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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ALEXSANDRA CONCEIÇÃO APOLINÁRIO

OBTENÇÃO DE PRODUTOS DE INTERESSE FARMACÊUTICO A PARTIR DO *Agave sisalana* PERRINE ex ENGELM.: UMA PROPOSTA DE REVITALIZAÇÃO DA CULTURA SISALEIRA NA PARAÍBA

CAMPINA GRANDE, MARÇO DE 2014

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Prof. Jose Alexsandro da Silva-Orientador

Prof. Adalberto Pessoa Junior/PPGCF/UEPB
Examinador interno

Profa. Carlota de Oliveira Rangel Yagui/USP/
Examinador externo

DEDICATÓRIA

Aos meus pais, Maria e Assis, com meu amor incondicional.

Porque me ensinaram com exemplos, me mostraram como agir não com palavras, mas com atitudes e, sobretudo, me fizeram enxergar o que eu poderia ser sem me dizer para ser.

As lições de compartilhar a qualquer momento o que temos de melhor com o próximo, de ver o lado bom de tudo, de gostar do cheiro de gente simples, de gostar de ir onde poucos gostam de ir, de ouvir e falar sobre a alegria ou dor de quem precisar, de ter sempre uma ação de levar esperança, sorriso e atenção a quem precisar, eu vi todos os dias diante de meus olhos na minha casa em vocês.

O melhor prazer da vida, o de alimentar alma daquilo que não se compra, mas se planta eu compreendi graças à humildade e sabedoria de vocês.

Epígrafe

Redigir uma dissertação de mestrado me levou a questionamentos que a ciência não me responderá, mas o tempo já me dá sinais que podem ser respostas. Em meio a gráficos, tabelas, *papers* eu entendi que esta tarefa poderia ser executada por qualquer pessoa, com acesso a bases científicas, com um bom programa estatístico e só um pouco de esforço, não precisaria de nada de especial para se chegar a uma dissertação concluída, qualquer outro em meu lugar seria capaz de fazê-la... Eu me perguntei: daqui há alguns anos o que vai me diferenciar entre tantos mestres em Ciências Farmacêuticas que o Programa de Pós-Graduação em Ciências Farmacêuticas da UEPB formará? E dentre tantos outros no nordeste e no Brasil? Já faz um tempo que entendi que não é preciso perfil ou dom ou dotes especiais para fazer pós-graduação... qualquer pessoa pode fazer mestrado e doutorado, será preciso apenas uma oportunidade em algum momento e um pequeno esforço que será possível adentrar em um programa ou até mesmo para alguns apenas sorte. E fazer uma boa pesquisa? Também não é preciso ser gênio, apenas usufruir das oportunidades comuns que temos hoje no Brasil, hoje há parcerias de intercâmbios acadêmicos nacionais e internacionais, há fomentos de diferentes fontes, há bolsas de estudos, verba do Programa de Apoio à Pós-Graduação (PROAP), enfim não há desculpas para não se fazer pelo menos um trabalho razoável. Eu mesma tive a oportunidade de usufruir de um Programa de Cooperação Acadêmica (PROCAD) com a USP, mas não foi apenas eu, foi eu e todos os meus colegas que quisessem usufruir disto... Mais uma vez vem o questionamento: o que me diferencia? Não estou falando em ser melhor, porque tenho convicção que todos nós temos algo de melhor e isso nos torna igual em termos sempre um ponto forte rodeado de pontos fracos... Estou tentando entender como daqui há alguns anos eu irei ter convicção de que tudo que fiz valeu à pena e de que não serei mais um igual a tantos que fizeram por fazer. Vi-me assim durante toda a escrita, dois anos de trabalho estavam no meu computador transformado em números e eu teria a missão juntar tudo aquilo em textos que deveriam explicar o que eu fiz, além disso, eu teria que conectar dados e ai estavam o dilema, meus dados são resultados de trabalhos feitos em lugares e tempos diferentes e auxiliados por pessoas diferentes, como conectar informações construídas em espaços tão longes? Foi aí que vi que não seria difícil fazer isso, os softwares e bases científicas me ajudariam, o difícil foi durante essa montagem de quebra-cabeça relembrar cada passo dado em cada momento deste trabalho, porque foram muitos... Começar do zero, refazer tudo de novo e de novo, improvisar quase tudo, precisar e precisar muito de ajuda do outro, ter ajuda do outro, porque fui acolhida e auxiliada por quem nem conhecia, ajudar também, pois passar por um período tão curto, mas tão intenso sem ajudar alguém, sem ceder um pouco de nosso melhor é realmente assustador e digo ajudar sem receber nada em troca, porque trocar nomes em publicações ou outra coisa do tipo é apenas uma forma inteligente de usar as pessoas sem se sentir culpado... E o que dizer dos momentos de buscar soluções e às vezes criar estas soluções? Entendi que a bancada é soberana, ela é mais forte que qualquer método teórico e sendo assim me conduz e seria muita prepotência minha acreditar que poderia iniciar uma pesquisa e chegar ao fim dela obtendo exatamente o que eu imaginei obter, mas seria muito mais

sábio e excitante deixar a natureza da pesquisa me guiar e me levar para onde nem imaginaria chegar e enxergar inesperadamente a beleza do NOVO. Isso foi capaz de me fazer voar longe e longe, pois eu vi que todos podem criar, deixar modelos, propagar o novo! Foram alguns "nãos" e alguns "sims", quase tudo tinha condições e nada era de graça, ou de qualquer jeito...Mas o mais importante eu entendia exatamente nos dias que escrevia esta dissertação...tudo passa, daqui a 100 anos não estarei mais por aqui, nada do eu conquiste eu levarei comigo para onde eu for se é que vamos para algum lugar ou apenas nos decompomos como toda matéria, mas eu via nestes dias que há formas de se eternizar e que daqui a 100 anos eu estaria aqui sim mas de outra forma por meio da ciência e do conhecimento que eu propagasse e não era isto que eu estava fazendo? Entendi que o que diferencia cada um de nós não é o título, pois pelo contrário ele será igual para todos...mas o caminho que fiz, esse será único. O que cada um tem que vencer, tem que superar e como faz isso, o que tem compartilhado na trajetória, porque o que compartilhamos também nos eterniza, essa é a diferença, o sucesso que eu espero está na trajetória, na caminhada, é ai que encontro o meu melhor, que encontro o meu prazer, o que ainda não tenho eu saboreio quando acontecer, mas no seu momento...não se faz pesquisa, pós-graduação pensando no que seremos, pois já somos! Qualquer um pode acumular títulos desde a Iniciação Científica até os mais seletos programas internacionais de aperfeiçoamento, assim se acumulam as gavetas de papéis e o *lattes* de palavras, mas o que me impulsiona é acumular aquilo que é o alimento para minha alma que são as emoções do dia dia na pesquisa (descobertas, a ansiedade do novo, a surpresa do inesperado), o prazer de ver ago feito por você e propagado por outros, (papers, resumos), a sensação de superação, a sensação de evolução...Enfim escrevendo a minha dissertação me vi tão igual a todos e me descobri paradoxalmente única como cada um de nós somos**. O TRABALHO PODE SER EXECUTADO POR QUALQUER UM, MAS A TRAJETÓRIA SERÁ SEMPRE DIFERENTE E ESTA TRAJETÓRIA DIZ SOBRE MIM MUITO MAIS QUE QUALQUER TÍTULO QUE EU VENHA A TER!**

PARAÍBA

Pê - a - pá
Erre - a - ra - í
Bê - a - bá
Paraíba
Paraíba do norte, do caboclo forte
Do homem disposto esperando chover
Da gente que canta com água nos olhos
Chorando e sorrindo, querendo viver
Do sertão torrado, do gado magrinho
Do açude sequinho, do céu tão azul
Do velho sentado num banquinho velho
Comendo com gosto um prato de angu
Acende o cachimbo, dá uma tragada
Não sabe de nada da vida do sul
Pê - a - pá
Erre - a - ra - í
Bê - a - bá
Paraíba
Paraíba do norte que tem seu progresso
Que manda sucesso pra todo país
Que sente a presença da mãe natureza
Que vê a riqueza nascer da raiz
Que acredita em deus, também no pecado
Que faz do roçado a sua oração
E ainda confia no seu semelhante
E vai sempre avante em busca do pão
O pão que é nosso, que garante a vida
Terrinha querida do meu coração
Pê - a - pá
Erre - a - ra - í
Bê - a - bá
Paraíba

(ZÉ RAMALHO)

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RESUMO

Agave sisalana é uma espécie amplamente cultivada no nordeste do Brasil, visando à produção da fibra sisal, sendo a Bahia e a Paraíba os maiores produtores mundiais. O processo de decorticação das folhas de *A. sisalana* implica no desperdício de mais de 95% da planta na forma dos resíduos sólido (bagaço) e líquido (suco), além disso, outras partes da planta, como o caule não são utilizadas. Aliado a isso, um declínio econômico na cultura sisaleira no Brasil é relatado por órgãos governamentais, o que tem direcionado à necessidade de agregar novos valores a esta espécie. Diante deste contexto, esta pesquisa objetivou obter e caracterizar diferentes produtos de interesse farmacêutico a partir do caule e folha (resíduos de decorticação) de *A. sisalana*. O caule e os resíduos (divididos em bagaço e suco) foram submetidos a etapas de processamento para obtenção de drogas vegetais e extratos, os quais foram caracterizados quanto a parâmetros farmacopéicos e metabólitos de interesse presentes foram quantificados (Capítulo 2). Após análise dos resultados obtidos pela caracterização inicial, os extratos aquosos do caule foram destinados à obtenção do polissacarídeo inulina, o qual foi isolado após etapas de precipitação por métodos físicoquímicos e purificação por cromatografia de troca iônica. As análises por diferentes técnicas instrumentais permitiram caracterizar o produto (Capítulo 3). Um *screening in vitro* preliminar de atividade prebiótica foi realizado nos extratos aquosos do caule secos por nebulização e polissacarídeos obtidos deste extrato também secos em spray dryer, ambas as amostras foram caracterizadas por termogravimetria e tiveram metabólitos de interesse quantificados (Capítulo 4). Polissacarídeos pécticos foram extraídos dos resíduos de decorticação e submetidos a diversas etapas de purificação que incluíram desproteinzação e cromatografia de troca iônica. Técnicas instrumentais foram empregadas para caracterização estrutural dos mesmos (Capítulo 5). A inulina obtida do caule apresentou características semelhantes àquelas comercialmente disponíveis obtidas de outras espécies. O *screening in vitro* indicou que o extrato seco do caule de *A. sisalana* apresentou potencial prebiótico e provavelmente seria como uma alternativa economicamente viável uma vez que o isolamento da inulina requer etapas que demandam tempo, geram alto custo e resultam em baixo rendimento. A caracterização dos polissacarídeos obtidos dos resíduos revelou estruturas comuns à pectina. Assim, esta pesquisa relata novas possibilidades de agregar valor ao *A. sisalana* por meio da obtenção de produtos de interesse farmacêutico.

PALAVRAS-CHAVE: *Agave sisalana*, Aproveitamento de resíduos, Caracterização de drogas vegetais, Inulina, Potencial prebiótico, Polissacarídeos pécticos

ABSTRACT

Agave sisalana is a specie widely grown in northeastern Brazil for production of sisal fiber, Bahia and Paraíba being the world's largest producers. The process of decortication of the leaves of *A. sisalana* implies loss of more than 95% of the plant in the form of solid waste (bagasse) and liquid waste (juice). In addition, parts of plant such as boles are not used. An economic decline in sisal crop from Brazil is also reported by government agencies, which have driven the need to add new values to this species. This study aimed to obtain and characterize different products of pharmaceutical interest from the boles and leaves (wastes from decortication) from *A. sisalana*. The boles and wastes (bagasse and juice) were subjected to processing steps for obtaining the extracts and herbal drugs, which were characterized according to pharmacopoeial parameters and metabolites of interest therein were screened (Chapter 2). After analyzing the results obtained by initial characterization, the aqueous extracts from boles were used for obtaining the polysaccharide inulin which was isolated after steps of precipitation by physicochemical methods and purified by ion exchange chromatography. Analyses by different instrumental techniques were used to characterize the product (Chapter 3). Preliminary *in vitro* screenings for prebiotic activity was carried out in the aqueous extracts and polysaccharides dried by spray drying. Both samples were characterized by thermogravimetry and metabolites of interest were quantified (Chapter 4). Pectic polysaccharides were extracted from the wastes and subjected to various steps of purification involving deproteinzation and ion exchange chromatography. Instrumental techniques were used for structural characterization of these samples (Chapter 5). The inulin obtained from boles showed characteristics similar to those available commercially obtained from other species. The *in vitro* screening indicated that the dried extract of *A. sisalana* exhibis prebiotic potential and could probably be an economically viable alternative because the isolation of inulin requires steps that demands time and high costs and result in low yields. The characterization of polysaccharides extracted from wastes indicated typical structures of pectin. Thus, this study reports new opportunities to add value to *A. sisalana* by recovering products of pharmaceutical interest.

KEYWORDS: *Agave sisalana*, Utilization of wastes, Herbal drugs characterization, Inulin , Prebiotic potential, Pectic polysaccharides

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LISTA DE ABREVIATURAS

1-FEH: frutano exohidrolase

1-FFT :frutano:frutano 1-frutosiltransferase

1-SST :sacarose:sacarose 1-frutosiltransferase

ANOVA: Análise de variância

CAM: Ciclo do ácido das crassuláceas

CD: Circular Dicrhoism

CG-MS- Cromatografia gasosa acoplada a espectrometria de massa

CIN: Crude inulin,

CLAE: por cromatografia líquida de alta eficiência

DAE: Dried aqueous extract

DCP: Dried crude polysaccharides

DEAE: dietilaminoetil

DNS: 3,5-dinitrosalicylic acid

DP: Degree of polymerization

DSC: Differential Scanning Calorimetry

DTA: Differential thermal analysis

EQ: equivalent

FT-IR: Fourier transform infrared spectroscopy

GAC: Grau de acetilação

GM: Grau de metoxilação

GP: Grau de polimerização

HG: homogalacturonanas

IDH: índice de desenvolvimento humano

IR: infra-red

INAS: inulin from *Agave sisalana*

LW: liquid waste

MAC: metabolismo do ácido das crassuláceas

MALDI: Matrix-assisted laser desorption/ionization

NMR: Nuclear Magnetic Ressonance

PLW: polysaccharides of liquid waste

PPT: precipitate

PSW: polysaccharides of solid waste

RG-I: ramnogalacturonanas do tipo I

RG-II: ramnogalacturonanas do tipo II

SW: solid waste

TGA: Thermogravimetric analysis

XGA: xilogalacturonanas

XRD: X-Ray diffraction

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1 INTRODUÇÃO

O gênero Agave, pertencente à ordem Asparagales e a família *Agavaceae*, reúne mais de 200 plantas monocotiledôneas e monocárpicas que crescem principalmente na China, Brasil, México, Tanzânia, África do Sul e Moçambique, embora seja nativo da América do Norte, com seu centro de origem no México (COLUNGA-GARCÍAMARÍN; EGUIARTE; ZIZUMBO-VILLARREAL, 2007; ESCAMILLA-TREVIÑO, 2011). Algumas espécies da família *Agavaceae* são extensivamente usadas para aplicações industriais como a obtenção de bebidas alcoólicas a exemplo da tequila produzida a partir do *Agave tequilana*, do qual é obtido também xarope de frutose e inulina para aplicação como substância prebiótica (ARRIZON et al., 2010; GOMEZ et al., 2010; KESTUR G. et al., 2013). O mescal é outra bebida destilada produzida a partir de algumas espécies como *Agave angustifolia*, *Agave salmiana*, *Agave americana* e *Agave durangensis* que apresentam ampla importância econômica no México (PEÑA-ALVAREZ., 2004).

No Brasil a espécie amplamente cultivada é o *Agave sisalana* do qual é obtido o sisal, principal fibra dura produzida no mundo, sendo o país o maior produtor e exportador desta matéria-prima (MARTIN; SILVA, 2009). O *A. sisalana* é extensivamente cultivado na região Nordeste, nos moldes de agricultura familiar, em especial nos estados da Bahia e da Paraíba, nos quais a cultura é responsável por geração de emprego e renda, principalmente, em localidades pobres, o que aumenta sua relevância no contexto socioeconômico (ESCAMILLA-TREVIÑO, 2011). No caso especifico da Paraíba há nos últimos anos um declínio significativo da cultura sisaleira tanto em áreas cultivadas como em produção. O baixo aproveitamento da planta aparece como um dos motivos para este declínio, já que apenas 5% dos produtos da desfibrilação das folhas de sisal para produzir a fibra dura são utilizados, sendo os 95%

restantes constituídos de resíduos sólidos (bagaço) e resíduos líquidos (suco do sisal) descartados. A única aplicação econômica para o *A. sisalana* na região é a obtenção da fibra e o estado da Bahia nos últimos anos destacou-se nesta produção (SANTOS et al., 2009).

Diante deste contexto é necessário pesquisar e efetivar novas aplicações para o *A. sisalana* na Paraíba de modo a revitalizar a sua cultura. Pesquisas apontam possibilidades de diferentes aplicações farmacológicas para espécie (DEBNATH et al., 2010; CERQUEIRA et al., 2012). Uma das abordagens mais difundidas é o isolamento de saponinas como a hecogenina e até a produção de corticosteroides a partir desta (MORS & SHARAPIN, 1973). Atividades anti-inflamatórias, analgésicas, antimicrobianas e anti-helmínticas também já foram relatadas (DUNDER et al., 2010; ADE-AJAYI, et al., 2011; MWALE et al., 2012). Outro aspecto estudado atualmente é o aproveitamento dos resíduos descartados recentemente ação antimicrobiana, antioxidante e imunológica (SANTOS et al., 2009; ZHANG, LIU & LIN, 2013) bem como a obtenção de materiais de importância para indústria farmacêutica como manitol e pectina a partir destes (BRANCO et al., 2010; SANTOS et al., 2013).

A observação de estudos realizados para todo gênero Agave apontam para o sucesso na obtenção de alguns produtos como bebidas e substâncias prebióticas já comercializados a partir de algumas outras espécies e pode ser um direcionamento para as pesquisas com *A. sisalana*, uma vez que algumas substâncias são produzidas pelo gênero em virtude de aspectos peculiares à fisiologia vegetal do mesmo (LÓPEZ & URIAS-SILVAS, 2007). Um aspecto ainda não explorado é a obtenção de polímeros de carboidratos do tipo frutanos, os quais são extensamente aceitos do ponto de vista econômico pelas suas aplicações como potentes prebióticos (BARCLAY et al., 2010).

As espécies de Agave apresentam o metabolismo do ciclo do ácido das crassuláceas por meio do qual frutanos são sintetizados e armazenados especialmente nos caules como carboidratos de reserva usados em situações de estresse térmico bem como fontes de carbono para o vegetal (BARCLAY et al., 2010; LIVINGSTON; HINCHA; HEYER, 2009; LÓPEZ & URIAS-SILVAS, 2007). Há uma extensa obtenção e comercialização destas substâncias a partir do *A. tequilana*, especialmente inulina e seus derivados, os frutooligossacarídeos, ambos com ação prebiótica e mesmo os produtos da hidrólise como a frutose (LOPEZ; MANCILLA-MARGALLI; MENDOZA-DIAZ, 2003; ARRIZON et al., 2010; GOMEZ et al., 2010).

Além dessa abordagem quimiotaxonômica que leva em consideração relatos das pesquisas em outras espécies de agave, um estudo mais profundo dos resíduos do *A.sisalana* precisa ser executado e compartilhado entre a comunidade científica, uma vez que as publicações até o momento não relatam diferenciação entre o bagaço e suco quanto ao seu conteúdo e aplicações. Desse modo esta pesquisa objetivou obter produtos de interesse farmacêutico a partir do caule e resíduos de decorticação do *Agave sisalana*, caracteriza-los e assim propor opções para revitalizar a cultura sisaleira.

CAPÍTULO 1

2 REFERENCIAL TEÓRICO

2.1 *Agave sisalana* Perrine: uma contextualização

Uma espécie amplamente conhecida no nordeste brasileiro é o *Agave sisalana* (sisal), representado na figura 1. Planta semi-xerófila que pertence ao gênero *Agave*, o qual faz parte da ordem Asparagales e da família Agavaceae que é formado por plantas monocotiledôneas que crescem principalmente em países como China, Brasil, México, Tanzânia, África do Sul e Moçambique, possuindo mais de 200 espécies, no entanto é nativo da América do Norte, sendo originário da Península de Yucatã no México com adaptação às regiões tropicais e subtropicais, suportando secas prolongadas (ESCAMILLA-TREVIÑO, 2011).

Figura 1 -*Agave sisalana* Perrine fotografada no munícipio de Pocinhos/Paraíba.

Fonte: Autor.

O *A. sisalana* é comercialmente importante pela produção da fibra de valor econômico em muitos países, especialmente no Brasil, que possui mais de 68% da produção mundial (SANTOS et al., 2013). A Paraíba que já foi de 1943 até 1976 a maior produtora mundial de sisal, hoje ocupa o segundo lugar, com uma produção inferior a da Bahia que é a maior produtora. A companhia Brasileira de Abastecimento

(CONAB) apresentou dados informando que no Brasil a produção de sisal na década de 1980 girou na casa das 200 mil toneladas por ano, em 1990 a produção caiu para 100 mil, em 2010 continuou declinando para 60 mil, em 2011 apresentou uma significante recuperação estimada em 111 mil toneladas por ano, mas em 2012, estima-se a produção tenha atingido o menor volume de sua história: 55 mil (CONAB 2013a, 2013b).

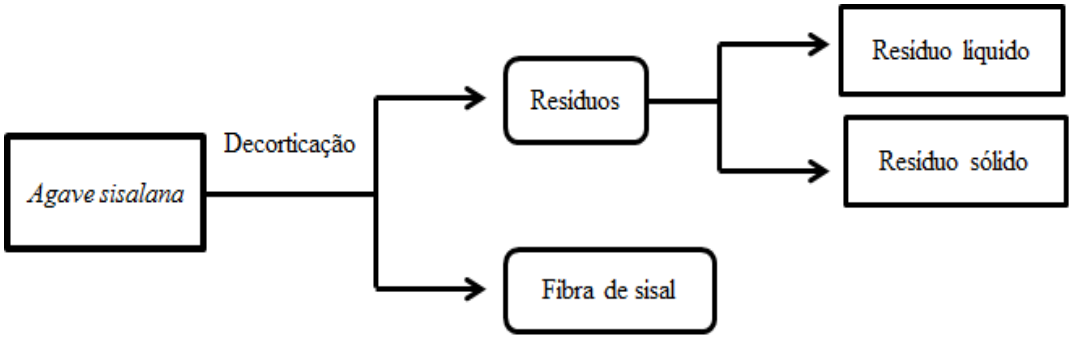
O sisal é o principal produto da agricultura familiar e do segmento agroindustrial do semiárido brasileiro, cujo Índice de desenvolvimento Humano (IDH) médio é 0,589. A agaveicultura se concentra em áreas de pequenos produtores, com predomínio do trabalho familiar. Embora, venha sendo utilizada de forma empírica pelos pequenos produtores rurais, a mesma se constitui como uma fonte de renda e emprego para um grande contingente de trabalhadores, bem como é um importante agente de fixação do homem na região do semiárido nordestino, sendo que em algumas dessas regiões é a única alternativa de cultivo com resultados econômicos satisfatórios (CUNHA, 2010).

A principal aplicação do *A. sisalana* é para obtenção da fibra que por sua vez tem suas aplicações na indústria automobilística e também na fabricação de cordas, barbante, cabos marítimos, tapetes, sacos, vassouras, estofamentos, e artesanato; além disso, tem utilização industrial na fabricação de pasta celulósica para produção do papel Kraft de alta resistência, e de outros tipos de papel fino, como para cigarro, filtro, absorvente higiênico, fralda (MARTIN et al., 2009). O aproveitamento do *A. sisalana* no Brasil é limitado exclusivamente a obtenção da fibra, não havendo outras aplicações do ponto de vista biotecnológico, alimentício ou farmacêutico (ADE-AJAYI et al., 2011; NARVÁEZ-ZAPATA, L.F. & SÁNCHEZ-TEYER, 2009).

Outro aspecto limitante que merece atenção é que o processo de decortificação das folhas de sisal para obtenção das fibras gera resíduos, que representam mais de 90 % de

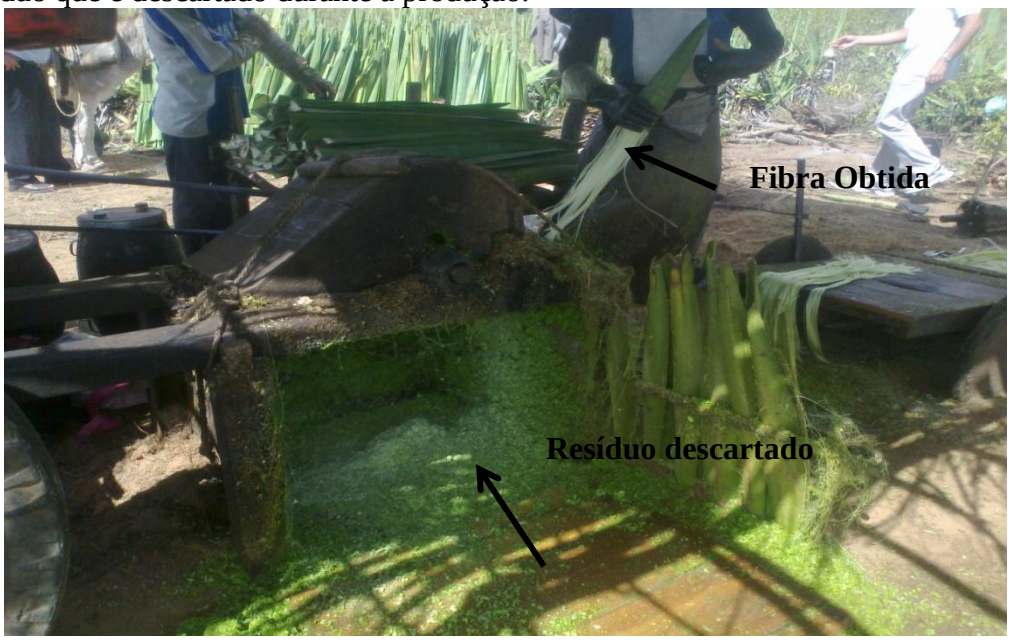
materiais restantes, os quais podem ser divididos em resíduos sólidos e resíduos líquidos (suco do sisal) que normalmente são descartados pelos produtores de sisal no próprio campo (SANTOS et al., 2009). A Figura 2 exibe o processo produtivo e seus produtos. Na Figura 3 pode-se observar o beneficiamento do *A. sisalana* para obtenção da fibra, nela é possível ver o resíduo que é descartado sem aproveitamento.

Figura 2-Esquema dos produtos gerados pelo processo de desfibrilação das folhas de *A. sisalana*.



Fonte: Autor

Figura 3- Beneficiamento do *A. sisalana* para obtenção da fibra no município de Pocinhos/Paraíba, com destaque para a fibra obtida para comercialização e para o resíduo que é descartado durante a produção.



Fonte: Autor.

Alguns estudos preliminares feitos com diferentes partes e inclusive os resíduos de *A. sisalana* bem como com outras espécies de agave, como as citadas anteriormente, indicaram a possibilidade de obtenção de excipientes farmacêuticos, agentes

antioxidantes e prebióticos destas espécies (ARRIZON et al., 2010; BRANCO et al., 2010; GOMEZ et al., 2010; HIGUERA, 2009; SANTOS et al., 2009; ZHANG; LIU; LIN, 2013). As pesquisas também relatam potencial para aplicações farmacológicas (CERQUEIRA et al., 2012; DEBNATH et al., 2010; DUNDER et al., 2010). Aliado a isto é notório que as plantas deste gênero apresentam a capacidade de armazenar grandes quantidades de açúcar, podendo ser matéria-prima para processos de fermentação, bem como potenciais fontes bioenergéticas para produção de etanol de segunda geração (ESCAMILLA-TREVIÑO, 2011).

Na Tabela 1 estão descritos alguns estudos com diferentes partes de diversas espécies de agave e suas potenciais aplicações farmacêuticas e biotecnológicas. O *A. sisalana* pode ser uma fonte potencial de polissacarídeos com diferentes aplicações de interesse às ciências farmacêuticas, mas ainda não explorada em suas reais possibilidades (BRANCO et al., 2010; SANTOS et al., 2013; ZHANG; LIU; LIN, 2013).

Tabela 1- Exemplos de possíveis aplicações de diferentes partes de plantas do gênero Agave.

Partes das plantas	**Espécie**	**Pesquisas**	**Referências**
Folhas	*Agave sisalana* Perrine ex Engelm	Atividades anti-inflamatória e analgésicas em processos agudos e crônicos	(DUNDER et al., 2010)
		Atividade antifúngica	(SANTOS et al., 2009)
		Extração de saponinas	(CERQUEIRA et al., 2012)
		Três flavonas e sete homoisoflavonas foram isolados	(CHEN et al., 2009)
		Efeitos gastroprotetores	(CERQUEIRA et al., 2012)
	Agave macroacantha	Extração de saponinas	(ESKANDER; LAVAUD; HARAKAT, 2010)

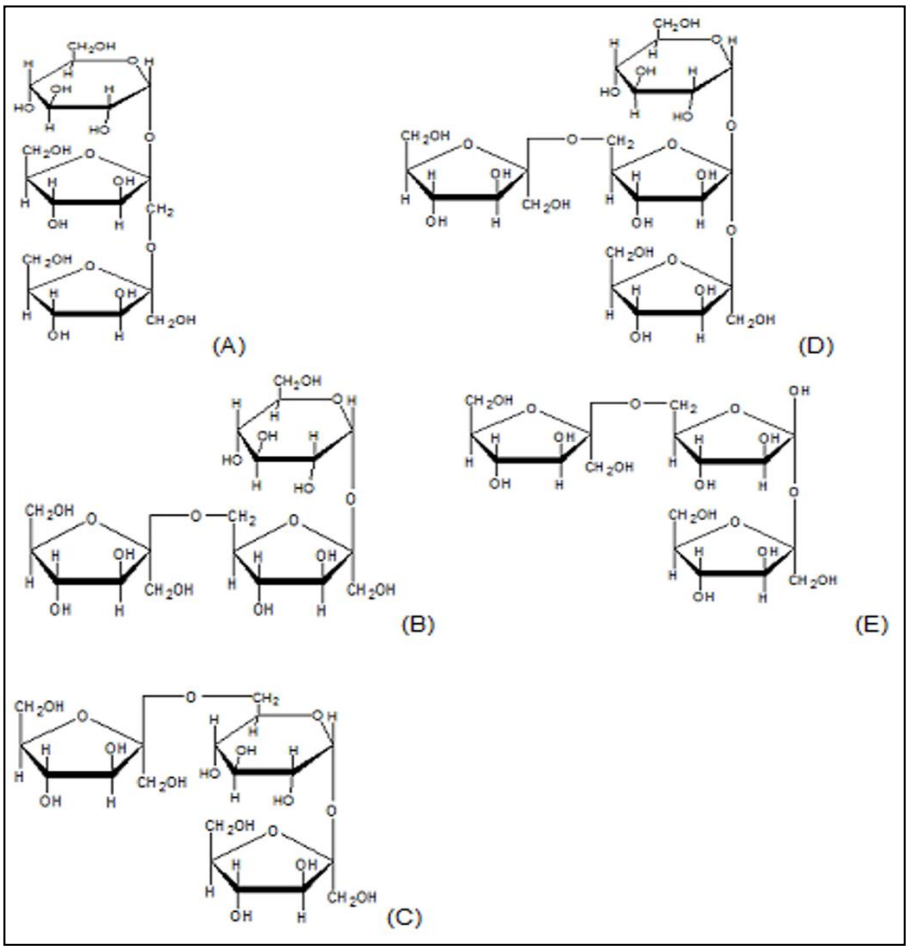
Folhas	*Agave americana*	Atividade antioxidante	(BEN HAMISSA et al., 2012)
	Agave attenuata		(RIZWAN et al., 2012)
	Agave intermixta Trel. (Maguey)	Atividades anti-inflamatória	(QUILÉZet al., 2004)
Sisal (fibra)	*Agave sisalana*	Produção de bioetanol	(LIMA et al., 2013)
		Extração de hemicelulose e lignina	(MEGIATTO et al., 2008)
Resíduos	*Agave sisalana*	Extração de manitol	(BRANCO et al., 2010)
		Extração de pectina	(SANTOS et al., 2013)
		Atividade antifúngica	(SANTOS et al., 2009)
		Atividade antioxidante	(ZHANG; LIU; LIN, 2013)
		Atividade imunologica	(ZHANG; LIU; LIN, 2014)
Caules	*Agave tequilana*	Xarope de frutose	(SOTO et al., 2011)
	A. tequilana	Produção da tequila	(ESCAMILLA-TREVIÑO, 2011)
	A. angustifolia	Produção do mezcal	
	Agave tequilana	Isolamento de inulina	(HIGUERA, 2009)/(SHARMA & VARSHNEY, 2012)
	Agave fourcroydes	Produção de bioetanol	(MARTÍNEZ-TORRES et al., 2011)

Fonte: Autor.

2.2 Síntese de frutanos: gênero agave e o metabolismo do ácido das crassuláceas

Os frutanos são carboidratos não-redutores formados por unidades frutosil que apresentam na sua estrutura um radical terminal de glicose. Sua estrutura pode ser linear ou ramificada, e são classificados em cinco grupos principais: frutanos do tipo inulina (1-cestose), do tipo levano (6-cestose), do tipo neosérie da inulina (neocestose), levanos do tipo misto (bifurcose) e frutanos da neosérie de lavanos também chamado levanos do tipo misto. Os representantes mais curtos destes frutanos têm as suas estruturas químicas ilustradas na Figura 4 (VAN LAERE & VAN END, 2002).

Figura 4-Diferentes tipos de frutanos, de acordo com a classificação de VAN LAERE & VAN DEN ENDE (2002): (a) 1-cestose (B) 6-cestose, (C) Neocestose, (D), Bifurcose, (E) Frutanos mistos.



