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Aplicação de tecnologias limpas para o aproveitamento integral das sementes de urucum (*Bixa orellana L.*) de reduzido teor lipídico

Clean technologies application for integral use of semi-defatted annatto seeds (*Bixa orellana* L.)

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Abandonar tudo por medo
Não transformar sonhos em realidade
Ter medo da vida e de seus compromissos
Não viver cada dia como se fosse um último suspiro!
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RESUMO

As sementes de urucum (*Bixa orellana* L.) são amplamente exploradas a nível industrial para obtenção do pigmento que possui aplicações em produtos alimentícios, cosméticos, têxteis, etc. Atualmente em escala industrial são utilizadas técnicas rudimentares e ineficientes para o aproveitamento sustentável das sementes de urucum. A extração com fluido supercrítico usando CO_2 (SFE-CO_2) como solvente foi usada para separar a fração lipídica rica em tocotrienóis das sementes de urucum, processo do qual foi obtida a matéria-prima desta pesquisa. Nesta tese foi estudada a influência de processos que envolvem tecnologias limpas na composição desta matéria-prima residuária, que apresenta potencial para usos futuros.

Inicialmente foi realizada a hidrólise ácida sobre as sementes de urucum *in natura* e sobre os resíduos dos processos de extração (SFE-CO_2 e Soxhlet) das sementes, junto com outras matérias primas (ginseng brasileiro e fibra de palma prensada) com o objetivo de se estudar a influência desses processos na estrutura dessas matérias primas para a produção de açúcares. A comparação morfológica dos materiais vegetais foi observada mediante a técnica de microscopia eletrônica; por meio da hidrólise foi verificada a influência da concentração de ácido na concentração resultante de açúcares obtidos em função do tempo de reação. As técnicas de extração servem como um pré-tratamento para a modificação da estrutura vegetal por favorecer a exposição de substratos para processos de conversão da biomassa lignocelulósica.

As sementes de urucum de reduzido teor lipídico, resíduo da SFE-CO_2, foram moídas e separadas em função do diâmetro de partícula (d_p) para estudos destinados a quantificação de bixina e dos polímeros presentes. Da fração das partículas de menor tamanho ($d_p \leq 300$ µm) foi verificada uma concentração relevante de bixina, fazendo com que estas partículas fossem direcionadas para a obtenção de extratos com maior pureza deste pigmento a partir da extração com etanol a baixa pressão. Dos resultados obtidos com as partículas maiores ($d_p > 300$ µm) foi verificado que essas partículas mantêm um conteúdo não relevante de bixina, mas sim uma concentração importante de amido, proteína e material lignocelulósico; fazendo com que estas partículas fossem destinadas a tratamento hidrotérmico modificando a estrutura da matéria-prima visando aplicações na indústria.

A aplicação das tecnologias limpas utilizadas neste trabalho conduz ao total aproveitamento das sementes de urucum residuária da extração com CO_2 supercrítico, por

contribuir com a obtenção de novos produtos que podem ser incorporados para aplicações futuras.

A técnica de extração com líquidos pressurizados foi estudada paralelamente para a obtenção de antocianinas usando como matéria-prima a polpa congelada de açaí. Extratos com maior concentração de antocianinas foram atribuídos à mistura de etanol e água, e a presença de ácido cítrico. Foi verificado que a temperatura e a pressão estudadas não exerceram influência relevante na concentração de antocianinas. Os resultados obtidos sugerem a necessidade de trabalhos futuros aumentando a proporção de água no solvente.

Palavras-chave: Bixina; Extração por solventes; Tratamento hidrotérmico; Hidrólise ácida; Resíduos agroindustriais.

ABSTRACT

Annatto seeds (*Bixa orellana* L.) are widely used industrially for obtaining a pigment with application in food, cosmetics and textiles, among others. Currently in an industrial scale are used rudimentary and inefficient techniques that do not allow the sustainable use of annatto seeds. The lipid fraction rich in tocotrienols from annatto seeds was extracted by supercritical fluid extraction technique using CO_2 (SFE-CO_2) as a solvent, the solid residue from this extraction was the raw material in this study. This thesis studied the influence in the composition of this residual material of processes that involves the use of clean technologies in order to create a potential use for this material.

Initially the acid hydrolysis of annatto seeds and their residue from extraction processes (SFE-CO_2 and Soxhlet) was performed, together with other raw materials (Brazilian ginseng and palm pressed fiber) for the purpose of studying the influence of extraction processes in the structure of these raw materials for the production of sugars. Morphological comparison of plant materials was observed by electron microscopy technique; the acid hydrolysis was evaluated by the influence of acid concentration in the production of sugars, obtained as a function of reaction time. The extraction technique serves as a pretreatment to modify the plant structure, enabling the exposure of substrate for lignocellulosic conversion.

The semi-defatted annatto seeds, residue from SFE-CO_2, were milled and separated according to the diameter of particle (d_p) for studies to quantify of bixin and polymers present. On the fraction of small particles size ($d_p \leq 300$ μm) was observed a significant concentration of bixin. These particles were used to obtain extracts with higher purity from this pigment using the low pressure solvent extraction (LPSE) with ethanol. On the results obtained with larger particles ($d_p > 300$ μm), it was observed that these particles have not a relevant bixin content, but a significant concentration of starch, protein and lignocellulosic material; destining these particles to an hydrothermal treatment designed to modify the vegetal structure of raw material aiming applications in industry.

The application of clean technologies used in this work leads to the total use of semi-defatted annatto seeds, residue from supercritical extraction with CO_2, for contributing to obtain new products that can be incorporated for future applications.

The pressurized liquid extraction (PLE) technique was studied in parallel for obtaining anthocyanins extracts from frozen pulp of acai. Extracts with higher concentration of anthocyanins were produced using a mixture of ethanol and water, without use of citric acid. Temperature and pressure did not exert a significant influence in the concentration of

anthocyanins. The results suggest the need of further studies increasing the proportion of water in the solvent.

Keywords: Bixin; Solvent extraction; Hydrothermal treatment; Acid hydrolysis; Agroindustrial residues.

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- CAPÍTULO 1 –

Introdução geral e objetivos

Neste primeiro capítulo estão abordados os principais tópicos desta tese que propõe o aproveitamento total das sementes de urucum (*Bixa orellana* L.) com reduzido teor lipídico. Formulou-se uma revisão do estado da arte das tecnologias usadas para tratamento das sementes de urucum, em que a extração do pigmento bixina (extração com solvente a baixa pressão) e a modificação física da estrutura vegetal (tratamento hidrotérmico) estão inclusas. As tecnologias propostas são tecnologias limpas e podem contribuir para a valoração deste resíduo agroindustrial mediante seu total aproveitamento.

CAPÍTULO 1 - INTRODUÇÃO GERAL E OBJETIVOS

1. INTRODUÇÃO

Este trabalho utiliza processos que empregam tecnologias limpas para o aproveitamento integral das sementes de urucum (*Bixa orellana* L.) com reduzido teor lipídico. A matéria-prima utilizada são as sementes de urucum residuárias do processo de extração usando CO_2 supercrítico (SFE-CO_2 - Supercritical Fluid Extraction using CO_2), processo que foi usado com a finalidade de extrair a fração lipídica rica em tocotrienóis. As tecnologias aplicadas neste estudo são: extração com solvente a baixa pressão (LPSE – Low Pressure Solvent Extraction) e tratamento hidrotérmico usando água pressurizada e CO_2 supercrítico.

A importância da execução deste trabalho planteou-se no intuito de englobar a cadeia produtiva do aproveitamento das sementes de urucum. A extração SFE-CO_2 foi aplicada nas sementes para se obter um óleo rico em substâncias antioxidantes; o resíduo desta extração é um material fonte de sustâncias de interesse para a indústria (pigmento bixina, carboidratos, proteínas e material lignocelulósico) e livre de contaminantes nocivos a saúde humana. Esta pesquisa foi originada em função da necessidade de se desenvolver uma continuidade da aplicação de tecnologias limpas para aproveitamento integral das sementes de urucum, a fim de propor o uso de técnicas alternativas que conduzam ao máximo aproveitamento desta matéria-prima seguindo uma política de sustentabilidade dos processos de extração sem geração de resíduos.

Esta tese é uma continuidade dos estudos realizados no LAboratório de tecnologia Supercrítica: Extração, Fracionamento e Identificação de extratos vegetais (LASEFI) aplicados na semente de urucum. Mediante um primeiro estudo foi estabelecido o processo de extração da fração lipídica das sementes de urucum inteiras usando CO_2 em estado

supercrítico (Albuquerque & Meireles, 2012), cuja matéria-prima residuária foi utilizada nesta tese. Sobre as sementes residuárias da SFE-CO_2 foi realizado um estudo paralelo que focou em extrair a maior quantidade de bixina contida nas sementes usando técnicas de extração com líquidos pressurizados (PLE – Pressurized liquid extraction), líquidos a baixa pressão (LPSE) e assistida por ultrassom (Rodrigues et al., 2014), cujos resultados não foram conclusivos para a extração do pigmento bixina. Em função disto houve a necessidade de se aperfeiçoar o processo de despigmentação da semente residuária.

Os objetivos propostos no projeto inicial englobavam a produção de açúcares fermentescíveis, através da hidrólise do material vegetal desengordurado por SFE-CO_2 e despigmentado a partir do uso de tecnologias de extração. Considerando que o processo de hidrólise envolveria altas temperaturas e pressões, a ponto de degradar o pigmento bixina e outros biocompostos presentes no material vegetal, e que as sementes a serem usadas ainda não estavam despigmentadas, foram feitos estudos prévios para retirar o pigmento a fim de obter um material do qual fosse possível a conversão em açúcares através do tratamento hidrotérmico que se trata de hidrólise em condições leves.

Com base nos trabalhos anteriores aplicados à sementes de urucum e na procura de acondicionar a matéria-prima residuária aos processos de despigmentação e ao aproveitamento dos polímeros de carboidratos presentes no material foi implementado um processo de moagem para verificar sua influência no rendimento em bixina no extrato usando a técnica de extração (LPSE), solvente e temperatura de extração recomendado por Rodrigues et al. (2014).

As sementes foram moídas e separadas em função do diâmetro de partícula (d_p) para estudos destinados à quantificação de bixina e dos polímeros presentes. A concentração de bixina quantificada em cada grupo estudado foi a principal caraterística levada em

consideração para a distribuição do material e os processos para os quais a matéria-prima foi destinada.

Da fração das partículas de menor tamanho ($d_p \leq 300\mu m$) foi verificada uma concentração de bixina maior do que nas sementes inteiras, fazendo com que este grupo de partículas fosse direcionado para um processo de extração para a obtenção de extratos com maior pureza deste pigmento. Foi usado etanol na extração com solvente a baixa pressão (pressão atmosférica) em que as partículas submergidas no solvente foram agitadas com auxílio de Agitador-Shaker. Para definição das condições experimentais foi desenvolvido um planejamento fatorial de 27 ensaios, e os parâmetros estudados foram: temperatura (40, 50 e 60 °C), relação solvente/matéria-prima (10, 15 e 20; g/g), e tempo de extração (5, 15 e 30 min). Os resultados obtidos derivaram num seguinte planejamento, usando temperatura (40 e 50 °C), relação solvente/matéria-prima (10 e 20), e tempo de extração (15, 30, 45 e 60 min). Foram também usadas neste trabalho sementes de urucum residuárias a partir de um novo lote e com maior conteúdo de bixina procedente de um estudo realizado por Silva et al. (2015).

Dos resultados obtidos com as partículas maiores ($d_p > 300\mu m$) foi verificado que o conteúdo de bixina nessas partículas não foi relevante quando comparado com a concentração de pigmento nas sementes inteiras ou nas partículas finas; mas os teores relevantes de amido, proteína e material lignocelulósico levaram com que estas partículas fossem destinadas ao tratamento hidrotérmico a fim de modificar a estrutura dessa matéria-prima como forma de se obter um produto promissor para aplicações na indústria.

O tratamento hidrotérmico foi proposto nas condições que propiciassem a modificação física dos polímeros no material vegetal e simultaneamente a produção de açúcares de menor grau de polimerização resultantes da hidrólise leve do polímero. Para cumprir este objetivo foi realizado o planejamento fatorial de 45 ensaios experimentais. Os parâmetros do processo considerados foram: temperatura (60, 70 e 80 °C), pressão (150, 200,

250, 300 e 350 bar) e a proporção água/CO_2 (20/80, 30/70 e 40/60, v/v). Durante a realização dos ensaios experimentais, ocorreu mau funcionamento do equipamento causado provavelmente pelas caraterísticas dos componentes da matéria-prima, que influenciou o entupimento das tubulações causado pelo inchamento dos grânulos de amido, o que resultou na suspensão do uso do equipamento após realização de apenas 11 ensaios. Os resultados e discussão do processo basearam-se nos resultados dos ensaios experimentais realizados.

1.1. Urucum (*Bixa orellana* L.)

O urucum (*Bixa orellana* L.) é um arbusto nativo de zonas tropicais da América do Sul, usado como cosmético e ingrediente na preparação de pratos tradicionais desde a época pré-colombiana. *Bixa Orellana* L. cultiva-se também na América Central, África e o sul da Ásia (Smith & Wallin, 2006). O pigmento procedente do urucum apresenta vasta aplicação nas indústrias de alimentos, química, cosmética e farmacêutica (Chisté et al., 2011; Das Saha & Sinha, 2012). Além da propriedade de atribuir cor, atualmente os pigmentos do urucum são muito apreciados pelos benefícios à saúde em função da sua atividade biológica (Giorgi et al., 2013).

A semente de urucum apresenta cerca de 2,0 a 4,8% de lipídios, 1,0 a 6,3% de pigmento, 9,6 a 13,3% de umidade, 12,1 a 17% de proteínas, 5,4 a 6,9% de cinzas e aproximadamente 50% de carboidratos totais (entre amido e material lignocelulósico) (Albuquerque & Meireles, 2012; Silva et al., 2008). A semente de urucum contém carotenóides e compostos fenólicos (Cardarelli et al., 2008), saponinas e taninos (Albuquerque & Meireles, 2011; Vilar et al., 2014).

O principal componente corante no urucum é o apocarotenoide bixina, um éster monometil dicarboxílico (9’-cis-6,6’-diapocarotenoide-6,6’-dioato metil hidrogênio), derivado do ácido dicarboxílico 9’-cis-norbixina (Scotter et al., 1998). A bixina representa mais que 80% do conteúdo das sustâncias corantes nas sementes do urucum e apresenta-se

normalmente na forma cis-. Entre os componentes corantes, encontra-se em menores proporções a trans-bixina, cis-norbixina e trans-norbixina (Preston & Rickard, 1980). A coloração produzida pelos pigmentos dá-se na faixa entre amarelo–laranja-vermelho, dependendo da concentração das sustâncias corantes acima mencionadas (Castro et al., 2011).

O conteúdo total do pigmento presente nas sementes varia de acordo com a variedade, cultura e tecnologias usadas, anteriores e posteriores à colheita (Albuquerque & Meireles, 2011; J. Smith & Wallin, 2006). Os pigmentos do urucum podem ser separados das sementes mediante várias técnicas, incluindo a imersão das sementes em óleo vegetal quente, soluções diluídas de sustâncias alcalinas e o uso de solventes (Scotter et al., 1998; J. Smith & Wallin, 2006; Vilar et al., 2014). O processo industrial de extração do pigmento das sementes de urucum comumente envolve o tratamento com solução alcalina, que se baseia em submergir as sementes de urucum em soluções de hidróxido de sódio ou de hidróxido de potássio. Mediante este tipo de extração, a bixina presente nas sementes de urucum in natura reage com a solução básica produzindo norbixina. O pigmento norbixina apresenta coloração mais amarelada e maior solubilidade em água (Scotter et al., 1998). Ainda que quimicamente a bixina e norbixina sejam muito similares, existem diferenças nas suas propriedades de solubilidade, estequiometria e estabilidade (Scotter, 2009).

A SFE-CO_2 modifica ligeiramente a composição das sementes por retirar grande parte da fração lipídica e preserva a maior parte do pigmento na semente residuária (Albuquerque & Meireles, 2012). Os polissacarídeos encontrados nas sementes de urucum são amido, celulose, hemicelulose e lignina (Vilar et al., 2014). Todos eles são considerados fontes de energia renovável, e atualmente há muitas pesquisas que abordam o aproveitamento sustentável dessas fontes de energia para produção de combustíveis, açúcares fermentescíveis, oligossacarídeos, etc. (Moreschi et al., 2006; Rogalinski et al., 2008a; Romero et al., 2010; Zhu et al., 2011). As tecnologias que normalmente são aplicadas para a conversão do material

vegetal envolvem inúmeros procedimentos, entre eles a hidrólise ácida, explosões de vapor e hidrólise enzimática (Cardenas-Toro et al., 2014a; Goldemberg, 2009). Atualmente as tecnologias hidrotérmicas, especialmente o uso de água subcrítica e supercrítica, têm mostrado excelentes resultados para a conversão do material vegetal (Brunner, 2009; Kus, 2012; Prado et al., 2012; Salak et al., 2013). A conversão por um processo de hidrólise envolvendo água, com ou sem a adição de CO_2 como catalizador da reação, apresenta como principal vantagem o uso de solventes não tóxicos e menores tempos de conversão quando comparados com métodos convencionais (Rogalinski et al., 2008a; van Walsum & Shi, 2004). Além disso, a seletividade do meio para a degradação de determinadas substâncias é efetuada por meio da manipulação dos parâmetros de temperatura e pressão (Brunner, 2009). Os produtos derivados da hidrólise são monossacarídeos, oligossacarídeos, ácidos orgânicos e outros produtos de interesse comercial para a indústria de alimentos, química e energética (Lu et al., 2009; Sheldon, 2014).

Nesta tese a semente de urucum de reduzido teor lipídico procedente do processo SFE-CO_2 foi selecionada como matéria-prima na qual foram aplicadas outras tecnologias limpas visando à continuação do seu uso industrial. As tecnologias usadas nesta pesquisa são abordadas nos seguintes itens e foram aplicadas sobre a matéria-prima procurando o melhor aproveitamento das caraterísticas de cada um dos componentes de interesse presentes na semente residuária a fim de gerar produtos de alto valor agregado.

1.2. Extração com solvente a baixa pressão (LPSE) - Método de extração do pigmento bixina.

O processo de extração é uma operação unitária com o objetivo principal de separação de compostos de interesse a partir das suas matrizes ou de misturas. Esta operação pode ser conduzida por métodos físicos, químicos ou mecânicos. Diferentes técnicas de extração representam alternativa importante para a recuperação de compostos provenientes de

plantas e outros materiais vegetais (Zabot et al., 2014). A maioria das técnicas de extração se baseia na capacidade do solvente e da aplicação de calor, incrementando a transferência de massa e a solubilidade dos compostos (Azmir et al., 2013). O objetivo dos processos de extração em matrizes vegetais é separar as substâncias de interesse das estruturas celulares nas quais elas se encontram mediante a ruptura dos tecidos vegetais ou mediante processo de difusão (Escribano-Bailón et al., 2002).

A técnica de extração que envolve o uso de solventes é considerada extração indireta. A matriz vegetal apresenta microestrutura complexa, formada por células, espaços intracelulares, capilares e poros. A extração pode ser influenciada pela estrutura molecular do soluto, tamanho, localização e sua ligação com outros componentes (Veggi et al., 2011).

A técnica de extração com solvente à baixa pressão (LPSE) é uma das técnicas mais utilizadas na indústria química, também chamada lixiviação, decocção ou eluição (Wang & Weller, 2006). O processo de extração sólido-líquido é conduzido à pressão atmosférica, e é caracterizado pelo contato entre o solvente e o material a ser extraído, ou soluto; depois da extração o solvente é removido e o extrato é concentrado (Leal et al., 2010; Rodrigues et al., 2014; Santos et al., 2012).

O rendimento da extração de compostos em matrizes vegetais pode ser influenciado pelas condições em que a operação é realizada. Executar um processo nas condições de operação adequadas depende da seleção dos parâmetros usados ao estabelecer as condições de temperatura, ação mecânica (agitação) e tipo de solvente empregado (Meireles et al., 2008). Os solventes comumente usados para extração de bixina com fins de análise são acetona, etanol, acetato de etila e hexano (Scotter, 2009). De acordo a Resolução CNNPA n° 44, os solventes e veículos autorizados para serem utilizados na elaboração e processamento de pigmentos para aplicações alimentares, restringem o número de solventes, permitindo para estes fins o uso de água, açúcares, etanol, amidos, glicerol, óleos e gorduras comestíveis entre

outros (BRASIL, 1978). Em vista disto e como sugerido por Rodrigues et al. (2014), o solvente selecionado para a extração do pigmento bixina neste estudo foi o etanol.

Aperfeiçoar o processo de extração de bixina é de fundamental importância, com o objetivo de não só aumentar o rendimento da operação como também minimizar a contaminação com subprodutos que afetem composição, capacidade de proporcionar cor e estabilidade do pigmento carotenoide (Scotter, 2009).

No decorrer deste trabalho, o processo LPSE foi aplicado sobre as sementes de urucum residuárias, que foram acondicionadas no intuito de aperfeiçoar o processo no quesito de aumentar a pureza do pigmento nos extratos. Neste processo de acondicionamento da matéria-prima foi feita a moagem das sementes e classificação pelo tamanho de partícula em finos e grossos. O grupo de partículas finas manteve-se como matéria-prima do processo de extração de bixina, logo o grupo de partículas grossas destinou-se à aplicação da tecnologia hidrotérmica visando o melhor aproveitamento dos demais componentes da semente (amido, proteínas e material lignocelulósico).

1.3. Tratamento hidrotérmico da fração grossa das sementes de urucum: hidrólise do material vegetal

São muitas as pesquisas que envolvem o uso de água como meio de reação para converter o material vegetal em novos materiais e/ou energia (Aqsha et al., 2012; Brunner, 2009; Rogalinski et al., 2005). Este tratamento apresenta excelentes resultados por se tratar de um processo rápido, seletivo e manipulável para com as variáveis: temperatura, pressão, tempo de processo e proporção solvente/matéria-prima (Kus, 2012; Zhao et al., 2011). Com a finalidade de acidificar o meio aquoso e acelerar o processo de hidrólise é possível adicionar dióxido de carbono (CO_2) ao meio. O CO_2 reage com a água formando ácido carbônico, o que atua como catalisador do processo ao diminuir o pH, propiciando a reação de hidrólise (Rogalinski et al., 2008a; Rogalinski et al., 2008b).

Os produtos de interesse a partir do processo de hidrólise dos polissacarídeos são pentoses e hexoses de vários graus de polimerização (Prado et al., 2014; Schacht et al., 2008). A maioria dos estudos de hidrólise foram realizados usando celulose e amido purificados. A aplicação do processo de hidrólise em matérias-primas mais complexas apresenta resultados diferentes aos das sustâncias puras. A diferença é causada pelo fato de que a hidrólise depende dos tipos de monossacarídeos presentes e das ligações entre eles (Oomori et al., 2004). Os polissacarídeos de reserva como o amido, são facilmente hidrolisáveis quando comparados com a hemicelulose, celulose e lignina que nos vegetais apresentam função estrutural (Iranmahboob et al., 2002).

Na aplicação do tratamento hidrotérmico sobre a fração de partículas grossas das sementes de urucum residuárias, foi planejado aplicar temperaturas consideradas brandas, visando à modificação e conversão parcial da fração amilósica presente na matriz vegetal. Por causa disso, a reação de hidrólise (consequência do tratamento hidrotérmico) não se conduz totalmente a fim de possibilitar a recuperação do leito vegetal para ser destinado a seu uso na indústria.

1.4. Tratamento hidrotérmico da fração grossa das sementes de urucum: Modificação física dos polímeros

A fração grossa da semente de urucum apresenta vários tipos de polímeros, entre proteínas, amido e material lignocelulósico (Albuquerque & Meireles, 2012; G. F. Silva et al., 2008). A modificação dos polímeros focou na modificação do amido presente nesta matéria prima, sem ser isolado; por isto nos seguintes capítulos a fração grossa das sementes de urucum foi chamada de farinha de urucum.

A necessidade da modificação do amido presente nas farinhas deve-se ao fato de que o polímero raramente é consumido na sua forma nativa. Os amidos nativos geralmente apresentam solubilidade limitada em água, o que restringe suas aplicações industriais (Singh

et al., 2007; Sweedman et al., 2013). São vários os métodos desenvolvidos para a modificação do amido, outorgando-lhe grande variedade de características e possibilidades de aplicação (Chen et al., 2014; Huang et al., 2010; Kapelko et al., 2013; Martinez et al., 2014; Sujka & Jamroz, 2013; Ulbrich & Floeter, 2014; Ulbrich et al., 2014). Todas as técnicas de modificação tendem a alterar as propriedades físico-químicas e atributos estruturais do amido para elevar seu valor tecnológico tanto para a indústria alimentícia e não alimentícia (Ashogbon & Akintayo, 2014).

A modificação física do amido se produz mediante a alteração das suas propriedades físico-químicas e da ordem cristalina do grânulo nativo que resulta no aumento da solubilidade em água, reduzindo o tamanho do grânulo (Ai & Jane, 2015; Zavareze & Dias, 2011). Os métodos físicos incluem diferentes combinações de temperatura, umidade, pressão, cisalhamento e irradiação (Lv et al., 2015; Majzoobi et al., 2011). A modificação física do grânulo de amido é simples, barata e segura quando comparada a outros métodos. Esta técnica não implica o uso de agentes químicos ou biológicos que possam ser prejudiciais à saúde humana (Ashogbon & Akintayo, 2014). O tratamento hidrotérmico é uma metodologia de modificação física, que resulta na modificação das propriedades físico-químicas do amido sem destruir a estrutura do grânulo (Brasoveanu & Nemtanu, 2014; Conde-Petit et al., 2001). Dependendo das caraterísticas intrínsecas da matéria-prima e da aplicação dos parâmetros envolvidos neste tratamento, as modificações do material vegetal estão refletidas nas propriedades de pasta conforme estudado nesta tese.

2. OBJETIVOS

2.1. Objetivo Geral

Aplicar tecnologias limpas que englobem a extração de bixina e a modificação da estrutura vegetal das sementes de urucum com reduzido teor lipídico, visando o total aproveitamento da matéria-prima.

2.2. Objetivos Específicos

- Avaliar o efeito do processo de extração com fluidos supercríticos na estrutura vegetal das sementes de urucum como pré-tratamento para a produção de açúcares mediante processos de hidrólise.
- Determinar as melhores condições no processo de extração com solvente a baixa pressão do pigmento bixina, favorecendo a obtenção de um extrato de maior concentração.
- Estudar as condições de tratamento hidrotérmico que favoreçam a modificação física da estrutura vegetal das sementes de urucum de reduzido teor lipídico, proporcionando melhoria nas suas características e propiciando seu uso e inclusão em outros processos industriais.

3. ESTRUTURA DO TRABALHO

Esta tese esta dividida em nove capítulos, nos quais o desenvolvimento da pesquisa de doutorado é apresentado com o objetivo de propor alternativas para o total aproveitamento das sementes de urucum residuárias da SFE-CO_2.

No **Capítulo 1 - Introdução geral e objetivos** são apresentados de forma geral o tema principal do trabalho realizado, o objetivo geral deste estudo e os objetivos específicos que foram estipulados para direcionar o andamento do trabalho. É apresentado um resumo explicativo sobre a matéria-prima principal deste trabalho, que se trata do uso total da semente de urucum, introduzindo-se os conceitos teóricos das operações empregadas para a recuperação dos pigmentos mediante técnicas de extração e a modificação da matriz vegetal mediante tratamento hidrotérmico.

Considerando que a matéria-prima desta tese foram as sementes de urucum de reduzido teor lipídico obtidas após um processo de extração surgiu a necessidade de estudar o impacto deste processo na estrutura da matriz vegetal e quais as consequências da reutilização deste material residuário em outros processos como o tratamento de hidrólise ácida, tema abordado no seguinte capítulo em que juntamente às sementes de urucum são incluídas outras matérias-primas residuárias para fins comparativos.

No **Capítulo 2 - Artigo científico: "Study of the effect of extraction process as pre-treatments for sugar production from acid hydrolysis"** é apresentado estudo da hidrólise ácida de resíduos de biomassas vegetais - ginseng brasileiro (*Pfaffia glomerata*), fibra de palma (*Elaeis guineensis*) e urucum (*Bixa orellana* L.)- provenientes de tratamentos de extração de óleos e biocompostos. A análise dos efeitos do processo de extração na estrutura das matrizes vegetais estudadas foi observada mediante microscopia eletrônica de varredura (SEM). O processo de hidrólise ácida foi interpretado mediante a quantidade de açúcares produzidos usando a técnica de análise espectrofotométrica segundo a metodologia

Somogyi-Nelson. Os resultados observados mediante SEM mostraram que os tratamentos de extração prévios à hidrólise ácida modificaram a matriz vegetal em relação a sua estrutura e proporção de componentes poliméricos. O processo de hidrólise ácida de cada matriz vegetal mostrou cinéticas de reação diferenciadas para cada matéria-prima e composição.

A necessidade de aprofundar o conhecimento dos componentes da estrutura vegetal das sementes de urucum e o comportamento desta estrutura após a aplicação do processo hidrotérmico fez com que fosse realizada uma pesquisa dessa problemática da qual foi derivado um artigo de revisão. No Capítulo 3 a ênfase foi dada no componente amido por ter vasta aplicabilidade e porque as sementes de urucum são uma potencial fonte não convencional. O artigo de revisão abordou as formas de modificação dos amidos provenientes de várias fontes convencionais e não convencionais.

No **Capítulo 3 - Review: "Physico-chemical properties, modifications and applications of starches from different botanical sources",** é apresentada uma revisão sobre as propriedades físico-químicas do amido (morfologia, tamanho, composição, propriedades reológicas e térmicas, etc.), além dos tratamentos de modificação (físicos e químicos) e a aplicação na indústria alimentícia e não alimentícia.

O **Capítulo 4 - Artigo científico: "Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation"** foi conduzido para o melhor aproveitamento do conteúdo do pigmento bixina presente nas sementes de urucum de reduzido teor lipídico. Para isto, as sementes foram moídas e as partículas separadas em função do seu tamanho. As frações foram agrupadas em: partículas finas ($d_p \leq 300\mu m$), destinadas à extração com solvente obtendo como produto um extrato solúvel em etanol e rico em pigmento bixina; e, partículas grossas ($d_p > 300\mu m$), destinadas ao estudo relatado no capítulo 5 desta tese.

No processo de extração com etanol (LPSE) nas partículas finas das sementes de urucum residuárias foram estudados os parâmetros temperatura, relação solvente/matéria-prima (m/m) e tempo de extração. O pigmento extraído foi quantificado mediante espectrofotometria. Os resultados da extração foram expressos como rendimento global (X_0, g extrato/100 g de matéria-prima), conteúdo de bixina (g bixina/100 g extrato ou g bixina/100 g matéria-prima) e rendimento da extração (porcentagem de extração de bixina relativa à matéria-prima). Foram desenvolvidos dois estudos em sequência. O primeiro estudo envolve a fração fina de sementes de urucum de reduzido teor lipídico resultante do processo de extração de Albuquerque & Meireles (2012) denominado "Lote 1" com porcentagem inicial de bixina e lipídios de 7,5% e 4,4% respectivamente. Este estudo foi conduzido nas temperaturas de 40, 50 e 60 °C, relação solvente/matéria-prima de 10, 15 e 20; e tempo de extração de 5, 15 e 30 min. Os resultados deste primeiro estudo mostraram que nas menores temperaturas e com o maior tempo de extração o extrato resultante continha maior quantidade de bixina.

Desta forma foi conduzido um segundo estudo de extração com solvente (LPSE) incluindo as partículas finas de um segundo lote de matéria-prima denominado "Lote 2" proveniente do processo SFE- CO_2 realizado por Silva et al. (2015), com porcentagem inicial de bixina e lipídios de 16,2% e 7,5% respectivamente. Este estudo foi conduzido nas temperaturas de 40 e 50 °C, relação solvente/matéria-prima de 10 e 20, e tempo de extração de 15, 30, 45 e 60 min. Os resultados mostraram que para ambas as temperaturas estudadas o maior tempo de contato entre o material e o solvente apresentou o maior rendimento global. No entanto os melhores resultados de concentração de bixina no extrato e de rendimento da extração (38% e 49% respectivamente para o Lote 1 e de 39% e 31% respectivamente para o Lote 2) foram obtidos na temperatura de 40 °C, sendo que todos estes resultados foram observados na relação solvente/matéria-prima de 20 e tempo de extração de 60 min.

No **Capítulo 5 - Artigo científico: "Hydrothermal modification treatment for the integral use of semi-defatted annatto seeds"** foi conduzido um tratamento hidrotérmico usando água pressurizada e CO_2 supercrítico sobre as partículas grossas (d_p > 300μm) denominadas farinha de urucum. Neste estudo foi usado o sistema de extração com fluidos em estado supercrítico (WATERS, Milford, USA), e os produtos deste processo são a modificação física da estrutura vegetal da farinha de urucum, que permanece no leito vegetal, e a produção de oligossacarídeos e monossacarídeos por hidrólise dos polímeros presentes na farinha, separados no extrato solúvel.

O processo de tratamento hidrotérmico usando água pressurizada e CO_2 foi conduzido à temperatura de 80°C, diferentes pressões (150, 200, 250, 300 e 350 bar) usando como solvente água/CO_2 em diferentes proporções (20/80, 30/70, 40/60) por um período de 30 min e vazão de 3 mL/min. O produto hidrolisado foi analisado quanto aos conteúdos de açúcares redutores e totais empregando a metodologia de Somogyi-Nelson, na qual os resultados são interpretados como glicose e sacarose, respectivamente. No leito vegetal, após o processo hidrotérmico, os polímeros foram modificados fisicamente. A modificação é quantificada mediante a análise do conteúdo de amido total remanescente e das mudanças qualitativas em suas propriedades de pasta, as quais diferem das propriedades originais.

No capítulo seguinte é estudada a obtenção de extratos obtidos pela aplicação da técnica de extração com líquidos pressurizados (PLE- Pressurized Liquid Extraction) da polpa congelada de açaí. Este trabalho foi realizado paralelamente ao trabalho de Rodrigues et al. (2014) que estudou a extração do pigmento bixina incluindo a mesma técnica. O objetivo deste capítulo foi aprofundar o conhecimento para outras metodologias de análise verificar a influencia dos parâmetros usados neste processo a fim de dar continuidade à aplicação de tecnologias limpas para a obtenção de biocompostos.

No **Capítulo 6 - Artigo científico: "Obtaining anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction"** foram obtidos extratos ricos em antocianinas a partir do processo PLE. Foram avaliados os efeitos das variáveis: temperatura (30 e 60 °C), pressão (20 e 80 bar), tipo de solvente (etanol puro e mistura etanol/água (50:50, v/v) e adição de ácido cítrico (0 e 0.3%, g/g) no solvente para a obtenção de extratos ricos em antocianinas a partir da polpa congelada de açaí (*Euterpe oleracea* Mart.). Os parâmetros com maior efeito sob a extração de antocianinas foram o tipo de solvente e a adição de ácido cítrico; no entanto, a pressão e a temperatura usadas não influenciaram significativamente os resultados do processo. O máximo rendimento global foi 63,5 ± 8,6 (%, b.s.) usando etanol (99,5%) e ácido cítrico (0,3% m/m). O conteúdo máximo de antocianinas foi 6,9 ± 1,4 (mg antocianina/g extrato, b.s.) usando etanol/água (50:50, v/v) sem adição de ácido cítrico.

No **Capítulo 7 – Discussão geral** é apresentada uma análise geral dos capítulos estudados e dos resultados experimentais obtidos ao decorrer desta tese.

No **Capítulo 8 – Conclusões Gerais e Sugestões para Trabalhos Futuros** são apresentadas as principais conclusões obtidas nos capítulos anteriores e as sugestões de pesquisa continuada no assunto.

O **Capítulo 9 - Memória do período de doutorado** inclui uma compilação das atividades desenvolvidas na formação acadêmica e os trabalhos realizados paralelamente à tese de doutorado.

- CAPÍTULO 2 –

Study of an extraction process as the pretreatment step for sugar production from acid hydrolysis

Recentemente a literatura científica tem enfatizado o aproveitamento de resíduos agroindustriais mediante conversão do conteúdo lignocelulósico dessas matérias-primas através da hidrólise para a obtenção de açúcares fermentescíveis. O equipamento para desenvolvimento do estudo da hidrólise com água pressurizada estava em processo de montagem, portanto neste trabalho foi usada a técnica de hidrólise ácida. Neste capítulo foram avaliados os efeitos dos processos de extração na estrutura das matérias-primas ginseng brasileiro, fibra de palma prensada e sementes de urucum como uma forma de pré-tratamento desses materiais residuais para posterior etapa de hidrólise. Foi comprovado que os processos extrativos modificam consideravelmente esses materiais a ponto de aumentar a exposição de substratos para processos de conversão da biomassa lignocelulósica.

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Study of an Extraction Process as the Pretreatment Step for Sugar Production from Acid Hydrolysis

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Abstract This work involves the residues of three plant species used in industry: Brazilian ginseng (*Pfaffia glomerata*), palm (*Elaeis guineensis*) and annatto (*Bixa orellana* L.). The studied plant residues come from oil and biocompounds extraction: Soxhlet extraction (SE), Pressurized liquid extraction (PLE) and Supercritical fluid extraction (SFE). The effects of these extraction processes on the structures of plant matrices were observed using scanning electron microscopy (SEM). Plant residues were subjected to acid hydrolysis. The hydrolysis process was conducted using a 0.5 L reactor at atmospheric pressure and the solvent at boiling temperature. Brazilian ginseng was hydrolyzed in hydrochloric acid solution (0.5, 2.5 and 5.0%, v/v) for 60 min. Palm pressed fiber and annatto were hydrolyzed in sulfuric acid solution (1.5 and 3.0%, v/v) for 90 min. Sugars produced by the hydrolysis were quantified and interpreted as Reducing sugars (RS) (g glucose/100 g raw material) and Total reducing sugars (TRS) (g sucrose/100 g raw material) by a spectrophotometric method. The results observed by SEM showed that the extraction treatments modified the vegetable matrix with respect to its structure and component ratio. The acid hydrolysis process of each vegetable matrix showed different reaction kinetics. The availability and source of the sugar polymers and the acid concentration were variables that affected the hydrolysis reaction.

Keywords Biomass, Hemicellulose, Cellulose, Starch, Acid Hydrolysis, Sugar

1. Introduction

Different extraction techniques are important alternatives for the recovery of compounds from plants and other vegetal materials [1]. Most extraction techniques are based on the abilities of solvents and heat to increase the mass transfer and solubility of compounds [2]. The objective of an extraction process in vegetable matrices is to separate the substances of interest from cellular structures by diffusion or breaking the vegetal tissues [3]. Conventional extraction techniques include Soxhlet extraction (SE), maceration, percolation and hydrodistillation [4]. Unconventional extractions include Microwave assisted extraction (MAE), Ultrasound-assisted extraction (UAE), Pulsed-electric field extraction (PEF), Enzyme-assisted extraction (EAE), Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE), etc. [2].

The SE process was designed primarily for lipid extraction; and, has found application in the extraction of valuable bioactive compounds from various natural sources [5]. The use of SE remains prevalent in industry despite needing a long process time and large amounts of solvent [4]. During SE, continuous contact between the vegetable matrix and the solvent is maintained using intermittent reflux. The solvent containing the dissolved material is drawn via a siphon and is discharged into a distillation apparatus, and this process occurs repeatedly until the complete extraction time [6].

A PLE process uses pressure to keep a solvent in the liquid phase above its normal boiling temperature [7]. The solubility of compounds and mass transfer rate increase at high temperature, lowering the viscosity and surface tension of the solvent, which improves the extraction rate [8]. Nonetheless, for some raw materials PLE is not a recommended technique as demonstrated by Rodrigues et al. [9].

Supercritical fluids are used as the extracting solvent in SFE; carbon dioxide is the most commonly used supercritical solvent. This state of matter is achieved when the solvent is subjected to a temperature and pressure above the critical point where there is no distinction between the liquid and gas phases [10]. Some properties of the supercritical fluid are similar to the gas phase, such as diffusion, viscosity and surface tension, whereas the density and solvation power are comparable to the liquid phase [11]. These properties increase the extraction rate and yield [12].

Extraction processes can be considered as pretreatments before further processing of the vegetal biomass [13, 14]. Extractions require preparatory treatments, such as drying and grinding [15]. Together these processes physically modify the vegetable matrix [16].

Several crops generate a large amount of biomass

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(lignocellulosic and starch). However, not all of this biomass is used by industry. An alternative is to use this biomass as a feedstock for the production of oligosaccharides and monosaccharides by hydrolysis due to the large availability and lower cost of vegetal biomass and the need to reduce waste production [17-19]. These sugar oligomers and monomers can be included in industrial processes for food, chemicals and sustainable energy production [20, 21].

Acid hydrolysis can be performed using concentrated or dilute acid solutions. Dilute acid solutions are especially useful when lignocellulosic biomass has a significant proportion of hemicellulose because the hemicellulosic fraction is more easily hydrolyzed than cellulose [22]. The hydrolysis process with dilute acid solutions facilitates the neutralization process but has a lower yield of glucose from cellulose [23]. Concentrated acid solutions require lower processing temperatures and pressures, and the hydrolysis reaction occurs at a lower process time [22]. Acid hydrolysis using concentrated acid solutions produces high yield of glucose from cellulose. In lignocellulosic hydrolysis from biomass, sulfuric acid is commonly used, although other mineral acids, such as hydrochloric, nitric and phosphoric acid are also used [24]. The major problems in acid hydrolysis processes are the utilization of corrosive and toxic solvents, the need to neutralize the reaction medium after the hydrolysis process and the subsequent solid waste disposal [25].

Ginseng is a common name used for various plants of the Panax genus, particularly Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolium*), and both species are the most commonly used in the pharmaceutical, cosmetic and food industries. Species of the Pfaffia genus, such as *Pfaffia glomerata*, belong to the Amaranthaceae family and are Brazilian substitutes for plants of the Panax genus; these species are popularly called Brazilian ginseng [26-29]. Brazilian ginseng roots (BGR) are used in herbal medicine and as a dietary supplement due to anti-inflammatory and analgesic properties [27], as well as cellular stress and immunological effects [28, 30].

Palm pressed fiber (PPF) is one of the major agroindustrial residues in Brazil. PPF is derived from palm oil production. Palm oil is an edible vegetable oil derived from the fruits of palm trees, primarily the African palm tree (*Elaeis guineensis*). This palm is grown in humid tropical climates. Palm oil, also known as dendê oil, has a reddish color because of its high carotenoids content [31]. Carotenoids are substances with potent biological and antioxidant activities and are considered precursors for the synthesis of vitamin A. The residues from palm oil extraction are usually burned as fuel despite containing a significant amount of remaining oil and can be a great source of lignocellulosic material [19, 32].

Annatto (*Bixa orellana* L.) is a shrub native to the tropical regions of South America. Annatto fruits are capsules named Cachopas, and these capsules open and have seeds arranged in series when ripe. On the surface of the seeds is the annatto pigment, ranging in color from orange to red [33]. Annatto has been used in traditional cooking since pre-Columbian times and is also used in cosmetic and pharmaceutical products. The principal pigment in Annatto seeds is apocarotenoid bixin, a derivate from dicarboxylic acid norbixin [34]. Currently, several extraction processes are available in industry to obtain this pigment. Extraction techniques include immersing the seeds in hot vegetal oil, using alkaline solutions and extraction with solvent. After removing coloring substances, the residue fraction represents approximately the entire biomass; however, these annatto seeds are considered waste after pigment extraction [9, 33, 35]. Vegetal residue formed by annatto seeds is extremely rich in starch and lignocellulosic material.

In this study, solid residues of the above-mentioned plant after SE, SFE and PLE processes were subjected to hydrolysis using acid solutions at different concentrations and compared to the original raw material. The aim of this study was to: i) determine whether pretreatment coupled with extraction modified the original lignocellulosic structure and ii) establish an association between pre-treatment and extraction processes that improve the production of oligomers and monomers of sugar by acid hydrolysis.

2. Material and Methods

2.1. Raw Material

The fresh BGR was obtained from CPQBA (Campinas, Brazil). The raw material was conditioned by washing. Next, the samples were dried with air-forced circulation at 40°C for 120 hours until a final moisture content of 8.9%. The washing and drying treatments of BGR enabled a subsequent grinding step using a knife mill (TECNAL, model TE-631, Piracicaba, Brazil). The residual raw material of BGR was obtained after the PLE process using water as the solvent. The conditions of the PLE process were a temperature of 60°C, pressure of 120 bar and Solvent/Feed ratio (S/F) of 4.5 (w/w).

A PPF sample was provided by Agropalma Company (Tâilandia, Brazil). Raw material was ground in a knife mill (TECNAL, model TE-631, Piracicaba, Brazil) for a few seconds. The PPF residue was obtained after a 6-hour SE process using petroleum ether as the solvent.

Annatto was obtained from the Instituto Agronômico de Campinas-IAC (Agronomic Institute of Campinas) (Campinas, Brazil). Defatted annatto seeds (DAS) were obtained by two processes: a 6-hour SE process using petroleum ether as the solvent and a SFE process using supercritical CO_2 as the solvent and parameters established by Albuquerque and Meireles [35]. Untreated annatto seeds and DAS obtained after fat extractions were ground in a knife mill (TECNAL, model TE-631, Piracicaba, Brazil) for a few seconds.

