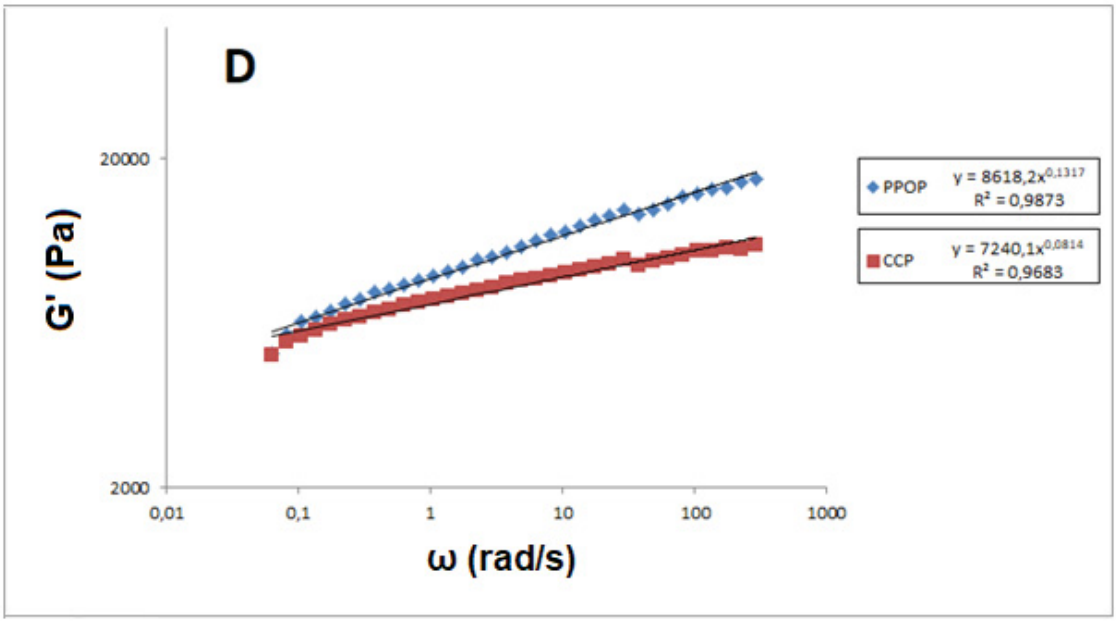


Figure S1 - G' plotted as function of the angular frequency (ω) for the different tested pH (A), pectin concentration (B) and sucrose concentration (C). The plot D shows a comparison between PPOP and the commercial citrus pectin (CCP) gels formed at the best conditions determined for gelation of PPOP. A non-linear regression Power model $G' = A\omega^B$ was applied for all data in order to obtain the parameters A and B.

CAPÍTULO 5

Artigo “Optimization of acid-extraction of pectin from grape (*Vitis vinifera cv. Chardonnay)* pomace, a winery waste”

Optimization of acid-extraction of pectin from grape (*Vitis vinifera cv. Chardonnay)* pomace, a winery waste

Cristiane Colodel [a]; Lúcia Cristina Vriesmann [a]; Reinaldo Francisco Teófilo [b]; Bianca Busato [a], Guilhermina Rodrigues Noleto [a], Carmen Lúcia de Oliveira Petkowicz [a].

[a] Universidade Federal do Paraná, Departamento de Bioquímica e Biologia Molecular, R. Francisco H. dos Santos, s/n, CP 19046, CEP 81531-980, Curitiba, PR, Brazil

[b] Universidade Federal de Viçosa, Departamento de Química, CEP 36570-000, Viçosa, MG, Brazil

Abstract

The Chardonnay grape pomace was evaluated as a source of pectin. A central composite design was applied in order to determine the effect of pH, extraction time and liquid: solid ratio on the yield and uronic acid (UA) content of the pectins extracted using boiling HNO_3 solution. The optimized extraction condition to reach the maximum yield and UA was established as pH=2.08, Et=135.23 min and LS=35.11 mL/g, resulting in theoretical yield of 12.8% and UA of 64.4%. The experimental yield of the pectin obtained under the optimized conditions (GPOP) was 11.1% and the UA was 56.8%. GPOP had ~25% glucose and was treated with α-amilase and amyloglucosidase resulting in the fraction α-GPOP. The purified pectin was composed of 63.5% UA, 7.8% Rha, 6.0% Ara and 13.6% Gal, besides minor amounts of other neutral monosaccharides. It was a low-methoxyl pectin (18.1%) with average molar mass (Mw) of 154.100 g/mol. Its structure major consisted of HG (55.7%), but had a high proportion of RG-I (35.2%). NMR analyses suggest that RG-I portion of α-GPOP is highly branched by short chains of even single residues of arabinose and galactose. α-GPOP was non-cytotoxic (100-500 µg/mL) for macrophages and induced the NO production at concentrations of 50 to 500 µg/mL, suggesting immunostimmulatory properties.

1. Introduction

Viticulture is one of the most important fruit crop, with a world production of more than 77 million tons in 2016 (FAOSTAT, 2018). About 80% of the production is destined to winemaking and the remaining is consumed *in natura* and processed into juices, jams, candies

and raisins (Maroun et al., 2017). The grape pomace is the main waste of winemaking and is composed of skins and seeds, and sometimes stalks are also present when they are not removed prior to pressing. The grape pomace corresponds to about 20% of the initial fruit mass, and comprises about 30% stalks, 30% seeds and 40% skin and pulp (Domínguez et al., 2017).

The waste remaining from winemaking can be a major problem from the environmental point of view, because it produces a large amount of organic matter that, if not destined or treated correctly, can cause pollution. Grape pomace is commonly dumped or used for animal feed or for soil fertilization (Mendes et al., 2013). However, these alternatives present some drawbacks. The discard of large quantities of winery waste in the soil can alter its physical, chemical and biological properties due to the low pH, high organic matter charge, high amounts of heavy metals (Bustamante et al., 2005), the presence of phytotoxic polyphenols and the antimicrobial activity (Domínguez et al., 2017). In the case of animal feeding, the high content of lignin makes the grape pomace somewhat indigestible (Domínguez et al., 2017).

On the other hand, grape pomace contains several compounds of great interest, which represents an alternative to reduce the pollution caused by this waste at the same time directing it towards a nobler end and generates greater economic advantage. The main compounds already obtained from grape pomace are the oil from seeds and citric acid, methanol and ethanol; grape pomace is also employed as substrate for xanthan gum production (Deng et al., 2011). Many studies have been carried out seeking to obtain products of higher value, most of them focusing on the phenolic compounds present in the grape pomace and its food, nutraceutical, pharmaceutical and chemical applications (Özkan et al., 2004; Spigno et al., 2007; Amendola et al., 2007; Rockenbach et al., 2011a; Rockenbach et al., 2011b; Fontana et al., 2013; Jara-Palacios et al., 2015; Tournour et al., 2015).

Grape pomace is a good source of dietary fibers with contents in the range of 19.9% to 40.8% depending on the variety of grape (*Vitis vinifera*) (González-Centeno et al., 2010). Dietary fibers include cell wall polysaccharides, namely cellulose, hemicellulose and pectins, in addition to lignin (Dhingra et al., 2012). The content and composition of polysaccharides also differ depending on the variety. Rondeau et al. (2013) evaluated the monosaccharide composition of grape pomace of red and white varieties from eight regions of France and althought the amount of each monosaccharide varied slightly among the varieties, based on the results, they concluded that the highest proportion was composed of glucans and xyloglucans, with a low proportion of pectic substances in the evaluated pomaces. The

varieties Cabernet Sauvignon, Syrah and Monastrell were studied by Apolinar-Valiente et al. (2015) and the authors also suggest the presence of glucans and xyloglucans, but they found considerable amounts of galacturonic acid, arabinose and galactose, monosaccharides typical from pectins. On the other hand, Gao et al. (2015) identified GalA as the major monosaccharide in the alcohol insoluble residue (AIR) composition of Cabernet Sauvignon grape pomace, suggesting that this waste can be a good source of pectins.

The term pectin refers to a group of acidic polysaccharides, which are divided into three classes. The most common and abundant are homogalacturonans (HG), which are composed of linear chains of →4(α-D-GalA)1→ and correspond to the "smooth region". In HG, the GalA units may be partially methylesterified at C-6 and partly acetylated at *O*-2 and/or *O*-3. The "hairy region" is formed by other classes of pectins, rhamnogalacturonan I (RG-I), rhamnogalaturonan II (RG-II) and xylogalacturonan. In RG-I, the backbone is composed of →4)α-D-GalA(1→2)α-L-Rha(1→ repeating units, where the rhamnose units may be substituted at *O*-4 by side chains of arabinans, galactans and arabinogalactans. In its turn, RG-II contains a HG main chain decorated by side chains composed of four complex and well-conserved oligosaccharides. Xylogalacturonan consists of a HG backbone in which some of GalA units is substituted at *O*-3 by β-Xyl residues (Mohnen, 2008; Maxwell et al., 2012).

Pectins are widely used in the food industry because of their thickening and gelling properties and may also present interesting biological effects. As soluble dietary fiber, they have the ability to reduce cholesterol absorption and decrease serum glucose levels (Flourie et al., 1984; Fuse et al., 1989; Naqash et al., 2017). Dietary pectins can be fermented in colon giving rise to short-chains fatty acids, which normalizes the gut microbiota and prevents pathogen overgrowth. They also can inhibit cell growth and enhance the immunological system. Pectins can modulate the immune system, causing either immunostimulation or immunosuppression, depending on its composition and structure (Popov and Ovodov, 2013). It has also been demonstrated that some pectins may display anti-tumor activity by binding to the pro-metastatic Gal-3 preventing cell adhesion, migration and inducing apoptosis (Zhang et al., 2015; Naqash et al., 2017).

Currently commercial pectins are extracted from apple pomace, which contains about 10-15% of pectins on dry weight, and citrus peel, with about 25-35%, both by-products of juice production (Ciriminna et al., 2015). These materials provide pectins with desirable characteristics, such as high yield, high content of galacturonic acid and high molar mass, whereas other alternative sources often have some undesirable structural properties (Willats et

al., 2006). However, Minjares-Fuentes et al. (2014) obtained a pectin with good characteristics from the grape pomace of the Cabernet Sauvignon variety. Using a Box-Behnken-type design, the authors optimized pectin extraction using ultrasound and citric acid, varying the temperature, extraction time and pH. Under the optimal conditions (75°C, 60 min and pH 2), the authors obtained a pectin with a yield of 32.3%, GalA content of about 97%, molar mass of 163,900 g/mol and DM of 55%.

Based on the findings of Minjares-Fuentes et al. (2014) and also on the evidence that the grape variety can influence its qualitative and quantitative composition, the proposal of this study was the optimization of the pectin extraction from grape pomace of Chardonnay variety, using conventional techniques of extraction. In addition, the effect of the enzyme-treated grape pomace optimized pectin (α-GPOP) on viability and nitric oxide production by mouse peritoneal macrophages was evaluated.

2. Materials and methods

2.1. Collect and preparation of the Chardonnay grape pomace

The Chardonnay grape pomace (CGP) was kindly provided by the winery Vinícola Araucária, in São José dos Pinhais, Parana, Brazil. The grapes were harvested in January, 2016 and the whole fruits were pressed to obtain the juice for the production of white wine. Thus, the CGP was composed of skins, seeds and stalks. After pressing, the pomace was immediately collected and frozen, lyophilized, milled and sieved (1 mm x 1 mm). The dried and powdered pomace was then treated with boiling ethanol (1:10 w/v) under reflux for 20 min. The resulting alcohol insoluble residue (AIR) was dried at room temperature and used for pectin extractions.

2.2. Pectin isolation and characterization

2.2.1. Pectin extraction

Pectin was extracted from AIR using boiling aqueous HNO_3 under reflux at different conditions according to an experimental design (Table 1). After centrifugation (4695.6 × *g*, 20 min, 4°C), the supernatant was filtered on a synthetic fabric, precipitated with absolute ethanol 1:2 (v/v) and then left to rest at 4°C overnight. The precipitate was recovered by filtration, rinsed with absolute ethanol and dried under vacuum.

2.2.2. Experimental design

Response surface methodology (RSM) was applied in order to determine the optimum conditions for pectin extraction from grape pomace. A central composite design (CCD) with three variables was used to determine the effect of pH, extraction time (Et) and liquid:solid ratio (LS) on yield and uronic acid content of pectin. The CCD consisted of 19 experiments including: eight factorial experiments (+1, -1 levels), six axial experiments (+α, -α levels), and five replicates of center point (0 levels) to make the estimation of possible pure error, using the independent variables as described in Table 1. All experiments were carried randomly to minimize the effect of possible systematic errors.

Multiple linear regression (MLR) was used for determination of the regression coefficients for the linear, quadratic and interaction terms. The significance of each effect and the regression coefficient was judged statistically by computing the *t*-value from associated errors. The regression coefficients were then used to generate the response surfaces, and the model was validated using the plot of observed vs. predicted values and the plot of observed vs. raw residuals (Teófilo and Ferreira, 2006). Calculations and graphics were performed using Statistica 7.0 software. A difference was considered statistically significant when $p < 0.05$.

2.2.3. Yield and uronic acid content

The pectin yield was determined as follows:

$$yield\ (\%) = \frac{dry\ pectin\ (g)}{AIR\ (g)} \times 100$$

The uronic acid content was measured by the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973), using galacturonic acid (5-100 µg/mL) as standard.

2.2.4. Obtention of the grape pomace optimized pectin (GPOP)

After defining the optimum conditions for extraction of pectin from grape pomace with the highest yield and uronic acid content, larger scale extractions were performed to obtain a higher amount of pectin to be characterized, which was named GPOP.

2.2.5. Enzymatic treatment of GPOP

In order to remove starch contamination, GPOP was subjected to an enzymatic treatment. GPOP was solubilized (10 mg/mL) in phosphate buffer (pH 7.0) and treated with 150 μL α-amylase (Sigma, Germany) and 100 μL amyloglucosidase (Sigma, Germany), at 55ºC for 3 h, when the maximum amount of reducing sugars was released according to a colorimetric test (Nelson, 1944), followed by boiling for 15 min for precipitation of the enzymes. The solution was filtered, dialyzed for 2 days against tap water in 10-12 kDa membrane and then lyophilized, giving rise to the sample named α-GPOP.