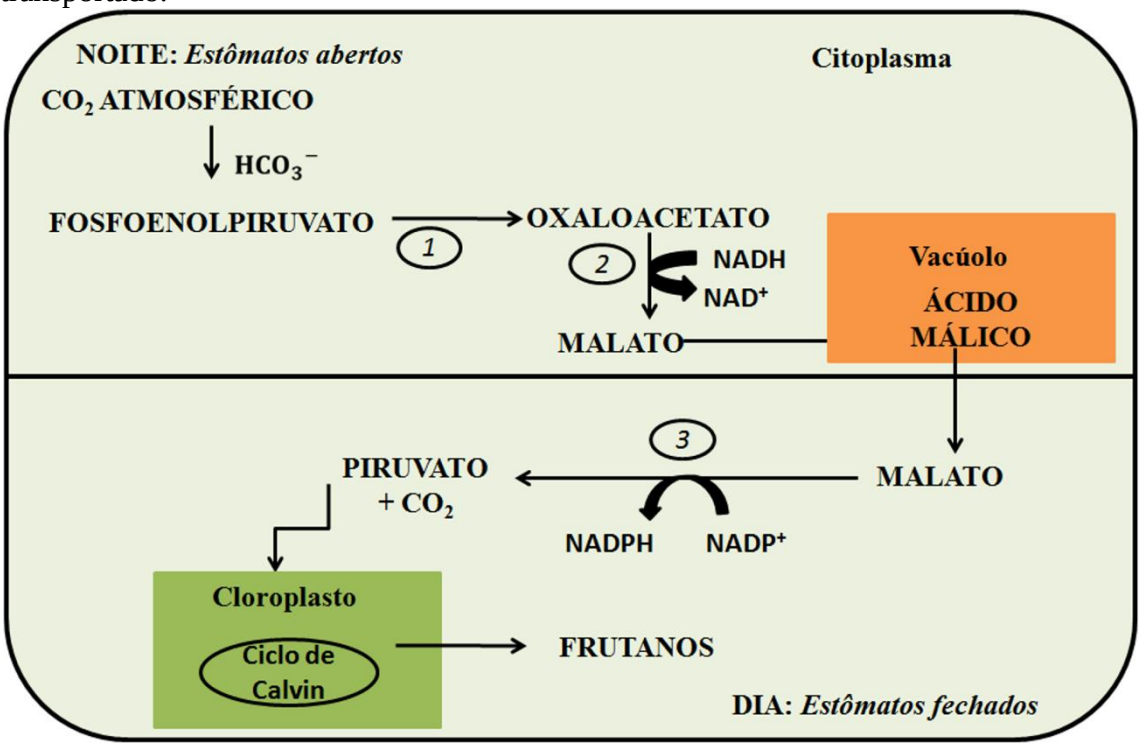
Fonte: Adapatado de LAERE & VAN DEN ENDE (2002).

Os frutanos são os principais produtos fotossintéticos do ciclo do ácido das crassuláceas e agem como osmoprotetor durante a seca (BORLAND, GRIFFITHS, HARTWELL, & SMITH, 2009). A principal função destas substâncias na planta é o armazenamento de energia e agir na tolerância ao estresse abiótico (ARRIZON et al., 2010; LOPEZ; MANCILLA-MARGALLI; MENDOZA-DIAZ, 2003).

Um grande número de espécies de agave apresenta o metabolismo do ácido das crassuláceas (MAC), sendo os frutanos o principal produto deste ciclo. O MAC é fundamentado na abertura de estômatos durante a noite e o seu fechamento durante o dia, isto envolve a redução do metabolismo e de perda de água por transpiração. Os mecanismos bioquímicos do MAC estão ilustrados na Figura 5 (BORLAND et al.,

2009). O MAC é o principal processo fisiológico adaptativo do gênero agave as condições abióticas do clima semiárido. Este processo metabólico explica por que os caules de plantas que pertencem a este gênero têm teores elevados de frutanos do tipo inulina (LOPEZ; MANCILLA-MARGALLI; MENDOZA-DIAZ, 2003).

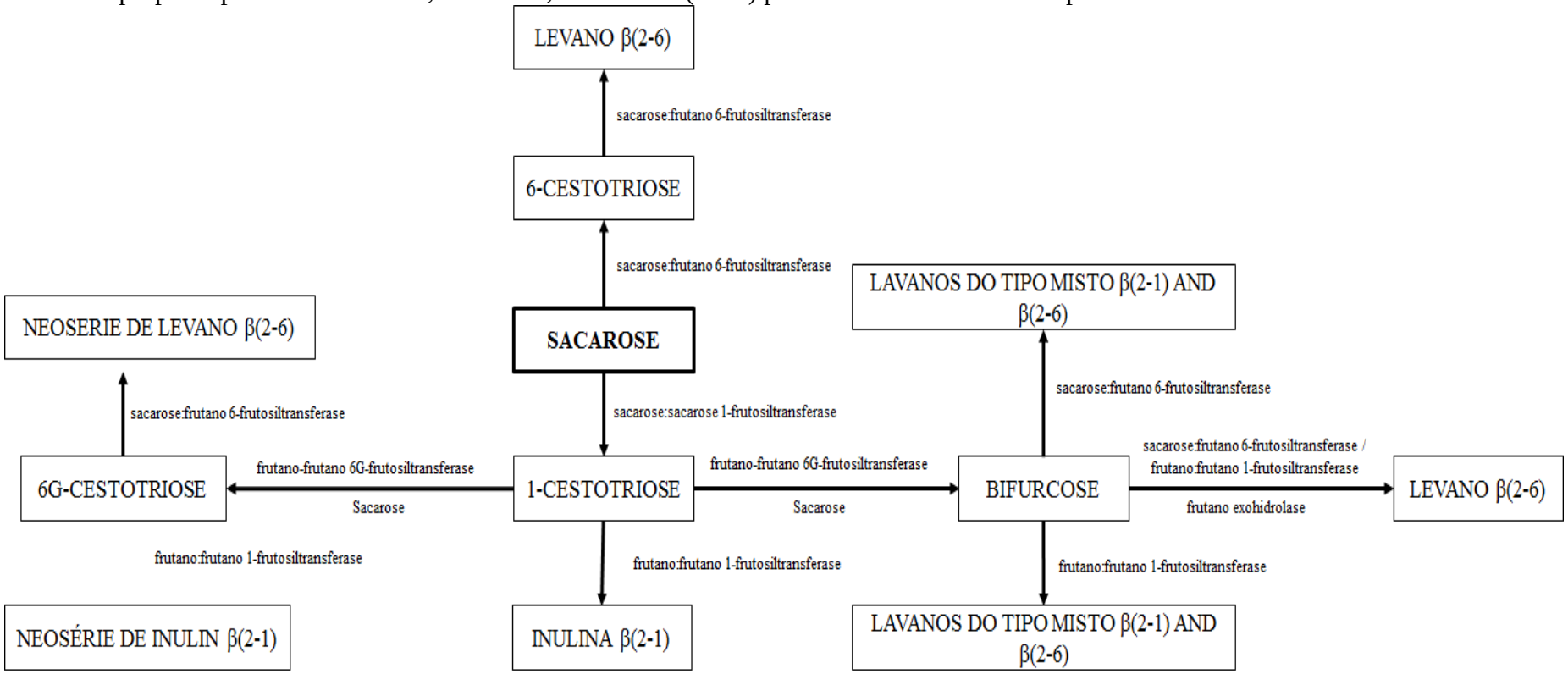
Figura 5-Ilustração do metabolismo do ácido das crassuláceas. 1- Ação da enzima fosfoenolpiruvato descarboxilase sobre fosfoenolpirvato resultando em oxaloacetato. 2- Ação da malato desidrogenase que converte o oxaloacetato em malato que é transportado.



Fonte: Autor

A síntese dos frutanos inicia-se quando a fotossíntese excede a demanda e a sacarose passa para um nível crítico (LIVINGSTON; HINCHA; HEYER, 2009) O processo é catalisado por três diferentes enzimas: sacarose:sacarose 1-frutosiltransferase (EC 2.4.1.99) (1-SST), frutano:frutano 1-frutosiltransferase (EC 2.4.1.100) (1-FFT) e frutano exohidrolase (EC 3.2.1.153) (1-FEH) (EDELMAN & JEFFORD, 1968). LIVINGSTON et al. (2009) propuseram um modelo de síntese que envolve duas novas enzimas: a sacarose:frutano 6-frutosiltransferase (6-SFT) e a frutano-frutano 6G-frutosiltransferase (6G-FFT) como é exibido na Figura 6.

Figura 6- Modelo proposto por LIVINGSTON, HINCHA, & HEYER (2009) para síntese de frutanos em plantas.



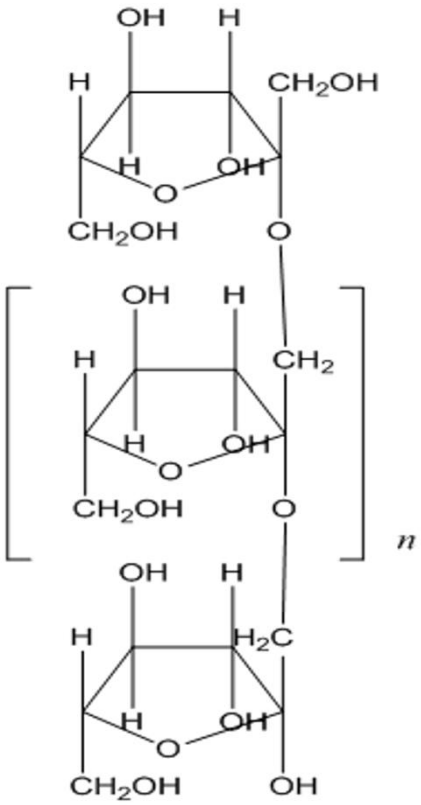
Fonte: Adaptado de LIVINGSTON, HINCHA, & HEYER (2009).

2.3 INULINA

2.3.1 Aplicações

A inulina possui uma estrutura complexa, como exibido na Figura 7 a sua cadeia é constituído por um número variável de unidades de frutose unidas por ligações β-(2→1) D-frutosil-frutose, e geralmente termina com apenas uma unidade de glucose ligadas através de uma α-D-glucopiranosilo ou uma ligação α-D-glucopiranosil ou α-(1→2) como na sacarose (BRUYN, ALVAREZ, SANDRA, & DE LEENHEER, 1992). Inulinas com uma unidade terminal de glicose são chamados α-D-glucopiranosil-[β-D-fructofuranosil] $_{[n-1]}$-D-frutofuranosides, enquanto que as que são constituídas apenas de frutose são denominadas de frutopiranosil-[α-D-frutofuranosil $_{[n-1]}$-D-fructofuranosides (RONKART et al., 2007).

Figura 7-Estrutura química da inulina.



Fonte: Autor

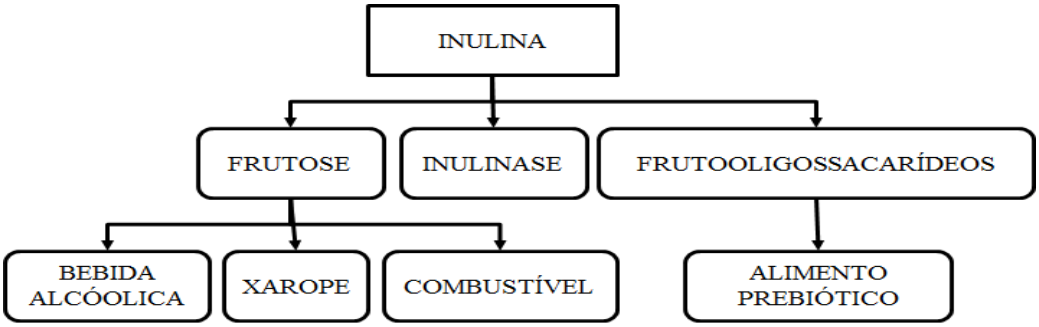
A inulina é um polissacarídeo de armazenamento de origem vegetal, com uma grande variedade de aplicações nas indústrias alimentícias e farmacêuticas. É um

substituto para o açúcar ou gordura possuindo um valor calórico muito baixo. Entre outras aplicações farmacêuticas possíveis estão a sua utilização na produção de medicamento de liberação colón específica. Também é largamente utilizada na produção de alimentos funcionais (BARCLAY et al., 2010).

Amplamente distribuída em uma variedade de plantas, a inulina está presente em mais de 30.000 produtos de origem vegetal, sendo frequentemente armazenados em folhas e outros órgãos que atuam como reserva de carboidratos (RITSEMA &; SMEEKENS, 2003; WICHIENCHOT et al., 2011). Há relatos na literatura da presença da inulina em algumas espécies de Agave, como *A. tequilana*, *A. americana*, *Agave atrovirens* Karw,*A. salmiana* e até um trabalho preliminar de triagem indicou a presença deste polissacarídeo nos resíduos do *A. sisalana* (ARRIZON et al., 2010; GOMEZ et al., 2010; HIGUERA, 2009; RAMÍREZ; GÓMEZ-AYALA; JACQUES-HERNÁNDEZ, 2006; SHARMA, S. & VARSHNEY, 2012)

A inulina é matéria-prima para a produção de bioetanol, xarope de frutose, proteína unicelular (substituto de alimentos ricos em proteínas) e óleo de uma única célula (transesterificação de triglicerídeos a partir de biomassa renovável), obtenção de frutooligossacarídeos (FOS) e outros produtos úteis (CHI et al., 2011). A Figura 8 expõe algumas das principais aplicações para inulina.

Figura 8-Possíveis aplicações para inulina.



Fonte: Autor.

2.3.2 Aplicação da inulina versus grau de polimerização

A inulina está presente em plantas como misturas heterogêneas, com diferentes graus de polimerização (GP) e estruturas químicas variadas. Os tipos de frutanos encontrados em plantas (moléculas oligoméricas ou polimérico) são dependente da espécie e relacionados com as condições ambientais e estágios de desenvolvimento da planta (MANCILLA-MARGALLI & LÓPEZ, 2006). As propriedades físico-químicas e funcionais da inulina estão ligados a GP assim como a presença de ramificações.

A fração de cadeia curta, oligofrutose, é muito mais solúvel e mais doce do que inulina nativa e de cadeia longa, e pode contribuir para melhorar a sensação na boca, porque as suas propriedades estão intimamente relacionadas com outros açúcares. Por exemplo, devido a um perfil de doçura semelhante ao da sacarose, mas o conteúdo calórico mais baixo (1-2 kcal/g) e poder edulcorante (30-35%) podem ser útil para substituir parcialmente a sacarose ou a substituí-la totalmente quando combinado com outros adoçantes não calóricos (GUGGISBERG et al., 2009; TÁRREGA & ROCAFULL; COSTELL, 2010).

A fração de cadeia longa é menos solúvel, mais viscosa, mais termoestável e pode atuar em propriedades reológicas e sensoriais de produtos lácteos, como substituto de gordura, pois atua como um agente de consistência do alimento da mesma maneira na forma de glóbulos de gordura (GUGGISBERG et al., 2009). A inulina de cadeia longa, quando colocada em água ou leite, tem a capacidade de formar microcristais, que podem interagir para formar uma textura suave e cremosa e proporcionar um paladar semelhante à gordura (LÓPEZ-MOLINA et al., 2005).

2.3.3 Aplicação da inulina na obtenção de produtos após a hidrólise

A inulina é uma promissora fonte de FOS e frutose resultante da ação inulinases. De acordo com suas ações estas enzimas podem ser classificadas endoinulinases (2,1-β-

D-frutano frutanohidrolases; EC 3.2.1.7), que hidrolisa as ligações entre as unidades de frutose localizado longe das extremidades da rede inulina liberando FOS, e exoinulinases (β-D-frutohidrolases; EC 3.2.1.80), que divide as unidades terminais de frutose em sacarose, rafinose e inulina liberando frutose (PESSOA; VITOLO, 1998).

Uma vez que a frutose é significativamente mais doce que a sacarose e glucose, a possibilidade de obter o xarope rico em frutose seria uma alternativa lucrativa para indústria de alimentos (SIRISANSANEEYAKUL et al., 2006).

2.3.4 Utilização em alimentos funcionais

Os alimentos funcionais têm sido desenvolvidos através da adição de inulina para aumentar o seu teor de fibra dietética. O efeito da inulina, como prebiótico é comprovado sobre padrões de culturas puras de *Streptococcus thermophilus* e *Bifidobacterium lactis* ou em co-cultura de fermentação (OLIVEIRA et al., 2012). As ligações glicosídicas do tipo β (2-1) da inulina apresentam resistência a hidrólise pelas enzimas salivares e intestinais, bem como promovem o crescimento de bactérias intestinais benéficas do tipo bifidobactérias, as quais apresentam diversas propriedades funcionais importantes à prevenção de doenças intestinais (KELLY, 2009). Desse modo, a inulina pode ser considerada um prebiótico e vem sendo amplamente utilizada com tal objetivo, especialmente diante da importância dos alimentos funcionais no contexto atual das pesquisas em nutrição e da indústria alimentícia. A Tabela 2 descreve os três principais mecanismo de ação da inulina no organismo que explicam suas propriedades nutricionais.

Tabela 2-Ações benéficas da inulina no trato gastrintestinal.

Baixa contribuição calórica	Influência em parâmetros fisiológicos do trato intestinal	Efeito prebiótico
A inulina não é hidrolisada no trato digestivo humano, não resultando em contribuição calórica neste processo. A degradação ocorre a nível de cólon por fermentação de bactérias.	A inulina afeta os parâmetros fisiológicos do trato digestivo, a exemplo do esvaziamento gástrico, tempo de trânsito, pH, e massa fecal, sendo considerado alimento funcional	A ingestão de inulina resulta em um significativo aumento das bifidobactérias. A flora *Bifidus* estimula o sistema imunológico, a absorção de minerais, e inibe o crescimento de bactérias nocivas ao organismo

Fonte: Autor

2.3.5 Sistemas de liberação de medicamentos

A inulina é um promissor agente de liberação de fármacos devido ao seu rápida solubilidade em água e estabilidade na presença das enzimas gástricas e intestinais. Uma ampla revisão publicada recente por IMRAN et al. (2012) aborda diversas aplicações na inulina como carreadores de fármacos.

Praticamente para todas as vias de administração há aplicações da inulina na liberação de fármacos. O fato da inulina não ser destruída no trato gastrointestinal torna este polímero um potente protetor de fármacos sensíveis bem como representa uma alternativa para proteger o trato intestinal de fármacos anti-inflamatório não-esteroidais (AINE'S), além disso, sua solubilidade favorece o aumento da biodisponibilidade de fármacos pouco solúveis (IMRAN et al., 2012).Outra aplicação da inulina nos sistemas de liberação de fármacos é como crioprotetor para vacinas devido suas altas temperatura de transição vítrea e baixa taxa de cristalização (AUDOUY et al., 2011).

2.3.6 Métodos de extração e isolamento da inulina de plantas

Devido a ampla distribuição em diferentes espécies de plantas, a extração e isolamento de inulina tem sido amplamente pesquisadas nos últimos anos (YANG; HU; ZHAO, 2011) . Muitos estudos apontam para um conjunto de condições ótimas de extração da inulina envolvendo principalmente temperatura, tempo de extração e taxa

solvente/matéria-prima, os quais podem influenciar no rendimento final do polímero (ABOU-ARAB; TALAAT; ABU-SALEM, 2011; ABOZED S. S., 2009; PASEEPHOL; SMALL; SHERKAT, 2007; SAENGKANUK et al., 2011; TONELI, 2007).

A solubilidade da inulina em água aumenta significativamente com o aumento da temperatura chegando a 35% (peso/volume) a 90^{o}C, desse modo a obtenção industrial da inulina é feita a partir da difusão em água quente (KIM; FAQIH; WANG, 2001). Em razão disso a os métodos de extração de inulina descritos na literatura são por meio de extração aquosa a quente com pequenas diferenças na temperatura e no tempo de extração.

Extração por ultrassom também já foi proposta para obtenção de inulina com maior rendimento em relação a outros métodos, neste caso as principais variáveis que devem ser controladas são amplitude da sonicação, temperatura e tempo (MILANI; KOOCHEKI; GOLIMOVAHHED, 2011). Entretanto deve-se ter cautela no uso deste método, pois alguns fragmentos de baixo peso molecular são formados durante a sonicação levando a uma mudança na estrutura química da inulina resultante da despolimerização da molécula, o uso de sonicação indireta (LINGYUN et al., 2007).

Após a extração da inulina, uma segunda etapa é necessária para o isolamento da mesma por processos de precipitação que podem ser realizados pelo abaixamento da temperatura ou por meio da utilização de diferentes solventes e envolvem variáveis como velocidade e tempo de centrifugação (ABOZED et al., 2009; LINGYUN et al., 2007).

Devido à baixa solubilidade a baixas temperaturas nos extratos ricos em inulina quando são resfriados e em seguida centrifugados ocorre a precipitação da inulina. TONELI et al. (2008) propuseram um novo método para precipitação da inulina por refrigeração e resfriamento do extrato seguido de centrifugação e nebulização para

obtenção da inulina em pó. No entanto este procedimento requer um significativo gasto de energia uma vez que o extrato aquoso deve ser concentrado em rotaevaporador antes da secagem. Gelo-degelo é outro método proposto para precipitação da inulina seguido de centrifugação (YANG; HU; ZHAO, 2011).

Inulina de cadeia longa pode também ser precipitada a partir de soluções aquosas na presença de altas concentrações de solventes orgânicos tais como metanol, etanol, propanol, acetonitrila e acetona, entre outros. Foi demonstrado que a acetona que é o melhor solvente para manter o GP natural seguido de etanol e metanol. O poder para forte precipitação de acetona de polissacarídeos é atribuída à sua capacidade para remover a água de solvatação destas biomoléculas, promovendo, assim, a desidratação e subsequente precipitação (DALONSO et al., 2009). Além disso, este solvente apresenta um ponto de ebulição muito baixo (56,5 °C), o que permite ser facilmente recuperado por destilação (MOERMAN; VAN LEEUWEN; DELCOUR, 2004). Apesar do etanol e acetona ser considerados os melhores solventes para precipitar a inulina (ABOZED, 2009) em geral, acetonitrila e acetona, são mais eficazes do que o etanol para a maioria inulinas (KU et al., 2003).

A Tabela 3 exibe alguns métodos para extração da inulina.

Tabela 3-Métodos de extração da inulina.

Planta	Tratamento da planta	Extração	Tratamento do extrato	Referência
Helianthus tuberosus L.	Globos *in natura* foram descongelados e cortados em fatias.	Doze (12) kg de plantas foram extraídos com 50 L de água destilada (80°C) em pH 6.8 (NaOH.)	O extrato foi filtrado e submetido a precipitação por gelo-degelo, e o precipitado foi centrifugado a 3000×*g* por 20 min.	(RONKART et al., 2007)
	Tubérculos foram lavados e cortados em pedaços para evitar o escurecimento a enzimática, as fatias foram mergulhadas em água ferventes acidificada com ácido ascórbico durante 2-3 min. Em seguida, as fatias foram colocadas em sacos de polietileno e armazenadas no congelador a -10 º C até o uso.	Um (1) kg de tubérculos foi transferido para um misturador com aquecimento e extraído com água quente (70ºC) durante 60 min com agitação constante.	O extrato foi filtrado e o resíduo foi re-extraido seguindo os mesmos passos.	(ABOU-ARAB; TALAAT; ABU-SALEM, 2011)
Cichorium intybus	A raíz seca foi usada como matéria-prima	A extração foi realizada a 70 ° C com agitação contínua. Água destilada e soluções alcoólicas foram testados como solvente para a extração de inulina	O extrato foi filtrado através em algodão	(DOBRE et al., 2008)

Helianthus tuberosus L.	Os tubérculos foram lavados e rinsados em 0,038 M de hipoclorito de sódio durante 30 min para eliminar os microrganismos. Os tubérculos restantes foram embalados em sacolas de polietileno e mantidas a18 °C	Oitenta e cinco gramas de água deionizada a 85 ° C foram adicionadas a 11,5 g de tubérculos e foram triturados, o extrato foi agitado a 130 rpm a 85 ° C durante 1 h em banho-maria.	Após arrefecimento até à temperatura ambiente, o volume final foi ajustada para 100 mL com água deionizada e a suspensão foi então centrifugada durante 20 min a 12.000 x g	(SAENGTHONGPINI; SAJJAANANTAKUL, 2005)
Helianthus tuberosus L.	Para extrair frutanos dos tubérculos, 2 kg do material foi descascado, cortadas e extraídos em 10 L de água quente contendo 100 ppm de metabissulfito de sódio para minimizar escurecimento a 95-98 ° C durante 10 min,	Extração foi realizada a 70 ° C com agitação contínua. Água destilada e soluções alcoólicas foram testados como solvente para a extração de inulina	O extrato resultante foi filtrado e então concentrado para 50% do volume original em rotaevaporador.	(PASEEPHOL; SMALL; SHERKAT, 2007)
Helianthus tuberosus L.	Os tubérculos foram lavados com água da torneira, cortados e foram imersas imediatamente em água fervente durante 5 min, seguido de imediata imersão em solução de ácido acético gelado (2%) para inibir a atividade da polifenoloxidase. Por fim, as fatias foram secas em estufa com circulação de ar.	Os tubérculos secos em pó foram misturados com água em diferentes relação pó / água (1:2,5 , 1:5, 1:10, 1:15 e 1:20 W / V) a diferentes temperaturas (65, 75, 85 e 95 ° C), bem como para diferentes tempos (40, 50, 60 e 70 min.)	Os extratos foram filtradoS	(ABOZED et al., 2009)

Morina officinalis	As raízes secas foram pulverizadas	Vinte (20) g de material foi extraída com etanol a 95% (400 mL) durante 1,5 h a 100 °C.	Os extratos foram filtrados e concentrados para 20 ml sob pressão reduzida a 50 ° C. O resíduo foi, em seguida, misturado com 20 mL de água	(YANG; HU; ZHAO, 2011)
Agave tequilana	Cinquenta (50) g de polpa foram obtidas a partir do corte transversal dos caules de *A. tequilana*	Os caules foram colocados em um misturador com 1,5 L de água destilada à temperatura de 80 ° C e agitados durante 5 min.	O extrato foi filtrado	(WALECKX et al., 2008)

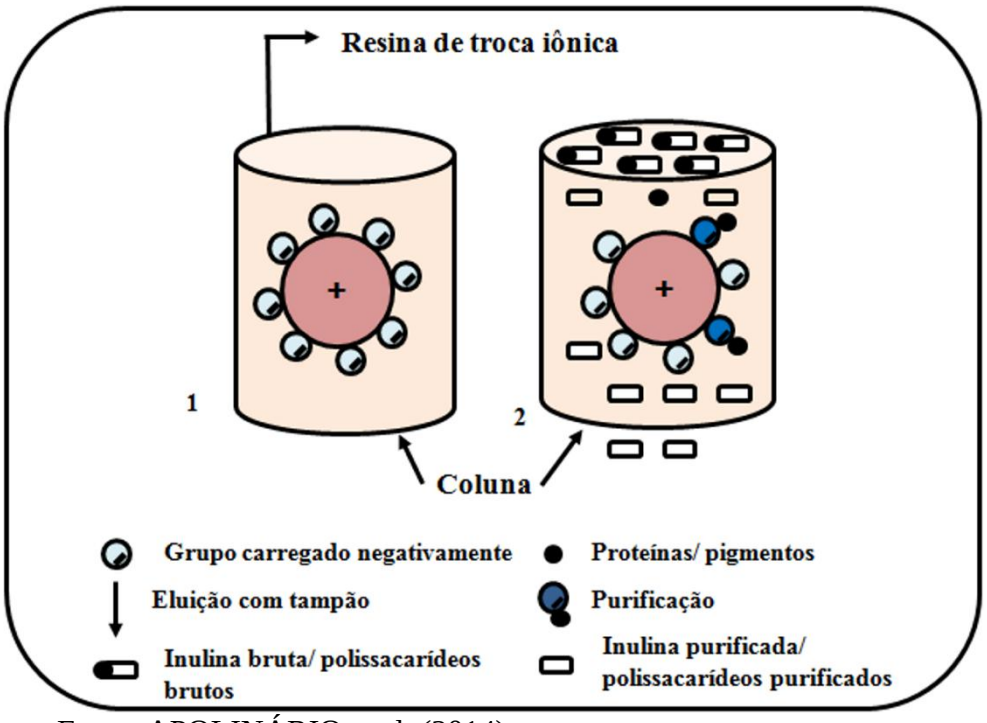
Fonte: APOLINÁRIO et al. (2014).

2.3.7 Os métodos de purificação da inulina

Os métodos de extração e precipitação acima descritos resultam geralmente em soluções que contêm uma mistura de inulina bruta, outros polissacarídeos, e partículas de material coloidal (a pectina, as proteínas e materiais de parede celular), assim as soluções devem ser purificadas para isolar o polissacarídeo específico de interesse (IZYDORCZYK, 2005) Para remover essas impurezas e purificar a inulina, vários métodos físico-químicos, bem como técnicas cromatográficas modernas e de custos elevados podem ser utilizados. A etapa inicial para quase todos os métodos é a dissolução do precipitado em água e a centrifugação para remover os materiais insolúveis (FANG; JIANG; WANG, 2006; HOLDERNESS et al., 2011). O tratamentos químico mais usual é a desproteinização com ácido tricloroacético e reagente de Sevag que consiste em uma mistura de clorofórmio e butanol (v/v, 4:1) e a remoção de taninos com $CaCl_2$ ou $Ca(OH)_2$ (PASEEPHOL et al., 2007; JI et al., 2011; XIN et al., 2012).

Muitos tipos de técnicas de cromatografia podem ser usados para separar inulina de outros tipos de polissacarídeos e de contaminantes. A técnica mais comum é a cromatografia de troca iônica, a qual pode ser influenciada pelo pH, força iônica do tampão de equilíbrio, natureza dos contra-íons, fluxo e temperatura. Para purificação de inulina as resinas de troca iônicas mais comuns são dietilaminoetil (DEAE) celulose a qual tem seu funcionamento descrito na Figura 9, a DEAE Shepharose e a DEAE Sephacel.

Figura 9- Esquema da purificação de inulina por resina de troca aniônica. 1. A resina de troca iônica DEAE celulose (constituída por um núcleo de catiônica e as cadeias externas negativas) é sequencialmente eluída e equilibrada com tampão. 2. O Sobrenadante (solução de inulina ou polissacarídeo brutos) resultante da centrifugação do precipitado re-dissolvido em água destilada ou tampão é fracionado na coluna contendo a resina equilibrada. A inulina ou polissacarídeos puros são obtidos em uma solução límpida. As etapas também podem ser realizadas com diferentes tampões de eluição para obter variadas frações de polissacárideos.



Fonte: APOLINÁRIO et al. (2014).

2.3.8 Técnicas analíticas

A quantificação da inulina pode ser feita diretamente nos extratos ricos em frutano. Uma vez que os frutanos são normalmente encontrados na forma de misturas complexas carboidratos com diferentes GP, composição de monômero e ligações glicosídicas, a sua análise é um passo fundamental para adquirir a informação básica sobre o próprio polímero, bem como para aprofundar a compreensão do seu mecanismo de ação, que é dependente da sua estrutura química. No entanto, a separação de misturas complexas de oligossaccarídeos não é fácil, devido à semelhança estrutural e o peso molecular e, além disso, também a sua identificação é dificultada pela falta de produtos

comerciais disponíveis (BROKL, HERNÁNDEZ-HERNÁNDEZ, SORIA, & SANZ, 2011).

Cromatografia gasosa acoplada a espectrometria de massa (CG-MS), ressonância magnética nuclear (RMN), ionização e dessorção a laser assistida por matriz (MALDI) (do inglês: Matrix-assisted laser desorption/ionization) e a espectrometria de massas tem sido bem sucedida para obtenção de informação estrutural de frutanos, principalmente GP. A cromatografia em camada delgada pode ser utilizada para avaliar composição de frutanos em plantas, no entanto, tem resolução limitada e baixas sensibilidade e precisão quando utilizadas para fins quantitativos. Cromatografia de troca aniônica de alto desempenho com detecção amperométrica pulsada (HPAEC-PAD) é aceito como o método mais importante para a determinação direta de inulina, pois fornece não apenas o conteúdo de inulina, mas também os perfis GP (LÓPEZ et al., 2003).

Embora devido a ausência de grupos cromóforos nas estruturas dos frutanos, o que limita a quantificação destes por técnicas convencionais de espectrofotometria (BROKL et al., 2011), alguns métodos indiretos foram desenvolvidos, os quais são baseados na hidrólise da inulina e derivatização da frutose e glicose com reagentes como o ácido dinitrossalicílico (DNS) fenol e antrona (ARRIZON et al., 2010; LINGYUN et al., 2007; PASEEPHOL; SMALL; SHERKAT, 2007). A inulina pode assim ser mensurada pela diferença entre os carboidratos totais e os açúcares redutores. Outro método espectrofotométrico indireto foi desenvolvido e validado para determinar o teor de inulina de alcachofra de Jerusalém, a técnica se baseia na oxidação de frutose liberada pelo excesso de periodato e subsequente quantificação do reagente restante, por medição da absorbância a 350 nm do complexo formado pelo triiodeto pela adição de iodeto de potássio (SAENGKANUK et al., 2011).

Oligômeros de inulina podem ser analisados por cromatografia líquida de alta eficiência (CLAE), utilizando diferentes técnicas de detecção, no entanto a detecção de UV / Vis fornece resultados pouco sensíveis, principalmente devido às propriedades sw fraca absorção de UV dos derivados de hidratos de carbono como já discutido antes, estes métodos requerem arranjos cromatográficas especiais. (SAENGTHONGPINIT; SAJJAANANTAKUL, 2005).