All the raw materials used in this study were kept under refrigeration (-18°C) and protected from light until used in

the acid hydrolysis experiments.

2.2. Characterization of Raw Materials

The moisture contents of vegetal raw materials were analyzed before the extraction processes [36]. On a dry basis, the lipid [37], protein [38] and ash contents were determined [39]. The carbohydrate content, including starch and lignocellulosic material, was estimated by taking the difference of the experimentally determined components.

2.3. Scanning Electron Microscopy (SEM)

Vegetal structures were imaged before and after undergoing SE, PLE and SFE processes using SEM in the Analytical Laboratory of Resources and Calibration (LRAC) at the School of Chemical Engineering (FEQ/UNICAMP, Campinas, Brazil). A metallic sputter coating was made (Sputter Coater POLARON SC7620, VG Microtech, Uckfield, England) with a thickness of 92 Å using gold as the metal and argon as the inert gas. To obtain the micrographs, a scanning electron microscope was coupled with an energy dispersive detector X-ray (Leo 440i, 6070, LEO Electron Microscopy/Oxford, Cambridge, England); an acceleration voltage equal to 15 kV and a beam current equivalent to 150 pA was used.

2.4. Acid Hydrolysis

Vegetal material (before and after extraction processes) was hydrolyzed at atmospheric pressure with the selected acid solution in a device designed with a glass flask (0.5 L) connected to a refrigeration column. For all the experiments, the raw material/acid solution ratio was 5/100 (w/w). The operating temperature was the boiling solution temperature at atmospheric pressure. The flask containing the acid solution was heated until boiling; the starting process time was taken immediately after adding the raw material.

2.4.1. Acid Hydrolysis Using Hydrochloric Acid Solution – BGR

The operating conditions were as follows: hydrochloric acid solution using three concentrations (0.5, 2.5 and 5.0% (w/w)), processing time from zero to 60 min. At each set time (10, 20, 30, 45 and 60 min), aliquots were removed in sufficient quantity for analysis.

2.4.2. Acid HYDROLYSIS ACID USING SULFURIC ACID SOLUTIon – PPF and DAS

The operating conditions were as follows: sulfuric acid solution using two concentrations (1.5 and 3.0% (w/w)), processing time from zero to 90 min. At each set time (10, 20, 30, 45, 60 and 90 min), aliquots were removed in sufficient quantity for analysis.

2.5. Reducing Sugars (RS) and Total Reducing Sugars (TRS)

During the hydrolysis processes, 1 mL aliquots were removed at each set of analysis time. These aliquots were analyzed for their concentrations of RS and TRS using the colorimetric method of Somogyi-Nelson [40] by spectrophotometry (Femto, model 800 XI, São Paulo, Brazil). For TRS quantification, the test samples were hydrolyzed with a hydrochloric acid solution (2 N) for 6 min at the boiling temperature, and then cooled and neutralized with a sodium hydroxide solution (2 N) to ensure that all the oligosaccharides were hydrolyzed into monosaccharides and would be detectable using the same colorimetric technique. RS and TRS concentrations were calculated using an external calibration curve; the RS and TRS concentrations were interpreted as glucose equivalent and sucrose equivalent, respectively. All the reagents used were of analytical grade.

3. Results and Discussion

3.1. Raw Materials Characterization

The centesimal composition of plant material before the extraction treatment, on a wet basis, is shown in Table 1. For all materials studied, the presence of carbohydrates was significantly high.

Table 1. Centesimal composition (wet basis, %)

	Brazilian ginseng roots (*Pfaffia Glomerata*)	Pressed palm fiber (*Elaeis guineensis*)	Annatto seeds (*Bixa orellana* L.)
Moisture	9.87±0.02	3.8±0.1	13.5±0.2
Lipids	0.56±0.02	6.7±0.9	2.0±0.2
Protein	2.7±0.3	6.5±0.2	13.0±0.2
Ash	3.1±0.2	3.2±0.2	3.9±0.7
Carbohydrates	84	80	68

The materials studied are potential substrates for the hydrolysis reaction before and after the extraction pretreatments. Important bioactive components must be extracted first due to the use of these compounds in the food, chemical and pharmaceutical industries.

3.2. Pretreatments Effects

The BGR was subjected to an extraction process using pressurized water. As shown in Figure 1, and comparing the plant material structures, the vegetal structure is visibly changed due to the PLE process. Some cellular components of BGR have been removed with the solvent during the extraction. Sample of BGR has a porous appearance and an expanded cellular structure, possibly due to the path in which the solvent diffuses into the vegetal matrix.

The cellular wall in Figure 1 (A) resembles the structure of cellulose and hemicellulose [14]. Considering the extraction temperature and pressure used during the PLE process, possibly, water has participated as a hydrolysis medium for hemicellulosic materials [41]. This result could be explained by Figure 1 (B), where the BGR sample cell wall had cellulosic order and spaces that were not visible by SEM

before the PLE process.

In Figure 2, the PPF raw material can be observed. A cell wall composed of cellulose fibers arranged in a certain order and presumably connected by lignin is shown [19; 20]. After the expelling (pressing) process, as shown in Figure 2 (A), PPF still has a significant amount of lipids. During the SE treatment, lipids remain going through these fibers and forming small pores in the lignocellulosic structure as shown in Figure 2(B). Lipid molecules are soluble in the extracting solvent and form a path through the lignocellulosic biomass. Apparently, the lignocellulosic material is not soluble in the solvent used for this extraction and did not react with this solvent.

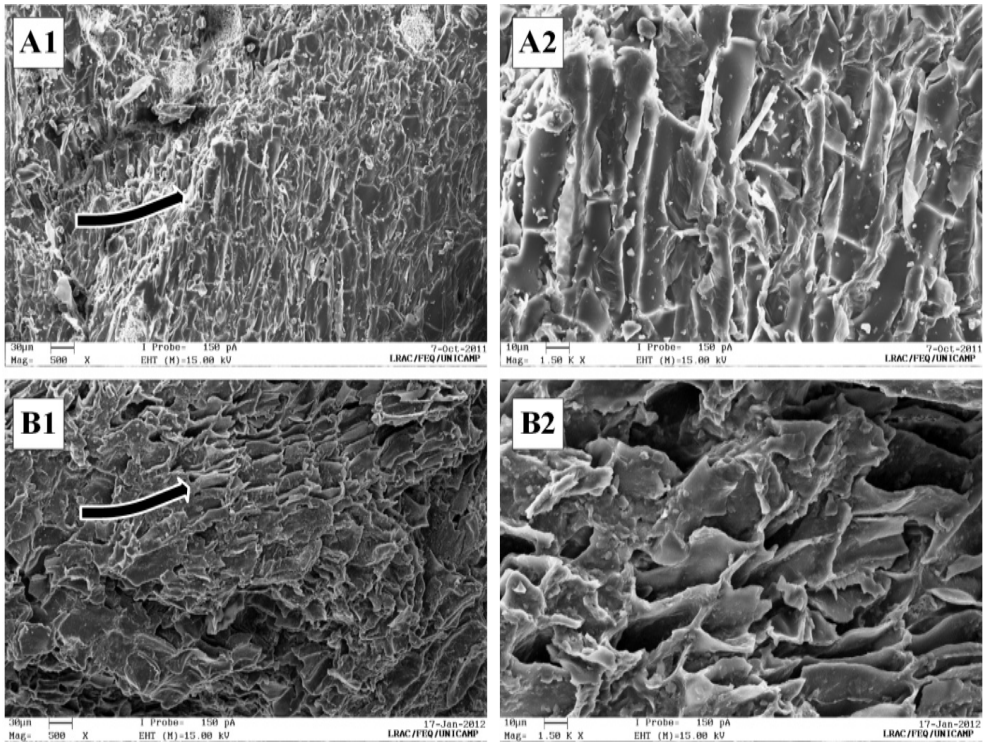

Figure 1. SEM images of (A) BGR *in natura* and (B) BGR after PLE. Numbers 1 and 2 correspond to magnification of 500x and 1500x, respectively

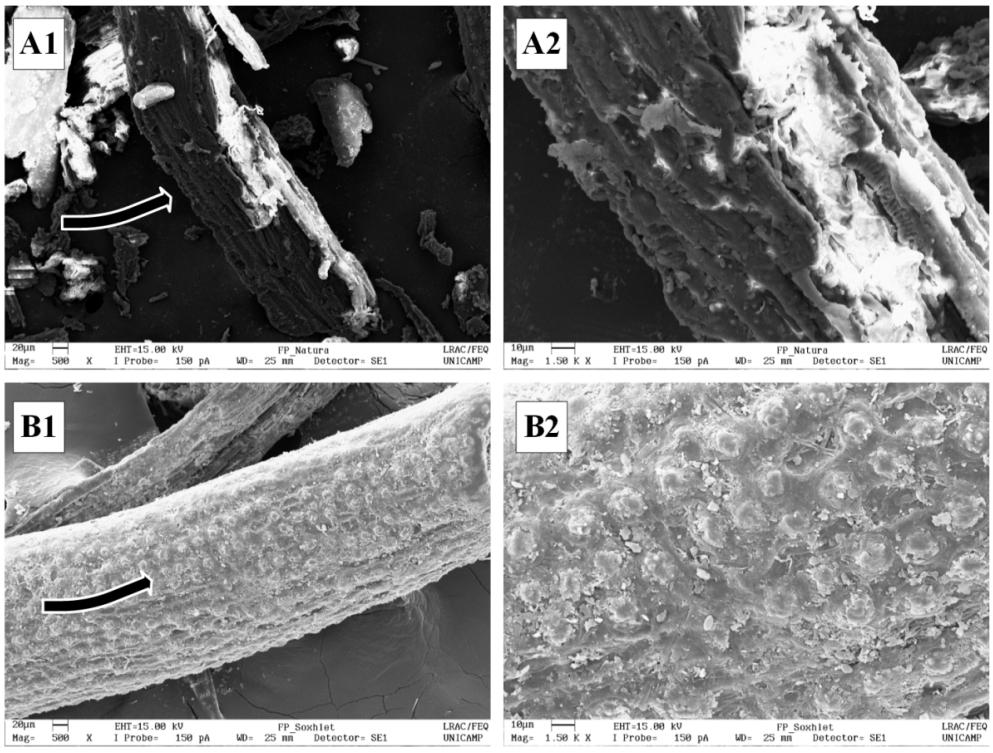

Figure 2. SEM images of (A) PPF before SE and (B) PPF after PLE. Numbers 1 and 2 correspond to magnification of 500x and 1500x, respectively

In Figure 3 the annatto seeds can be observed before and after the SE and SFE processes. In both cases, the vegetal material was penetrated by the solvent after the extraction process, partially dragging lipids and starches from the annatto seeds. Starch in the vegetal structures is deposited in the form of relatively dense granules that range in size between 1- 100 μm, depending on the plant species. Starch is synthesized in special cell compartments formed by cellulose called amyloplasts [42, 43]. As observed in Figure 3 (A), the interior morphology of the annatto seeds showed an amyloplast structure that is formed when cellulose creates symmetrical spaces where granules of starch are hosted [43]. Starch in annatto seeds exhibits a form of round granules.

Studying the formation of the starch granules, lipids molecules form starch-lipid complexes in some plant species such as cereals. In a different way, for example in tubers and oilseed, starch granules are not associated with the lipid molecules in its structure. The three types of lipids that are associated with starch granules are triacylglycerides, diacylglycolipids and phospholipids [42, 44].

Braga et al. [45] studied the influence of a SFE process on ginger (*Zingiber officinale* R.) and turmeric (*Curcuma longa* L.) starches, two roots that possess approximately 40-45% starch. The authors concluded that the extraction process using supercritical CO_2 as the solvent did not alter the starch content. In contrast, after a SFE process (Figure 3 C) that uses the same solvent and DAS as the feedstock, starch was dragged with the lipid fraction from the seeds. Starch is also dragged in Figure 3 (B), so annatto seeds likely have a lipid fraction associated with the starch granules.

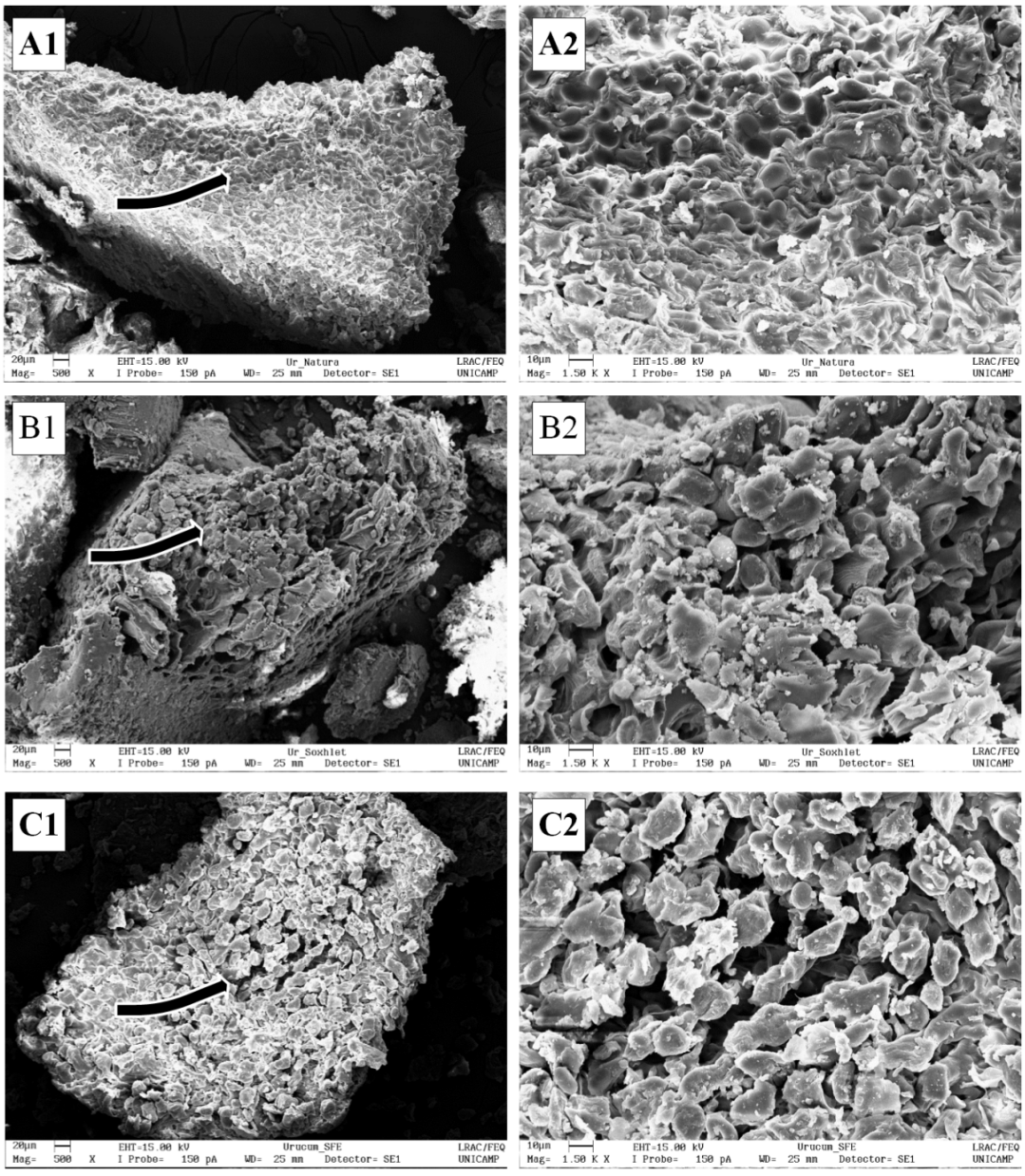

Figure 3. SEM images of (A) Annatto seeds *in natura*, and (B) DAS after SE and (C) DAS after SFE. Numbers 1 and 2 correspond to magnification of 500x and 1500x, respectively

3.3. Hydrolysis Yield

According to the results obtained for BGR, the best yields of RS and TRS for the hydrolysis process were obtained from BGR *in natura*. BGR pretreatment by the PLE process using water as the solvent most likely removed sugars present in the biomass along with the compounds of interest for the extraction. The pretreatment process may have caused a thermos-hydrolysis process at first because of the self-ionization properties of water. The BGR hydrolysis kinetics behavior is shown in Figure 4, for BGR *in natura* the sugars originally present in the biomass are most likely oligomers and monomers that are easier to hydrolyze as shown by both curves (RS and TRS yield). Hydrolysis kinetics for BGR in nature has a higher yield than those of BGR after the PLE process. The analysis of the kinetics behavior of the hydrochloric acid solutions showed that for the most dilute solution (0.5% w/w), acid hydrolysis increased during the initial processing time and kept growing throughout the operation. Despite this behavior, yields of sugar did not exceed those yields observed using higher acid concentrations. A reaction with an intermediate concentration (2.5% w/w) showed better results from the start of the reaction. The hydrolysis yield at a higher acid concentration (5.0% w/w) showed a high rate of RS and TRS production from hydrolysis at first, but this production rapidly decayed because the degradation rates of sugar monomers was greater than the oligomer hydrolysis at this acid concentration.

The products of interest generated by hydrolysis of vegetal biomass are pentoses and hexoses with various degrees of polymerization. Degradation of glucose due to the hydrolysis reaction produces compounds such as 5- hydroxymethyl furfural (5-HMF) and organic acids that have 5-HMF as a precursor, such as levulinic and formic acid [23].

The kinetics of the hydrolysis of PPF, shown in Figure 5, does not indicate significant differences between PPF material before and after SE pretreatment. Extraction treatment did not alter the physical morphology and composition of the lignocellulosic compounds found in the PPF raw material. The PPF structure is highly resistant due to strong polysaccharides, such as cellulose and lignin. Regarding the acid concentrations used for PPF acid hydrolysis, the observed kinetics behavior between the acid concentration and hydrolysis yield is proportional. The hydrolysis rate is maintained with increasing operation time [24].

In Figure 6, the hydrolysis kinetics for Annatto seeds is shown. These curves suggest that defatted, SFE pretreated material was more easily hydrolysable than raw material in nature and also defatted, SE pretreated material, according to RS and TRS yields. The kinetic hydrolysis of Annatto and micrographs of its structure (Figures 5, 6 and 7) can be explained by SE pretreatment removing more of the starch-lipid complex than SFE treatment [35].

Romero et al. [15] studied acid hydrolysis of residues generated by pruning olive trees using sulfuric acid solutions in concentrations from 0 to 32% (w/w) and temperatures between 60 and 90°C. These results showed that the cellulose and hemicellulose fractions that remain insoluble during the hydrolysis operation decrease proportionally with the increase in the acid solution concentration. When using dilute solutions, the authors observed that only the hemicellulose content decreased, whereas the cellulose fraction remained unchanged. When the acid concentration was greater than 4.5% (w/w) of sulfuric acid, hydrolysis of cellulose started. On the other hand, the lignin content of these raw materials remained constant.

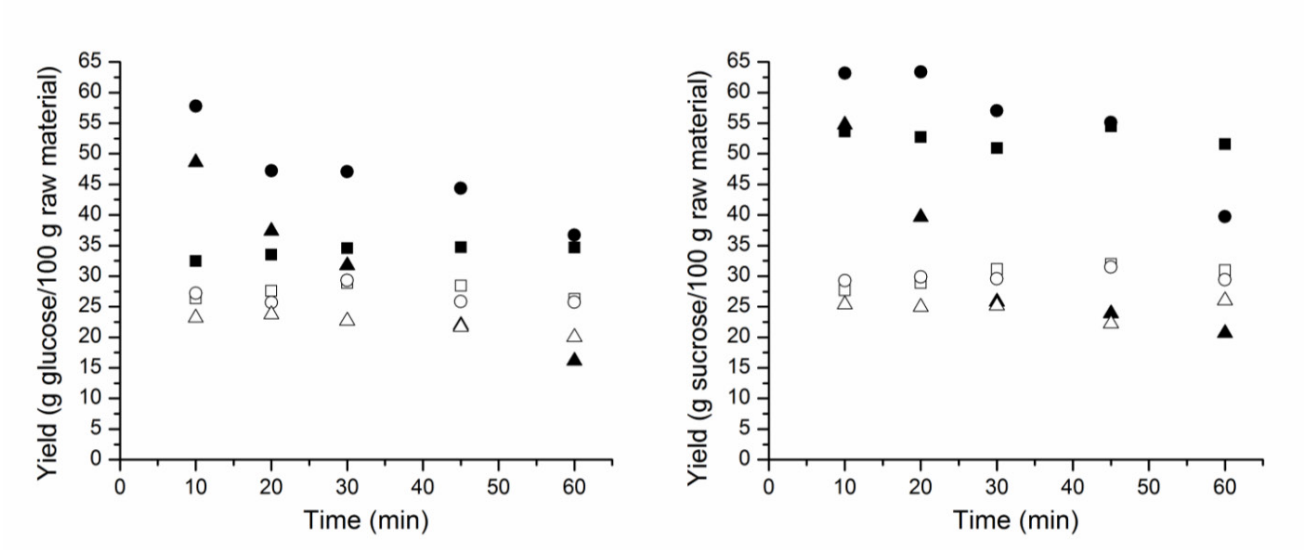

Figure 4. Acid hydrolysis of BGR *in natura* (■, ●, ▲) and after PLE (□, ○, Δ) using hydrochloric acid solution 0.5% (■, □), 2.5% (●, ○) and 5.0% (▲, Δ), (w/w); respectively. a) RS yield (%) and b) RTS yield (%)

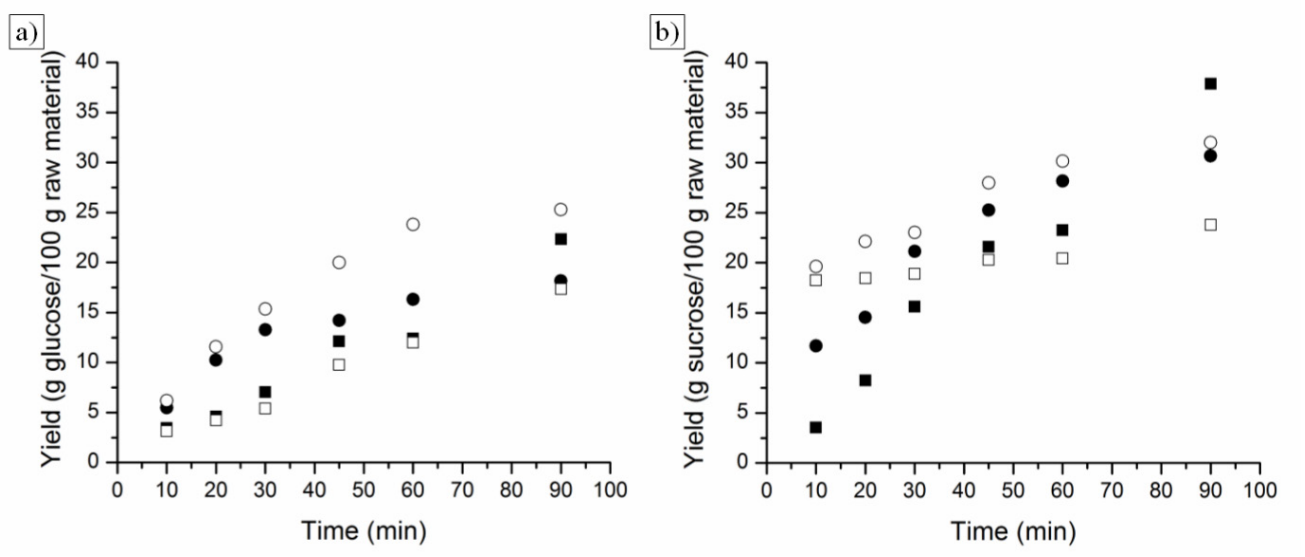

Figure 5. Acid hydrolysis of PPF (■, ●) and PPF after SE (□, ○) using sulfuric acid solution 1.5% (■, □) and 3.0% (●, ○), (w/w); respectively. a) RS yield (%) and b) RTS yield (%)

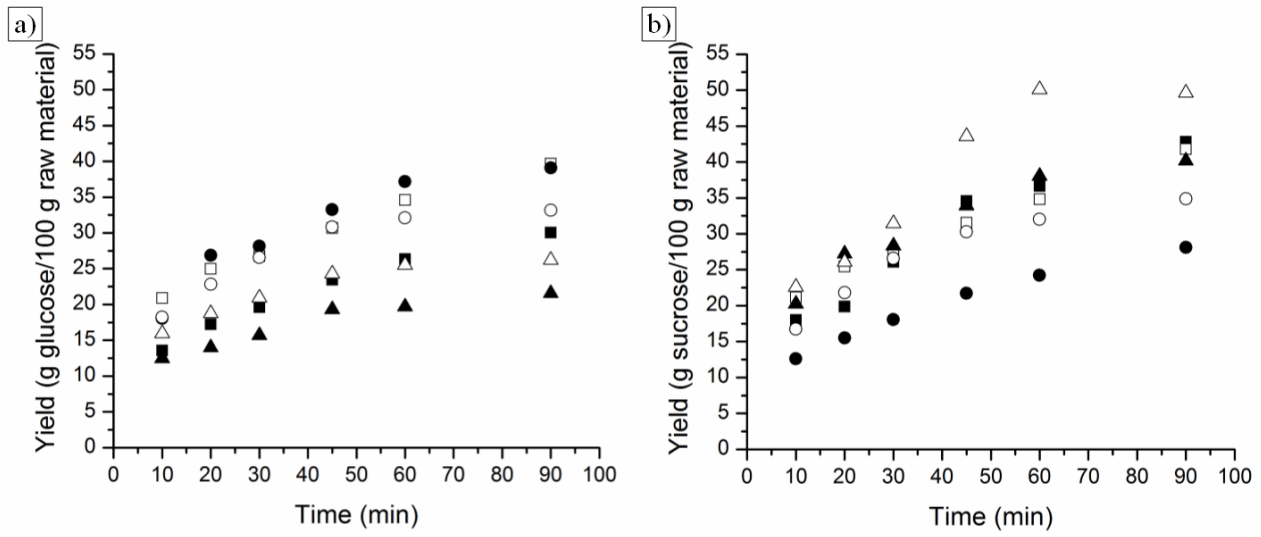

Figure 6. Acid hydrolysis of annatto seeds *in natura* (■,□), DAS after SE (●, ○) and DAS after SFE (▲, Δ) using sulfuric acid solution 1.5% (■, ●,▲) and 3.0% (□,○, Δ), (w/w); respectively. a) RS yield (%) and b) RTS yield (%)

4. Conclusions

Analysis of the observations from micrographs showed that the morphologies of the different biomasses changed after the extraction processes. The surfaces of the treated raw materials showed a considerable increase in porosity and roughness when compared with untreated materials.

The acid hydrolysis process showed different behaviors for each material that was hydrolyzed. All the plant materials studied belong to different vegetal groups, and the cell structures of these plants have polysaccharides in different forms and proportions. Acid hydrolysis parameters depended on the acid concentration and polysaccharide in the plant.

Extraction treatments preceding the acid hydrolysis process modified vegetal matrices in different ways. In BGR material, PLE pretreatment removed a large number of water-soluble molecules including structural polysaccharides. Brazilian ginseng *in natura* showed better yield results than raw material after PLE. The SE pretreatment of PPF modified the cellular structure and composition of the sample, although the polysaccharides in the PPF structure needed a more invasive treatment (mainly lignin) to have a more perceptible impact on hydrolysis. Hydrolysis kinetics in annatto seeds showed that SE and SFE pretreatments had different effects on the plant matrix.

5. Perspectives and Future Trends

The starch presence in the structure of annatto seeds has special importance, because of not only the oligomers and monomers produced but also because the starch market is constantly growing and requires a continuous search for

products with features that satisfy industry needs. In addition to modified starch, the natural pigment bixin is also derived from annatto seeds.

The hydrolysis of vegetal matrices with special properties like the raw materials in this study could result in products with special aromas and colors. These materials have potential application in food formulations, and the sugars produced by these processes could be used as substrates in a wide range of processes.

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Acronyms

Soxhlet extraction (SE), Pressurized liquid extraction (PLE), Supercritical fluid extraction (SFE), Scanning electron microscope (SEM), Reducing sugar (RS), Total reducing sugar (TRS), Microwave assisted extraction (MAE), Ultrasound-assisted extraction (UAE), Pulsed-electric field extraction (PEF), Enzyme-assisted extraction (EAE), Brazilian Ginseng Roots (BGR), Pressed Palm Fiber (PPF), Defatted Annatto Seeds (DAS), Solvent / Feed ratio (S/F).

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- CAPÍTULO 3 –

Physicochemical properties, modifications and applications of starches from different botanical sources

Estudos em torno da semente de urucum realizados no LASEFI (Albuquerque & Meireles, 2012; Rodrigues et al., 2014; Moraes et al. 2015) mostram uma tendência para futuros trabalhos que enfatizem o aproveitamento total da semente de urucum. As sementes de urucum de reduzido teor lipídico possuem teores relevantes de bixina e amido. Neste artigo de revisão o foco está na utilização promissora do amido de urucum e nos possíveis processos de modificação deste polissacarídeo para usos futuros.

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Physicochemical properties, modifications and applications of starches from different botanical sources

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Abstract

Present trends towards technologies and processes that increase the use of residues make starchy vegetal biomass an important alternative material in various applications due to starch's versatility, low cost and ease of use when its physicochemical properties are altered. Starch is increasingly used in many industrial applications and as a renewable energy resource. Starch can be modified to enhance its positive attributes and eliminate deficiencies in its native characteristics. In this article, the state of knowledge on conventional and unconventional starches and their properties, characteristics, modifications and applications are reviewed.

Keywords: starch modification; starch granules; paste properties; conventional starch; unconventional starch; starch biomass; food.

Practical Application: Use of unconventional starches and vegetal residues containing starch in industry.

1. Starch

Starch is the most abundant carbohydrate reserve in plants and is found in leaves, flowers, fruits, seeds, different types of stems and roots. Starch is used by plants as source of carbon and energy (Smith, 2001). The biochemical chain responsible for starch synthesis involves glucose molecules produced in plant cells by photosynthesis. Starch is formed in the chloroplasts of green leaves and amyloplasts, organelles responsible for the starch reserve synthesis of cereals and tubers (Smith, 2001; Tester et al., 2004). Starch production in the chloroplast is diurnal and performed rapidly by the plant. Conversely, starch reserves produced by amyloplasts are deposited over several days, or even weeks. Starch is stored and cyclically mobilized during seed germination, fruit maturation and the sprouting of tubers (Ellis et al., 1998). The main location of starch synthesis and storage in cereals is the endosperm. Major starch sources are cereals (40 to 90%), roots (30 to 70%), tubers (65 to 85%), legumes (25 to 50%) and some immature fruits like bananas or mangos, which contain approximately 70% of starch by dry weight (Santana & Meireles, 2014). The accumulation pattern of starch granules in each plant tissue, shape, size, structure and composition is unique to each botanical species (Smith, 2001).

Starch synthesized by plant cells is formed by two types of polymers: amylopectin and amylose. Amylopectin consists of linear chains of glucose units linked by α-1,4 glycosidic bonds and is highly branched at the α-1,6 positions by small glucose chains at intervals of 10 nm along the molecule's axis; it constitutes between 70 to 85% of common starch (Durrani & Donald, 1995). Amylose is essentially a linear chain of α-1,4 glucans with limited branching points at the α-1,6 positions and constitutes between 15-30% of common starch. Starch's structural units, amylose and amylopectin, are shown in Figure 1. The polymodal distribution of α-glucans chains of different sizes and the grouping of branch points in the amylopectin molecule allow the formation of double helical chains. Amylose and amylopectin can be arranged in a semicrystalline structure forming a matrix of starch granules with alternating amorphous (amylose) and crystalline (amylopectin) material, which is known as the growth rings in superior plant starch (Jenkins et al., 1993).

Several types of starches are known as "waxy" starches due to the waxy appearance of the endosperm tissue from which they are derived; these tissues contain a minimal amount of amylose in their granule composition (<15%). Waxy starch requires high energy for gelatinization due to its high crystallinity (Hung et al., 2007). Other starches have a high content of amylose (>30%); these starches can also contain other polysaccharide molecules and exhibit a slight deformation in granule appearance.

Cereal starches contain lipid molecules in their structures in the form of phospholipids and free fatty acids; they are associated with the amylose fraction (Ellis et al., 1998; Tester et al., 2004). The presence of lipid complexes in starch granules is observed as a hydrophobic nucleus situated within helices formed by amylose chains. The lipid complexes vary between 0.15 to 0.55% of the amylose fraction in cereal starches (Tester et al., 2004). Lipids in starch granules, despite representing a small fraction, can significantly reduce the swelling capacity of the starch paste (Morrison & Azudin, 1987). Starch contains approximately 0.6% of protein associated with the molecule. The origins of protein and lipids on starch are situated on the granule surface. Lipids and proteins in starch granules can raise its functionality. In wheat for example, the associated protein in the starch granules receives a lot attention due to its association with grain

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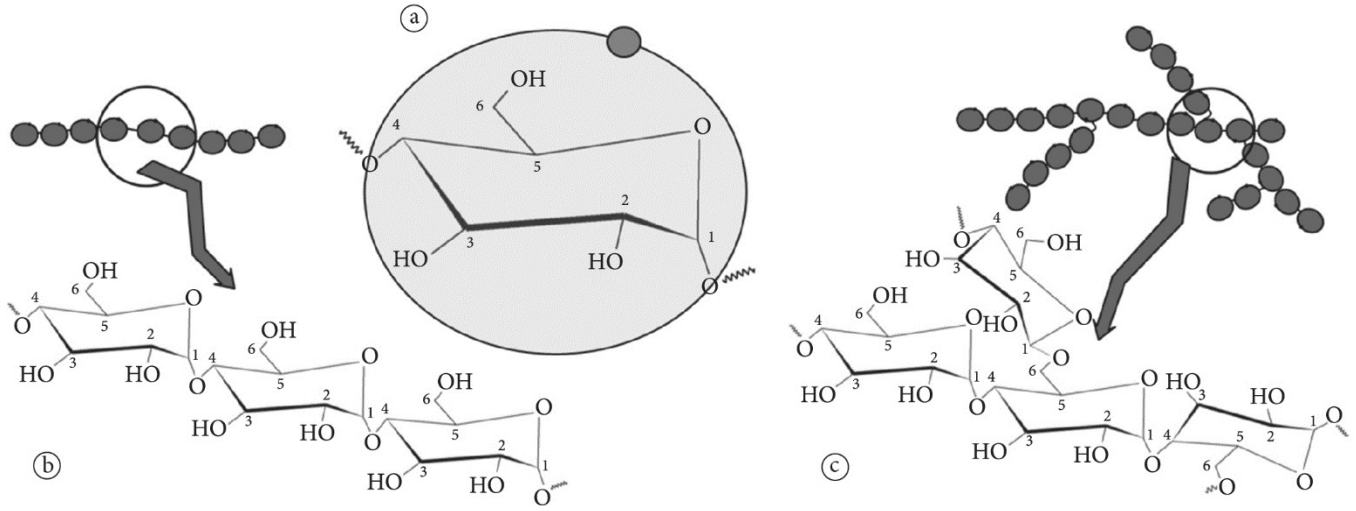

Figure 1. Basic structural design of (a) glucose units, (b) amylose and (c) amylopectin, along with the labeling of the atoms and torsion angles. Extension of the basic units to macromolecular structures was adapted from Pérez & Bertoft (2010).

hardness. Starch also contains a relatively small quantity (<0.4%) of minerals (calcium, magnesium, phosphorus, potassium and sodium). Among these, phosphorus is of primary importance and is present in starch in three main forms: monophosphate esters, phospholipids and inorganic phosphate.

The physical and chemical aspects of starch synthesis and the composition of amylose and amylopectin have been discussed in detail in other reviews by Smith (2001), Tester et al. (2004), Pérez & Bertoft (2010) and Conde-Petit et al. (2001).

2. Physicochemical properties of starch

The length of the α-glucan chains, amylose-amylopectin ratio and branching degree of amylopectin define the size, structure and particular utility of starch granules in each plant species. Other characteristics associated with the granule such as form, surface type and phosphate groups influence the starch's properties and uses (Smith, 2001).

2.1. Characteristics of starch granules: morphology, size, composition and crystallinity

Starch granules have microscopic sizes with diameters ranging from 0.1 to 200 μm, and its morphology varies between different shapes, such as oval, ellipsoidal, spherical, smooth, angular and lenticular, depending on the botanical source (Buléon et al., 1998; Hoover, 2001; Singh et al., 2003). Size distribution can be uni-, bi-, or polymodal. In amyloplasts, starch granules are present individually or in groups (Jane et al., 1994; Pérez & Bertoft, 2010). Common cereals such as wheat, barley and rye contain two types of starch granules: (i) A-type, lenticular shape and large size and (ii) B-type, spherical shape and small size (Tester et al., 2006; Vamadevan & Bertoft, 2015). The physicochemical properties of certain native starches are listed in Table 1. Starch granules are typically isolated before microscopic observation, and the isolation method is important because it can potentially affect the starch's original size (Gao et al., 2014; Lawal et al., 2011). Granule morphology typically contains a central line known as the hilum or "Maltese cross". Each starch granule may contain one or more Maltese crosses, and this characteristic reduces the birefringence ability of the starch granule (Jiang et al., 2010).

The amount of amylose present in the granule significantly affects the physicochemical and functional properties of starch. The amylose content can vary within the same botanical variety because of differences in geographic origin and culture conditions (Gao et al., 2014). Researchers have highlighted the role of amylose in the initial resistance of granules to swelling and solubility, as swelling proceeds rapidly after leaching of amylose molecules. The capacity of amylose molecules of form lipid complexes prevents their leaching and consequently the swelling capacity (Singh et al., 2003). Amylose is anhydrous and can form excellent films, which are important characteristics for industrial applications. Films formed by amylose are very strong, colorless, odorless and tasteless (Campos et al., 2011).

Phosphorus is one of the non-carbohydrate components present in the starch granule and significantly affects its functional characteristics. Phosphorus is present as monoester phosphates or phospholipids in various types of starches. Monoester phosphates are associated with the amylopectin fraction by covalent bonds, increasing the clarity and viscosity of the paste, whereas the presence of phospholipids results in opaque and low viscosity pastes (Craig et al., 1989). The phospholipid content in starch granules is proportionally related to amylose. Phospholipids tend to form complexes with amylose and long branches of amylopectin, resulting in starch granules with limited solubility (Morrison et al., 1993; Singh et al., 2003). The nature of the phosphorus in starch granules has an important influence on the transmittance of the paste. Starches such as wheat and rice with high phospholipid contents produce pastes with low transmittance power compared to potato or corn starch pastes because the latter starches contain less phospholipids. Potato starch demonstrates high transmittance due to its phosphate monoester content (Singh et al., 2003).

Table 1. Morphology and composition of native starch granules from various sources.

Species	Variety/ Designation	Granule Shape	Diameter (μm)	Lipid (%w/w)	Protein (%w/w)	Phosphorus (%w/w)	Amylose (%w/w)	Degree of crystallinity/ Crystalline type
Wheat	Wild type	Spherical and lenticular (Singh et al., 2003) A-type: Spherical or ellipsoidal (Zhang et al., 2013) B-type: Disc-shape and lenticular	< 30 (Singh et al., 2003) 22-36 (Jane et al., 1994) A-type: >10 (Zhang et al., 2013) B-type: < 10 (Zhang et al., 2013)	0.08-0.12 (Buléon et al., 1998) 0 (Morrison et al., 1984)	0.2-0.3 (Šubarić et al., 2012)	0.40 (Sang et al., 2007)	25.6±1.0 (Hung et al., 2007)	27.7±2.7/A (Hung et al., 2007)
	Waxy						1.0±0.5 (Hung et al., 2007)	30.0±2.1/A (Hung et al., 2007)
	High amylose						37.5±1.0 (Hung et al., 2007)	9.4±2.5/C (Hung et al., 2007)
Barley	Wild type	A-type: Disc-shape (Ellis et al., 1998) B-type: lenticular (Ellis et al., 1998)	A-type:10-25 (Ellis et al., 1998) B- type: 5 (Ellis et al., 1998)	0.7-1.2 (Morrison et al., 1984)	0.2-0.4 (Ellis et al., 1998)		19-22.1 (Ellis et al., 1998) 25.3-30.1 (Morrison et al., 1984)	
	Waxy		A-type:10-25 (Ellis et al., 1998) B- type: 5 (Ellis et al., 1998)	0.3-0.5 (W. Morrison et al., 1984)	0.06-0.15 (Ellis et al., 1998)		1.8-3.6 (Ellis et al., 1998) 2.1-8.3 (Morrison et al., 1984)	
	High amylose		A-type: 15-32 (Ellis et al., 1998) B-type :2-3 (Ellis et al., 1998)	1.0-1.7 (Morrison et al., 1984)			18.2-24.1 (Ellis et al., 1998) 38.4-44.1 (Morrison et al., 1984)	
Sorghum	Wild type	Polygonal, dented and round (Wankhede et al., 1989)	8-14,5 polygonal, 8-10 round (Wankhede et al., 1989)	0.8 (Emmambux & Taylor, 2013)	2.3 (Emmambux & Taylor, 2013)		23.7-27.6 (Boudries et al., 2009)	26-30/A (Boudries et al., 2009)
	Waxy	Larger than normal sorghum (Lawal et al., 2011)	5-25 (Sang et al., 2008)		< 0.3 (Sang et al., 2008)		3.5 (Sang et al., 2008) 0 (Sang et al., 2008)	
	Heterowaxy						14 (Sang et al., 2008)	
Rice	Wild type	Angular, polygonal (Hoover et al., 1996)	< 20 (Singh et al., 2003) 2-8 (Hoover et al., 1996; Patindol et al., 2015)	0.6-1.4 (Dhital et al., 2011)	0.1 (Hoover et al., 1996)	0.1 (Lim et al., 1994)	21-25 (Patindol et al., 2015)	
	Waxy						0-2 (Patindol et al., 2015)	
	High amylose						> 25 (Patindol et al., 2015)	
Corn	Wild type	Angular (Singh et al., 2003)	11.5± 0.3 (López et al., 2010)	0.6-0.8 (Dhital et al., 2011) 0.3-0.53 (Morrison et al., 1984)	0.4 (Dhital et al., 2011)	0.31-0.35 (Sang et al., 2007)	28.5 Nuwamanya et al., 2013) 23.86 ± 0.66 (López et al., 2010) 25.8-32.5 (Morrison et al., 1984)	19.2 ±1.4/A (López et al., 2010)
	Waxy			0.01-0.05 (Morrison et al., 1984)			1.4-2.7 (Morrison et al., 1984)	
	High amylose	Polygonal and Angular (Ellis et al., 1998)		0.38-0.67 (Morrison et al., 1984)		0.02 (Lim et al., 1994)	70 (Lim et al., 1994) 42.6-67.8 (Morrison et al., 1984)	

Lipid, protein and phosphorus contents are shown as % of total dry weight.

Table 1.Continued...

Species	Variety/ Designation	Granule Shape	Diameter (µm)	Lipid (%w/w)	Protein (%w/w)	Phosphorus (%w/w)	Amylose (%w/w)	Degree of crystallinity/ Crystalline type
Potato		Smooth-surfaced, oval and irregular (Singh et al., 2003)	< 110 (Singh et al., 2003)	0.1 (Dhital et al., 2011)	0.1 (Dhital et al., 2011)	0.6 (Dhital et al., 2011)	29.3±0.2 (Yuan et al., 2007)	45.9±0.6/B (Yuan et al., 2007)
Sweet Potato		Polygonal (Jane et al., 1994)	5-25 (Jane et al., 1994)				28.9±0.35 (Tan et al., 2006)	
Cassava		Flake-shape and irregular (Falade & Akingbala, 2010)	5-25 (Jane et al., 1994)	0.2 (Vasanthan & Hoover, 1992)	0.3 (Leelavathi et al., 1987)	0.01 (Lim et al., 1994)	23.7±0.1 (Yuan et al., 2007) 8-25 (Paes et al., 2008)	48±0.2/A (Yuan et al., 2007) 12-15/B (Paes et al., 2008)
Turmeric		Polygonal and Angular (Braga et al., 2006)	5-70 (Braga et al., 2006)		0.6±0.1 (Braga et al., 2006)		48±3 (Braga et al., 2006)	
Ginger		Polygonal and Angular (Braga et al., 2006)	5-40 (Braga et al., 2006)		0.53±0.01 (Braga et al., 2006)		34±2 (Braga et al., 2006)	
Dioscorea			3-22 (Yuan et al., 2007)				26.3±0.2 (Yuan et al., 2007)	48.5±0.3/C (Yuan et al., 2007)

Lipid, protein and phosphorus contents are shown as % of total dry weight.