2.2.6. Monosaccharide composition

Neutral monosaccharide composition was determined by gas-liquid chromatography (GLC) after hydrolysis and derivatization. GPOP was hydrolyzed using 2 mol/L trifluoroacetic acid (TFA) (8 h, 100 °C) and evaporated to dryness. The monosaccharides were reduced with $NaBH_4$ (16 h, 4°C) (Wolfrom and Thompson, 1963a) and then acetylated with pyridine-acetic anhydride (1:1 v/v, 16 h, 25°C) (Wolfrom and Thompson, 1963b). The resulting alditol acetates were extracted with $CHCl_3$ and analyzed using a 5890 S II Hewlett–Packard gas chromatograph at 220°C (flame ionization detector and injector temperature, 250°C) with a DB-210 capillary column (0.25 mm internal diameter × 30 m), film thickness 0.25 μm, using nitrogen as the carrier gas (2.0 mL/min).

Uronic acid content was measured as described in section 2.4 and the identity of uronic acid was determined by ion exchange chromatography with pulse amperometric detection (HPAEC-PAD). After hydrolysis with 2 mol/L TFA (8 h, 100°C) and acid removal, the sample (1 mg/mL) was filtered through a membrane of 0.22 μm and analyzed in a Thermo Scientific Dionex ICS-5000 chromatograph (Thermo Fisher Scientific, USA) with CarboPac PA20 column (3 ×150 mm) using gradient of 1 mol/L NaOH and 1 mol/L NaOAc as eluent (Nagel et al., 2014) in N_2 atmosphere in a flow of 0.2 mL/min at 30 °C. Data were collected and analyzed using the Chromeleon™ 7.2 Chromatography Data System software.

2.2.7. High Pressure Size Exclusion Chromatography (HPSEC)

The molar mass distribution of the pectin was obtained by HPSEC. The sample (1.0 mg/mL) was solubilized in 0.1 mol/L $NaNO_2$ containing 0.5 g/L NaN_3, filtered (Millipore,

Canada, 0.22 μm) and analyzed using a Waters unit coupled to a refractive index (RI), a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector. Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled to the multidetection instrument. A solution of 0.1 mol/L $NaNO_2$ and 0.5 g/L NaN_3 was used as eluent at a flow of 0.6 mL/min.

2.2.8. Spectroscopic methods

The degree of methyl-esterification (DM) was determined by Fourier transformed infrared (FT-IR). Spectroscopic grade KBr powder was used and discs were prepared using a 99:1 salt/sample ratio. The spectra were collected at the absorbance mode in the frequency range of 4000–400 cm^{-1} using a Vertex 70 spectrophotometer (Bruker, Germany), at 4 cm^{-1} resolution. The degree of methyl-esterification was calculated as the ratio between the area of the methyl-esterified band (1749 cm^{-1}) and the sum of methyl-esterified (1749 cm^{-1}) and non-esterified (1630 cm^{-1}) bands, as previously described by Vriesmann and Petkowicz (2009). The spectra were obtained using OriginPro 8 software.

For heteronuclear single quantum coherence nuclear magnetic resonance (HSQC-NMR) analysis, 20 mg of the sample was dissolved in D_2O (40 mg/mL) and analyzed in a Bruker Avance DRX400 spectrometer (Bruker, Germany) at 70°C. Chemical shifts are expressed in δ (ppm), using acetone (δ 30.2/2.22) as reference.

2.2.9. Colorimetric methods

Colorimetric methods were used to determine: the total phenolic content (Singleton, Rossi, 1965), expressed as gallic acid equivalents; protein content (Bradford, 1976), using bovine serum albumin as standard; and the acetyl content (Hestrin, 1949), using erythritol acetate as standard. The degree of acetylation (DA) was calculated as described by Colodel et al. (2017).

2.3. Biological assays

2.3.1. Peritoneal macrophage isolation

Peritoneal macrophages were obtained from female albino swiss mice (6-8 week-old) according to standard protocol described by Noleto et al. (2002). Briefly, mice was euthanized by inhaled anesthetic, and then 10 mL ice-cold sterile phosphate-buffered saline solution

(HBSS) were infusing in their peritoneal cavity. The exudate collected was centrifuged at 300 × g, at 4 ºC for 20 min and the pellet was re-suspended with RPMI 1640 medium containing 5% fetal bovine serum (FBS) and antibiotics (50 μg/mL gentamicin, 100 U/mL penicillin and 100 μg/mL streptomycin). The cells were counted and plated into 96-well plates in an density (2×10^5 cells/well) for cell viability and (5×10^5 cells/well) for nitric oxide assay. After 2 hours at 37 ºC under 5% CO_2, the non-adherent cells were removed by washing once with HBSS at 37 ºC and adherent macrophages obtained.

All recommendations of the Brazilian National Law (n. 6.638, November 5, 1979) for scientific management of animals were respected. In addition, animal handling procedures for scientific research were approved by the Animal Ethics Committee of UFPR number 1069.

2.3.2. Cell viability

The cell viability was determined by 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method as described by Mosmann (1983) and Noleto et al. (2002). Adherent macrophages were incubated in absence (control) or presence of α-GPOP at concentrations 100, 250 and 500 μg/mL diluted in medium for 48h, in the same conditions. Next, the medium was replaced by MTT 500 μg/mL in HBSS and the plate incubated in the dark for 10 minutes. After addition of dimethyl sulfoxide for dilution of formazan crystals, the absorbance in 550 nm was determined. Control (macrophages incubated only in the medium) was considered as 100% of viability.

2.3.3. Nitric oxide production

The nitric oxide production was indirectly assessed by measuring nitrite in the culture medium using the Griess reaction with some modifications, as previously described by Keller et al. (1990). Adherent macrophages were incubated for 48h in absence (control) or presence of lipopolysaccharide from *Escherichia coli* (LPS) (positive control) or α-GPOP at concentrations 100, 250 and 500 μg/mL diluted in medium for 48h, in the same conditions. After that, 100 μL of supernatants were mixed with an equal volume of Griess reagent and incubated at 25 ºC for 10 minutes. Absorbance was measured at 550 nm in microplate reader. The nitrite concentration was calculated from a $NaNO_2$ standard curve and the results are expressed as μmol.

2.3.4. Statistical analysis

The results of biological assays were submitted to analysis of variance (ANOVA) test, followed by Tukey's test for average comparison, using GraphPad Prism® statistic program. Experimental values were expressed as mean ± standard error of the mean (SEM) or mean ± stardard deviation (SD) of at least two independent experiments in triplicate. Significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Characteristics of the Chardonnay grape pomace (CGP)

According to the winery that provided the material, the pomace corresponded to about 20-25% of the mass of the fresh pressed fruits of the Chardonnay grape, which is in agreement with data previously reported (Yu and Ahmedna, 2013). CGP had a moisture content of 65.3%, close to the 63.9% reported by González-Centeno *et al*. (2010) for the same variety and to others varieties (Minjares-Fuentes *et al*., 2014), and lower than the 97.4% reported by Zietsman et al. (2017) for Chardonnay pomace. The AIR yielded 71.2% of the CGP, higher than the ~60% reported by González-Centeno et al. (2010).

3.2. *Model building for the optimization of the pectin extraction and fitting evaluation*

Although grape pomace is a residue globally produced in large amounts in the wine production, only one study was found in the literature regarding its use as a source of pectin (Minjares-Fuentes et al., 2014). Considering that the chemical composition may vary between different grape varieties, this study aims to evaluate the effect of pH, extraction time and liquid: solid ratio on the yield and uronic acid content of pectin from Chardonnay grape pomace.

The experimental yields and uronic acid contents of the pectins extracted from the AIR obtained from Chardonnay grape pomace are presented in Table 1 and the coefficients and statistical parameters obtained from these results are given in Table 2.

Table 1 – Decoded and coded levels of variables and responses for CCD for pectin extractions from Chardonnay grape pomace.

Assay	pH	Et (min)	LS (mL/g)	Yield (%)	Uronic acid (%)
5	2 (-1)	36 (-1)	30 (+)	10.3	56.2
15	2.5 (0)	73 (0)	22.5 (0)	10.1	27.3
7	2 (-1)	110 (+1)	30 (+1)	10.9	60.5
3	2 (-1)	110 (+1)	15 (-1)	10.0	33.4
18	2.5 (0)	73 (0)	22.5 (0)	7.9	36.6
11	2.5 (0)	10.77 (-α)	22.5 (0)	6.7	15.1
6	3 (+1)	36 (-1)	30 (+1)	5.4	21.0
4	3 (+1)	110 (+)	15 (-1)	7.9	29.8
8	3 (+1)	110 (+)	30 (+1)	9.1	33.0
17	2.5 (0)	73 (0)	22.5 (0)	9.8	29.0
10	3.34 (+α)	73 (0)	22.5 (0)	5.7	27.6
14	2.5 (0)	73 (0)	35.11 (+α)	8.1	31.2
12	2.5 (0)	135.23 (+α)	22.5 (0)	9.6	33.2
1	2 (-1)	36 (-1)	15 (-1)	9.9	45.2
19	2.5 (0)	73 (0)	22.5 (0)	8.2	33.1
13	2.5 (0)	73 (0)	9.89 (-α)	6.9	24.8
2	3 (+1)	36 (-1)	15 (-1)	5.9	14.8
9	1.66 (-α)	73 (0)	22.5 (0)	10.1	62.3
16	2.5 (0)	73 (0)	22.5 (0)	8.8	30.1

Et = extraction time; LS = liquid to solid ratio.
The assays were carried out in random order.

Table 2 - Coefficient estimates, statistical parameters and ANOVA for CCD models of pectin yield and uronic acid content for pectins extracted from Chardonnay grape pomace.

	Yield				**Uronic acid**			
	Coeff.	Std.Err.	t(9)	p	Coeff.	Std.Err.	t(9)	p
Mean	***8.92***	0.39	23	0	***31.05***	2.35	13.16	0
pH	***-1.48***	0.23	-6.3	1.4×10^{-4}	***-11.36***	1.43	-7.94	2.3×10^{-5}
pH^2	-0.18	0.23	0.78	0.45	***5.78***	1.43	4.04	0.0029
Et	***0.83***	0.23	3.51	0.0066	***3.66***	1.43	2.56	0.031
Et^2	-0.09	0.23	-0.4	0.69	-1.58	1.43	-1.11	0.3
LS	0.29	0.23	1.25	0.24	***4.27***	1.06	2.98	0.015
LS^2	-0.33	0.23	-1.4	0.2	-0.22	1.43	-0.16	0.88
pH × Et	0.62	0.31	2.03	0.072	***4.31***	1.87	2.31	0.046
pH × LS	-0.07	0.31	-0.2	0.81	-3.58	1.87	-1.92	0.087
Et × LS	0.27	0.31	0.89	0.39	1.63	1.87	0.88	0.4
	ANOVA Yield				**ANOVA Uronic acid**			
	df	MS	F	p	df	MS	F	p
Regression	9	***5.13***	6.80	0.0043	9	***328.3***	11.76	0.0006
Lack of fit	5	0.61	0.65	0.6772	5	39.4	2.92	0.1606
Pure error	4	0.93			4	13.5		
Total SS	18				18			

Values in bold and italics are significant at $\alpha = 0.05$ with 9 degrees of freedom.

The Pareto's charts were used as a tool to graphically observe the significance and importance of the tested variables on the yield and uronic acid content of pectins and are depicted in the Fig. 1.

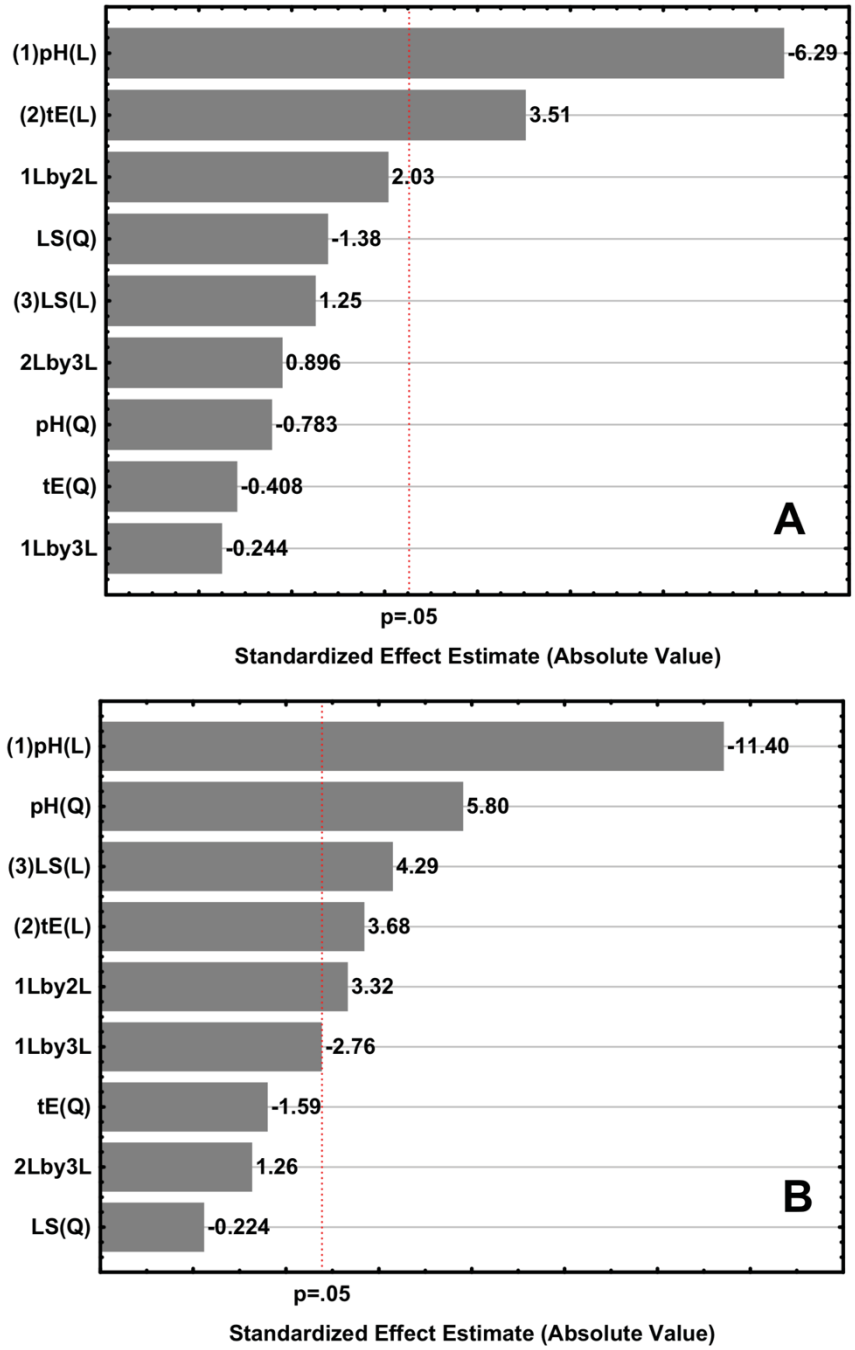


Fig. 1 – Pareto's chart showing the significance of the variables of the quadratic models for the (A) yield and (B) uronic acid content of the pectins extracted from Chardonnay grape pomace. L = linear model; Q = quadratic model.