2.4 Substâncias pécticas

Pectinas são carboidratos naturais considerados funcional e estruturalmente os mais complexos, sendo compostas de uma mistura de heteropolissacarídeos compostos por no mínimo 17 monossacarídeos diferentes sendo os principais ácido galacturônico (GalA) e açúcares neutros como L-ramnose (L-Ram), L-arabinose (L-Arab), D-xilose (D-Xil), L-Fucose (L-Fuc) e D-galactose (D-Gal)(KOŠŤÁLOVÁ; HROMÁDKOVÁ; EBRINGEROVÁ, 2013). A pectina pode conter três sequências de ligações como pode ser visto na Figura 10.

As ligações existentes podem formar quatro tipos de polissacarídeos pécticos: homogalacturonanas (HG), ramnogalacturonanas do tipo I (RG-I), que incluem as cadeias de arabinogalactanas e arabinanas, ramnogalacturonanas do tipo II (RG-II) e/ou as xilogalacturonanas (XGA) covalentemente ligados HG é composta de ligações do tipo α 1→4 parcialmente esterificada com grupos metil álcool (YAPO, 2011).

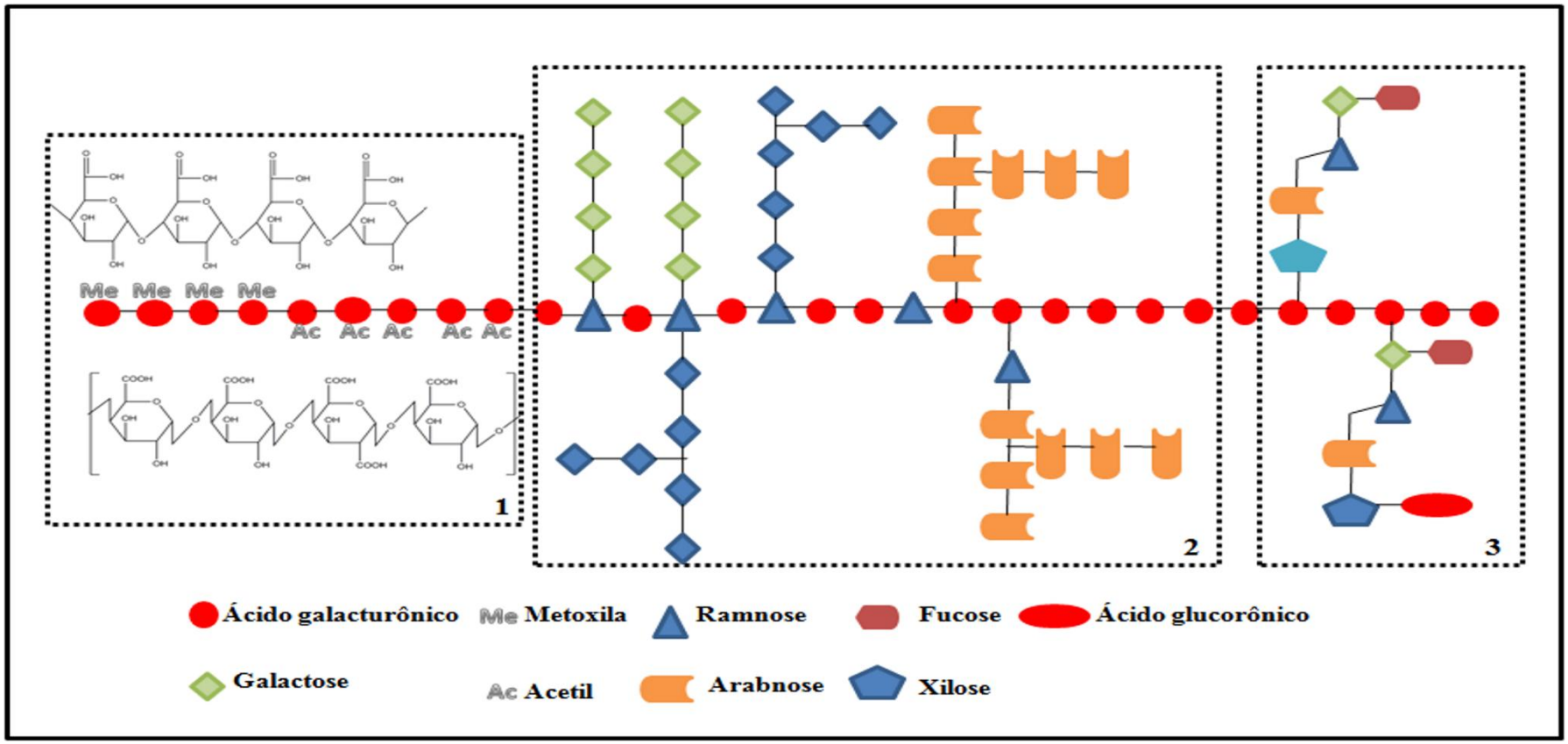
HG é a maior porção das moléculas de pectina, os resíduos de GalA podem ser parcialmente esterificados em C-6 e acetilado em O-2 e /ou O-3. A presença de grupos metoxilas nos carboidratos pécticos determina o grau de metoxilação (GM), pectinas com grau de metoxilação maior que 50 % são denominadas de altamente metoxiladas (YAPO, 2011). As RG-I são formadas por uma cadeia principal de unidades alternantes de ácido α-D-galacturônico ligado (1→4), e α- L-ramnose ligada (1→2), à qual se ligam

cadeias laterais de polissacarídeos neutros, tais como arabinanas, galactanas e arabinogalactanas. RG-II são polissacarídeos pécticos bastante complexos, de baixo peso molecular, contendo na sua cadeia principal 7 a 10 unidades de GalpA com ligação α-(1→4), substituídos em O-2 e/ou O-3 por cadeias laterais heteropoliméricas. Estas cadeias laterais das RG-II contêm cerca de 10 açúcares diferentes e 20 ligações distintas. Os açúcares mais comuns são L-Rha, L-Ara, D-Gal.

2.4.1 Propriedades dos polissacarídeos pécticos

As propriedades de troca iônica, capacidade de ligação de água e ligações de hidrogênio de polissacarídeos pécticos dependem geralmente do número e distribuição dos grupos metil e acetil ao longo da cadeia péctica. O GM corresponde à percentagem de grupos carboxílicos esterificados com metanol. O grau de acetilação (GAC) é definido como a percentagem de resíduos de galactoronosil esterificado com um grupo acetil (LEVIGNE et al., 2002). A planta de origem e as condições selecionadas para o isolamento e purificação da pectina interferem nas propriedades físico-químicas da mesma (CHAN; CHOO, 2013) .

Figura 10- Esquema da estrutura da pectina: **1**- HG chamada de região lisa ou *smooth region* constituída por homopolímeros de unidades de ácido D-galacturônico unidas por ligações glicosídicas do tipo α-(1→4) com porção metilada e acetilada. **2**- RG-1 chamada de região ramificada ou *hairy region*, consiste de uma cadeia principal de unidades alternantes de ácido D-galacturônico ligadas α-(1→4) e ramnose ligadas α-(1→2), à qual se ligam cadeias laterais neutras tais como arabinanas e arabinogalactanas. **3**- RG-1 também chamada de região ramificada ou *hairy region* são polissacarídeos altamente ramificados, com cadeias de unidades de β-D-galactopiranose unidas por ligações (1→3) e (1→6). As ligações (1→3) predominam nas cadeias internas, enquanto que as ligações (1→6) principalmente nas cadeias externas.



Fonte: Autor

3 OBJETIVOS

3.1 Objetivo geral

- Obter novos produtos de interesse farmacêutico a partir do *Agave sisalana* Perrine

3.2 Objetivos específicos

- Caracterizar as matérias-primas vegetais e os seus derivados obtidos a partir do processamento do caule e dos resíduos de *A. sisalana*
- Extrair e purificar inulina do caule de *A.sisalana*
- Obter e caracterizar estruturalmente polissacarídeos a partir dos resíduos de decorticação de *Agave sisalana*
- Avaliar preliminarmente a capacidade prebiótica de produtos obtidos tecnologicamente do *A. sisalana*

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CAPÍTULO 2

ARTIGO 1 **Physicochemical Quality Parameters of Herbal Products from *Agave sisalana***

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Physicochemical Quality Parameters of Herbal Products from *Agave sisalana*

Alexsandra Conceição Apolinário[a]; Morgana Lopes do Nascimento[b]; Juliana Patrícia de Luna Vieira[a]; Camila de Oliveira Melo[b], Felipe Fernandes Santos[b], Bolívar Ponciano Goulart de Lima Damasceno[a,b]; Attilio Converti[c], Adalberto Pessoa[d]; José Alexsandro da Silva[a,b*]

[a]*Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Brazil*

[b]*Department of Pharmacy, State University of Paraíba, Brazil*

[c]*Department of Civil, Chemical and Environmental Engineering, Chemical Engineering Pole, Genoa University, Italy*

[d]*Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Brazil*

***Corresponding author:** Professor José Alexsandro da Silva

Mailing Address: Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, CEP 58429-600, Campina Grande, Paraíba, Brazil

Telephone: (+55 11) 99595-8427 / 3315-3300 (Branch line: 3516)

Abstract

Agave sisalana components have great potential in different pharmaceutical applications, but the quality of herbal raw materials is essential to reach the desired product specifications. In this work we investigated the physicochemical quality parameters of bole and wastes from decortication of *A. sisalana* leaves. The statistically significant variations among products suggest different pharmaceutical applications for each of them.

Keywords: *Agave sisalana*; decortication; wastes; herbal drug; thermal analysis.

1. Introduction

Agave sisalana Perrine, popularly known as sisal, belongs to the Agavaceae family and is a monocotyledonous plant from Mexico. It is well adapted to the semiarid region of Brazilian Northeast that is currently the largest producer of sisal fiber in the world. However only 5% of mass resulting from decortication of sisal leaves is exploited to produce a hard fiber that is used for various purposes, the remaining 95% consisting of a solid waste (bagasse) and a liquid waste (juice) that are normally discarded by sisal farms

Studies demonstrated the potential of *A. sisalana* in pharmaceutical applications because of its anti-inflammatory and antimicrobial activities and revealed that its different parts contain many secondary metabolites, especially steroidal sapogenins, which are used for the synthesis of corticosteroids (Mendes et al. 2003; Pereira et al. 2006). Moreover, this species may be a source of important carbohydrates for pharmaceutical industry such as inulin-type fructans (Apolinário et al. 2014).

However, additional efforts are needed to characterize different materials that can be harnessed from *A. sisalana*, especially wastes. Safety assessments should be a main concern for pharmaceutical industries and suppliers of botanicals as ingredients, not only for a sense of responsibility to the consumers, but also to guarantee agreement with the rigorous statutory requirements (Zöllner & Schwarz 2013).

Based on this background and taking into account quality tests recommended for herbal drugs by the Brazilian National Agency for Sanitary Surveillance (ANVISA), the present work aimed at analyzing and comparing some physicochemical quality parameters of different herbal products from *Agave sisalana*.

2. Results and discussion

2.1 Characterization of materials

Three different materials from *Agave sisalana* were analyzed in this study, namely the liquid waste (I) obtained directly from solid waste by a decortication machine, separated by manual squeezing and lyophilized, the bole (II) and the leaf solid waste (III). As shown in Table 1, the herbal drugs II and III have significantly different physicochemical parameters, for which the pharmacopoeic parameters would point out different possible applications.

Table 1-Physicochemical quality parameters of herbal drugs from Agave sisalana bole (II) and solid leaf waste (III).

Herbal drug	Loss on drying (%)	Ash content (%)	Extractives content in water (%)	pH	Density ($g.mL^{-1}$)
II	4.3 ± 0.1	6.4 ± 0.12	79.3 ± 18.5	5.8 ± 0.1	0.43 ± 0.01
III	6.4 ± 0.1	16.4 ± 0.7	31.5 ± 9.1	9.0 ± 0.1	0.55 ± 0.03

$p < 0.05$ for all values.

Determination of loss on drying highlighted an acceptable percentage of moisture according to the quality standards for plant materials, which limits to 14% its maximum acceptable value in herbal drugs (Da Costa et al. 2013). The higher value of leaf waste (6.4 ± 0.1%) compared to bole (4.3 ± 0.1%) could have partly been due to the faster leaf metabolism and growth.

The granulometry distribution illustrated in Figure 1 evidenced for III larger particle size than for II. Ash content of the two herbal drugs was higher than the limit allowed by the Brazilian Pharmacopoeia (5%), likely due to the presence in III of impurities coming from defibration (Lima et al. 2013).

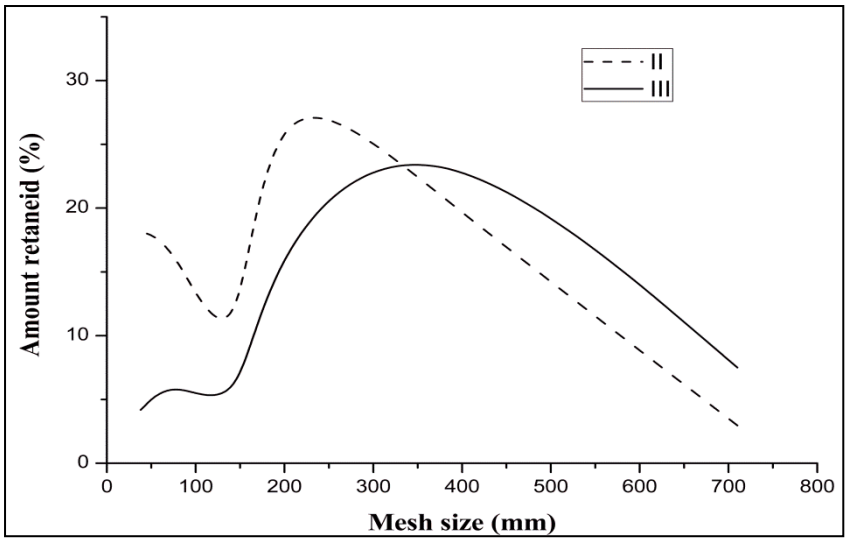

Figure 1-Granulometric distribution of herbal drugs from *Agave sisalana*.

The larger content of extractives in the aqueous extract of bole (II-AE) compared with that of leaf waste (III-AE) may be explained by the high level of water-soluble carbohydrates in the boles of all agave species (Arrizon et al. 2010), which is in agreement with its higher values of dry residue and °Brix (Table 2).

Table 2-Physicochemical quality parameters of aqueous extracts of *Agave sisalana* bole (II-AE) and solid leaf waste (III-AE).

Extract of herbal drug	Volume (mL)	Dry residue (%)	pH	Density ($g.mL^{-1}$)	°Brix
II-AE	268.0	6.1 ± 0.4	5.0 ± 0.3	1.01 ± 0.01	5.5 ± 0.6
III-AE	206.5	2.5 ± 0.2	8.8 ± 0.1	1.00 ± 0.01	2.3 ± 0.5

$p < 0.05$ for all values, except for density ($p = 0.222$)

Some physicochemical quality parameters were also investigated for I either in powder form or diluted in water, whose results are listed in Table 3.

Table 3-Physicochemical quality parameters of the aqueous liquid lyophilized waste from *Agave sisalana.*

Loss on drying (%)	pH 10 % (w/v)	Density ($g.mL^{-1}$)	Brix 10 % (w/v)
10.73 ± 0.29	5.11 ± 0.01	0.57 ± 0.03	3.83 0.23

These results highlight significantly different compositions between the above extracts, with particular concern to pH and °Brix. Although both were obtained by decortication of leaves, these differences may be explained by the fact that the process of manual squeezing to obtain juice separated from bagasse could have dragged water-

soluble components in addition to the lyophilization process used to concentrate these compounds.

2.2 Spectrophotometric scanning

The three products (I, II and III) exhibited maximum absorption in the UV region, the two main bands laying between 200–300 nm. Whereas III and I had similar profiles, the former showing peaks at 669 nm (0.065), 307 nm (0.490) and 271 nm (0.901) and the latter at 671 nm (0.112), 307 nm (0.652) and 271 nm (1.221), maximum absorption of II occurred at 307 nm (0.282) and 269 nm (0.759). The high absorption around 270 nm can be ascribed in all cases to π–π transition of aromatic rings present either in proteins or phenolic compounds (Mezzomo et al. 2011), while that at 307 nm can be assigned to carbohydrates and that at about 670 nm to chlorophyll that is absent in boles (Subramoniam et al. 2012).

2.3 Thermal analysis

Thermogravimetric analysis (TGA) is routinely employed to study any physico-chemical changes in various products including herbal drugs as well as preformulation or drug excipient compatibility (Choudhary & Sekhon 2011). Knowledge of thermal decomposition kinetics of herbal drugs or biomass is essential for efficient design of thermochemical processes for the conversion of these materials into products and energy (Damartzis et al. 2011). The TGA thermograms of I, II and III are illustrated in Figure 2.

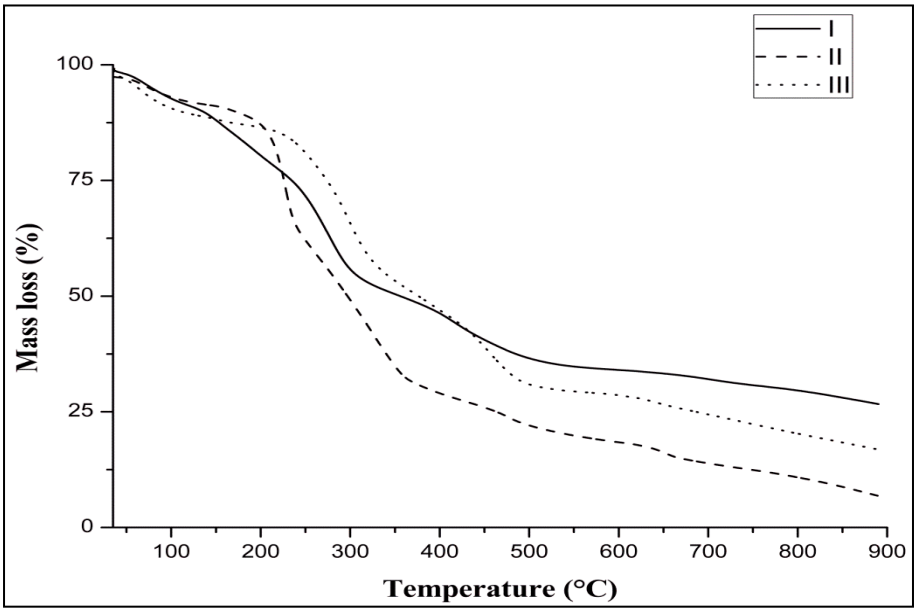

Figure 2-Thermogravimetric curve of herbal drugs of *Agave sisalana.*

The decomposition processes involved several events of mass loss. The first decomposition stage, occurred in the temperature range of 36–100°C, was due to volatile compounds, mainly water, after an initial moisture removal of 7.2% at 95.78°C, 6.0% at 100.38°C and 10.6% at 98.03°C from I, II and III, respectively. As suggested by Da Costa et al. (2013), the differences between thermal analyses and gravimetric methods may be due to the heating rate applied to obtain the TGA curves, whereas the gravimetric technique is an isothermal one.

II showed a second event that implied a 29.3% mass loss in the thermal decomposition range between 213 and 236°C. The largest mass loss during the second event was that of III (35.4%), that took place between 257 and 322°C and corresponded to thermal decomposition of carbohydrates, while the one of I (28.2%) at lower temperature range (250-302°C). As suggested by Espinosa-Andrews et al. (2012), mass loss associated with thermal decomposition around 200°C could in fact be ascribed to decomposition of branched chain fructans that are abundant in all agave species.

As far as II is concerned, its main decomposition, corresponding to the third event that implied a 32.9% loss and occurred between 297 and 360°C, may be assigned to breaking of aliphatic structures of some polysaccharides, while the fourth one, took place between 462 and 498°C (7.6% mass loss), to thermal breakdown of aromatic and more stable structure of lignin. Finally, the fifth event, occurred between 639 and 668°C (6.9% mass loss), may be ascribed to decomposition of carbon-rich residue leading to ash (Carballo et al. 2008) that constituted no less than 6.8% of the overall mass. The data obtained from TG taken together match the results of Aragon et al. (2002), who observed a so high sugar content of *Cissampelos sympodialis* Eichl. that the thermal behavior of the whole plant material followed that of the main polysaccharide fraction.

On the other hand, I and III showed a third event in the temperature ranges of 397-486°C and 434-486°C that implied 15.6 and 16.6% mass losses, respectively, thereby pointing out the removal of gaseous products. The mineral residue of III following the fourth event (17.0%) was substantially higher than the one earlier mentioned for II (6.8%), and that of I (26.7%) even more, hence suggesting that the process of leaf decortication and the manual squeeze may have dragged additional impurities to the liquid that was not filtered before lyophilization. The lower mass losses occurred for I and III compared with II demonstrate that the boles have higher thermal stability than both liquid as solid wastes.

2.4 Total and free sugars, total phenolics and flavonoids

In agreement with spectrophotometric scanning, I, II-AE and III-AE exhibited different contents of total and free sugars, total phenolics and flavonoids (Table 4). In particular, I showed the highest contents of all these metabolites likely because, as earlier mentioned, manual squeezing adopted to obtain it dragged most of the compounds present in the waste. Therefore, bagasse and juice of *A. sisalana* had different quantitative compositions.

It was also observed that boles are sources of carbohydrates as it occurs in other agave species (Arrizon et al. 2010). It was also observed that boles are sources of carbohydrates as it occurs in other agave species (Arrizon et al. 2010). This content of sugars is within the range found to others species which are considered with potential to food and pharmaceutical industry in accordance to Vidanarachchi et al. (2009) which isolated water-soluble prebiotic compounds and showed to four species (*Arthropodium cirratum*, *Cordyline australis*, *Undaria pinnatifida* and *Acacia pycnantha)* values of total sugars between 250–794 mg/g dry matter. To others species already used as sources of polysaccharides as *Agave tequilana*, higher values were found about 867 mg/g (Waleckx et al. 2008). However taking into account that after decortication of leaves from *A. sisalana* 95% are normally discarded, the quantifications pointed to a new application and utilization of others parts of plant as boles and wastes.

Table 3-Quantification of total and free sugars and total phenolics and flavonoids of liquid waste (I), aqueous extracts of bole (II-AE) and solid leaf waste (III-AE) from *Agave sisalana*.

Sample	Total Sugars ($mg.g^{-1}$)	Free Sugars ($mg.g^{-1}$)	Total Phenolics ($mg.g^{-1}$)	Flavonoids ($mg.g^{-1}$)
I	$451.44^{a} \pm 32.65$	$103.39^{a} \pm 12.16$	$58.11^{a} \pm 0.41$	$5.99^{a} \pm 0.51$
II-AE	$347.65^{b} \pm 47.36$	$27.63^{b} \pm 0.77$	$2.23^{b} \pm 0.10$	$0.48^{b} \pm 0.02$
III-AE	$28.12^{c} \pm 0.68$	$8.13^{c} \pm 0.87$	$8.31^{c} \pm 0.26$	$4.55^{c} \pm 0.19$

$p < 0.05$ for all values. Means in the same column followed by different letters were statistically different.

3. Experimental

3.1 Plant materials

Agave sisalana was collected in the germplasm bank of an experimental farm by a research-unit of Embrapa (Brazilian Agricultural Research Corporation) in Monteiro-PB, Brazil (7°52'40.50" S and 37°07'34.91" W) on January 2013. A voucher was deposited at the "Manoel de Arruda Câmara" herbarium of State University of Paraíba, Campina Grande-PB, Brazil, under the number 210. The liquid and solid wastes utilized

in this study were collected directly from a decortication machine in a sisal farm processing leaves of 6-year old sisal plants. One portion of the resulting liquid waste was submitted to lyophilization. Both the solid waste and boles were dried in a forced circulation oven at 40°C until constant weight to give the corresponding herbal drugs. Dried samples of solid waste and bole were stored in sealed polyethylene bags in an oven at room temperature, the liquid waste in amber bottles at -60°C and the freeze dried sample of liquid waste in the sealed polyethylene bags at 4°C. The storage time of the samples after processing was about two months until the end of all analyzes.

Herbal drugs were extracted with distilled water by dynamic maceration to obtain the corresponding liquid extracts. Figure 3 shows the steps followed to achieve all materials (herbal drug and extracts) from *A. sisalana*.

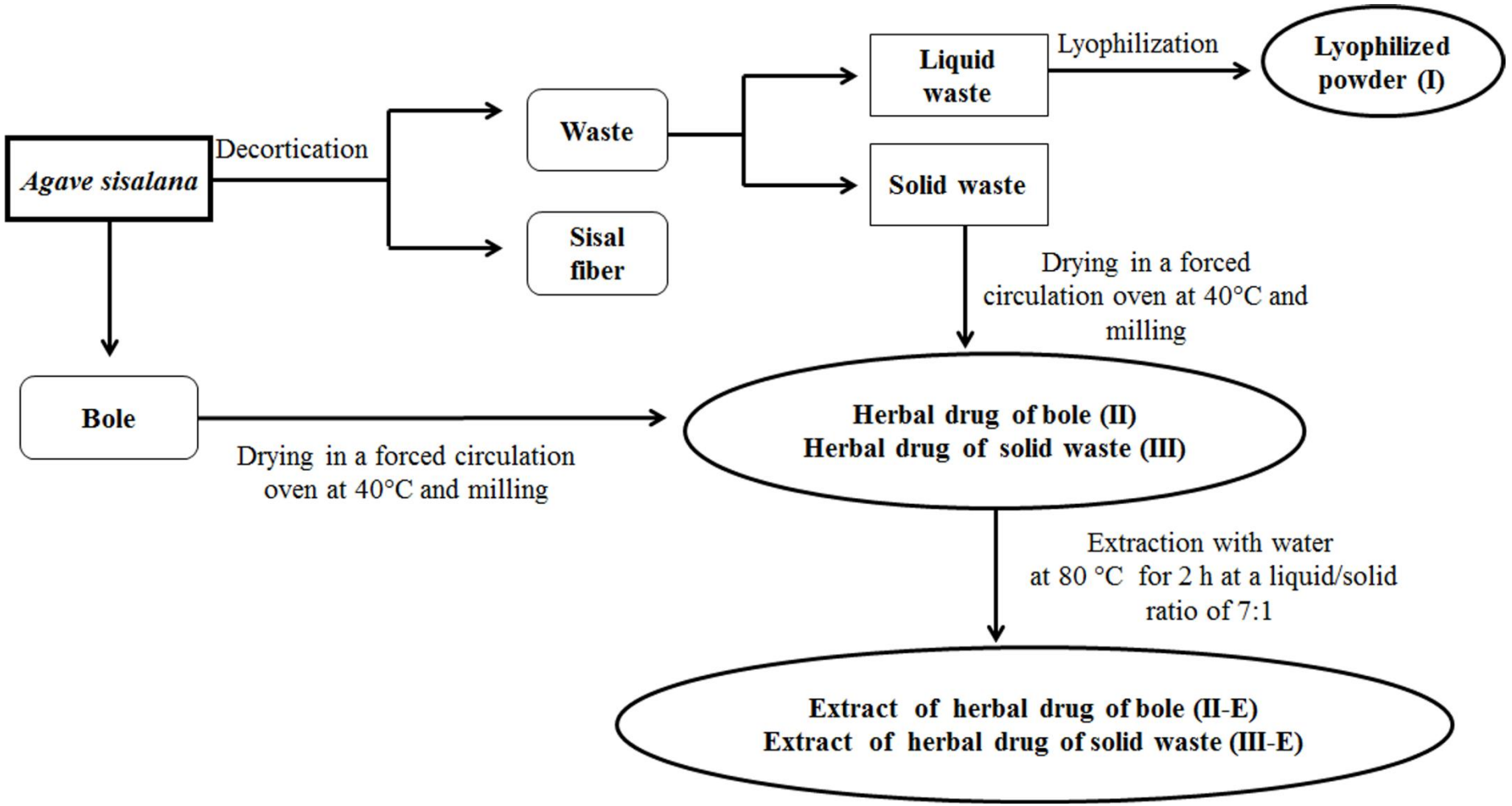

Figure 3-Scheme of achievement of botanic materials.

3.2 Characterization of materials

The procedures of Brazilian Pharmacopoeia (2010) were followed to perform physic-chemical analyses on both II and III, specifically pH, grain size, density, loss on drying, ash content and extractives. Samples of I were subject to determinations of pH and °Brix, after dilution with water to one-tenth of their original concentrations, grain size, density, loss on drying, ash content. II-AE and III-AE were submitted to determinations of pH directly in the extracts, density by pycnometer and °Brix by refractometer utilizing water for calibration.

3.3 Spectrophotometric scanning

Diluted samples of I (0.5 $mg.mL^{-1}$), II-AE (25 $\mu L.mL^{-1}$) and III-AE (10 $\mu L.mL^{1}$) were submitted to spectrophotometric scanning in the wavelength range of 200-800 nm by a UV-Vis spectrophotometer, model UV-mini-1240 (Shimadzu, Kyoto, Japan), quartz cuvettes with 1.0 cm-pathlength and water as a blank.

3.4 Thermal analysis

Thermogravimetric analysis of raw materials (I, II and III) was made by a differential scanning calorimeter, model DSC Q20 (TA Instruments, New Castle, DE, USA), on samples having mass of 5.0 ± 0.05 mg. Rising temperature experiments were conducted in the temperature range of 25–900°C, at heating rate of 10 $°C.min^{-1}$ in synthetic air and under nitrogen flow of 50 $mL.min^{-1}$, respectively. The apparatus was calibrated with calcium oxalate monohydrate.

3.5 Quantification of total and free sugars, total phenolics and flavonoids

Total carbohydrates were determined by the phenolsulfuric acid method (Dubois et al. 1956) using sucrose as a standard. Free reducing sugars were determined using 3,5-dinitrosalicylic acid as reagent and glucose as a standard (Miller, 1959). Total phenolics content of samples was determined using the Folin-Ciocalteau reagent and gallic acid monohydrate as a standard (Saha et al. 2013). Total flavonoids were determined through the formation of a flavonoid-aluminum complex (Mbaebie et al. 2012) using quercetin as a standard. All reagents were purchased from Sigma-Aldrich (São Paulo, Brazil).

3.6 Statistical analyses

All analyses were performed in triplicate, and the results expressed as mean ± standard deviation (SD). Statistical comparison of data was performed by one-way analysis of variance (ANOVA) and Tukey's test using the Origin Pro 8.0® software

(OriginLab, Northampton, MA, USA), and *p*-values < 0.05 were considered as statistically significant.

4. Conclusions

A. sisalana liquid waste, bole and leaf solid waste showed relevant differences in their physicochemical parameters along with spectral and thermal behaviors. In addition, spectrophotometric quantifications demonstrated quantitative differences in the composition of agave bagasse and juice. Results suggested new applications of this species for herbal drug production, with special concern to the liquid waste that exhibited the highest contents of total and free sugars, total phenolics and flavonoids.

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CAPÍTULO 3

ARTIGO 2- Physicochemical characterization of biopolymer inulin isolated from boles of *Agave sisalana*

Artigo submetido ao periódico **CARBOHYDRATE POLYMERS**

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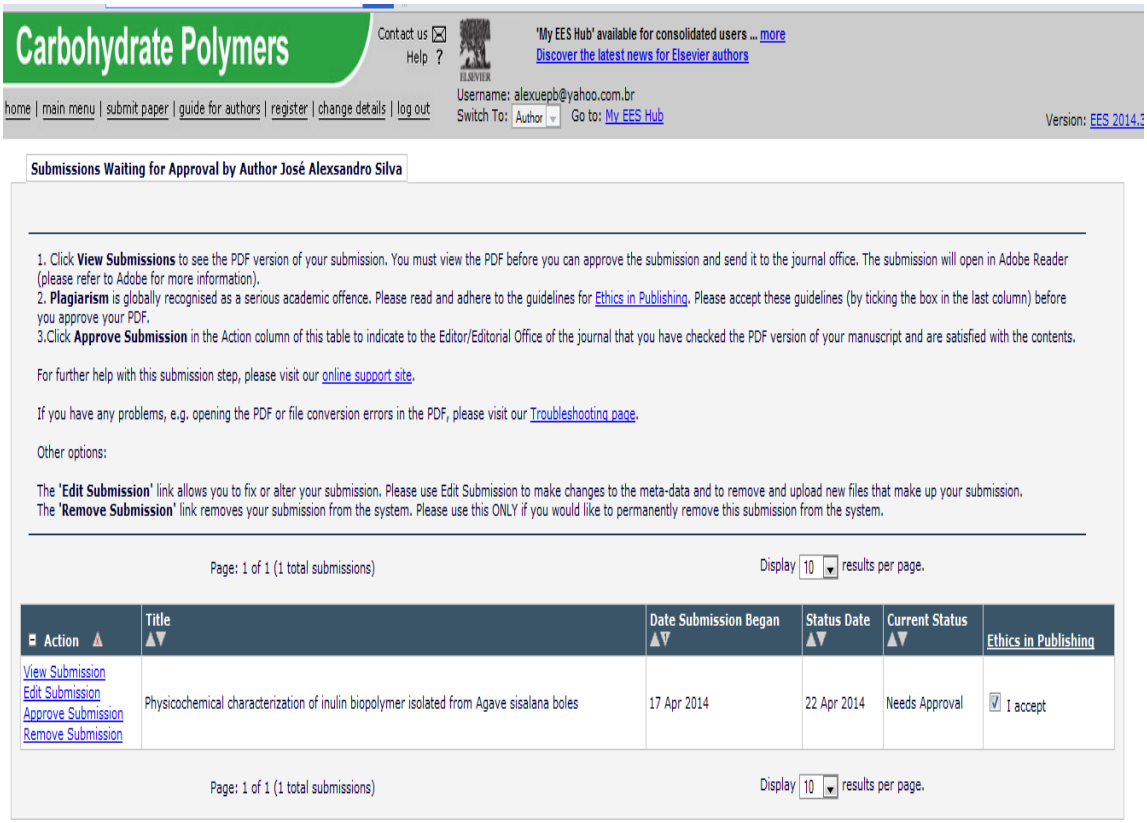


"A ciência, como um todo, não é nada mais do que um refinamento do pensar diário."
Albert Einstein

Physicochemical characterization of biopolymer inulin isolated from boles of *Agave sisalana*

Author Names: Alexsandra Conceição Apolinário[a], Camila de Oliveira Melo[b], Morgana Lopes[b], Erika Martins de Carvalho[c], Bolivar Ponciano Goulart de Lima Damasceno[a,b]Attilio Converti[d], Adalberto Pessoa[e], José Alexsandro da Silva[a,b]

Authors Affiliation:

[a]Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, 58429-600, Campina Grande, Paraíba, Brazil, acapolinario@gmail.com; bolivarpgld@pq.cnpq.br; alexuepb@pq.cnpq.br

[b]Department of Pharmacy, State University of Paraíba, Brazil, camillamello-@hotmail.com; morganaldn@gmail.com

[c]Institute of Pharmaceutical Technology, Farmanguinhos, Fundação Oswaldo Cruz – Fiocruz, Rua Sizenando Nabuco, 100, 21041-250, Rio de Janeiro, RJ, Brasil, erikamc@far.fiocruz.br

[d]Department of Civil, Chemical and Environmental Engineering, Chemical Engineering Pole, Genoa University, Via Opera Pia 15, 16145, Genova, Italy, converti@unigi.it

[e]Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580 – Bloco 16, Cidade Universitária, 05508-000, São Paulo, Brazil,pessoajr@usp.br

***Corresponding author:** José Alexsandro da Silva

Mailing Address: Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, CEP 58429-600, Campina Grande, Paraíba, Brazil

Telephone: (+55 11) 99595-8427 / 3315-3300 (Branch line: 3516)

E-mail: alexuepb@pq.cnpq.br

Highlights

- Boles from *Agave sisalana* were submitted to extraction with hot water.
- Crude inulin was precipitated from aqueous extract.
- Inulin was purified with ion exchangers in diethylaminoethylcellulose.
- Inulin from *A.sislana* was characterized by several analytical techniques.
- Temperature of glass transition and polymerization degree of inulin were determined

Abstract

Agave sisalana Perrine is a species common in the Brazilian Northeast region where it is exploited as a resource of hard fiber (sisal). Taking into account that some agave species are sources of fructans, this paper aimed at extracting and isolating inulin from *A. sisalana*. After preparation of aqueous extract of boles, such a biopolymer was precipitated using water as a solvent at low temperature, and powder was obtained after purification with exchange ionic resin by freeze-drying. Infrared analysis and magnetic nuclear resonance allowed shedding light on the chemical structure of fructose polymer. X-ray diffraction, thermal analysis, circular dichroism and Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) analysis clarified significant aspects of physicochemical characteristics of this polysaccharide allowed determining its polymerization degree and revealed features similar to those of inulin extracted from others sources.