Starch granules have very complex structures. The complexity is built around variations in their composition (α-glucans, moisture, lipids, proteins and phosphorylation), component structure and variation between amorphous and crystalline regions. Amylose associated with large branches of amylopectin molecules comprise the amorphous region of granules, and amylopectin molecules with short branches comprise the crystalline region; therefore, a higher proportion of amylopectin in starch granules results in greater crystallinity (Cheetham & Tao, 1998). There are three types of crystalline structures: A-type characteristics from cereal starches, B-type found in tubers and C-type present in legumes (Singh et al., 2003). Crystalline structures are based on the double helix formed by the amylopectin molecule. In A-type structures, the amylopectin branches are short (polymerization degree of 6-15) and linked by α-1,6 bonds. A-type is characteristic of amylopectin ramifications. In B-type, the glucose chains are more polymerized and can act as bases where the branches are A-type or form branched amylopectin molecules. B-type chains are subdivided into B1, B2, B3 and B4. B1 chains have a polymerization degree between 15 and 25, and B2 chains are typically between 40 and 50; B3 and B4 are the highest. C-type crystallinity is a combination of the A- and B-types and consists of amylopectin molecules with non-reduced ends (Cheetham & Tao, 1998).

2.2. Birefringence and glass transition temperature (Tg)

Birefringence is the ability to doubly refract polarized light. All starch granules in their native form exhibit birefringence that is proportional to their crystalline structure. Birefringence patterns in starch granules represent the radial arrangement of amylopectin molecules, and their chains form 90° angles with the reduced ends in the direction of the hilum or starch granule center. Weak birefringence patterns are indicative of disorganization of the crystalline region (BeMiller & Whistler, 2009). Loss of birefringence in starch granules is associated with deformation due to its modification (Liu et al., 1991).

T_g is an important parameter affecting the physical properties of polymers. Glass transition occurs similar to a thermodynamic second order transition, where the specific volume and enthalpy are functions of temperature (Biliaderis et al., 1999). T_g describes the induction temperature of the progressive transition from an amorphous state to a rubbery state as the material is heated, generally in the presence of a solvent or plasticizer when referring to polysaccharides (Tester & Debon, 2000). Because starch consists of an amorphous and a crystalline region, the exact T_g is detected with difficultly.

2.3. Swelling capacity and solubility of starch granules

One of the most important structural characteristics of starch is that it passes through several different stages from water absorption to granule disintegration. Water absorption and consequent swelling of the starch granule contribute to amylopectin-amylose phase separation and crystallinity loss, which in turn promotes the leaching of amylose to the inter-granular space (Conde-Petit et al., 2001). When starch molecules are heated in water excess, the semi-crystalline structure is broken, and water molecules associate by hydrogen bonding to hydroxyl groups exposed on the amylose and amylopectin molecules. This association causes swelling and increases granule size and solubility (Singh et al., 2003). The swelling capacity and solubility of starch illustrate the interactions of the polymeric chains comprising the amorphous and crystalline granule fractions (Zhang et al., 2005). The extent of this interaction is influenced by the amylose-amylopectin proportion and is characteristic of each molecule depending on the polymerization degree, length and grade of chain branching, molecular weight and molecular conformation (Hoover, 2001; Ratnayake et al., 2002). The swelling capacity of starch is directly associated with the amylopectin content because the amylose acts as a diluent and inhibitor of

swelling (Singh et al., 2003). Some species of starch that contain amylose-lipid complexes exhibit swelling capacity and solubility restrictions (Morrison et al., 1993).

The swelling stage of starch granules is the initial step of all other paste characteristics. Initially, granule swelling is reversible, increasing its volume up to 30% (Gryszkin et al., 2014). Water absorption and heating of the starch dispersion breaks the hydrogen bonds responsible for granule cohesion, partially solubilizing the starch (Hoover, 2001). Water penetrates the interior of the starch granule, hydrating the linear fragments of amylopectin (Xie et al., 2008). This process leads to irreversible swelling, increasing the granule size several fold and the paste viscosity. Paste viscosity is essentially the principal measure of the potential application of starch in industry (Gryszkin et al., 2014; Sarker et al., 2013).

2.4. Gelatinization and retrogradation properties of starch

Starch, when heated in the presence of excess water, undergoes a transition phase known as gelatinization, and there is a characteristic temperature interval for gelatinization corresponding to each starch species. Gelatinization occurs when water diffuses into the granule, which then swells substantially due to hydration of the amorphous phase causing loss of crystallinity and molecular order (Donovan, 1979; Jenkins et al., 1993; Jiménez et al., 2012). The gelatinization process starts at the hilum and quickly spreads throughout the periphery. Gelatinization occurs initially in the amorphous region, favored by the weak hydrogen bonds present in this area. The process then extends to the crystalline region. Amylose presence reduces the fusion point in the crystalline region and the amount of energy necessary to initiate gelatinization (Sasaki et al., 2000). The gelatinization process is represented by transition temperatures and gelatinization enthalpies in the paste, and these measures are characteristic for each species. High transition temperatures correspond to a high degree of crystallinity, high stability and resistance of the granule structure to gelatinization (Tester et al., 2004). Gelatinization of starch granules is associated with a loss of birefringence and crystalline order due to the breaking of the double helix in the crystalline region and the leaching of amylose (Donovan, 1979; Evans & Haisman, 1982). This transitions starch from a semi-crystalline form (relatively indigestible) to an amorphous form that is easily digestible (Tester & Debon, 2000). Similar to water, other solvents are also used to promote gelatinization. The principal consideration with solvents is their capacity to form hydrogen bonds with the molecules in the starch granules (i.e., liquid ammonia, formamide, formic acid, chloroacetic acid and dimethyl sulfoxide). The gelatinization process is affected by solvent type and starch/solvent proportions (Jiménez et al., 2012). Gelatinization is necessary for particular processes, e.g., textile and hydrolyzed starch industries. Gelatinization affects the rheological properties and viscosity of the paste, making the starch granule more accessible to enzymatic action. When starch granules swell and its components are in solution, the medium properties change from a simple starch granules suspension to a starch paste. Amylose and amylopectin form separate phases because of thermodynamic immiscibility. In food, starch is typically in combination with other polymeric ingredients, such as proteins and other polysaccharides, forming different phases (Conde-Petit et al., 2001). Gelatinization of granules is caused by many processes or the manufacturing of products from raw materials based on starch, especially cereals. Gelatinization progress along the granule is determined by the physicochemical properties of the starch, the presence of other ingredients, the availability of water and process parameters applied (i.e., temperature, time and mechanical energy) (Schirmer et al., 2015).

The molecular interaction produced after gelatinization and cooling of the paste is known as retrogradation (Hoover, 2000). During retrogradation, amylose molecules associate with other glucose units to form a double helix, while amylopectin molecules re-crystallize through association of its small chains (Singh et al., 2003). After retrogradation, starch exhibits lower gelatinization and enthalpy compared to native starch because its crystalline structure has been weakened (Sasaki et al., 2000). Initially, the amylose content exercises a strong influence over the retrogradation process; a large amount of amylose is associated with a strong tendency for retrogradation. Amylopectin and intermediate materials influence the retrogradation process during storage under refrigeration; each polymer has a different recrystallization rate (BeMiller, 2011; Conde-Petit et al., 2001). Recrystallization of starch easily occurs at temperatures under 0 °C, but also occurs above 100 °C. Starch retrogradation is intensified by repeated freezing and thawing of paste (Leszczyñski, 2004). Some research has shown that paste components such as proteins influence the retrogradation properties of paste through emulsification. Proteins form complexes with starch that retards the retrogradation process during refrigerated storage (Wu et al., 2010). The presence of other components in addition to proteins and lipids, such as other carbohydrates, salts and polyphenols, significantly affects retrogradation (Fu et al., 2015). In general, retrogradation in starch pastes, as well as foods containing starch, is unfavorable in terms of food quality, causing syneresis of gels or hardness. Currently, retrograded starch is classified as a form of resistant starch (RS) (Zięba et al., 2011) and is used in industry for different purposes described in other sections of this review.

2.5. Rheological and thermal properties

Starch paste forms immediately after gelatinization, and starch granules are increasingly susceptible to disintegration by shearing because they are swollen. The paste obtained is a viscous mass consisting of one continuous phase of solubilized amylose and/or amylopectin and one discontinuous phase of the remaining starch granules (Ambigaipalan et al., 2011). Starch functionality is directly related to gelatinization and the properties of the paste. All of these properties affect the stability of products, consumer acceptance and production reliability (Šubarić et al., 2012). The characteristics of the native starch, the effects of the physical or chemical modifications of the granules, the process parameters and the botanical sources of the starch are all critical factors governing the behavior and characteristics of the starch paste. The transformation of starch during manufacturing depends on the temperature-time-mixture ratio and the modification ratio during processing (Conde-Petit et al., 2001).

Starch granules are insoluble in cold water due to the hydrogen bonds and crystallinity of the molecule. When starch is dispersed in hot water below its T_g, the starch granules swell and increase several times in size, breaking the molecules and consequently leaching amylose to form a three-dimensional network and increase the paste's viscosity (Sarker et al., 2013). Starch paste can contain un-swollen granules, partially swollen granules, aggregates of swollen starch granules, fragments and molecules of retrograded starch and starch that has dissolved or precipitated (BeMiller & Whistler, 2009).

The presence of relatively short chains of amylose and amylopectin adds opacity to starch suspensions and foods containing them. In products such as sources, dressings and puddings, this opacity is not a disadvantage; however, jellies and fruit fillings require starch suspensions with high clarity (Eliasson, 2004). Paste clarity is commonly determined by the percent transmittance from a dilute solution of starch (1% w/w) at a wavelength of 650 nm (Bello-Pérez & Paredes-López, 1996; Ulbrich et al., 2015). Products like potato starch have transmittances between 42 to 96% and are considered high clarity pastes, followed by cassava starch at 51-81%. Common cereals generally present transmittances of 13 to 62% (Craig et al., 1989; Nuwamanya et al., 2013). Low clarity in common cereals (non-waxy) is due to the presence of traces of swollen starch granules (Craig et al., 1989) and amylose-lipid complexes (Bello-Pérez & Paredes-López, 1996). Clarity in starch suspensions is modified during storage, decreasing due to amylose and/or amylopectin molecules (Jacobson et al., 1997; Waterschoot et al., 2015a).

Rheological properties describe the behavior of materials subjected to shearing forces and deformation, which are considered viscoelastic complexes. The basic feature of starch rheology is its viscosity. Other rheological characteristics involve texture, transparency or clarity, shear strength and the tendency for retrogradation. All of these features play important roles in the commercial applications of starch (BeMiller & Whistler, 2009; Berski et al., 2011). Rheological starch properties are studied through the behavior of viscosity curves, which are influenced by temperature, concentration and shear stress (Singh et al., 2003). "Paste properties" is the term used to describe the changes that occur in starch after gelatinization in excess water. Instruments like the Rapid Visco Analyzer (RVA) describe the viscosity parameter as functions of temperature and time. The RVA describes paste behavior in three periods: (i) a controlled heating period, increasing the temperature of the suspension from room temperature to a maximum that is generally determined at 95 °C; (ii) an isothermal period, maintaining the suspension at the maximum temperature for analysis; and (iii) a cooling period, decreasing the temperature to approximately 50 °C. Throughout the analysis, the suspension is subjected to shear forces. Suspensions typically exhibit a peak in viscosity that starts after gelatinization and increases as the granules swell, followed by a decrease in viscosity due to granule disintegration and polymer realignment. A "Breakdown" is defined by a difference between the viscosity peak and the minimum viscosity at the maximum analysis temperature. During the cooling period, amylose leaching forms a gel or three-dimensional network. Gel formation further increases the viscosity, called the "cold paste viscosity". The difference between the paste viscosity at the end of the cooling period and the minimum viscosity at 95 °C is termed the "setback" (Saunders et al., 2011; Wang & Weller, 2006).

Starch gels are composed of amylose chains and intermediate materials dispersed in a starch suspension after granule disintegration in a three-dimensional network structure. The level and nature of the leached material and molecular interactions determine the viscoelastic properties. Methods used to describe the viscoelastic parameters of the paste include equipment such as a dynamic rheometer, an amylograph or a viscoamylograph (Schirmer et al., 2015). Several parameters describe the viscoelastic behavior of pastes: (G'), the measure of recovered or accumulated energy in each deformation cycle and an indicator of the elastic behavior of the paste; the dissipated energy (G"), the loss of energy in each deformation cycle that describes the viscosity behavior of the material; and the modulus or tangent (G"/G'), describing the material's behavior (high values (›1) indicate liquid-like behavior, and low values indicate solid-like (<1) behavior) (BeMiller & Whistler, 2009; Waterschoot et al., 2015b). Gel resistance is determined using a texture analyzer, in which parameters such as peak force [N] define the resistance of the three-dimensional network (Ulbrich et al., 2015).

The main factors affecting the rheological properties of starches are their source and the presence of other polymers (Sarker et al., 2013; Schirmer et al., 2015). Many polymers coexist with starch in aqueous mixtures and interact in different ways to produce several attributes influencing the stability, texture and quality of food products. Starch paste viscosity is associated with lipids, mainly phospholipids, that complex with amylose and hinder or reduce the granule's swelling capacity. Other effects associated with paste viscosity are decreased amylose solubility, increased formation time and limited gelling properties. Amylose-lipid complexes require high temperatures for dissociation (Singh et al., 2003). Rheology is widely recognized for its effect on the quality of food and its sensory characteristics. The rheological properties of starch determine its potential application as a thickener or gelling agent (Berski et al., 2011). Determining the thermal properties of starch involves terms such as the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), difference between T_c and T_o, and enthalpy of fusion, all of which can be measured using equipment like a differential scanning calorimeter (DSC) (Kong et al., 2012) and depend on the starch concentration (Waterschoot et al., 2015b). During heating, T_o is the temperature at which the paste viscosity starts to increase; T_p is the maximum viscosity temperature; and T_c is the final temperature of the viscosity increment. (T_c-T_o) is a comparative measure, and an increase in this value indicates a high amount of granule modification in the amorphous and crystalline regions (Jenkins et al., 1993; Kong et al., 2012). Some researchers include retrogradation among the thermal properties of starch. This paste property typically begins 20 °C lower than its gelatinization temperature (T_{gel}), and retrogradation is proportional to the presence of amylopectin (Tan et al., 2006; Yuan et al., 1993). Variations in a starch's thermal properties after gelatinization and throughout refrigerated storage can be attributed to variations in the amylose/amylopectin ratio, the size and shape of the granule, and the presence or absence of lipids and proteins (Singh et al., 2003; Tan et al., 2006).

3. Starch modification

Starch is rarely consumed in its intact form and frequently used by industry in its native form. Most native starches are limited in their direct application because they are unstable with respect to changes in temperature, pH and shear forces. Native starches show a strong tendency for decomposition and retrogradation (Berski et al., 2011). Additionally, some starch granules are inert, insoluble in water at room temperature, highly resistant to enzymatic hydrolysis and consequently lacking in functional properties. Native starches are often modified to develop specific properties such as solubility, texture, adhesion and tolerance to the heating temperatures used in industrial processes (Singh et al., 2007; Sweedman et al., 2013).

Several methods have been developed to produce modified starches with a variety of characteristics and applications. All of these techniques alter the starch polymer, making it highly flexible and changing its physicochemical properties and structural attributes to increase its value for food and non-food industries (López et al., 2010). The starch modification industry is constantly evolving. Modifications of starch include physical, chemical and enzymatic methods (Yadav et al., 2013). Physical methods involve the use of heat and moisture, and chemical modifications introduce functional groups into the starch molecule using derivatization reactions (e.g., etherification, esterification, crosslinking) or involve breakdown reactions (e.g., hydrolysis and oxidation) (Singh et al., 2007; Wurzburg, 1986).

3.1. Physical modification of starch

Physical modifications of starch can improve its water solubility and reduce the size of the starch granules. Physical methods to treat the native granules include: different combinations of temperature, moisture, pressure, shear and irradiation. Physical modification of starch granules is simple, cheap and safe. These techniques do not require chemical or biological agents, and are therefore preferred when the product is intended for human consumption (Ashogbon & Akintayo, 2014). Table 2 gives examples of different research on the physical modification of starch.

Pre-gelatinized starch (PGS)

Pre-gelatinized starch (PGS) is starch that has undergone a cooking process until complete gelatinization and a simultaneous (or subsequent) drying process. Drying methods include drum drying, spray drying and extrusion. The main consequence of this treatment is the destruction of the granular structure, resulting in complete granular fragmentation, and the absence of birefringence properties. The principal properties of PGS are an increase in swelling capacity, solubility and cold water dispersion. PGS functionality depends on the cooking conditions, drying and the starch source (Ashogbon & Akintayo, 2014). Of the physically modified starches, PGS is primarily used as a thickener in many instantaneous products, such as baby food, soups and desserts, due to its ability to form pastes and dissolve in cold water. The use of PGS is preferred in sensible foods because it does not require heating to form a paste (Majzoobi et al., 2011).

Hydrothermal modification

This physical modification involves changes in the physical and chemical properties of the starch without destroying the granule structure (Zavareze & Dias, 2011). Physical modifications occur at temperatures above the T_g and below the T_{gel} of the starch granules (Tester & Debon, 2000). Essentially, hydrothermal modification can only occur when starch polymers transition from the amorphous region to the semicrystalline region. Starch in its native form exhibits amylopectin ramifications by forming a double helix chain, and this behavior imparts a crystalline structure to the starch molecule. Heat treatment involving temperatures between the T_g and melting temperature (T_m) may not alter the double helix conformation or degree of starch crystallinity; these conditions are present when starch pastes are drying. Physical modification of starch performance improves starch paste characteristics such as texture and plasticity by reducing T_g and consequently relaxing the hydrogen bonds and polymer-polymer interactions. The presence of water reduces the T_m (Conde-Petit et al., 2001). Hydrothermal modification is differentiated into annealing and hydrothermal treatments (Collado & Corke, 1999).

a) **Annealing (ANN)** is a physical treatment of starch granules in which the parameters of moisture, temperature and heating time determine the results obtained (Tester & Debon, 2000). The ANN process requires an excess of water (76% w/w) or an intermediate containing water (40% w/w) (Jacobs & Delcour, 1998). The objective of this treatment is to improve the molecular mobility. ANN is associated with a physical reorganization of the starch granule in the presence of water (Tester & Debon, 2000). Water is a suitable plasticizer for starch. Starch granule hydration causes a transition from a glassy to a static state, increasing the mobility of the amorphous regions to a crystalline state. These changes generate tangential and radial movements in the crystalline and amorphous regions, and physicochemical modifications increase chain interaction in the crystallinity region (Chen et al., 2014). The ANN process is associated with partial gelatinization of starch Tester &Debon (2000) state that the ANN term can only be used when the temperature of the process does not reach T_{gel} and therefore gelatinization does not occur. In ANN starch, the T_{gel} after modification is not less than the T_{gel} of the native starch. The ANN process has important industrial applications, imparting different characteristics to products due to an increase in starch granule size, thermal stability, T_{gel} and the availability of starch to digestion by enzymes such as α-amylase. However, there is no justification for its use in terms of energy and time because many cheaper chemical processes can modify starch properties rapidly and selectively, making them more competitive than ANN.

b) **Hydrothermal treatment (HMT).** This treatment includes a thermal application in the presence of a limited amount of water (typically less than 35% w/w) and a process time between 15 min to 16 h (Jacobs & Delcour, 1998). The effects of this treatment on the morphological and physicochemical

Table 2. Physical modification of starch.

Type of physical modification	Starch source	Parameters studied	Observed results	References
Pre-Gelatinization	Rice flour Rice starch	Temperature (100 °C) Time (20 min) Suspension[a] 35% (w/v) Storage (4 °C /7 days) Drying by lyophilization	The retrogradation process was delayed; rice flour was more susceptible to modification.	(Wu et al., 2010)
	Commercial wheat starch	Suspension[a] 10% (w/v) Drying by double drum: temperature (158 °C), pressure (5 bar)	Increased solubility and swelling capacity; viscosity initiated at 25°C[b].	(Majzoobi et al., 2011)
	Wheat starch	Suspension[a] 37% (w/v) Drying by drum: temperature (150 °C), pressure (5 bar)	Increased solubility, swelling capacity and viscosity[b].	(Li et al., 2014)
Hydrothermal modification				
Annealing	Water chestnuts (*Eleocharis dulcis*)	Temperature (65 °C) Time (24 h) Suspension[a] 35% (w/v)	Reduction of swelling capacity, solubility and viscosity[b].	(Yadav et al., 2013)
	Yam starch	Temperature (50 °C) Time (24 h) Suspension[a] 33% (w/v)	Reduction of granule density and swelling capacity[b].	(Falade & Ayetigbo, 2015)
	Polylactic acid (PLA) and commercial corn starch mixture	Suspension[a] 40% (w/v) Temperature (50, 60, 80, 100 and 120 °C)	Increase in crystallinity associated with the process temperature. Increased thermal stability and mechanical properties[b].	(Lv et al., 2015)
Hydrothermal Treatment (HTM)	Water chestnuts (*Eleocharis dulcis*)	Temperature (110 °C) Time (16 h) Suspension[a] 70, 75 and 80% (w/v)	Reduction of stability, solubility and viscosity of the starch paste[b].	(Yadav et al., 2013)
	Cassava (*Manihot esculenta* Crantz), Arrowroot (*Maranta arundinacea*), Sweet potato (*Ipomoea batata*)	Temperature (80, 100 and 120 °C) Time (6,10 and 14 h) Suspension[a] 50, 70 and 85% (w/v)	Reduction of paste clarity and swelling capacity of starches. Increase in solubility and stability[b].	(Jyothi et al., 2010)
	Potato Starch	Temperature (120 °C) Time (3 h) Suspension[a] 70, 75 and 80% (w/v) pH (5.6 and 6.5)	Reduction in swelling capacity of granules and amylose leaching. Acidity during treatment decreased the modification time and promoted acid hydrolysis in starch with lower levels of RS[b].	(Kim & Huber, 2013)
	Yellow sweet potato (*Ase jantan*) flour Purple sweet potato (*Ayamurasaki*) flour	Temperature (50 and 77 °C) Time (3 and 6 h) Suspension[a] 70% (w/v)	Increasing the operating temperature decreased the paste viscosity. HMT showed a reduced swelling capacity in the granules and amylose leaching[b].	(Putri et al., 2014)
	Rice Starch Rice flour	Temperature (100 °C) Time (16 h) Suspension[a] 70, 75 and 80% (w/v)	HMT primarily affected the paste properties of rice flour. The authors attributed these differences to presence of protein in the rice flour.	(Puncha-Arnon & Uttapap, 2013)
	Ginger and turmeric (Residues of supercritical fluid extraction-SFE)	Treatment assisted with high pressure using subcritical water and CO_2 Suspension[a] 33% (w/v) Pressure (150 bar) Time (11 min) Ginger: Temperature (200 °C) Turmeric: Temperatures (130, 150 and 180 °C)	Subcritical water was acidified by CO_2, producing hydrothermolysis. Modification of starch was monitored by sugar production (reducing and total reducing sugar).	(Moreschi et al., 2006)

[a]based on starch dry weight; [b]compared to native starch.

Table 2. Continued...

Type of physical modification	Starch source	Parameters studied	Observed results	References
Non-hydrothermal modification	Ginger (*Zingiber officinale* R.) Turmeric (*Curcuma longa* L.)	Treatment of SFE using CO_2 and a co-solvent. Ginger: Co-solvent (isopropyl alcohol, 1.5% v/v), temperature (35 °C), pressure (250 bar) Turmeric: Co-solvent (ethanol/isopropyl alcohol (1:1) mixture, 10% v/v), temperature (30 °C), pressure (300 bar).	SFE treatment did not alter granule morphology or amylose/amylopectin ratio. For ginger, the modification process reduced T_{gel} and the maximum viscosity. Turmeric starch showed the opposed behavior.	(Braga et al., 2006)
	Potato starch Wheat starch Corn starch Rice starch	Treatment using ultrasound Medium: Water and alcohol	Starches treated by ultrasound showed depolymerization of the granules. Modification of the paste properties[b]. Water was a better medium for starch modification.	(Sujka & Jamroz, 2013)
	Tapioca	Treatment with argon gas as a plasma Medium: Water Suspension[a] 40% (w/w)	During modification, two reactions occurred, crosslinking and depolymerization of the starch that may replace the chemical reactions.	(Wongsagonsup et al., 2014)

[a]based on starch dry weight; [b]compared to native starch.

properties of starch granules include important changes in crystalline structure, swelling capacity, gelatinization, paste properties and retrogradation (Hoover, 2010; Hormdok & Noomhorm, 2007; Jyothi et al., 2010). Structural and physicochemical changes generated by HMT are directly influenced by the botanical source of the starch granule with respect to its composition and organization of amylose and amylopectin. HMT is also used as a pre-treatment because of the structural modification into amorphous and crystalline regions on the granules. These alterations make the granule susceptible to chemical and enzymatic modifications and acid hydrolysis (Zavareze & Dias, 2011).

Non-thermal physical modification

Some processes in food production are applied to extend the life of a product using thermic treatments at boiling temperatures (or even higher) for seconds or minutes. Traditional treatments cause a loss of some vitamins and nutrients and alter their organoleptic properties. Non-thermal modification is an alternative to traditional processes that also eliminates pathogenic microorganisms and spores. Non-thermal techniques involve the use of high pressure, ultrasound, microwaves (Anderson & Guraya, 2006; Braşoveanu & Nemtanu, 2014; Hódsági et al., 2012; Mollekopf et al., 2011) and electric pulses. The high pressure technology in industry uses pressure from 400 to 900 MPa. High pressure generally restricts the swelling capacity and consequently decreases paste viscosity. Other technologies use pressure several times bellow atmospheric pressure (vacuum pressures); this technology uses gas in a plasma state and is the most recent technology used for starch granule modification (Deeyai et al., 2013; Wongsagonsup et al., 2014). Plasma is an ionized gas composed of several types of active ionic species: electrons, ions, excited atoms and protons (Bogaerts et al., 2002). For this treatment, the gases used include ethylene, hydrogen, oxygen, ammonia, air, methane or argon in a plasma state. This treatment modifies the starch in different ways, including its hygroscopicity, degree of polymerization and oxidation (Wongsagonsup et al., 2014).

Modification of starch using microwaves involves several interacting mechanisms, such as irradiation, furnace dimensions and the characteristics of the starch. In the microwave irradiation process, the most important parameters are moisture and temperature, which influence the dielectric properties of the starch (Braşoveanu & Nemtanu, 2014). Starch modification by microwaves results from the rearrangement of starch molecules that generates changes in solubility, swelling capacity, rheological behavior, T_{gel} and enthalpies (Iida et al., 2008; Yu et al., 2013; Zuo et al., 2012). Depending on the starch source and moisture, modification by microwave also produces variations in morphology and crystallinity in the granule (Braşoveanu & Nemtanu, 2014).

The use of ultrasound is also considered a physical modification treatment. This treatment is applied to starches in suspension and starches that have undergone previous gelatinization (Iida et al., 2008; Yu et al., 2013; Zuo et al., 2012). Ultrasound primarily affects the amorphous region, while maintaining the granule's shape and size. The starch surface becomes porous, and properties such as the swelling capacity, solubility and viscosity of the paste are modified (Luo et al., 2008). Ultrasonic modification depends on the sound, frequency, temperature, process time and the starch suspension properties (i.e., concentration and botanical source of starch) (Zuo et al., 2009).

Other physical modification methods: grinding and extrusion

Large-scale extraction of starch from cereals such as wheat require grinding. Substantial granule damage occurs during grinding, and shear forces that compress the granule structure are generated. Damage to the starch granule is visible as cracks

in the starch surface. Grinding reduces the crystallinity of the amylopectin molecule and its double helix conformation severely. Fragmentation of the amylopectin molecule eliminates restrictions on the swelling capacity, thereby facilitating subsequent gelatinization. The easy gelatinization of the starch granules after grinding decreases parameters such as T_0 and enthalpies in the final starch product.

Extrusion is defined as a process at high temperatures and in short amounts of time (HTST), wherein starch granules are subjected to mechanical shearing forces in a relatively low moisture environment (Camire et al., 1990). Extrusion increases the T_{gel} of starch, changing the molecular extension and its associations, such as the amylose-lipid complex structure. The extrusion process also affects starch digestibility and can reduce the RS content (Martínez et al., 2014).

3.2. Chemical modification of starch

Chemical modification involves the introduction of functional groups on the starch molecule without affecting the morphology or size distribution of the granules. Chemical modifications generate significant changes in starch behavior, gelatinization capacity, retrogradation and paste properties (López et al., 2010). Food and non-food industries expand starch properties and improve them through chemical modifications. Table 3 shows examples of different chemical modifications of starch.

Cationic starch

Cationic starches are generally produced by reacting starch with compounds containing tertiary or quaternary ammonium, imino, amino, sulfuric or phosphate groups. Free hydroxyl ions present in the native starch molecule are commonly altered using cationic monomers such as 2,3-epoxypropyl trimethyl ammonium chloride (ETMAC) or 3-chloro-2-hydroxypropyl trimethyl ammonium chloride (CTA) in dry or wet processes. In dry cationic modification, in the absence of a liquid phase, the reactive is sprayed onto the dry starch during extrusion. The semi-dry method for cationization involves a mixture of starch and spray reagent prior to the thermal treatment. Wet cationization includes a homogeneous reaction with dimethyl sulfoxide (DMSO) or a heterogeneous reaction in alkaline solution. The physicochemical properties of the starch and granular structure are altered after the cationic process, particularly when the process involves a high degree of substitution. The cationic reaction reduces the paste temperature, increases the viscosity peak and results in various changes in starches from different sources. Among modified starches, cationic starch materials are preferred by the textile industry because the positive charge introduced in the molecular chains conform to the electrostatic bonds between the negative charges of the cellulose fibers (Hubbe, 2007). There are several applications for cationic starches (i.e., in water treatment as flocculants and as additives in textile products, paper and cosmetics), preferred for their low cost, excellent fit, biocompatibility and rapid degradation (Zhang, 2001).

Cross-linked starch

Crosslinking of a polymer occurs when linear or branched chains are covalently interconnected and is known as cross-linking or cross-ligation. The reagents used form ether or ester bonds with hydroxyl groups in the starch molecules (Singh et al., 2007). This modification increases the polymer's rigidity by forming a three-dimensional network. Crosslinking in starch increases the degree of polymerization and molecular mass; starch molecules lose water solubility and become soluble in organic solvents. Several agents are used to crosslink native starch: sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), epichlorohydrin (ECH) and phosphoryl chloride ($POCl_3$), among others (Woo & Seib, 2002).

Depending on the reagent used for crosslinking, the final product is classified in one of three types: i) monostarch phosphate produced by starch esterification with orthophosphoric acid, potassium or sodium orthophosphoric or STPP; ii) distarch phosphate produced when native starch reacts with STMP or $POCl_3$; or iii) phosphated distarch phosphate, resulting from combined treatments of monostarch and distarch phosphates (Gunaratne & Corke, 2007; Jyothi et al., 2006).

The source of starch granule, methods and parameters used for crosslinking modification has an important influence on the properties of the final product. Starch properties affected by crosslinking modification include the paste clarity and swelling capacity. Some authors suggest that both properties are linked, and a reduction in swelling is responsible for the decrease in paste clarity (Kaur et al., 2006; Koo et al., 2010). The degree of crosslinking also reduces the moisture, lipids and proteins associated with the native starch granule; these changes are produced by all of the aforementioned crosslinking agents in different proportions (Carmona-Garcia et al., 2009). In the food industry, cross-linked starch is associated with formulations of frozen products due to its stabilizing, thickening, clarity and retrogradation resistance properties of the pastes formed. Its uses also extend to other industries such as plastics (López et al., 2010).

Acetylated starch

Acetylation is a modification of polymeric starch molecules through the introduction of functional acetylated groups (CH_3CO) that react with free hydroxyl groups present in the branched chains of the starch polymer to produce a specific ester (Sweedman et al., 2013). Acetylation is the more common chemical modification method, resulting in native starch esterification using reactive reagents such as anhydrous acetic acid, vinyl acetate or OSA in the presence of an alkaline catalyst (NaOH, KOH, $Ca(OH)_2$, Na_2CO_3) (Wang & Wang, 2002). Starch modified with OSA is an effective emulsifier used in the food, pharmaceutical and cosmetic industries; in this modification, OSA adds hydrophobic chains to the hydrophilic structure of starch (Chen et al., 2014). The introduction of acetyl groups reduces the resistance of bonds between the starch molecules. Acetylated starch increases the swelling capacity and solubility compared to native starch (Berski et al., 2011). The presence of hydrogen bonds in acetylated starch is restricted due to electrostatic repulsion forces on the starch molecule (Lawal &

Table 3. Chemical modifications of starch.

Type of chemical modification	Starch source	Parameters studied	Observed results	References
Cationization	Waxy maize starch	Esterification reaction. Chemical reactive: 2,3-epoxypropil trimethyl ammonium chloride (EDMAC). pH (9.0)	Reduction in starch granule size and fragmentation. Esterification reaction is produced on the granule surface[a].	(Liu et al., 2015)
	Commercial potato starch	Halogenation and amination reactions. Chemicals reactive to halogenation: Epichlorohydrin and hydrochloric acid. Temperature (100 °C) Time (1 h) Amination agents: putrescine, histamine, cadaverine and tyramine. Temperature (60, 80 and 100 °C) Time (4, 8 and 12 h)	Researchers studied the effects of amine groups in cationization reactions. Due to sensible amine structures, lower temperatures gave better results.	(Anthony & Sims, 2013)
Crosslinking	Oat starch	Chemical reactive: Sodium tripolyphosphate (STPP) and sodium phosphate (STMP)	Reduction in the amylose content due to alkaline conditions; increase in the swelling capacity of starch granules; viscosity increase at high temperatures; and rapid development of viscosity at cooling temperatures[a].	(Berski et al., 2011)
	Commercial starches from wheat, corn, oat, rice, banana and potato.	Starch suspension (≈33%, w/w) Chemical reactive: mixture of STMP:STPP (99:1) Temperature (25-70 °C) pH (10.5 -12.3) Time (5-24 h)	RS production was ≤ 100% in all studied materials. RS produced was less water-soluble at 95 °C and increased the T_{gel} and enthalpies of starch paste[a].	(Woo & Seib, 2002)
	Commercial corn and wheat starch	Starch suspension (50 g / 70 ml of water) 12% mixture of STMP:STPP (99:1) 10% sodium sulfate. Temperature (38-70 °C) pH (10-12) Time (3 h)	Researchers studied the application of parameters such as temperature and pH to produce RS. At higher pH, the RS production was increased for both starches.	(Kahraman et al., 2015)
Acetylation	Commercial potato starch	Pretreatment: gelatinization and retrogradation Chemical reactive: anhydrous acetic acid.	Increased resistance to enzymatic action over retrograded starch.	(Zięba et al., 2011)
	Commercial potato starch	Pretreatment: enzymatic hydrolysis and extrusion Chemical reactive: anhydrous acetic acid. Catalyst: sodium hydroxide.	Increase in solubility and decrease in swelling capacity caused by both pretreatments[a]. Acetylation effects were directly dependent on the pretreatment.	(Kapelko et al., 2013)
	Commercial corn starch	Hydrothermal pretreatment: Suspension[a] 65% (w/v) Temperature (48, 52, 57, 60 and 62 °C) Time (3 h) Chemical reactive: Octenyl succinic anhydride (OSA). Catalyst: Sodium hydroxide.	Pretreatment increased the swelling capacity of granules and the peak of viscosity. Decrease in paste temperature[a].	(Chen et al., 2014)
	Oat starch	Chemical reactive: anhydrous acetic acid Catalyst: Sodium hydroxide.	Reduction in amylose content, temperature of the paste and viscosity at high temperature. Rapid development of viscosity upon cooling[a].	(Berski et al., 2011)
	Commercial corn starch	Chemical modification Chemical reactive: OSA. Catalyst: Sodium hydroxide. Temperature (35 °C) Time (2 h)	Improved performance of starch for textile applications. Reduction in surface tension, impregnation, wetting and diffusion of starch in textile fibers[a].	(Zhang et al., 2014)

[a]when compared with native starch.

Table 3.Continued...

Type of chemical modification	Starch source	Parameters studied	Observed results	References
Acid hydrolysis	Wheat (30% starch) Potato (25% starch) Peas (38% starch)	Acid solutions: chlorhydric acid and sulfuric acid (0.36 and 0.72 N). Starch suspension: 40% (w/w, dry basis). Temperature (40 °C) Time (4 and 24 h)	Hydrolysis reduced granule size and paste viscosity. The amylopectin depolymerization increased the quantity of linear chains similar to amylose, favoring gel formation and its strength.	(Ulbrich et al., 2014)
	Sorghum (*Sorghum bicolor*)	Acid solution: chlorhydric acid (0.1 N). Starch suspension: 40% (w/w, dry basis). Temperature (50 °C) Time (1.5 h).	Granule surface was highly porous, reducing its swelling capacity[a].	(Ali & Hasnain, 2014)
	Amaranth starch	Acid solution: chlorhydric acid (0.5 N). Starch suspension: 66% (w/w, dry basis). Temperature (35 °C) Time (0-72 h).	Reduction in size and molecular mass of granules. Increase in solubility, and reduction in gel formation[a].	(Kong et al., 2012)
	Commercial wheat starch (87.5% starch, dry basis) Commercial potato starch (84.9% starch, dry basis) Commercial pea starch (89.3% starch, dry basis)	Acid solutions: chlorhydric acid and sulfuric acid (0.36 and 0.72 N). Starch suspension: 2.5% (w/w, dry basis). Temperature (21 °C) Time (4 and 24 h).	Modification of starch by acid hydrolysis was different for each source. Amylopectin depolymerization increased the quantity of linear chains similar to amylose, favoring gel formation and its strength.	(Ulbrich et al., 2015)
	Yam starch	Acid solution: chlorhydric acid (6%, w/v). Starch suspension: 1/2 (w/v, dry basis). Temperature (27 °C) Time (192 h). Neutralization with sodium hydroxide (10%, w/v)	Increase in granule density. Breakdown or fragmentation of granules. Increase in swelling capacity[a].	(Falade & Ayetigbo, 2015)
Oxidation	Sorghum (*Sorghum bicolor*)	Starch suspension: 35% (w/w, dry basis). Chemical reactive: sodium hypochlorite. Catalyst: Sodium hydroxide. pH (9.5) Time (10 min)	Decrease in swelling capacity and viscosity of the paste[a].	(Ali & Hasnain, 2014)
	Commercial potato starch	Comparison between microwave use and conventional oxidation. Chemical reactive: hydrogen peroxide. Catalyst: sodium tungstate ($Na_2WO_4 \bullet 2H_2O$)	Decrease in the viscoelastic properties of the paste due to oxidation. Microwave uses are not significantly difference from the conventional method.	(Ptaszek et al., 2013)
	Oat Starch	Chemical reactive: sodium hypochlorite.	Apparent increase in linear molecules due to amylopectin depolymerization. Reduction in viscosity at high temperature and faster development of viscosity upon cooling[a].	(Berski et al., 2011)
Dual modifications	Sorghum (*Sorghum bicolor*)	Chemical modifications: oxidation and acetylation.	Increase in paste clarity[a].	(Ali & Hasnain, 2014)
	Commercial corn starch	Chemical modifications: oxidation and alkali treatment. Chemical reactive for oxidation: sodium hypochlorite. Alkaline reactive: sodium hydroxide.	Combined treatments generated several damages to the surfaces of the starch granules, making them more susceptible to enzymatic hydrolysis.	(Spier et al., 2013)
	Wheat starch	Chemical reactive: mixtures of i) succinic acid and acetic anhydride and ii) Azelaic acid and acetic anhydride	Decreased T_{gel} and retrogradation tendency. Increase in paste stability during storage.	(Šubarić et al., 2012)
	Potato Potato peel (industrial residue)	Application of ultrasound and microwave. Medium of dispersion: i) neutral water, ii) acidified water (sulfuric acid, 3 M) and iii) alkaline water (sodium hydroxide, 1 M)	Starch depolymerization was quantified by reducing sugar production. Ultrasound method in acidified water showed the highest degree of depolymerization.	(Hernoux et al., 2013)

[a]when compared with native starch.

Adebowale, 2005). In acetylated starch, hydroxyl groups and anhydrous glucose have been converted to acetylated groups (Huang et al., 2010). Acetylated starch with a low degree of substitution (0.01-0.2) has several applications in conforming films, adherents, thickeners, stabilizers, texturizers and encapsulation agents (Elomaa et al., 2004). Bello-Pérez et al. (2000) studied the acetylation process in banana starches. Acetylation modified the starch granule, decreasing the retrogradation tendency and increasing the solubility and swelling capacity of banana starch considerably compared to native starch. Acetylated banana starch also increased the paste viscosity.

Other methods of chemical modification

The oldest chemical modification technique is acid modification. Products of acid modification have several applications and uses in the food, paper, textile and pharmaceutical industries (Hoover, 2000). Acid modification methods involve the application of acidic solutions (commonly HCl and H_2SO_4) to form a concentrated paste (35–40% of solids) at a temperature below T_g for a specific duration depending on the desired viscosity or conversion degree (Amaya-Llano et al., 2008; Thirathumthavorn & Charoenrein, 2005). The mechanism of acid modification is also known as acid hydrolysis (Amaya-Llano et al., 2008). Hydrolysis is produced randomly, breaking the α-1,4 and α-1,6 links and shortening the polymeric chains. Acid hydrolysis of starch develops in two stages: an early stage in which hydrolysis preferentially attacks the amorphous regions of granules at a high reaction rate and a subsequent stage in which hydrolysis occurs in the crystalline region at a slower rate (Wang & Wang, 2001). The hydrolysis rate and starch modification are in proportion to the amylose:amylopectin ratio, as well as to the size and conformation of granules (Hoover, 2000). After acid hydrolysis, the molecular mass of the starch granules decreases, and its crystallinity increases (Zuo et al., 2014). Singh & Ali (2000) studied the influence of various acids used in starch modification (HCl, HNO_3, H_2SO_4, H_3PO_4); the starch sources were wheat, corn, peas, tapioca and potato. These authors concluded that H_3PO_4 produced a lower hydrolysis rate, whereas HCl and HNO_3 resulted in a higher reduction in molecular mass and consequently a higher hydrolysis rate. Acid hydrolysis reduces the amylose content in starch granules, and this reflects an increase in paste temperature and gelatinization enthalpies (Lawal, 2004). The use of dilute acid solutions for starch modification improves gel consistency and reduces paste viscosity due to depolymerization of the starch granule (Pérez & Bertoft, 2010; Ulbrich et al., 2015).

Another important process for starch modification is oxidation, a process in which functional groups such as carboxyl and carbonyl groups are introduced in the starch molecule and depolymerize the molecule (Kuakpetoon & Wang, 2001). During oxidation, it is important to maintain the appropriate parameters, such as temperature and pH. The reactive oxidants used include hydrogen peroxide, per-acetic acid, potassium permanganate, sodium hypochlorite, chromic acid and nitrogen dioxide (Sánchez-Rivera et al., 2005; Sandhu et al., 2008; Wang & Wang, 2003). In recent years, starch modified by oxidation has had great use in the food industry to form adherent surfaces and coatings (Lawal et al., 2005).

Dual modifications

Dual modifications include chemical modifications and different types of modifications combined. Dual modifications have been used in industry to optimize modified starch functionality (Ashogbon & Akintayo, 2014). This new approach involves the combination of chemical and physical agents (e.g., acetylation assisted by microwave, phosphorylation assisted by high pressures). Specifically, dual chemical modifications involve two processes of chemical modification (e.g., acetylation/oxidation, crosslinking/acetylation, crosslinking/hydroxypropylation) (Adebowale & Lawal, 2002; Carmona-Garcia et al., 2009; Huang et al., 2010). Starches modified by two chemical methods, such as emulsifiers, agglutinants and thickeners, are commonly used in the food industry and are included as adsorbents of heavy metals in the non-food industry (Ashogbon & Akintayo, 2014).

4. Resistant starch (RS)

Starch is quantitatively the major source of energy in the human diet. Starch digestibility is attributed to the interaction of several factors, including the vegetal source, granule size, amylose/amylopectin ratio, degree of molecular association between components, degree of crystallinity, amylose chain length and presence of amylose-lipid complexes on starch granules (Cummings & Englyst, 1995). Lipids are fatty acids specifically interacting with amylose to form complexes and reduce starch digestibility because their presence decreases enzymatic hydrolysis by amylase (Taylor et al., 2015).

Until recently, starch was considered completely digested after cooking. This concept has been challenged by observations that some of starch crosses the colon, where it is subject to bacterial fermentations. Today, this indigestible starch is recognized as part of the dietary fiber fraction of food (Conde-Petit et al., 2001). Most polysaccharides of interest for nutrition (e.g., starch, dextrin, glycogen and cellulose) are unions of glucose units, differing only in the type of linkage. As a group, polysaccharides may contain monosaccharides in addition to glucose, either alone or combined.

Starch digestion begins in the mouth, with α-amylase enzymes present in saliva. Enzyme activity is partially preserved until reaching the stomach. However, most starch is digested in the small intestine by enzymes from the pancreas. Degradation products of amylose are maltose and maltotriose, whereas amylopectin degradation produces dextrins and oligomers formed by α-1,6 linkages. Until the end of the intestine is reached, all of these polymers are degraded to glucose by enzymes such as α-glycosidase and oligo-α-1,6-glucosidase. Glucose absorption is followed by an immediate increase in glucoses levels in blood (Perera et al., 2010).