The results of the analysis of variance (ANOVA) presented in Table 2 show that both models was set at $p < 0.05$ for the regression and $p > 0.05$ for lack of fit. The coefficients of determination (R^2) for uronic acid and yield responses were 87.71 and 90.60, respectively.

3.3. Factors affecting the yield

The yield of pectins extracted from the Chardonnay grape pomace varied between 5.4% and 10.9%, as shown in Table 1. From the Table 2, it can be seen that the yield of pectin was affected by the linear effect of the pH followed by the extraction time, but not by the liquid:solid ratio. No interactive or quadratic effects were significant. In the study carried out by Minjares-Fuentes et al. (2014) evaluating the effect of temperature, extraction time and pH in the ultrasound-assisted extraction of pectin from Cabernet Sauvignon grape pomace, the results showed that both the linear as the quadratic effect of the pH was significant, but the coefficient of the linear effect was positive, contrary to what was observed in this study. However, the range of pH tested by the authors (1.0 – 2.0) was lower than the tested in the present work (1.66 – 3.34), and it may be the reason of the difference in the effect of pH, since although pH reduction usually increases the yield of pectins, extremely acidic media may decrease the yield due to hydrolysis of the polymer, which hence does not precipitate with the addition of ethanol (Emaga et al., 2008).

As shown in the Pareto's chart depicted in Fig. 1-A, the pH was the variable with the major impact on the yield. The negative effect of the pH (-1.48, Table 2) indicates an inversely proportional relationship between the pH of the solvent and the yield as a response, i.e. among the pH range tested, the more acid the extraction medium the greater the yield achieved (Fig.1). Insoluble pectins are more prone to undergo hydrolysis in more acidic media, increasing the pectin recovery from plant material (Hosseini et al., 2016). The same trend was observed for pectin extracted from sugar beet pulp (Levigne et al., 2002; Yapo et al., 2007), apple pomace (Wang et al., 2007), orange peel (Maran et al., 2013) and banana peels (Emaga et al., 2008).

Extraction time was the second most influent variable on yield, as shown in Fig.1-B. The Et coefficient of 0.83 (Table 2) demonstrates that the yield is positively affected by the extraction time, as illustrated in Fig.1, and increase with the increasing of the Et. Jafari et al. (2017) commented that the increase in yield as a result of the increase in extraction time is probably due to the longer reaction time, allowing a greater mass transfer from the particles to the solvent. The extraction time was also significant and directly related to the yield of pectins from Cabernet Sauvignon grape pomace (Minjares-Fuentes et al., 2014). Pectins from sugar beet pulp (Yapo et al., 2007), banana peels (Emaga et al., 2008), passion fruit peel (Seixas et al., 2014), dragon fruit peel (Muhammad et al., 2014) and *Citrus medica* peel (Pasandide et al., 2017) also had the yield increased by the increase in the extraction time.

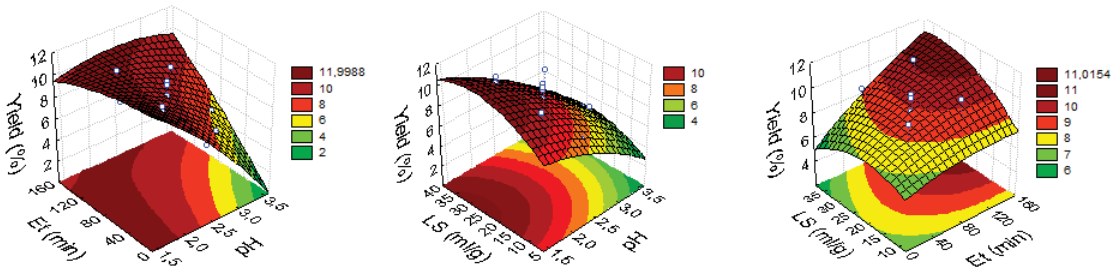


Fig. 2 - Response surfaces showing the effect of the variables pH, extraction time (Et) and liquid:solid ratio (LS) on the yield for pectins extracted from Chardonnay grape pomace.

3.4. Factors affecting the uronic acid (UA) content

The uronic acid content is an important feature for pectin quality. In fact, only pectins containing >65% of GalA meet the regulations for commercial purposes (Willats et al., 2006).

In the present work, the amount of UA in pectins extracted from the Chardonnay grape pomace varied between 14.8% and 62.3% (Table 1). The linear effect of all the studied variables was significant for the UA content, as well as the quadratic effect of the pH and the interaction pH x Et (Table 2).

As shown in Pareto's chart (Fig 1-B), the pH was the most influent variable on the UA content predominantly by its linear followed by its quadratic effect. The negative coefficient of the linear effect of pH (Table 2) shows that a decrease in pH causes an increment in the UA content. This behavior is illustrated in the response surfaces (Fig. 3), where it is notable that although the quadratic effect of pH has been also significant, the UA content was enhanced with higher acid strength. Oliveira et al. (2016) observed the same trend for extraction of pectin from banana peels using citric acid and applying a CCD where the effect of pH, temperature and extraction time was evaluated. They observed that both linear and quadratic effect of pH was significant to the uronic acid content, causing an inflection on the surface but reaching higher values at lower pH levels. Emaga et al. (2008) also extracted pectins from banana peels, however they found that the increase in pH resulted in higher levels of GalA.

Both the extraction time and the liquid:solid ratio had directly proportional effects to the UA, increasing UA the longer the time and proportion of the solvent employed, as demonstrated by the coefficients in Table 2. When Liu et al. (2010) investigated the effect of HCl concentration, extraction time and liquid:solid ratio on the extraction of mulberry bark pectins, they observed that the UA tended to increase using higher extraction times and higher

liquid:solid ratio. Similar results were reported for pectins extracted from cocoa husks (Chan and Choo, 2013).

The increment in the UA caused by lower pH, longer extraction times and higher liquid:solid ratios can be understand as a result of a combination of better extraction capacity and concomitant degradation of the pectin chains. At the same time that more acidic medium makes the chains bound to the cell wall more prone to hydrolysis, longer extraction times promote more mass transference to the solvent whereas the higher liquid:solid ratio facilitates the solubilization. In the same way that affect the yield, the low pH and long reaction time also cause further release of the side chains of pectins, augmenting the uronic acid content (Chan and Choo, 2013; Oliveira et al., 2016).

The interaction pH × Et means that the simultaneous increase or decrease in pH and in extraction time results in higher levels of uronic acid, as illustrated in Fig. 3.

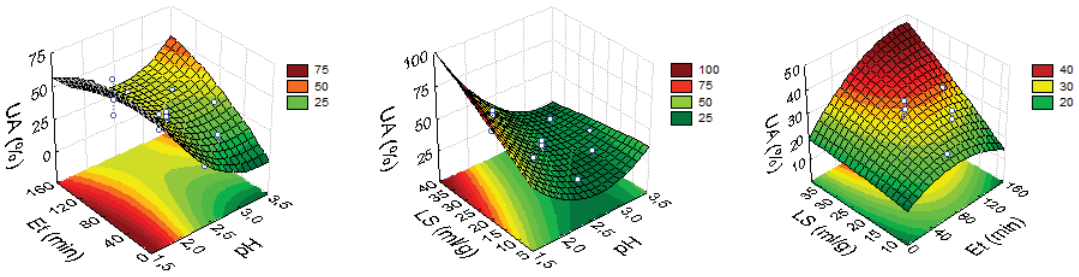


Fig. 3 - Response surfaces showing the effect of the variables pH, extraction time (Et) and liquid:solid ratio (LS) on the uronic acid content for pectins extracted from Chardonnay grape pomace.

3.5. Definition of the optimal conditions

The optimum conditions to obtain the pectin with the highest yield and the highest uronic acid content were determined by applying the simultaneous analysis of the responses using the desirability function of Derringer and Suich (1980). The analysis showed that the optimal conditions were pH=2.08, Et=135.23 min and LS=35.11 mL/g, resulting in theoretical yield of 12.8% and UA of 64.4%.

3.6. Characterization of the GPOP

The pectin extracted under the optimal conditions (GPOP) was characterized. The data regarding its yield, composition and structural characteristics are presented in Table 3.

Table 3 – Yield, composition and structural characteristics of GPOP and its subfraction α-GPOP.

		GPOP	α-GPOP
	Yield (%)	11.1 ± 0.4 [a]	74.8 [b]
Monosaccharide composition (%)	Rha	2.7 ± 0.2	7.8 ± 0.3
	Fuc	0.2 ± 0.0	1.1 ± 0.4
	Ara	2.9 ± 0.1	6.0 ± 0.5
	Xyl	2.5 ± 0.3	1.9 ± 0.7
	Man	2.2 ± 0.04	3.5 ± 0.0
	Gal	7.2 ± 0.2	13.6 ± 0.3
	Glc	25.5 ± 0.04	2.5 ± 0.4
	GalA	56.8 ± 0.3	63.5 ± 1.1
	DM	43.3 ± 1.5	18.1 ± 0.4
	Acetyl content (%) / DA (%)	5.1 ± 0.2 / 0.4 ± 0.02	4.3 ± 0.3 / 0.3 ± 0.02
	HG (%)	-	55.7
	RG-I (%)	-	35.2
	(Ara+Gal)/Rha (%)	-	2.5
	Phenolics (%)	3.6 ± 0.4	2.8 ± 0.04
	Proteins (%)	nd	nd
	M_w (g/mol)	1.597×10^5	1.541×10^5

[a] Based on AIR. [b] Based on GPOP.

nd = not detected.

Neutral monosaccharides were determined by GLC as alditol acetates. GalA was measured by colorimetric method and identified by HPAEC-PAD. DM The degree of methylesterification (DM) was determined by FT-IR. Acetyl content and phenolics was determined by colorimetric methods, and degree of acetylation (DA) was calculated as described by Colodel *et al*. (2017). M_w (average molar mass) was determined by HPSEC/MALLS. The percentage of HG and RG-I was calculated as described by M'sakni et al. (2006).

The experimental yield of GPOP was 11.1%, lower than the predicted 12.8%. This yield is close to optimized yield obtained from some fruits wastes, such as cacao pod husks (10.1%) (Vriesmann et al., 2012) and banana peel (9.02% - 14.2%) (Maran et al., 2017; Oliveira et al., 2016). With respect to the uronic acid content, the experimental value was 56.8%, lower than the 64.4% predicted by the model and it does not attend to the minimum required to commercial applications.

The results obtained in the present study differ greatly from those reported by Minjares-Fuentes et al. (2014), who obtained an optimized pectin with 34% yield with 96% GalA from Cabernet Sauvignon pomace. However, the differences may lie in the extraction

method and in the composition of the grape pomace used in the two studies. Minjares-Fuentes et al. (2014) employed ultrasound assisted extraction, and it has already been shown that ultrasound-assisted extractions may produce higher yields compared to conventional techniques (Xu et al., 2014; Wang et al., 2015). Furthermore, the Cabernet Sauvignon pomace used by the authors is described as being composed only of peels and seeds, while the Chardonnay pomace used in the present study also contained a large amount of stalks.

Some previous studies have shown that the cell wall of skins of different grape varieties contains galacturonic acid as the major monosaccharide, followed by arabinose and galactose, evidencing the high content of pectic components (Lecas and Brillouet, 1994; Vicens et al., 2009; Deng et al., 2011; Apolinar-Valiente et al., 2015). On the other hand, stalks are composed mainly of cellulose (30.3% – 37.88%), lignin (17.4% - 39.6%) and hemicelluloses (14.93% – 24.5%) but there is no pectin in its composition (Spigno et al., 2008; Ping et al., 2011; Prozil et al., 2012). Thus, the presence of stalks can reduce the yield of pectin as well as decrease the content of uronic acids due to the possible co-extraction of other components.

The monosaccharide composition of GPOP (Table 3) shows that the major component is pectin, due to the presence of GalA as the major monosaccharide besides some galactose, rhamnose and arabinose, which are characteristic of pectic polysaccharides. However, the presence of a considerable amount of glucose evidenced the co-extraction of a glucan, which revealed to be starch by the Lugol test.

Accordingly, the HPSEC elution profile of GPOP (Fig. 4) shows a bimodal profile, suggesting the presence of two distinct polysaccharide families. A predominant peak was identified at ~ 47 min, probably corresponding to the pectin, whereas a peak of lower intensity, probably corresponding to the starch, appeared in ~ 59 min.

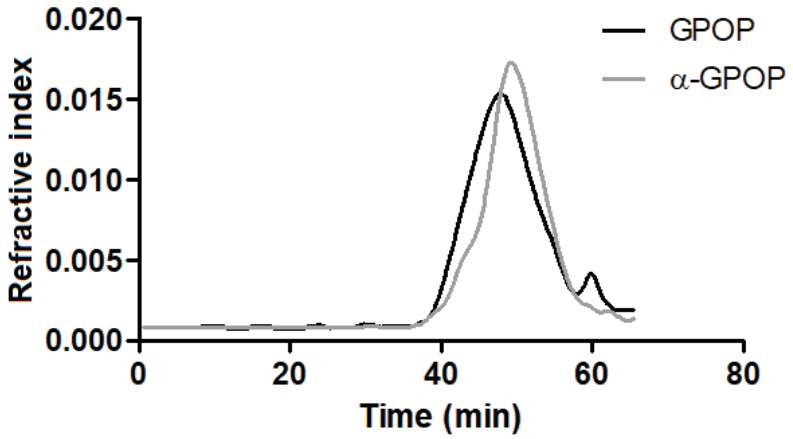


Fig. 4 – Comparison between the elution profile by HPSEC of the crude GPOP (black line) and the starch-free α-GPOP (gray line).