Keywords:

Agave sisalana, Inulin extraction, Polymerization degree, Amorphous material

Abbreviations: INAS: Inulin from *Agave sisalana*, **PPT:** Precipitate, **CIN:** crude inulin, **EQ:** equivalent

1. Introduction

Among the various species of the genus *Agave*, *Agave sisalana* Perrine is a species common in the Brazilian Northeast region where it is exploited only as a source of hard fiber (sisal). However some studies demonstrated the potential of *A. sisalana* in different pharmaceutical applications. Important secondary metabolites, biological activities and new products of interest for the industry of drugs and foods have in fact been described in recent researches that approached new uses of this plant (Branco et al., 2010; Cerqueira et al., 2012; Dunder et al., 2010; Santos, Espeleta, Branco, & de Assis, 2013; Zhang, Liu, & Lin, 2013).

Studies performed on some agave species demonstrated that they can be a source of fructans such as inulin. The most important commercial source of inulin is chicory that accumulates between 13 and 17% (w/w) of fructan by fresh weight, i.e. a content similar to the that found in mature agave species (Ávila-Fernández, Galicia-Lagunas, Rodríguez-Alegría, Olvera, & López-Manguía, 2011). In these species, fructose polymers are present as reserve of carbohydrates, which are synthesized and stored mainly in the stems (Arrizon, Morel, Gschaedler, & Monsan, 2010). Agave fructans, particularly that of *Agave tequilana*, are branched fructose biopolymers with a degree of polymerization (DP) ranging from 3 to 29 and a large number of β(2–6) linkages; therefore, they are classified as mixed fructans and neoseries fructans (Lopez, Mancilla-Margalli, & Mendoza-Diaz, 2003).

In an in deep review Apolinário et al. (2014) described that inulin-type fructans, which are photosynthetically produced by the crassulacean acid metabolism (CAM), act as osmoprotectants during drought; for this reason, inulin is present as storage carbohydrate in more than 30,000 vegetable species. This polysaccharide is a water-soluble fructose-based polymer resulting from extended sucrose metabolism, which is

composed of 20–30 fructose unit chains; its DP determines its applications and hence the value of the crop (Guggisberg, Cuthbert-Steven, Piccinali, Bütikofer, & Eberhard, 2009).

This paper aimed at describing extraction, isolation and physicochemical characterization of inulin isolated from boles of *A. sisalana*.

2. Material and methods

2.1 Preparation of the extract

The botanical material was collected in Monteiro, Paraíba-PB (7°52'40.50" S and 37°07'34.91" W) on January 2013. A voucher was deposited at the Herbarium Manoel de Arruda Câmara (Campina Grande, Paraíba, Brazil) under number 210. The boles were collected from six years old plants, dried in a forced circulation oven at 40°C until constant weight and milled.

The extraction was performed twice at 80°C in a thermostatic bath (model SL 155/10, Solab, SP, Brazil) for 2 hours in distilled water at a 7:1(w/v) ratio. Dynamic maceration was allowed by agitation at 1600 rpm using a mechanical stirrer (model 713, Fisatom, SP, Brazil). To obtain final aqueous extracts, samples were filtered through qualitative paper.

2.2 Total and free reducing sugars quantification in the extract

Total carbohydrate content of the extract was determined by the phenolsulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) at 480 nm, using a reference curve obtained in triplicate with pure inulin (HP® Orafti Group, Tienen, Belgium) as a standard whereby was obtained equation $Y=0.0092X+0.011$ ($R^2=0.9929$).

Determination of free reducing sugars in the extract was performed using 3,5-dinitrosalicylic acid (DNS) as a reagent (Sigma-Aldrich®, SP, Brazil) then, they were

quantified comparing the absorbance of samples at 540 nm against a reference curve obtained in triplicate using fructose (Sigma-Aldrich®) as a standard (Miller, 1959) using following equation $Y=0.6018X + 0.0481$ ($R^2= 0.9954$).

2.3 Isolation and purification of inulin

Polysaccharides of aqueous extract were precipitated with acetone (Vetec® Duque de Caxias, RJ, Brasil) (1:2 v/v) overnight at 4°C. The crude precipitate (crude ppt) was pelletized by centrifugation (Excelsa I 206 BL, Fanem, Guarulhos, SP, Brazil) at 1117.8 *g* for 20 min and submitted to four cycles of dissolution in distilled water at 80°C (1:2 w/v) and centrifugation under the same conditions as above. This solution was then purified with diethylaminoethyl (DEAE)-cellulose resin (Sigma-Aldrich®) in a 1.5 x 12 cm prolypropylene column (Econo-Pac® 732-1010, Bio-Rad, Hercules, CA, USA) equilibrated with 0.05 M Tris HCl buffer (Vetec®) (pH 7.0). The unbound fraction was precipitated with acetone (1:2 v/v) and centrifuged, and the precipitate was re-dissolved in distilled water (1:2 w/v) and lyophilized. Figure 1 shows the steps of isolation of inulin from *A. sisalana* (INAS).

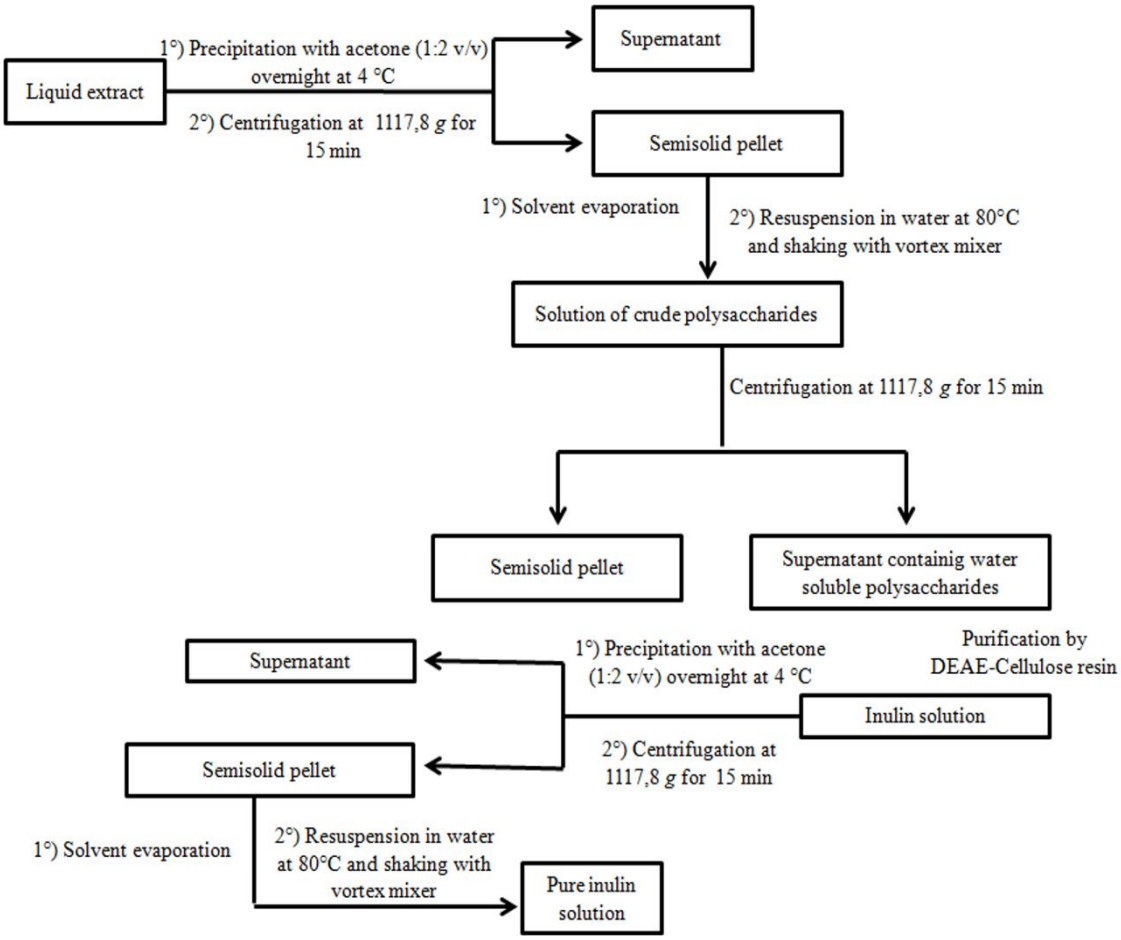


Figure 1- Scheme of isolation of inulin from from *A.sisalana* boles.

2.4 UV-Vis scanning of inulin powder

Diluted INAS at concentration of 1000 µg.mL^{-1} was submitted to UV-Vis scanning in the wavelength range of 200-800 nm to make quality control of the final product. To this purpose, readings were performed in triplicate using 1.0 cm-pathlength quartz cuvettes in a UV/Vis spectrophotometer (model 1240, Schimadzu, Kyoto, Japan) against water as blank.

2.5 Infrared analysis

The infrared (IR) spectra of inulin from *A. sisalana* were done using a IR spectrophotometer (Vertex 70 interferometer, Bruker Optics, Ettlingen, Germany). Five milligrams of inulin were homogenized with KBr, and the resulting mixture was pressed

139 to form tablets and subjected to analysis in the wave number range between 4,000 and
140 400 cm^{-1}.

141 2.6 X-ray diffraction

142 X-ray diffraction (XRD) patterns of inulin samples in powder form were
143 recorded at room temperature using a diffractometer (Miniflex Goniometer, Rigaku,
144 Tokyo, Japan). Diffraction spectra were collected within 2 h in the range from 10° to
145 80° with a constant step of 0.04° and a counting time of 1 s/step.

146 2.7 Thermal analysis

147 Thermogravimetric (TG) analysis and differential thermal analysis (DTA) of
148 inulin were carried out using an Exstar TG/DTA (model 7200, SII Nanotechnology Inc.,
149 Tokyo, Japan). Samples were heated to 500°C at a rate of 20°C/min under N_2 flowing
150 (100 $mL.min^{-1}$). Differential Scanning Calorimetry (DSC) curves were obtained through
151 the same equipment during heating of samples from 25 to 300°C at a rate of 5°C/min
152 using aluminum pan.

153 2.8 Circular dichroism

154 A spectropolarimeter (model J-810, Jasco, Tokyo, Japan) was used to collect
155 circular dichroism (CD) spectra of inulin samples at a concentration of 0.6 $mg.mL^{-1}$.
156 Spectra were collected at 25°C in the wavelength range from 200 to 800 nm at 1 nm
157 intervals. Samples were heated from 40 to 80°C and a new scanning was carried out.

158 2.9 MALDI-TOF analysis

159 Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF)
160 analysis was operated in positive linear mode using an Autoflex III (Bruker Daltonics,
161 Billerica, MA, USA). Samples were dissolved in water (4 $mg.mL^{-1}$) and solubilized in a

matrix of alpha-cyano-4-hydroxycinnamic acid and acetonitrile (10 $mg.mL^{-1}$); then, 1.0 mL of mixture was applied into the probe and quickly dried under vacuum.

2.10 Nuclear Magnetic Resonance

^{1}H and ^{13}C NMR spectra of inulin were recorded on a spectrometer (model Avance 500, Bruker, Bremen, Germany) (500 MHz for ^{1}H and 125 MHz for ^{13}C) in D_2O at 30 ± 0.1°C using a 30 pulse (12.5 μs for ^{1}H and 7.0 μs for ^{13}C) and a 5 mm switchable probe. ^{1}H NMR spectra were acquired by 1024 scans with a relaxation delay of 2.0 s, 16K data points, an 8278.1 Hz spectral width using a digital resolution of 0.30 Hz. The ^{13}C ones were acquired by 386440 scans with 23980.8 Hz spectral width using a digital resolution of 1 Hz and 32K data points.

3 Results and discussion

3.1 Total and free sugar contents of the extract

As a first characterization attempt, the sugar general profile of the extract, either in terms of free reducing or total sugars, was determined spectrophotometrically (Dubois et al., 1956; Miller, 1959) before isolating inulin. The values of free reducing and total sugars, 27.73±0.1 and 347.65±0.1, respectively expressed in mg of sugar/g of dried bole are the ranges reported in the literature for other plants that are considered as potential sources of prebiotics (Vidanarachchi, Iji, Mikkelsen, Sims, & Choct, 2009). Higher values of total sugar concentration were reported by Waleckx, Gschaedler, Colonna-Ceccaldi, & Monsan (2008) for *Agave tequilana*, but no similar study was made about these metabolites in *A. sisalana*. Nonetheless, these promising results suggest that boles of this species could be a potential source of water-soluble carbohydrates like those of other agave species.

3.2 Isolation and purification of inulin

As is well known, a factor that greatly influences the inulin content of vegetal species is the age of plant; it was suggested a direct relationship between this factor and the activity of 1,2-α-fructan 1-fructosyltransferase (1-FFT), one of the enzymes that catalyze fructan biosynthesis, i.e., the inulin-type fructan content is the highest in plants older than 3 years (González-Cruz, Jaramillo-Flores, Bernardino-Nicanor, & Mora-Escobedo, 2011). For this reason, 6-years old plants were used in this study.

In addition, the final yield of inulin isolated from different plant species is influenced by factors related to the extraction process such as temperature, extraction time, solvent/solid ratio and precipitation conditions to recover inulin (solvent type, velocity and time of centrifugation); therefore, the optimal extraction conditions reported in the Materials and Methods section were selected on the basis of a previous literature survay (Apolinário et al., 2014).

Based on this background, crude inulin (CIN) was obtained as a brown pellet by precipitation of the extract with acetone, with a yield of 4.8% of dry bole, while pure INAS was obtained as white powder with a yield of 0.52% of the raw material and 10.87% of CIN. No absorbance of INAS at 270 nm wavelength suggested the absence of any proteins in this material.

3.3 Infrared analysis of *A. sisalana* inulin

As shown in Figure 2, the IR spectrum of INAS revealed typical bands of inulin-type fructans with the presence of hydroxyl and carbonyl groups.

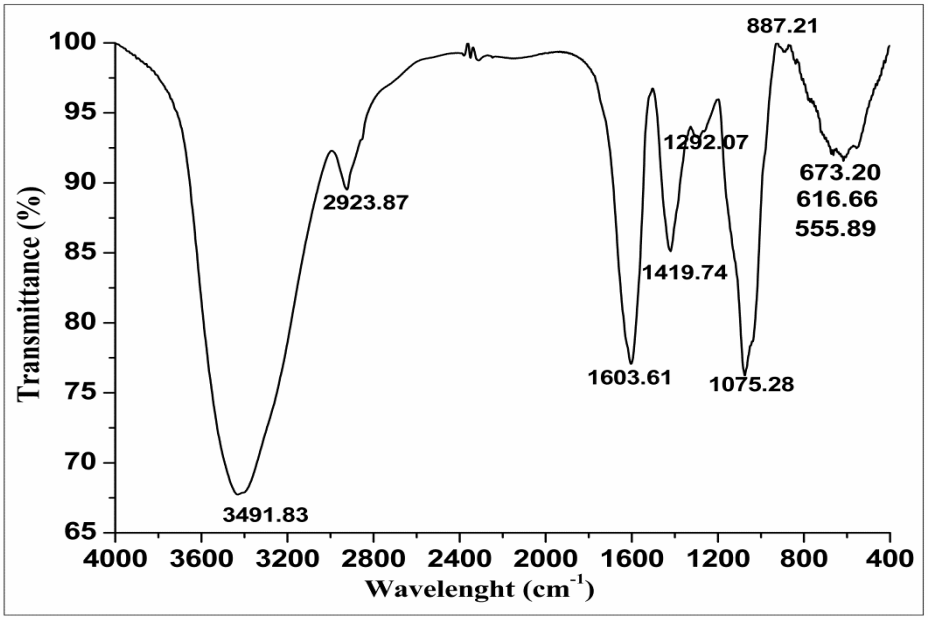


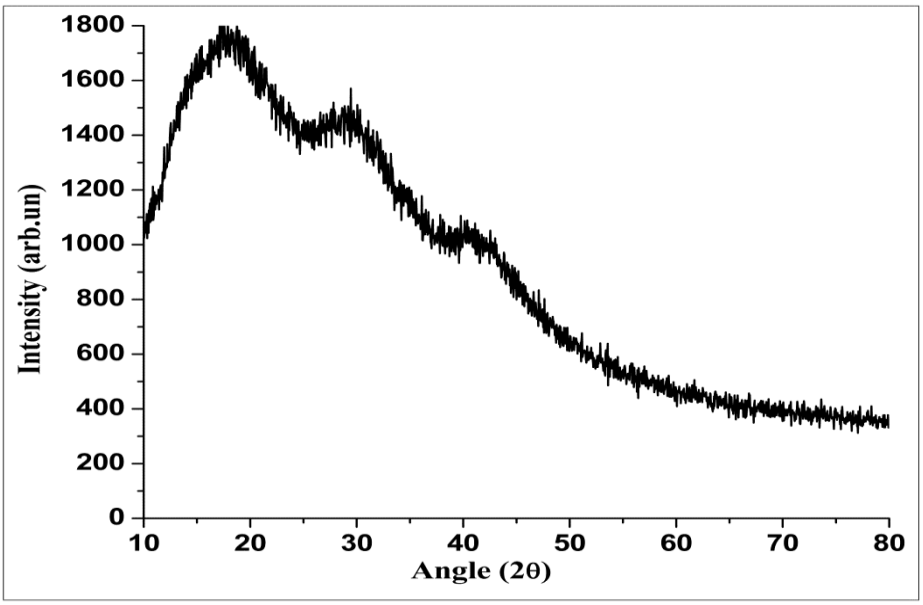
Figure 2 - Infrared spectrum of *A. sisalana* inulin.

The analysis showed bands of the so-called finger-print region of inulin molecule (Panchev, Delchev, Kovacheva, & Slavov, 2011), namely at 3431.93 and 2923.87 cm^{-1} (in the 3600-2500 cm^{-1} region), at 1603.61 cm^{-1} (in the 2500-1550 cm^{-1} region) and at 1419.74, 1292.07, 1075.28, 887.21, 673.20, 616.66 and 555.89 cm^{-1} (in the 1500-900 cm^{-1} region).

A major broad stretching peak around 3491.83 cm^{-1} indicated the presence of hydroxyl groups, and the small band at around 2923.87 cm^{-1} was attributed to C-H stretching and bending vibrations. The relatively strong absorption peak at around 1603.61 cm^{-1} corresponded to the absorption of the C-O bond of glycosides (Chen et al., 2011), while the one at 1419.74 cm^{-1} was assigned to C-OH deformation vibration with contribution of O-C-O symmetric stretching vibration of carboxylate groups. The IR bands between 1229.07 and 887.21 cm^{-1} were typical of the polysaccharide structure with C-O-C bonds between the monomers forming the polymer (Dalonso et al., 2009), while that at 1055.31 cm^{-1} could be assigned to stretching vibrations of pyranose ring of inulin (Gómez-Ordóñez & Rupérez, 2011).

224 3.4 X-ray diffraction

225 The results of X-ray diffraction (XRD) analysis of isolated inulin are illustrated in
226 Fig. 3. It is noteworthy the absence of any sharp diffraction peak, a feature that is
227 typically observed in materials present in amorphous state, whereas it can be noticed a
228 peak of 17.25° at 2θ that is characteristic of the polymeric structure of inulin (Yi, Ha,
229 Lee, & Chung, 2013).



230
231 Figure 3- X-rays diffractogram of *A. sisalana* inulin.

232 This physical state was the likely result of the process used to isolate inulin from
233 *A. sisalana*; amorphous materials are in fact formed through the rapid cooling of a
234 liquid to a certain temperature, so that the molecules do not have enough time to
235 rearrange and are frozen in their original position. This physical state can also be
236 obtained when a solution is rapidly dried by freeze dryer (Ronkart, Paquot, Fougnies,
237 Deroanne, & Blecker, 2009). The molecular weight of inulin is another factor that plays
238 a crucial role in crystallinity, in that inulins with degree of polymerization (DP) > 25 are
239 relatively crystalline, while smaller DPs are usually associated to amorphous state
240 (Zimeri & Kokini, 2002).

Most commercial inulin products obtained as dry powders by techniques like spray dryer can be amorphous or semi-crystalline (Glibowski & Pikus, 2011). Depending on moisture and/or temperature of storage, the amorphous products can be subjected to physical modifications to improve stability (Ronkart et al., 2009).

3.5 Thermal analysis

Thermogravimetric (TG) analysis and differential thermal analysis (DTA) and The Differential Scanning Calorimetry (DSC) are illustrated in Figure 4. TG and DTA of INAS highlighted two thermal events that are similar to those observed by Espinosa-Andrews & Urias-Silvas (2012) in fructans isolated from *A. tequilana* (Fig. 4A).

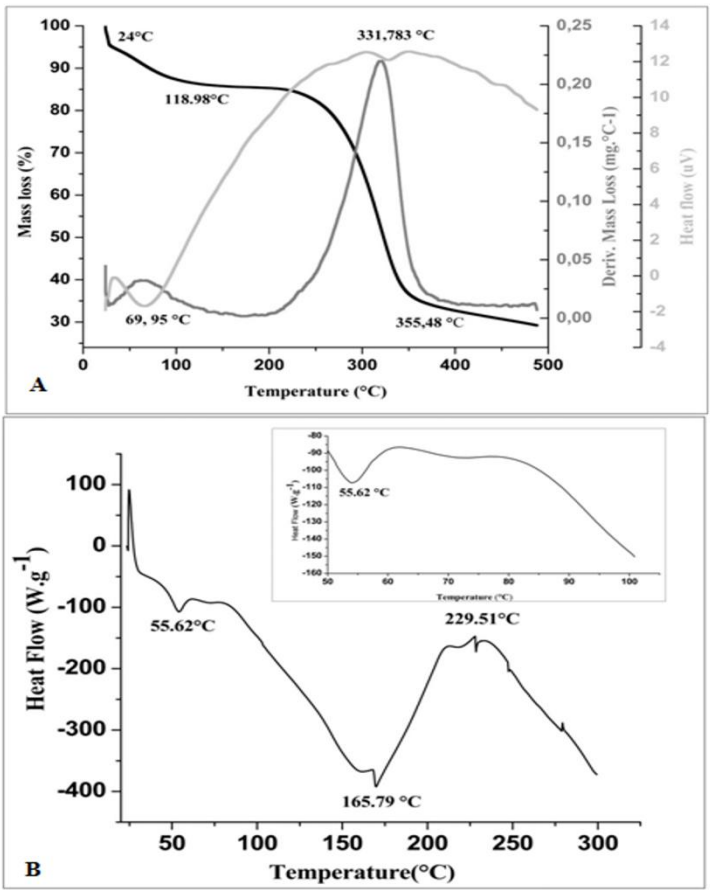


Figure 4 – Thermogravimetric, differential thermal analysis and differential scanning calorimetry profiles of of *A. sisalana* inulin.

The former event, occurred since the beginning of the run from 24 to 118.98°C, showed a maximum peak at 66.6°C and a mass loss of 13.10%, probably due to

evaporation of bound water (Ronkart, Deroanne, Paquot, Fougnies, & Blecker, 2010). The latter and major event, occurred between 246.98 and 355.48°C with a peak at 322.27°C, implied a mass loss of 47% and was likely related to decomposition of branched chains of agave fructans (Espinosa-Andrews & Urias-Silvas, 2012). Both peaks appeared in the TG curve well correlate to the endothermic events evidenced by the DTA thermogram at 69.95°C and 331.783°C. Consistently with these observations, Dan, Ghosh, & Moulik (2009) observed by DTA two endothermic events in inulin, the latter being associated to thermal decomposition of the biopolymer. In addition, endothermic peaks at temperature ranges very close to the lower one detected in this work (69.95°C) were reported for inulin from others sources (Panchev et al., 2011).

As is well known, temperature of glass transition (Tg) is the temperature assigned to a region above which a material is fluid or rubbery and below which it is immobile and inflexible, simply frozen in a disordered, non-crystalline state (Kawai, Fukami, Thanatuksorn, Viriyarattanasak, & Kajiwara, 2011). Different studies pointed out that the factor mostly influencing inulin Tg is water content. Depending on the relative moisture, a caking phenomenon may in fact occur when glass transition temperature is below the storage temperature, because the increase in water activity of inulin promotes its crystallization (Ronkart et al., 2006, 2009).

The DSC profile of INAS depicted in Figure 4B showed a Tg in the range between 50 and 55.82°C with a small peak around 55.62°C. The above Tg range is practically coincident with that reported by Panchev et al. (2011) for inulin (51-55°C) and consistent with most of data reported in the literature for the same material (Hinrichs, Prinsen, & Frijlink, 2001; Ronkart et al., 2006, 2007, 2009, 2010; Zimeri & Kokini, 2003).

Chiavaro, Vittadini, & Corradini (2007) reported for Raftilose® Tg in the temperature range between 44.7±0.5 and 55.5±0.6 ºC, the highest values being associated to the highest molecular weights. Consistently, Hinrichs et al. (2001) observed an increase in the range of Tg with increasing the molecular weight.

3.6 Circular Dichroism

Results of Circular Dichroism (CD) shown in Figure 5 revealed no modifications in the INAS structure with increasing temperature, without variations of cotton effects or positive and negative ellipticity and suggested good thermal stability (Zhang, Chen, Ma, & Zhang, 2013).

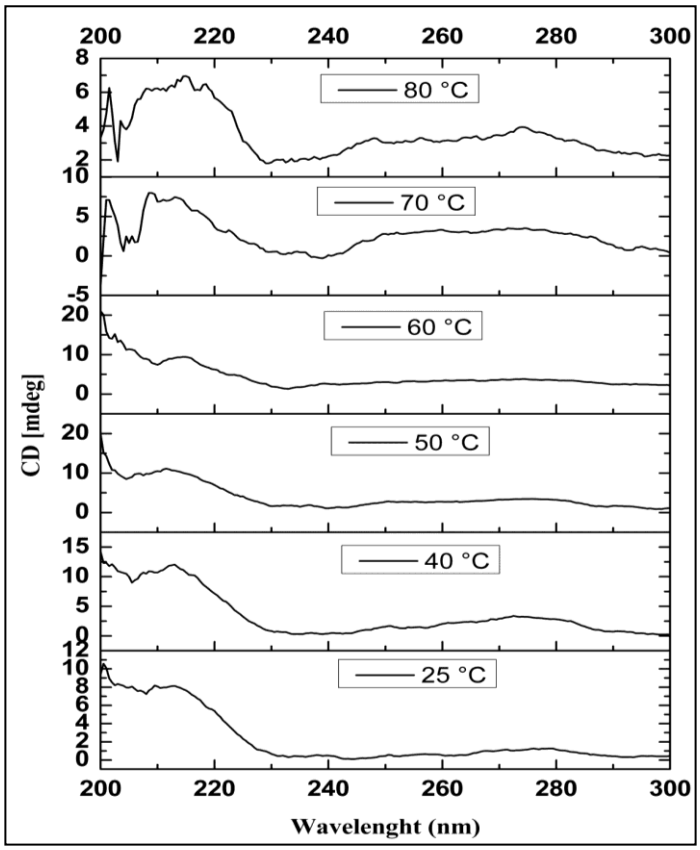


Figure 5 - Circular dichroism spectra of *A. sisalana* inulin obtained at different temperatures in the range between 25 and 80°C.

However, it was possible to note that ellipticity in the range of highest absorbance (around 200-215 nm) decreased from 20 to about 5 millidegrees (mdeg) when temperature was increased from 60 to 80ºC. This effect, which is consistent with the

295 results of DSC that pointed out modifications in INAS structure with glass transition
296 above 50°C, could be related to conformational change (Yang & Zhang, 2009).

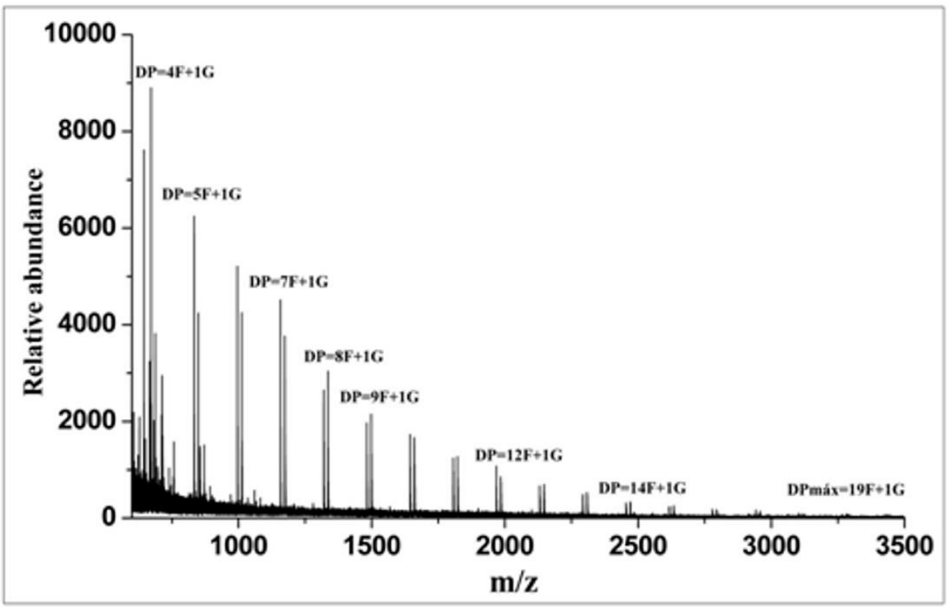
297 3.7 MALDI-TOF analysis

298 As is known, the properties of inulin are greatly influenced by DP, in that short-
299 chain inulin (average DP up to 11 units) is thermally less stable, more soluble and less
300 viscous than the long-chain inulin (between 23 und 25 units). Long-chain inulin is used
301 as a fat substitute because of its capacity to form microcrystals that interact with each
302 other forming small aggregates able to capture an excessive amount of water, so
303 creating a soft and creamy texture (Guggisberg et al., 2009).

304 In its turn, inulin DP is a function of the precipitation method, in that short-chain
305 fructans are usually precipitated by lowering temperature, whereas long-chain fructans
306 by using organic solvents (Apolinário et al., 2014). Another crucial factor influencing
307 fructan DP is the plant age. A previous study addressed to the effect of *A. tequilana* age
308 on fructan structure pointed out an increase in DP in 4-year old plants compared to the
309 2-year old ones, and a subsequent reduction in 6-year old plants, which was associated
310 to partial depolymerisation of longer fructans to lower length fructans (Arrizon et al.,
311 2010). Since there was no clear information in the literature about these issues for *A.*
312 *sisalana* inulin, we preferred to isolate it from boles of 6-year old plants combining
313 these requirements, i.e. by precipitation with acetone overnight a 4°C, and to perform
314 Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) analysis to
315 classify it as a short- or long-chain polymer.

316 MALDI-TOF analysis of INAS illustrated in Figure 6 revealed molar masses
317 between 671-3283 Da corresponding to DP in the range 5-20. There was an increase in
318 intensity of peaks around 671-1157 Da corresponding to compounds with DP between 5
319 and 7. These results as a whole suggest that inulin from *A. sisalana* should be classified

320 as a short-chain inulin as a possible alternative to sucrose because of its well-known
321 solubility and sweetness.



322
323 Figure 6- MALDI-TOF spectrum of *A. sisalana* inulin

324 This DP range is similar to that detected for fructans isolated from 6-year old
325 plants of *A. tequilana* ($4 \leq DP \leq 24$) (Arrizon et al., 2010).

326 3.8 Nuclear Magnetic Resonance

327 Both the ^{1}H and ^{13}C NMR spectra of INAS are shown in Figure 7. Proton and
328 carbon signals were assigned by comparison with Inulin HP® taken as a reference
329 material and chemical shift of previous studies (Oliveira et al., 2011).