Factors that affect starch digestibility include the structural characteristics of the starch (i.e., the amylose:amylopectin ratio, degree of gelatinization, retrogradation and formation of amylose complexes), the structural characteristics of the food and the presence of other components such as soluble dietary fiber (Conde-Petit et al., 2001). RS is defined as the sum of starch or the sum of starch degradation products that are not absorbed by the small intestine in healthy individuals (Champ et al., 2003).

RS is divided into five types that have been substantially affected by the transformation process (Homayouni et al., 2014). The first group (RS I) is the product of treatments in which starch is physically inaccessible and the breakdown of the granular structure does not occur (Hasjim & Jane, 2009). The second group, RS II, consists of gelatinized starch (i.e., the starch has lost its crystalline conformation and is composed primarily of amylose); this type is very common in most starchy foods (Fuentes-Zaragoza et al., 2011). On the other hand, RS III is formed during starch retrogradation, which occurs after manufacturing in the presence of water, cooling and storage (Sanz et al., 2009; Yao et al., 2009). Chemical modifications to produce gelling and emulsification agents result in RS IV. Starch containing amylose-lipid complexes and requiring high temperatures of gelatinization are recognized as RS V, which is water insoluble (Cummings & Englyst, 1995; Jiang et al., 2010).

The RS is undigested starch that reaches the end of the digestive system, where it is the substrate of fecal microflora. Fermentation products from the RS are short chain fatty acids with different physiological and probiotic effects (Conde-Petit et al., 2001). The scientific interest in RS has increased significantly in recent decades because of its capacity to produce high levels of butyrate throughout the colon (Fuentes-Zaragoza et al., 2011). Butyrate is the most important energy source for colonocytes and has demonstrated beneficial effects on metabolism and cell growth; it also inhibits a variety of factors that propagate the initiation, progression and growth of colon tumors (Champ et al., 2003). The RS associated with small chains of fructooligosaccharides act synergistically in the digestive system to cause a prebiotic effect that benefits human health (Fuentes-Zaragoza et al., 2011).

5. Unconventional starches

The overall starch market is continually expanding, and the current demand is covered by four conventional sources: wheat, corn, potato and cassava. There are significant differences in the starch properties of these conventional groups in addition to the differences in their amylose-amylopectin ratios and the characteristics of these molecules. Non-amylosic components such proteins, lipids and phosphate groups are also important differences in the characteristics of conventional and unconventional starches.

Emmambux & Taylor (2013) studied the starch properties of cereals, legumes and tubers grown in Africa. The starch granules of certain cereals and beans possessed the common characteristics of small size, slightly porous surfaces and special paste properties, making them an interesting alternative for industry. These properties suggest treatments involving shearing operations or mimetic agents of fats because of their organoleptic characteristics and texture (D'Silva et al., 2011; Wokadala et al., 2012). Starch from beans (*Vigna unguiculata*) had a very high degree of retrogradation, which makes it suitable for incorporation into gluten-free pastes because this feature helps to maintain the texture of the product.

Zhang et al. (2005) studied the production, physicochemical properties and digestibility of banana starch. Pulp in immature bananas contains between 70-80% starch (dry basis); this amount is comparable to starch in the endosperm of corn grains and potato pulp. Banana starch is resistant to digestive enzymes (Faisant et al., 1995), which makes its use viable and competitive in a market of low-carb food consumption and products with reduced calories.

Almeida et al. (2013) studied the use of unconventional starches and commercial starches in the manufacturing of English cake and compared their behavior in this product. Unconventional starches incorporated into this formulation increased the sensory quality of the product. Starch from beans, followed by Peruvian carrots, yielded better results compared to commercial starch in terms of the technological quality of the paste during beating. Starch present in chickpeas and beans showed similar characteristics to that of commercial starch in terms of sensory properties, texture and moisture.

Today, chemical modifications of starch remain necessary. The industry requires these chemical processes to meet the demand of consumers favoring natural products. A simple way to impact starch properties is by mixing different types of native and/or physically modified starch (Sandhu et al., 2010; Waterschoot et al., 2015b). The use of these mixtures, as well as enabling improvements in the starch properties and pastes, also provides economic advantages when the replacement starch source is cheaper than the conventional source. However, there are limited studies on the physicochemical properties and functionality of starch mixtures (Waterschoot et al., 2015b) and the use of unconventional starches.

Currently, unconventional starches are often ignored or wasted during the isolation or separation of bioactive compounds from raw materials such as seeds and legumes (Braga et al., 2006; Yuan et al., 2007). These starches are subjected to unit operations that often involve thermal or hydrothermal treatments, causing alterations in the structural characteristics of the starch and physical modifications and improving the physicochemical properties and characteristics of the paste conformation. Additionally, raw materials from the extraction processes remain in the extraction residues including the starch fraction and a small fraction of bioactive compounds, which increase the technological and nutritive value (Braga et al., 2006; Santana & Meireles, 2014). Starches present in legumes, rhizomes, herbs and seeds are considered unconventional and may be used as ingredients in the same manner as starches from cereals and tuber due to their similar physicochemical and functional properties. These properties are improved by modification treatments and may be used to develop new processes and consequently new products (Santana & Meireles, 2014).

6. Starch applications in the food industry

The biological function of starch in plants is as a reserve of carbon and energy. As food, starch is the most abundant and important digestible polysaccharide. The starches in food are commonly derived from grains or seeds (wheat, corn, rice, and barley), tubers (potato) and roots (cassava) (Buléon et al., 1998; Waterschoot et al., 2015a). Starch provides 70 - 80% of the calories consumed by humans worldwide.

As food, starch functions as a structural agent because of the modifications introduced during manufacturing. Starch is used in the food industry mainly as a modifier of texture, viscosity, adhesion, moisture retention, gel formation and films (Waterschoot et al., 2015a).

An important utilization of starch in the food industry is in baking flour. Among the bakery products, cakes and breads are the most important due to their high consumption. In the formulations of the baking industry, starch is one of the components responsible for the structure and properties of the final products. Other industrial processes include starch in small quantities as a food additive or a thickening and gelling agent.

Starch is often used in granular form and is thus is included in the confectionery industry as a molding powder for the various forms of sweets, which can be reused many times. Starch is also used in the preparation of diverse types of pasta in the preparation of noodles and those intended for extrusion and in the formulation of instant foods and fried foods.

In the food industry, edible films are barriers that prevent moisture transfer, gas exchange, oxidation and the movement of solutes, while maintaining their organoleptic properties (Dhall, 2013). During manufacturing, films are incorporated as plasticizers, flavors, colors, sweeteners, antioxidants and antimicrobials. Edible films have received much attention due to their advantages over synthetic films. Edible films are produced from renewable materials; they can be consumed together with coated food and otherwise do not contribute to pollution because their degradation is faster than synthetic films. Their main disadvantage lies in their mechanical and permeable properties (Bourtoom, 2008). The basic materials used to produce edible films are cellulose, starch, gums and chitosan; the linear configuration of polymers can produce films with flexible, transparent and oil resistant properties. For these reasons, amylose is the most important fraction in starch granules. Typically, the starch granule is composed of 25% amylose and 75% amylopectin. Edible films require starches with a high amylose content (≥ 70%). The amylopectin molecule cannot adequately form films; the branched structure imparts poor mechanical properties to the film, reducing its tensile strength and elongation (Bourtoom, 2008; Dhall, 2013).

Polysaccharides are typically hygroscopic and therefore are poor barriers to moisture and gas exchange. The use of plasticizers in the film composition improves the barrier against moisture exchange and restricts microbial activity. The starch used in edible film preparation is incorporated to partially or completely replace the plastic polymers. Native starch does not produce films with adequate mechanical properties and requires pretreatment, the use of a plasticizer, mixture with other materials, genetic or chemical modification, or a combination of these treatments. Among the plasticizers, for hydrophilic polymers, such as starch, are glycerol and other low-molecular weight polyhydroxy-compounds, polyether, and urea. Processes such as extrusion adjust the parameters of temperature and mechanical energy over the starch paste, making it a thermoplastic material that is also suitable for the production of edible films (Dhall, 2013).

7. Starch destined for non-food applications

New processing techniques and the current demands of biodegradable and renewable resources have highlighted the versatility of starch and introduced it to new markets. Furthermore, starch is a chemical feedstock for conversion into numerous products with considerable value (Ellis et al., 1998).

In the pharmaceutical industry, starch is used as an excipient, a type of bonding agent to active drugs. Because of its content of amylose, starch is capable of forming an inclusion complex with many food ingredients, such as essential oils, fatty acids and flavoring ingredients. It therefore acts as an encapsulant and increases the shelf life of products.

Plastics obtained from oil are being replaced by natural polymers; starch is known for its ability to form films in food packaging applications (Jiménez et al., 2012). Edible and biodegradable starch films can be obtained from native starch or its components amylose or amylopectin by two main techniques: a wet method that includes a starch suspension and posterior drying or a dry method that involves a thermoplastic process (Paes et al., 2008). Modified starches can also be used in film production (Bourtoom, 2008; Campos et al., 2011; Dhall, 2013; López et al., 2010)

For new industrial applications of starch, especially in plastic polymer production, the hygroscopicity of starch is a disadvantage because the main feature of plastics films is their hydrophobic property. Starch granule size, its form and associated molecules influence film production. Wheat starch is typically associated with a significant amount of protein, which may result in a Maillard reaction and cause bleaching; therefore, this type of starch is not used in to manufacture biodegradable plastics films (Ellis et al., 1998).

In the textile industry, starch films are also used during textile production as fiber coatings. Native starch forms rigid and brittle films due to its cyclic structure. Brittle films are not advantageous because they reduce protection, increase friction and thus damage the thread. The polarity of native starch minimizes the adhesion of synthetic fibers, affecting the tensile strength and abrasion. Starch is commonly modified to improve the physical properties, emulsifying ability and film formation (Zhang et al., 2014).

Many industrial processes use starch after partial or complete destruction of its structure. When this occurs, the properties of its components and the relationships between them increase their importance. Differences between the amount and type of lipids originally present in the native starch may cause two starches with the same amylose-amylopectin ratio to have different physical properties, such as viscosity (Ellis et al., 1998).

Starch solutions are viscous, and the ability of starch to change the viscosity of other solutions and pastes is well known and exploited in the food industry. This property is also used in the oil drilling industry, where starch is used to adjust the viscosity of the mud used during drilling operations. Highly viscous starch solutions are desirable for industrial processes involving starch pastes for mechanical manipulation, such as the paper, corrugated and textile industries (Ellis et al., 1998).

Several studies have concluded that is possible to produce a new generation of detergents in which the surfactants and bleaching components are derived entirely from starch. An estimated 50 to 60% of chemical products in formulations for powder detergents and 65 to 75% of liquid detergent formulations could be substituted with products derived from starch.

High viscosity is important in the adhesive field. Most native starches do not maintain a stable viscosity when transformed to pastes and or subjected to high shear velocity or longer heating periods. However, chemically modified starch behaves properly under these conditions (Ellis et al., 1998).

The production of biodegradable plastics is still young when compared to the petrochemical plastic industry. Starch will play an important role in its growth in container production and in the form of biodegradable materials that conform to suitable matrices because it is a relatively inexpensive material compared to other polymers (Bourtoom, 2008; Dhall, 2013).

In recent years, starch has been studied for the production of nanoelements as nanocrystals that result from the breakdown of the amorphous region in semicrystalline starch granules by acid hydrolysis or for the production of nanoparticles from gelatinized starch (Le Corre et al., 2010). These nano compounds have unique properties due to their nano size compared to conventional size materials. Nanoparticles can be used as fill material in filtration and form effective barriers in flexible packaging (Bondeson et al., 2006).

8. Conclusions, perspectives and future trends

Starch has a major role in the food industry not only in for its nutritional value, but also for its broad technological functionality. The amylose and amylopectin polymers, lipids, proteins and phosphorus present in granules have significant effects on the physicochemical properties and functionality of starch.

Starch is rarely consumed in its native form; this form is also not commonly used in industry because native starches have restricted solubility in water, which limits industrial applications. Several methods have been developed for the production of modified starch, with a variety of features and applications.

Modification processes can greatly improve the characteristics of native starch by altering its physicochemical properties and structural attributes and increasing its technological value. Starch characteristics depend on the modification used and are necessary for its use in industry; they include cold water solubility, viscosity and swelling capacity after cooking, retrogradation tendency, loss of structural order after gelatinization and consequent syneresis of systems conformed by starch. The industry of starch modification is constantly evolving. Starch is a highly flexible polymer, and there are several ways to modify its structure and obtain a functional product with adequate properties for specific industrial applications, increasing its added value.

Physical modification of starch can enhance its water solubility and reduce the size of the starch granules. Physical methods for the treatment of native granules include combinations of temperature and moisture, pressure, shear and irradiation. Physical modification is simple, inexpensive and safe. These modification techniques are preferred because they do not require chemical or biological agents that may be harmful to health.

Physical modifications of starch consist of three categories. i) PGS is produced by cooking until complete gelatinization with subsequent drying, which destroys the granular structure and increases the swelling capacity, solubility, viscosity and dispersion capacity in cold water. ii) Hydrothermal modifications do not destroy the granular structure and occur at temperatures above T_g and below T_{gel} of the granule (these parameters are different for each botanical source) in the presence of water. Two processes for the latter modification were considered: ANN, which requires water in intermediate quantities or excess (40-76%, w/w), and HTM, which uses a restricted water content in a dispersion medium (≤ 35%, w/w). Hydrothermal modifications reduce starch solubility, swelling capacity and amylose leaching and increase the crystallinity and T_{gel} of the starch granules. In starch pastes, modifications reduce the viscosity and increase the stability. iii) Physical non-thermal modifications are preserve the quality of nutrients that may be contained in the starch paste and are susceptible to heat. The use of high pressure reduces the swelling capacity and viscosity of starch; vacuum pressures have the opposite effect, increasing the swelling capacity and reducing the degree of polymerization. Microwaves modify the dielectric properties of starch and its morphology and crystallinity, and ultrasound modifies the swelling capacity of granules and pastes. Industrial processes such as grinding and extrusion also physically modify starch granules.

Chemical modification of starch involves the introduction of functional groups to the starch molecule without affecting the morphology or granule size distribution. Cationization modifies the dielectric properties of granules depending on their substitution degree, reducing the paste temperature and increasing its viscosity. Crosslinking of polymeric chains increases the degree of polymerization in starch granules, modifying its solubility in organic solvents and reducing its swelling capacity. Acetylation results in the esterification of starch, increasing its swelling capacity and solubility. Other chemical modifications, such as acid hydrolysis and oxidation, reduce the degree of polymerization of starch and the paste viscosity.

Not all starch is digestible, and the indigestible portion is part of the fraction of dietetic fiber or RS. Chemical modifications such as crosslinking are used to increase the amount of RS, and these starches are included in paper and textile processes.

The starch industry is in constant expansion, and modification processes increase its versatility. When starch is physically or chemically modified, it can be adapted for different purposes in food and/or non-food industries. Applications of starch modifications (physical or chemical) can increase the use of unconventional starches and vegetal residues containing starch in industry. Depending on cost and accessibility, the use of conventional starch can be replaced in whole or in part by unconventional starches in industrial processes when appropriate. Determining the required characteristics of starch for each process is necessary to select the best modification method according to the application requirements, market trends, availability, structural characteristics and cost.

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Acronyms: Glass transition temperature (T_g), Resistant Starch (RS), Rapid Visco Analyzer (RVA), Onset temperature (T_o), Peak temperature (T_p), Conclusion temperature (T_c), Differential Scanning Calorimeter (DSC), Gelatinization temperature (T_{gel}), Supercritical fluid extraction (SFE), Pre-gelatinized starch (PGS), Melting temperature (T_m), Annealing (ANN), 2,3-epoxypropil trimethyl ammonium chloride (EDMAC), Sodium tripolyphosphate (STPP), Sodium phosphate (STMP), Octenyl succinic anhydride (OSA), 2,3-epoxypropyl trimethyl ammonium chloride (ETMAC), 3-chloro-2-hydroxypropyl trimethyl ammonium chloride (CTA), Dimethyl sulfoxide (DMSO), Sodium trimetaphosphate (STMP), Sodium tripolyphosphate (STPP), Epichlorohydrin (ECH), Phosphoryl chloride ($POCl_3$).

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- CAPÍTULO 4 –

Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation

Neste capítulo discute-se o uso das sementes de urucum para a obtenção de bixina. As sementes com reduzido teor lipídico foram moídas e classificadas em função do tamanho de partícula a fim de se avaliar o desempenho da obtenção do pigmento. A matéria-prima foi separada mecanicamente; as partículas foram classificadas em grossas (d_p > 300 µm) e finas ($d_p \leq$ 300 µm). Em seguida foi realizada a extração de bixina com solvente a baixa pressão a partir das partículas menores. A análise econômica classificou ambas as técnicas como um processo integrado em que o método mecânico pode ser usado para a aquisição de um produto com alto valor agregado.

Artigo que será submetido após revisão.

Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation

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ABSTRACT

This work involves the application of physical separation methods in order to concentrate the pigment of semi-defatted annatto seeds (DAS), a noble vegetal biomass rich in bixin pigments. DAS are the residue produced after the extraction of the lipid fraction from annatto seeds using supercritical fluid extraction (SFE). Physical methods such as i) mechanical fractionation method (MFM) and ii) integrated process of MFM and low pressure solvent extraction (LPSE) were studied. An integrated process named MFM+LPSE was proposed for processing two different semi-defatted annatto material denominated batches 1 and 2. The cost of manufacture (COM) was calculated for two different production scales (5 and 50 L) considering the MFM+LPSE integrated process and on the other hand only considering the MFM. The integrated MFM+LPSE process showed a significantly higher COM than MFM. This work suggests the MFM as an adequate and minor cost process to obtain a rich-pigment product from DAS.

Keywords: *Bixa orellana* L.; Bixin; Biocompounds recovery; Mechanical separation; Low pressure solvent extraction; Cost of manufacture.

1. Introduction

Annatto (*Bixa orellana* L.) is a native shrub from South America, used as cosmetic and ingredient on traditional food since pre-Columbian time. Annatto is also cultivated in Central America, Africa and the South of Asia (Smith & Wallin, 2006). Annatto pigments are widely used in food, chemical, cosmetic and pharmaceutical industries (Chisté et al., 2011). Nowadays, besides being used as coloring agents, annatto pigments are being investigated due to their biological activity and to the possible health benefits (Giorgi et al., 2013).

Annatto seeds are typically composed of 2.0 to 4.8% of lipids, 1.0 to 6.3% of pigments, 9.6 to 13.3% of moisture, 12.1 to 17% of proteins, 5.4 to 6.9% of ash and approximately 50% of total carbohydrates (starch and lignocellulosic material) (Albuquerque & Meireles, 2012; Silva et al., 2008). Annatto seeds contain carotenoids and phenolic compounds (Cardarelli et al., 2008), saponins and tannins (Vilar et al., 2014).

The major coloring matter in annatto seeds is the apocarotenoid bixin, a dicarboxylic monomethyl ester (9'-cis-6,6'-diapocarotenoid-6,6'-dioato methyl hydrogen), derived from 9'-cis-norbixina dicarboxylic acid (Scotter et al., 1998). Bixin represents more than 80% of colorant substances content in annatto seeds. Bixin is normally present in their cis- form, in minor proportion are present trans-bixin, cis-norbixin and trans-norbixin forms (Preston & Rickard, 1980). Depending of the pigment concentration, annatto pigments color ranges between yellow to red (Castro et al., 2011). Total content of pigment present in seeds varies according to the variety, culture, and the pre or post-harvest technique used (Smith & Wallin, 2006). Annatto pigments can be separated from seeds by various techniques, including immersion in hot vegetal oil, dilute alkaline solutions and other organic solvents (Scotter et al., 1998; Smith & Wallin, 2006; Vilar et al., 2014).

Industrial processes of annatto pigment extraction commonly use alkaline solutions of sodium hydroxide and potassium hydroxide. In the industrial process, bixin pigment reacts with the basic solution modifying its structure to norbixin. Norbixin and bixin are chemically similar, although norbixin is yellowish; they have different solubility, stereochemistry and stability (Scotter et al., 1998). Generally, bixin extraction techniques produce a crude concentrate with low quantity of bixin. A key factor for bixin extraction process is to obtain an increase in the bixin yield as well as minimize contamination by sub-products that affect the extract composition, which harms the stability and power of coloring (Scotter, 2009). The extraction involving the use of solvents is considered an indirect extraction method. The microstructure of vegetal matrix is a complex. Is formed by cells, intracellular and capillary spaces and pores, therefore, the extraction is influenced by the molecular structure of the solute, molecular size, location and its ligation with other components (Veggi et al., 2011). The LPSE technique is a technique used in chemical industry, also called leaching, decoction or elution (Wang & Weller, 2006). The solid-liquid extraction process is conducted at atmospheric pressure, placing the solvent in contact with the material to be extracted. After extraction, the solvent is removed and the extract is concentrated (Leal et al., 2010; Rodrigues et al., 2014; Santos et al., 2012).

However, in today's market, processes to obtain pigment-rich products must be not only efficient but also relatively cheap to be competitive. Factors such as performance (obtain as much product as possible), productivity (require the least amount of processing time) and selectivity (obtain a product rich in the substance of interest) should be considered when determining the economic viability of a process (Prado et al., 2011). On the other hand, integration of processes could be an attractive approach for the production of valuable products of higher quality at lower costs (Cardenas-Toro et al., 2015; Fujii, 2012; Mendes et al., 2009).

Supercritical fluid extraction (SFE) removes mostly the lipid fraction present in annatto seeds without extract a significant amount of pigment (Albuquerque & Meireles, 2012; Moraes et al., 2015). The raw material for this work was the semi-defatted annatto seeds (DAS). DAS are the residue produced after the extraction of the lipid fraction from annatto seeds using SFE. Reutilization constitutes a viable alternative to revalorize industrial residues and to reduce their contaminant capacity (Díaz et al., 2013; Galanakis, 2013).

Therefore, the aim of this study was to generate an alternative method for annatto pigment extraction through the use of clean technologies leading to concentrate the pigment from DAS. The pigment concentration was conducted using four phases: i) a preliminary study to establish the best sequence of operations over integer DAS; from this preliminary study, the MFM process was established; ii) a first extraction study on fine particles (FP) fraction integrating the LPSE to MFM process in order to identify significant parameters in the solvent extraction process; iii) a second study of pigment extraction in order to optimize the integrated process and finally, (iv) the process economic feasibility was evaluated, comparing MFM and MFM+LPSE.

2. Material and methods

2.1. Raw material

The raw material used in this work – denominated Batch1 and Batch 2 – was produced from previous work done in our laboratory by Albuquerque and Meireles (2012) and Silva et al. (2015), respectively.

Annatto seeds (AS) from Batch 1 were donated by the Agronomic Institute of Campinas (IAC - Instituto Agronômico de Campinas, São Paulo, Brazil) in 2012 and Batch 2 was obtained from Estação dos Grãos Ltda. (São Paulo, Brazil) in 2014. AS were partially defatted by SFE using CO_2 (99.9% of purity, Gama Gases Especiais Ltda., São Bernardo do Campo, Brazil) in a commercial SFE-2x5LF-2-FMC system (Thar Technologies, Pittsburgh,

USA) obtaining semi-defatted annatto seeds (DAS). Conditions of SFE extraction were temperature of 40 °C and pressure of 200 bar, and these parameters were determined by Albuquerque and Meireles (2012) for the extraction of the annatto lipid phase with a high content of γ e δ- tocotrienols.

For Batch 1, the SFE was performed using a solvent mass to feed mass ratio (S/F) of 3.5 suggested as the best condition for annatto lipid fraction extraction, condition in which a high content of bixin in DAS was maintained (Albuquerque & Meireles, 2012). For Batch 2, a S/F of 11 was used, with the objective of extracting the highest amount of lipid fraction from AS, with subsequent studies about formation of emulsions (Silva et al., 2015). The DAS were storage at freezing temperature (-18 °C) and protected from light before use as the raw material in this work.

2.2. Raw material characterization

The AS and DAS were characterized in terms of moisture (Sluiter et al., 2008), lipids (Thiex et al., 2003) and bixin (Smith & Wallin, 2006) content. Other materials content (carbohydrates, proteins and ash) was calculated by difference. The raw material was milled using a knife mill (TECNAL, Piracicaba, Brazil). A sieve (CATEL, Piracicaba, Brazil) was used to separate the fractions according to the diameter of particle (d_p). Sieves used correspond to 16, 24, 32 and 48 Mesh, Taylor Standard (d_p of 1000, 700, 500 and 300 μm, respectively). The material was classified according the particle size. Particles with diameter lower than 48 Mesh ($d_p \leq 300$ μm) were named FP and the particles with diameter larger than 48 Mesh ($d_p > 300$ μm) were named large particles (LP).

2.3. Preliminary study of the DAS

A preliminary study was conducted only with DAS from Batch 1. Experiment runs for extraction of the pigment from DAS were performed using LPSE technique. The paths followed for this study are shown in Figure 1.

Path A followed the operations of mill and sieving of DAS; path B and C include the LPSE technique for pigment extraction from DAS. Path B proposed the LPSE process using milled DAS as raw material, whereas in path C proposed the LPSE process over integer DAS and only after LPSE the annatto seeds are milled.

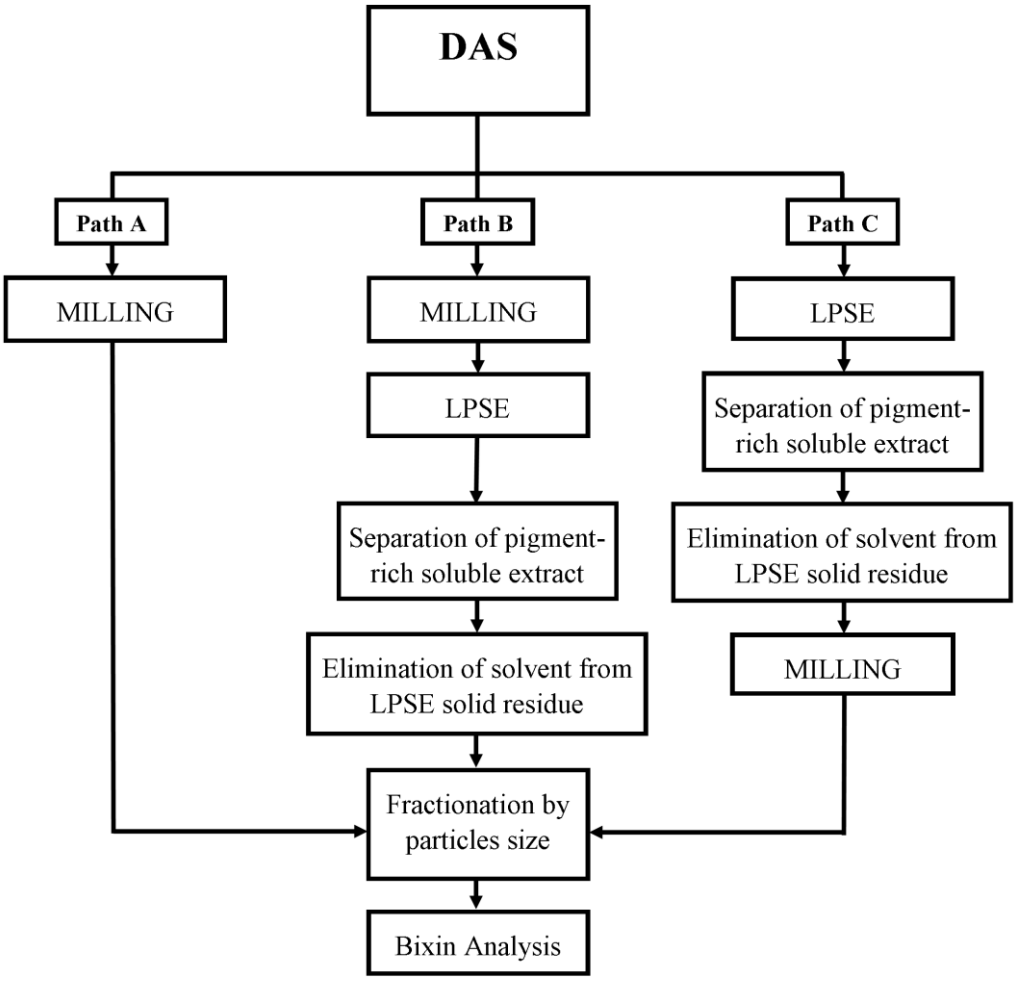

Figure 1: Preliminary study for pigment extraction from integer DAS.

2.4. Solvent extraction method (LPSE)

DAS (20 g) were placed in Erlenmeyer flasks (250 mL) and extracted with ethanol PA (Chemco, Hortolandia, Brazil). The Erlenmeyer flasks were placed into an Incubator-Shaker (MARCONI, Piracicaba, Brazil) at 295 RPM. The S/F ratio was 8, and the extraction was performed for 120 min at a temperature of 50 °C and atmospheric pressure Rodrigues et al. (2014). Each assay was conducted in triplicate and every flask was sealed using plastic paraffin film to restrict the loss of solvent. Then the extract was filtered through a filtration system using a filter paper (LABCENTER, São Paulo, Brazil), with nominal porosity of 15 µm. For quantitative determinations, the solvent was evaporated from the remaining extract using a vacuum-controlled rotary evaporator (Laborota, Vertrieb, Germany) at 35 °C and protected from light. The pigment dry extract obtained was maintained in a desiccator until constant weight. The extracts were kept at -18 °C to determine the overall

yields and quantification of the total pigment (expressed as bixin) and the total phenolic compounds (expressed as gallic acid equivalents).

2.4.1. First study of pigment extraction in FP fraction

In these experimental runs, FP from Batch 1 were used as raw material. Three temperatures (40, 50 and 60 °C), three S/F ratios (10, 15 and 20), and three agitation times (5, 15 and 30 min) were evaluated. The experiments were conducted using a full-randomized factorial, totalizing 27 experiments. Due to the small particle size of the FP, a porous sealed bag where the fine material (1 g) remains suspended was developed. The porous sealed bag was confectioned using a filter paper (Qualy, São Paulo, Brazil), with nominal porosity of 15 µm. The porous sealed bag was placed in Erlenmeyer flasks (150 mL) and extracted in the Incubator-shaker. After 2 min, the solvent was drained from the porous sealed bag and the extract was recovered.

2.4.2. Second study of pigment extraction in FP fraction

In these experimental runs, FP from Batches 1 and 2 were used as raw material. Two temperatures (40 and 50 °C), two S/F ratios (10 and 20 g/g), and four agitation times (15, 30, 45 and 60 min) were evaluated. The experiments were performed using the methodology described previously in section 2.4.1. The experiments were conducted using a full-randomized factorial, totalizing 32 experiments

2.5. Analysis and quantification of raw material and extracts from LPSE

2.5.1. Determination of overall yield (X_0)

Overall yield, X_0 (g extract/100g raw material), was calculated using Eq. 1 as the ratio of the total mass of the extract ($m_{extract}$) and the initial mass of the sample (m_{sample}) in dry basis.

$$X_0 = \frac{m_{extract}}{m_{sample}} \times 100 \qquad \textbf{Eq.1}$$

2.5.2. Determination of pigment content (Bixin)

The amount of pigment, Bixin (g bixin/100 g extract or g bixin/100 g raw material), was determined according to the methodology described by Smith and Wallin (2006). For raw material analysis, bixin was extracted from integer DAS (approximately 0.1 g) with acetone PA-ACS (Synth, Diadema, Brazil) until complete discoloration. For extracts obtained from the extractions, a sample (approximately 10 µg) was diluted in acetone. Acetone solutions from raw material and extracts were diluted until concentrations appropriate for analysis. The absorbance of the diluted samples were measured at 487 nm

using a UV/VIS spectrophotometer (FEMTO, model 800 XI, São Paulo, Brazil) and the percentage of bixin present in raw material and extracts was calculated using Eq.2.

$$Bixin = \frac{A}{E} \times \frac{V_1}{m} \times \frac{V_2}{v_1} \qquad \textbf{Eq. 2}$$

Eq. 2 was adapted from Smith and Wallin (2006), were *A* is the absorbance, *E* is the specific absorbance determined for bixin as 3090, *m* is the mass (g) of sample for analysis, v_1 (mL) is an aliquot for dilution (when required), V_1 and V_2 (mL) are the volumes of first and second (when required) dilution in order to get the absorbance reading below 1.0 (Lambert-Beer law).

2.5.3. Determination of efficiency of the extraction (BY)

The BY (% of bixin yield relative to raw material) is interpreted as the percentage of bixin extracted from the original quantity of pigment presented in the raw material. BY was calculated using Eq. 3, which uses values of X_0, pigment content in the extract ($Bixin_{extract}$) and the pigment content in the original sample for extraction ($Bixin_{sample}$).

$$BY(\%) = \frac{X_0 \times Bixin_{extract}}{Bixin_{sample}} \qquad \textbf{Eq. 3}$$

2.5.4. Determination of total phenolic content (Phenolic)

Total phenolic content of FP and extracts was estimated using the Folin-Ciocalteau method, based on a colorimetric oxidation/reduction reaction of phenols developed by Singleton and Rossi (1965) and adapted for vegetal extracts by Singleton et al. (1999). After reaction, the absorbance of samples were measured at 760 nm using a UV/VIS spectrophotometer (FEMTO, model 800 XI, Sao Paulo, Brazil). Quantitative measurements were performed based on a standard calibration curve of gallic acid (mg/mL). The total phenolic content was expressed as percentage of gallic acid equivalents (GAE) in extracts using Eq.4, where *V* (mL) is the volume of dilution for analysis and *m* (mg) is the weight of dry material for analysis.

$$Phenolic = \frac{GAE_{mg/mL} \times V}{m} \times 100 \qquad \textbf{Eq.4}$$

2.6. Statistical analysis

The analysis of variance (ANOVA) and the Tukey test determined using the Minitab® 16 software with a confidence interval of 95% ($p_{value} \leq 0.05$).

2.7. Process simulation: Technical and economic evaluation

Simulations of the MFM and the integrated MFM+LPSE processes were performed using the SuperPro Designer® 8.5® software. The flowsheet of the integrated process is described in Figure 2 and the process is divided in two sections, one responsible by

mechanical fractionation (MFM) and another responsible for the production of a pigment-rich extract using LPSE. Initially the MFM was simulated; for this process, the DAS are comminuted (P-1/GR-101) and afterwards, LP and FP fractions are obtained using a vibratory screen (P-3/VSCR-101). For the MFM+LPSE process, once obtained the full fraction of the FP, the LPSE process is performed in the extractor (P-2/V-101). The solvent is fed using a solvent pump (P-6/PM-101) and once finished the extraction process, the solvent is eliminated using a distiller (P-4/C-101). The solvent is reused and stored in a tank (P-7/V-102). While the pigment-rich extract is recovered and the extraction system is cleaning, the MFM is performed. Input parameters and process conditions were based in optimal conditions described on section 3.3.

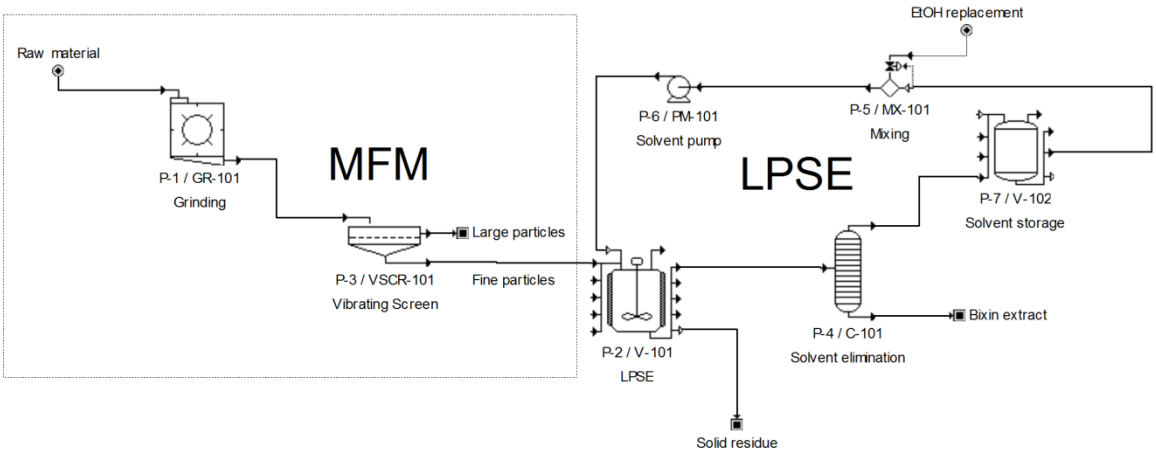

Figure 2: Flowsheet of the MFM+LPSE integrated process, designed by the SuperPro Designer® 8.5 software.

2.7.1. Economic evaluation and process scale-up

The cost of manufacturing (COM) of the pigment-rich FP via MFM process and the pigment-rich extract obtained by the integrated MFM+LPSE processes was calculated. For MFM and MFM+LPSE processes, COM was estimated using the information described in the extraction procedures section. COM can be determined by the sum of three main components: direct costs, fixed costs and general expenses. COM was estimated according to the methodology proposed by Turton et al.(2009), where the three major components of COM are estimated in terms of five major costs: raw materials, operating costs, utilities, waste treatment, and initial investment by the equation:

$$COM = 0.304FCI + 2.73COL + 1.23(CUT + CWT + CRM) \qquad \textbf{Eq. 5}$$

Where FCI is the fixed capital of investment, COL is the cost of operational labor, CUT is the cost of utilities, CWT is the waste treatment cost and CRM is the cost of raw material (DAS and extraction solvent). FCI involves expenses related to the implementation of the production line (extraction units and other equipment). COL is related to the operators of the extraction units (1operator to the 5 and 50 L extraction units). CUT considers the energy used in the solvent cycle for steam generation, water refrigeration and electricity requirements. CRM consists of the raw material cost and the cost of the solvent. Finally, CWT was considered zero because the waste generated by the process can be considered harmless and clean and may be reused in other applications or simply disposed of as ordinary vegetable waste. More details can be found in Rosa and Meireles (2005) and Albuquerque and Meireles (2012).

Although there is a difficulty to obtain equipment quotations, values based in past vendor quotations and literature are a good alternative (Silla, 2003). In this work, to scale the equipment cost to the required capacity, Eq. 6 was used. C_1 is the equipment cost with capacity Q_1; C_2 is the known base cost for equipment with capacity Q_2 and n is a constant depending on equipment type. Values of n were collected from literature (Green & Perry, 2007; Silla, 2003; R. Smith, 1995; Turton et al., 2009). In Table 1 are presented the base costs used is this work.

$$C_1 = C_2\left(\frac{Q_2}{Q_1}\right)^n \qquad \textbf{Eq. 6}$$

For the process scale-up, a procedure similar to that described by Santos et al. (2012) was used: we assumed that the yield and extract composition obtained at the laboratory scale would also be obtained at the industrial scale when the same processing conditions were used (temperature, pressure, S/F ratio, bed porosity, etc.). The process was designed to operate for 7920 h per year, which corresponds to 3 daily shifts for 330 days per year (35 days per year can be used to other operations such as preventive maintenance or repairs). LPSE extractor volumes of 5 and 50 L were considered. The solvent loss during the distillation process was assumed 2 %, which was the loss from a distiller calculated by the simulator. The cost of the raw material (DAS) was assumed zero because the raw material can be considered as the residue of the SFE process as mentioned previously. Considering these aspects, Table 2 provides important information about data used for COM simulation.

Table 1: Base cost for equipment composing the extraction plant

Equipment	n [a]	Unit base cost (US$) [b]
Mechanical fractionation		
Milling machine	0.8	1,500.00
Fractionation machine	0.9	550.00
Structural material for supporting the equipment [c]	0.6	507.00
LPSE		
Jacketed extraction vessel	0.5	500.00
Electric liquid pump	0.6	3,920.00
Separation vessel	0.5	1,460.00
Block valve	0.6	220.00
Temperature controller	0.6	310.00
Piping, connectors, crossheads, mixers and splitters [c]	0.6	610.00
Structural material for supporting the equipment	0.6	1,015.00
Solvent storage tank	0.6	300.00
Total Mechanical fractionation + LPSE process	–	**10,892.00**

[a] n constant depending on equipment type based on references (Green and Perry (2007), Silla (2003), R. Smith (1995), Turton et al. (2009));[b] Based on an operating plant with extractor of 1 L; [c] total cost.

Table 2: Input economic parameters used in the SuperPro Designer 8.5® software

	5 L	50 L
Fixed capital investment (FCI)		
Mechanical fractionation + LPSE unit extraction [a]	US$ 34,830.00	US$ 152,234.00
Depreciation rate [b]	10 %/year	10 %/year
Annual maintenance rate [b]	6 %/year	6 %/year
Operational labor (COL) [c]	US$ 5.95 h^{-1}	US$ 5.95 h^{-1}
Number of workers	1	1
Cost of the raw material (CRM)		
Ethanol [e]	US$ 0.85 kg^{-1}	US$ 0.85 kg^{-1}
Utilities (CUT)		
Electricity [e]	US$ 0.20 $(kWh)^{-1}$	US$ 0.20 $(kWh)^{-1}$
Water steam (high pressure) [f]	US$ 20 t^{-1}	US$ 20 t^{-1}
Water [f]	US$ 0.05 t^{-1}	US$ 0.05 t^{-1}
$CaCl_2$ (refrigerant fluid) [f]	US$ 0.25 t^{-1}	US$ 0.25 t^{-1}

[a] Estimated cost using the Eq. (6); [b] based on Peters et al. (2003); [c] Bureau of Labor Statistics, http://www.bls.gov/fls/country/brazil.htm, USA, last accessed on 15/07/2015; [d] base on Veggi et al. (2011); [e] direct quotation; [f] SuperPro designer 8.5® database

3. Results and discussions

3.1. Preliminary study and establishment of MFM for bixin concentration from integer DAS

Annatto seeds were selected for this study initially in order to continue the previously work conducted by our group of investigation, specifically by the works performed by Albuquerque and Meireles (2012) and by Rodrigues et al. (2014).

Albuquerque and Meireles (2012) determined the parameter conditions for the SFE of the lipid fraction from integer annatto seeds and Rodrigues et al. (2014) used the residue of SFE in order to extract the pigment from integer DAS due to it was considered that the pigments were on the surface of seeds.

In order to completely use the DAS biomass, our initial purpose was to extract the maximum amount of pigment from integer DAS and use the solid residue from pigment extraction (with a reduced pigment concentration) in other processes (e.g., using hydrothermal technologies) to obtain other products from DAS. Another possibility is the implementation of a milling operation to increment the reaction surface between the raw material and the solvent. According to these initial purposes, the better path to insert the milling operation was studied. In Figure 1 were shown the three paths of the preliminary studies. Part A only includes the milling of DAS, Path B introduces the milling the DAS before pigment extraction and Path C includes the milling operation after extraction and solvent evaporation. For a better comparison between all paths, the particle size fractionation was conducted because the raw material – solid residue from path B and C – still contains extractable pigments. The experimental values expressed as bixin content and bixin distribution obtained for each group of the particle size are shown in Table 3. Rodrigues et al. (2014) obtained an extraction efficiency of 28.5% from integer DAS using ethanol as solvent at 60 °C and S/F ratio of 8. The results obtained in this work are superior, for example, after LPSE process, paths B and C had a BY of 37.1% and 32.7%, respectively.

Table 3: Paths for the preliminary study on DAS

Particle Size (d_p, µm)	Path A			Path B			Path C		
	Size distribution	**Bixin content**	**Bixin distribution[a]**	**Size Distribution**	**Bixin content**	**Bixin distribution[b]**	**Size distribution**	**Bixin content**	**Bixin distribution[c]**
> 1000	9 ± 0.5	0.9 ± 0.1	3 ± 0.4	10 ± 0.3	0.7 ± 0.1	5 ± 0.6	6 ± 0.5	0.2 ± 0.01	0.9 ± 0.1
700 – 1000	32 ± 0.8	0.6 ± 0.1	8 ± 0.5	30 ± 0.7	0.7 ± 0.1	14 ± 0.6	27 ± 0.2	0.3 ± 0.01	5 ± 0.2
500 – 700	22 ± 0.4	1 ± 0.4	10 ± 0.4	24 ± 0.3	0.8 ± 0.1	12 ± 0.1	22 ± 0.7	0.4 ±0.01	6 ± 0.1
300 - 500	14 ± 0.8	2 ± 0.6	10 ± 0.2	22 ± 0.3	1 ± 0.1	12 ± 0.3	17 ± 0.6	0.8 ± 0.01	8 ± 0.6
≤ 300	23 ± 0.3	8 ± 0.7	69 ± 0.9	14 ± 0.8	6 ± 0.2	57 ± 0.8	28 ± 0.3	3 ± 0.1	80 ± 2

Values presented as mean ± standard deviation. Size distribution (%, relative to sample). Bixin (g bixin/100g material); Bixin distribution (%, relative to total pigment content on sample). The subscripts a, b and c, corresponded to values of pigment content of 2.3, 1.6 and 1.5 g bixin/100g of sample. Sample term correspond to: [a]DAS milled without pigment extraction (Path A), [b]DAS residue after milling and solvent extraction of pigment (Path B) and [c]DAS residue after solvent extraction of pigments and milled (Path C).