The average molar mass relative to the peak at ~ 47 min was calculated as 1.597×10^5 g/mol. This value is lower than that of the optimized pectin obtained from the Carbernet Sauvignon pomace (207.000 g/mol), but is within the range of values obtained in the different conditions of the experimental design (109.630 – 203.300 g/mol) (Minjares-Fuentes et al., 2014).

The DM of GPOP, as determined by FT-IR, was 43.3%, characterizing it as low methoxyl (LM) pectin. As for the molar mass, the DM of the GPOP was lower than that of the optimized pectin obtained from the Cabernet Sauvignon pomace (64.1%), but it is in great agreement with the values found for pectins obtained under the different conditions of the experimental design (16.57% – 57.47% with an average value of 41.3%) (Minjares-Fuentes et al., 2014).

GPOP exhibited a low DA (0.4%), lower than pectins from apple (5%) and citrus (1.4% - 1.6%) (Leroux et al., 2003), which are common sources of commercial pectins. Highly acetylated pectins, such as from sugar beet, have low gelling ability and it has been shown that removal of the acetyl groups improves the ability of pectins to form gels (Matthew et al., 1990).

Although grape pomace is rich in phenolic compounds (Özkan et al., 2004), GPOP had a low phenolic content, only 3.6%, and protein was not detected.

3.7. Characterization of the α-GPOP

Once the presence of starch as contaminant was identified in GPOP, a treatment with α-amylase and amyloglucosidase was carried out, resulting in the subfraction named α-GPOP. The elution profile of α-GPOP by HPSEC (Fig. 4) showed the disappearance of the peak

eluted at ~59 min in GPOP chromatogram, confirming it was due to starch. After the enzymatic treatment the glucose content was reduced from 25.5% to 2.5% (Table 3). As a result, the proportion of the monosaccharides typical of pectins was increased. The rhamnose content increased from 2.7% to 7.8%; of arabinose, from 2.9% to 6.0%; galactose from 7.2% to 13.6% and galacturonic acid from 56.8% to 63.5%, close to the value of 65% needed for commercial purposes.

A great reduction (-58.2%) in the degree of esterification occurred after the enzymatic treatment, resulting in a DM of 18.1%. In the conditions of pH and temperature used in the enzymatic treatment, pectins may undergo demethylation and β-elimination and these reactions prove to be competitive (Renard and Thibault, 1996). As there was only a slight reduction (-3.5%) in molar mass, from 1.597×10^5 g/mol to 1.541×10^5 g/mol, it suggests that there was no considerable degradation of the chains. Possibly this absence of degradation is due to the low DM of GPOP, since methyl esterification is required for β-elimination reaction to occur (Kravtchenko et al., 1992; Renard and Thibault, 1996).

The bidimensional ^{1}H-^{13}C HSQC NMR analysis was used to obtain information regarding the structure of α-GPOP (Fig. 5).

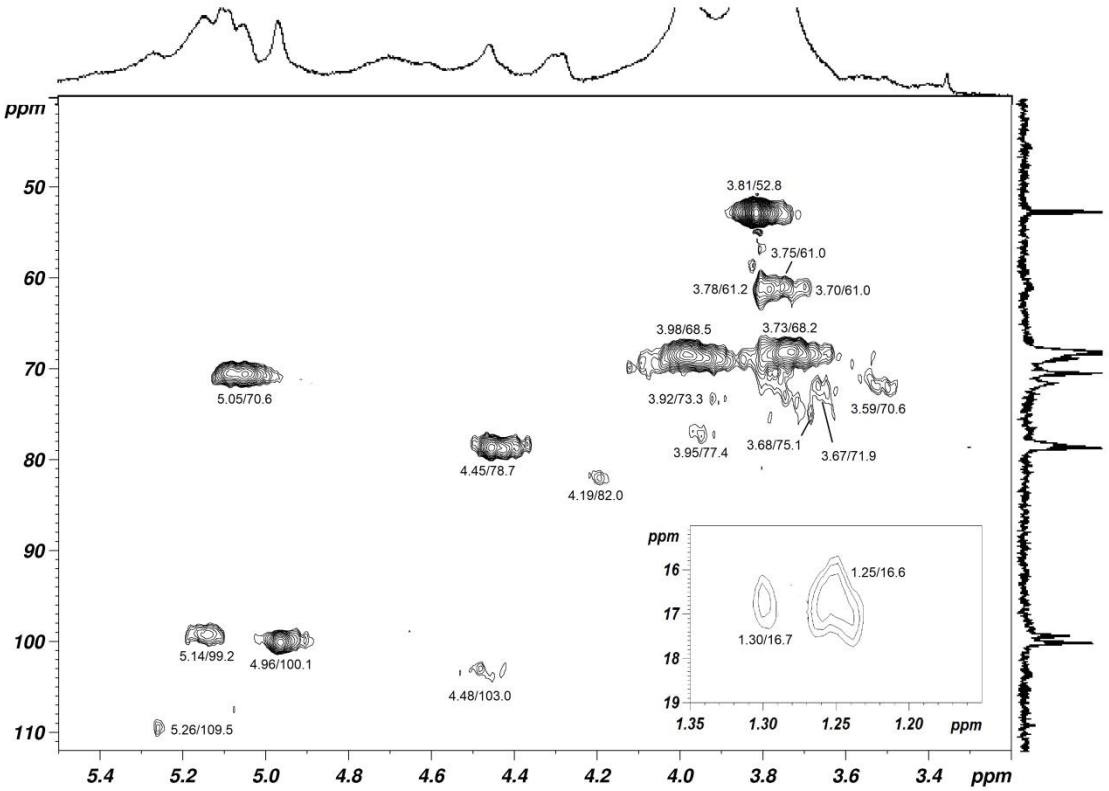


Fig. 5. ^{1}H-^{13}C HSQC NMR correlation map of α-GPOP in D_2O at 70ºC.

Only the anomeric signal from the non-esterified →4)α-D-GalA*p*(1→ units were identified at δ4.96/100.1, corroborating the low DM of α-GPOP, although the signal attributed to the CH_3-C6 from methyl-esterification was found at δ3.81/52.8. The H2/C2 – H5/C5 correlations of →4)α-D-GalA*p*(1→ was found at δ3.73/68.2, δ3.98/68.5, δ4.45/78.7 and δ5.05/70.6, respectively (Petersen et al., 2008).

The anomeric signal of →2)α-L-Rha*p*(1→ units appeared at δ5.14/99.2, and the signals related to H6/C6 of unsubstituted →2)α-L-Rha*p*(1→ and to branched →2,4)α-L-Rha*p*(1→ units from RG-I backbone were found at δ1.25/16.6 and δ1.30/16.7, respectively (Ovodova et al., 2005). The higher intensity of the H6/C6 signal from →2,4)α-L-Rha*p*(1→ compared to →2)α-L-Rha*p*(1→ demonstrates that the RG-I domain of α-GPOP is highly branched by side chains. Concerning these side chains, they seem to be composed of single units or very short chains of galactose and arabinose, since only the signals of terminal units of these monosaccharides were observed. *t*-α-L-Ara(1→ units were identified by the correlations at δ5.26/109.5, δ4.19/82.0, δ3.95/77.4 and δ3.78/61.2, attributed to H1/C1, H2/C2, H3/C3 and H5/C5, respectively (Habibi et al., 2004), whereas the correlations at δ4.48/103.0, δ3.59/70.6, δ3.67/71.9, δ3.92/73.3, δ3.68/75.1 and δ3.70-3.75'/61.0 were identified, respectively, as the H1/C1 – H6-H6'/C6 of the *t*-β-D-Gal(1→ units (Golovchenko et al., 2007).

The results obtained by the analysis of ^{1}H-^{13}C HSQC NMR are in great agreement with the ratio of HG (55.7%), RG-I (35.2%) and the low proportion of side chains calculated by the ratio (Ara + Gal)/Rha (2.5%) found in the α-GPOP. These data suggest that most of the pectin is composed of regions of HG, but it contains a large proportion of RG-I, and the side chains present in these regions are in fact short.

3.8. Cytotoxic effect on macrophages and nitric oxide production

Plant polysaccharides are very promising candidates as therapeutics to improve the immune response. Several plant-isolated polysaccharides have been shown to have potent immunomodulatory activity. Compared to polysaccharides extracted from bacteria and synthetic compounds, they have the advantage of low toxicity and few side effects (Schepetkin and Quinn, 2006). Popov and Ovodov (2013) reviewed the immunomodulatory properties of pectins isolated from different plants in Russia and northern Europe and found a relationship between the structure and immunomodulatory effect of pectins. They concluded that pectins containing <75% of GalA have an immunostimulatory effect, that the RG-I

domain is the most responsive region to immunostimulation and that the structure of the side chains of the RG-I region play an important role in stimulating the immune response. Regarding the pectin side chains, linear chains of arabinans and galactans appear to be important for immunostimulation (Popov and Ovodov, 2013).

Considering the review by Popov and Ovodov (2013), α-GPOP has structural features that meet the two main requirements for immunostimmulation, i.e, less than 75% of GalA and high proportion of RG-I domains (35.2%). In the present study its ability to stimulate NO production by mouse peritoneal macrophages was evaluated.

Initially, the effect of α-GPOP on viability of mouse peritoneal macrophages was evaluated. As can be seen in the Fig. 6-A, none of the concentrations tested (100-500 µg/mL) was cytotoxic to the macrophages compared to the control.

Upon confirmation of non-toxicity of α-GPOP, the ability to stimulate NO production by macrophages was tested. The results displayed in the Fig. 6-B demonstrate that α-GPOP increased the macrophage secretion of NO relative to the control, mainly at lower concentrations (50 – 250 µg/mL), which had statistically the same stimulatory effect as LPS, used as a positive control.

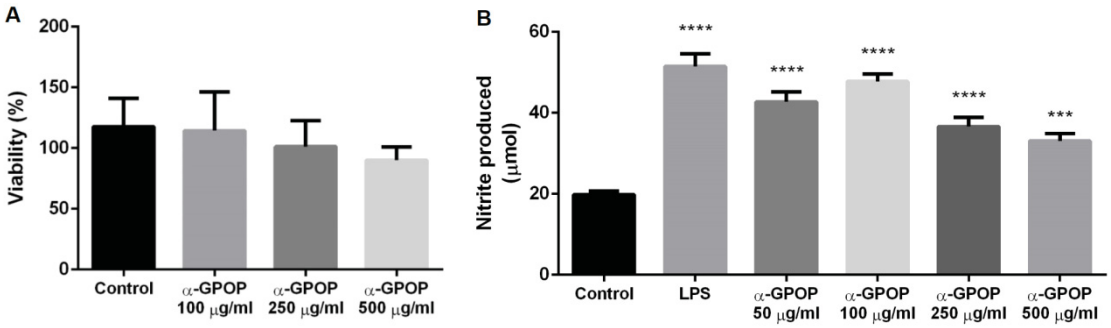


Fig. 6 – Effect of α-GPOP on (A) viability and (B) nitric oxide production in peritoneal macrophages. Results are expressed as mean ± SD (n = 2, each experiment in duplicate), ANOVA $p < 0.05$ significant difference from the control (medium) group. *** $p < 0.001$.

Differently from the pectins described by Popov and Ovodov (2013) with long and, in some cases, bulky side chains, α-GPOP presented only terminal units of α-L-Ara→ and β-D-Gal→, indicating the presence of very short side chains. Nevertheless, α-GPOP has been shown to be capable of stimulating macrophages to produce NO. It is possible that the immunostimulatory effect of α-GPOP is due to the non-reducing residues of arabinose and galactose present in the short side chains. In fact, Zhang et al. (2012) and Li et al. (2018)

demonstrated that the removal of arabinose at non-reducing terminal positions in the side chains of RG-I pectin from the roots of *Panax ginseng* and from the pollen of *Nelumbo nucifera* led to a decreasing in the ability of stimulate NO production by peritoneal macrophages, evidencing that these residues play an important role on the immunostimmulatory activity of pectins. α-GPOP presented slightly higher efficiency than these two pectins, resulting in 48 μmol of NO produced when the cells were treated with 100 μg/mL sample, whereas at the same concentration, *Panax ginseng* and *Nelumbo nucifera* pectins produced ~40 μmol and ~34 μmol of NO, respectively (Zhang *et al.,* 2012; Li *et al.,* 2018).

Conclusions

Under the optimized extraction conditions (pH=2.08, Et=135.23 min and LS=35.11 mL/g), the Chardonnay grape pomace provided a pectin with yield of 11.1% and 56.8% galacturonic acid, and even after the enzymatic treatment for removal of starch, the galacturonic acid content was 63.5%, lower than required for commercial pectins. However, the optimized pectin α-GPOP displayed interesting biological effects, being non-cytotoxic at the tested concentrations (100-500 μg/mL) and stimulating NO secretion by macrophages at concentrations 50-500 μg/mL, which suggest immunostimulatory properties. Further investigation is needed to prove the immunostimulatory effects of GPOP.

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CAPÍTULO 6

Testes de remoção de espécies metálicas potencialmente tóxicas em meio aquoso pelas pectinas otimizadas obtidas da casca de ponkan (PPOP) e do bagaço de uva (αGPOP)

TESTES DE REMOÇÃO DE ESPÉCIES METÁLICAS POTENCIALMENTE TÓXICAS EM MEIO AQUOSO PELAS PECTINAS OTIMIZADAS OBTIDAS DA CASCA DE PONKAN (PPOP) E DO BAGAÇO DE UVA (α-GPOP)

INTRODUÇÃO

Espécies metálicas potencialmente tóxicas, tais como íons de cádmio, zinco, cobre, níquel, chumbo, mercúrio e cromo, são lançadas ao ambiente por meio de diversas atividades humanas. Pesticidas, herbicidas e fertilizantes usados na agricultura, processos envolvidos na mineração e refinamento de minérios, fundição, fabricação e descarte de baterias, lançamento de efluentes e rejeitos domésticos e industriais, tintas e pigmentos e até mesmo atividades militares são fontes de contaminação por espécies metálicas potencialmente tóxicas (NGAH; HANAFIAH, 2008; NAGAJYOTI; LEE; SREEKANTH, 2010; RAHMAN et al., 2016).