330

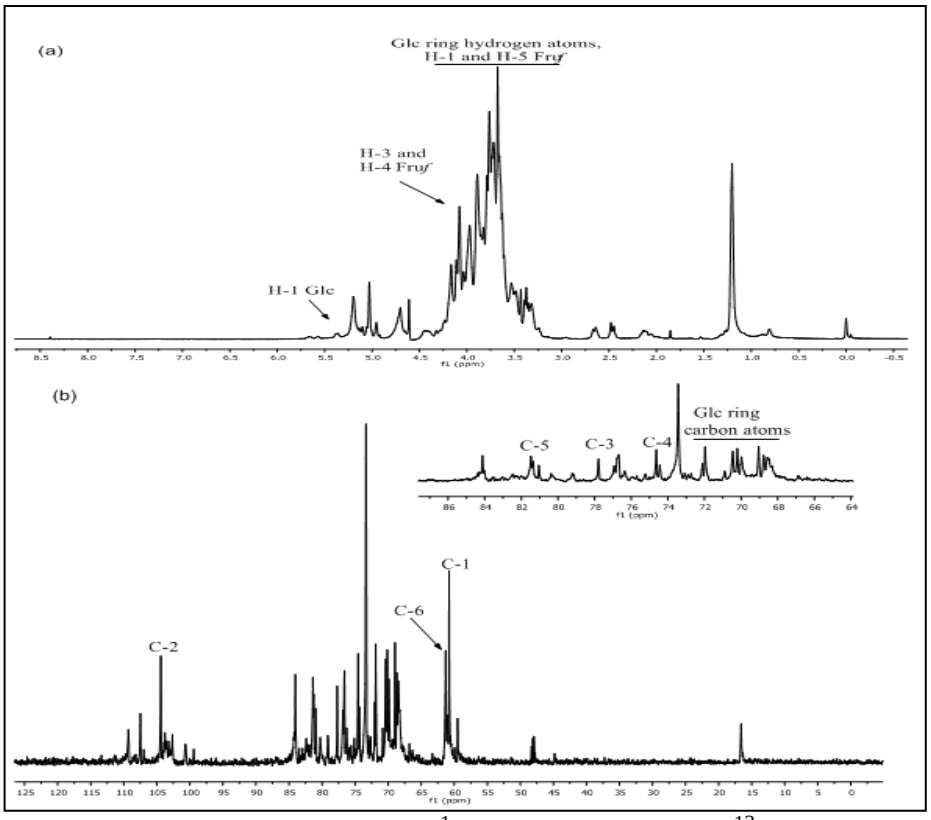


Figure 7- NMR spectra of INAs. (a)^{1}H NMR spectrum. (b)^{13}C NMR spectrum.

The ^{1}H NMR spectrum of INAS showed the presence of one signal in the anomeric region at δ = 5.36 ppm and the others between 3.15 and 4.24 ppm, while the main results of ^{13}C NMR spectrum are summarized in Table 1.

Table 1 - ^{13}C NMR chemical shifts (ppm) of Inulin HP® and *A. sisalana* inulin.

Carbon	**Inulin HP®**	***A. sisalana* inulin**
	β-Fructofuranose	
C-1	61.31	60.90
C-2	103.30	104.45
C-3	77.48	77.87
C-4	74.90	74.71
C-5	81.33	81.57
C-6	62.27	61.58
	β-Glucopyranose	
C-1	92.59	nd[a]
C-2	72.99	72.15
C-3	74.48	74.44
C-4	71.47	70.50
C-5	72.66	71.92
C-6	69.76	69.03

[a]not detectated

The signal at δ = 104.46 corresponded to the anomeric region of the C-2 of β-D-Fruf residues. Lopez et al. (2003) reported a chemical shift at 104.54 ppm due to a C-2 of an internal β-Fructofuranose (β-D-Fruf) unit with (2→1) linkage, but no resonance was detected around 63 ppm to confirm this linkage. Nonetheless, it was also reported that the resonance downfield at δ = 64.00 may be attributed to C-1 and C-6 with β-D-Fruf residues to a β(2→6) linkage, which in this case were assigned as C-1 (δ 60.90 and C-6 δ 61.58 ppm. Another important region is that from δ 79 to 84 where C-5 signals can be found. The INAS spectrum showed one signal at 81.45 ppm, which, according to Lopez et al. (2003), could also be attributed to β(2→6) linkage.

4 Conclusions

Inulin was isolated from *A. sisalana* boles with characteristics similar to inulin from others sources. The structure of this polysaccharide was identified by FT-IR and NMR spectra. There was a clear correlation between the amorphous state of inulin, confirmed by X-ray diffraction, with data from the thermal analysis by DSC that indicated a temperature of glass transition (55.62°C) typical of amorphous materials. This result appears to be consistent with the structure changes evidenced by circular dichroism. These features are expected for materials that have passed through steps of processing including precipitation with solvent and freeze drying. The analysis of DP made through MALDI-TOF also revealed correlation between the amorphous state and the temperature range of glass transition. Future studies to optimize the operational conditions of each stage through factorial designs will be important to improve the yield of inulin from *A. sisalana*.

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CAPÍTULO 4

ARTIGO 3- Prebiotic potential of *Agave sisalana*: a preliminar screening

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"Esta é a essência da ciência: faça uma pergunta impertinente e cairá no caminho da resposta pertinente".
J. Bronowski

SHORT COMMUNICATION

Prebiotic potential of *Agave sisalana*: a preliminar screening

Author Names: Alexsandra Conceição Apolinário[a]; Antonio Diogo Silva Vieira[b], Susana Marta Isay Saad[b], Bolivar Ponciano Goulart de Lima Damasceno[a], Attilio Converti[c], Adalberto Pessoa Jr[b], José Alexsandro da Silva[a,b]

Authors Affiliation:

[a]Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, 58429-600, Campina Grande, Paraíba, Brazil, acapolinario@gmail.com; bolivarpgld@pq.cnpq.br; alexuepb@pq.cnpq.br

[b] Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580 – Bloco 16, Cidade Universitária, 05508-000, São Paulo, Brazil, antdiogovieira@gmail.com; susaad@usp.br; pessoajr@usp.br

[c]Department of Civil, Chemical and Environmental Engineering, Chemical Engineering Pole, Genoa University, Via Opera Pia 15, 16145, Genova, Italy, converti@unigi.it

***Correspondingauthor:** José Alexsandro da Silva

Mailing Address: Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, CEP 58429-600, Campina Grande, Paraíba, Brazil

Telephone: (+55 11) 99595-8427 / 3315-3300 (Branch line: 3516)

E-mail: alexuepb@pq.cnpq.br

Highlights

- Aqueous extract of *Agave sisalana* was spray dried.
- Crude polysaccharides of aqueous extract extract of *Agave sisalana* were isolated and spray dried.
- Crude polysaccharides presented larger concentration of inhibiting metabolites.
- Dried extract presented a higher quantity of sugars which may have resulted higher facility to fermentation

Abstract

This work aimed at evaluating the prebiotic potential of the extract and crude polysaccharides from *Agave sisalana* boles by a preliminary *in vitro* qualitative screening. Crude polysaccharides were obtained from the aqueous bole extract by precipitation using acetone as a solvent and re-suspended in water. The original liquid extract and the polysaccharide solution were then spray dried and submitted to thermal analysis to quantify the more interesting metabolites. Qualitative screening of prebiotic activity was performed on seven probiotic strains belonging to *Lactobacillus* and *Bifidobacterium* genera using inulin, fructo-oligosaccharides, fructose, and glucose as positive controls. The extract powder exhibited higher potential of fermentation compared with that of crude polysaccharides, likely due to larger concentration of inhibiting metabolites in the latter fraction resulting from its process of achievement.

Keywords: *Agave sisalana*, Aqueous extract, Crude polysaccharides, Prebiotic activity

Abbreviations: DAE: Dried aqueous extract, **DCP:** Dried crude polysaccharides

1. Introduction

Prebiotics are defined as a "selectively fermented ingredients that allows specific changes in the composition and/or gastrointestinal microbiota activity that confer benefits upon host well being and health" (Gibson et al., 2004; Roberfroid, 2007). Non-digestible carbohydrates are considered as prebiotics when they respect the following criteria: a) resistance to gastric acidity and mammalian enzymes; b) vulnerability to fermentation by gut bacteria, and c) capacity to enhance the viability and/or action of beneficial microorganisms (Al-Sheraji et al., 2013).

The emergent demand for herbal extracts is observed in food and pharmaceutical industries, because they can be used as natural supplements prolonging the stability and shelf life of food products (Khaleel & Haddadin, 2013). Many new functional foods have been manufactured with health promotion purposes by combining traditional foods with herbal extracts in the form of dietary supplements or dairy foods (Al-Sheraji et al., 2013).

A study of Apolinário et al. (2014) revealed that aqueous extract from *Agave sisalana* boles has a high content of sugars and this could suggest a prebiotic application of this species. Therefore, this work aimed at evaluating the prebiotic potential of dried aqueous extract and crude polysaccharides obtained by spray drying boles of *Agave sisalana* by a preliminary *in vitro* qualitative screening.

2. Methodology

2.1 Achievement of dried extract and crude polysaccharides

The boles of *Agave sisalana* were collected from six years-old plants in Monteiro, PB, Brazil (7°52'40.50" S and 37°07'34.91" W), in January 2013 (voucher number 210 deposited at the Manoel Arruda Câmara herbarium). Samples were dried in a forced circulation oven at 40°C until a constant weight and milled. The extraction was

performed with water at 80°C for 2 h. Polysaccharides of the aqueous extract were precipitated with acetone (Vetec, Duque de Caxias, RJ, Brazil) (1:2 v/v), maintained at 4°C overnight, centrifuged (Excelsa II 206 BL, Fanem, Guarulhos, SP, Brazil) at 2795*g* for 20 min, submitted to four cycles of dissolution in distilled water at 80°C (1:2 w/v) and finally centrifuged under the same conditions as above. Both aqueous extract and crude polysaccharides were powdered using a Spray Dryer (MSD 0.5, Ribeirão Preto, SP, Brazil) with a concurrent flow regime and a pneumatic (two-fluid) spray nozzle with an inlet orifice diameter of 1.0 mm, pump setting of 0.3 $L.h^{-1}$, atomization air flow rate of 40 $L.min^{-1}$, aspirator setting of 3.8 $m^3.min^{-1}$ and inlet temperature of 70 ± 2 ºC. Figure 1 shows the setup of the protocol used to get dry powders of both aqueous extract (DAE) and crude polysaccharides (DCP).

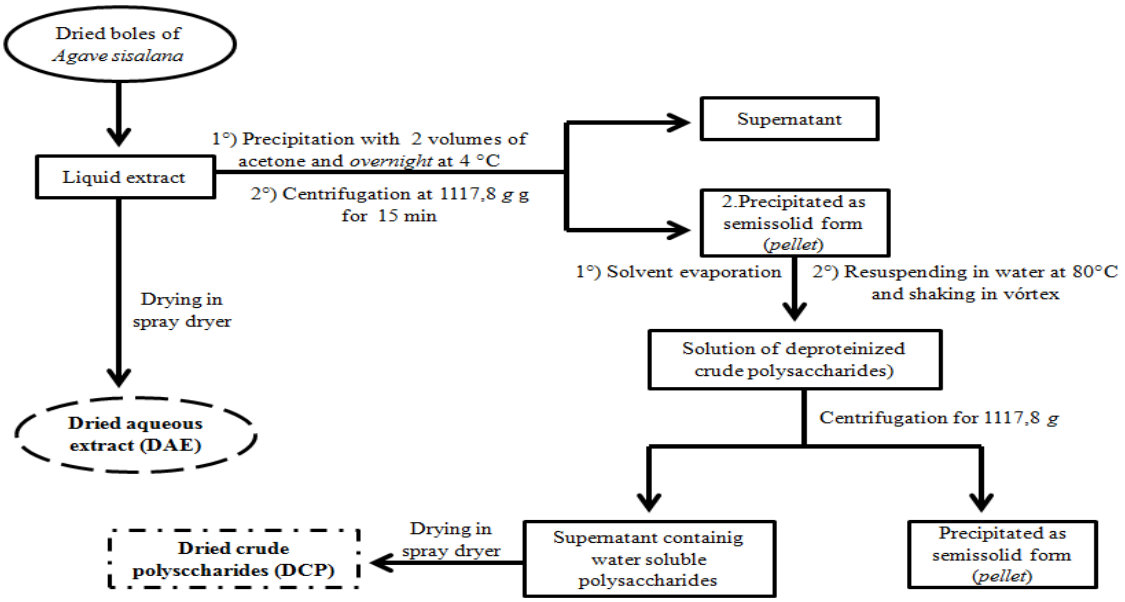

Figure 2- Setup of the protocol used to get dry powders of both aqueous extract (DAE) and crude polysaccharides (DCP).

2.2 Thermal gravimetric analysis of dry powders of aqueous extract and crude polysaccharides

The thermal behavior of DAE and DCP samples was studied using thermal gravimetric analysis (TGA) (STA 1500, TA Instruments, New Castle, DE, USA).

Rising temperature experiments were conducted in the temperature range of 25–900°C, at heating rate of 10 °C.min^{-1} in synthetic air and under nitrogen flow of 50 mL.min^{-1}.

2.3 Metabolites quantification dry powders of aqueous extract and crude polysaccharides

Total carbohydrate content of both DAE and DCP was determined by the colorimetric method of Dubois, Gilles, Hamilton, Rebers, & Smith (1956) against a standard curve of HP inulin, while that of free reducing sugars by the one of Miller (1959) against a standard curve of fructose. Total phenolics content of samples was determined using the Folin-Ciocalteau reagent and gallic acid monohydrate as a standard (Saha et al., 2013). Total flavonoids were determined through the formation of a flavonoid-aluminum complex (Mbaebie, Edeoga, & Afolayan, 2012) using quercetin as a standard. All reagents were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). All analyses were performed in triplicate, and the results expressed as mean ± standard deviation (SD). Statistical comparison of data was performed by one-way analysis of variance (ANOVA) and Tukey's test using the Origin Pro 8.0 software (OriginLab, Northampton, MA, USA), and p-values < 0.05 were considered as statistically significant.

2.4 Fermentation screening

Qualitative screening was performed following the method of Watson et al. (2013). For this purpose, seven bacterial strains were tested for their ability to utilize DAE and DCP from *A. sisalana* as substrates, namely *Lactobacillus acidophilus* LA-5 (Christian Hansen, Hørsholm, Denmark), *Lactobacillus casei* Lc11, *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* LPC-37, *Bifidobacterium animalis* subsp. *lactis* HN-019 (Danisco, Madison, WI, USA), and *Bifidobacterium animalis* subsp. *lactis* BB-12

(Chr. Hansen). *Lactobacillus rhamnosus* GG isolated from capsules (Culturelle, Amerifit, EUA) was also employed.

The screenings of DAE and DCP were compared with those performed using fructooligosaccharides (95 % of purity with 5% of sucrose, glucose and fructose, Orafti, GR inulin, 92 % of purity with 8% of sucrose, glucose, and fructose, Orafti), fructose (Sigma-Aldrich) and glucose (Cinética, Brazil). The above microorganisms were inoculated in specific media in accordance to Vieira (2013).

The fermentation was performed on MRS medium containing phenol red as indicator (Buriti et al., 2014). All steps of analysis are summarized in Figure 2. An aliquot of 4.5 ml of modified MRS medium containing phenol red as indicator was transferred to Falcon-type tubes to which 0.5 ml of sterile solution of DAE, DCP and control carbohydrates were added in *milli Q*® water (50 mg. mL^{-1}) previously filtered through a 0.22 mM filter to obtain a sample concentration of 5 mg. mL^{-1}broth, a broth absent of carbohydrates (negative control) was also used. To prepare the inoculum of microorganisms, 20 mg of each probiotic culture were weighed and dissolved in 10 ml of de Man-Rogosa-Sharpe broth (MRS, Oxoid) for *Lactobacillus* strains, modified MRS supplemented with cysteine (L (+)- Cysteine HCl, Cromoline®, 0.5 g. L^{-1}) strains of *B. animalis* and *B. lactis*. In order to obtain the approximate value of the number of colony forming units (CFU) that would be inoculated, serial dilutions from 10^{-1} to 10^{-6} were made in 0.1% peptone water (bacteriological tryptone 1g.$L^{-1}$from each initial inoculums). All tubes were incubated and growth at 37 ± 1°C under anaerobic conditions (Anaerobic System Anaerogen, Oxoid). Visually the color changes of the medium indicated by phenol red from red (A2) into yellow (B2) was the initial parameter used to indicate fermentation The samples before incubation (t=0) and 24 hours after incubation were collected to determine qualitative fermentation by

measuring the absorbance at 620 nm in a spectrophotometer Spectra Max Plus (Molecular Devices). An aliquot containing 100 µL of microorganisms diluted inoculum (approximately 6 log CFU) was added to modified MRS medium and phenol red or with no added carbohydrates. To confirm the inoculated amount and assess possible growth arising from contamination, 10µL of the last four above dilutions were plated. The plates were incubated anaerobically for 48 hours at 37°C, when counting of colonies proceeded.

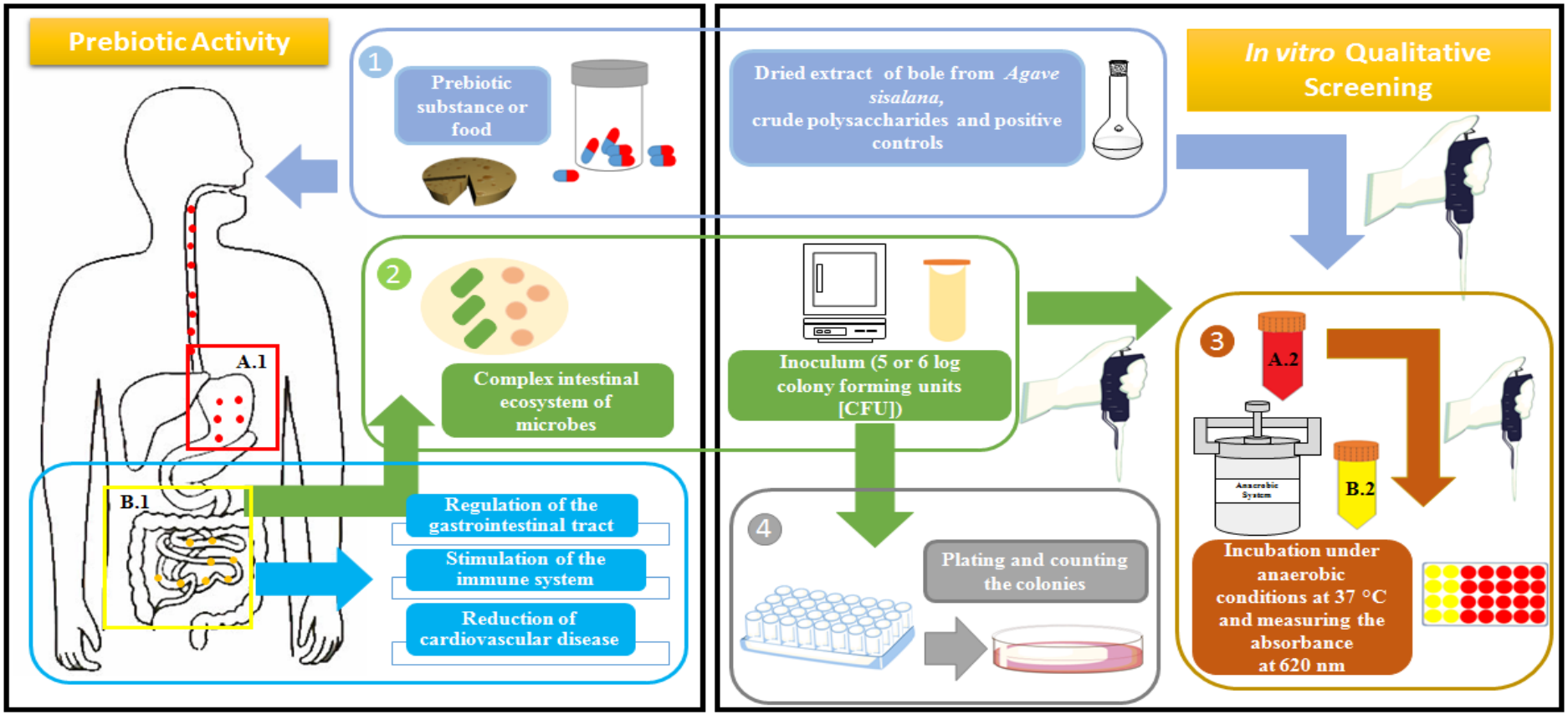

Figure 2*- **Scheme of *in vivo* prebiotic behavior and *in vitro* screening activity**. **1-**Prebiotic compounds can be consumed in the pharmaceutical formulation or in the food products. **2-** Bacteria from the intestinal microbiota and prebiotic compounds support the growth of probiotic microbiota and suppress the proliferation of harmful microorganisms. **3-** Resistance to gastric acidity and enzymes and vulnerability (A1) to fermentation by gut bacteria (B1) are two preliminary characteristics of prebiotics. **4-** Confirmation of the inoculums by plating.

***Figure was draw with software 2D ChemBioDraw Ultra 12.0 (CambridgeSoft, 1986–2009)**

3 Results and discussion

3.1 Thermal gravimetric analysis of dry powders of aqueous extract and crude polysaccharides

According to the thermograms (Fig. 3), decomposition of the dry powder of aqueous extract (DAE) occurred in three steps, while that of crude polysaccharides (DCP) in four steps.

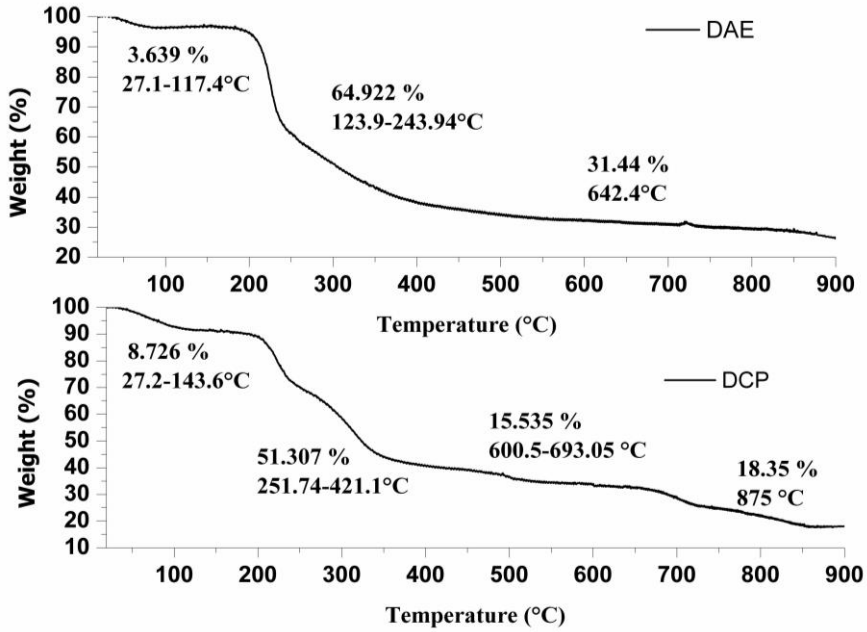

Figura 3- Thermograms of dry powders of aqueous extract (DAE) and of crude polysaccharides (DCP).

The small initial mass loss represents the first event, which can be attributed to the water loss. The highest value observed for DCP can be explained with the desorption of moisture as hydrogen bound water from the saccharide structure (Bothara & Singh, 2012).

The second mass loss event pointed out relevant differences between the analyzed powders, in that DAE exhibited smaller stability than DCP, likely due to decomposition of additional thermosensitive metabolites (proteins, pigments, phenolics, etc.) extracted by water. Contrary to DAE, this decomposition phase happened in DCP in two steps: a)

the former showed the typical behavior of polysaccharides that can be ascribed to thermal decomposition of branched chains of agave fructans with depolymerization; b) the latter can be related to final pyrolytic decomposition of polysaccharides that were not purified and then contained contaminants (Espinosa-Andrews & Urias-Silvas, 2012; Xie et al., 2013) .

The mineral residue content resulting from carbonization of DAE (last event) was higher than that of DCP likely because the process to obtain crude polysaccharides implied the precipitation only of water soluble substances. The temperature ranges of decomposition events are indicative of good thermostability of both materials, event though polysaccharides were more stable.

3.2 Metabolites quantification in dry powders of aqueous extract and crude polysaccharides

Table 1 lists the contents of different classes of metabolites detected either in DAE or DCP. Lower content of total sugars were found in DCP compared to DAE, which can be explained by fact that precipitation with acetone to recover crude polysaccharides consisted of various steps all implying significant material losses. On the other hand, the higher contents of the other metabolites in DCP are the likely result of the fact that all soluble compounds present in the starting liquid extract were subject to precipitation, hence concentrating in this powder.

Table 2-Contents (mg/g) of different classes of metabolites contained in dry powders of aqueous extract (DAE) and of crude polysaccharides (DCP).

Samples	Total phenols	Flavonoids	Total sugars	Free sugars
DAE	5.67^{a}± 0.09	0.6^{b} ±0.02	859.30^{b} ±129.9	47.96^{b} ±1.11
DCP	16.32^{b}± 0.09	10.94^{a} ±0.2	531.06^{a} ±35.47	99.02^{a} ±2.96

$p < 0.05$ for all values. Means in the same column followed by different letters were statistically different.

3.3 Fermentation screening

Table 2 lists the results of qualitative fermentation screening of different *Lactobacillus* and *Bifidobacterium* strains for their ability to consume the carbon sources contained either in DAE or DCP, using different carbohydrates as positive controls and the MRS medium as the negative one. As expected, crude polysaccharides always behaved as a worst carbon source than positive controls, whereas DAE exhibited an intermediate performance.

This may be related to the results found for quantification of metabolites in the samples because the obtaining of DCP concentrated others metabolites besides polysaccharides and monosaccharides in relation to extract and this may have interfered in the fermentation since that DAE presented a higher quantity of sugars which may have resulted higher facility to fermentation. No effects were observed for bifidobacteria for two samples and the carbohydrates used as positive controls, even after repeating the test. This fact was attributed to the inherent strains problems.

Crude polysaccharides could be purified and unwanted substances removed but this would lead to important losses and lower yield. Therefore, these results suggesting that aqueous extract of bole from *A. sisalana* could be sources of prebiotic compounds should be confirmed, as well as others tests of safety and efficiency are necessary. Use of extracts, juices or others complexes with prebiotic properties were already reported in many studies and emphasizes that it is not necessary to invest in expensive and time-consuming steps of isolating and purifying carbohydrates, but only to use materials with high fructans or others polysaccharides contents. In this study DAE of *A. sisalana* presented potentially prebiotic properties, which can be largely studied to optimize the production.

Studies with others species resulted in good perspectives. For example, *Agave tequilana* stems were grounded and diluted in water, the juice obtained with high brix was filtered, clarified and spray-dried and this material was considered a by-product (dietary fibre), the remaining stem was used to obtain the agave insoluble dietary fibre showed promising effects as functional ingredients (Sáyago-Ayerdi et al., 2014). Prebiotic properties of onion (*Allium cepa* L.) was investigated by the evaluation the fermentation effects on the cecum of rats treated with diets containing lyophilized onion. The *in vitro* and *in vivo* fermentation was also evaluated and resulted in increase the production of short-chain fatty-acids and in the molar proportion of propionate and butyrate and decrease in the molar proportion of acetate and in pH (Pascoal, Filisetti, Alvares, Lajolo, & Menezes, 2013). A study investigated the *in vivo* and *in vitro* prebiotic effects of an aqueous extract of *Anoectochilus formosanus* commonly used in the folk medicine in Asia and exhibited prebiotic effects including a decrease in cecum pH and increases of calcium absorption and fecal bifidobacteria (Yang, Lin, & Lu, 2012).

Table 3-Qualitative screening of consumption of different carbohydrates by various strains of *Lactobacillus* and *Bifidobacterium*.

Samples	***Lactobacillus***					***Bifidobacterium***	
	***L. acidophilus*-LA-5**	***L. acidophilus*-NCFM**	***L. paracasei*-LPC -37**	***L.rhamnosus*-GG**	***L. casei* Lc11**	***B. animalis* subsp. *lactis* BB-12**	***B. animalis* subsp. *lactis* HN 019**
FOS	+	++	+++	-	-	-	-
Inulin	+	+	+++	-	-	-	-
Fructose	+	++	++	+++	+	-	-
Glucose	+	+++	++	+++	+++	-	-
DAE	+	++	++	-	+	-	-
DCP	+	+	+	-	-	-	-
mMRS	-	-	-	-	-	-	-

- indicates final absorbance at 620 nm < 0.2; + indicates final absorbance = 0.2–0.4; ++ indicates final absorbance = 0.5-0.7; +++ indicates final absorbance ≥ 0.8.

4 Conclusion

The preliminary results of this study suggest that the extract of boles from *Agave sisalana* could have a potential prebiotic effect linked to its sugar content; therefore, it could be an economic viable material for both the pharmaceutical and food industries, whereas isolation and purification of crude polysaccharides would require a large number of steps implying high costs and low yield of final products. However, complete fermentation tests, *in vivo* assays, and studies of optimization of the technology involved should be carried out to obtain a final standardized product.

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CAPÍTULO 5

ARTIGO 4- Structural characterization of pectic polysaccharides extracted of liquid and solid wastes of *Agave sisalana* by a combination of conventional methods

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"A verdadeira ciência ensina sobretudo a duvidar e a ser ignorante."

Miguel Unamuno

Structural characterization of pectic polysaccharides extracted of liquid and solid wastes of *Agave sisalana* by combination of conventional methods

Author Names: Alexsandra Conceição Apolinário[a], Maria Sallett Rocha de Souza[b]; Bolivar Ponciano Goulart de Lima Damasceno[a] Attilio Converti[c], Adalberto Pessoa[d], José Alexsandro da Silva[a]

Authors Affiliation:

[a]Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, 58429-600, Campina Grande, Paraíba, Brazil, acapolinario@gmail.com; bolivarpgld@pq.cnpq.br; alexuepb@pq.cnpq.br

[b]Department of Pharmacy, Federal University of Paraíba, Brazil, sallett@gmail.com

[c]Department of Civil, Chemical and Environmental Engineering, Chemical Engineering Pole, Genoa University, Via Opera Pia 15, 16145, Genova, Italy, converti@unigi.it

[d]Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580 – Bloco 16, Cidade Universitária, 05508-000, São Paulo, Brazil, pessoajr@usp.br

***Corresponding author:** José Alexsandro da Silva

Mailing Address: Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N - Bairro Universitário, CEP 58429-600, Campina Grande, Paraíba, Brazil

Telephone: (+55 11) 995958427 / 3315-3300 (Branch line: 3516)

E-mail: alexuepb@pq.cnpq.br

Highlights

- Wastes from decortications of *Agave sisalana* were fractioned in solid and liquid wastes.
- Bagasse of *A. sisalana* was dried and submitted to extraction with hot water.
- Extract from bagasse and the juice were used to obtain pectic polysaccharides.
- Different composition and yield have been observed between polysaccharides obtained from solid and liquid wastes.

Abstract

The process of decortication of *Agave sisalana* leaves to obtain fiber utilizes no more than 5% by weight of the plant, thereby large amounts of wastes are produced. Wastes of *A. sisalana* can be divided into 2 types: liquid waste (juice) and solid waste (bagasse).This work aimed at extracting and isolating polysaccharides from either juice or bagasse of *A. sisalana*. A new method was proposed to extraction of polysaccharides from wastes and different analytical techniques were employed to characterize samples. It was possible to confirm that polysaccharides have a typical structure of pectic substances, but the significantly different physicochemical profiles pointed out that different protocols should used for bagasse and juice.

Keywords: *Agave sisalana*, Wastes, Purification, Pectic polysaccharides

Abbreviations: SW: solid waste, **LW:** liquid waste, **PSW:** polysaccharides of solid waste, **PLW:** polysaccharides of liquid waste.

1. Introduction

Agave sisalana Perrine (sisal) is a monocotyledonous plant belonging to the Asparagales order and the Agavaceae family that has perennial cycle. Sisal fibers obtained from the plant leaves of the plant and they are extensively used for many applications. Brazil northeastern is the world's largest producer and exporter of these sisal fibers[1].

Process of decortication of leaves to obtain fiber exploits no more than 5% by weight of the plant, thereby large amounts of wastes are produced. Wastes of *A. sisalana* can be divided into 2 types a liquid waste (juice) and solid waste (bagasse). Some researches pointed to potential pharmaceutical applications of thse wastes linked to their as antimicrobial activity and content of polysaccharides such as mannitol, pectin and new water-soluble polysaccharides with biological activity [2-4].

Different protocols of polysaccharides extraction and isolation are described in the literature, which differ considerably one another in their application and sequence. Anion-exchange chromatography is largely used to the purify crude samples, but it must be preceded by steps of deproteinization and depigmentation or at least by precipitation to remove contaminants[5]. Type of solvents to remove fats, techniques of dialysis, number of centrifugations, drying by atomization or freeze drying are steps that widely vary among different methods [6].

Wastes biomasses produced by decortication of *A.sisalana* have shown to be promising raw materials for polysaccharides industrial production [7-8]. However many steps for purification steps are required because of their complex composition and the presence of large amount of impurities resulting from of uncontrolled conditions during handicraft sisal farms activities. Thereby, the objective of this study was to extract and

to isolate polysaccharides of bagasse and juice from *A. sisalana* leaves by modification of conventional methods and to characterize the resulting substances.

2. Materials and methods

2.1 Materials and chemicals

Agave sisalana was collected in Monteiro/PB, Brazil (7°52'40.50" S and 37°07'34.91" W), in January 2013. A voucher was deposited at the "Manoel de Arruda Câmara" herbarium of the State University of Paraíba, Campina Grande-PB, Brazil, under the number 210. Fig. 1 illustrates the protocols employed to obtain both liquid and solid residues collected directly from a decortication machine in a sisal farm processing leaves of 6-year old plants. Juice was removed from bagasse by manual pressing.