According to the values presented in Table 3 for the pigment concentration remained in DAS (Path B and C), it is possible to deduce: i) LPSE has better performance that the process evaluated by Rodrigues et al. (2014), ii) LPSE process of integer DAS needs large amounts of solvent to enhance the extraction; therefore in order to optimize the quantity of solvent used, it may be considered that the largest quantity of pigment is found on seed surface and after milling, this tissue is found mainly on fraction of FP, iii) after milling and fractionation in path A, approximately 70% of pigments from integer DAS were directly concentrated on FP, without using solvents or other energy necessary to evaporate the solvent.

Thereby, the present work proposes an alternative process for the complete use of DAS, as shown in Figure 3. The MFM is used to divide the material in two groups according to the particle size, in FP which contains most of the pigment and LP which includes the remaining particles.

Table 4 shows the composition of all raw materials analyzed in this study: annatto seeds before SFE (AS), DAS and LP and FP fractions obtained using MFM from Batch 1 and 2. The composition between integer AS and DAS shows a slight difference between Batches, due to different origin and treatment. As expected, the lipid content of integer DAS decreased after SFE for both batches. On the other hand, other substances such as the pigments were increased. After MFM, a higher amount of lipid and bixin were remained in the FP. LP fraction contains larger amounts of other materials such as protein, starch and lignocellulosic compounds; therefore, this fraction is not appropriate as raw material to extraction and could be used as a special flour – whit bioactive properties - since it is a source of protein and starch.

According to this preliminary study, MFM appears to be the best technique for pigment concentration from DAS. The pigment was redistributed mainly in the FP fraction, the bixin content increased from the integer DAS to FP from 2.3 to 7.6 (g bixin/100g raw material) for batch 1, as showed in Table 3. On the other hand, paths B and C are not adequate, since a great quantity of pigment remains in the solid residue.

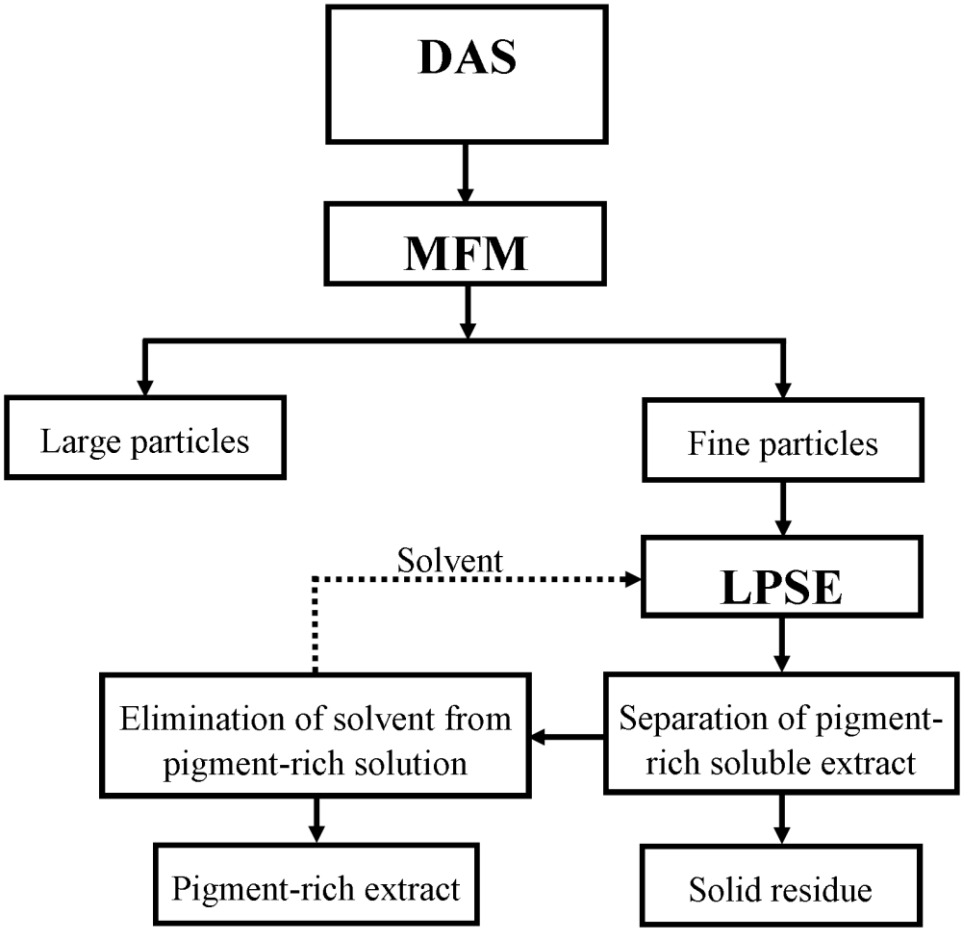

Figure 3. Flowchart of integrated use of DAS

Table 4. Composition of Annatto seeds (wet basis %)

	Batch 1				Batch 2			
	AS	**DAS**	**LP**	**FP**	**AS**	**DAS**	**LP**	**FP**
Moisture[a]	13 ± 0.1	12 ± 0.1	8 ± 0.1	6 ± 0.1	14 ± 0.1	13 ± 0.2	11 ± 0.5	7 ± 0.1
Lipids[a]	2 ± 0.3	1 ± 0.1	1 ± 0.2	4 ± 0.1	4 ± 0.3	1 ± 0.2	1 ± 0.2	8 ± 0.1
Other material*[a]	83	85	90	79	80	83	87	65
Bixin[b]	2 ± 0.4	2 ± 0.1	0.9 ± 0.01	8 ± 0.4	2 ± 0.2	3 ± 0.1	0.8 ± 0.1	17 ± 0.9
Phenolic[c]	-	-	-	3 ± 0.4	-	-	-	3 ± 0.1

Values presented as mean ± standard deviation. Composition of integer annatto seeds in natura (AS), integer semi-defatted annatto seeds (DAS), Large particle fraction of semi-defatted annatto seeds (LP) and Fine particle fraction of semi-defatted annatto seeds (FP); (*) Calculated by difference; (-) values not determined. [a]All results are expressed in percentage (%), [b](g bixin/100g raw material), [c](% of GAE).

The next step in this work involves the pigment extraction from FP fraction through the integration of MFM and LPSE processes in order to optimize the extraction process and evaluate the use of ethanol as extraction solvent.

3.2. Extraction studies using the MFM + LPSE integrated process

Two consecutive groups of experiments of solvent extraction studies were developed. The first study, integrates the LPSE process with MFM using FP from batch 1. The experimental values obtained for X_0, Bixin, BY and phenolic content from the extracts at various experimental conditions considered in this first study are presented in Table 5.

The results show that all response variable increased as the extraction time increased. Over the conditions studied, the temperature, S/F ratio and time had a significant impact on all response variables ($p_{value}<0.05$). Is important to highlight that the extraction time was not enough to achieve the maximum extraction efficiency and therefore, larger extraction time had to be studied in the subsequent assays.

An individual analysis of temperature and S/F ratio on the first study of pigment extraction after 30 minutes of extraction were performed using a Tukey's test. Although in the range studied of S/F ratio was not significantly different ($p_{value}>0.05$), as the values of the S/F ratio showed the maximum efficiency, in the second study, the minimum and maximum S/F ratios (10 and 20, respectively) were studied. The temperature had a significantly impact ($p_{value} \leq 0.05$) on the Bixin and Phenolic responses. A temperature of 40 °C appears to be the best condition for extraction, only X_0 showed a good performance when the process was performed at 60 °C. This result can be explained because temperature affects the solvent behavior, increasing the solubility of other substances in ethanol whereas the selectivity for the pigment decreases. According to this fact, for further experiments, temperatures of 40 and 50 °C were evaluated. The results of X_0, Bixin, BY and phenolic content obtained after the second study are presented in Table 6.

Table 5. Results of first study of LPSE using FP.

Conditions of extract		Response variables			
Temperature (°C)	**Time (min)**	**X_0 (%)**	**Bixin (%)**	**BY (%)**	**Phenolic (%)**
S/F = 10					
40	5	3	13	5	2
40	15	6	17	12	2
40	30	6	28	23	3
50	5	3	11	5	1
50	15	6	15	13	2
50	30	8	15	16	2
60	5	4	12	5	2
60	15	8	14	14	2
60	30	10	14	18	2
S/F = 15					
40	5	5	13	9	0.9
40	15	5	20	15	3
40	30	9	22	27	2
50	5	4	7	3	1
50	15	5	17	11	2
50	30	8	18	18	2
60	5	5	13	7	1
60	15	5	14	10	2
60	30	8	15	15	2
S/F = 20					
40	5	3	15	6	2
40	15	9	31	37	2
40	30	9	33	39	3
50	5	3	12	5	2
50	15	5	16	11	2
50	30	8	15	15	3
60	5	5	12	8	3
60	15	8	20	21	3
60	30	10	23	32	2

X_0, (g extract/100g raw material, ±0.8); **Bixin**, (g bixin/100g extract, ±3); **BY**, (% of bixin yield relative to raw material, ±4); and **Phenolic**, (% of GAE, ±0.2). Standard deviation was calculated from ANOVA.

Table 6. Results of second study of LPSE using FP.

Conditions of extract		Response variables			
Temperature (°C)	**Time (min)**	**X_0 (%)**	**Bixin (%)**	**BY (%)**	**Phenolic (%)**
				Batch 1	
S/F = 10					
40	15	4	15	8	3
40	30	6	25	18	3
40	45	7	29	27	3
40	60	8	32	32	2
50	15	4	10	6	2
50	30	8	12	13	3
50	45	8	15	17	3
50	60	8	15	16	3
S/F = 20					
40	15	5	30	20	3
40	30	8	34	36	3
40	45	10	37	46	6
40	60	10	38	49	5
50	15	4	17	9	2
50	30	7	18	17	3
50	45	9	28	32	7
50	60	9	20	24	5
				Batch 2	
S/F = 10					
40	15	7	21	9	3
40	30	8	24	12	4
40	45	9	28	16	5
40	60	10	27	17	4
50	15	7	11	4	2
50	30	10	16	9	3
50	45	10	23	14	3
50	60	11	22	15	3
S/F = 20					
40	15	9	31	16	2
40	30	11	36	25	3
40	45	11	41	29	4
40	60	13	39	31	3
50	15	6	12	4	4
50	30	11	22	15	5
50	45	15	33	31	6
50	60	18	27	30	5

X_0, (g extract/100g raw material, ±0.9[a], ±3[b]); **Bixin**, (g bixin/100g extract, ±4[a], ±5[b]); **BY**, (% of bixin yield relative to raw material, ±5[a], ±5[b]); and **Phenolic**, (% of GAE, ±0.2[a], ±0.6[b]). Standard deviation was calculated from ANOVA, [a]Batch1 and [b]Bach2, respectively.

The results show that Bixin and BY increased to 45 min and after this time, these values did not continue increasing. It is possible that due to the application of temperature during a prolonged time, the compounds have been affected and their chemical characteristics (cis- and trans- forms) have changed. The phenolic content has a similar behavior, after 45 min the concentration of total phenolic compounds stopped.

The temperature and S/F ratio were statistically evaluated using the experimental values of extraction obtained after 45 min. Due to differences between lipid composition and pigment content of batches 1 and 2, the statistical analysis were performed separately. (Table 3). The ethanol is a good solvent for the lipid phase and although the initial concentration in bixin was higher in Batch 2, the pigment extraction was not proportional to initial concentration for both sources. For example, although the Bixin presented in extracts is higher in batch 2, the process had a better efficiency when the batch 1 was used, because the Bixin reached a similar value even starting from a raw material with less bixin concentration.

The results for this second study show that the temperature had not a significantly impact in extraction process (p_{value}>0.25). However, bixin content and BY showed the best performance at 40 °C, whereas for X_0 and phenolic, the process had better performance at 50 °C. Therefore, with the objective of optimizing the extraction process it is important to take into consideration the selection of the process temperature. The S/F ratio had a significant impact only in batch 1 for phenolic (p_{value}=0.02) and in batch 2 for BY (p_{value}<0.01). According to Table 6, the process had a better performance using a S/F ratio of 20.

The results obtained from the second study also suggest that although ethanol can extract a certain amount of pigment, there were a significant amount of pigments remained on the raw material before extraction. This fact is a limitation of the LPSE process because only with the mechanical fractionation process is possible to concentrate the pigment without the use of solvents.

3.3. Economic evaluation and process scale-up

The cost of manufacturing (COM) of the pigment-rich extract via integrated MFM+LPSE process and the obtaining of pigment-rich FP by MFM process were calculated. For both processes, COM was estimated using the information described in the results and discussion section. Based in experimental measurements, for the MFM process, the time to comminute 1 kg of DAS used in simulation was 12 min and the time of sieving the comminuted material in a 5 L system was 180 min and 360 min for batch 1 and 2, respectively. For a 50 L system was 360 min and 540 min for batch 1 and 2, respectively. LPSE technique is described below. The simulation of the LPSE for both batches was

performed at 40 °C, S/F ratio of 20 and 45 min of extraction time. Two different production scales considering the MFM+LPSE integrated process and other process only considering the MFM were economically analyzed. Two different production scales (5 L and 50 L) were considered as shown in Figure 4. It is possible to observe that COM decreases as production scale increases for all batches and processes. For example, using the batch 1, the MFM COMs were estimated to be US$ 40.28 kg^{-1} and US$ 21.58 kg^{-1} for a process with capacity of 5 L and 50 L respectively. On the other hand, as the quantity of DAS in batch 2 required for reaching a similar proportion of FP obtained is two times higher than in batch 1; the process time increased. Consequently, the COM is higher as shown in Figure 4 and COM for batch 2 is 9 and 4 times larger than batch 1, for a process with capacity of 5 L and 50 L, respectively. Although after the integrated MFM+LPSE process, the bixin content of the pigment-rich extract is 5.8 and 6.4 times higher than pigment-rich FP obtained by MFM for batches 1 and 2, respectively, the COM for the MFM+LPSE process is quite larger. For batch 1 in a 5 L production scale, the COM is US$ 1153.60 kg^{-1}, which compared with MFM process in the same conditions, is 96% more expensive than to produce a kg of pigment-rich FP after MFM. This result can be explained due to the higher productivity of the MFM process and the costs generated by a larger number of equipment used in MFM+LPSE process, the longer process time and the quantity of solvents required to perform the extractions.

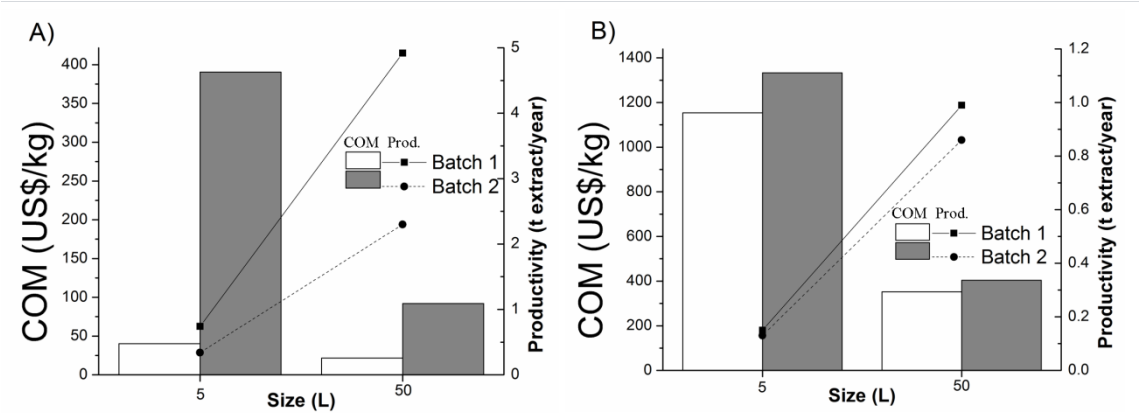

Figure 4: Influence of system capacity on the A) MFM and B) MFM+LPSE processes COMs

As shown in Figure 5, as the raw material was considered as a residue with value zero, the contribution of CRM in the COM for the MFM process is zero. MFM process does not use raw materials with economic cost, whereas in MFM+LPSE process, the solvent contributes with a slight increase in the participation of the CRM in the COM (Figure 5). As the raw material changed (Batches 1 and 2), the COL increased its impact on the COM for the

MFM process and conversely, for MFM+LPSE process, the COL decreased. In MFM process, the CUT almost have no participation in COM, whereas in the MFM+LPSE process is observed as a slight increase. The MFM+LPSE process requires solvents as raw materials, a longer processing time and higher energetic expenditures and energy requirements. These factors contribute with the increment of CUT in the COM. In the MFM process, as is required more time of sieving in the vibratory screen for batch 2, the participation of the COL in the COM is higher due to the longer time and therefore, higher quantity of labor to obtain the same quantity of FP. On the other hand, in the MFM+LPSE process, it was considered the same number of workers and in this process is possible to obtain the fine particle while the LPSE process is clean up and the pigment-rich extract is collected.

In summary, when both processes are compared, although the MFM+LPSE produces an extract with higher content of bixin, the MFM process is a shorter process and its COM is significantly lower than the MFM+LPSE process. The possibility of using pigment-rich FP as substitute of the pigment-rich extract obtained after LPSE according its COM have been demonstrated.

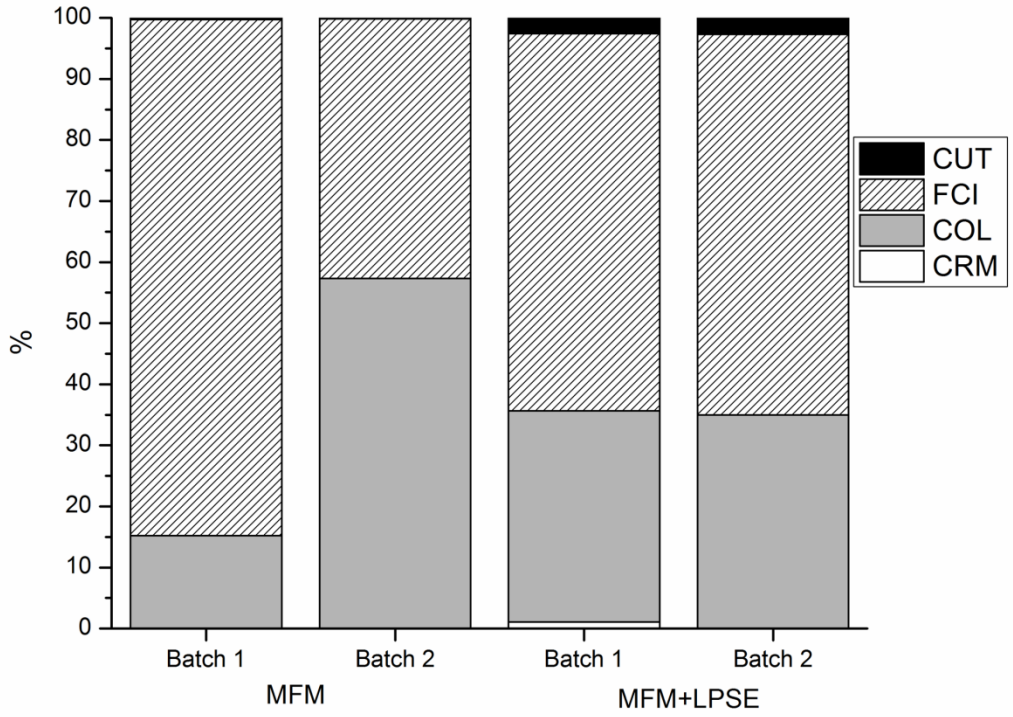

Figure 5: Influence of raw material (Batch 1 and 2) on the contribution of each component in COM in MFM and MFM+LPSE process for system capacity of 50 L.

4. Conclusions

In this work, the semi-defatted annatto seeds resulting from lipid extraction are simultaneously a residue and a feedstock. MFM process allows obtain a FP fraction with a significant amount of bixin. The bixin content in the FP increased from 2.9 to 7.6 (g bixin/100g raw material) and from 3.0 to 16.6 % (g bixin/100g raw material) for batches 1 and 2, respectively. The results show that all response variable increased as the extraction time increased. The results indicate that the bixin extraction was affected primarily by the extraction temperature, S/F ratio and time. Specifically, increasing the extraction time improved the extraction efficiency. A temperature of 40 °C is the best condition for extraction. Higher temperatures increase the solubility of other substances in ethanol, increasing the X_0 but decreasing the bixin content. The COM for the integrated MFM+LPSE process is quite expensive when compared with MFM process. For batch 1 in a 5 L production scale, the COM is US$ 1153.60 kg^{-1}, which compared with MFM process in the same conditions, is 96% more expensive than to produce a kg of pigment-rich FP after MFM. Thus, MFM process appears to be an attractive and economically feasible technique for obtaining bixin-rich particles from DAS.

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The authors have declared no conflict of interest.

Acronyms: Supercritical fluid extraction (SFE), Solvent mass to feed mass ratio (S/F), Annatto seeds (AS), Semi-defatted annatto seeds (DAS), Mechanical fractionation method (MFM), Low pressure solvent extraction (LPSE), Fine particles (FP), Overall yield (X_0), Bixin percentage (Bixin), bixin yield (BY), Total phenolic content (Phenolic), Cost of manufacturing (COM), Fixed capital of investment (FCI), Cost of operational labor (COL), Cost of utilities (CUT), Waste treatment cost (CWT), Cost of raw material (CRM).

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- CAPÍTULO 5 –

Hydrothermal modification treatment for the integral use of semi-defatted annatto seeds

Neste capítulo foi desenvolvida uma metodologia para a modificação física das sementes de urucum através do tratamento hidrotérmico das partículas grossas (de maior tamanho), que foram denominadas por “farinha de urucum”. O grau de modificação desta matéria-prima foi avaliado a partir dos resultados das propriedades de pasta, produção de açúcares redutores e totais e teor de amido.

Artigo que será submetido após revisão.

Hydrothermal modification treatment for the integral use of defatted annatto seeds

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ABSTRACT

Hydrothermal technology using pressurized water assisted by supercritical CO_2 from semi-defatted annatto seeds (DAS) was performed. The lipid fraction of annatto seeds was initially removed by extraction with supercritical CO_2. DAS was milling and fractionated according to particle size in fine particles (FP) and annatto seed flour (ASF). The hydrothermal modification treatment (HMT) of the ASF was performed at 80 °C, using a total solvent flow rate of 3 mL/min during 30 min, and the independent variables pressure (150, 200, 250, 300 and 350 bar) and water/CO_2 ratio (20/80, 30/70 and 40/60). The results indicate that the best results for monomers and oligomers of sugar were a water/CO_2 ratio of 40/60 and 350 bar. The HMT caused the modification of polymers (mainly starch) in all experimental conditions causing a decrease in the pasting temperature, peak and final viscosity. The application of HMT contributes to adding value to non-conventional sources of starch such as DAS through the incorporation of the ASF in the formulation of novel products.

Keywords: Annatto flour; Starch modification; Unconventional starch; Hydrolysis; Treatment of residues; Biocompounds recovery.

1. Introduction

Annatto (*Bixa orellana* L.) is a plant native from Brazil, and also is cultivated in other regions of South and Central America such as Peru, Mexico and Ecuador (Smith & Wallin, 2006). Annatto is used in cosmetics and as coloring agent in traditional food since pre-Columbian time (Elias et al., 2002). Due to its coloring power, annatto has been widely used in food, textile, cosmetic and pharmaceutical industries (Chisté et al., 2011; Vilar et al., 2014). On the other hand, annatto has been investigated owing to its benefits on human health and their bioactive properties (Giorgi et al., 2013).

Semi-defatted annatto seeds (DAS) are the residue produced after the extraction of the lipid fraction from annatto seeds (AS) using supercritical fluid extraction (SFE)

(Albuquerque & Meireles, 2012). The raw material for this work was the fraction with size larger than 48 Mesh (Taylor standard) from milling DAS.

The CO_2 in supercritical state (CO_2-SC), solvent used in SFE process, is an inert, odorless, tasteless, environment-friendly and GRAS (generally regarded as safe) solvent. Furthermore, in the extraction process with CO_2-SC, there is no solvent residue in the extract or in the solid SFE residue, since it is a gas in the ambient conditions, for this reason CO_2-SC is the most common supercritical fluid solvent used in food applications (Brunner, 2005). The solubility of the compounds in CO_2-SC was summarized by Brunner (2005) and by Del Valle and Aguilera (1999).

Commonly the treatment of agroindustrial residues is focused in the conversion in a suitable form of energy, using innumerable methods (Goldemberg, 2009). Nowadays, the conversion of the lignocellulosic material from vegetable residues in new materials and/or energy through use of water as reaction medium has become a key factor in developing of sustainable technologies. Hydrothermal technologies especially those that use subcritical and supercritical water have shown excellent results as conversion treatments. Hydrothermal treatments have an excellent performance because the properties of the solvent are easily manipulated through changes in temperature, pressure, processing time and proportion of solids (Kus, 2012; Zhao et al., 2011). Furthermore, water properties can be changed in order to increase the selectivity of specific substances and enhance the conversion process (Brunner, 2009).

Native starches are rarely consumed or used in their native form. Their application is limited because its instability (e.g. temperature or pH effects), additionally they are insoluble in water at room temperature and consequently lacking in functional properties, showing a strong tendency for decomposition and retrogradation (Berski et al., 2011; Sweedman et al., 2013). Native starches are commonly modified in order to develop specific properties such as solubility, texture, adhesion and tolerance to temperatures used in industrial processes (Singh et al., 2007; Sweedman et al., 2013). Starch modification includes physical, chemical, enzymatic and genetic methods (Yadav et al., 2013). The hydrothermal modification treatment (HMT) produces a physical modification of starch, which involves the use of heat and moisture (Singh et al., 2007; Wurzburg, 1986). The change of physical and chemical properties do not destroy the granule structure (Zavareze & Dias, 2011), even improves the texture and plasticity of the starch paste, raising their technology value (Ashogbon & Akintayo, 2014; Conde-Petit et al., 2001). Physical modification of starch is

considered simple, cheap and safe because chemical or biological agents are not used (Ashogbon & Akintayo, 2014).

HMT includes a thermal application in the presence of a limited amount of water (typically less than 35%, w/w). Among the different techniques of HMTs, the hydrolysis reaction is also known as starch modification treatment (Kim & Huber, 2013; Moreschi et al., 2006; Ulbrich et al., 2014). Hydrolysis processes with or without using water and CO_2 as reaction catalyzer has as advantages the use of nontoxic solvents and shorter process times when compared with other hydrolysis methods (Rogalinski et al., 2008a; Rogalinski et al., 2008b). The final products of the hydrolysis from starch and lignocellulosic materials are monosaccharides, oligosaccharides, organic acids and other products. It is important to highlight that chemical and renewable energy industries have great interest in this type of products (Sheldon, 2014). Current trends are directed to the development of technologies and processes that increase the use of vegetable residues such as starch. This material is an important alternative for different industrial applications due to its versatility, low cost and simplicity with their physicochemical properties are modified (Brasoveanu & Nemtanu, 2014; Santana & Meireles, 2014).

Annatto seed flour (ASF) is an important material that can be reintroduced in food processing or to be used in other industries. In this context, the objective of this study was to evaluate the modification of polymers (mainly starch) and the production of monomers and oligomers of sugar (separated into the soluble extract) from ASF by HMT process. This research was developed in order to contribute with the sustainability of annatto industry through the total use of seeds and the valorization of agroindustrial residues.

2. Material and methods

2.1. Raw material

The raw material in this study was product from previous work done in our laboratory by Albuquerque and Meireles (2012). AS were donated by the Agronomic Institute of Campinas (IAC - Instituto Agronômico de Campinas, São Paulo, Brazil). Integer AS were partially semi-defatted by SFE using CO_2 (99.9% of purity) (Gama Gases Especiais Ltda., São Bernardo do Campo, Brazil) in a commercial SFE system (Thar Technologies, Pittsburgh, USA). The SFE parameters were determined by Albuquerque and Meireles (2012) for the partial extraction of the annatto lipid phase with a high content of γ e δ- tocotrienol. The SFE was performed using temperature of 40 °C and pressure of 200 bar, and a solvent mass to feed mass ratio (S/F) of 3.5; suggested as the best condition for annatto lipid fraction extraction

maintaining a high contained of pigment in DAS. The vegetal residue from SFE was storage at freezing temperature (-18 °C) and protected from light until used in this work.

2.2. Composition of raw material

The AS, DAS and DAS fractions were characterized in terms of moisture (Sluiter et al., 2008a), protein (Thiex et al., 2002), lipids (Thiex et al., 2003), ash (Sluiter et al., 2008b), starch (McCleary et al., 1997) and bixin (Smith & Wallin, 2006) contents. The composition on other carbohydrates (cellulose, hemicellulose and lignin) was calculated by difference.

2.3. Preparation of raw material

The raw material for hydrothermal treatment used in this study was the ASF (large particle fraction from DAS). Integer DAS was milled using a knife mill (TECNAL, Piracicaba, Brazil). Then, the material was classified according to the particle size using a sieve (CATEL, Piracicaba, Brazil). The fraction with diameter of particle lower than 48 Mesh ($dp \leq 300$ μm) was denominated as fine particles (FP).

2.4. HMT of ASF using pressurized water and supercritical CO_2

HMT process was performed in a MV-10 ASFE system (WATERS, Milford, USA). An extraction cell with nominal capacity of 25 cm^3 was used. The sample represented approximately 2/3 of the total volume of the extraction cell. The extraction cell was not totally filled due to that swelling capacity of starch could produce an increment in the particles volume, leading the clogging of solvent tube and consequently, the process interruption.

In this process, the carbohydrate polymers (starch mainly) present in ASF is simultaneously modified and hydrolyzed. The modified starch remains in ASF and at the same time, the monosaccharides and oligosaccharides produced by hydrolysis are dragged out by the solvent. The hydrothermal process was performed at 80 °C, using a total solvent flow rate of 3 mL/min during 30 min. Five pressures (150, 200, 250, 300 and 350 bar) and four water/CO_2 ratios (20/80, 30/70 and 40/60) were evaluated.

2.5. Chemical characterization and calculations

2.5.1. Determination of starch percentage (Starch)

The starch content was determined in all raw materials and in ASF obtained before and after the hydrothermal treatment. The analysis was conducted using the methodology developed by McCleary et al. (1997). Thermostable α-amylase and amyloglucosidase enzymes were used in a colorimetric analysis (Megazyme, Wicklow, Ireland). The method was developed to quantify the glucose content of the digestible and resistant starches. The analyses were performed in triplicate. The absorbance of each sample

and the D-glucose control were measure at 510 nm using a spectrophotometer (Beckman Coulter, California, USA). Total starch was considered as the sum of the digestible starch and the resistant starch and the results for each sample were expressed in dry basis percentage.

2.5.2. Pasting properties

The pasting properties were measured using a Rapid Visco Analyzer (RVA) (Newport Scientific Pty Ltd., New South Wales, Australy). All materials for analysis were reduced to powder using a ball-mill and the moisture content was adjusted (14% basis). The sample (3.5 g) was dispersed in 25 mL of distilled water and the total weight was 30 g. The annatto samples in suspension were stored at 5 °C during 24 h before analysis. The RVA analysis was adjusted for raw material with *Standard I* set-up and for experimental samples using *Extrusion I* set-up. The profiles were continually recorded using the Thermocline for Windows® v. 3 software (TCW). The analyses were performed in triplicate. According to Saunders et al. (2011), in the paste behavior can be distinguished mainly three periods: a first period called controlled heating period (95 °C), followed by an isothermal period and finally a cooling (50 °C). Pasting temperature (°C - temperature from which the viscosity begins to increase during heating), peak time (min - time at which peak of viscosity occurred), peak viscosity (cP - maximum viscosity after the heating portion of the test), trough viscosity (cP - lowest viscosity after the peak viscosity just before it begins to increase again), breakdown (cP - a difference between viscosity peak and the minimum viscosity at maximum temperature of analysis); during cooling period, final viscosity (cP - viscosity at the end of the test), and setback (cP - difference between paste viscosity at end of cooling period and trough viscosity) were determined (Saunders et al., 2011; Wang & Weller, 2006).

The viscosity curves produced during heating and cooling of the sample suspension generally show a similar characteristic pasting curve as showed in Figure 1.

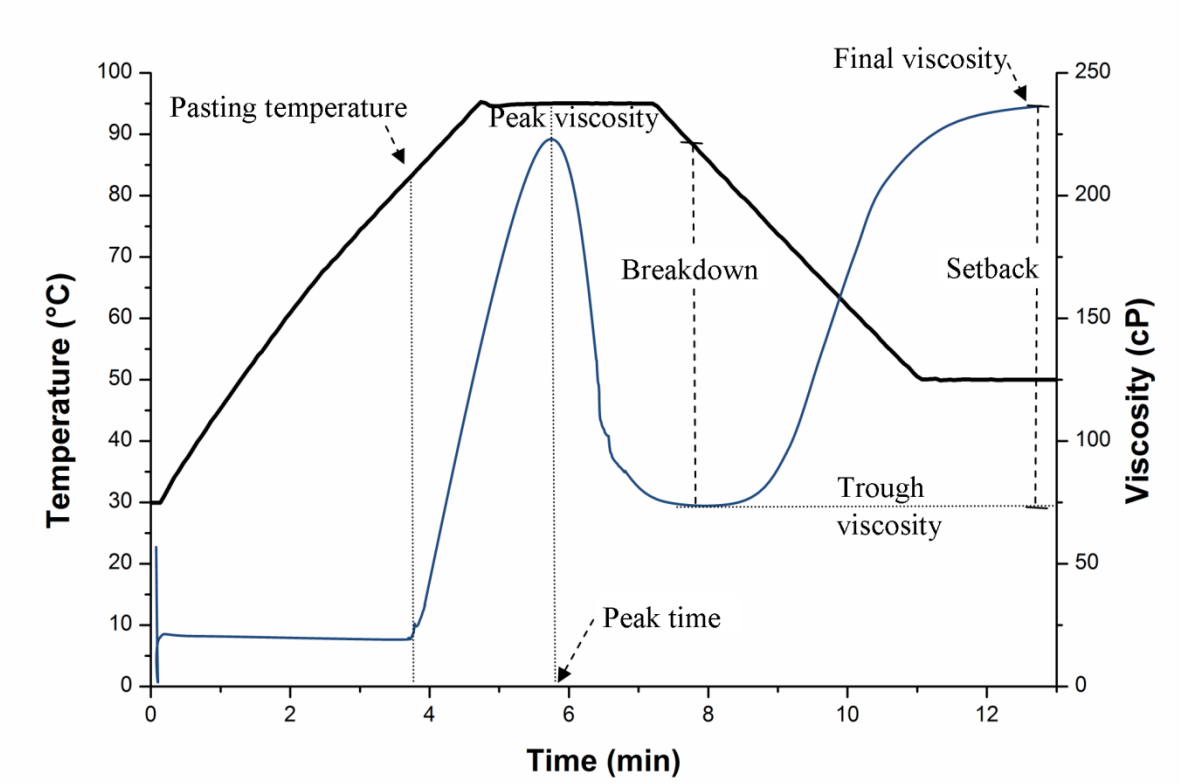

Figure 1: Typical RVA pasting curve showing the commonly measured parameters, adapted from Saunders et al. (2011)

2.5.3. Determination of reducing sugars (RS) and total reducing sugars (TRS)

The hydrolyzed solutions obtained from HMT of ASF were lyophilized (LIOBRAS, São Carlos, Brazil). RS and TRS concentration were determined using the colorimetric method of Somogyi-Nelson (Nelson, 1944) by spectrophotometry (FEMTO, Sao Paulo, Brazil). For TRS quantification, the calibration curve (sucrose solution) and the test samples were hydrolyzed with a hydrochloric acid solution (2 N) for 6 min at boiling temperature, and then cooled and neutralized with a sodium hydroxide solution (2 N) to ensure that all the oligosaccharides were hydrolyzed into monosaccharides. Quantitative measurements were performed based on a standard calibration curve of sugar (mg/mL), RS and TRS concentrations were interpreted as glucose equivalent and sucrose equivalent, respectively. All the reagents used were of analytical grade.

3. Results and discussions

3.1. Composition of raw material

Table 1 shows the characterization of AS in natura (before SFE), DAS (after SFE) and DAS fractions obtained as ASF ($d_p > 300$ µm) and FP ($d_p \leq 300$ µm).

Table 1. Composition of Annatto raw material

	Annatto (*Bixa orellana* L.)			
	AS	**DAS**	**ASF**	**FP**
Moisture	13 ± 0.1	12 ± 0.1	8 ± 0.1	6 ± 0.1
Lipids	2 ± 0.3	1 ± 0.1	1 ± 0.2	4 ± 0.1
Protein	15 ± 0.2	16 ± 0.5	16 ± 1	20 ± 0.1
Ash	5 ± 0.3	6 ± 0.5	6 ± 0.4	8 ± 0.4
Starch	20 ± 1	18 ± 2	18 ± 2	11 ± 0.5
Other Carbohydrates[a]	42	45	50	43
Pigment[b]	2 ± 0.4	3 ± 0.1	0.9 ± 0.1	8 ± 0.4

Values expressed in percentage (%, wet basis) and presented as mean ± standard deviation; for annatto seeds (AS), semi-defatted annatto seeds (DAS), Annatto flour (ASF) and the fine particles (FP) from DAS. ([a]) Calculated by difference and interpreted as hemicellulose, cellulose and lignin content; ([b]) expressed as bixin.

After SFE process it can be stated that: i) CO_2-SC dissolves non polar or slightly polar compounds (e.g. mono and sesquiterpenes, glycerol, saturated lipids up to C_{12}); ii) pigments are even less soluble; iii) water has a low solubility at temperatures below 100 °C; and iv) proteins, polysaccharides, sugars and mineral salts are insoluble.

In Table 1 is clearly observed a decrease on the lipid content between AS and DAS. This result can be explained due to CO_2-SC selectivity, when the lipid fraction and lipid soluble compounds (e.g. tocotrienols) are selectively extracted. A decrease in starch fraction also was observed, this behavior is not related with selectivity of the CO_2-SC (Braga et al., 2006), It can be inferred that the starch content decreased possibly due to the fact that annatto starch contains an amylose-lipid complex, and this complex could be dragged by the CO_2 during the SFE process (Alcázar-Alay et al., 2015). Torres et al. (2015) studied the bixin and phenolic extraction from DAS using a high turbulence extraction method assisted by ultrasound. When ethanol was used as solvent, the annatto starch was dragged by the solvent. The DAS composition also showed a proportionally increment of other components (e.g. pigment, protein and other carbohydrates) due to the redistribution of this compounds between the two fractions.

After milling and fractionation of DAS in two fractions, the ASF fraction represents about 80% of the two fractions, therefore, it was expected a similar composition between DAS and ASF.

The principal difference between DAS and ASF is the pigment content that was concentrated mainly in FP. On the other hand, besides the pigment, other compounds as lipids

and protein increased notoriously in the FP fraction, whereas the starch and lignocellulosic materials content decreased significantly.

As can be observed in Table 1, annatto shows an interesting composition of dietary fiber (other carbohydrates). The dietary fiber is an important component of diet and in human nutrition, also associated to an antioxidant capacity from phenolic compounds (Bhaskar et al., 2012; Guo & Beta, 2013; Mrabet et al., 2012) . A dietary fiber is defined as complex carbohydrates and represents the edible part of plants that are resistant to digestion and absorption in the small intestine (Cummings & Englyst, 1995; Taylor et al., 2015). Insoluble dietary fiber is made up of cellulose and other non-starch polysaccharides, lignin and cutin; whereas pectins, β-glucans, and other polysaccharides are typical soluble dietary fiber (Saura-Calixto, 2011). The demand for by-products from fruits and vegetables as sources of dietary fiber has been increasing due to these sources offer higher nutritional quality, lower caloric content and stronger antioxidant capacity; dietary fiber plays an important role in human health such in prevention of several diseases, constipation, colon cancer, atherosclerosis, obesity and diabetes (Al-Farsi et al., 2007; O'Shea et al., 2012; Rodríguez et al., 2006; Saura-Calixto, 2011). Several studies has been developed about the addition of dietary fiber from different sources in food (Adebowale et al., 2013; Almeida et al., 2013; Hemdane et al., 2015; Mildner-Szkudlarz et al., 2013; Sánchez-Zapata et al., 2009), improving the food quality and nutritional benefits. Therefore, as ASF is a material rich in starch and lignocellulosic materials, the ASF produced in this study could be a feasible source of dietary fiber to the food industry, mainly in the formulation of functional foods with potential health-beneficial effects.

As ASF maintains a small quantity of pigment and is a material rich in starch and lignocellulosic materials and the FP fraction can be used a raw material for a subsequent research about annatto-pigment extraction (Taham et al., 2015), in order to complete use of the DAS, the present work propose a new process whose flow process chart is shown in Figure 2.

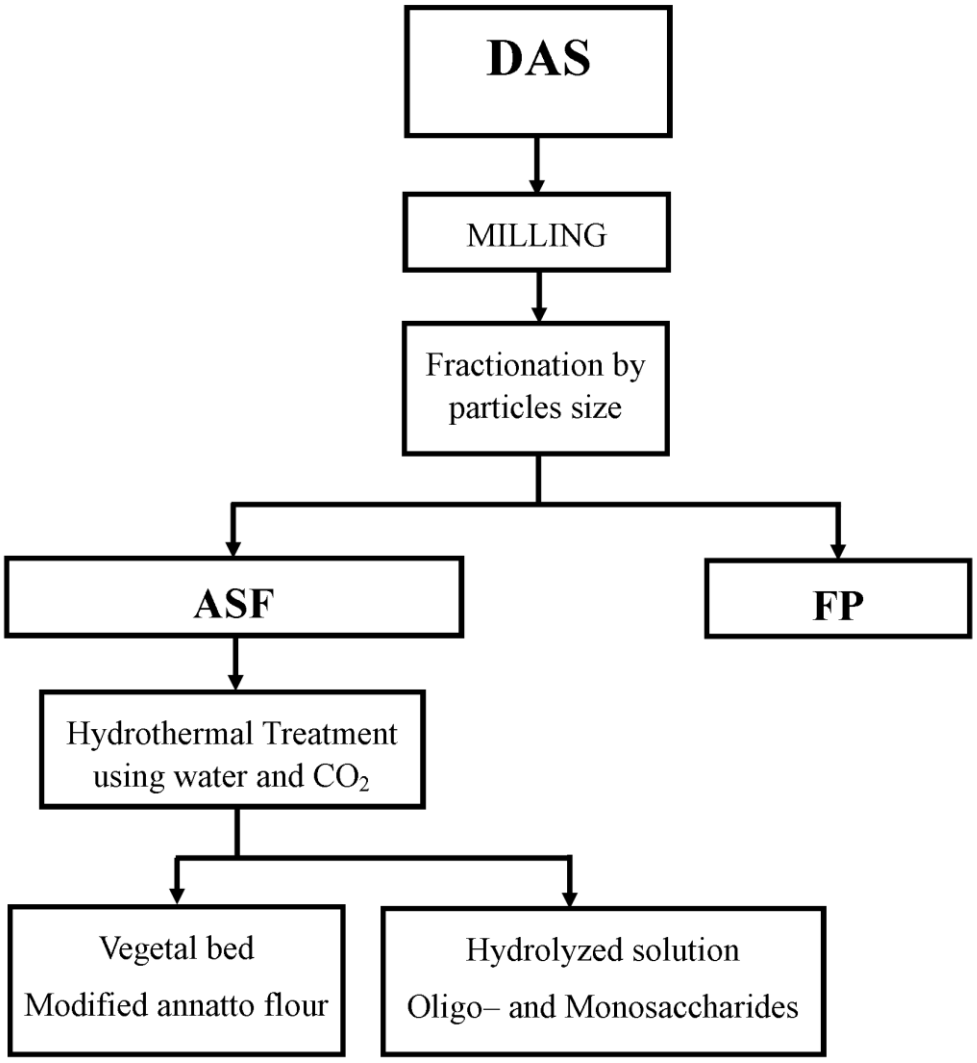

Figure 2: Flowchart of integrated use of DAS

3.2. HMT using water and CO_2

The number of experimental runs studied in the HMT where reduced due to some mechanical problems on equipment caused by raw material characteristics; due to this limitations, 11 experiment runs from a total of 15 were performed.

Using the extraction system was possible to obtain different annatto by-products from the hydrothermal treatment of ASF: i) water soluble hydrolyzed - oligomer and monomers of sugar - dragged with the solvent; and ii) the modified annatto flour remained in the extraction cell. Hydrolyzed water solutions were lyophilized and the RS and TRS were quantified. Results obtained from analysis and quantifications for all experimental runs are presented in Table 2.