Estas espécies metálicas contaminam o solo e a água e como não se degradam no ambiente, acumulam-se causando impactos negativos nos ecossistemas e na saúde dos seres vivos (NGAH; HANAFIAH, 2008; CAROLIN et al., 2017). Por este motivo, é muito importante que estes íons metálicos sejam removidos e diversas metodologias já foram desenvolvidas para este fim, cada uma apresentando vantagens e desvantagens (CAROLIN et al., 2017).

Uma destas metodologias baseia-se no uso de sistemas capazes de sorver as espécies metálicas carregadas. Entre os biosorventes mais investigados, encontram-se materiais provenientes de fontes vegetais, como, por exemplo, sementes, folhas, cascas e bagaços (JAIN; MALIK; YADAV, 2016), e os grupos carboxílicos presentes nas pectinas, carregados negativamente, são apontados como um dos principais sítios de interação entre as espécies metálicas e o biosorvente (PATHAK; MANDAVGANE; KULKARNI, 2015).

Diversos sistemas contendo pectinas já demonstraram boa eficiência na remoção de espécies metálicas, entre eles, beads de xerogéis (MATA et al., 2009; JAKÓBIK-KOLON et al., 2017; LESSA et al., 2017) e hidrogéis (MATA et al., 2009; GUILHERME et al., 2010).

A literatura científica descreve também diversos estudos que avaliaram a capacidade de ligação de metais pesados a pectinas isoladas (PASKINS-HURLBURT et al., 1977; DRONNET et al., 1996; KARTEL, KUPCHIK, VEISOV, 1998; KHOTIMCHENKO, KOVALEV, KHOTIMCHENKO, 2007; BALARIA, SCHIEWER, 2008; KHOTIMCHENKO, KOLENCHENKO, KHOTIMCHENKO, 2008; MATA et al., 2010; SCHIEWER, IQBAL, 2010; WAI, ALKARKHI, EASA, 2010; CELUS et al., 2017). De

maneira geral, pectinas de baixo grau de metil esterificação, que apresentam mais grupos carboxílicos negativamente carregados, apresentam maior eficiência de interação com as espécies metálicas, demonstrando que esta interação ocorre principalmente por interações eletrostáticas (KHOTIMCHENKO, KOVALEV, KHOTIMCHENKO, 2007; BALARIA, SCHIEWER, 2008; KHOTIMCHENKO, KOLENCHENKO, KHOTIMCHENKO, 2008; SCHIEWER, IQBAL, 2010; WAI, ALKARKHI, EASA, 2010; CELUS et al., 2017).

A maior parte dos estudos que avaliaram a capacidade de remoção de espécies metálicas por pectinas utilizaram Pb^{2+} (PASKINS-HURLBURT et al., 1977; KARTEL, KUPCHIK, VEISOV, 1998; KHOTIMCHENKO, KOVALEV, KHOTIMCHENKO, 2007; BALARIA, SCHIEWER, 2008; MATA et al., 2010; WAI, ALKARKHI, EASA, 2010). Outros metais divalentes também foram testados, tais como Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Ca^{2+}, Fe^{2+} e Co^{2+} (DRONNET et al., 1996; KARTEL, KUPCHIK, VEISOV, 1998; KHOTIMCHENKO, KOLENCHENKO, KHOTIMCHENKO, 2008; MATA et al., 2010; SCHIEWER, IQBAL, 2010; WAI, ALKARKHI, EASA, 2010; CELUS et al., 2017). Com base nos seus resultados, Dronnet et al. (1996) propuseram a seguinte ordem de seletividade: $Cu^{2+} \sim Pb^{2+} >> Zn^{2+} > Cd^{2+} \sim Ni^{2+} \geq Ca^{2+}$, em ordem descrescente de afinidade, que difere ligeiramente da ordem de seletividade proposta por Kartel, Kupchik e Veisov (1998): $Pb^{2+} >> Cu^{2+} > Co^{2+} > Ni^{2+} >> Zn^{2+} > Cd^{2+}$. Apesar da divergência entre os dois estudos, é um consenso entre as investigações de que o Pb^{2+} e o Cu^{2+} são os metais que apresentam maior afinidade pela pectina, fato corroborado também em outras investigações (MATA et al., 2010; WAI, ALKARKHI, EASA, 2010).

Alguns estudos sugerem que a capacidade das pectinas de se ligar a metais pesados pode ser aplicada na área médica, como agentes de desintoxicação (PASKINS-HURLBURT et al., 1977; KOHN, 1982; KARTEL, KUPCHIK, VEISOV, 1998; KHOTIMCHENKO, SERGUSCHENKO, KHOTIMCHENKO, 2006; WAI, ALKARKHI, EASA, 2010), enquanto outros estudos propõem o uso de pectinas para a remoção de metais tóxicos de efluentes industriais (DRONNET et al., 1996; KHOTIMCHENKO, KOVALEV, KHOTIMCHENKO, 2007; BALARIA, SCHIEWER, 2008; KHOTIMCHENKO, KOLENCHENKO, KHOTIMCHENKO, 2008; MATA et al., 2010).

Considerando a habilidade das pectinas de interagir com espécies metálicas potencialmente tóxicas e de removê-las do meio aquoso, esta etapa do trabalho teve como objetivo avaliar a capacidade da PPOP, isolada da casca de ponkan conforme descrito no artigo "Extraction of pectin from ponkan (*Citrus reticulata* Blanco *cv. Ponkan*) peel: Optimization and structural characterization" (COLODEL et al., 2018) e da α-GPOP, isolada

do bagaço de uva Chardonnay conforme descrito no artigo "Optimization of acid-extraction of pectin from grape (*Vitis vinifera cv. Chardonnay)* pomace, a winery waste" (ainda não publicado), ambos apresentados anteriormente nos capítulos 3 e 5, respectivamente, de remover as espécies Cu^{2+} e Pb^{2+} em solução.

MATERIAIS E MÉTODOS

As concentrações das soluções de espécies metálicas tóxicas foram baseadas na Resolução CONAMA 430/2011 (CONAMA, 2011). Para a realização dos testes de remoção de espécies metálicas, 40 mg de pectina (PPOP, α-GPOP ou pectina cítrica CP Kelco DM = 30%, usada como referência) foram solubilizados em 10 mL de H_2O destilada em tubos falcon de 50 mL e o pH foi ajustado para 6,0 com NaOH 0,1 M. Foram preparadas soluções estoque de Pb^{2+} na concentração de 10 mg/L usando $Pb(NO_3)_2$ e de Cu^{2+} na concentração de 20 mg/L usando $Cu(NO_3)_2.3H_2O$, e ambas foram ajustadas até pH 6,0 com HNO_3 0,1 M. 10 mL da solução estoque de Pb^{2+} ou Cu^{2+} foram então adicionados ao tubo falcon, resultando em concentração final da espécie metálica de, respectivamente, 5 mg/L e 10 mg/L e de pectina de 2 g/L. Os tubos foram então mantidos sob agitação de 180 rpm por 2h. Em seguida, as soluções foram centrifugadas a 5000 rpm por 15 min a 25ºC e o sobrenadante foi analisado por espectrometria de emissão atômica por plasma acoplado indutivamente (ICP-OES) em um espectrômetro Thermo Scientific ICAP 6500, operando com visão axial, radiofrequência de 40 MHz e potência de 1150 W, vazão do gás do plasma de 12 L/min, vazão do gás auxiliar de 1 L/min e vazão do gás no nebulizador de 0,7 L/min, com nebulizador concêntrico. As linhas analíticas utilizadas foram de 327, 395 nm para o Cu^{2+} e 220,353 nm para o Pb^{2+}. Os reagentes utilizados foram HNO_3 65% (Merck) e solução padrão contendo 1000 mg/L de Cu^{2+} e de Pb^{2+} (UltraScientific) em água deionizada. A curva analítica multielementar, incluindo o branco, foi preparada em meio de HNO_3 1% (v/v) na faixa de concentração de 0,010 mg/L a 2,0 mg/L. Todas as análises foram realizadas em triplicata.

RESULTADOS E DISCUSSÃO

Conforme a legislação brasileira, definida pela Resolução CONAMA 430/2011, os limites aceitáveis de concentração de Pb^{2+} e Cu^{2+} para o lançamento de efluentes são,

respectivamente, 0,5 mg/L e 1,0 mg/L (CONAMA, 2011). Foram utilizadas concentrações arbitrárias de Pb^{2+} e Cu^{2+} 10 vezes maiores que o limite permitido pela legislação. A Fig. 1 apresenta os resultados dos testes de remoção destes metais pela pectina otimizada da casca da ponkan (PPOP), pela pectina otimizada da casca de uva e tratada com α-amilase (α-GPOP) e pela pectina LM cítrica comercial. Observa-se que, nas condições testadas, nenhuma das pectinas foi capaz de remover nem o Pb^{2+} e nem o Cu^{2+} do meio aquoso, visto que os resultados foram iguais para as duas espécies metálicas comparadas ao controle, sem adição de pectinas.

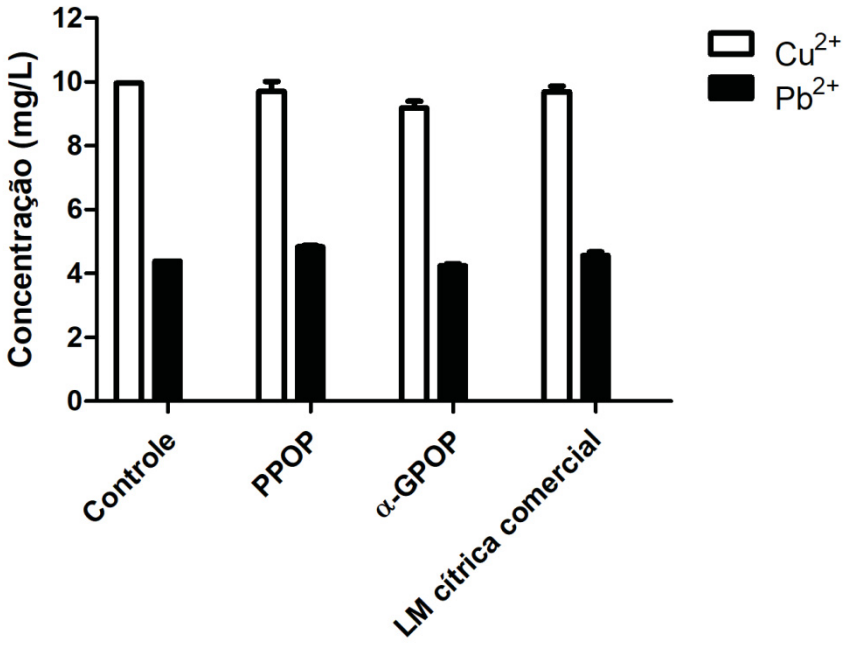


Fig. 1 – Concentração de Cu^{2+} (barras brancas) e Pb^{2+} (barras pretas) após o tratamento com diferentes pectinas, comparadas ao controle (sem tratamento). Todas as análises foram realizadas em pH 6.

Considerando-se os dados descritos na literatura e os resultados obtidos experimentalmente, pode-se sugerir que a ineficiência demonstrada pelas pectinas testadas em ligar-se ao Pb^{2+} e Cu^{2+} deve-se à baixa concentração inicial das espécies metálicas. Os trabalhos que obtiveram resultados positivos de remoção de espécies metálicas tóxicas utilizaram concentrações iniciais de Pb^{2+} entre 20 mg/L (BALARIA, SCHIEWER, 2008), e 2000 mg/L (WAI, ALKARKHI, EASA, 2010), respectivamente 4 e 400 vezes mais altas que a concentração de Pb^{2+} utilizada neste estudo, e de Cu^{2+} entre 100 mg/L (MATA et al., 2010) e 630 mg/L (WAI, ALKARKHI, EASA, 2010), respectivamente 10 e 63 vezes mais altas que a concentração de Cu^{2+} utilizada neste estudo. As concentrações de pectina relatadas na

literatura variam de 0,1 g/L (BALARIA, SCHIEWER, 2008) e 2 g/L (KHOTIMCHENKO, KOVALEV, KHOTIMCHENKO, 2007), similar à concentração usada neste estudo, que foi de 2 g/L.

Levando em conta os padrões estruturais das pectinas PPOP, α-GPOP e LM cítrica comercial e a relação já estabelecida na literatura entre o grau de metil-esterificação e a capacidade de sorção de espécies metálicas, esperava-se que ao menos a α-GPOP, com DM = 18,1% e a LM cítrica comercial, com DM = 30%, fossem capazes de ligar-se às espécies metálicas. Como a α-GPOP não demonstrou habilidade de interagir com Ca^{2+} nos testes de formação de gel, a pectina LM cítrica comercial foi inserida no experimento como um controle positivo, pois foi eficiente no teste de gelificação com Ca^{2+}, evidenciando possuir estrutura favorável à interação com cátions bivalentes. No entanto, nenhuma das pectinas testadas foi eficiente na remoção dos íons metálicos, sugerindo que tal ineficiência não se deve a características químicas e estruturais das pectinas.

CONCLUSÃO

Diante dos resultados observados, pode-se sugerir que as pectinas não foram eficientes nos testes de remoção de espécies metálicas nas condições testadas, devido à baixa concentração destas espécies, o que dificulta a interação com a pectina e impede a formação de ligações estáveis que permitam a formação de um complexo que possa ser removido do meio aquoso.