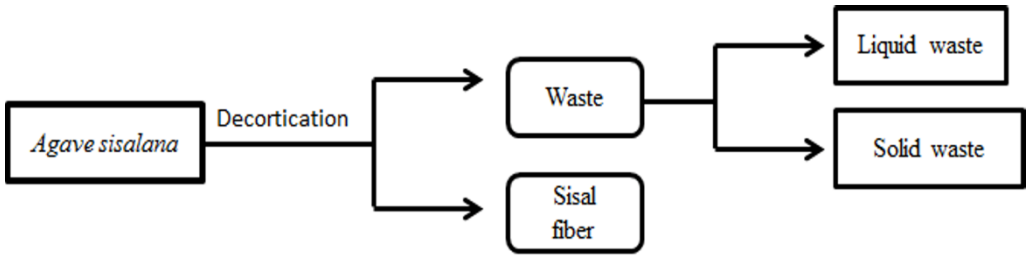

Figura 1- Experimental protocols employed to obtain liquid and solid wastes from decortications of *Agave sisalana* leaves.

Diethylaminoethyl (DEAE)-cellulose D3764, the standard monosaccharide (d-fructose) and activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MI, USA), HP inulin was purchased from Beneo GR, Orafti, (Oreye, Belgium). Galic acid, acetone, chloroform and butanol from Vetec® (Duque de Caxias, RJ, Brasil). All reagents used in this study were of analytical grade.

2.2 Extraction of polysaccharides

Fig. 2 shows all steps following the extraction and isolation of polysaccharides from wastes.

The solid waste (SW) was submitted to extraction with hot distilled water in thermostated bath (SL 155/10, Solab, SP, Brasil) for 2 hours twice in a 7:1(w/v) ratio, shaking at 1600 rpm in a mechanical stirrer (model 713, Fisatom, São Paulo, Brazil). Liquid waste (LW) was filtered with qualitative filter paper.

To remove impurities, the pH of juice and extract was increased to about 10-12, these materials were mixed with a 5% slurry of calcium hydroxide for 30 min at 50°C. After centrifugation (Excelsa II 206 BL, Fanem, Guarulhos, São Paulo) at 2500 RPM by 20 min, 10% phosphoric acid was added to the supernatant with vigorous stirring to adjust its pH to 8–9, causing the precipitation of excess calcium. The mixture was allowed to stand at 50°C for 2–3 h before other centrifugation. The clarification process was repeated twice.

After this step samples were precipitated with acetone (1:2 v/v) and submitted an overnight at 4°C. The crude precipitate (crude ppt) was pelleted by centrifugation and it was submitted at four cycles of resuspension in distilled water (1:5) at 80°C (1:5 w/v) and centrifugation by 20 minutes. This solution was mixed with Sevag reagent (chloroform:butanol, 4:1, v/v) at a ratio of 3:1 (v/v) to deproteinization. The mixture was shaken vigorously for 10min at room temperature and centrifuged at 2500 RPM by 20 min. After centrifugation, supernatant was precipitated with acetone (1:2 v/v) and submitted an overnight at 4°C and it was performed new cycle of resuspension and centrifugation.

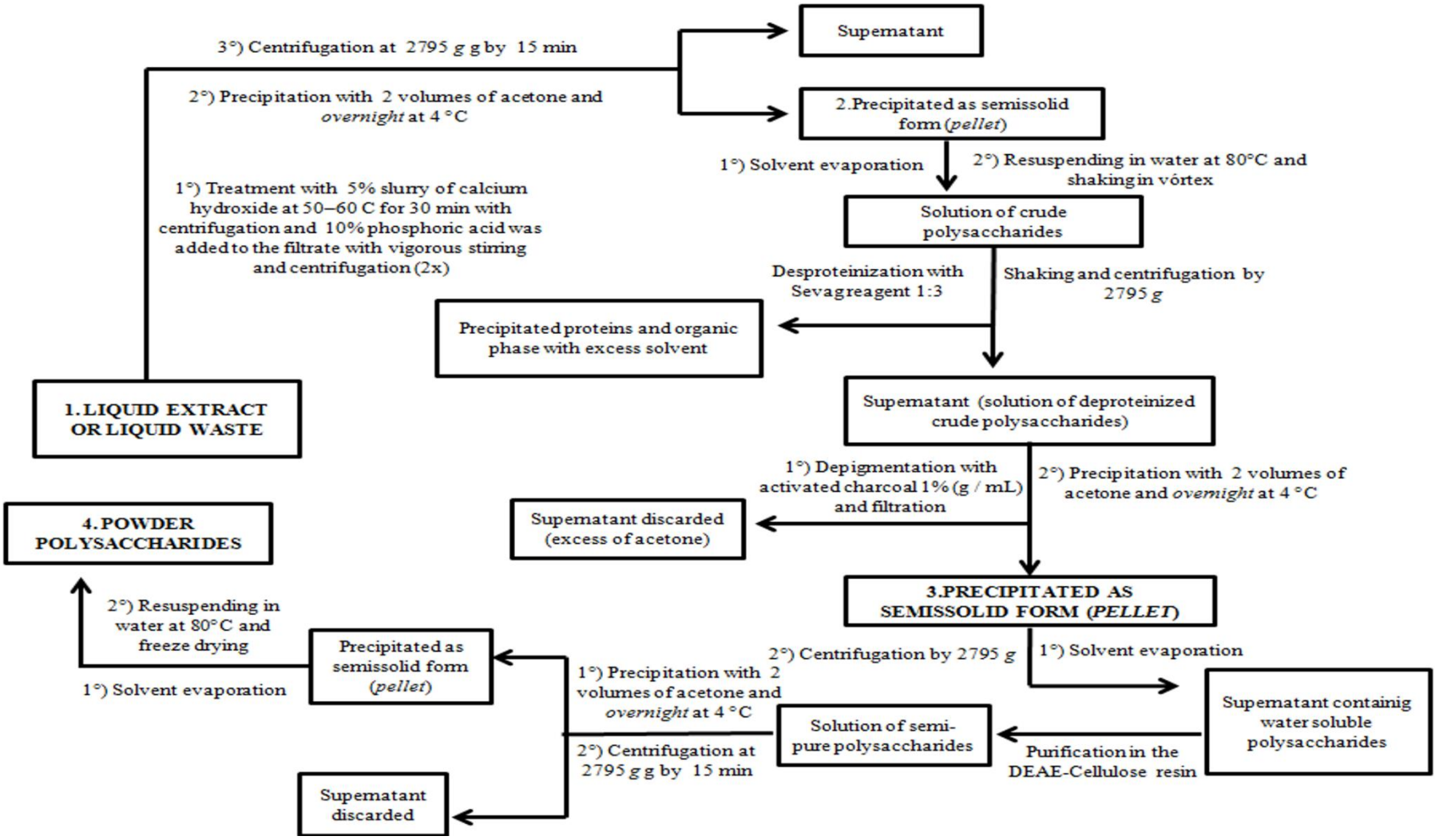

Figura 2- Experimental protocols employed to extract, isolate and purify polysaccharides from liquid and solid wastes from decortications of *Agave sisalana* leaves.

All supernatants were mixed and depigmented with activated charcoal 1% (g. mL $^{-1}$) at 50°C with agitation in shake (TE 420, Tecnal, Piracicaba, SP, Brazil) and filtration and it was purified with DEAE-cellulose resin into a 1.5x 12 cm prolypropylene column (Econo-Pac® 732-1010, BioRad, USA) equilibrated with 0.05 M Tris-HCl buffer (pH 7.0). The unbound fraction was precipitated in under the same conditions above and the precipitate was re-dissolved in distilled H_2O (1:5 w/v) and freeze-dried. The polysaccharides were obtained in the form a white powder to polysaccharides of solid waste (PSW) and yellowish powder to polysaccharides of liquid waste (PLW).

2.3 Quantification of total and free sugars, total phenolics and flavonoids in the wastes

Total carbohydrates were determined by the phenolsulfuric acid method using sucrose as a standard[9]. Free reducing sugars were determined using 3,5-dinitrosalicylic acid as reagent and glucose as a standard[10].Total phenolics content of samples was determined using the Folin-Ciocalteau reagent and gallic acid monohydrate as a standard[11]. Total flavonoids were determined through the formation of a flavonoid-aluminum complex using quercetin as a standard[12]. All reagents were purchased from Sigma-Aldrich (São Paulo, Brazil).

2.4 Process control

To evaluation of deproteinization of samples and others substances, scanning UV-vis of freeze dried sample diluted (1000 µg.mL^{-1}) was performed in a spectrophotometer UV/VIS (Schimadzu1240, Kyoto, Japan) in a quartz cuvette 1.0 cm pathlength, in the range of 200-800 nm, utilizing water as blank and performed in triplicate.

2.5 Elemental analysis

Elemental analysis (carbon, hydrogen, nitrogen) was performed with an elemental analyzer (AD6, Perkin Elmer, New York, USA). Sulfur analysis was made by Plasma Atomic Emission Spectrometry (ICP-AES) (Arcos SOP, Spectro, USA).

2.6 Infrared Analysis (IR)

The infrared spectra of inulin from *A. sisalana* were taken in KBr tablets with spectrophotometer (Subtech Spectrum ASCII PEDS 4.0, Perkin–Elmer, New York, USA). Polysaccharides were homogenized with KBr; each mixture was pressed to form tablet and subject to analysis in the range 4.000–400 cm^{-1}.

2.7 X-ray diffraction (XRD)

X-ray diffraction patterns of inulin sample (in powder form) at room temperature were registered on diffractometer (Miniflex Goniometer, Rigaku, Tokyo, Japan). X-ray diffraction spectrum was collected within the 2h range from 10° to 80° with a constant step 0.04° and counting time 1 s/step.

2.8 Dynamic light scattering

The average particle size and distribution of polysaccharides in solutions were determined by the dynamic light scattering (DLS) method using a Nanotrac Wave® (Microtrac, York, USA). The light source was a diode pump solid-state laser (DPSS) with a wavelength of 780 nm. The solutions were diluted to a concentration of 1 $mg.mL^{-1}$ with deionized water and all measurements were carried out at 25 °C.

2.9 Thermal analysis

Simultaneous thermal analysis that consisted in the termogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC) of the samples was carried out (STA 1500, TA Instruments, USA). The sample was heated to 500°C at a rate of

20°C/min under the flowing N_2 gas (100 mL. min^{-1}). DCS curves were obtained during heating of the samples from 25 to 300 °C with heating speed of 5°C/min and it was used aluminum pan.

2.10 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI TOF was operated in positive linear mode (Autoflex III Bruker Daltonics, Billerica, MA, USA). The sample was dissolved in purified water (4mg.mL^{-1}) and it was solubilized in a matrix of alpha-cyano-4-hydroxycinnamic acid and acetonitrile (10 mg. mL^{-1}), 1 mL of mixture was applied into the probe and quickly dried under vacuum.

2.11 Nuclear Maganetic Ressonance (NMR)

The1H,13C NMR solution spectra were acquired in a spectrometer (Avance 500, Bruker, Bremen, Germany) in D2O at 30±0.1°C using a 5 mm switchable probe. The 1H NMR spectra were acquired by 500 MHz and the 13C NMR spectra were acquired by 125 MHz.

3. Results and discussion

3.1 Extraction of polysaccharides and process control

A difference of yield was observed between wastes, LW presented higher yield to freeze-dried polysaccharides and higher content of water soluble sugars (Table 1).

Table 1-Yield of the samples obtained at different stages.

Samples	Crude polysaccharides	Crude and Deproteinized polysaccharides	Freeze dried polysccharides after purification with exchange ionic resin
SW	47.74 g	9 g	169.7 mg
LW	31.652 g	20.323 g	3.9g

Purification steps resulted in reduced yields to both samples, however, extraction of pectic polysaccharides of wastes from *A.sisalana* which were not subjected to purification steps resulted in even lower yields to crude polysaccharides [13]. Therefore the method chosen was favorable because it allows the removal of undesirable substances considered impurities

Higher yield to LW is explained by the fact that despite SW and LW being product of decortication of a leaves from *A. sisalana*, LW was removed to SW by manual squeezing and this process is an extraction method that can drag most of the compounds present in the waste. Therefore, bagasse and juice of *A. sisalana* have different quantitative compositions and water soluble substances (Table 2).

Table 2-Profile of content of some important metabolities in the wastes of *A. sislana*.

Samples	**Total Sugars ($mg.mL^{-1}$)**	**Free Sugars ($mg.mL^{-1}$)**	**Total Phenolics ($mg.mL^{-1}$)**	**Flavonoids ($mg.mL^{-1}$)**
LW	$231.39^{a} \pm 7.50$	$79.17^{a} \pm 0.64$	$5.12^{a} \pm 0.55$	$1.23^{a} \pm 0.022$
SW	$3.39^{b} \pm 0.08$	$1.08^{b} \pm 0.09$	$1.64^{b} \pm 0.05$	$0.60^{b} \pm 0.0.3$

Scanning UV-vis presented minimum absorption in the region of 200-400 nm with a small peak at 271 (0.093) to PSW in the UV spectrum, indicating that most of the pigments and proteins were removed by this method. To PLW was demonstrated high absorbance in the 216nm (3.180) that confirms pectin presence and 274nm (0.977) pointed to a polysaccharide-protein complexes [14-15]. Thus the protein can't be removed completely to PLW and this is related with differences cited above to two wastes, it is possible to understand the process of deproteinization should be repeated to this sample since metabolites content to LW is always greater to SW. The literature reports that the protein contents decrease after purification but are not completely eliminated[16-17].

3.2 FTIR

As shown in Fig. 3, the IR spectra of PSW and PLW revealed typical bands to polysaccharides with hydroxyl and carbonyl groups [16, 18-20].

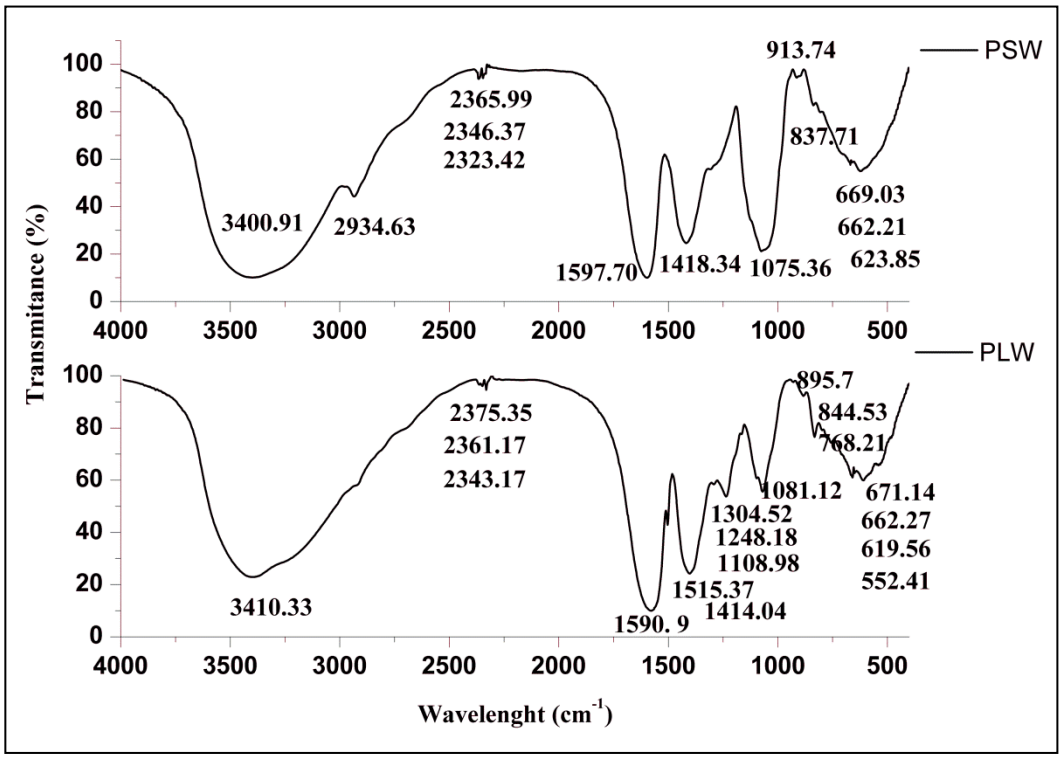

Figure 3- Infrared spectra of polysaccharides.

The IR analysis of polysaccharides showed a strong band at 3400 cm^{-1}attributed to the hydroxyl stretching vibration of the molecule. The peak at 2934,63 cm^{-1} was due to C-H stretching vibration from methyl esters of galacturonic acid to PSW, bands at 2300cm^{-1} in the far-infrared region to two polysaccharides shows the presence N–H stretching of some amidated salts[21]. The small peaks at 1597.7cm^{-1} confirmed that the freeze- dried contained low amounts of protein to PSW. More characteristic and more intense peaks to proteins were also related to PLW: 1590.9, 1515.37 and 1304.52[22]. Absorptions at 1418.34 to PSW and 1414.04 to PW indicated presence of uronic acids [23]. These bands agrees the presence of free carboxylic acids in pectin with low methyl ester contents [24].

Absorption peak at 1248 cm^{-1} only PLW was assigned to the stretching vibrations of S=O, an evidence of sulfate ester, indicating that PLW could contain sulfated portion[25]. Typical bands of arabinogalactans were seen, the broader band of 1108.98 cm^{-1} and 1081.12 cm^{-1} was representative of C- O- C and OH in pyranose structure to PLW. The absorptions at 1075.36 cm^{-1} are related to C-O stretching to PSW and suggested that the monosaccharides in PSW and PLW had a pyranose ring[26].

FT-IR spectra in the wave number between 850 and 1200 cm^{-1} is considered as the "finger print" region for carbohydrates, which is unique to a compound. To PSW, the bands at 837.74 and 913.74 cm^{-1} were characteristic of α-configurations The and absorption at 844 and 895.7 cm^{-1} indicated α- and β-glycopyranosidic linkages of the sugar units concurrently existing to PLW[27].

The bands in the range of 500–600 cm^{-1} are assigned to skeletal modes of pyranose rings[28]. All date presented suggested that PSW and PLW may be heteropolysaccharides. Uronic acid presence is a common component of polysaccharides as pectins that are present in the PLW as shown in the scanning UV vis. amidated salts and proteins are others aspects in accordance to scanning spectrophotometer.

3.3 Elemental analysis

The carbon, hydrogen, nitrogen and sulfur weight percentages were determined (Table 3), respectively, confirmed that PSW and PLW contains protein and sulfate. PSW and PLW presented similar ratio of carbon to hydrogen indicating the polysaccharide composition[29], but differences in the content of nitrogen which are consistent with the results discussed above and indicated higher content of proteins to PLW.

Table 3- Elemental analysis of polysaccharides % (w/w).

Polysaccharides	% Hidrogen	% Carbon	% Nitrogen	% Sulfur
PSW	28.64	5.05	0.84	<0.3
PLW	29.57	4.13	1.49	<0.3

3.4 X-ray diffraction (XRD)

X-ray diffraction (XRD) patterns were shown in Fig.4. The hales of polysaccharides, named "bun-shaped" curve, exhibited a non-crystalline state [30]. PSW only had a very broad peak around 2θ=20–45° (Fig.5A) and typical of amorphous polymers with the characteristic peaks observed to PLW (Fig.5B) at 10°, 20° and 40°, respectively. Peaks around 10° and 20°C shown PLW diffratogram are generally observed with pectin [31-33].

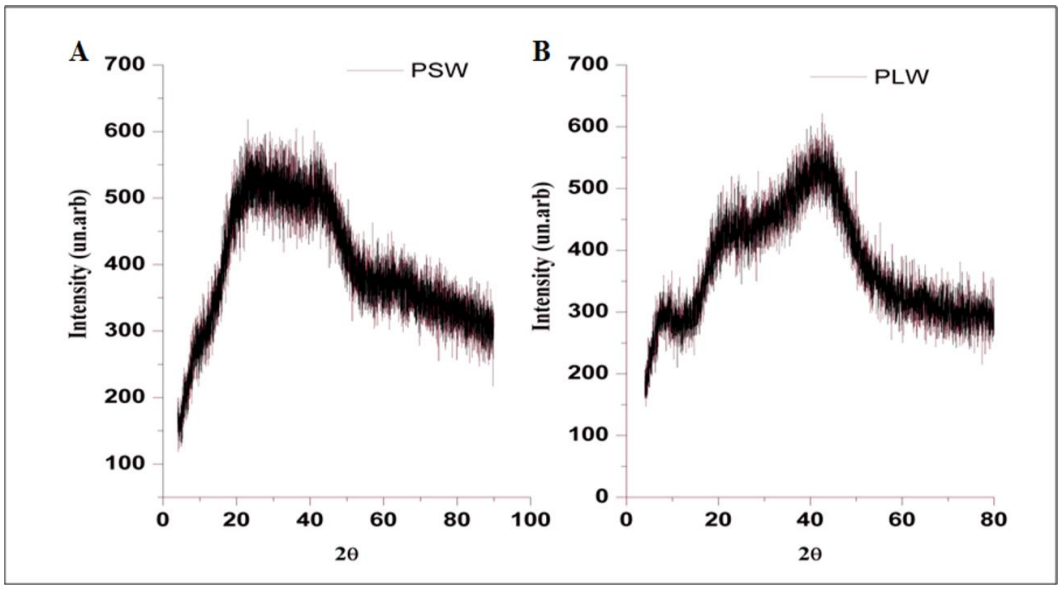

Figure 4- X-rays diffratogram spectra of polysaccharides.

This physical state is resulting of processing of polysaccharides from *A. sisalana* which included many steps of precipitation and drying. Freeze-drying consists in a rapid cooling that leads to the existence of an extremely viscous state before the solute molecules have time to rearrange and orient into a crystalline construction, and the system remains amorphous [31].

3.5 Size distribution of particles

PSW presented a distribution with higher intensity (%) around 690 nm, while PLW presented a distribution with higher intensity around 420 nm (Fig. 5). Size varied from 210 nm to 6 μm to PSW and to PLW the variation was from 105 nm to 3 μm.

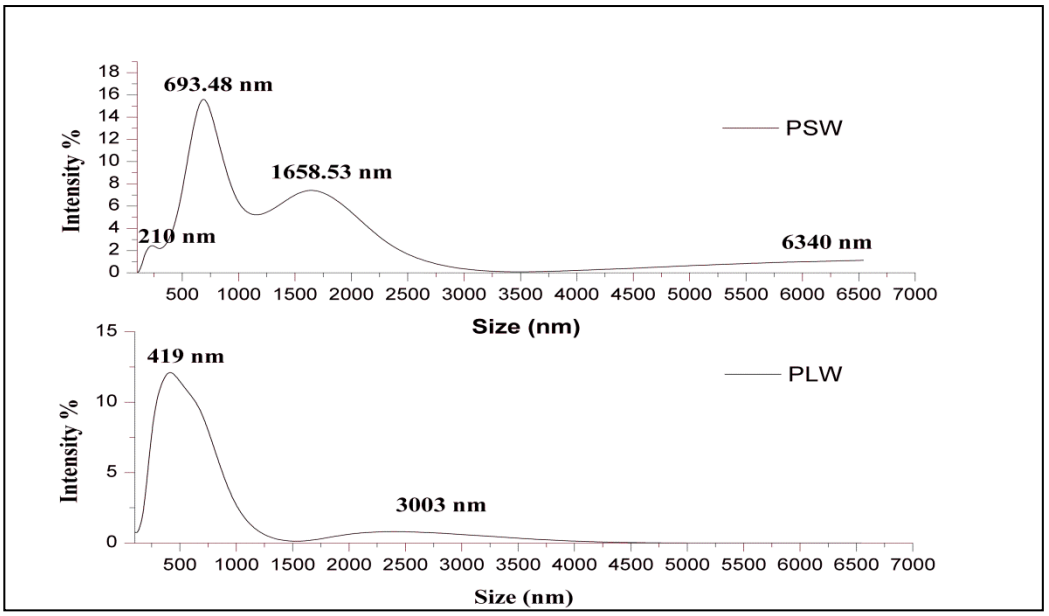

Figure 5-Size distribution profiles of polysaccharides.

DLS measures hydrodynamic diameter has tendency to select zones of clear, uniformly distributed particles and tends to yield larger particle diameter due to known bias for the larger diameter particles [34]. However is possible infers by this method that particles of PLW have a more homogeneous size distribution as well as smaller particles which will influence the solubility coefficient of the powder [15].

3.6 Thermal analysis

The thermal proprieties of the polysaccharides were studied using TGA and DSC was employed to analyze particulars as range of glass transition of samples. According to the thermograms (Fig. 6A and 6B), PSW and PLW decomposition occurred in two significant steps.

The initial drops in mass to two samples represented the loss of water desorption of moisture and suggested hydrogen bound water to the polysaccharide structure [35].

To PSW the loss was of 7.637% and this step extended to 181.8°C with peak at 56.19°C. To PLW this initial event was complexes as seen in the DTG because some small events occurred and process extended to 207.6°C with loss of 11.129%.

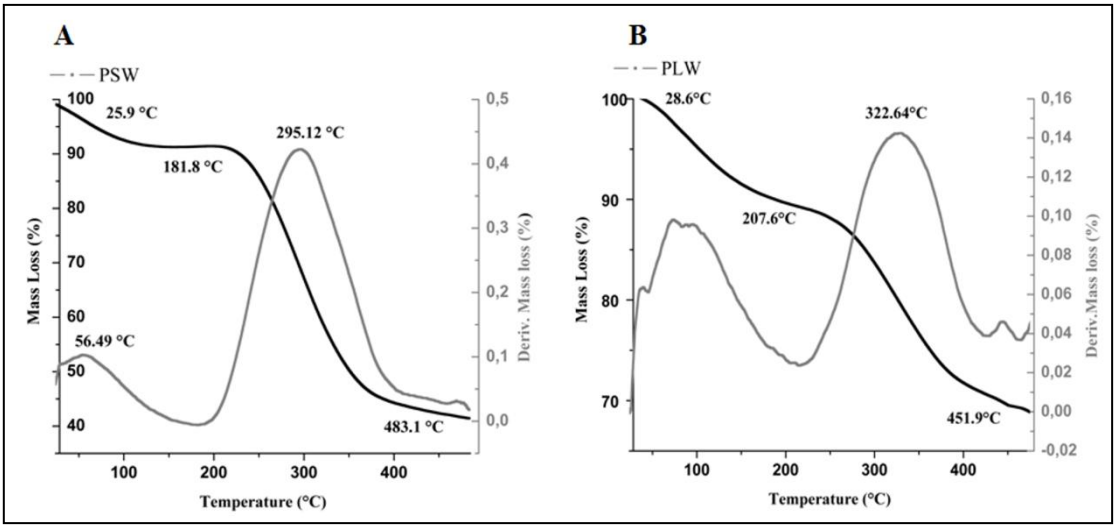

Figure 6-Thermogravimetry analysis curves of polysaccharides.

Polysaccharides did not show any significant weight loss below 300°C, the second event to two polysaccharides could be attributed to initial thermal decomposition. PSW had mass loss of 49.913 % between 295.12 and 483.1°C. PLW decomposition process presented mass loss of 19.868% between 322.61 and 451.9°C [35-37].

The precise estimation of residual mass was not possible in the heating range used because higher temperatures around 900°C should be used [38-40]. However at final temperature used, it was viewed a residual mass of 41.42% to PSW and this value was higher to PLW ranging 68.41%. This thermal behavior is related to date presented in FTIR analysis that indicated that polysaccharides could be pectin structure with uronic acid and also sulfate groups to PLW which in accordance with literature had high residual masses in the thermogravimetric analysis [41-43].

DSC curves (Fig.7) showed one endothermic peak to polysaccharides, reflecting the transition from the glassy to the rubbery state which is common to amorphous

powders[44]. Endothermic event to PSW occurred from 37.44 to around 126.90°C with peak at 68°C and heat of fusion of 150.5 $J.g^{-1}$ and to PLW this event occurred from 40.79 to 136.70°C with peak at 76.41°C and a higher heat of fusion 199 $J.g^{-1}$.

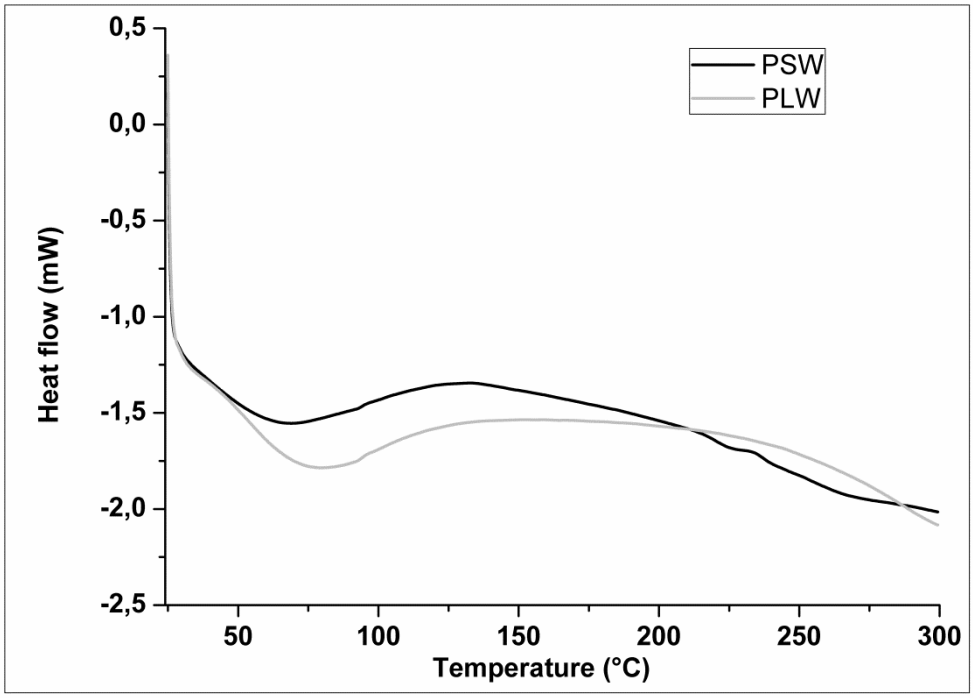

Figure 7-Differential scanning calorimetry curve of polysaccharides.

Changes in the reaction enthalpy to samples are related to differences in their quantitative composition of two wastes previously reported. Thus the increase of endothermic transition temperature and heat of fusion to PLW was possibly because its higher water content described in TGA curve and others components as shown in the spectrophotometric quantifications already reported [45].

Exothermic peaks reflects the sample degradation properties and they are not present in the thermogram because degradation ranges temperatures to these polysaccharides are above 300°C as reported in the TGA curves [45].

These thermal proprieties exhibited by DSC suggested that PSW and PLW are complex biopolymers and they have others components in the structures beyond pectin confirming others analyzes reported in this study that indicated proteins and salts presence.

3.7 MALDI TOF

Fig. 8 showed MALDI mass spectra of PSW. The analysis was performed with non-hydrolyzed polysaccharides (native) and compounds were detected with high peak intensities and almost no fragment ion peaks of the analytes could be observed [46]. The highest degree of methoxylation was observed to oligomers with the most intense signals[47].

Despite the difficulty of finding fragmentation points, some pentoses residues are presented in the spectra (*m*/z: 485 and 616.75, 511.99 and 643.90, 598.84 and 730.99, 628.38 and 760.54, 639.38 and 771.41) with mass differences between the signals around 132 Da and with degrees of polymerization between 4 and 6. Hexoses residues (*m*/z: 598.84 and 760.54) with mass differences of 162 Da and DP of 4 and 5. Masses difference between 550.5 and 730.99 around 180 Da was attributed to galactose. Mass spectroscopy cannot distinguish stereo isomers, therefore the presence of ions having a mass difference of *m*/z 150 (*m*/z: 639.38 and 788.88) may be related to pentoses as arabinose or xylose with DP from 5 to 6 and mass difference of m/z 164 indicated presence of hexoses as rhamnose or fucose (*m*/z: 415.579.19, 607.27 and 771.41).

Only mass difference around 176 was (m/z: 415.6 and 590.76) was associated with galacturonic acid residues. No formation of fragments related into these substances very high peak intensities at *m*/z 521, 522.5 and 550.5 indicating no fragmentation and highest degree of methoxylation [47]. This is in accordance to previous results that suggested significant presence of these acids in the TGA final residues, hydrolysis of sample could be reveal fragments.