Table 2: Hydrothermal treatment of ASF

# Exp.	Solvent relation (Water:CO_2)	Pressure (bar)	Soluble hydrolyzed Extract			Solid Matrix
			Product yield (%)	RS (%)	TRS (%)	Starch (%)
1	20:80	150	8	4 ± 0.1	6 ± 0.1	12 ± 0.5
2	20:80	200	5	4 ± 0.4	4 ± 0.1	17 ± 4
3	20:80	250	11	8 ± 0.6	8 ± 0.1	17 ± 0.7
4	20:80	300	4	1 ± 0.1	2 ± 0.2	19 ± 1
5	20:80	350	6	4 ± 0.1	5 ± 0.1	20 ± 3
6	30:70	150	15	8 ± 0.8	11 ± 0.4	15 ± 0.8
7	30:70	200	8	5 ± 0.6	5 ± 0.1	17 ± 0.3
8	40:60	200	12	3 ± 0.7	7 ± 0.8	19 ± 2
9	40:60	250	15	10 ± 0.8	12 ± 0.1	20 ± 1
10	40:60	300	10	6 ± 0.8	8 ± 0.6	20 ± 1
11	40:60	350	16	14 ± 2	16 ± 0.1	23 ± 2

Product yield expressed as g hydrolyzed product/100 g raw material; reducing sugar (RS) expressed as g glucose/100 g hydrolyzed product; total reducing sugar (TRS) expressed as g sucrose/100 g hydrolyzed product; starch expressed as g starch/100g modified annatto flour. Values calculated in dry basis and presented as mean ± standard deviation.

An important increment in relation with the increment of water (40:60) can be observed from results obtained for the hydrolyzed material (product yield, RS and TRS). On the other hand, the pressure parameter did not show a clear behavior in relation to experimental values.

After the HMT, the ASF keeps inside the extraction vessel. The starch content on the modified ASF was quantified and presented in Table 2. The results show that the starch content increased as the water and pressure increased. The granule of starch is formed by two types of polymers: amylopectin and amylose. The amylopectin consist of linear chains of glucose units linked by α-1,4 glycosidic bonds and is highly branched at the α-1,6 positions by small glucose chains, constituting between 70 to 85% of common starch. Amylose is essentially a linear chain of α-1,4 glucans with limited branched points at the α-1,6 positions (Durrani & Donald, 1995; Jenkins et al., 1993). This result was not expected because the hydrolysis should have decreased the starch content in the ASF due to the production of sugar oligomers and monomers as products of the hydrolysis. Despite that fact, an increment of starch content in other hydrolysis processes was also observed by other researchers (Marco Ulbrich et al., 2014; M. Ulbrich et al., 2015) , who used acid hydrolysis modifications (chemical modification). These researchers explained the increased in starch content due to

the amylopectin depolymerization. Hydrolysis increases the quantity of similar linear chains with the amylose chains. For example, in this study, the enzymatic method (by amylose enzymes) used to starch quantification could have triggered this behavior.

The paste profiles of raw materials are shown in Figure 3. Differences between AS and DAS was observed. SFE process caused an slight increment in the peak viscosity and retrogradation tendency. This result can be explained by the possibility that the SFE process has caused a starch modification due to the temperature used during the process.

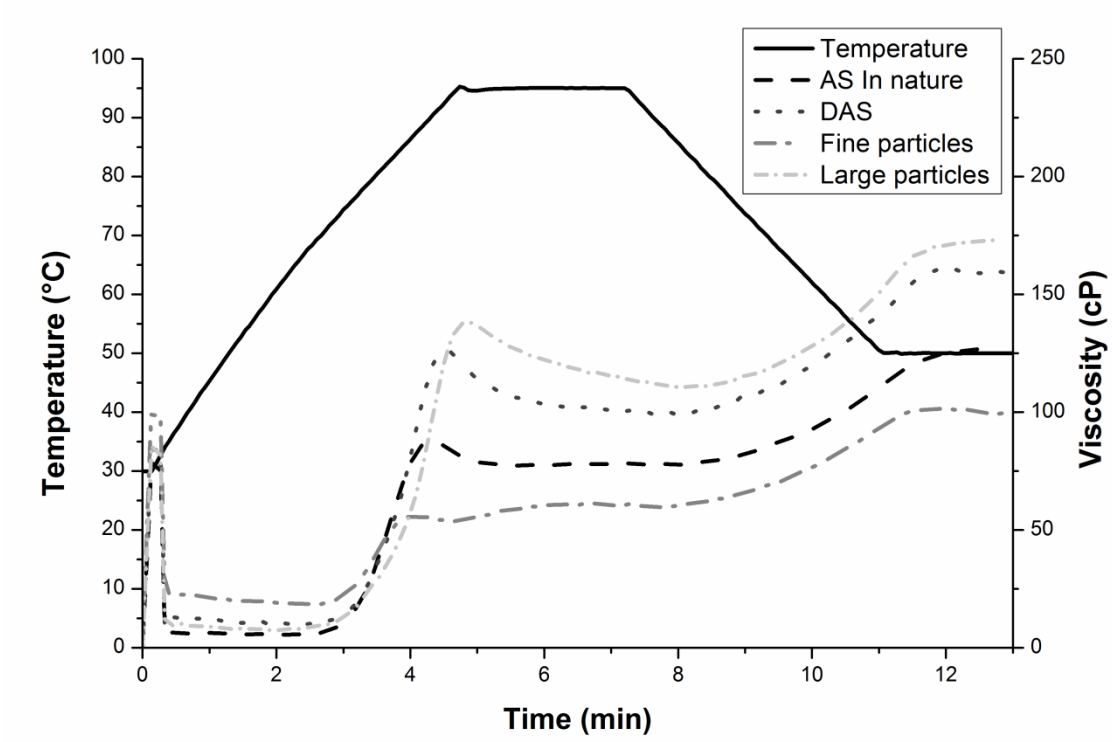

Figure 3: RVA pasting profiles of: AS (Annatto seeds) in natura, DAS (semi-defatted annatto seeds), fine particles and annatto flour (large particles) from DAS.

The paste properties describe the changes that occur in starch after gelatinization in excess of water. The methodology used in RVA analysis describes the paste viscosity as a function of temperature and time (Saunders et al., 2011). The pasting profile showed by annatto material in Figures 3 and 4 are different from the typical pasting curve showed in figure 1, signal of particular properties of annatto. The main factors that affect the paste properties are the starch source and the presence of other polymers (Sarker et al., 2013; Schirmer et al., 2015). The differences on paste behavior between AS, DAS, ASF and LP are possibly caused by the differences on the composition as is shown on Table 1. Paste properties are affected by lipid, protein and lignocellulosic contents as well as its variations.

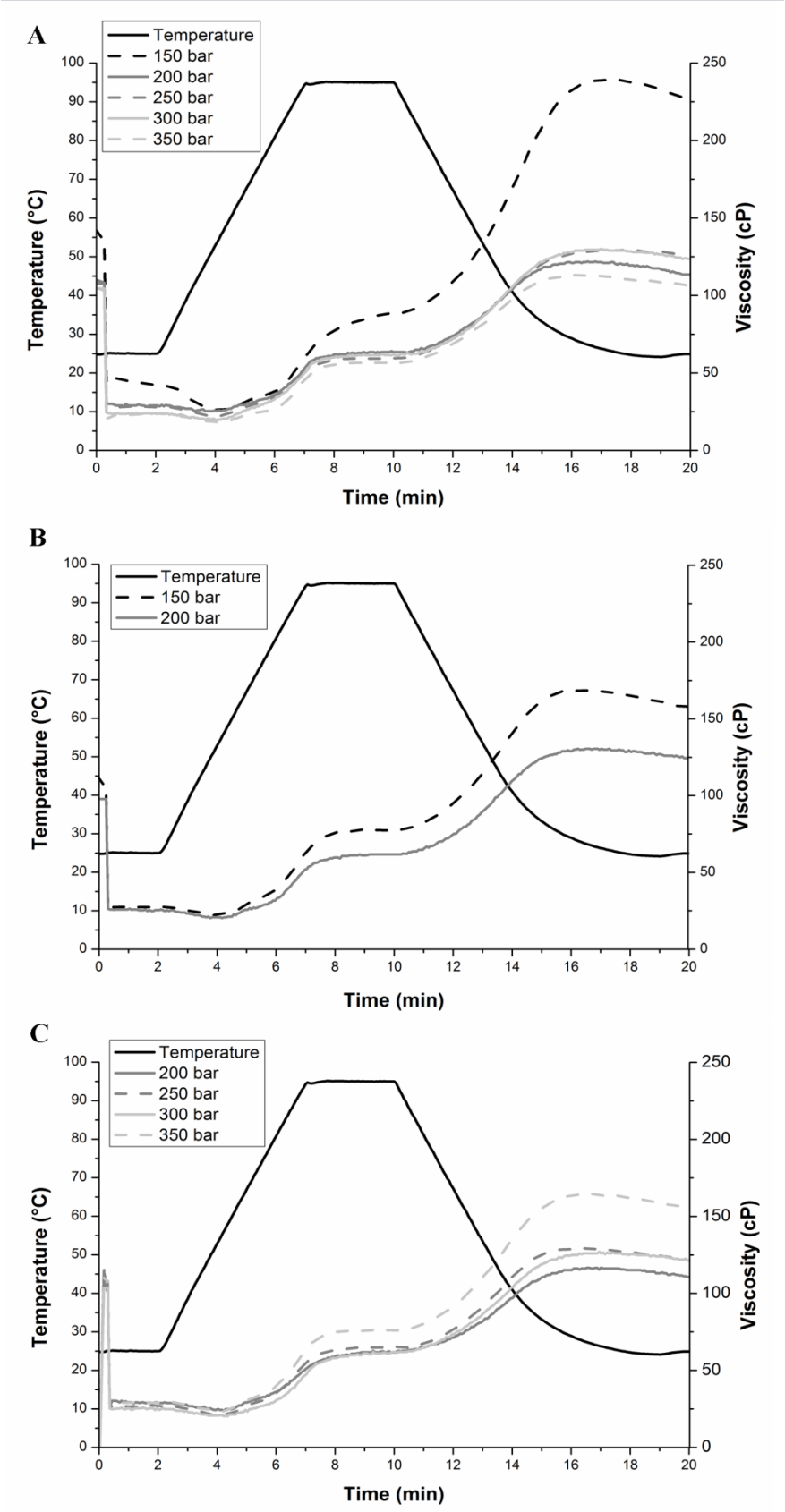

Figure 4: RVA pasting profiles of modified annatto flour; using the solvent proportion water: CO_2 of (A) 20:30, (B) 30:70 and (C) 40:60.

Table 3: Pasting properties of raw materials and modified starch from Annatto seeds

Material			Pasting Parameters						
			Pasting temperature (°C)	Peak time (min)	Peak viscosity	Trough viscosity	Breakdown	Final viscosity	Setback
					(cP)				
AS			72 ± 1	4 ± 1	88 ± 1	77±3	11	127 ± 4	50
DAS			74 ± 1	5 ± 1	126 ± 4	100±2	26	159 ± 4	59
ASF			76 ± 1	5 ± 1	138 ± 2	110±1	28	174 ± 1	64
FP			74 ± 1	4 ± 1	56 ± 4	54±4	2	102 ± 6	48
Experiment points									
# Exp.	**Solvent relation (Water:CO_2)**	**Pressure (bar)**							
1	20:80	150	67 ± 1	10 ± 1	89 ± 1	88 ± 1	1	240 ± 3	152
2	20:80	200	66 ± 1	9 ± 1	64 ± 3	64 ± 2	0	122 ± 6	59
3	20:80	250	68 ± 1	9 ± 1	60 ± 7	60 ± 8	0	130 ± 20	71
4	20:80	300	65 ± 1	9 ± 1	62 ± 1	62 ± 1	0	130 ± 3	69
5	20:80	350	79 ± 1	9 ± 1	57 ± 1	57 ± 1	0	113 ± 1	57
6	30:70	150	68 ± 1	9 ± 1	79 ± 8	78 ± 8	1	170 ± 13	93
7	30:70	200	68 ± 1	9 ± 1	62 ± 9	62 ± 9	0	131 ± 7	69
8	40:60	200	69 ± 1	9 ± 1	62 ± 8	62 ± 7	0	117 ± 10	55
9	40:60	250	69 ± 1	9 ± 1	66 ± 3	65 ± 3	1	132 ± 9	67
10	40:60	300	67 ± 1	9 ± 1	61 ± 2	61 ± 2	0	128 ± 2	67
11	40:60	350	71 ± 1	9 ± 1	76 ± 1	76 ± 1	0	161 ± 4	85

Table 3 summarizes the principal pasting parameters evaluated in RVA analysis. Unmodified starch granules are generally insoluble in water below 50 °C, which is denominated as critical temperature, characteristic for each starch species (Donovan, 1979). In Table 3 can be observed that the critical temperatures of annatto starch before and after modification are in the range of 65 to 79 °C. These results agree with the critical temperature values obtained for starch from waxy maize and rice (BeMiller & Whistler, 2009). When starch granules are heated in water above its critical temperature, the granules absorb a large amount of water and the size is increased as a product of swelling (Conde-Petit et al., 2001; N. Singh et al., 2003). Above the critical temperature, starch granules experiment an irreversible process known as gelatinization. Water diffuses into the granule and due to hydration; the starch granule loses its crystallinity and molecular structure, completing the starch solubilization (Jenkins et al., 1993; Jiménez et al., 2012).

As expected, when the temperature exceeds the gelatinization temperature, the starch granules begin to swell and viscosity is increased. The temperature in which the viscosity is raised is known pasting temperature and is an indication of the minimum temperature required to cook the starch sample. This temperature has implications in the stability of other components in the development of formulations containing starch (Saunders et al., 2011). The gelatinization process is necessary for processes such as textile and hydrolyzed starch industries. When starch granules swell and its components are in solution, the properties change from simple starch suspension to starch paste(Jiménez et al., 2012). The gelatinization progress along the granule is determined by the own properties of starch granule, the presence of other ingredients (e.g., proteins and polysaccharides) and process parameters (Schirmer et al., 2015). In RVA analysis, the pasting temperature in which the viscosity begins to increase, shows the start of starch gelatinization and paste formation. In this work, the hydrothermal modification of ASF caused the reduction of pasting temperature. The reduction of pasting temperature is an important modification, for example, the ASF obtained is this work can be used for applications in sensible food, because it does not require heating to form a paste (Majzoobi et al., 2011).

Above pasting temperature the majority of starch granules become swollen; as the temperature increased, the granules rupture and amylose is dragged out, a three-dimensional network is formed and viscosity increases rapidly (Sarker et al., 2013). Granule rupture and subsequent polymer alignment due to the mechanical shear reduces the apparent viscosity of the paste. These combined processes that follow gelatinization are known as pasting. The starch paste formed after gelatinization is a viscous mass consisting of one continuous phase

of solubilized amylose and/or amylopectin and one discontinuous phase with remaining starch granules (Ambigaipalan et al., 2011). In flour, starch remains typically in combination with other polymers such as protein and other polysaccharides, forming different phases (Conde-Petit et al., 2001).

The peak viscosity occurs at the equilibrium point between swelling that causes an increase in viscosity and the rupture and alignment of the polymers that causes a decrease in the viscosity. The rate and degree of swelling and breakdown is characteristic of each starch source and each modification. On modified annatto starch the peak viscosity decreased. This decreased can be explained because the peak viscosity on starch paste is increased due to starch concentration, but decreases with starch amylose content (Ai & Jane, 2015; Nguyen et al., 1998). The amylose content can have been incremented due to hydrolysis reaction over annatto flour, affecting the peak viscosity behavior caused by amylopectin depolymerization in the modified ASF.

Before HMT process, the ASF pasting curve shows a slight breakdown of viscosity during the hold period. After HMT process, modified ASF shows a particular paste behavior. The breakdown of viscosity was not identified in all experiments, therefore, the peak viscosity is almost constant during all isothermal period (maximum temperature of analysis) and the viscosity restarts a continuous increment during the cooling period. A similar behavior was observed by Puncha-Arnon and Uttapap (2013), in rice starch modified with a hydrothermal treatment. In that study, the trough viscosity could not be clearly defined and therefore, pasting parameters such as peak viscosity, breakdown and setback could not be determined. The authors suggested that the protein composition on starch material could have been denatured by the heat treatment and these changes would have affected the paste properties. As shown in Table 1, for all raw materials, proteins are the second major component after carbohydrates and their presence would influence the paste properties.

After the controlled heating and the isothermal period of analysis the mixture is subsequently cooled, re-association between starch molecules occurs, and viscosity normally will be increased to a final viscosity. This phase of the pasting curve is commonly referred to as the setback region, and involves the starch retrogradation. During retrogradation, amylose molecules are associated with other glucose units to form a three-dimensional network and then, amylopectin molecules are re-crystallized through association of small chains and a gel is formed (N. Singh et al., 2003). The presence of other components different of starch such as proteins, lipids, other carbohydrates and salts may significantly affect the retrogradation property of the paste (Fu et al., 2015; Wu et al., 2010). In general, retrogradation in starch

pastes is unfavorable in terms of food quality, causing syneresis of gels or hardness. Setback parameter is directly associated with syneresis tendency; HMT process do not show a significant modification on retrogradation tendency in ASF.

4. Conclusions

HMT appears to be an attractive technique to cause physical modification of ASF components, mainly starch. After modification, ASF demonstrated to have paste properties that can be compared with conventional starch sources and therefore, presents many potential uses in different industries, where the modified properties might be adaptable and advantageous. Modified ASF also have pigment characteristics from annatto seeds, this property in some way could be also considered as an added value in ASF. Annatto by-products of hydrolysis such as oligomers and monomers of sugars from polysaccharides depolymerization can be incorporate in formulations of special food or on third-generation energy production.

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Acronyms: Semi-defatted annatto seeds (DAS), Supercritical fluid extraction (SFE), Annatto seeds (AS), Annatto seeds flour (ASF) Hydrothermal treatment (HMT), Solvent mass to feed mass ratio (S/F), Reducing sugar (RS), Total reducing sugar (TRS), Rapid Visco Analyzer (RVA).

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- CAPÍTULO 6 –

Obtaining of anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction

Este trabalho foi realizado simultaneamente aos estudos de parceria para extração de bixina das sementes de urucum de reduzido teor lipídico através da técnica de extração com líquidos pressurizados. Neste estudo foi desenvolvido um processo focado na extração e recuperação de antocianinas, substâncias antioxidantes presentes na polpa congelada de açaí (*Euterpe oleracea* Mart.).

Artigo que será submetido após revisão.

Obtaining of anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction

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Abstract

Açai (*Euterpe oleracea* Mart.) is considered a functional food because besides being a source of energy and fiber, it is a valuable source of bioactive compounds such as anthocyanins, minerals and fatty acids. In the present work, antioxidant-rich extracts from açai pulp were obtained using pressurized liquid extraction (PLE). The effects of the independent variables solvent (pure ethanol and ethanol/water (50:50 v/v)), citric acid (0 and 0.3%, w/w), pressure (20 and 80 bar) and temperature (30 and 60 °C) were evaluated using a full factorial design. The anthocyanin extraction was affected primarily by the extraction solvent and the citric acid percentage. The temperature and pressure had not a significant effect in the extraction process. The results indicate that the maximum overall yield (X_0) was 63.5 ± 8.6 (%, d.b.) when the process was performed using ethanol (99.5%) and citric acid (0.3% w/w). The maximum total anthocyanin content (TA) and anthocyanin recovered from raw material were 6.9 ± 1.4 (mg anthocyanin/g extract, d.b.) and 10.6 ± 2.0 (%, d.b.), respectively.

Keywords: Flavonoids; Phenolic compounds; Antioxidant; Biocompounds recovery; Clean technology.

1. Introduction

Açai (*Euterpe oleracea Mart.*) is a palm tree commonly found in northern South America, mainly in the Brazilian Amazon (Oliveira & Santos, 2011). A wide variety of marketable products are produced from this plant, being the spherical fruits the most

important edible products (Del Pozo-Insfran et al., 2004). Recently, açai has attracted worldwide interest due to its exotic flavor and the bioactive properties such as antioxidant capacity and anti-inflammatory activity (Kang et al., 2011; Pacheco-palencia et al., 2007; Schauss et al., 2006b). The Açai pulp is a product with high content of phenolic compounds such as anthocyanins, proanthocyanidins, other flavonoids and lignans (Schauss et al., 2006a). Anthocyanins contribute to prevent some degenerative diseases, and their effects on human health have been mainly attributed to their antioxidant ability to reduce free radicals and prevent cardiovascular problems and other diseases. (Kruger et al., 2014; Spada et al., 2009). The anthocyanins are a group of flavonoid compounds that are responsible for the red, violet and blue color of fruits and vegetables such as strawberry, grape and cranberry, among others (Newsome et al., 2014). Therefore, they are considered a possible substitute of synthetic dyes used in foods, due to their attractive color and to its high solubility in aqueous mediums. Additionally, anthocyanins are considered nontoxic and safe additives (Pina et al., 2012). However, there are some limitations for commercial use of anthocyanins due to the availability of feedstock (quantity and quality), difficulty in the purification of the extracts, high cost of raw materials and low stability during processing and storage (Del Pozo-Insfran et al., 2004; Liazid et al., 2014).

The growing interests in the recovery of biocompounds such anthocyanins from vegetal matrices with possible application in food, pharmaceutical and cosmetic industries represent an important opportunity (Machado et al., 2014). Anthocyanins are generally extracted using methanol, ethanol, acetone, water or a mixture of these solvents (Welch et al., 2008). Anthocyanins are relatively unstable pigments and the temperature is the main factor that triggers the degradation of the anthocyanins, other factors such as their chemical structure, pH, presence of light, ions, enzymes or oxygen, can trigger their degradation (Liazid et al., 2014; Sui et al., 2014). However, the color stability of the anthocyanins is increased under acid presence due to anti-browning effects (Altunkaya & Gökmen, 2009). In this context, to improve the extraction yield, the addition of small quantities of acid (e.g. hydrochloric, formic and citric acid) can be considered to enhance the extraction efficiency (Adjé et al., 2010).

PLE is an environmental friendly extraction technique that allows overcoming the inconveniences of traditional techniques whereas extracts with high quality and purity are obtained. Nowadays, PLE process has become a feasible alternative to recover bioactive compounds from vegetal feedstock (Liazid et al., 2014; Liu et al., 2014; Machado et al., 2014). PLE uses elevated temperatures (30 – 200 °C) and moderate to high pressures (35 –

200 bar) to facilitate and enhance the extraction process. PLE is also called pressurized fluid extraction (PFE), accelerated solvent extraction (ASE), pressurized solvent extract ion (PSE) or enhanced solvent extraction (ESE). As the extraction process is performed using high pressures, the solvent remains at liquid state, even when are used temperatures above the boiling point of the solvent (Richter et al., 1996). Thus, PLE can operate at high temperatures increasing the solubility and desorption of the target compounds from the matrix (Nieto et al., 2010). In the PLE process, pressure and temperature can be adjusted to improve the selectivity of the extraction solvent over a particular group of compounds (Carabias-Martínez et al., 2005; Liu et al., 2014). In the PLE process, solvents generally recognized as safe (GRAS) such as water, ethanol or their mixture are commonly used in phenolic and anthocyanin extraction (Mane et al., 2015; S. Rodrigues et al., 2015).

In this context, the objective of this study was to evaluate PLE for obtaining anthocyanins from frozen açai pulp. Process parameters such as temperature, pressure, solvent and citric acid were evaluated in order to obtain anthocyanin-rich extracts. Pure ethanol and ethanol/water (50:50 v/v) were used as solvents because they are GRAS solvents. In order to evaluate the acid citric effect on the anthocyanin extraction process, citric acid was added into the extraction solvent.

2. Material and Methods

2.1. Chemicals

Ethanol (Dinâmica, Diadema, Brazil) and citric acid (Synth, Diadema, Brazil) were used. Ultra-pure water was obtained from a Mili-Q water purifier system (Millipore, Bedford, USA). The chemicals and solvents used in anthocyanin analysis were methanol (Merck, Darmstadt, Germany) and formic acid (Panreac, Barcelona, Spain) in high-performance liquid chromatography (HPLC) grade. Cyanidin chloride (Sigma–Aldrich Chemical Co., St. Louis, USA) was used as anthocyanin standard.

2.2. Raw material

Frozen açai pulp (De Marchi, São Paulo, Brazil) was obtained from a local market located in Campinas, Brazil. The raw material was kept at -18 °C before use as the raw material in the extractions. The moisture content was determined by lyophilization (LIOBRAS, São Carlos, Brazil). The moisture percentage was calculated by weight the difference before and after lyophilization. The dry matter was characterized according the anthocyanin content using the UHPLC method.

2.3. Pressurized liquid extraction (PLE) process

PLE was performed in homemade equipment as shown in Figure 1. Frozen açai pulp (5.5 g) was placed inside the extraction cell (5 cm^3). The extraction vessel was heated by a heating jacket. The extraction cell assembly containing the raw material was heated during 3 minutes; filled with ethanol and pressurized using a HPLC pump (Thermoseparation Products, California, USA). After reaching the desired pressure, the extraction cell was maintained at the desired temperature for a static period of 5 min. Then the block and back-pressure valves were opened, and carefully adjusted to maintain the system pressure. The dynamic extraction time was 11 min, and the solvent-to-feed ratio (S/F ratio) was 8.5. After the PLE process, the extract was rapidly cooled and protected from light to prevent the degradation of the compounds. After extraction, an aliquot (2 mL) of the extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis.

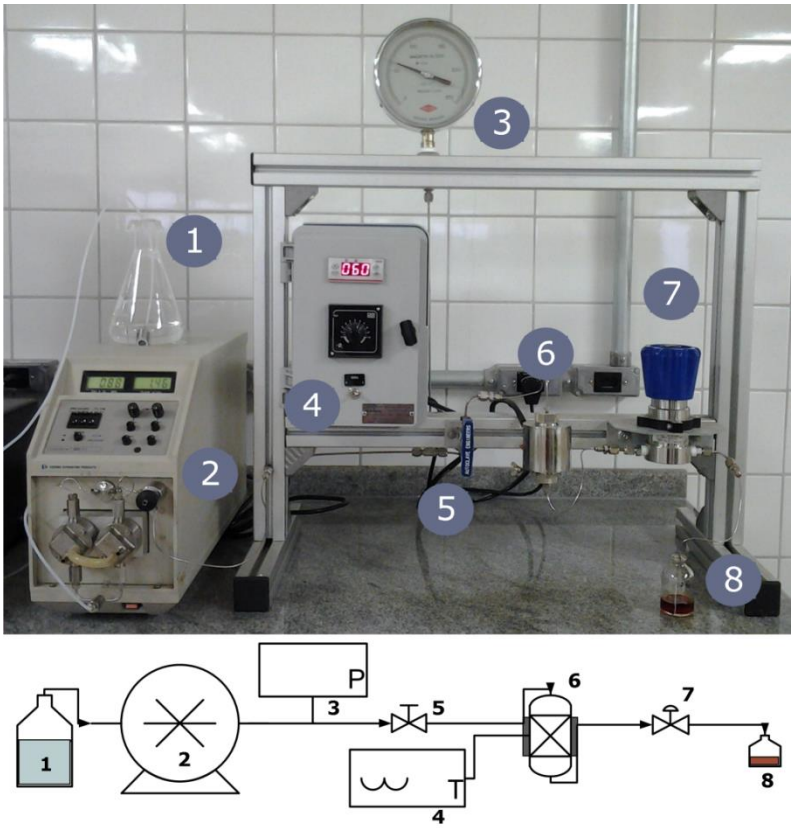

Figure 1: Schematic diagram for the PLE experimental apparatus. 1- solvent reservoir; 2 - HPLC pump; 3 - manometer; 4 - temperature controller; 5 - blocking valve; 6 – extractor column; 7 – back pressure valve; 8 – sampling bottle.

2.5. Determination of overall yield (X_0)

The overall yield (X_0) was expressed in dry basis using the Eq. 1. X_0 was calculated as the ratio of the total mass of the extract ($m_{extract}$) and the initial mass of the extraction sample (m_{sample}) in dry basis.

$$X_0(\%) = \frac{m_{extract}}{m_{sample}} \times 100 \qquad \textbf{Eq.1}$$

2.6. Anthocyanin analysis

2.6.1. Sample preparation

The extracts from the açai samples (raw material) were obtained using an ultrasound-assisted extraction technique. Ultrasonic irradiation was carried out using a UP200S sonifier (200 W, 24 kHz) (Hielscher Ultrasonics, Teltow, Germany), with the sample immersed in a water bath coupled to a temperature controller (Frigiterm-10, J.P. Selecta, S.A., Barcelona, Spain). The following extraction parameters were used to extract the anthocyanins: extraction solvent: methanol:water (pH 2) (50:50); temperature: 30 °C; output amplitude of the nominal amplitude of the transducer: 100% (200 W); duty cycle: 0.5 s; solvent volume: 25 mL; extraction time: 10 minutes; amount of sample: 0.5 g. The extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solutions, Dallas, USA) prior to chromatographic analysis.

2.6.2. Identification of anthocyanins

Anthocyanins were identified by ultra-performance liquid chromatography (UPLC) coupled to quadrupole-time-of-flight mass spectrometry (Q-ToF-MS) (Waters Corp., Milford, USA). The injection volume was set to 3 µl. The chromatographic separation was performed on a reverse-phase C18 analytical column (Waters Corp., Milford, USA) of 2.1 mm x 100 mm and 1.7 µm particle size. For the identification of anthocyanins, water (2% formic acid) as solvent A and methanol as solvent B, as mobile phases at a flow rate of 0.4 mL min^{-1} were used. The gradient employed was as follows: 0 min, 15% B; 3.30 min, 20% B; 3.86 min, 30% B; 5.05 min, 40% B; 5.35 min, 55% B; 5.64 min, 60% B, 5.94 min, 95% B; 7.50 min, 95% B. Total run time was 12 min, including 4 min for re-equilibration. The determination of the analytes was carried out using an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 700 L h^{-1}, desolvation temperature = 500 ºC, cone gas flow = 10 L h^{-1}, source temperature = 150 ºC, capillary voltage = 700 V, cone voltage = 20 V and trap collision energy = 4 eV. Full-scan mode was used (m/z = 100-1200). The anthocyanins identified in the extracts of açai were: cyanidin-3-O-glucoside (C3Gl), cyanidin-3-O-rutinoside (C3R), peonidin-3-O-glucoside

(Peo3Gl) and peonidin-3-O-rutinoside (Peo3R). The molecular ions for these anthocyanins presented the following m/z ratios: cyanidin-3-O-glucoside, 449; cyanidin-3-O-rutinoside, 595; peonidin-3-O-glucoside, 463; peonidin-3-O-rutinoside, 609.

2.6.3. Separation and quantification of anthocyanins

The separation and quantification of anthocyanins were performed on an elite ultra-high performance liquid chromatography (UHPLC) LaChrom Ultra system (Hitachi, Tokyo, Japan) consisting of an L-2200U Autosampler, an L2300 Column Oven, an L-2160U Pumps and an L-2420U UV-Vis Detector. The column oven was adjusted at 50 ºC for the chromatographic. UV-Vis Detector was set at 520 nm for the analysis. Anthocyanins were analyzed on a HaloTM C18 Hitachi LaChrom column (100 x 3 mm I.D., particle size 2.7 µm). A gradient method, using acidified water (5% formic acid, solvent A) and methanol (solvent B), working at a flow rate of 1.0 mL min^{-1}, was employed for the chromatographic separation. The gradient employed was as follows: 0 min, 15% B; 1.50 min, 20% B; 3.30 min, 30% B; 4.80 min, 40% B; 5.40 min, 55% B; 5.90 min, 60% B; 6.60 min, 95% B; 9.30 min, 95% B; 10 min, 15% B.

2.6.4. UHPLC calibration

The UHPLC method was used to obtain a calibration curve for cyanidin chloride (y = 300568.88x – 28462.43), which is the anthocyanidin standard commercially available for cyanidin anthocyanins. Regression equation and correlation coefficient (R_2 = 0.9999) were calculated using Microsoft Office Excel 2010. The limit of detection (0.198 mg L^{-1}) and quantification (0.662 mg L^{-1}) were also calculated using Microsoft Office Excel 2010. The four anthocyanins present in açai (cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, peonidin-3-O-glucoside and peonidin-3-O-rutinoside) were quantified using the calibration curve for cyanidin chloride, taking into account the molecular weight of the anthocyanins analyzed. All analyses were carried out in duplicate.

2.7. Determination of anthocyanin yield (AY)

Anthocyanin yield (AY) expressed in dry basis was calculated using Eq. 2. AY was calculated multiplying the value of X_0 by the total anthocyanins in the extract ($Anthocyanins_{extract}$(mg/g)) and dividing by the total anthocyanins in original sample for extraction ($Anthocyanins_{sample}$ (mg/g)).

$$AY(\%) = \frac{X_0(\%) \times Anthocyanins_{extract}(mg/g)}{Anthocyanins_{sample}(mg/g)} \qquad \textbf{Eq. 2}$$

2.8. Statistical analysis

A full factorial design was used to determine the effects of extraction parameters temperature (30 and 60 °C), pressure (20 and 80 bar), solvent (pure ethanol and ethanol/water (50:50 v/v)) and the addition of citric acid (0 and 0.3% w/w). The experiments were performed in duplicate. Minitab® 16 software was used to analyze the effects of the extraction conditions (temperature, pressure, solvent and percentage of citric acid) on the overall yield (X_0), total anthocyanin content (TA) and anthocyanin yield (AY) with a confidence interval of 95% ($p_{value} \leq 0.05$).

3. Results and discussion

3.1. Raw material characterization

The moisture content (wet basis) of the frozen açai pulp was 89% ± 2%. This result agrees with the result obtained by Oliveira and Santos (2011) in a similar sample (89% ± 1% of moisture). This author also determined other components in wet basis, including ash (0.4% ± 0.03%), protein (3% ± 0.1%) lipids (6% ± 0.1%), total sugar (2% ± 0.7%), fibers (4% ± 0.1%) and minor components as calcium and iron. Table 1 list the anthocyanin content of frozen açai pulp expressed in dry basis.

Table 1. Anthocyanin composition on frozen açai pulp (in dry basis)

	Anthocyanin content (mg/g)
C3Gl	0.6 ± 0.01
C3R	10 ± 0.3
Peo3Gl	0.3 ± 0.01
Peo3R	0.6 ± 0.01
Total Anthocyanins	11 ± 0.3

Values presented as mean ± standard deviation. Total Anthocyanins: cyanidin-3-O-glucoside (C3Gl), cyanidin-3-O-rutinoside (C3R), peonidin-3-O-glucoside (Peo3Gl) and peonidin-3-O-rutinoside (Peo3R).

According to the quality standard, the açai pulp products are derived from the edible fraction of the açai fruit after recovery the pulp through appropriate technological processes (Oliveira & Santos, 2011). Açai pulp is classified according to the amount of total solids. There are three types: thick (A) which has 14% of dry matter and a very dense appearance; average (B) which has 11% to 14% of dry matter and a dense appearance and fine (C) which contains 8% to 11% of dry matter and a thin appearance (Moraes, 2000).

According to the amount of total solids, the raw material used in this can be classified as type C. For PLE process, the frozen açai pulp was not dehydrated in order to preserve the integrity of target biocompounds.

3.2. PLE anthocyanin-rich extracts

Table 2 lists the experimental conditions and the overall yield and anthocyanin yields, and total anthocyanin content obtained by PLE.

3.2.1. Overall yield (X_0) of PLE

In the temperature, pressure, solvent and citric acid ranges studied, only the combination of solvent and citric acid effects ($p_{value} < 0.03$) had a significant impact on the extraction yield. The overall yield varied from 14.5% d.b. to 63.5% d.b. The results show that the extraction yield has an increment directly proportional to the increment in concentration of ethanol and citric acid as can be clearly observed in the contour plot presented in Figure 2. The ethanol and citric acid contribute to increase overall yield, as consequence of the ethanol polarity and the acid medium that caused the increase of the extraction of a higher quantity of components from the matrix and reduce the selectivity of process. For example, the highest yield obtained was result of a process that used pure ethanol and 0.3% of citric acid (pH 3.7).

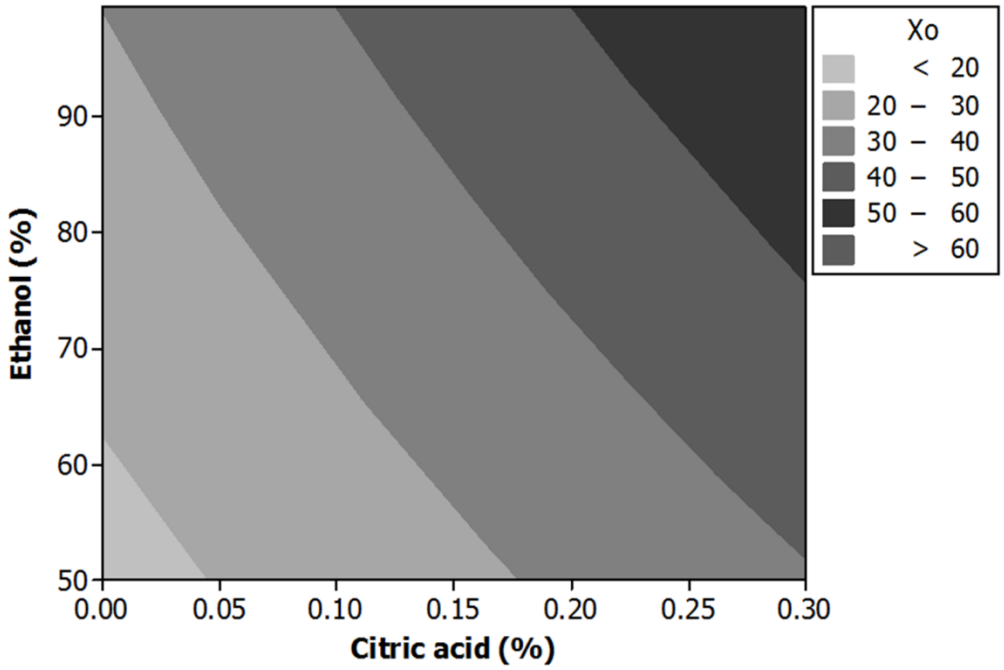

Figure 2: Contour of plot of ethanol (%) and citric acid (%) for X_0.

Table 2. Results obtained from PLE process

#	Conditions of PLE process				Response variables						
	Temperature (°C)	Pressure (bar)	Ethanol (%)	Citric acid (%)	X_0 (%)	C3Gl* ($\times 10^{-1}$)	C3R*	Peo3Gl* ($\times 10^{-1}$)	Peo3R* ($\times 10^{-1}$)	TA*	AY (%)
1	30	20	50	0	16 ± 1	7 ± 2	6 ± 1	2 ± 0.3	3 ± 0.8	7 ± 1	10 ± 1
2	60	20	50	0	15 ± 2	6 ± 1	5 ± 1	2 ± 0.1	3 ± 0.4	6 ± 1	8 ± 0.5
3	30	80	50	0	18 ± 3	7 ± 1	5 ± 0.9	2 ± 0.1	3 ± 0.5	6 ± 1	11 ± 2
4	60	80	50	0	17 ± 3	7 ± 0.8	5 ± 0.9	2 ± 0.3	3 ± 0.7	6 ± 1	10 ± 0.1
5	30	20	99.5	0	30 ± 5	3 ± 0.6	2 ± 0.4	0.8 ± 0.2	1 ± 0.4	2 ± 0.6	6 ± 0.4
6	60	20	99.5	0	36 ± 7	3 ± 0.9	2 ± 0.5	0.8 ± 0.1	1 ± 0.3	3 ± 0.6	9 ± 0.4
7	30	80	99.5	0	31 ± 3	3 ± 0.2	2 ± 0.2	0.8 ± 0.1	2 ± 0.2	3 ± 0.2	8 ± 0.1
8	60	80	99.5	0	24 ± 0.2	4± 0.4	2 ± 0.3	1 ± 0.1	2 ± 0.2	3 ± 0.4	7 ± 0.7
9	30	20	50	0.3	34 ± 1	2 ± 0.3	1 ± 0.2	0.6 ± 0.1	1 ± 0.1	2 ± 0.3	6 ± 0.6
10	60	20	50	0.3	41 ± 1	2 ± 0.5	2 ± 0.4	0.6 ± 0.1	1 ± 0.1	2 ± 0.4	9 ± 1
11	30	80	50	0.3	42 ± 5	2 ± 0.5	1 ± 0.3	0.5 ± 0.1	1 ± 0.1	2 ± 0.3	7 ± 1
12	60	80	50	0.3	40 ± 5	2 ± 0.2	2 ± 0.2	0.6 ± 0.1	1 ± 0.1	2 ± 0.2	7 ± 0.1
13	30	20	99.5	0.3	58 ± 2	0.7 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	3 ± 0.6
14	60	20	99.5	0.3	61 ± 8	0.8 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	3 ± 0.9
15	30	80	99.5	0.3	58 ± 3	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	3 ± 0.8
16	60	80	99.5	0.3	64 ± 9	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	3 ± 0.2

*Expressed as mg/g extract, d.b.; X_0 overall extraction yield (%, d.b.), TA the amount of total anthocyanins in extract and AY the extraction yield anthocyanin (%, d.b.) from PLE process. Values presented as mean ± standard deviation. Total Anthocyanins: cyanidin-3-O-glucoside (C3Gl), cyanidin-3-O-rutinoside (C3R), peonidin-3-O-glucoside (Peo3Gl) and peonidin-3-O-rutinoside (Peo3R).

In this study the temperature had not a significant effect on the anthocyanin extraction process from açai pulp by PLE. This result disagrees with the observed by Machado et al. (2014) in an anthocyanin extraction process from blackberry residues when temperature had a positive effect in the overall yield. The anthocyanins identified in blackberry extracts showed higher yields when the PLE process was performed using acidified water (pH 2.5) and temperatures between 60 and 80 °C at 75 bar. Although in some PLE processes are commonly used high temperatures to obtain higher yields, in this work, moderate temperatures were considered in order to maintain the integrity of the anthocyanins. According to Liu et al. (2014), phenolics compound such as anthocyanins are thermolabile and therefore, the use of high temperatures should be avoid to prevent the degradation of the compounds.

3.2.2. Anthocyanin composition in the PLE extracts

In this study, to determine the composition of the extracts, two major anthocyanins (C3Gl and C3R) were selected. C3Gl is known as one of the most predominant anthocyanins in nature and in açai fruit (Del Pozo-Insfran et al., 2004) and C3R has been reported as one of the most thermally stable anthocyanins (Rubinskiene et al., 2005; Sui et al., 2014). As shown in Table 1, the anthocyanin C3R represents the highest content in the total composition of anthocyanins in the frozen açai pulp. This behavior is not common in açai fresh pulp and blackberry (*Rubus fruticosus* L.) residues according to values reported by Del Pozo-Insfran et al. (2004) and Machado et al. (2014), respectively, where the anthocyanin C3Gl is the predominant compound in the extracts. This difference can be explained due to process conditions through which the raw material used in this work was obtained and the water content in the raw material (Welch et al., 2008; Yamaguchi et al., 2015).

Yamaguchi et al. (2015) highlight the presence of C3Gl and C3R in açai extracts. Both compounds contribute with biological properties in the extracts such as antioxidant, anti-inflammatory, anti-proliferative and cardio protective activities. The presence of these anthocyanins (C3Gl and C3R) in the frozen açai pulp extracts as shown in Table 1 is an indicator the antioxidant potential activity of the extracts obtained by PLE process as presented in Table 2. On the other hand, another two anthocyanins with minor concentration were identified: Pe3Gl and Pe3R.

Total anthocyanin (TA) content is given as a function of the process parameters in Table 2. It should also be noted that over the temperature and pressure ranges studied, TA was not significantly affected by any of this factors. Instead, once again the TA was significantly affected by the combination of solvent and citric acid effects ($p_{value} < 0.01$).

Vieira et al. (2013) evaluated the influence of ultrasonic and agitated bed extraction methods on the anthocyanin composition of extracts obtained from jussara (*Euterpe edulis*) pulp. For authors, anthocyanin contents were significantly affected by the extraction time, ethanol concentrations in acidified water and S/F ratios, but not by the extraction temperature or between extraction methods studied.

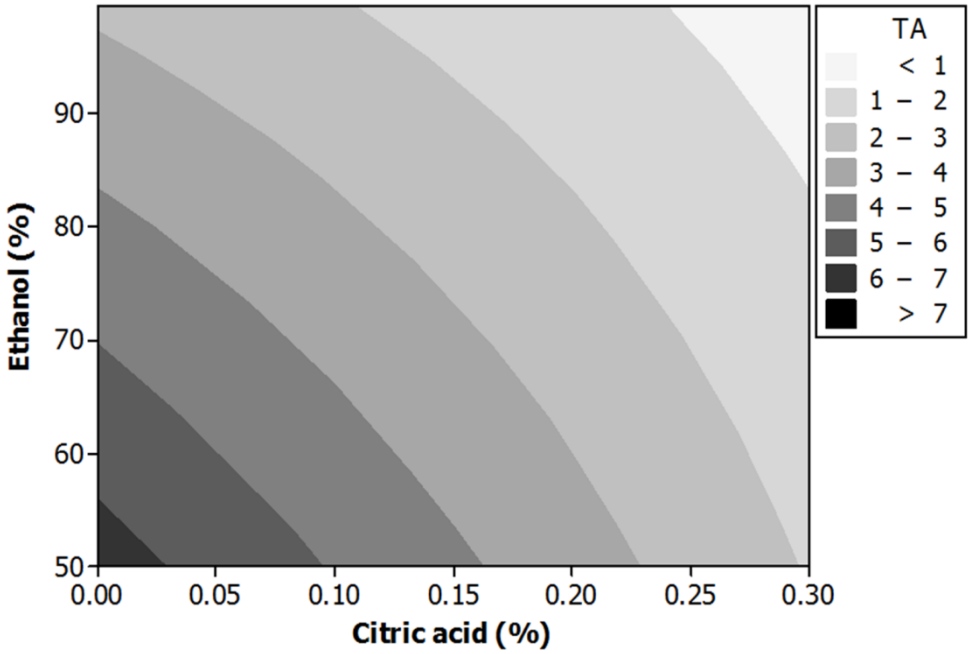

Figure 3: Contour plot of ethanol (%) and citric acid (%) for TA.