CAPÍTULO 7

Sorção de corantes catiônicos pelo bagaço de uva (*Vitis vinifera cv. Chardonnay*)

SORÇÃO DE CORANTES CATIÔNICOS PELO BAGAÇO DE UVA (*Vitis vinifera cv. Chardonnay*)

INTRODUÇÃO

Os corantes são utilizados em diversos setores, como a indústria de tintas, de papel, e principalmente na indústria têxtil. Estes setores geram grandes volumes de efluentes, através dos quais os corantes podem causar séria contaminação. Mínimas concentrações de corante são suficientes para causar coloração visível na água, tornando-a imprópria para consumo. Além de ser prejudiciais aos seres vivos devido à toxicidade, a presença de corantes em ambientes aquáticos pode prejudicar a absorção de luz e desequilibrar os ecossistemas. Portanto, a remoção de corantes dos efluentes é de extrema importância (SALLEH et al., 2011).

Existem diversos métodos para remoção de corantes em meio aquoso, tais como coagulação/floculação, biodegradação, troca iônica, oxidação, separação por membranas e adsorção em carvão ativado, porém, estes métodos possuem limitações como: produção de grande quantidade de lodo ao final do processo, baixa eficiência, seletividade, alto custo energético ou econômico. Neste contexto, a utilização de resíduos agroindustriais como biosorventes para a remoção de corantes vem se apresentando uma boa alternativa, uma vez que são de baixo custo, muitos vêm mostrando boa capacidade de adsorção e ainda contam com a possibilidade de serem reutilizados com eficiência após vários ciclos de adsorção/dessorção (CIRINI, 2006; YAGUB et al., 2014).

Considerando os resultados obtidos anteriormente e descritos no capítulo 5, que demonstraram que o bagaço de uva Chardonnay contém pectinas de baixo DM, podendo estar negativamente carregadas, dependendo do pH, e que resíduos agroindustriais na sua forma natural costumam ser mais eficientes na sorção de corantes catiônicos do que para outros tipos de corantes, conforme aponta Salleh et al. (2011), a capacidade de sorção do bagaço de uva Chardonnay foi testada para cinco diferentes corantes catiônicos. Os corantes catiônicos ou básicos são empregados principalmente na indústria têxtil e possuem coloração bastante intensa, de grande visibilidade mesmo quando em concentrações muito baixas (YAGUB et al., 2014). Embora alguns tenham aplicações na área da saúde, como por exemplo, o cristal violeta, o verde malaquita e o azul de metileno, que possuem efeitos antibacterianos e antifúngicos (MALEY, ARBISER, 2013; ALDERMAN, 1985; WAINWRIGHT, CROSSLEY, 2002), a exposição frequente a estes compostos pode ser muito prejudicial à

saúde tanto humana quanto à biota aquática, pois apresentam efeitos mutagênicos, carcinogênicos, e poderem causar disfunções no sistema reprodutivo e nervoso (SALLEH et al, 2011; YAGUB et al., 2014). Portanto, é de grande importância que sejam removidos do ambiente aquático e das águas para consumo.

MATERIAIS E MÉTODOS

O bagaço de uva Chardonnay foi coletado, liofilizado, moído e peneirado conforme descrito no capítulo 5 e foi nomeado como GPP (do inglês, *grape pomace powder*). A capacidade de biosorção do GPP foi avaliada para os corantes catiônicos: azul de metileno, cristal violeta, safranina, auramina O e verde malaquita, cujas estruturas estão representadas na Fig. 1 enquanto a Tabela 1 apresenta algumas de suas propriedades.

Tabela 1 – Identificação, massa molar e comprimento de onda de absorção máxima dos corantes catiônicos utilizados nos testes de sorção pelo GPP.

Corante	**Color Index (CI)**	**Massa molar (g/mol)**	**Absorção máxima (λ_{max}, nm)**
Auramina O	41000	303,85	432
Azul de metileno	52015	319,90	665
Cristal violeta	42555	407,99	585
Safranina	50240	350,85	518
Verde malaquita	42000	364,90	617

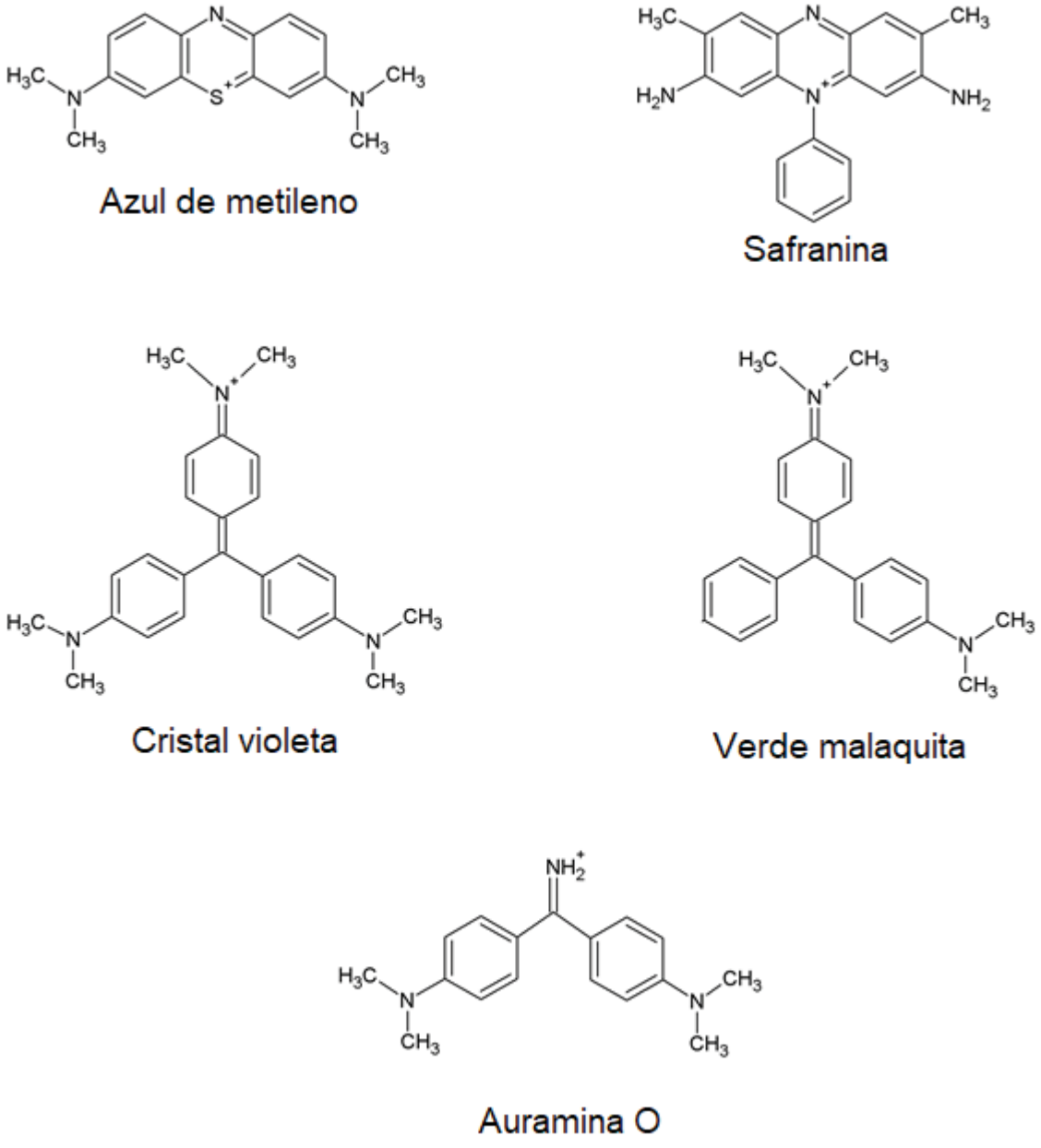


Fig. 1 – Estrutura química dos corantes catiônicos utilizados nos testes de sorção pelo GPP. FONTE: A AUTORA.

Para a determinação do ponto de carga zero do GPP, foram preparados tubos contendo 50 mL de solução de KCl 0,1 M em diferentes pHs na faixa de 2 a 10. Em cada tubo cujo pH havia sido previamente ajustado foi adicionado 50 mg do GPP. Os tubos foram agitados em vórtex e mantidos em repouso a 20ºC por 24 h e em seguida o pH do sistema foi medido. O ponto de carga zero foi definido como o valor de pH onde não houve diferença entre o pH inicial e o pH final. A análise foi realizada em triplicata para cada pH.

Para avaliar o efeito do pH na sorção dos corantes pelo GPP, foram preparadas soluções dos corantes em concentração de 100 mg/L e o pH foi ajustado para valores entre 2 e 10 usando NaOH 0,1 M ou HNO_3 0,1 M. 1 mL da solução do corante foi adicionado a eppendorfs contendo 10 mg de GPP e estes foram submetidos à agitação de 140 rpm por 120 min a 20ºC.

Para avaliar o efeito da concentração inicial do corante, foram preparadas soluções dos corantes em concentrações variando entre 100 e 1000 mg/L, que foram ajustadas ao pH 6 com HNO_3 0,1 M. 1 mL da solução do corante foi adicionado a eppendorfs contendo 2 mg de GPP, que foram submetidos à agitação de 140 rpm por 120 min a 20ºC.

Para avaliar o efeito do tempo de agitação, 1 mL da solução do corante em pH 6 foi adicionado a eppendorfs contendo 2 mg de GPP, que foram submetidos à agitação de 140 rpm a 20ºC em tempos variando entre 1 e 240 min. Para os corantes azul de metileno, cristal violeta e verde malaquita, foi utilizada solução com concentração inicial de 800 mg/mL, enquanto que para os corantes safranina e auramina O foi empregada concentração inicial de 300 mg/mL.

Após cada experimento, as amostras foram centrifugadas a 8000 rpm por 15 minutos e a absorbância lida em espectrofotômetro. Foram efetuadas diluições quando necessário. Curvas de calibração foram construídas para cada pH testado em faixa de concentração de 1 a 20 mg/L. A capacidade de sorção (q_e, mg/g) foi calculada pela equação abaixo, onde C_i (mg/L) é a concentração inicial do corante, C_f (mg/L) é a concentração final do corante após a sorção, V (L) é volume da solução de corante empregado e m (g) é a massa de GPP empregada:

$$q_e = \frac{(C_i - C_f) \, . \, V}{m \, (g)}$$

RESULTADOS E DISCUSSÃO

Para avaliar o efeito do pH na capacidade de remoção dos corantes pelo GPP, os experimentos foram realizados na faixa de pH de 2 a 10. Como pode ser observado na Fig. 2, em pH 2 todos os corantes testados tiveram a menor eficiência de remoção. Isso pode estar relacionado à protonação das pectinas presentes no bagaço de uva, uma vez que pectinas apresentam pKa variável entre 3 e 4 (SRIAMORNSAK et al., 2007). Assim, em pH acima de

3-4, as pectinas encontram-se negativamente carregadas, sendo capazes de interagir com espécies de carga positiva, como é o caso dos corantes catiônicos. Outro fenômeno que pode reduzir a sorção dos corantes em pH 2 é a competição dos íons H+ do meio pelos sítios de sorção (SAEED, SHARIF, IQBAL, 2010).

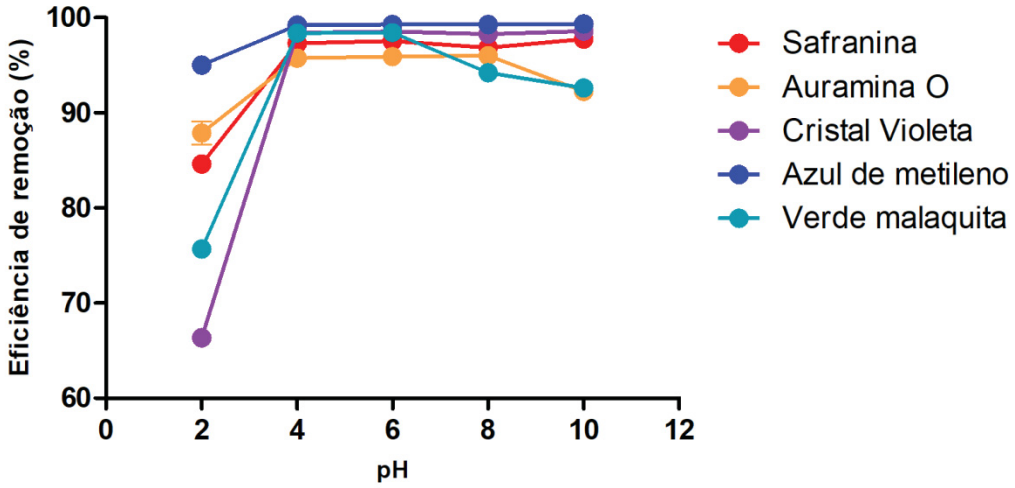


Fig. 2 – Efeito do pH do meio na eficiência da remoção dos corantes safranina, auramina O, cristal violeta, azul de metileno e verde malaquita (100 mg/L) pelo bagaço de uva Chardonnay (10 mg). FONTE: A AUTORA.

Os resultados dos experimentos de remoção dos corantes em diferentes pHs são bastante concordantes com os resultados obtidos no experimento de determinação de ponto de carga zero, apresentados na Fig. 3. No gráfico mostrado na Fig. 3, nota-se que o GPP não possui carga em valores de pH menores que 4, uma vez que o pH não foi alterado durante o tempo de contato do material com a solução de KCl 0,1 M. Por outro lado, em pH > 4, o GPP passa a apresentar carga negativa, acidificando o meio em relação ao pH inicial da solução por meio da liberação de íons H^+ (desprotonação).