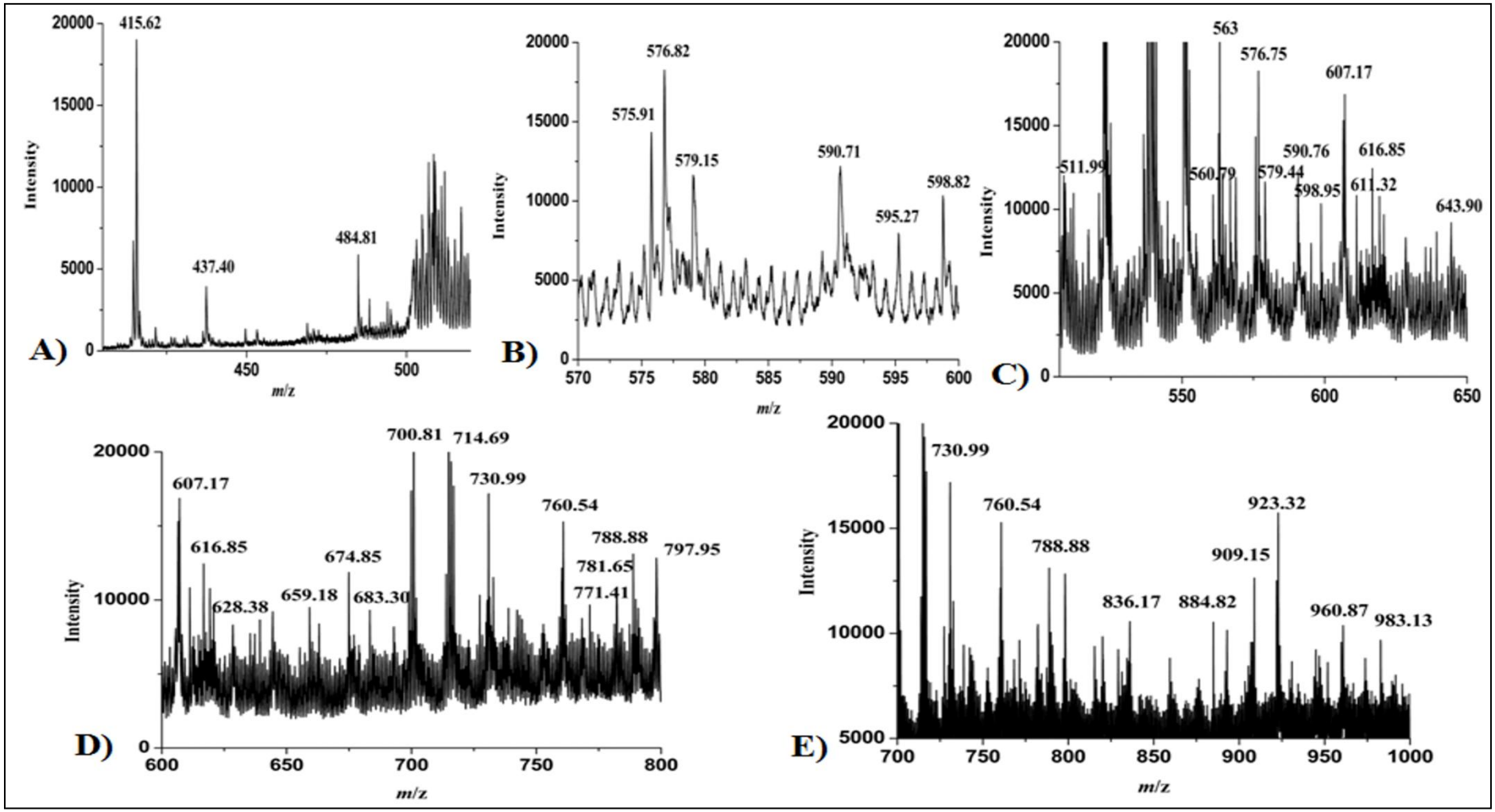

Figure 8-MALDI TOF spectra of PSW.

It is possible that no fragments related to this substances were peaks formed and this could associated with in the 521, 522.5 and 550.5 could be related to galacturonic or glucoronic acid which are in high quantity highest degree of methoxylation and hexoses presence. Fig.09 showed maldi tof spectra to PLW and were some noisy, indicating that purification of the samples was still insufficient as already discussed.

As well as to PSW, almost no fragment ion peaks could be observed to PLW and high intensity of peak at m/z 538.9 could be related to galacturonic or glucuronic acids contents which are not fragmented. A residue of hexose was attributed to mass difference 162 m/z (m/z: 649.15 and 841.15) with DP around 4 to 6. Difference of 190 m/z (908.46 and 1098.19) units was observed and it was associated with its methyl ester derivative. Dimmer of carbohydrate was observed to hexose with 328 m/z (m/z: 388.38 and 716.4) and to galacturonic or glucuronic acids with m/z 352 (m/z: 378 and 730.87).

Spectra showed mass differences of 22 Da that can be explained by the presence of salt adducts as $[M+H]^+$, $[M+Na]^+$, $[M-H^+2Na]^+$ often found in anionic carbohydrates. Besides these date are in accordance to others researches that showed galactose, glucose, mannose, rhamnose, arabinose and galacturonic acid as polysaccharides of wastes from *A. sisalana* [48-49].

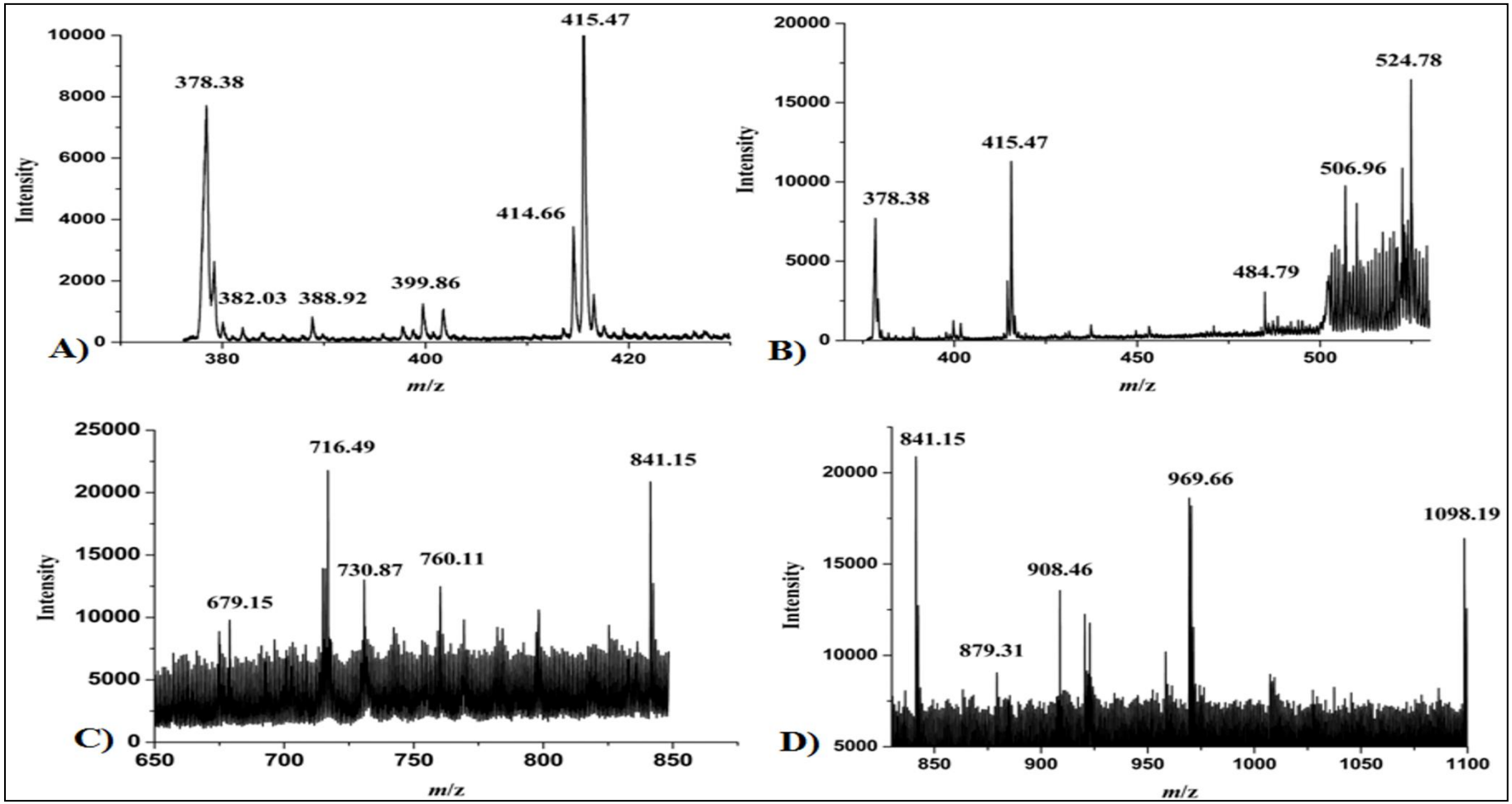

Figura 9-MALDI TOF spectra of PLW.

3.8 NMR

PSW and PLW 1H and 13C NMR spectra are given in Fig. 10. Most of the α-anomeric protons usually appear in the δ 5–6 ppm region while most of the β-anomeric protons in the δ 4-5 ppm range. Based on literature values the main chemical shifts are shown in the Tables 3

The presence of peaks around 40 ppm (δ 47.64 ppm to PSW and δ 42.74 ppm and δ 44.52 ppm to PLW) confirm the incomplete removal of proteins bound to the polysaccharides [50]. The presence of carbon atom signal in the range of δ 170–180 ppm proved that samples contain uronic acid or glycoprotein [51].

Methylation analysis was performed according to Rosenbohm et al. (2002)[52] with equation 1 and values were estimated to be 31.043% to PSW , to polysaccharides extracted of wastes from *A. sisalana* with composition similar to pectin were found values of 38.6%, however authors attributed . To PLW was not possible to calculate it using the equation the result is negative and this can be attribuited impurities of this sample as happened to Maldi Tof.

Equation 1: Determination of the degree of methylation (DM)

$$DM = \frac{(I_{COOMe} + I_{H1}) - I_{COO^-}}{(I_{COOMe} + I_{H1}) + I_{COO^-}}$$

I_{COOMe}: Integrals of H-5 adjacent to esters

I_{H1}: Integrals of H-5 adjacent to esters

I_{COO^-}: Integrals H-5 adjacent to carboxylates

Acetylation analysis was performed according to methylation analysis was performed according to Bédouet et al. (2003)[52] with equation 2 and values were estimated to be 10.66% to PLW and in to PSW 27.66%.

Equation 2: Determination of the degree of acetylation (DAc)

$$DAc = \frac{A_{CH3}}{3_{H2}}$$

A_{CH3}: Integrals of H-4 protons of 4-linked α-Gal*p*A
$_{H2}$: Integrals of *O*-acetyl prótons δ 2.2 – 2.0 ppm

For pectins, determination of the DA is limited by the absence of well identified signals from Gal*p*A protons which resonate near or overlap signals of protons from α-arabinosyl and β-galactosyl residues [52].

Chain of galacturonic acid residues of pectin which can be methoxy-esterified at C-6 and/or acetylated on O-2 and O-3. The ion-exchange properties, water-binding capability, cross-linking through calcium ions and hydrogen bonding of pectic polysaccharides depend usually on the number and distribution of methyl and acetyl groups along the pectic backbone. The DM corresponds to the percentage of carboxyl groups esterified with methanol. The DAc is defined as the percentage of galacturonosyl residues esterified with one acetyl group[53]. The plant source and conditions selected for isolation and purification of pectin interferes in the physicochemical properties of pectin [54].

Tabela 4-The chemical shift data for related glycosyl residues of PSW and PLW.

	PSW						PLW					
Glucosyl residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6α, 6β C-6	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a, 6b C-6
→4)--D-GalpA-(1→	4.83/ ND	3.64/72.40	3.82/ND	4.25/78.7	4.40/72.98	ND	4.80/ND	3.50/ND	3.91/ND	4.34/79.92	4.42/72.92	ND
→2)--l-Rhap-(1→	ND	4.05/77.7	3.81/ND	ND	3.84/ND	ND	ND	4.04/76.47	ND	ND	3.75/ND	ND
→3)-α-D-Manp-(1→	ND	3.61/ND	4.08/78.7	3.87/ND	3.81/74.30	3.44/ND	ND	ND	ND	ND	ND/74.31	3.50/ND
→4)-β-D-Galp-(1→	ND	3.45/72.40	3.64/74.30	4.05/77.70	3.60/76.27	3.64 and 3.71/ ND	ND	3.45/72.92	3.70/ND	ND	ND/76.47	ND
→5)-α-l-Ara f-(1→	ND	4.06/ND	3.93/77.70	4.14/ND	4.12/ND	ND	ND	4.04/ND	3.91/76.47	4.14/84.93	4.14/ND	ND

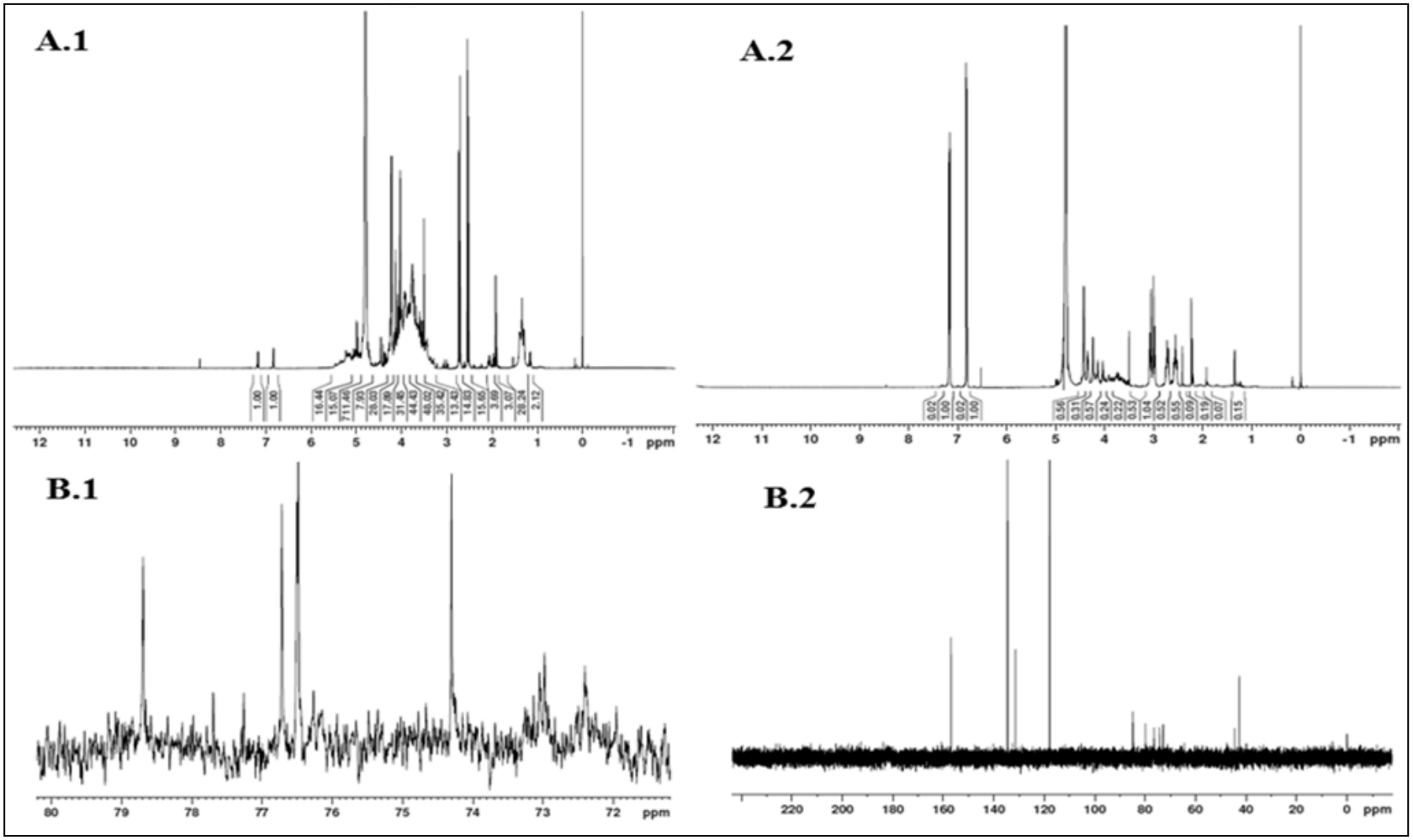

Figure 10- NMR spectra of polysaccharides. **A.1**: 1H NMR spectrum (D2O) of PSW. **A.2**- 1H NMR spectrum of PLW.**B-1**- 13C NMR spectrum of PSW. **B-2** 13C NMR spectrum of PLW.

4. Conclusão

It was possible to obtain pectic polysaccharides of solid and liquid waste of decortication of *A. sisalana*. This paper reported that the wastes obtained decortication from the agave show differences that reflect in the products obtained from these. To juice the presence of impurities difficult to analyze some important parameters and it was observed that to this waste more effective methods of purification are required however the bagasse.

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CONSIDERAÇÕES FINAIS

Os dados apresentados e discutidos permitem afirmar que o *Agave sisalana* pode ser usado para obtenção de diferentes produtos além da fibra sisal e que a utilização de outras partes além das folhas, principalmente os resíduos de decorticação podem levar a uma revitalização da cultura no Nordeste que atualmente encontra-se em declínio econômico. De modo a consolidar a obtenção de produtos de interesse farmacêutico a partir do *A.sisalana* serão necessários experimentos que permitam otimização dos processos utilizados e que garantam eficácia e segurança dos mesmos.

Finalmente, pode-se asseverar que o Programa de Cooperação Acadêmica (PROCAD) entre a Universidade Estadual da Paraíba e Universidade de São Paulo permitiu estabelecer um pilar necessário a consolidação da pós-graduação e pesquisa científica no semi-árido paraibano por meio de novas atribuições e aplicações para o *Agave sisalana*.

APÊNDICE 1-Artigo publicado

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Review

Inulin-type fructans: A review on different aspects of biochemical and pharmaceutical technology

Alexsandra Conceição Apolinário[a], Bolívar Ponciano Goulart de Lima Damasceno[a], Napoleão Esberard de Macêdo Beltrão[b], Adalberto Pessoa[c], Attilio Converti[d], José Alexsandro da Silva[a,*]

[a] Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N – Bairro Universitário, 58429-600 Campina Grande, Paraíba, Brazil
[b] Brazilian Agricultural Research Corporation, National Center for Cotton Research, Rua Oswaldo Cruz, 1143, Cx. Postal 174, 58428-095 Centenário, Campina Grande, Paraíba, Brazil
[c] Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580 – Bloco 16, Cidade Universitária, 05508-000 São Paulo, Brazil
[d] Department of Civil, Chemical and Environmental Engineering, Chemical Engineering Pole, Genoa University, Via Opera Pia 15, 16145 Genova, Italy

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ABSTRACT

Inulin is a natural storage polysaccharide with a large variety of food and pharmaceutical applications. It is widely distributed in plants, being present as storage carbohydrate in more than 30,000 vegetable products. Due to their wide distribution in nature and significant role in industry, the extraction, isolation and characterization of inulin-type fructans are gaining attention in recent years. Inulin sources have recently received increasing interest as they are a renewable raw material for the production of bioethanol, fructose syrup, single-cell protein and single cell oil, obtainment of fructooligosaccharides and other useful products. This review focuses on the state-of-the-art of biochemical and pharmaceutical technology of inulin-type fructans.

Contents

* Corresponding author at: Graduation Program in Pharmaceutical Sciences, State University of Paraíba, Rua Juvêncio Arruda, S/N – Bairro Universitário, CEP 58429-600 Campina Grande, Paraíba, Brazil. Tel.: +55 83 9131 8427/3315 3300x3516.
E-mail addresses: acapolinario@gmail.com (A.C. Apolinário), bolivarpgld@pq.cnpq.br (B.P.G. de Lima Damasceno), napoleao.beltrao@gmail.com (N.E. de Macêdo Beltrão), pessoajr@usp.br (A. Pessoa), converti@unige.it (A. Converti), alexuepb@pq.cnpq.br (J.A. da Silva).

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1. Introduction

Inulin is a natural, plant-derived storage polysaccharide with a large variety of food and pharmaceutical applications. It is a substitute for sugar or fat having a very low caloric value, acts in a similar way as dietary fibers and contributes to improve gastrointestinal system conditions. Among other possible pharmaceutical applications are its use as an orally delivered drug targeting the colon, to delay absorption of drugs with adverse effects on stomach, or the treatment of diseases that show a peak in symptoms in the early morning (Barclay, Ginic-Markovic, Johnston, Cooper, & Petrovsky, 2012). Because of these properties, food and pharmaceutical industries have been finding applications of inulin and its derivatives such as fructooligosaccharides (FOS) in the production of functional foods, nutritional composites and drugs (Barclay, Ginic-Markovic, Cooper, & Petrovsky, 2010; Barclay et al., 2012; Cummings, Macfarlane, & Englyst, 2001; Judprasong, Tanjor, Puwastien, & Sungpuag, 2011; Laparra, Tako, Glahn, & Miller, 2008; Matusek, Meresz, Le, & Oersi, 2009; Morris & Morris, 2012).

Inulin is widely distributed in a variety of plants as storage carbohydrate, being present in more than 30,000 vegetable products (Wichienchot et al., 2011), among which are the tubers of *Helianthus tuberosus* (Jerusalem artichoke), *Cichorium intybus* (chicory), *Dahlia pinnata* (dahlia) and *Polymnia sonchifolia* (yacon) (Braz de Oliveira et al., 2011). It was discovered by the German scientist Valentine Rose, in the early 1800s, as a carbohydrate source from the roots of *Inula helenium*, and after named inulin by Thomson in 1817. The plant physiologist Julius Sachs, who was a pioneer in fructan research, was able in 1864 to detect, using a microscope, the spherocrystals of inulin in the tubers of *D. pinnata*, *H. tuberosus* and *I. helenium* after precipitation with ethanol (Franck & De Leenheer, 2005). It was shown to be a mixture of oligo- and/or polysaccharides composed of fructose units with β-configuration of the anomeric C_2, which makes inulin-type fructans resistant to hydrolysis by human intestinal digestive enzymes that have specificity for α-glycosidic bonds. For this reason all these compounds have been classified as nondigestible oligosaccharides (Roberfroid, 2007).

Plant inulin has chains incorporating from 2 to 100 fructose units, whose length, composition and polydispersity depend on the plant species, the phase in its life cycle, the harvesting date and the extraction and post-extraction procedures (Barclay et al., 2010; Ronkart et al., 2007). Inulin can be hydrolyzed by both endo- and exo-inulinases. The exo-inulinases remove the terminal fructose residues from the non-reducing end of chain, while the endo-inulinases act on the internal linkages (Braz de Oliveira et al., 2011; Ertan, Ekinci, & Aktac, 2003; Ronkart et al., 2007).

This review focuses on the state-of-the-art of biochemical and pharmaceutical technology of inulin-type fructans with emphasis in their biosynthesis. Moreover, the methods for isolation and characterization of inulin from different vegetal species are described and biotechnological applications of these carbohydrates are related.

2. Fructans: origin and role in plants

Inulin-type fructans are water-soluble fructose-based polymers that result from extended sucrose metabolism (Weyens et al., 2004). In plants, they are frequently stored in leaves and others organs acting as carbohydrate reserve (Ritsema & Smeekens, 2003). These fructan-containing plant species are found in a number of mono and dicotyledonous families such as Liliaceae, Amaryllidaceae, Gramineae and Compositae. In Liliaceae, Amaryllidaceae and Compositae, inulins are usually stored in bulbs, tubers and tuberous roots (Braz de Oliveira et al., 2011). Besides this, fructans have been reported to play a fundamental role also in osmoregulation, to act as protectants against dehydration induced by drought or freezing and to be involved in abiotic stress–tolerance (Livingston, Premakumar, & Tallury, 2006; Ritsema & Smeekens, 2003).

These substances play an important role in the quality control of fruits because several pathways that link the synthesis and breakdown of these carbohydrate reserves are in dynamic equilibrium and determine fruit quality during storage. Alterations in the pattern of soluble sugars are often associated with increased cold hardiness in a wide range of plant species (Gibson, 2005). The stress/tolerance response by changes in FOS accumulation in tablet grape was monitored after high CO_2 treatment, during low temperature storage, and the results showed an increasing FOS accumulation (Blanch, Sanchez-Ballesta, Escribano, & Merodio, 2011)

Some species growing in arid habitats develop photosynthetic adaptive processes such as the crassulacean acid metabolism (CAM) that allow them to efficiently uptake CO_2 at night and use water. Fructans are photosynthetic product of CAM and act as osmoprotectants during drought (Borland, Griffiths, Hartwell, & Smith, 2009). Fructans of these species include inulin, levans, neo-series inulin and highly branched structures (Waleckx, Gschaedler, Colonna-Ceccaldi, & Monsan, 2008), whose main function is energy storage and to act in abiotic stress tolerance in plants (Arrizon, Morel, Gschaedler, & Monsan, 2010; López, Mancilla-Margalli, & Mendoza-Diaz, 2003; Leach & Sobolik, 2010).

A large number of agave species possess a CAM, which explains the recent efforts to achieve inulin-type fructans from them (López & Urías-Silvas, 2007). Heads of plants belonging to the Agave genus have high contents of fructan oligomers, composed mainly of fructose units linked to a sucrose molecule, which can be easily degraded by thermal or enzymatic treatments leading to free sugars, mainly fructose. Many patents have been granted on the use of fructans from Agave species as a raw material for many purposes (Narvávez-Zapata & Sânchez-Teyer, 2009).

3. Chemical structure of fructans

Fructans are present in plants as heterogeneous mixtures with different degrees of polymerization (DP) and various chemical structures. The type of fructans found in plants (oligomeric or polymeric molecules) and the presence of a specific type of fructan are species-dependent and related with the environmental conditions and developmental stage of the plant (Mancilla-Margalli & Lopez, 2006). Five types of fructans with different structures were described in higher plants: inulin-type fructans (1-kestose), levan-type fructans (6-kestose), fructans of the inulin neoseries (neokestose), mixed-type levans (bifurcose) and fructans of the levan neoseries also called mixed-type levans (mixed-type F_3 fructan), whose shortest representatives, mentioned between brackets, have their chemical structures illustrated in Fig. 1 (Van Laere & Van den Ende, 2002).

These authors reported that inulin-type fructans are fructose polymers that have mostly or exclusively β-(2→1) fructosyl-fructose linkages, whereas levan-type fructans have mostly or exclusively β-(2→6) fructosylfructose linkages. Although these fructan types are essentially linear molecules, a low degree of branching can occur through β-(2→6) linkages in the case of inulins or β-(2→1) linkages in levans. In case the terminal glucose molecule is absent (Fn-type fructans), there are reducing compounds in contrast to the regular type fructans (G-Fn), and the terms inulo-n-oses [β-(2→1) linkages] and levan-n-oses [β-(2→6) linkages] are used.

Without any doubt, inulin is the best-known and studied fructan (Van Laere & Van den Ende, 2002), and the

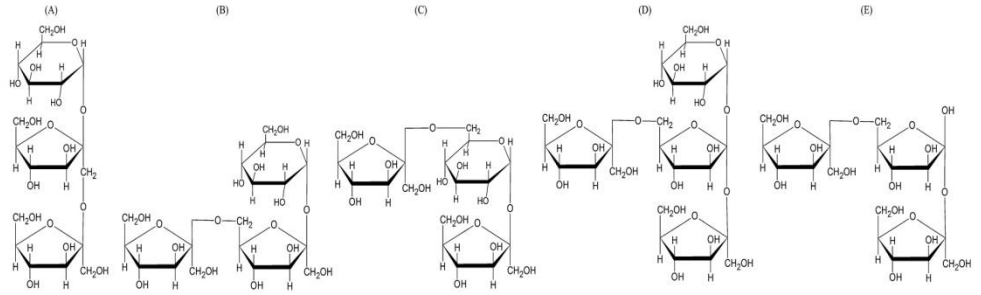

Fig. 1. Different types of fructans from higher plants according to the classification of Van Laere and Van den Ende (2002): (A) 1-kestose; (B) 6-kestose; (C) neokestose; (D) bifurcose; (E) mixed type F3 fructan.

number and distribution of different oligomers were shown to be characteristic of the inulin producing plant (Kiss & Forgo, 2011). Native inulin always is extracted from fresh plants, taking precautions either to inhibit the plant own inulinase activity or to prevent acid hydrolysis, but no fractionation procedure is applied to eliminate the smaller oligosaccharides and monomers that are naturally present, as it occurs for commercially available products (Franck & De Leenheer, 2005).

Inulin has not a simple structure; its chain constituted by a variable number of fructose units, linked by β-(2→1) D-fructosyl-fructose bonds, usually terminates with only one glucose unit linked through an α-D-glucopyranosyl or α-(1→2) bond as in sucrose (Bruyn, Alvarez, Sandra, & De Leenheer, 1992). Inulins with a terminal glucose unit are called α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (or FOS), while those made up only of fructose fructopyranosyl-[α-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (or inulooligosaccharides) (Ronkart et al., 2007).

Based on the polymeric structure described above, it is evident that the main aspect of inulin structure is associated with its β-(2→1) bonds, which prevent inulin from being digested like a typical carbohydrate and are responsible for its low caloric value along with its behavior as a dietary fiber (Roberfroid & Slavin, 2000).

The physico-chemical and functional properties of inulin are linked to DP as well as the presence of branches. The short-chain fraction, oligofructose, is much more soluble and sweeter than native and long-chain inulin, and can contribute to improve mouthfeel because its properties are closely related to those of other sugars. For example, owing to a sweetness profile similar to that of sucrose, but lower caloric content (1–2 kcal/g) and sweetening power (30–35%), it can be useful to partially replace sucrose or to replace it totally when combined with other non-caloric sweeteners (Guggisberg, Cuthbert-Steven, Piccinali, Bütikofer, & Eberhard, 2009; Tárrega, Rocafull, & Costell, 2010). The long-chain fraction is less soluble, more viscous and more thermostable than native inulin and can act in rheological and sensory properties of dairy products as a fat substitute in low-fat or reduced-fat products; in these cases inulin acts as a filler or as breaker of structure in the same way as fat globules do (Guggisberg et al., 2009). It was reported that long-chain inulin, when sheared in water or milk, has the ability to form microcrystals, which can interact to form a smooth creamy texture and provide a fat-like mouth sensation (López-Molina et al., 2005).

Other physico-chemical properties that are influenced by DP include the melting, glass transition temperature, the capability of gel formation and gel strength (Bot, Erle, Vreeker, & Agterof, 2004). The interaction with other food components such as starch or hydrocolloids is also influenced (Meyer, Bayarri, Tarrega, & Costell, 2011). Therefore, fractions with variable DP can be used to formulate specialty food products (Hernalsteens & Maugeri, 2008; Yi, Zhang, Hua, Sun, & Zhang, 2010).

4. Biosynthesis of inulin

Fructan synthesis starts when photosynthesis exceeds the demand and sucrose reaches a critical level in sink organs, but differences exist among species regarding linkages, branching patterns and sizes (Livingston, Hincha, & Heyer, 2009).

Fructan biosynthesis in plants is catalyzed by three different classes of enzymes: sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST), fructan:fructan 1-fructosyltransferase (EC 2.4.1.100) (1-FFT) and fructan exohydrolase (EC 3.2.1.153) (FEH). Inulin is synthesized from a starting molecule of sucrose, which explains the presence of only one glucose unit in its chain. The relative inertness of glucose confers certain protection to the polymer, because it does not break down spontaneously. Enzymes then gradually transfer fructose from another sucrose molecule to perform the polymerization. The attachment of the incoming fructose takes place on the relatively reactive primary hydroxyl group linked to the anomeric carbon through the methylene group at C_1 of fructose moiety of sucrose (Barclay et al., 2010). In 1968, it was proposed a model for the biosynthesis of inulin in *H. tuberosus* that assumes no phosphorylated precursor, sucrose as the only substrate and the action of both 1-SST and 1-FFT. 1-SST transfers a fructose moiety from sucrose to the C-1 of a fructose in another sucrose molecule yielding the trisaccharide 1-kestose (GF + GF → GFF + G) in an essentially irreversible reaction, and similar transferases lead to 6-kestose and neokestose (Edelman & Jeeeord, 1968). Then, 1-FFT transfers fructose moieties from 1-kestose (or larger fructans) to sucrose or other fructans (GFn + GFm → GF(n + 1) + GF(m − 1) with $n \geq 1$, $m \geq 2$) (Van Laere & Van den Ende, 2002).

A further model was proposed (Livingston et al., 2009) for the synthesis in plants (Fig. 2) of the earlier five types of fructans from sucrose illustrated in Fig. 1.

It involves other two fructosyltransferases in addition to 1-SST, FEH and 1-FFT, specifically a sucrose:fructan 6-fructosyltransferase (6-SFT) and a fructan:fructan 6G-fructosyltransferase (6G-FFT). 6-SFT is able to catalyze the synthesis of (a) 6-kestose from sucrose and its subsequent elongation to higher levans, (b) fructans of levan neoseries from neokestose, (c) bifurcose from 1-kestose and sucrose, and (d) mixed-type levans from bifurcose. On the other hand, 1-SST catalyzes the synthesis of 1-kestose from sucrose, 6G-FFT that of neokestose from 1-kestose and sucrose, and 1-FFT the elongations of (a) neokestose to higher fructans of the inulin neoseries, (b) 1-kestose to inulin, and (c) bifurcose to mixed-type levans. Finally, levan-type fructans can also be produced from bifurcose by the concerted action of 6-SFT, 1-FFT and FEH.

Some researchers drew attention to differences between inulins of bacterial or fungal origin, which range from high molecular-weight fructans to oligosaccharides, and inulins of plant origin. The synthesis of inulin takes place in bacteria and fungi spores by transfer of additional fructose moiety of sucrose to the terminal fructose unit of 1-kestose or higher inulin-type fructans and is performed by

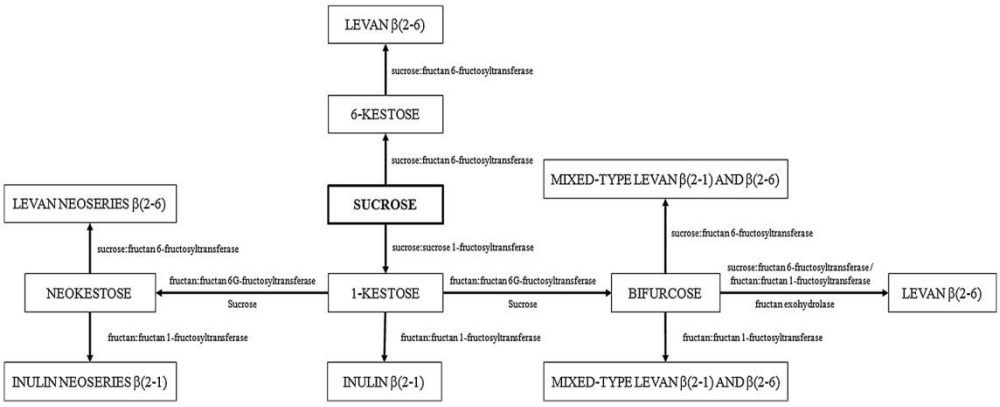

Fig. 2. Model proposed by Livingston et al. (2009) for the synthesis of fructans in plants involving four fructosyltransferases and a fructan exohydrolase.

sucrases (inulosucrase or levansucrase). Since the acceptor reactivity increases with the length of its chain, inulin from these sources usually has very high DP (Franck & De Leenheer, 2005).