Contrary to the behavior shown by the overall yield, the TA composition presented a constant increment inversely proportional to the increment in concentration of ethanol and citric acid as can be observed in Figure 3. The highest amount of TA was obtained when ethanol/water (50:50 v/v) was used as a solvent (pH 6.1), which demonstrate the solubility of anthocyanins in aqueous solutions. (Pina et al., 2012). Although small quantities of citric acid can improve the extraction efficiency once the color stability of the anthocyanins is increased under acid conditions (Adjé et al., 2010), in this work, the addition of citric acid did not show a positive effect on the quantity of anthocyanins extracted. On the other hand, the effects of the solvent and citric acid on X_0 and TA in PLE extracts were different. Due to higher quantities of ethanol and citric acid increase the extraction of other components from raw material and then, the TA in the extract is reduced.

3.2.3. Anthocyanin yield (AY)

The anthocyanin yield is given as a function of temperature, pressure, solvent and citric acid in Table 2. The yield varied from 2.7% d.b. to 10.6% d.b. The anthocyanin yield was significantly affected by the combination of solvent and citric acid (p_{value} = 0.01). It is important to highlight that in the range of temperature and pressure tested, neither of two parameters had a significant effect on the extraction process. The interaction between ethanol and citric acid appears to be the key factor in the extraction process. In Figure 4 is presented the contour plot of the interaction of ethanol and citric acid. Better anthocyanin yields were obtained when the solvent had a great proportion of water and with no citric acid addition. As was stablished previously, a lower content of ethanol contributes with the extraction process. In the same way, the process had a better performance when lower quantities of citric acid were used. As established previously, a lower content of ethanol contributed with the extraction process because the solubility of the anthocyanins is increased in aqueous solutions. In the same way, the process had better performance when lower quantities of citric acid were used. The citric acid in this study increases the release of other compounds, and as consequence, a lower purity of the extract and a decrease in the anthocyanin yield.

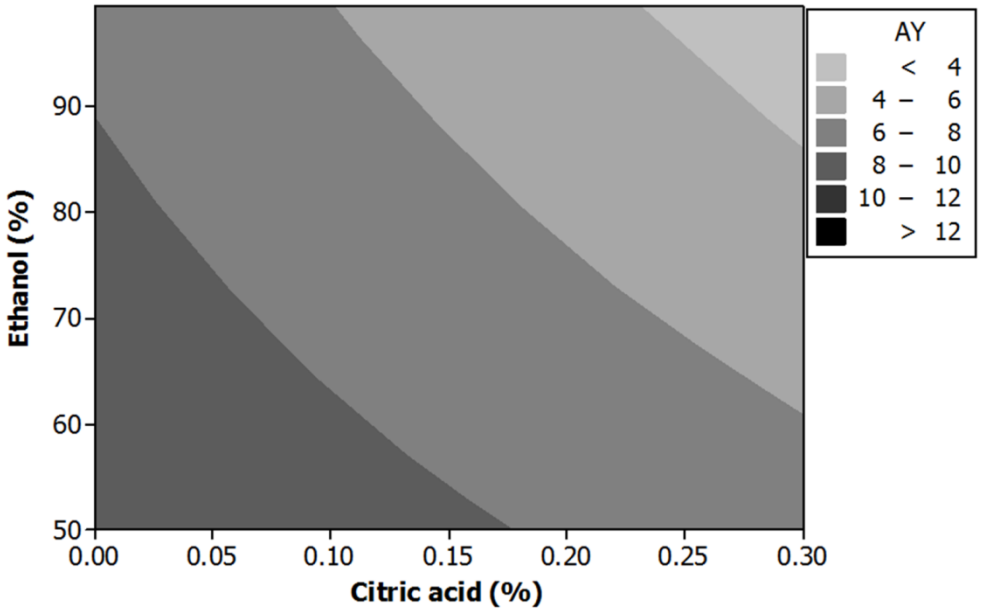

Figure 4: Contour of plot ethanol (%) and citric acid (%) for AY.

However, the use of citric acid in other researches has been successful. For example, Adjé et al. (2010) studied the ultrasound-assisted extraction of anthocyanins from flowers of *Delonix regia* using water as extraction solvent. In that work, the researchers

extracted the anthocyanins using acidified water (sulfuric acid (0.01N) and citric acid (0.01N)). The results obtained showed better results when the process was performed using higher concentration of citric acid. Although the use of citric acid seems have a better protective effect on anthocyanins than other acids such as sulfuric acid, the use of citric acid in anthocyanin extraction is a topic that still being discussed. For example, the acid citric have not a positive effect in all anthocyanin extraction researches. According to Pacheco-palencia et al. (2007), the citric acid presence in rich-anthocyanin systems can promote the degradation of the compounds and a negative effect in the color retention. Therefore, further research about the use of citric acid in anthocyanin extraction should be conducted in order to clarify the effects of the citric acid in the extraction process. On the other hand, after performing the statistical analysis and due to the AY values obtained in this work, it is recommended to perform subsequent studies increasing the proportion of water in the extraction solvent.

In this study, the temperature and pressure had not a positive neither negative effect in the extraction process, therefore, is possible to establish that the use of the PLE process in anthocyanin extraction is not recommended at least in the evaluated range of temperature and pressure. However, the results obtained in this work are relevant because it contributes with the purpose of obtain anthocyanin-rich extracts from frozen açai fruit pulp. A similar behavior was observed by L. M. Rodrigues et al. (2014). The authors recommended instead the PLE process, the use of the low pressure solvent extraction (LPSE) technique in order to obtain better results.

4. Conclusions

The results of this study show that anthocyanin-rich extracts can be obtained from frozen açai pulp an environmentally friendly process such as the PLE process. The results indicate that the anthocyanin extraction was affected primarily by the extraction solvent and the acid citric percentage. The temperature and pressure had not a significant effect in the extraction process. Specifically, increasing the water proportion in the extraction solvent improved the extraction efficiency of the anthocyanin, while increasing the citric acid negatively affected the extraction process. The best results for the anthocyanin extraction were ethanol/water (50:50 v/v) without citric acid to acidulate the extraction solvent. The application of PLE process to recovery anthocyanin biocompounds from frozen açai pulp was not determined conclusively, due to that in the evaluated range of temperature and pressure, neither of two parameters had a significant effect in the anthocyanin extraction. Although, this study proved that anthocyanins from frozen açai pulp are present in a fully functional form in

the extracts obtained; further research is necessary in order to understand the effect of the process conditions. Another range of temperature and pressure, minor concentration of citric acid and larger quantities of water in the extraction solvent could be evaluated in order to optimize the process.

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The authors have declared no conflict of interest

Acronyms

Pressurized liquid extraction (PLE); Overall yield (X_0); Total anthocyanin (TA); Anthocyanin yield (AY); Cyanidin-3-O-glucoside (C3Gl); Cyanidin-3-O-rutinoside (C3R); Peonidin-3-O-glucoside (Peo3Gl); Peonidin-3-O-rutinoside (Peo3R).

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- CAPÍTULO 7 –

Discussão geral

Neste capítulo é apresentada uma análise geral dos capítulos estudados e dos resultados experimentais obtidos ao decorrer desta tese.

CAPÍTULO 7 – DISCUSSÃO GERAL

No **Capítulo 2 - "Study of the effect of extraction process as pre-treatments for sugar production from acid hydrolysis"** foi estudado o efeito dos processos de extração (PLE, Extração Soxhlet e SFE-CO_2) sobre a estrutura vegetal das matérias-primas sementes de urucum, a fibra de palma prensada e o ginseng brasileiro através de comparação para com os materiais que antecederam à etapa de extração realizada no trabalho e foi comprovado que cada matéria-prima e processo de extração aplicado neste estudo refletem diferentes efeitos na estrutura residuária dependendo das caraterísticas intrínsecas de cada matéria-prima e da seletividade do solvente usado.

As análises de composição centesimal destes materiais mostraram uma proporção considerável de carboidratos (amido e/ou material lignocelulósico) que tornam esses resíduos em potenciais substratos para processos de conversão mediante hidrólise ácida.

Mediante as observações da morfologia de cada material estudado foram observadas estruturas celulares diferenciadas. O ginseng brasileiro apresenta uma estrutura celular constituída aparentemente por hemicelulose e celulose (Gomez et al., 2008); o tratamento PLE usando água fez com que esse solvente, ao carregar as sustâncias de interesse viesse a atravessar as paredes celulares resultando no relaxamento da estrutura do material e simultaneamente favoreceu a hidrólise do mesmo. A fibra de palma prensada apresenta uma estrutura celular em que o material lignocelulósico é constituído principalmente por celulose e lignina (Cardenas-Toro et al., 2014b; Chin et al., 2014); após o tratamento de extração Soxhlet foi visualizada porosidade na superfície do material, sugerindo que todo o material de natureza lipídica foi arrastrado pelo solvente (éter de petróleo), e que não houve nenhuma interação entre o material lignocelulósico e o solvente. As micrografias das sementes de urucum mostram uma estrutura celular organizada, na qual é possível observar a conformação

celulósica chamada de amiloplastos (Ellis et al., 1998; Smith, 2001) evidenciando a presença de amido nesta matéria-prima. Os tratamentos de extração aplicados nas sementes de urucum refletiram na morfologia da semente uma mudança que aparentemente envolve o arrastamento do material amilósico pelos solventes. Foi verificado por Braga et al. (2006) nos estudos sobre cúrcuma e gengibre que a extração SFE-CO2 não altera a composição lignocelulósica e de amido presente nas estruturas vegetais, logo o comportamento do solvente na extração da fração lipídica das sementes de urucum através do arrastamento aparente dos grânulos de amido supõe que, entre as caraterísticas próprias do amido presente na semente de urucum está envolvido o complexo amilose-lipídico.

Os resultados obtidos da hidrólise ácida aplicada aos materiais estudados foram diferenciados por causa da natureza dos componentes lignocelulósicos e quantidades disponíveis para hidrólise em cada uma das matérias-primas, e pelos efeitos dos diferentes processos de extração de onde os materiais a hidrolisar foram obtidos. Para o ginseng brasileiro os melhores resultados de concentração de açúcares redutores (AR) e açúcares redutores totais (ART) corresponderam ao material anterior à extração, provavelmente pelo fato da água ter favorecido a reação de hidrólise e o arraste dos açúcares disponíveis na matéria-prima original, portanto o material após extração possui uma menor quantidade de açúcares disponíveis para hidrólise ácida. A fibra de palma prensada e as sementes de urucum tiveram pequeno aumento na quantidade de AR e ART na hidrólise do material após os processos de extração aos quais foram submetidas.

Nos resultados apresentados no **Capítulo 4 - "Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation"**, visando o maior aproveitamento da matéria-prima residuaria para obtenção de produtos de valor comercial foi aplicado um estudo preliminar de acondicionamento do material mediante a moagem e agrupamento em partículas finas ($d_p \leq$

300 µm) e grossas (d_p > 300 µm); a concentração de bixina foi o critério utilizado para definir os destinos de cada grupo de partículas em função da aplicabilidade desse pigmento como corante. Neste capítulo as fontes de partículas finas correspondem a dois lotes de processos de extração: do Lote 1 (SFE-CO_2, S/F 3,5) foram obtidos aproximadamente 23% dessas partículas e do Lote 2 (SFE-CO_2, S/F 11), 10 %. As diferenças de aquisição de partículas finas entre os lotes estão possivelmente associadas à quantidade de CO_2 e tempo de extração aplicados no material vegetal (Albuquerque & Meireles, 2012). O tratamento LPSE foi estudado neste grupo de partículas na procura de obter extratos ricos em bixina considerando que este material disponibilizava maior quantidade de bixina e maior superfície de contato para o solvente. Na avaliação econômica foi estudada viabilidade do processo de separação mecânica (MFM) e a integração deste com o processo de extração (MFM+LPSE) e foram estabelecidas comparações dos custos finais de aquisição dos produtos de cada etapa: as partículas finas obtidas pelo processo MFM e os extratos obtidos pelo processo integrado. Os resultados da análise de custo mostraram que o processo mecânico é mais vantajoso do que o processo integrado por ter menor custo.

No **Capítulo 5 - Artigo científico: "Hydrothermal modification treatment for the integral use of semi-defatted annatto seeds"** foi aplicada a tecnologia hidrotérmica nas partículas grossas das sementes de urucum obtidas no capítulo anterior pelo método de separação mecânica. A modificação física da estrutura dos polímeros presentes na matéria-prima foi estudada através da influencia dos parâmetros do processo (temperatura, pressão e relação agua/CO_2, refletidas nas propriedades de pasta. Ao tempo que se produz as mudanças dos polímeros presentes nas partículas grossas das sementes de urucum, acontece uma hidrólise parcial gerada pelas caraterísticas da água utilizada e a acidificação do meio causado pela presença de CO_2; desta hidrólise leve é obtida uma solução aquosa que apresenta AR e

ART os mesmos que foram quantificados e considerados como um subproduto do processo estudado.

Na obtenção de extratos de biocompostos através da técnica de extração com líquidos pressurizados (PLE- Pressurized Liquid Extraction) da polpa congelada de açaí realizada no **Capítulo 6 - "Obtaining anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction"** os parâmetros estudados (temperatura, pressão, solvente e adição de ácido cítrico) exerceram efeitos diferenciados nos resultados de rendimento global, concentração de antocianinas e eficiência da extração. Ao estudar o rendimento da extração e a concentração de antocianinas no extrato, os parâmetros que apresentaram efeitos significativos foram a seleção do solvente e a adição de ácido cítrico no meio. O rendimento global da extração aumentou em função do aumento da concentração de etanol e ácido cítrico no meio de extração, enquanto que a quantidade de antocianinas presente no extrato diminui. A variável estudada como eficiência da extração representa a porcentagem de antocianinas quantificadas no extrato quando comparadas com a quantidade de antocianinas presentes na matéria prima antes da extração. A eficiência da extração de antocianinas novamente apresenta como parâmetros significativos a escolha do solvente e a adição de ácido cítrico no meio, e pelo fato de ser calculada considerando-se o rendimento da extração e a quantidade de antocianinas no extrato é sugerido que a análise da superfície de resposta produzida pela interação do uso de solvente e da quantidade de ácido cítrico presente no meio seja levada em consideração para trabalhos futuros.

Os parâmetros temperatura e pressão adotados no processo PLE não influenciaram a extração de antocianinas; este comportamento também foi evidenciado na extração do pigmento bixina nas sementes de urucum de reduzido teor lipídico conforme Rodrigues et al. (2014), no qual é sugerido o uso de extração a baixa pressão no lugar da extração com líquidos pressurizados.

- CAPÍTULO 8 –

Conclusões gerais e Sugestões para trabalhos futuros

Os capítulos anteriores e alguns resultados foram resumidos a fim de evidenciar as informações de maior importância e sugerir trabalhos futuros.

CAPÍTULO 8 – CONCLUSÕES GERAIS E SUGESTÕES PARA TRABALHOS FUTUROS

No **Capítulo 1** foi abordado de forma sucinta o objetivo desta pesquisa que trata da utilização de técnicas de extração e tratamento hidrotérmico que aborda o aproveitamento total da semente de urucum residuária do processo de extração de tocotrienóis com CO_2 supercrítico. As principais tecnologias abordadas neste capítulo foram a extração com solvente a baixa pressão (LPSE) e o tratamento hidrotérmico com água pressurizada e CO_2 supercrítico. Foi comprovado que a técnica LPSE com solvente etanol é adequada para a extração do pigmento bixina. O tratamento hidrotérmico é uma técnica adequada para a modificação da estrutura da matéria-prima vegetal para fins industriais. Ambos os processos são considerados tecnologias limpas.

No **Capítulo 2** foi estudado o efeito dos tratamentos de separação física na estrutura de diferentes matrizes vegetais: extração com fluidos pressurizados no ginseng brasileiro, extração Soxhlet na fibra de palma prensada e nas sementes de urucum, e a extração supercrítica nas sementes de urucum, usada no decorrer desta tese. Os efeitos dos processos de extração resultaram na exposição de materiais lignocelulósicos que podem ser convertidos em açúcares fermentescíveis através de hidrólise ácida.

O estado da arte sobre a modificação de amido foi apresentado no **Capítulo 3.** A revisão da literatura mostrou a importância do amido nas indústrias alimentícia e não alimentícia, tanto pelas caraterísticas nutricionais como pelas propriedades funcionais do amido e a versatilidade das suas diferentes aplicações. Foram abordadas as metodologias de modificações físicas e químicas de amidos procedentes de fontes convencionais e não convencionais. A revisão bibliográfica evidencia que dependendo do custo e disponibilidade, o uso de amido convencional poderia ser substituído parcial ou totalmente por amidos não convencionais sempre que estes apresentem caraterísticas apropriadas para sua aplicação. Uma vez que estas características podem ser alteradas para determinados fins mediante a aplicação de tratamentos de modificação, existe enorme potencial para o aproveitamento de sementes de urucum que possuem teor considerável de amido em sua composição.

Nos **Capítulos 4 e 5** foram usados processos visando o total aproveitamento das sementes de urucum provenientes do processo de extração da fração lipídica com CO_2 supercrítico. Foi desenvolvido um fluxograma de processo que objetiva a formulação de produtos que possam ser incorporados em diversos processos na indústria. Nesta tese o aproveitamento global das sementes de urucum de reduzido teor lipídico é baseado na

moagem e no fracionamento do material vegetal em função do tamanho de partículas (método mecânico).

O **Capítulo 4** integra o processo de separação mecânica e o processo de extração com solvente a baixa pressão para a obtenção do pigmento bixina a partir das partículas de menor tamanho ($dp \leq 300$ μm). Foi obtido extrato com quantidade significativa de bixina. A análise econômica mostrou que a extração com solvente aumenta o custo do pigmento bixina obtido por LPSE sem apresentar maiores vantagens do que a bixina existente na fração fina das sementes separada por método mecânico. Portanto, o método mecânico é uma forma atrativa e economicamente viável para aquisição de bixina.

No **capítulo 5** o material correspondente às partículas de maior tamanho (grossas), denominado neste estudo "farinha de urucum", foi submetido ao tratamento hidrotérmico para a modificação das propriedades do material vegetal visando seu aproveitamento para fins industriais. Os produtos obtidos foram: i) extrato aquoso rico em oligômeros e monômeros de açúcar, que poderiam ser usados na alimentação ou na produção de energia de terceira geração; ii) a farinha modificada de urucum, cujas mudanças foram avaliadas através das informações geradas pelas propriedades de pasta e teor de amido.

No **Capítulo 6** foi realizada a extração com fluidos pressurizados para a obtenção de antocianinas da polpa congelada de açaí. Foram estudadas as influências da pressão, da temperatura e dos solventes usados. As variáveis temperatura e pressão não exerceram influência relevante na obtenção de antocianinas. A variação da proporção de solventes e a presença de ácido cítrico foram determinantes na obtenção de extratos com maiores e menores concentrações de antocianinas.

Finalmente pode ser concluído que esta tese cumpriu com os objetivos propostos que levaram ao aproveitamento total e sustentável das sementes de urucum de reduzido teor lipídico.

Sugestões:

1) Devido à descontinuidade dos estudos do tratamento térmico sobre a farinha do urucum, sugere-se realizar a avaliação completa desses fatores (temperatura, pressão e proporção de água/CO_2) na modificação dos polímeros presentes na farinha;

2) Após o isolamento do amido presente na semente de urucum, sugere-se o estudo aprofundado deste material que venha comprovar a hipótese sugerida nesta tese de que este amido apresenta um complexo amilose-lipídico que faz com que o mesmo seja arrastado

pelos solventes usados nos processos de extração realizados no LASEFI sobre esta matéria-prima (CO_2 e etanol);

3) Usar as partículas finas provenientes deste estudo como ingrediente na formulação de produtos cárneos, derivados lácteos e outros tipos de alimentos, onde este material possa ser usado para conferir cor e substituir os corantes de origem sintética;

4) Usar a farinha de urucum como ingrediente na formulação de alimentos como alternativa de substituição parcial de farinhas convencionais em que as propriedades da farinha de urucum sejam apropriadas;

5) Desenvolver estudos que deem continuidade à obtenção de extratos ricos em antocianinas da polpa congelada de açaí a fim de aperfeiçoar o processo de extração estudado nesta tese para obter maiores rendimentos em compostos bioativos.

- CAPÍTULO 9 –

Memória do período de doutorado

Neste capítulo é apresentado um resumo da trajetória do doutorado. Estão mencionadas as disciplinas que foram cursadas e os trabalhos realizados a parte desta tese, frutos de parceria com outros integrantes do LASEFI.

CAPÍTULO 9 – MEMÓRIA DO PERÍODO DE DOUTORADO

A doutoranda Sylvia Carolina Alcázar Alay ingressou no programa de doutorado em Engenharia de Alimentos (DEA/FEA/UNICAMP) no segundo período de 2011 e desenvolveu as atividades de pesquisa com o auxilio financeiro da CAPES (bolsa de doutorado PEC-PG, processo 5532116). Por ter cursado o mestrado em engenharia de alimentos pela mesma instituição, as disciplinas obrigatórias oferecidas pelo programa de doutorado foram convalidadas. Para alcançar o número mínimo de créditos requisitados e complementar a formação intelectual e técnica foram cursadas as disciplinas optativas. Em 2011, foram cursadas as disciplinas TP121 - Tópicos Especiais em Engenharia de Alimentos - ciclo de aprendizagem PDSA (Plan, Do, Study, Act) e TP132 - Métodos Matemáticos na Engenharia de Alimentos. Em 2012 foram cursadas as disciplinas TP199 - Seminários e TP121 - Tópicos em Engenharia de Alimentos (Estatística experimental), e em 2013, TP106 - Análise Sensorial e Instrumental. A doutoranda participou do Programa de Estágio Docente grupo C (PED C) com atividades de apoio parcial à docência na disciplina TA 331-A - Termodinâmica (2º Semestre 2011 e 1º Semestre 2012, sob supervisão da Professora Doutora Maria Angela de Almeida Meireles Petenate) e na disciplina TA731-A - Operações Unitárias II (1º Semestre de 2014, sob supervisão da Professora Doutora Tânia Forster Carneiro) totalizando 33 créditos.

No inicio do doutorado o projeto de pesquisa foi intitulado "Hidrólise da biomassa lignocelulósica presente no resíduo de extração CO_2-SC das sementes de urucum com água sub/supercrítica e CO_2: produção de oligossacarídeos, avaliação técnica e econômica", e até o final de 2012 o equipamento de hidrólise foi montado no laboratório, como parte de uma nova linha de pesquisa com ênfase nas reações químicas e hidrólise de biomassa visando a produção de combustível de terceira geração. Como consequência foram desenvolvidos vários trabalhos em parceria que resultaram em apresentações de congresso[13-15]* e duas publicações no periódico Food and Public Health[2,4], sendo uma delas o segundo capítulo desta tese. Até o final da elaboração desta tese foi obtida uma conscientização aprimorada das várias possibilidades de agregação de valor às sementes de urucum de reduzido teor lipídico, redirecionando esta pesquisa para o uso de tecnologias de extração de pigmento e modificação física da matéria-prima vegetal.

As sementes de urucum de reduzido teor lipídico foram obtidas do material residual do processo de extração supercrítica com CO_2, processo estabelecido para a melhor

*Os números entre colchetes estão relacionados às referências listadas ao final desta memória.

extração de tocotrienóis da semente de urucum, por Albuquerque e Meireles, publicado em Janeiro de 2012. A ênfase dada a importância do pigmento bixina nas sementes desengorduradas gerou a necessidade de despigmentar as sementes antes de serem submetidas ao processo de hidrólise, pois o emprego de altas temperaturas degradaria o pigmento, que é um produto de grande valor biológico e econômico. A partir disso foi desenvolvido o trabalho de dissertação de Liara Macedo Rodríguez, sobre a extração da bixina com líquidos pressurizados, que foi defendido em setembro de 2013. Os resultados deste trabalho de mestrado foram publicados em colaboração, apresentados como trabalhos completos em congressos[6,7] e uma publicação no periódico Comptes Rendus Chimie[5]. Outros trabalhos foram desenvolvidos com o objetivo de recuperar biocompostos com a técnica de extração com fluídos pressurizados[8,9], sendo que um deles está no terceiro capítulo deste trabalho e o outro publicado na revista Food and Bioproducts Processing[3]. O processo que usa líquidos pressurizados não foi o mais adequado para a extração de pigmento bixina.

Foram desenvolvidos trabalhos que abordaram a extração de bixina com o solvente etanol a baixa pressão, a moagem e o fracionamento das partículas das sementes de urucum (capítulo 5), a introdução de uma metodologia para modificação da estrutura vegetal do urucum a partir do processo hidrotérmico (capítulo 6) e um artigo de revisão que mostra metodologias de modificação do amido[1], polissacarídeo que se encontra em quantidade considerável nas sementes de urucum.

As pesquisas referentes tanto ao projeto de doutorado quanto às colaborações com outros integrantes do grupo de pesquisa do LASEFI são:

Artigos completos publicados em periódicos

[1] **S.C. Alcázar-Alay**, M.A.A. Meireles, "Physicochemical properties, modifications and applications of starches from different botanical sources – a review", Food Science and Technology, vol. 35, no. 2, pp. 215-236, 2015.

[2] **S.C. Alcázar-Alay**, F.P. Cardenas-Toro, D.T. Santos, M.A.A. Meireles, "Study of an Extraction Process as the Pretreatment Step for Sugar Production from Acid Hydrolysis", Food and Public Health, vol. 5, no. 2, pp. 47-55, 2015.

[3] F.P. Cardenas-Toro, **S.C. Alcázar-Alay**, J.P. Coutinho, H.T. Godoy, T. Forster-Carneiro, M.A.A. Meireles, "Pressurized liquid extraction and low-pressure solvent extraction of carotenoids from pressed palm fiber: Experimental and economical evaluation", Food and Bioproducts Processing, vol 94, pp. 90-100, 2015.

[4] F.P. Cardenas-Toro, **S.C. Alcázar-Alay**, T. Forster-Carneiro, M.A.A. Meireles, "Obtaining Oligo-and Monosaccharides from Agroindustrial and Agricultural Residues Using Hydrothermal Treatments", Food and Public Health, vol.4, no. 3, pp. 123-139, 2014.
[5] L.M. Rodrigues; **S.C. Alcázar-Alay**; A.J. Petenate; M.A.A. Meireles. "Bixin extraction from defatted annatto seeds", Comptes Rendus Chimie, vol.17, pp. 268-283, 2014.

Trabalhos completos publicados em anais de congressos

[6] **Sylvia C. Alcázar-Alay**; Liara M. Rodrigues; Tania Forster-Carneiro; M. Angela A. Meireles. "Pressurized water extraction (PWE) of annatto pigment". In: III Iberoamerican Conference on Supercritical Fluids, April 1 - 5, 2013, Cartagena de Indias - Colombia.
[7] Liara M. Rodrigues, **Sylvia C. Alcázar-Alay**, M. Thereza M. S. Gomes; M. Angela A. Meireles. "Pressurized liquid extraction (PLE) of bixin from defatted annatto seeds". In: III Iberoamerican Conference on Supercritical Fluids, April 1 - 5, 2013, Cartagena de Indias-Colombia.

Resumos publicados em anais de congressos

[8] Fiorella P. Cardenas-Toro; **Sylvia C. Alcázar-Alay**; M. Angela A. Meireles. "Low-pressure solvent extraction of saponins from quinoa (*Chenopodium Quinoa Willd*)". In: 31 Congreso Latinoamericano de Química (CLAQ-2014) XXVII Congreso Peruano de Química, October 14 - 17, 2014, Lima - Peru.
[9] Fiorella P. Cardenas-Toro; **Sylvia C. Alcázar-Alay**; Gerardo Fernandez Barbero; M. Angela A. Meireles. "Obtaining of anthocyanin rich-extracts from açai fruit pulp by pressurized liquid extraction". In: 31 Congreso Latinoamericano de Química (CLAQ-2014) XXVII Congreso Peruano de Química, October 14-17, 2014, Lima - Peru.
[10] **Sylvia C. Alcázar-Alay**; Caroline J. Steel; M. Angela A. Meireles. "Valorização integrada do subproduto da extração com CO2 supercrítico de sementes de urucum". In 1° Simpósio Brasileiro de Compostos Bioactivos, October 06 - 08, 2014, São Paulo - Brasil.
[11] **Sylvia Alcázar-Alay**; M. Angela A. Meireles, **"**Extração dos pigmentos de urucum (*Bixa orellana* L.) usando etanol a baixa pressão e agitação a partir das sementes com reduzido teor lipídico e menor tamanho de partícula". In XXIV CBCTA - Congresso Brasileiro de Ciência e Tecnologia de Alimentos (CBCTA), September 25 - 29, 2014, Sergipe - Brasil.
[12] Fiorella P. Cardenas-Toro, **Sylvia C. Alcázar-Alay**, M. Angela A. Meireles, "Presurized liquid extraction of carotenoid and phenolic compounds from pressed palm fiber". In: 10

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[13] **Sylvia C. Alcázar-Alay**; Tania Foster-Carneiro; M. Angela A. Meireles. "Subcritical water hydrolysis of annatto (*Bixa orellana* L.) seed residues". In: Workshop on Supercritical Fluids and Energy. December 8 - 11, 2013, São Paulo - Brazil.

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Apêndices

Apêndice A

PRESSURIZED WATER EXTRACTION (PWE) OF ANNATTO PIGMENT

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PRESSURIZED WATER EXTRACTION (PWE) OF ANNATTO PIGMENT

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Abstract. Annatto (*Bixa orellana L.*) contains the natural pigment bixin which is not soluble in water under ambient conditions. The industrial process used to obtain bixin from annatto seeds is the extraction with alkaline solution; this process is known to transform bixin (ester) into norbixin (dicarboxylic acid) that is soluble in water. The overall aim of this work was to use pressured water extraction (PWE) technique to increase the efficiency of the extraction process. For this purpose PWE was conducted on defatted annatto seeds using pure water and alkaline solutions (NaOH) of pH 10 and 14. Temperatures of 303, 353 and 393 K and pressures of 2, 4 and 20 MPa were used. A low-pressure solvent extraction (LPSE) was also performed. Bixin and norbixin on extracts were determined by UV at 470 and 482 nm; the molar extinction coefficient used was $E_{1cm}^{1\%}$= 2826 and 2870, respectively. The largest yields in bixin were obtained for the LPSE process: 20.9% (pure water), 10.5% (pH = 10) and 2.95% (pH = 14). For pure water at 303 K, the yields were 0.13% and 0.08% for 2 and 4 MPa, respectively. For pure water at 393 K, the yields were 0.37% and 0.20% for 2 and 20 MPa, respectively. At 353 K and 2 MPa, the yields were 0.22%, 0.58% and 0.40% for pure water, water at pH = 10 and water at pH = 14, respectively. Therefore, this work shows that PWE is not an efficient process for the extraction bixin from defatted annatto seeds.

Keywords: *Bixa orellana L.*, bixin, pressured liquid extraction, pressured water extraction.

1. Introduction

Over the last decade, the technology of pressurized fluid extraction (PFE) using water (or subcritical water) has emerged as a sustainable alternative for the extraction of antioxidants from natural sources. Subcritical water extraction (PWE) is an environmentally friend technology that is fast, selective can be automated and uses none or small amounts of organic solvents [1, 2].

There are many important studies of the extraordinary properties of subcritical and supercritical water for chemical reactions, the properties of superheated water, subcritical water, and supercritical water change with temperature and density [3].

Many of the anomalous properties of water are due to the very strong hydrogen bonding. Over the superheated temperature range the extensive hydrogen bonds break down, changing the properties more than usually expected by increasing temperature alone [3].

Water has several distinguishing properties that make it one of the most ideal solvents in natural products studies. First, water is the greenest, cheapest and most easily available solvent. Secondly, by adjusting pH or adding certain salts water can provide very selective extraction, finally, the polarity of water can vary significantly with temperature changes so that water may be used to extract a variety of compounds and behaves more like an organic solvent, such as methanol or ethanol with different polarities. Water can be used as a solvent, reagent, and catalyst in industrial and analytical applications, including extraction, chemical reactions, and cleaning [3, 4].

However, high temperature is not always desirable for natural products studies because unwanted reactions such as oxidation, decomposition, degradation or rearrangement reactions may occur at elevated temperatures [4].

The main pigments present in the seeds of annatto (*Bixa orellana L.*) are bixin and norbixin, whose structures are shown in Figure 1. These pigments are carotenoids of coloration yellow and red, colors of importance in the food, pharmacological and cosmetic industries [5, 6]. In food industries these natural pigments are often used in cheeses, sausages, meats and candies.

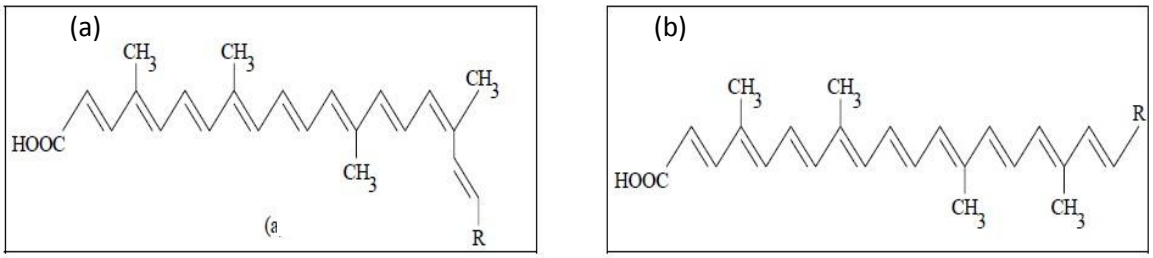

Figure 1. Molecular structures of bixin (R=COOCH3) and norbixin (R=COOH): (a) α.-(cis) and (b) ß-(trans).

Normally, three main methods can be used to extract the pigment from the annatto seeds: vegetable oil extraction, alkaline solution extraction and organic solvent extraction. In the first case, the pigment is obtained by abrasion of the exocarp submerged in warm vegetable oil (340 K). When extracted by organic solvent, such as acetone and methanol, a product with higher pigment concentrations, can be obtained. In this case, after extraction, the solvent is removed and then the powder pigment is dissolved in vegetable oil. The water-soluble form of this pigment is produced by abrasion of the annatto seed exocarp in alkaline solution, and the resultant product is the salt of norbixin (cis and trans) [5, 7, 8, 9]. Therefore, bixin is easily converted into norbixin by dissolving the bixin in an alkaline medium.

The chemical, toxicological, and antioxidant properties, and degradations of bixin and norbixin have been extensively studied. Considering the restrictions placed on the use of synthetic pigments by the World Health Organization, interest in natural pigments is increasing. The annatto seed pigments, bixin and norbixin, are amongst those most used in the food, pharmacological and cosmetic industries due to the intensity of their colors, their greater stability and the wide variety of tones from yellow to red. This range of colors is an additional advantage of the annatto carotenoids over other carotenoids, such as those of the carrot and beetroot, which only show their respective color [9].

The objectives of this work was to study the effects of temperature (303–393 K) and pressures (2–20 MPa) on the extraction of bixin and norbixin from defatted annatto seeds using PWE; and, to compare the extraction efficiency of PWE with that of conventional extraction methods.

2. Materials and methods

2.1 Materials

Sample preparation. Annatto seeds (*Bixa orellana L.*) was obtained from Institute Agronomic of Campinas (IAC, Brazil); the whole seeds were subjected to supercritical fluid extraction (SFE) with supercritical CO_2 (99.9% CO_2, Gama Gases Especiais Ltd., São Bernardo do Campo, Brazil) in a commercial extraction system (Spe-ed SFE Laboratory System, 7071, Applied Separations, Allentown, USA), equipped with an electric oven and a pneumatic pump [10]. SFE was conducted at conditions of 313 K and 20 MPa, and resulted in the extraction of a lipidic fraction, with high content of γ e δ-tocotrienol [9, 10, 11, 12]. The residue from this process consisted of seeds with a small lipid content [10, 14]; the amount of bixin and norbixin removed from the seeds were very small. The defatted seeds, which were the raw material for this study, were maintained at 255 K and protected from light.

2.2 Extraction procedures

Pressurized water extraction (PWE). The pressurized liquid extraction unit is shown in Figure 2. The solvent was pumped by a HPLC pump (Thermoseparation Products, Model ConstaMetric 3200 P/F, Fremoni, USA) into the extraction cell, which was placed in an electrical heating jacket at a desired temperature, until the required pressure was obtained. All connections within the system were made using stainless steel tubes [13].

Defatted annatto seeds (4.0 g) were placed in a 6.57-cm^3 extraction cell (Thar Designs, Pittsburg, USA) containing a sintered metal filter at the bottom and upper parts. The cell containing the sample was heated, filled with extraction solvent and then pressurized.

The sample was placed in the heating system for 5 min to ensure that the extraction cell would be at the desired temperature (303–393 K) during the filling and pressurization procedure.

Thereafter, the blocking and micrometric valves were carefully opened, keeping the pressure at an appropriate level for the desired flow (1.67 cm^3/min), to rinse the extraction cell with fresh extraction solvent for 30 min (dynamic extraction time).

After PWE, the extracts were rapidly cooled and maintained at 255 K and protected from light to prevent pigment degradation prior to analysis.

The extraction solvents were: pure water and alkaline solutions (NaOH) at pH 10 and 14.

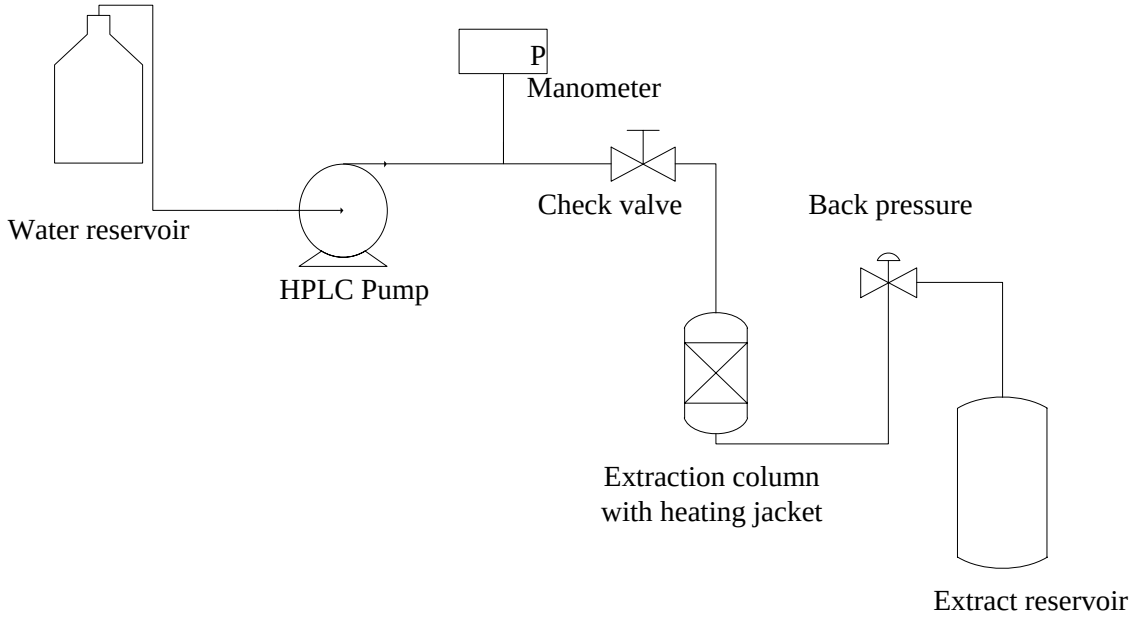

Figure 2. Pressured water extraction unit.

Conventional low-pressure solvent extraction (LPSE). Conventional solid–liquid extraction was performed at room temperature and pressure, immersing 4.0 g of defatted annatto seeds into Erlenmeyer flasks containing 30 cm^3 of solvent (pure water and alkaline solutions (NaOH) of pH 10 and 14). Extractions were carried out with agitation for 30 min. After extraction, the solvent was separated from the annatto seeds and the extracts were rapidly cooled and maintained at 255 K temperature and protected from light.

2.3 Analysis of the extract

The bixin and norbixin in the extracts were determined according to the method issued by Joint FAO / WHO Expert Committee on Food Additives Monographs [5]. The bixin and norbixin were exhaustively extracted from the seeds by maceration and with acetone (Merck, Darmstadt, Germany) until discoloration of seed at room temperature.

The liquid extracts of PWE and LPSE were lyophilized and after dilute in acetone to yield suitable concentrations of bixin or norbixin for analysis.

The yield bixin and norbixin extracts (%) of the defatted annatto seeds and of the extracts were determined by UV (Spectrophotometer Hitachi U 3010) at 470 and 482 nm (A); the molar extinction coefficient used was = 2826 and 2870, respectively with Equation 1. V_1 and V_2 are volumes of dissolution, v_a is an aliquot volume and m_{EXT} is the extract weight (μg).

3. Results and discussion

3.1 Bixin content

The results for PWE are shown in Table 1. The highest yield of 0.37% bixin extract was showed at temperature of 393 K and pressure of 2 MPa. At 393 K, the content of bixin was much higher for the extraction done at 2 MPa (0.37%) than that done 20 MPa (0.20%). The final extracts under these conditions (393 K and 20 MPa) showed a brownish coloration, in contrast with the coloration of bixin characteristic of yellow-red. The extracts also had a strong odor of caramel due at hydrolysis reaction and degradation of the sugars present in annatto seeds. At 303 K, the bixin content varied from 0.13% (2 MPa) to 0.08% (4 MPa). However in this case, both extracts showed extracts a characteristic color (yellow). In this study the increase of pressure decreased the yield, thus, further experimental work is needed to understand the role of pressure over the system pressurized water + defatted annatto seeds. At 2 MPa, bixin contents were 0.13% (303 K), 0.22% (353 K) and 0.37% (393 K) indicating a favorable effect of temperature. Higher temperatures were not tested considering the unwanted reactions and degradation of pigment.

Table 1. Global yield and Bixin content (%)

	PWE extract (K/MPa)					LPSE extract (K/MPa)
	303/2	353/2	393/2	303/4	393/20	303/0.1
Global yield	2.61	5.41	11.86	2.62	12.59	2.90
Bixin content	0.13	0.22	0.37	0.08	0.20	20.90

The largest bixin content in the extract was obtained by technique LPSE (20.90%), therefore, this work shows that PWE is not an efficient process for the extraction bixin from defatted annatto seeds.

3.2 Norbixin content

Norbixin was quantified in the process performed using alkaline solutions (pH 10 and pH 14) for PWE done at 353 K/2 MPa and the LPSE (Table 2).

Table 2. Global yield and norbixin content (%)

	PWE extract (353K/2MPa)		LPSE extract (303K/0.1MPa)	
	Global yield	Norbixin content	Global yield	Norbixin content
Alkaline Solution (pH 10)	5.65	0.58	2.28	10.50
Alkaline Solution (pH 14)	15.60	0.40	13.25	2.95

The largest content of norbixin (10.50%) was obtained by LPSE using the alkaline solution at pH 10; increasing the alkaline solution pH to 14 decreased the yield. For the PWE process the same trend with respect to the effect of pH was observed, nonetheless, the yields were two orders of magnitude smaller.

4. Conclusion

This study demonstrated that PWE is not an adequate method for the extraction of pigments from defatted annatto seeds as compared to LPSE.

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Apêndice B

Avaliação do conteúdo de açúcares redutores (AR) e açúcares redutores totais (ART)

Metodologias de análise

Avaliação das metodologias DNS e Somogyi-Nelson

Visando a avaliação do conteúdo de açúcares redutores (AR) e açúcares redutores totais (ART) nos hidrolisados foram avaliadas duas metodologias de análise, ambas as tecnologias referem-se a princípios de oxido-redução.

Os açúcares redutores são todos aqueles que possuem um ión hidroxila no carbono anomérico livre, ou seja, sem estar comprometida em uma ligação química, podendo então reduzir outras moléculas com características oxidantes. Esta habilidade pode ser utilizada como instrumento para a quantificação de açúcares.

*Foi feita uma adaptação das metodologias para assim determinar o teor de ART mediante hidrolise de solução padrão de sacarose (60 g/L). Adicionou-se em 10 mL da solução padrão um volume igual de solução HCl 2N e incubou-se em banho-maria à temperatura de ebulição por 5 minutos. Depois, neutralizou-se a solução ácida com 10 ml de solução NaOH 2N e resfriou-se rapidamente em banho de gelo até a temperatura ambiente.

Método DNS

- Sensibilidade do método: 100-1000 mg/L
- Principio da análise

O ácido 3,5-dinitro salicílico (DNS) é um composto aromático que reage com açúcares redutores formando ácido 3-amino-5-nitro salicílico.

O reativo DNS contém fenol, metabisulfito de sódio e hidróxido de sodio (a reação se desenvolve em meio básico), e tartarato de sódio e potásio (responsável da estabilidade da coloração obtida).

Os açúcares redutores foram quantificados por espectrofotometria com comprimento de onda de 540nm.