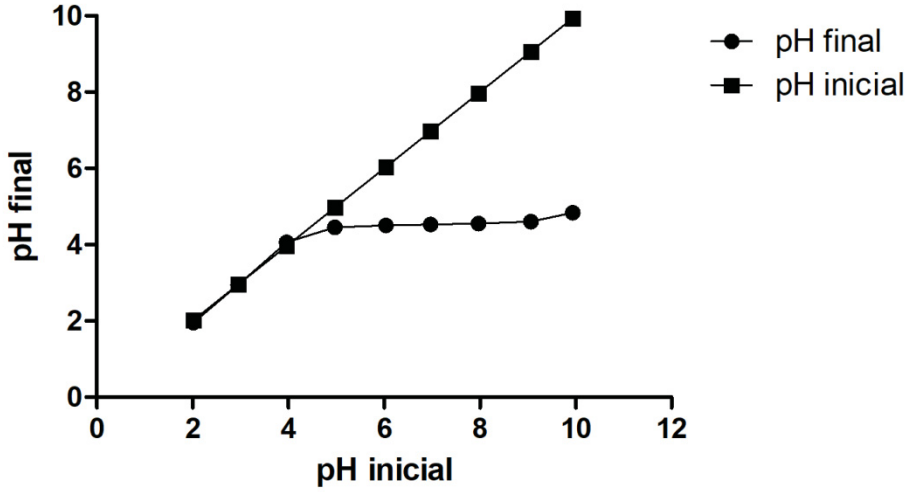


Fig. 3 – Determinação do ponto de carga zero do bagaço de uva Chardonnay (GPP). Os pontos representados pelos quadrados indicam a correlação linear entre o pH inicial e o pH final caso não houvesse alteração do pH por interferência do GPP, enquanto os círculos representam o valor final do pH após 24 h de contato do GPP com a solução de KCl 0,1 M.

É notável que existem diferenças entre os comportamentos dos diferentes corantes avaliados conforme a variação do pH. Pode-se observar que o azul de metileno, o cristal violeta e a safranina mantiveram sua eficiência máxima de remoção ao longo de toda a faixa de pH entre 4 e 10, alcançando 99%, 98% e 97% de eficiência, respectivamente. Este comportamento foi bastante semelhante ao obtido por Gong et al. (2005), avaliando a capacidade de sorção de três corantes catiônicos (azul de metileno, vermelho neutro e azul de cresil brilhante) em cascas de amendoim, sendo que a máxima sorção foi obtida e mantida em pH de 4 – 11. O mesmo perfil também foi observado para o resíduo de girassol utilizando cristal violeta como adsorvato (HAMEED, 2008). No entanto, para a auramina O e para o verde malaquita, a faixa de pH de máxima eficiência foi mais restrita. Para a auramina O, a eficiência máxima, de 96%, foi alcançada apenas na faixa entre 4 e 8, reduzindo em pHs mais básicos, em concordância com o pKa de 9,8, acima do qual a molécula passa ao seu estado neutro, desprotonado (FIDELES, 2017). Já para o verde malaquita, a eficiência máxima foi de 99%, e foi alcançada apenas na faixa de pH de 4 a 6, o que é condizente com o seu comportamento em meio aquoso, onde em pH 4, encontra-se 100% ionizado, em pH 6,9, 50%, em pH 7,4, apenas 25% e em pH 10,1 a totalidade das moléculas encontram-se na forma não-ionizada (ALDERMAN, 1985).

Como em pH 6 a máxima eficiência de remoção foi alcançada para todos os corantes, todos os demais experimentos foram realizados neste pH. Foram realizados ensaios para testar o efeito da concentração inicial do corante, e os resultados estão ilustrados na Fig. 4. Para

todos os corantes, foi observado um aumento na quantidade de corante adsorvido por unidade de adsorvato com o aumento da concentração do corante, até se observar um platô, sugerindo que a saturação dos sítios de sorção do GPP foi alcançada. No entanto, isso se deu em diferentes proporções para os diferentes corantes, e a concentração de saturação para cada corante teve um valor específico.

A auramina O e a safranina apresentaram a menor concentração de saturação, atingindo o platô a partir de 400 mg/L. Para o cristal violeta, o azul de metileno e o verde malaquita, a saturação foi alcançada na concentração de 800 mg/L. Embora a concentração de saturação para os três corantes tenha sido a mesma, o verde malaquita apresentou uma relação linear entre a concentração inicial do corante e a capacidade máxima de sorção na faixa de concentração de 100 a 800 mg/L, após a qual atingiu a saturação, enquanto o azul de metileno e o cristal violeta descreveram uma hipérbole linear, com perfis muito semelhantes.

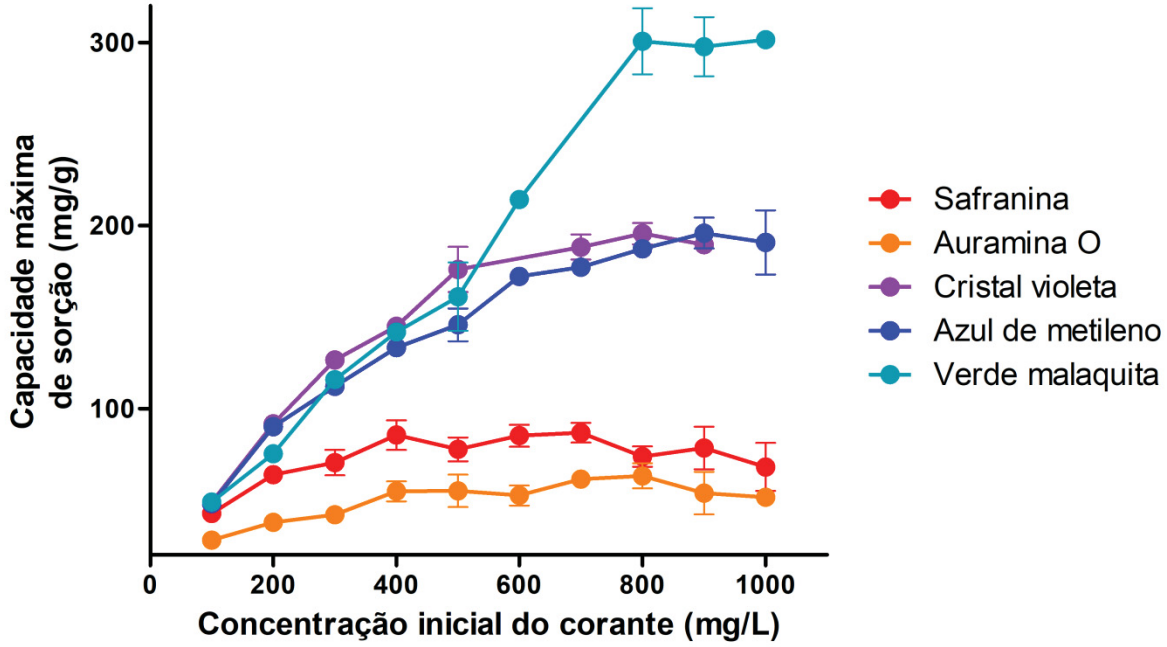


Fig. 4 – Efeito da concentração inicial do corante na capacidade sorção dos corantes safranina, auramina O, cristal violeta, azul de metileno e verde malaquita pelo bagaço de uva Chardonnay (2 mg) em pH 6. FONTE: A AUTORA.

Para a avaliação do efeito do tempo de contato na sorção dos corantes pelo bagaço de uva Chardonnay, foram utilizadas as concentrações de saturação determinadas pelo experimento anterior, em tempos de 1 a 240 min. A Fig. 5 ilustra os resultados obtidos. Assim

como nos testes variando a concentração dos corantes, foram observados comportamentos semelhantes entre a auramina O e a safranina, e entre o cristal violeta e o azul de metileno, enquanto o verde malaquita apresentou comportamento distinto, destacando-se dos demais. Para todos os corantes, o aumento no tempo de contato levou ao aumento na capacidade de sorção, até atinge um platô indicando a saturação do GPP. Para todos os corantes, a saturação foi atingida em 120 min, exceto para a auramina O, para a qual a saturação se deu em 60 min.

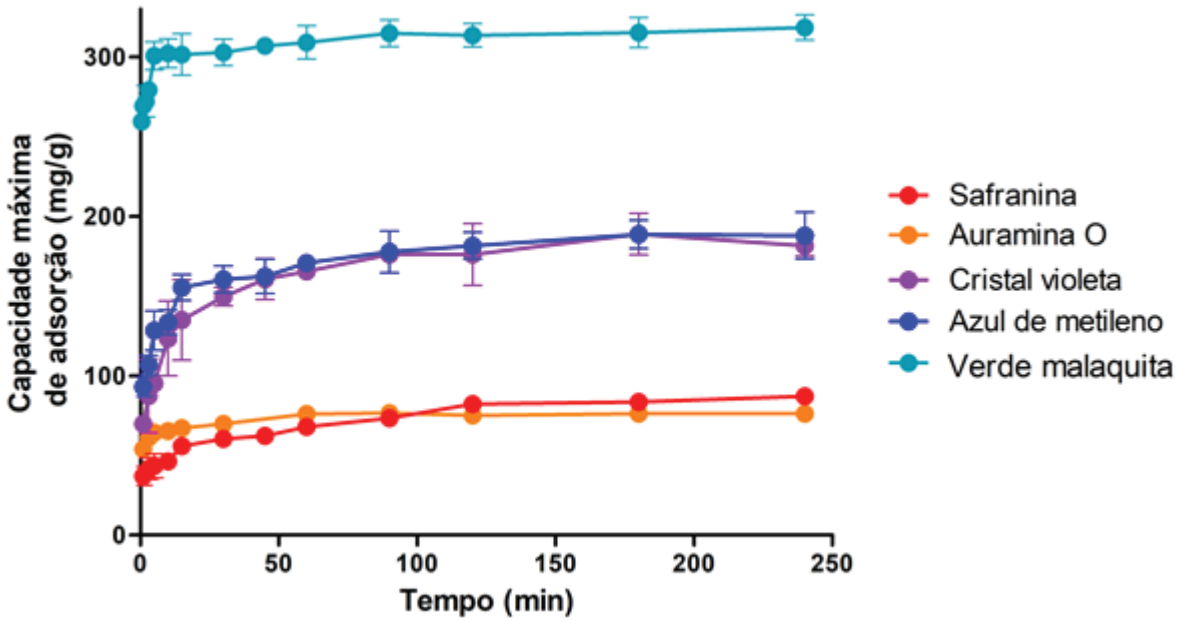


Fig. 5 – Efeito do tempo de contato na sorção dos corantes safranina, auramina O, cristal violeta, azul de metileno e verde malaquita pelo bagaço de uva Chardonnay (2 mg), em pH 6 e usando a concentração de saturação máxima de cada corante. FONTE: A AUTORA.

A capacidade máxima de sorção dos corantes pelo GPP foi determinada pela quantidade de corante adsorvido (mg) por grama de GPP na condição de equilíbrio após a saturação ser atingida. A capacidade máxima de sorção para cada corante pode ser visualizada de maneira comparativa na Fig. 6.

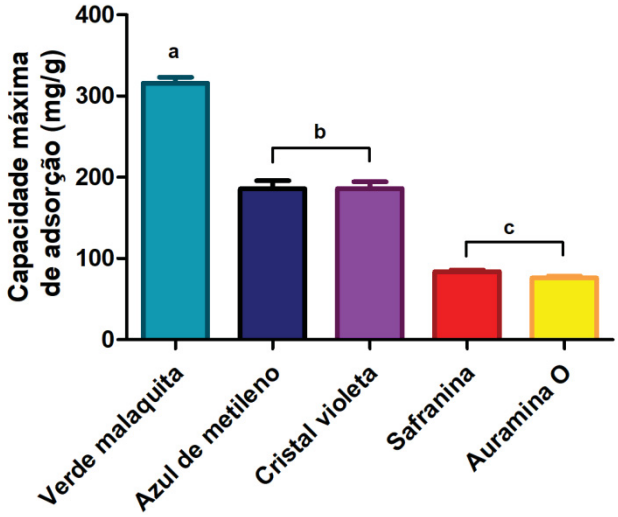
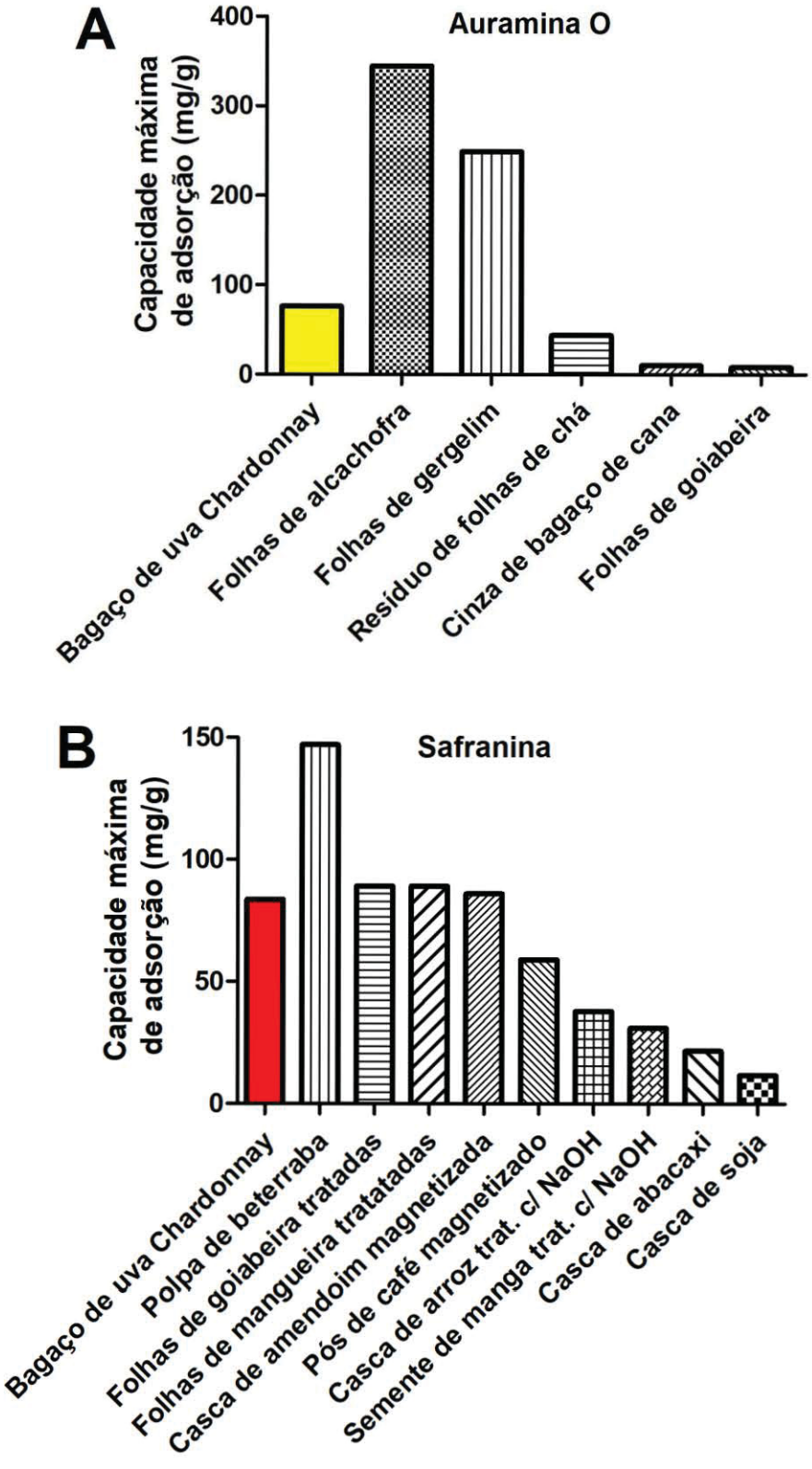