5. Natural factors influencing the chemical structure and content of inulin-type fructans

The chemical structure of fructan greatly depends on the species. Inulin from garlic has a 2,1-linked β-D-fructosyl back bone with 2,6-linked β-D-fructosyl side chains, and the same applies to *Agave tequilana* (Ávila-Fernández, Galicia-Lagunas, Rodríguez-Alegría, Olvera, & López-Munguía, 2011). In addition, plants that naturally accumulate fructan can also degrade it in order to remobilize the stored carbon, which is the major drawback for inulin recovery.

The inulin DP depends upon different factors: plant species, climate and growing conditions, harvesting maturity and storage time after harvest (Chi et al., 2011). For instance, inulin stored by chicory has a rather low mean DP. Higher-DP inulins are found in artichoke, globe thistle (*Echinops ritro*) and *Viguiera discolor*. Variation in the chain length of inulin polymers in different Asteraceae species could be the result of different enzymatic characteristics (Vergauwen, Van Laere, & Van den Ende, 2003). Throughout the period of artichoke storage there occur a decrease in inulin content and mean DP, owing to its depolymerisation (Leroy, Grongnet, Mabeau, Le Corre, & Baty-Julien, 2010).

It has been observed in chicory that inulin DP in the early growing season is much higher than at harvest, because the activities of 1-FFT and 1-FEH depend on both plant and environmental factors. Several solutions for this breakdown problem have been proposed, including attempts to obtain new fructan-accumulating plants by genetic modification of crops that originally do not synthesize fructan. The main advantage of using highly productive non-fructan-accumulating plant species with well-established husbandry and processing chain is that plants that naturally accumulate fructan can also degrade the polymer in order to remobilize stored carbon. Moreover, the introduction of fructan biosynthesis in non-fructan species would render new types of fructan with different DP not yet present in natural fructan-producing plants, i.e. the so-called tailor-made fructan (Van Arkel et al., 2013).

When the water-soluble sugar content of 2-, 4- and 6.5-year old *A. tequilana* plants for linkage analysis was investigated, the youngest plants exhibited the highest levels of free monosaccharides and low molecular weight fructans (DP = 3–6) with potential application as prebiotics, while the DP reached a maximum of 3–30 in 4-year-old plants and then decreased to 4–24 in the oldest ones (Arrizon et al., 2010). Besides, it was established a relationship between plant age and fructan synthesis in *Agave atrovirens* Karw by identifying changes in fructan composition in its leaves at three ages (3, 6 and 9 years) and quantifying the fructosyltransferase enzymatic complex activity, thus determining the growth stage with the highest fructan content (Leopoldo, Maria Eugenia, Aurea, & Rosalva, 2011). The content of non-structural carbohydrates was the highest in the leaves of the youngest plants and decreased by 86% in those of the oldest ones. The main non-structural carbohydrate was sucrose in the youngest plants, while inulin-type fructans, glucose and fructose predominated in the oldest ones.

6. Inulin extraction and precipitation

Due to their wide distribution in nature and significant role in industry, the extraction, isolation and characterization of inulin-type fructans are still gaining attention in recent years (Yang, Hu, & Zhao, 2011). Many investigations were developed to set optimum extraction conditions in order to improve inulin extraction from plants, and temperature, extraction time, and solvent/solid ratio were identified as the most important factors influencing the yield (Abou-Arab, Talaat, & Abu-Salem, 2011; Abozed, Abdelrashid, El-Kalyoubi, & Hamad, 2009; Paseephol, Small, & Sherkat, 2007; Saengkanuk, Nuchadomrong, Jogloy, Patanothai, & Srijaranai, 2011; Toneli, Park, Ramalho, Murr, & Fabbro, 2008). Solubility of inulin in water remarkably increases with temperature, being almost insoluble at 25 °C and reaching about 35% (weight/volume) at 90 °C; therefore, the industrial production process is based on diffusion in hot water (Kim, Faqih, & Wang, 2001).

Following this concept almost all of inulin extraction methods described in the literature make use of hot water as a solvent, with only small differences in temperature and extraction time. For example, inulin was extracted from Jerusalem artichoke ground tubers by a pretreatment step involving boiling water treatment for 10–15 min (Paseephol et al., 2007), from dry chicory roots by hot water diffusion at an average temperature of 80 ± 2 °C for 1 h with continuous stirring (Toneli et al., 2008), from globe artichoke by distilled water (80 °C) at pH 6.8 (by NaOH) to avoid inulin hydrolysis at pH < 6 (Ronkart et al., 2007), and from *H. tuberosus* L. by hot deionized water at 85 °C for 1 h (Saengthongpinit & Saijaanantakul, 2005).

Ultrasound-assisted extraction has been recently proposed to improve inulin extraction yield compared to the above traditional methods, the main independent variables being sonication amplitude, temperature and time. In the extraction of inulin from the body of Burdock root (*Arctium lappa*), a raise in the amplitude or

the extraction time increased the yield, while temperature had a minor effect, and the optimum extraction conditions were shown to be a sonication time of 25 min, a sonication amplitude of 83.22% and a temperature of 36.76 °C (Milani, Koocheki, & Golimovahhed, 2011). However, caution is needed in using sonication to extract inulin, because some low-molecular-weight fragments are formed by the direct action of ultrasounds and changes occur in the chemical composition of the inulin; therefore, their direct use has been suggested for inulin depolymerization to get a diffluent short inulin, while indirect sonication would be more suited to extract natural inulin (Lingyun et al., 2007). In the direct method a probe is directly inserted into a sample vessel, whereas the indirect sonication is performed by immerging the sample in an ultrasound cleaning bath and shaking it periodically in orbital shaker.

A new method has recently been proposed to extract inulin from Jerusalem artichoke tubers, consisting in a three-stage homogenate extraction that ensured an extraction yield (16.39 g per 100 g of tuber) about 14% higher than the conventional hot water extraction (Li, Meng, & Sun, 2012). After tuber washing with pressurized water to remove oils, drying in air at ambient temperature and treating with hot water at 80 °C for 10 min to inactivate the polyphenol oxidases, they were suspended in distilled water, shredded and extracted three times, and the resulting filtered liquors subjected to a clarification process.

Inulin recovery usually starts with its precipitation after extraction, which can be performed by either lowering temperature or using different solvents and involves variables such as centrifugation speed and time (Abozed et al., 2009; Lingyun et al., 2007). Due to its low solubility at low temperature, when an inulin concentrated solution is cooled or frozen it undergoes a process of phase splitting. Toneli et al. (2008) proposed a new method of inulin precipitation by cooling or freezing the extract followed by centrifugation and spry drying to obtain inulin in powder form. However, since this method implies significant energy expenditure, the liquid extract must be concentrated by evaporation before drying, and the temperature lowered to favor inulin precipitation. Freezing/thawing is another method proposed to precipitate inulin followed by centrifugation (Yang et al., 2011).

Long chain inulin can be alternatively precipitated from aqueous solutions in the presence of high concentrations of organic solvents such as methanol, ethanol, propanol, acetonitrile and acetone among others. Acetone was shown to be the best solvent to keep the natural DP, followed by ethanol and methanol. The strong precipitating power of acetone with regard to polysaccharides was ascribed to its ability to remove the water of solvation of these biomolecules, thereby promoting dehydration and subsequent precipitation (Dalonso et al., 2009). In addition, it has a very low boiling point (56.5 °C), which allows it to be easily recovered by distillation (Moerman, Van Leeuwen, & Delcour, 2004). Even though ethanol and acetone were shown to be the best solvents to precipitate inulin from Jerusalem artichoke tubers (Abozed et al., 2009), in general acetonitrile and acetone are more effective than ethanol for most inulins (Ku, Jansen, Oles, Lazar, & Rader, 2003). Nonetheless, for safety reasons, ethanol is recognized to be the best choice in food applications (Paseephol et al., 2007). With ethanol, recovered chicory inulin had a DP of 25 and the dahlia one of 40 (Moerman et al., 2004).

Table 1 summarizes the most significant methods of inulin extraction from different sources.

7. Purification methods

The extraction and precipitation methods described above usually result in solutions containing a mixture of crude inulin, other polysaccharides, non-carbohydrate compounds, particulate and colloidal matter (i.e. pectin, proteins, and cell wall materials), which have to be further purified to isolate the specific polysaccharide of interest (Izydorczyk, 2005). To remove these impurities and purify inulin, various physicochemical methods as well as modern and expensive chromatographic techniques can be used.

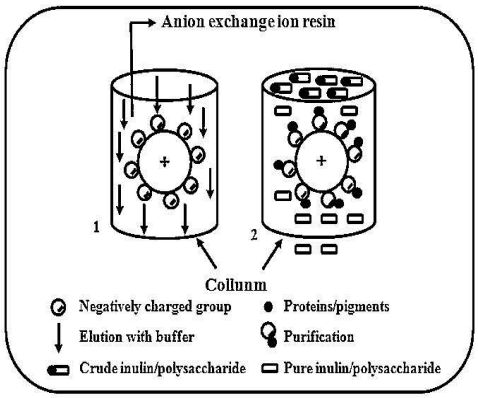

Fig. 3. Schematic of inulin purification by anion-exchange resins. 1. The DEAE cellulose anion-exchange resin (made up of a cationic nucleus and external negative chains) is sequentially eluted and equilibrated with buffer. 2. The supernatant (crude inulin) resulting from centrifugation of re-dissolved precipitate in water or buffer is fractionated using ion-exchange chromatography resin and the pure inulin/polysaccharide is obtained in a clear solution. Further steps can also be used using different buffers to obtain various fractions of polysaccharides.

The initial step common to almost all methods is dissolving the precipitate in distilled water and centrifuging it to remove any insoluble material (Fang, Jiang, & Wang, 2006; Holderness et al., 2011). Chemical treatments usually precede the separation through chromatographic techniques. Among these, deproteination with trichloroacetic acid has been reported by different authors (Chen et al., 2011; Fang et al., 2006; Feng, Jia, Shi, & Chen, 2010). In some cases, crude polysaccharides were further treated with cetylpyridinium chloride, amyloglucosidase and protease to remove almost completely proteins, uronic acids, starch-like α-D-glucans and hexosamines (Li et al., 2012). To obtain sulfated polysaccharide from *Sargassum pallidum* (Turn.) C. Ag., and two acidic polysaccharide fractions from *Polygala tenuifolia*, 10% $CaCl_2$ was added and kept overnight to precipitate tannins (Ji et al., 2011; Xin et al., 2012).

Several types of chromatographic techniques can be used to separate inulins from each other and/or from non-carbohydrate contaminants. Among them is the ion-exchange chromatography, whose effectiveness is well known to be influenced by pH, ionic strength of the buffer, nature of the counter-ion, flow rate and temperature. For inulin purification, the most commonly used anion-exchange resins are diethylaminoethyl (DEAE) cellulose (Fig. 3), DEAE Sepharose and DEAE Sephacel.

A large number of studies describe the isolation and purification of polysaccharides by means of DEAE cellulose. By this method, single water-soluble polysaccharides were obtained from the roots of *Cudrania tricuspidata* (Carr) (Lei, 2010) and *Ophiopogon japonicus*, a traditional Chinese medicinal herb (Chen et al., 2011), from the fruit of açai (*Euterpe oleracea*) (Holderness et al., 2011), from *Inonotus obliquus*, a well-known medicinal plant traditionally used for its antihyperglycemic effects, from *Grifola frondosa*, which belongs to the family of Meripilaceae and is marketed in China, Japan and other Asian countries as a medicinal and edible fungus (Chen, Ma, Liu, Liao, & Zhao, 2012), and from *Ginko biloba sarcotesta* (Wu et al., 2011); two different polysaccharide fractions were isolated and purified from the aqueous extract of *Prunella vulgaris* L. (PV) (Feng et al., 2010); and even three fractions were successfully purified

Table 1
Inulin type-fructans extraction methods from different sources.

Plant	Plant's treatment	Extraction	Extract's treatment	Reference
Helianthus tuberosus L.	Fresh frozen globes were thawed and then grated into slices	Twelve kg of plants were extracted by 50 L distilled water (80 °C) at pH 6.8 (by NaOH)	The extract was filtered through 1 mm and 5 μm filters, frozen/thawed and the precipitate was centrifuged at 3000 × *g* for 20 min	Ronkart et al. (2007)
Helianthus tuberosus L.	Tubers were washed to remove undesirable materials and cutted into slices. In order to avoid enzymatic browning, the slices were dipped in boiling water acidified with ascorbic acid and boiled for 2–3 min. Then, the slices were kept in polyethylene bags and stored in freezer at −10 °C until used	One kg of tubers was transferred into a warming blender and extracted with five-fold excess of hot water (70 °C) for 60 min at 70 °C with constant stirring	The suspension was filtered and residue was re-extracted using the same steps	Abou-Arab et al. (2011)
Cichorium intybus	Dried grounded root and root were used as the starting material	Batch extraction was performed at 70 °C with continuous stirring. Distilled water and alcoholic solutions were tested as solvent for inulin extraction	The suspension was filtered through a cloth	Dobre et al. (2008)
Helianthus tuberosus L.	Tubers were washed and soaked in 0.038 M sodium hypochlorite for 30 min to eliminate soil and reduce micro-organisms. The remaining tubers were packed in sealed polyethylene bags and kept in duplicates at 5, 2, and −18 °C	Eighty-five grams of deionized water at 85 °C were added to 11.5 g of crushed tubers, and the slurry was shaken at 130 rpm at 85 °C for 1 h in a water bath	After cooling to room temperature, the total weight was adjusted to 100 g with deionized water and the slurry was then centrifuged for 20 min at 12,000 × *g*	Saengthongpinit and Saijaanantakul (2005)
Helianthus tuberosus L.	To extract fructans of the tubers, 2 kg lots of peeled tubers were chopped into fine pulp in 10 L of hot water containing 100 ppm sodium metabisulphite to minimize browning at 95–98 °C for 10 min	Batch extraction was performed at 70 °C with continuous stirring. Distilled water and alcoholic solutions were tested as solvents for inulin extraction	The resulting extract was filtered through muslin cloth and then concentrated to 50% of the original volume using a single-stage climbing film evaporator	Paseephol et al. (2007)
Helianthus tuberosus L.	Tubers were washed with tap water and any deteriorated parts were removed, then the tubers were sliced. The sliced tubers were immersed immediately in boiling water for 5 min, following by immediate dipping in cold acetic acid solution (2%) to inhibit polyphenoloxidase activity. After that, slices were dried in electronic air oven	The dried powdered tubers were mixed with water at different powder tubers/water ratio (1:2.5, 1:5, 1:10, 1:15 and 1:20, w/v) at different temperatures (65, 75, 85 and 95 °C) as well as for different times (40, 50, 60 and 70 min)	The suspension was filtered	Abozed et al. (2009)
Morina officinalis	The dried roots were powered	Twenty g of material were extracted with 95% ethanol (400 mL) for 1.5 h at 100 °C	Once filtered, the extracts were concentrated to 20 mL under reduced pressure at 50 °C. The residue was then mixed with 20 mL water	Yang et al. (2011)
Agave tequilana	Fifty g of pulp were produced from the transversal cutting of six mature *A. tequilana* heads	The heads were placed in a mixer with 1.5 L of distilled water at 80 °C and agitated for 5 min	The obtained suspension was then filtered in preparation for analysis	Waleckx et al. (2008)

from the crude polysaccharide of *Dendrobium denneanum* (Fan et al., 2009).

DEAE Sepharose was successfully employed in other efforts. A water-soluble polysaccharide was isolated from the rhizome of *Menispermum dauricum* DC (Menispermaceae), a traditional Chinese medicinal herb that is widely used for treating sore throats, colitis, dysentery and rheumatic arthralgia (Lin et al., 2013); polysaccharides were purified from the seed of Longan (*Dimocarpus longan* Lour.), a non-climacteric subtropical fruit with high commercial value (Jiang et al., 2013); inulin-type oligosaccharides (DP < 10) were isolated from *Morinda officinalis*, a plant of the traditional Chinese herbal medicine (Yang et al., 2011); sulfated polysaccharides were purified from *Turbinaria conoides* (Chattopadhyay et al., 2010); and a crude polysaccharide was isolated from the rhizome and roots of *Rhodiola rosea* L. and fractionated (Cai et al., 2012).

Although less commonly used, also DEAE Sephacel was successful to isolate and fractionate similar polysaccharides from the roots of *Polygala tenuifolia* and from *Glycyrrhiza glabra* L., a ligneous perennial shrub growing in the Mediterranean region, Asia Minor and Middle East, which is also cultivated widely in southern Russia (Wittschier, Faller, & Hensel, 2009).

In these studies, the type and concentration of the buffer to equilibrate the resin in the column were varied so as to give rise different polysaccharide fractions. For instance, using 0.2 mol/L NaCl, Hu, Liu, Ni, & Lu (2012) isolated a complete and single fraction from *Inonotus obliquus* that might have been a single compound, while multiple fractions were obtained with 0.5 mol/L NaCl, with

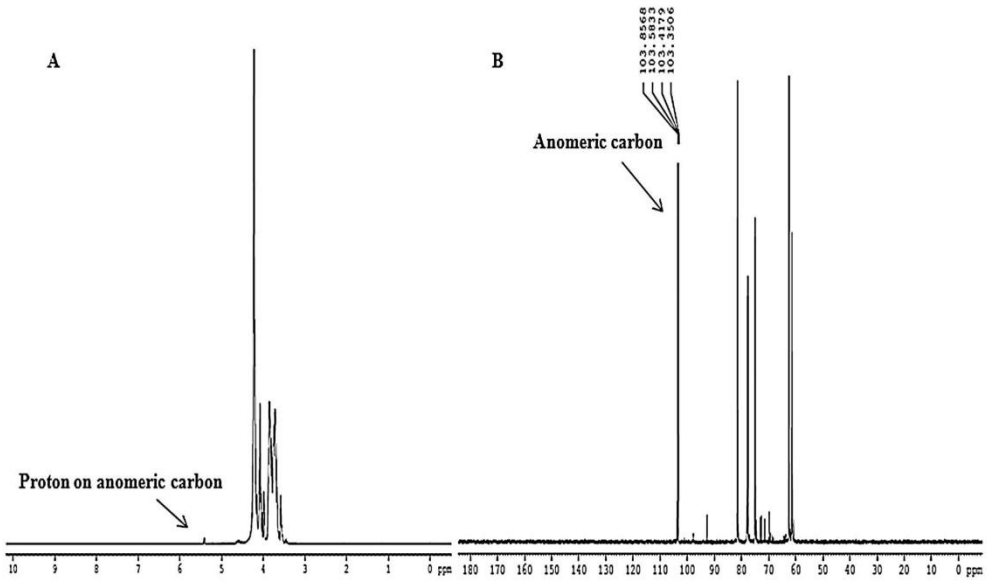

Fig. 4. Typical ^{13}H NMR (A) and ^{13}C NMR (B) spectra of inulin HP®.

the second peak displaying the highest polysaccharide concentration. Polysaccharides were successfully recovered from leaves of *Panax ginseng* CA Meyer using a DEAE Cellulose column eluted by a stepwise gradient of NaCl solutions (0.0, 0.1, 0.25 and 0.5 mol/L), and the appropriate fractions pooled, dialyzed and lyophilized to give four fractions (Ni et al., 2010). Polysaccharides from powdered açai were passed through a DEAE cellulose column and sequentially eluted with 0.05 mol/L Tris–HCl buffer and 2 mol/L NaCl, with minimal loss (Holderness et al., 2011).

8. Analytical techniques

Quantification of inulin type-fructans may be performed in the extracts so as to provide a preliminary assessment of plant fructan contents. Since fructans are usually found as complex mixtures of carbohydrates with different DP, monomer composition and glycosidic linkages, their analysis is a fundamental step to acquire basic information on the polysaccharide itself as well as to deepen understanding of its action mechanism, which is dependent on its chemical structure. However, the separation of complex mixtures of oligosaccharides is not straightforward, because of structural and molecular weight similarity; in addition, their identification is also hampered by the lack of available commercial standards (Brokl, Hernández-Hernández, Soria, & Sanz, 2011).

Gas chromatography–mass spectrometry (GCMS), nuclear magnetic resonance (NMR), and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry have been successful to obtain structural information on fructans, mainly DP. Fig. 4, panel A, illustrates a typical ^{1}H NMR spectrum of inulin HP®, in which one can see the chemical shift of glucose anomeric carbon, while panel B clearly shows four signals from 10.35 to 103.86 ppm of the ^{13}C NMR spectrum that suggest a high DP (>20). The presence of β-D-fructofuranosyl linkage is confirmed by the signal at 81.43 ppm. On the other hand, Fig. 5 shows the MALDI-TOF spectrum of inulin HP®, where the sharp peaks correspond to different values of molecular weight and then of DP.

Thin-layer chromatography (TLC) can be used to assess both the level and the composition of fructans in plant tissues. However, the last technique has limited resolution and low sensitivity and accuracy when used for quantitative purposes. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been accepted as the most powerful method for direct determination of inulin, for it furnishes not only the content of inulin but also the DP profiles (López et al., 2003).

Although the absence of a chromophore or fluorophore group in oligosaccharide structures limits their direct detection by conventional spectrophotometric detectors (Brokl et al., 2011), some indirect spectrophotometric methods have been developed, which are based on inulin hydrolysis, derivatization of the released fructose and glucose with various reagents such as dinitrosalicylic acid (DNS) and p-hydroxybenzoic acid hydrazide (PHBAH), phenol and anthrone, and final measurement of the reaction products. Many reports on enzymatic hydrolysis and detection by different analytical methods have been published (Arrizon et al., 2010; Lingyun et al., 2007; Paseephol et al., 2007). The inulin content of different plant materials is finally measured as the difference between total carbohydrate and reducing sugars, which are often preliminarily quantified in their extracts.

To provide only a few examples, an indirect method was proposed (Paseephol et al., 2007) to quantify the fructan content of a concentrated Jerusalem artichoke extract, where total carbohydrate was assayed colorimetrically by the phenol–sulfuric acid method and reducing sugars spectrophotometrically using PHBAH. The same was done by Lingyun et al. (2007) on tubers of the same plant, but using the DNS method for reducing sugar determination. The same way, Arrizon et al. (2010) determined the fructan content of *A. tequilana* using the anthrone and DNS methods to assess its total carbohydrate and free reducing sugar contents, respectively. Another indirect spectrophotometric method was developed and validated to determine the inulin content of Jerusalem artichoke, which is based on the oxidation of released fructose by excess periodate and subsequent quantification of the remaining reactant by measuring the absorbance at 350 nm of the triiodide complex formed by the addition of potassium iodide (Saengkanuk et al., 2011).

Nonetheless, attempts with direct, conventional spectrophotometric methods on extracts are also reported in the scientific literature. For example, inulin content of dry and grounded chicory

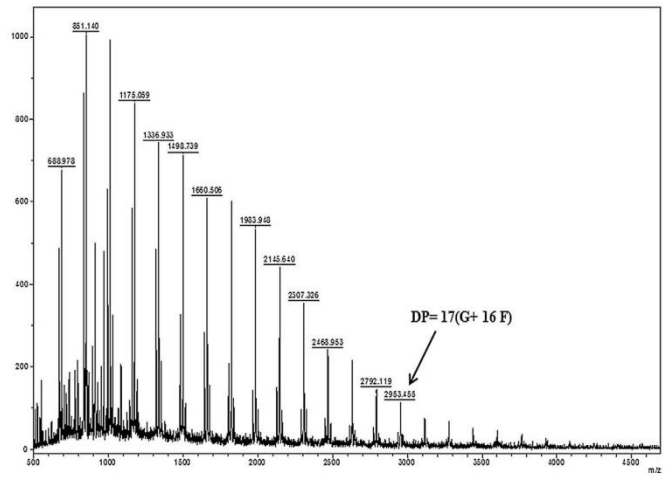

Fig. 5. Positive ion MALDI-TOF-MS spectrum of inulin HP® recorded with 2,5-dihydroxybenzoic acid as the matrix.

root was determined by a simple method based on the fact that vanillin, in presence of concentrated sulfuric acid, forms with inulin a deep red color complex that yields a characteristic absorption (Dobre, Stroescu, Stoica, Draghici, & Antohe, 2008), and the same was done for *A. sisalana* juice using HCl and resorcinol reagent (Sharma & Varshney, 2012).

Inulin oligomers have been analyzed by high-performance liquid chromatography (HPLC) using different detection techniques, however UV/vis detection gave poor results in terms of sensitivity, mainly due to the weak UV-absorbing properties of native carbohydrate derivatives. HPAEC–PAD has been applied to the analysis of inulin samples along with refractive index (RI) detection methods. However, these methods require special chromatographic arrangements, because PAD and RI detections are sensitive to eluents and the applied gradient elution (Saengthongpinit & Saijaanantakul, 2005).

A reliable HPLC method was set up using a carbohydrate column and evaporative light scattering detection (HPLC-ELSD) for proper qualitative and quantitative analysis of inulin oligomers, in order to determine the composition of thermally treated samples of chicory, Jerusalem artichoke and food products, as well as to lay the basis of assessment of the prebiotic effect (Kiss & Forgo, 2011). Thermal treatments were carried out at two high temperatures (180 and 210 °C) so as to characterize diversely inulin thermal degradation, and the released oligomers were identified by HPLC–MS and mass spectrometric detection using atmospheric pressure chemical ionization in positive ion mode.

These spectrophotometric techniques can also be used in combination to get more reliable information. For instance, Arrizon et al. (2010) investigated the effect of *A. tequilana* age on its fructan content and structure for economical purposes by HPLC, HPAEC-PAD, MALDI-TOF-MS and GC–MS, with HPLC allowing for separation of polymerized and non-polymerized sugars, HPAEC-PAD for determination of distribution of fructans with approximate DP ranging from 3 to 6 and longer fructans compared with Dahlia tubers inulin, MALDI-TOF-MS for detection of differences in oligosaccharide distribution induced by plant age, and GC profile for linkages analysis.

Despite of the increase in the inulin yield from Jerusalem artichoke tubers by direct sonication, by means of HPAEC-PAD it was verified the reduction of inulin DP and of some low-molecular-weight compounds (Lingyun et al., 2007). By the same technique, five FOS were identified in table grapes, namely 1-kestose, neokestose, nystose, nystose and kestopentaose, whose patters were shown to be differently influenced by storage and CO_2 treatment (Blanch et al., 2011). Researchers isolated inulin-type oligosaccharides with different DP from the traditional Chinese medicine plant *Morina officinalis* by size-exclusion chromatography, and determined their purity by HPLC–ELSD equipped with cyclodextrin-bond column (Yang et al., 2011). The elution flow rate was shown to be a crucial factor influencing the separation of oligosaccharides, in that an increase from 0.2 to 0.4 mL/min narrowed the fraction array from DP < 10 to DP < 6.

Magnetic nucler resonance (NMR) has recently been used to determine DP of fructans in aqueous extracts of roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni that belongs to the family of Asteraceae (Oliveira et al., 2011). Whereas the ^{1}H NMR spectrum of FOS from roots showed the presence of one signal in the anomeric region, all resonances present in the ^{13}C NMR spectrum of leaves could be assigned to FOS. Moreover, the C-2 resonance of fructofuranose indicated ketose residues, while chemical shifts of ^{1}H and ^{13}C of the main residues in the 2D NMR spectra were fully assigned, based on literature data, to D-fructofuranosyl units with β-configuration. The same technique has been successful in combination with MS and literature data to confirm the structures of inulin-type oligosaccharides from *M. officinalis* (Yang et al., 2011). Analyses showed that glucose was present in both α-glycopyranose and β-glycopyranose forms, while fructose as α-fructofuranose, β-fructofuranose and β-fructopyranose. ^{1}H NMR and ^{13}C NMR spectra of oligosaccharides with 2 < DP < 10 were similar to those of sucrose and revealed they were inulin-type oligosaccharides made up of the same α-glycopyranose and β-fructofuranose residues, differing in the number of fructofuranose residues. Finally, NMR has recently been proposed in a new method to monitor inulin hydrolysis in real time (Barclay et al., 2012) based on the progressive variation of its DP.

9. Biotechnological applications of inulin oligosaccharides

Inulin sources have recently received increasing interest as they are a renewable raw material for production of bioethanol, fructose syrup, single-cell protein (substitute for protein-rich foods) and single cell oil (transesterification of triacylglycerols from renewable biomass), obtainment of FOS and other useful products (Chi et al., 2011). Functional foods have been developed by addition of inulin to increase their dietary fiber content (Komatsu et al., 2013). The effect of inulin, as one of the most attracting prebiotics in functional food preparation, on the fermentation patterns either

of pure cultures of *Streptococcus thermophilus* and *Bifidobacterium lactis* or in co-culture was investigated by Oliveira, Perego, Oliveira, and Converti (2012). These authors showed that the addition of inulin significantly reduced the time to complete the fermentation, enhanced biomass growth and increased the levels of lactic and acetic acids, diacetyl and acetoin in both pure cultures and co-cultures.

Inulin is a promising source for oligosaccharide production as a result of the action of inulinases. According to their mode of action, such enzymes can be classified into endoinulinases (2,1-β-D-fructan fructanohydrolases; EC 3.2.1.7), which specifically hydrolyze bonds between fructose units located away from the ends of inulin network releasing fructooligosaccharides, and exoinulinases (β-D-fructohydrolases; EC 3.2.1.80), which split terminal fructose units in sucrose, raffinose and inulin releasing fructose. Strong endoinulinases act on inulin in the absence of exoinulinase or invertase activities. Inulin can be easily hydrolyzed to fructose by exoinulinase that progressively removes the terminal fructose units from the non-reducing end of inulin in only one step. Taking into account that exoinulinases are encoded by only one gene in most of microorganisms, it is evident the interest of food industry for this biopolymer as raw material for fructose production (Pessoa & Vitolo, 1998, 1999). Since fructose is significantly sweeter than table sugar (sucrose) and glucose, the possibility to obtain high-fructose-corn-syrup (HFCS) from inulin-containing materials rather than by isomerization of corn starch hydrolyzates (Sirisansaneeyakul, Worawuthiyanan, Vanichsriratana, Srinophakun, & Chisti, 2007) would be an interesting challenge for the food industry.

In the last decades a large number of fungal, yeast and bacterial strains were used for inulinase production, among which *Kluyveromyces marxianus* (Yépez Silva-Santisteban, Converti, & Maugeri Filho, 2006; Yépez Silva-Santisteban, Converti, & Maugeri Filho, 2009) and *Aspergillus niger* (Paixão, Teixeira, Silva, Teixeira, & Alves, 2013) were reported as the most common and preferred sources. Inulinases catalytic properties are greatly influenced by the molecular weight, optimum pH, optimum temperature and stability, which in turn depend especially upon their provenience (Neagu & Bahrim, 2011; Singh & Gill, 2006). The simultaneous occurrence of inulinase (I) and invertase (S) activities in many cases could be explained by an enzymatic complex, which would mainly function as inulinase or invertase when $I/S > 10^{-2}$ or $<10^{-4}$, respectively (Sharma & Varshney, 2012).

Some studies have been focused on the development of enzymatic processes to reduce energy consumption and increase sugar recovery from *A. tequilana* fructans (ATF). In a study comparing the ATF hydrolysis by fructozyme (a mixture of endo and exoinulinases from *A. niger*) with that of inulin, it was found a lower enzyme specificity for ATF (V_{max} = 32.1 U/mL, k_M = 27 mmol/L) than for inulin (V_{max} = 34.1 U/mL, k_M = 7.2 mmol/L), which was ascribed to the complex ATF structure with high branching degree as well as to a different ratio of β-(2→1) and β-(2→6) linkages (Muñoz-Gutiérrez, Rodríguez-Alegría, & Munguía, 2009).

Direct fermentation of inulin extracts into ethanol has been investigated using several inulinase-producing yeasts mainly belonging to the *Kluyveromyces* and *Saccharomyces* genera. In another interesting two-step approach, the inulin extract was first hydrolyzed by bacteria or fungi and then fermented to ethanol (Bonciu, Tabacaru, & Bahrim, 2010). To this purpose, yeast strains from different sources were isolated and characterized for their ability to growth in medium having fructose as a carbon source and to ferment inulin hydrolyzates.

Another promising application of inulin is the production of difructose anhydrides (DFAs,) cyclic disaccharides consisting of two fructose units linked at their reducing carbons with potential function as food additives (Saito & Tomita, 2000). Two kinds of DFAs have been produced by inulin degradation by microbial enzymes, namely DFA I (α-D-fructofuranose-β-D-fructofuranose-1,2′:2,1′-dianhydride) by DFA I-forming inulin fructotransferase (EC 4.2.2.17) and DFA III (α-D-fructofuranose-β-D-fructofuranose-1,2′:2,3′-dianhydride) by DFA III-forming inulin fructotransferase (EC 4.2.2.18). DFA III was shown to have half the sweetness but only 1/15 the calories of sucrose, thus enhancing the absorption of calcium and other minerals in the small and large intestines of rats and in humans (Zhao et al., 2011).

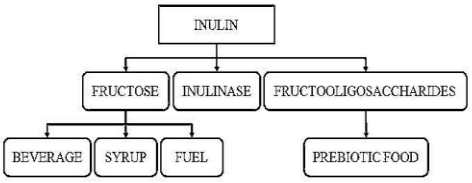

Fig. 6. Possible biotechnological applications of the inulin obtained from plants.

The flowsheet in Fig. 6 illustrates some biotechnological applications of inulin from plants.

10. Conclusions

In this review it was shown that the aspects of the biosynthetic origin of inulin type-fructans and their chemical structures are already well known. The process of hot-water extraction, followed by precipitation, is equally widespread in the literature; however, due to the large number of variables involved in this preliminary processing step, a lot of optimization studies based on statistical tools were carried to find the best temperature and solvent/raw material ratio as well as the most effective recovery technique. One of the bottlenecks in terms of cost and standardization is the purification of these products. The analyzed studies devoted to their purification describe methods consisting of a variety of different operations, but in general anionic exchange resins are the most widely and successfully employed. Analytical techniques have their complexity justified by the fact that the DP of oligosaccharides may largely vary depending on the plant species or even on the phase of its reproductive cycle, thus requiring sensitive techniques to highlight this aspect clearly. Regarding the broad applications of inulin type-fructans, to which a specific chapter of this review was addressed, there is a clear need for additional efforts to disseminate low-cost methods of extraction, purification and analysis reproducible on an industrial scale.

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