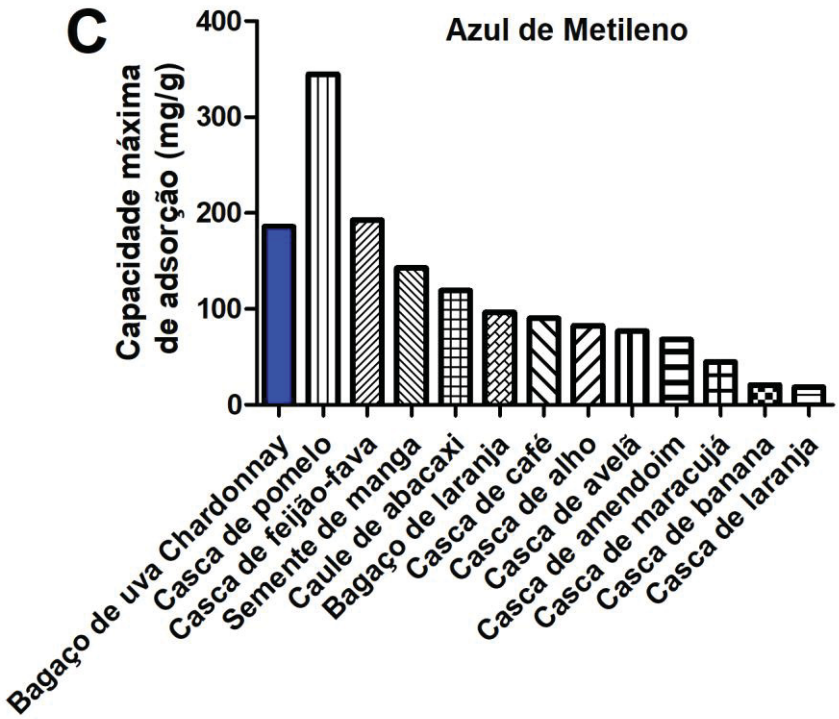
Fig. 6 – Capacidade máxima de sorção pelo GPP para os corantes verde malaquita, azul de metileno, cristal violeta, safranina e auramina O. As letras a, b e c referem-se à análise estatística, onde letras diferentes indicam diferença significativa a $p<0,05$ (one-way ANOVA seguido de teste de Tukey). FONTE: A AUTORA.

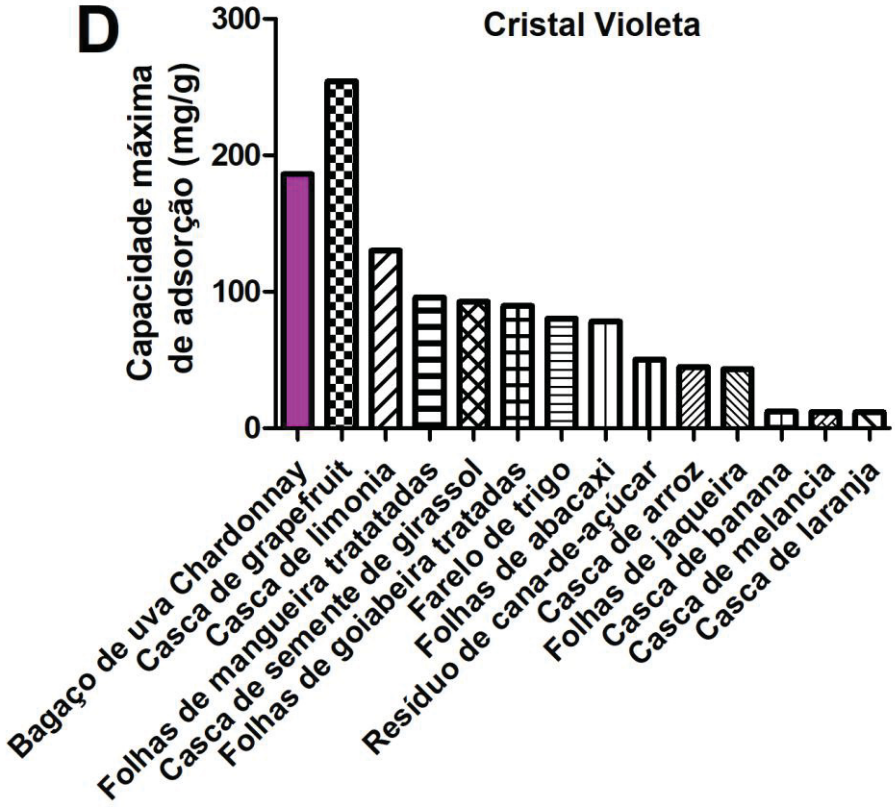
Para os corantes auramina O e safranina, a capacidade máxima de sorção foi de, respectivamente, 76,0 mg/g e 82,3 mg/g. Estes valores foram muito semelhantes e não apresentaram diferença significativa de acordo com a análise estatística. Para o azul de metileno e o cristal violeta, os valores foram de 177,6 mg/g e 160,5 mg/g, respectivamente, e também não apresentaram diferença significativa entre si. A mais alta capacidade de sorção, de 307,0 mg/g, foi observada para o verde malaquita.

Os dados apresentados na Fig. 7, onde a capacidade de sorção do GPP para os corantes testados é comparada a outros resíduos agroindustriais, evidenciam a alta eficiência do GPP como adsorvente, visto que este material mostrou desempenho comparável e na maioria dos casos superior a diversos outros resíduos, inclusive a resíduos que passaram por tratamentos químicos buscando o melhoramento das suas capacidades adsortivas (GONG et al., 2006; HAMEED, EL-KHAIARY, 2008; DAHRI, KOOH, LIM, 2014; AHMAD, KUMAR, 2010; BANERJEE et al., 2016; SONAWANE, SHRIVASTAVA, 2009; KUMAR, 2007; KOÇER, ACEMIOGLU, 2015; KUSHWAHA, GUPTA, CHATTOPADHYAYA, 2014; PARIMALADEVI, VENKATESWARAN, 2011; GUPTA, KUSHWAHA, CHATTOPADHYAYA, 2016; GUECHI, HAMDAOUI, 2016; MALEKBALA et al., 2012a; SWAMI, BUDDHI, 2006; SAFARIK, SAFARIKOVA, 2010; SAFARIK et al., 2012; CHOWDHURY et al., 2012; MALEKBALA et al., 2012b; MOHAMMED, IBRAHIM, SHITU, 2014; CHANDANE, SINGH, 2014; GAIKWAD, KINLDY, 2009; MALL,

SRIVASTAVA, AGARWAL, 2007; KHOSLA, KAUR, DAVE, 2013; LIU et al., 2013; LARIBI, SAHMOUNE, 2016; YAGUB et al., 2014; BHARATHI, RAMESH, 2012).




C
Azul de Metileno
Capacidade máxima de adsorção (mg/g)
400
300
200
100
0
Bagaço de uva Chardonnay
Casca de pomelo
Casca de feijão-fava
Semente de manga
Caule de abacaxi
Bagaço de laranja
Casca de café
Casca de alho
Casca de avelã
Casca de amendoim
Casca de maracujá
Casca de banana
Casca de laranja


D
Cristal Violeta
Capacidade máxima de adsorção (mg/g)
300
200
100
0
Bagaço de uva Chardonnay
Casca de grapefruit
Casca de limonia
Folhas de mangueira tratatadas
Casca de semente de girassol
Folhas de goiabeira tratadas
Farelo de trigo
Folhas de abacaxi
Resíduo de cana-de-açúcar
Casca de arroz
Folhas de jaqueira
Casca de banana
Casca de melancia
Casca de laranja

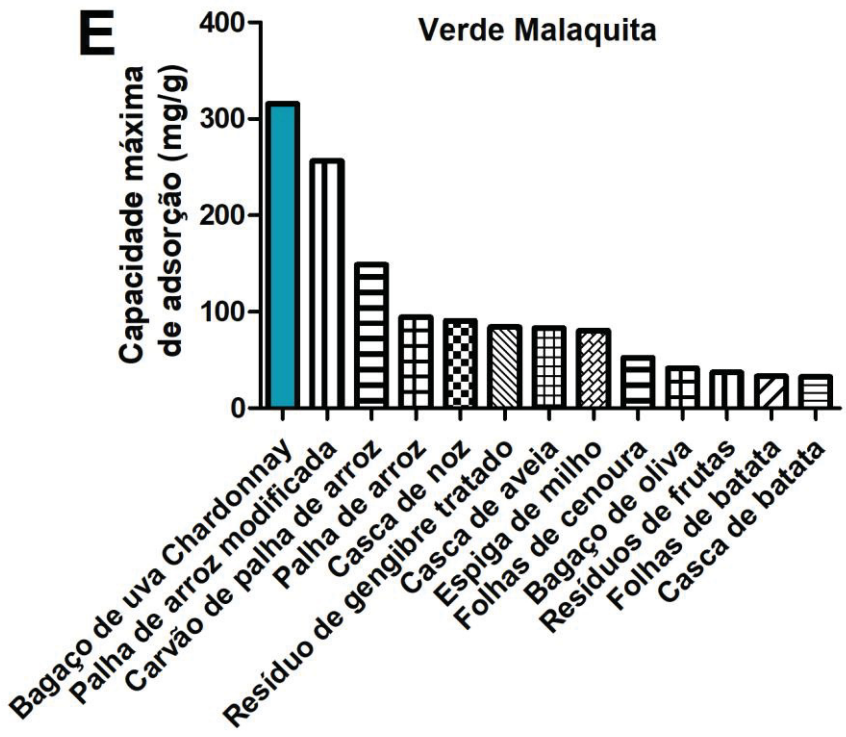


Fig. 7 – Comparação da capacidade de sorção do GPP e de outros adsorventes provenientes de resíduos agroindustriais descritos da literatura para os corantes (A) auramina O; (B) safranina; (C) azul de metileno; (D) cristal violeta e (E) verde malaquita. FONTE: A AUTORA.

CONCLUSÃO

Os resultados obtidos demonstram que o bagaço de uva Chardonnay apresenta um grande potencial de aplicação como bioadsorvente para remoção de corantes em meio aquoso. Isso é vantajoso do ponto de vista econômico, pois o direcionamento de um resíduo, que costuma ser simplesmente descartado nas lavouras como adubo, para fins mais nobres gera um retorno financeiro. Além disso, a alta eficiência de remoção de corantes do bagaço de uva Chardonnay em seu estado natural representa custos muito menores comparados a outros adsorventes que precisam passar por tratamentos prévios para melhorar sua capacidade adsortiva. Também do ponto de vista ambiental existem vantagens, pois agregando valor financeiro ao resíduo, espera-se que ele seja direcionado a fins mais úteis e deixe de representar um potencial poluente em épocas de safra, além do próprio resíduo também poder ser utilizado para a remediação de efluentes poluídos por corantes.

Mais experimentos precisarão ser realizados para uma investigação mais aprofundada a respeito dos mecanismos de sorção para cada corante, tais como a identificação

dos grupos funcionais envolvidos no processo de sorção e a determinação dos modelos cinéticos adequados.

5 CONSIDERAÇÕES FINAIS

Os resultados obtidos neste trabalho demonstram que a casca da ponkan é fonte de pectinas com características estruturais diversas, dependendo do método de extração e purificação aplicados. A extração ácida fervente usando solução de HNO_3 em proporção de 36 mL/g de material em pH 1,6 por 100 minutos fornece pectina com características estruturais e propriedades reológicas semelhantes às pectinas cítricas comerciais, evidenciando que a casca de ponkan pode ser utilizada para a produção industrial de pectinas nas regiões onde o fruto é produzido em grande escala. Extrações aquosas a frio e a quente proporcionaram frações pécticas com composição e DM semelhantes, mas quando purificadas mostraram-se bastante discrepantes quanto às suas características estruturais e propriedade de estimulação de produção de NO por macrófagos.

O bagaço de uva Chardonnay mostrou não ser apropriado para a produção de pectinas para uso comercial, pois a pectina obtida por extração ácida, mesmo após remoção de contaminantes (amido), não alcançou o teor mínimo de GalA de 65%. Por outro lado, a pectina obtida deste material contém alta proporção de RG-I, favorável para aplicações biológicas, tais como as MCPs, e os resultados dos testes de estimulação de produção de NO por macrófagos sugerem que ela possui propriedade imunoestimulatória. Além disso, o bagaço bruto demonstrou alta capacidade de sorção de corantes catiônicos em meio aquoso, sugerindo que este material tem potencial uso como material de baixo custo para tratamento de efluentes contendo esta classe de corantes.

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ANEXO 1 – DIREITOS AUTORAIS PARA PUBLICAÇÃO DO ARTIGO "CELL WALL POLYSACCHARIDES FROM PONKAN MANDARIN (*Citrus reticulata* Blanco *cv. Ponkan*) PEEL" NA TESE

ANEXO 2 – DIREITOS AUTORAIS PARA PUBLICAÇÃO DO ARTIGO "EXTRACTION OF PECTIN FROM PONKAN (*Citrus reticulata* Blanco *cv. Ponkan*) PEEL: OPTIMIZATION AND STRUCTURAL CHARACTERIZATION" NA TESE

ANEXO 3 – DIREITOS AUTORAIS PARA PUBLICAÇÃO DO ARTIGO "RHEOLOGICAL CHARACTERIZATION OF A PECTIN EXTRACTED FROM PONKAN (*citrus reticulata* Blanco *cv. Ponkan*) PEEL" NA TESE