Método Somogyi-Nelson

- Sensibilidade do método: 20-200 mg/L
- Principio da análise

Os açúcares redutores da amostra, aquecidos em meio alcalino, transformam-se em enodióis que reduzem o íon cúprico (Cu^{+2}) presente no reativo de Somogyi, a íon cuproso (Cu^{+1}). O óxido cuproso (Cu_2O), assim formado reduz o composto arsênio-molibídico para

óxido de molibdênio resultando numa coloração azul cuja intensidade é proporcional à quantidade de açúcares redutores existentes na amostra.

Os açúcares redutores foram quantificados por espectrofotometria com comprimento de onda de 540nm

Hipóteses

Questionamentos a esclarecer:

- É possível a análise de açúcares totais com o uso destas metodologias?
- As metodologias apresentam repetitividade?
- As equações obtidas das soluções padrão apresentam linearidade?
- Quais são as diferenças do uso de cada método?
- Quais são as vantagens ao usar cada método?

Planejamos resolver estas perguntas aplicando ambas as metodologias em soluções padrão de glicose (açúcares redutores) e sacarose (açúcares redutores totais), para proceder ao estudo dos dados experimentais de absorbância obtidos.

Experimentação

Foram analisadas as soluções padrão usando ambos os métodos de análise como se mostra nas Figuras 1 e 2, a coloração gerada demonstra que é possível quantificar açúcares redutores totais usando o tratamento de hidrólise ácida e neutralização da solução padrão sacarose.

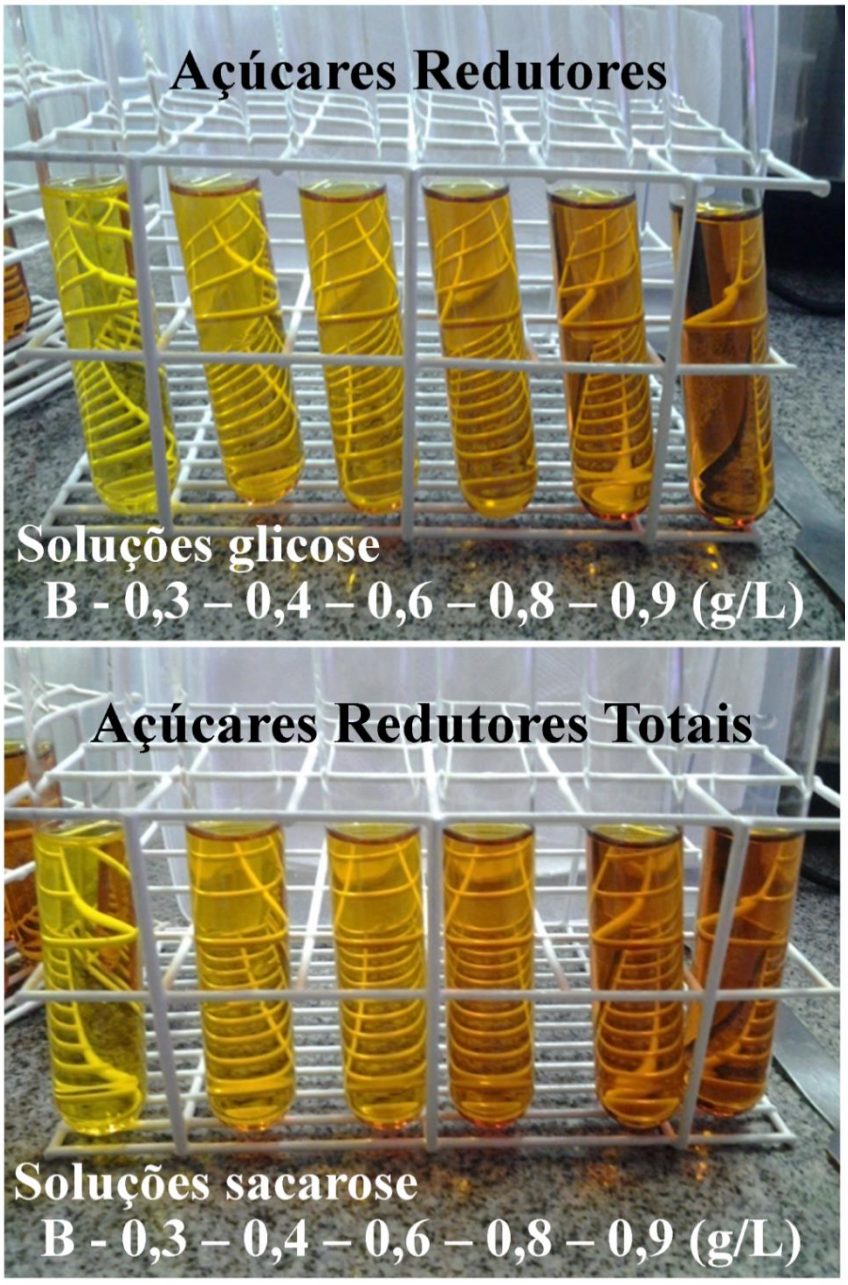

Figura 1: Método DNS

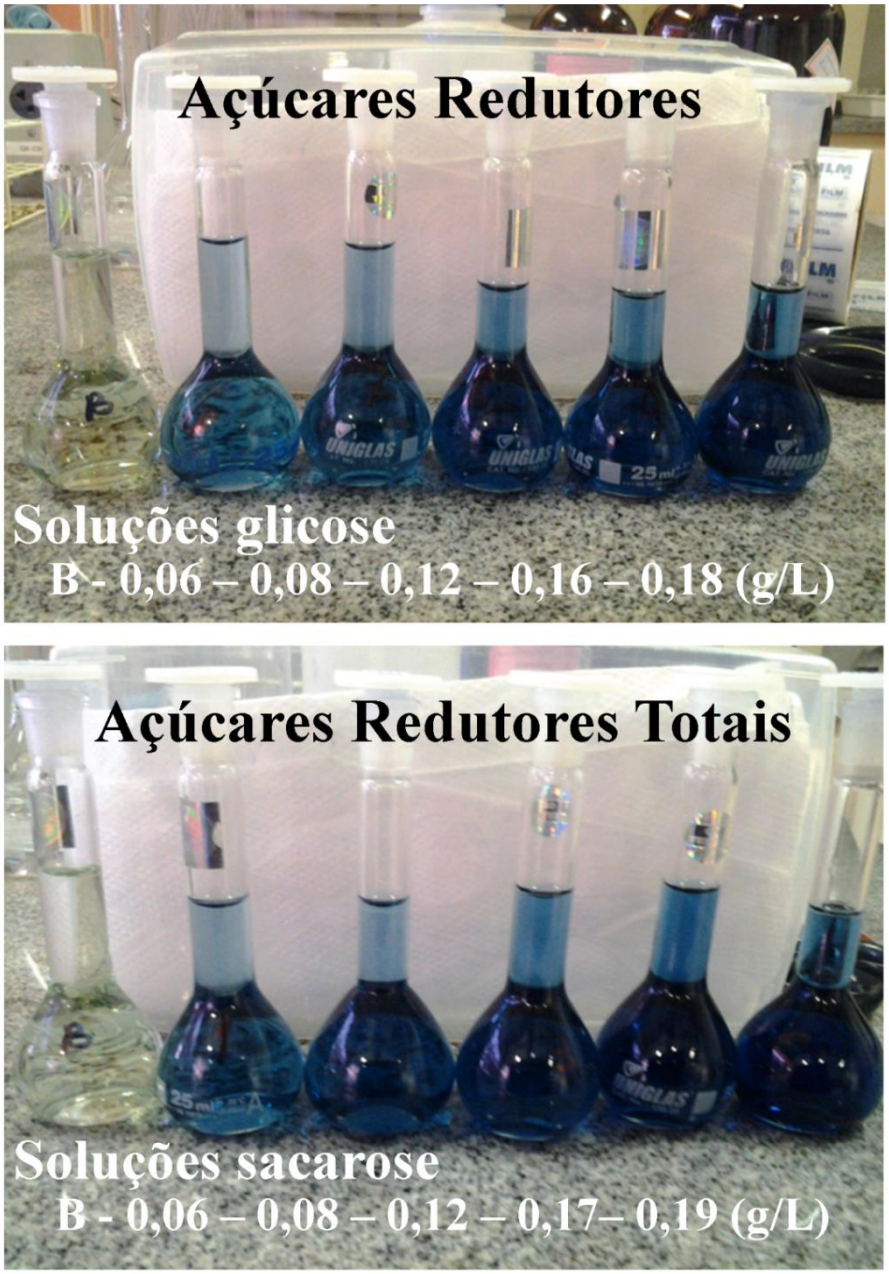

Figura 2: Método Somogyi-Nelson

Ambas as metodologias foram estudadas para gerar uma reta de 5 pontos, cada ponto apresenta quatro repetições. Os dados de absorbância obtidos foram analisados usando o software Minitab® 16, a fim de determinar se o modelo matemático ajusta-se aos dados experimentais usando o p-valor. Na tabela 1 se apresentam as equações das retas e os resultados obtidos da análise estatística.

Tabela 1: Análise dos resultados

Método	**Solução Padrão**	**Equação da reta**	**R^2**	**p-valor**
DNS	Glicose (AR)	Abs = -0.0190+0.382(g/L)	0.961	0.994
	Sacarose (ART)	Abs = -0.0282+0.460(g/L)	0.989	0.902
Somogyi-Nelson	Glicose (AR)	Abs = 0.0324+3.43(g/L)	0.926	0.136
	Sacarose (ART)	Abs = 0.0111+4.13(g/L)	0.977	0.283

O ajuste da equação das retas é interpretado mediante o p-valor, o nível de significância usado foi de 0.05 (α); por tanto se o p-valor for menor ou igual do que α assume-se que o modelo não é linear. Já que os p-valor foram maiores do que α conforme Tabela 1, todos os modelos são lineares e ajustam-se aos dados experimentais.

Conclusões

Dos resultados obtidos concluímos que:

- As metodologias estudadas, ambas podem ser usadas na quantificação de AR e ART.
- As equações das curvas são lineares e o experimento tem repetitividade.
- A vantagem do uso de cada metodologia está referida à sensibilidade do método, logo o método a ser usado depende da concentração de açúcares na amostra.

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Apêndice C

Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation

Analise estatística

First study of LPSE using fine particles

	C1	C2	C3	C4	C5	C6	C7	C8	C9
	RunOrder	Blocks	S/F	Temperature	Time	Xo	Bixin	BY	Phenolic
	1	1	10	40	5	3.1	12.5	5.2	2.2
	2	1	10	40	15	5.5	16.7	12.2	2.3
	3	1	10	40	30	6.1	27.9	22.6	2.9
	4	1	10	50	5	3.4	11.2	5.1	1.4
	5	1	10	50	15	6.3	15.3	12.9	1.6
	6	1	10	50	30	7.9	15.1	15.8	2.3
	7	1	10	60	5	4.2	12.2	5.1	1.5
	8	1	10	60	15	7.6	14.1	14.2	1.7
	9	1	10	60	30	9.6	14.0	17.8	2.2
	10	1	15	40	5	5.3	12.6	8.8	0.9
	11	1	15	40	15	5.4	20.4	14.6	2.5
	12	1	15	40	30	9.2	22.1	27.1	2.4
	13	1	15	50	5	3.6	6.5	3.1	1.1
	14	1	15	50	15	4.8	16.7	10.6	1.8
	15	1	15	50	30	7.6	17.6	17.8	2.2
	16	1	15	60	5	4.5	12.5	7.4	1.3
	17	1	15	60	15	5.4	14.1	10.1	1.6
	18	1	15	60	30	7.7	14.7	15.1	1.9
	19	1	20	40	5	3.1	14.7	6.0	1.7
	20	1	20	40	15	9.0	31.2	37.0	2.2
	21	1	20	40	30	9.1	32.8	39.5	2.7
	22	1	20	50	5	2.9	11.7	4.5	1.7
	23	1	20	50	15	5.2	15.6	10.7	2.0
	24	1	20	50	30	7.5	15.3	15.2	2.5
	25	1	20	60	5	5.3	11.7	8.3	2.5
	26	1	20	60	15	8.0	19.5	20.6	2.7
	27	1	20	60	30	10.2	23.4	31.6	2.3

General Linear Model: Xo versus S/F, Temperature, Time

Factor	Type	Levels	Values
S/F	fixed	3	10, 15, 20
Temperature	fixed	3	40, 50, 60
Time	fixed	3	5, 15, 30

Analysis of Variance for Xo, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
S/F	2	3.3274	3.3274	1.6637	2.37	0.156
Temperature	2	9.8274	9.8274	4.9137	6.99	0.018
Time	2	86.9919	86.9919	43.4959	61.83	0.000
S/F*Temperature	4	11.2681	11.2681	2.8170	4.00	0.045
S/F*Time	4	7.1437	7.1437	1.7859	2.54	0.122
Temperature*Time	4	0.5837	0.5837	0.1459	0.21	0.927
Error	8	5.6274	5.6274	0.7034		
Total	26	124.7696				

S = 0.838705 R-Sq = 95.49% R-Sq(adj) = 85.34%

Unusual Observations for Xo

Obs	Xo	Fit	SE Fit	Residual	St Resid
19	3.1000	4.0370	0.7036	-0.9370	-2.05 R
20	9.0000	8.0481	0.7036	0.9519	2.08 R

R denotes an observation with a large standardized residual.

General Linear Model: Bixin versus S/F, Temperature, Time

Factor	Type	Levels	Values
S/F	fixed	3	10, 15, 20
Temperature	fixed	3	40, 50, 60
Time	fixed	3	5, 15, 30

Analysis of Variance for Bixin, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
S/F	2	106.02	106.02	53.01	5.21	0.036
Temperature	2	276.31	276.31	138.15	13.58	0.003
Time	2	359.70	359.70	179.85	17.68	0.001
S/F*Temperature	4	51.18	51.18	12.79	1.26	0.361
S/F*Time	4	31.43	31.43	7.86	0.77	0.573
Temperature*Time	4	78.35	78.35	19.59	1.93	0.200
Error	8	81.38	81.38	10.17		
Total	26	984.37				

S = 3.18952 R-Sq = 91.73% R-Sq(adj) = 73.13%

General Linear Model: BY versus S/F, Temperature, Time

Factor	Type	Levels	Values
S/F	fixed	3	10, 15, 20
Temperature	fixed	3	40, 50, 60
Time	fixed	3	5, 15, 30

Analysis of Variance for BY, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
S/F	2	273.24	273.24	136.62	6.90	0.018
Temperature	2	333.24	333.24	166.62	8.41	0.011
Time	2	1249.83	1249.83	624.92	31.56	0.000
S/F*Temperature	4	205.07	205.07	51.27	2.59	0.118
S/F*Time	4	125.11	125.11	31.28	1.58	0.269
Temperature*Time	4	106.33	106.33	26.58	1.34	0.334
Error	8	158.42	158.42	19.80		
Total	26	2451.25				

S = 4.45004 R-Sq = 93.54% R-Sq(adj) = 79.00%

Unusual Observations for BY

Obs	BY	Fit	SE Fit	Residual	St Resid
20	37.0000	31.9407	3.7330	5.0593	2.09 R
22	4.5000	-0.4370	3.7330	4.9370	2.04 R

R denotes an observation with a large standardized residual.

General Linear Model: Phenolic versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       3  10, 15, 20
Temperature  fixed       3  40, 50, 60
Time         fixed       3  5, 15, 30


Analysis of Variance for Phenolic, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS   Adj MS      F      P
S/F                2  1.17630  1.17630  0.58815   9.79  0.007
Temperature        2  0.58741  0.58741  0.29370   4.89  0.041
Time               2  2.82296  2.82296  1.41148  23.49  0.000
S/F*Temperature    4  0.81926  0.81926  0.20481   3.41  0.066
S/F*Time           4  0.51704  0.51704  0.12926   2.15  0.166
Temperature*Time   4  0.48593  0.48593  0.12148   2.02  0.184
Error              8  0.48074  0.48074  0.06009
Total             26  6.88963


S = 0.245138   R-Sq = 93.02%   R-Sq(adj) = 77.32%
```

Second study of LPSE using fine particles

	C1	C2	C3	C4	C5	C6	C7	C8	C9
	RunOrder	Blocks	S/F	Temperature	Time	Xo	Bixin	BY	Phenolic
	1	1	10	40	15	4.0	15.2	8.1	2.6
	2	1	10	40	30	5.5	24.8	18.2	2.8
	3	1	10	40	45	6.9	29.0	26.6	2.9
	4	1	10	40	60	7.6	31.5	31.8	2.1
	5	1	10	50	15	4.3	10.1	5.7	2.2
	6	1	10	50	30	8.2	11.5	12.5	2.5
	7	1	10	50	45	8.3	15.2	16.6	3.2
	8	1	10	50	60	8.3	14.6	15.9	2.7
	9	1	20	40	15	4.9	30.4	19.6	2.8
	10	1	20	40	30	8.0	33.9	36.1	3.0
	11	1	20	40	45	9.5	36.6	45.9	5.8
	12	1	20	40	60	9.7	37.9	48.8	5.3
	13	1	20	50	15	4.1	17.2	9.4	2.3
	14	1	20	50	30	7.0	18.3	16.9	3.0
	15	1	20	50	45	8.5	27.8	31.3	6.5
	16	1	20	50	60	8.9	20.1	23.8	5.0
	17	2	10	40	15	7.1	21.3	9.4	3.1
	18	2	10	40	30	7.9	23.6	11.6	4.0
	19	2	10	40	45	9.0	27.8	15.6	4.9
	20	2	10	40	60	10.2	27.1	17.2	3.8
	21	2	10	50	15	6.5	10.8	4.4	2.4
	22	2	10	50	30	9.6	15.5	9.2	2.6
	23	2	10	50	45	9.8	22.8	13.8	3.2
	24	2	10	50	60	10.9	21.9	14.7	3.2
	25	2	20	40	15	8.6	30.8	16.4	2.3
	26	2	20	40	30	11.1	36.3	24.9	3.0
	27	2	20	40	45	11.3	40.9	28.7	4.3
	28	2	20	40	60	12.9	38.8	30.9	3.0
	29	2	20	50	15	5.9	11.8	4.3	4.2
	30	2	20	50	30	10.8	22.3	14.9	5.4
	31	2	20	50	45	15.0	32.8	30.5	5.9
	32	2	20	50	60	18.4	26.7	30.4	5.1

General Linear Model: Xo versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for Xo, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS      F      P
S/F                1   9.7656   9.7656  9.7656  11.03  0.045
Temperature        1   2.1756   2.1756  2.1756   2.46  0.215
Time               3  11.7969  11.7969  3.9323   4.44  0.126
S/F*Temperature    1   3.3306   3.3306  3.3306   3.76  0.148
S/F*Time           3   6.5319   6.5319  2.1773   2.46  0.240
Temperature*Time   3  20.5119  20.5119  6.8373   7.72  0.064
Error              3   2.6569   2.6569  0.8856
Total             15  56.7694


S = 0.941077   R-Sq = 95.32%   R-Sq(adj) = 76.60%
```

General Linear Model: Bixin versus S/F, Temperature, Time

Factor	Type	Levels	Values
S/F	fixed	2	10, 20
Temperature	fixed	2	40, 50
Time	fixed	4	15, 30, 45, 60

Analysis of Variance for Bixin, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
S/F	1	108.68	108.68	108.68	5.63	0.098
Temperature	1	0.68	0.68	0.68	0.04	0.863
Time	3	177.86	177.86	59.29	3.07	0.191
S/F*Temperature	1	1.50	1.50	1.50	0.08	0.798
S/F*Time	3	294.56	294.56	98.19	5.09	0.107
Temperature*Time	3	626.60	626.60	208.87	10.82	0.041
Error	3	57.90	57.90	19.30		
Total	15	1267.78				

S = 4.39325 R-Sq = 95.43% R-Sq(adj) = 77.16%

General Linear Model: BY versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for BY, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS      F      P
S/F                1     1.44     1.44    1.44   0.07  0.809
Temperature        1    89.30    89.30   89.30   4.31  0.129
Time               3   496.23   496.23  165.41   7.99  0.061
S/F*Temperature    1    34.22    34.22   34.22   1.65  0.289
S/F*Time           3   537.77   537.77  179.26   8.65  0.055
Temperature*Time   3  1280.12  1280.12  426.71  20.60  0.017
Error              3    62.14    62.14   20.71
Total             15  2501.24


S = 4.55128   R-Sq = 97.52%   R-Sq(adj) = 87.58%
```

General Linear Model: BY versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for BY, using Adjusted SS for Tests

Source            DF   Seq SS   Adj SS  Adj MS      F      P
S/F                1     1.44     1.44    1.44   0.07  0.809
Temperature        1    89.30    89.30   89.30   4.31  0.129
Time               3   496.23   496.23  165.41   7.99  0.061
S/F*Temperature    1    34.22    34.22   34.22   1.65  0.289
S/F*Time           3   537.77   537.77  179.26   8.65  0.055
Temperature*Time   3  1280.12  1280.12  426.71  20.60  0.017
Error              3    62.14    62.14   20.71
Total             15  2501.24


S = 4.55128   R-Sq = 97.52%   R-Sq(adj) = 87.58%
```

General Linear Model: Xo versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for Xo, using Adjusted SS for Tests

Source            DF   Seq SS  Adj SS  Adj MS     F      P
S/F                1   19.803  19.802  19.802  3.24  0.170
Temperature        1    2.102   2.102   2.102  0.34  0.599
Time               3   72.822  72.822  24.274  3.97  0.143
S/F*Temperature    1    5.062   5.062   5.062  0.83  0.430
S/F*Time           3   16.502  16.503   5.501  0.90  0.533
Temperature*Time   3   17.223  17.223   5.741  0.94  0.520
Error              3   18.322  18.322   6.107
Total             15  151.837


S = 2.47134   R-Sq = 87.93%   R-Sq(adj) = 39.66%
```

General Linear Model: Bixin versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for Bixin, using Adjusted SS for Tests

Source            DF   Seq SS  Adj SS  Adj MS     F      P
S/F                1    59.29   59.29   59.29  2.14  0.239
Temperature        1     0.49    0.49    0.49  0.02  0.903
Time               3   519.76  519.76  173.25  6.27  0.083
S/F*Temperature    1     2.25    2.25    2.25  0.08  0.794
S/F*Time           3    31.38   31.38   10.46  0.38  0.777
Temperature*Time   3   480.11  480.11  160.04  5.79  0.092
Error              3    82.94   82.94   27.65
Total             15  1176.24


S = 5.25817   R-Sq = 92.95%   R-Sq(adj) = 64.74%
```

General Linear Model: BY versus S/F, Temperature, Time

Factor	Type	Levels	Values
S/F	fixed	2	10, 20
Temperature	fixed	2	40, 50
Time	fixed	4	15, 30, 45, 60

Analysis of Variance for BY, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
S/F	1	149.45	149.45	149.45	5.65	0.098
Temperature	1	0.02	0.02	0.02	0.00	0.982
Time	3	682.97	682.97	227.66	8.60	0.055
S/F*Temperature	1	16.20	16.20	16.20	0.61	0.491
S/F*Time	3	118.72	118.72	39.57	1.49	0.375
Temperature*Time	3	196.16	196.16	65.39	2.47	0.239
Error	3	79.42	79.42	26.47		
Total	15	1242.93				

S = 5.14512 R-Sq = 93.61% R-Sq(adj) = 68.05%

General Linear Model: Phenolic versus S/F, Temperature, Time

```
Factor       Type   Levels  Values
S/F          fixed       2  10, 20
Temperature  fixed       2  40, 50
Time         fixed       4  15, 30, 45, 60


Analysis of Variance for Phenolic, using Adjusted SS for Tests

Source            DF   Seq SS  Adj SS  Adj MS     F      P
S/F                1   3.2400  3.2400  3.2400  8.08  0.065
Temperature        1   1.3225  1.3225  1.3225  3.30  0.167
Time               3   3.3450  3.3450  1.1150  2.78  0.212
S/F*Temperature    1   3.4225  3.4225  3.4225  8.54  0.061
S/F*Time           3   1.8950  1.8950  0.6317  1.58  0.359
Temperature*Time   3   4.0225  4.0225  1.3408  3.35  0.174
Error              3   1.2025  1.2025  0.4008
Total             15  18.4500


S = 0.633114   R-Sq = 93.48%   R-Sq(adj) = 67.41%
```

Results for: Worksheet 4

Apêndice D

Obtaining bixin from semi-defatted annatto seeds by mechanical method and solvent extraction: process integration and economic evaluation

Analise econômica

Economic Evaluation Report
for Lote1_LPSE_tanque_5L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	286,000 $
Capital Investment Charged to This Project	286,000 $
Operating Cost	173,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	149.58 kg MP/yr
Unit Production Cost	1,153.60 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 51.71 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Bixin extract'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	9,000	9,000
		Size/Capacity = 5.00 kg/h		
1 / 0 / 0	V-101	Blending Tank	26,000	26,000
		Vessel Volume = 36.25 L		
		Unlisted Equipment		9,000
			TOTAL	44,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	44,000
2. Installation	17,000
3. Process Piping	15,000
4. Instrumentation	17,000
5. Insulation	1,000
6. Electrical	4,000
7. Buildings	20,000
8. Yard Improvement	7,000
9. Auxiliary Facilities	17,000
TPDC	142,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	36,000
11. Construction	50,000
TPIC	85,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	227,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	11,000
13. Contingency	23,000
CFC = 12+13	34,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	261,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,195	121,394	100.00
TOTAL		13,195	121,394	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Ethyl Alcohol	0.850	666	kg	566	100.00
Annato	0.000	3,214	kg	0	0.00
Bixin	0.000	84	kg	0	0.00
TOTAL				566	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	468	kW-h	43	3.14
Steam	12.000	2	MT	29	2.12
Chilled Water	0.400	3,252	MT	1,301	94.74
TOTAL				1,373	100.00

7. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	1,000	0.33
Labor-Dependent	121,000	70.35
Facility-Dependent	49,000	28.53
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	1,000	0.80
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	173,000	100.00

Economic Evaluation Report
for Lote1_LPSE_tanque_50L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	1,220,000	$
Capital Investment Charged to This Project	1,220,000	$
Operating Cost	351,000	$/yr
Main Revenue	0	$/yr
Other Revenues	0	$/yr
Total Revenues	0	$/yr
Cost Basis Annual Rate	997.03	kg MP/yr
Unit Production Cost	351.84	$/kg MP
Unit Production Revenue	0.00	$/kg MP
Gross Margin	- 1.00	%
Return On Investment	- 19.80	%
Payback Time	N/A	
IRR (After Taxes)	N/A	
NPV (at 7.0% Interest)	0	$

MP = Total Flow of Stream 'Bixin extract'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	62,000	62,000
		Size/Capacity = 33.33 kg/h		
1 / 0 / 0	V-101	Blending Tank	91,000	91,000
		Vessel Volume = 362.55 L		
		Unlisted Equipment		38,000
			TOTAL	190,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	190,000
2. Installation	77,000
3. Process Piping	67,000
4. Instrumentation	76,000
5. Insulation	6,000
6. Electrical	19,000
7. Buildings	86,000
8. Yard Improvement	29,000
9. Auxiliary Facilities	76,000
TPDC	625,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	156,000
11. Construction	219,000
TPIC	375,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	1,000,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	50,000
13. Contingency	100,000
CFC = 12+13	150,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	1,150,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,193	121,371	100.00
TOTAL		13,193	121,371	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Ethyl Alcohol	0.850	4,441	kg	3,775	100.00
Annato	0.000	21,425	kg	0	0.00
Bixin	0.000	563	kg	0	0.00
TOTAL				3,775	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	3,119	kW-h	287	3.14
Steam	12.000	16	MT	194	2.12
Chilled Water	0.400	21,674	MT	8,669	94.74
TOTAL				9,151	100.00

7. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	4,000	1.08
Labor-Dependent	121,000	34.60
Facility-Dependent	217,000	61.72
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	9,000	2.61
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	351,000	100.00

Economic Evaluation Report
for Lote1_Separacion mecanica_5L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	78,000 $
Capital Investment Charged to This Project	78,000 $
Operating Cost	30,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	741.09 kg MP/yr
Unit Production Cost	40.28 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 29.60 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Fine particles'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	9,000	9,000
		Size/Capacity = 5.00 kg/h		
		Unlisted Equipment		2,000
			TOTAL	12,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	12,000
2. Installation	6,000
3. Process Piping	4,000
4. Instrumentation	5,000
5. Insulation	0
6. Electrical	1,000
7. Buildings	5,000
8. Yard Improvement	2,000
9. Auxiliary Facilities	5,000
TPDC	39,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	10,000
11. Construction	14,000
TPIC	24,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	63,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	3,000
13. Contingency	6,000
CFC = 12+13	9,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	72,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	1,760	16,192	100.00
TOTAL		1,760	16,192	100.00

5. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	460	kW-h	42	100.00
TOTAL				42	100.00

6. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.00
Labor-Dependent	16,000	54.24
Facility-Dependent	14,000	45.62
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	0	0.14
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	30,000	100.00

Economic Evaluation Report
for Lote1_Separacion mecanica_50L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	505,000 $
Capital Investment Charged to This Project	505,000 $
Operating Cost	107,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	4,941 kg MP/yr
Unit Production Cost	21.58 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 12.08 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Fine particles'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	62,000	62,000
		Size/Capacity = 33.33 kg/h		
		Unlisted Equipment		15,000
			TOTAL	77,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	77,000
2. Installation	39,000
3. Process Piping	27,000
4. Instrumentation	31,000
5. Insulation	2,000
6. Electrical	8,000
7. Buildings	35,000
8. Yard Improvement	12,000
9. Auxiliary Facilities	31,000
TPDC	261,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	65,000
11. Construction	91,000
TPIC	156,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	417,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	21,000
13. Contingency	42,000
CFC = 12+13	63,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	480,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	1,760	16,192	100.00
TOTAL		1,760	16,192	100.00

5. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	3,064	kW-h	282	100.00
TOTAL				282	100.00

6. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.00
Labor-Dependent	16,000	15.19
Facility-Dependent	90,000	84.55
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	0	0.26
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	107,000	100.00

Economic Evaluation Report
for Lote2_LPSE_tanque_5L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	286,000 $
Capital Investment Charged to This Project	286,000 $
Operating Cost	172,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	129.01 kg MP/yr
Unit Production Cost	1,333.14 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 51.52 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Bixin extract'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	9,000	9,000
		Size/Capacity = 5.00 kg/h		
1 / 0 / 0	V-101	Blending Tank	26,000	26,000
		Vessel Volume = 72.19 L		
		Unlisted Equipment		9,000
			TOTAL	44,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	44,000
2. Installation	17,000
3. Process Piping	15,000
4. Instrumentation	17,000
5. Insulation	1,000
6. Electrical	4,000
7. Buildings	20,000
8. Yard Improvement	7,000
9. Auxiliary Facilities	17,000
TPDC	142,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	36,000
11. Construction	50,000
TPIC	85,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	227,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	11,000
13. Contingency	23,000
CFC = 12+13	34,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	261,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,190	121,348	100.00
TOTAL		13,190	121,348	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Ethyl Alcohol	0.850	40	kg	34	100.00
Annato	0.000	3,213	kg	0	0.00
Bixin	0.000	84	kg	0	0.00
TOTAL				34	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	468	kW-h	43	3.10
Steam	12.000	3	MT	31	2.21
Chilled Water	0.400	3,282	MT	1,313	94.69
TOTAL				1,386	100.00

7. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.02
Labor-Dependent	121,000	70.55
Facility-Dependent	49,000	28.62
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	1,000	0.81
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	172,000	100.00

Economic Evaluation Report
for Lote2_LPSE_tanque_50L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	1,220,000 $
Capital Investment Charged to This Project	1,220,000 $
Operating Cost	347,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	859.76 kg MP/yr
Unit Production Cost	403.91 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 19.52 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Bixin extract'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	62,000	62,000
		Size/Capacity = 33.33 kg/h		
1 / 0 / 0	V-101	Blending Tank	91,000	91,000
		Vessel Volume = 721.92 L		
		Unlisted Equipment		38,000
			TOTAL	190,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	190,000
2. Installation	77,000
3. Process Piping	67,000
4. Instrumentation	76,000
5. Insulation	6,000
6. Electrical	19,000
7. Buildings	86,000
8. Yard Improvement	29,000
9. Auxiliary Facilities	76,000
TPDC	625,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	156,000
11. Construction	219,000
TPIC	375,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	1,000,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	50,000
13. Contingency	100,000
CFC = 12+13	150,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	1,150,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,185	121,302	100.00
TOTAL		13,185	121,302	100.00

5. MATERIALS COST - PROCESS SUMMARY

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Ethyl Alcohol	0.850	264	kg	224	100.00
Annato	0.000	21,412	kg	0	0.00
Bixin	0.000	563	kg	0	0.00
TOTAL				224	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

6. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	3,117	kW-h	287	3.10
Steam	12.000	17	MT	204	2.21
Chilled Water	0.400	21,869	MT	8,748	94.69
TOTAL				9,238	100.00

7. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.06
Labor-Dependent	121,000	34.93
Facility-Dependent	217,000	62.34
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	9,000	2.66
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	347,000	100.00

Economic Evaluation Report
for Lote2_Separacion mecanica_5L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	87,000 $
Capital Investment Charged to This Project	87,000 $
Operating Cost	135,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	345.95 kg MP/yr
Unit Production Cost	390.52 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 147.11 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Fine particles'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	9,000	9,000
		Size/Capacity = 5.00 kg/h		
		Unlisted Equipment		2,000
			TOTAL	12,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	12,000
2. Installation	6,000
3. Process Piping	4,000
4. Instrumentation	5,000
5. Insulation	0
6. Electrical	1,000
7. Buildings	5,000
8. Yard Improvement	2,000
9. Auxiliary Facilities	5,000
TPDC	39,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	10,000
11. Construction	14,000
TPIC	24,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	63,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	3,000
13. Contingency	6,000
CFC = 12+13	9,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	72,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,200	121,440	100.00
TOTAL		13,200	121,440	100.00

5. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	460	kW-h	42	100.00
TOTAL				42	100.00

6. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.00
Labor-Dependent	121,000	89.89
Facility-Dependent	14,000	10.08
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	0	0.03
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	135,000	100.00

Economic Evaluation Report
for Lote2_Separacion mecanica_50L

1. EXECUTIVE SUMMARY (2015 prices)

Total Capital Investment	515,000 $
Capital Investment Charged to This Project	515,000 $
Operating Cost	212,000 $/yr
Main Revenue	0 $/yr
Other Revenues	0 $/yr
Total Revenues	0 $/yr
Cost Basis Annual Rate	2,306 kg MP/yr
Unit Production Cost	91.87 $/kg MP
Unit Production Revenue	0.00 $/kg MP
Gross Margin	- 1.00 %
Return On Investment	- 32.29 %
Payback Time	N/A
IRR (After Taxes)	N/A
NPV (at 7.0% Interest)	0 $

MP = Total Flow of Stream 'Fine particles'

2. MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2015 prices)

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	GR-101	Grinder	62,000	62,000
		Size/Capacity = 33.33 kg/h		
		Unlisted Equipment		15,000
			TOTAL	77,000

3. FIXED CAPITAL ESTIMATE SUMMARY (2015 prices in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	77,000
2. Installation	39,000
3. Process Piping	27,000
4. Instrumentation	31,000
5. Insulation	2,000
6. Electrical	8,000
7. Buildings	35,000
8. Yard Improvement	12,000
9. Auxiliary Facilities	31,000
TPDC	261,000

3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	65,000
11. Construction	91,000
TPIC	156,000

3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	417,000

3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	21,000
13. Contingency	42,000
CFC = 12+13	63,000

3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	480,000

4. LABOR COST - PROCESS SUMMARY

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	9.20	13,200	121,440	100.00
TOTAL		13,200	121,440	100.00

5. UTILITIES COST (2015 prices) - PROCESS SUMMARY

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.092	3,064	kW-h	282	100.00
TOTAL				282	100.00

6. ANNUAL OPERATING COST (2015 prices) - PROCESS SUMMARY

Cost Item	$	%
Raw Materials	0	0.00
Labor-Dependent	121,000	57.32
Facility-Dependent	90,000	42.55
Consumables	0	0.00
Waste Treatment/Disposal	0	0.00
Utilities	0	0.13
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	212,000	100.00

Apêndice E

Obtaining of anthocyanin-rich extracts from frozen açai (*Euterpe oleracea* Mart.) pulp using pressurized liquid extraction

Analise estatística

Anthocyanins

	RunOrder	Temperature (°C)	Pressure (bar)	Ethanol (%)	Citric acid (%)	Xo	TA	AY
	1	30	20	50.0	0.0	15.04	7.90	10.83
	2	60	20	50.0	0.0	13.19	6.86	8.24
	3	30	80	50.0	0.0	18.52	7.15	12.06
	4	60	80	50.0	0.0	15.43	7.11	9.99
	5	30	20	99.5	0.0	26.21	2.76	6.58
	6	60	20	99.5	0.0	31.27	3.13	8.93
	7	30	80	99.5	0.0	28.89	3.07	8.08
	8	60	80	99.5	0.0	23.51	3.61	7.73
	9	30	20	50.0	0.3	34.47	1.61	5.04
	10	60	20	50.0	0.3	40.41	2.58	9.49
	11	30	80	50.0	0.3	39.01	2.16	7.66
	12	60	80	50.0	0.3	37.01	2.03	6.83
	13	30	20	99.5	0.3	57.09	0.60	3.15
	14	60	20	99.5	0.3	55.01	0.75	3.75
	15	30	80	99.5	0.3	59.36	0.61	3.32
	16	60	80	99.5	0.3	57.41	0.61	3.19
	17	30	20	50.0	0.0	17.02	5.95	9.22
	18	60	20	50.0	0.0	15.77	5.20	7.47
	19	30	80	50.0	0.0	18.04	5.58	9.17
	20	60	80	50.0	0.0	19.34	5.65	9.96
	21	30	20	99.5	0.0	33.91	1.93	5.95
	22	60	20	99.5	0.0	40.54	2.26	8.35
	23	30	80	99.5	0.0	32.61	2.75	8.17
	24	60	80	99.5	0.0	23.74	3.10	6.70
	25	30	20	50.0	0.3	32.57	1.99	5.90
	26	60	20	50.0	0.3	42.41	1.93	7.45
	27	30	80	50.0	0.3	44.02	1.43	5.73
	28	60	80	50.0	0.3	43.39	1.72	6.81
	29	30	20	99.5	0.3	59.19	0.43	2.29
	30	60	20	99.5	0.3	66.04	0.41	2.44
	31	30	80	99.5	0.3	55.73	0.44	2.24
	32	60	80	99.5	0.3	69.57	0.46	2.95

Factorial Fit: Xo versus Temperature (°C), Pressure (bar), ...

Estimated Effects and Coefficients for Xo (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		36.429	0.7300	49.90	0.000
Temperature (°C)	1.398	0.699	0.7300	0.96	0.353
Pressure (bar)	0.340	0.170	0.7300	0.23	0.819
Ethanol (%)	17.153	8.576	0.7300	11.75	0.000
Citric acid (%)	26.229	13.114	0.7300	17.96	0.000
Temperature (°C)*Pressure (bar)	-2.245	-1.123	0.7300	-1.54	0.144
Temperature (°C)*Ethanol (%)	0.365	0.182	0.7300	0.25	0.806
Temperature (°C)*Citric acid (%)	2.329	1.164	0.7300	1.59	0.130
Pressure (bar)*Ethanol (%)	-2.645	-1.322	0.7300	-1.81	0.089
Pressure (bar)*Citric acid (%)	1.949	0.974	0.7300	1.33	0.201
Ethanol (%)*Citric acid (%)	3.611	1.806	0.7300	2.47	0.025
Temperature (°C)*Pressure (bar)*Ethanol (%)	-0.108	-0.054	0.7300	-0.07	0.942
Temperature (°C)*Pressure (bar)*Citric acid (%)	0.834	0.417	0.7300	0.57	0.576
Temperature (°C)*Ethanol (%)*Citric acid (%)	0.074	0.037	0.7300	0.05	0.960
Pressure (bar)*Ethanol (%)*Citric acid (%)	1.541	0.771	0.7300	1.06	0.307
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	3.299	1.649	0.7300	2.26	0.038

S = 4.12968 PRESS = 1091.47
R-Sq = 96.80% R-Sq(pred) = 87.21% R-Sq(adj) = 93.80%

Analysis of Variance for Xo (coded units)

Source	DF	Seq SS
Main Effects	4	7873.79
Temperature (°C)	1	15.62
Pressure (bar)	1	0.92
Ethanol (%)	1	2353.67
Citric acid (%)	1	5503.58
2-Way Interactions	6	275.45
Temperature (°C)*Pressure (bar)	1	40.32
Temperature (°C)*Ethanol (%)	1	1.07
Temperature (°C)*Citric acid (%)	1	43.38
Pressure (bar)*Ethanol (%)	1	55.97
Pressure (bar)*Citric acid (%)	1	30.38
Ethanol (%)*Citric acid (%)	1	104.33
3-Way Interactions	4	24.70
Temperature (°C)*Pressure (bar)*Ethanol (%)	1	0.09
Temperature (°C)*Pressure (bar)*Citric acid (%)	1	5.56
Temperature (°C)*Ethanol (%)*Citric acid (%)	1	0.04
Pressure (bar)*Ethanol (%)*Citric acid (%)	1	19.00
4-Way Interactions	1	87.05
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	1	87.05
Residual Error	16	272.87
Pure Error	16	272.87
Total	31	8533.87

Source	Adj SS	Adj MS
Main Effects	7873.79	1968.45
Temperature (°C)	15.62	15.62
Pressure (bar)	0.92	0.92
Ethanol (%)	2353.67	2353.67
Citric acid (%)	5503.58	5503.58
2-Way Interactions	275.45	45.91
Temperature (°C)*Pressure (bar)	40.32	40.32
Temperature (°C)*Ethanol (%)	1.07	1.07

Temperature (°C)*Citric acid (%)	43.38	43.38
Pressure (bar)*Ethanol (%)	55.97	55.97
Pressure (bar)*Citric acid (%)	30.38	30.38
Ethanol (%)*Citric acid (%)	104.33	104.33
3-Way Interactions	24.70	6.18
Temperature (°C)*Pressure (bar)*Ethanol (%)	0.09	0.09
Temperature (°C)*Pressure (bar)*Citric acid (%)	5.56	5.56
Temperature (°C)*Ethanol (%)*Citric acid (%)	0.04	0.04
Pressure (bar)*Ethanol (%)*Citric acid (%)	19.00	19.00
4-Way Interactions	87.05	87.05
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	87.05	87.05
Residual Error	272.87	17.05
Pure Error	272.87	17.05
Total		

Source	F	P
Main Effects	115.42	0.000
Temperature (°C)	0.92	0.353
Pressure (bar)	0.05	0.819
Ethanol (%)	138.01	0.000
Citric acid (%)	322.71	0.000
2-Way Interactions	2.69	0.053
Temperature (°C)*Pressure (bar)	2.36	0.144
Temperature (°C)*Ethanol (%)	0.06	0.806
Temperature (°C)*Citric acid (%)	2.54	0.130
Pressure (bar)*Ethanol (%)	3.28	0.089
Pressure (bar)*Citric acid (%)	1.78	0.201
Ethanol (%)*Citric acid (%)	6.12	0.025
3-Way Interactions	0.36	0.832
Temperature (°C)*Pressure (bar)*Ethanol (%)	0.01	0.942
Temperature (°C)*Pressure (bar)*Citric acid (%)	0.33	0.576
Temperature (°C)*Ethanol (%)*Citric acid (%)	0.00	0.960
Pressure (bar)*Ethanol (%)*Citric acid (%)	1.11	0.307
4-Way Interactions	5.10	0.038
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	5.10	0.038
Residual Error		
Pure Error		
Total		

Unusual Observations for Xo

Obs	StdOrder	Xo	Fit	SE Fit	Residual	St Resid
16	16	57.4100	63.4900	2.9201	-6.0800	-2.08R
32	32	69.5700	63.4900	2.9201	6.0800	2.08R

R denotes an observation with a large standardized residual.

Estimated Coefficients for Xo using data in uncoded units

Term	Coef
Constant	14.4086
Temperature (°C)	-0.460852
Pressure (bar)	-0.176531
Ethanol (%)	0.052795
Citric acid (%)	-107.105
Temperature (°C)*Pressure (bar)	0.00800979
Temperature (°C)*Ethanol (%)	0.00803816
Temperature (°C)*Citric acid (%)	3.84917
Pressure (bar)*Ethanol (%)	0.00406229
Pressure (bar)*Citric acid (%)	2.74237
Ethanol (%)*Citric acid (%)	2.33199
Temperature (°C)*Pressure (bar)*Ethanol (%)	-1.52918E-04
Temperature (°C)*Pressure (bar)*Citric acid (%)	-0.0676233

Temperature (°C)*Ethanol (%)* Citric acid (%)	-0.0487018
Pressure (bar)*Ethanol (%)* Citric acid (%)	-0.0375084
Temperature (°C)*Pressure (bar)* Ethanol (%)*Citric acid (%)	0.000987280

Least Squares Means for Xo

	Mean	SE Mean
Temperature (°C)		
30	35.73	1.032
60	37.13	1.032
Pressure (bar)		
20	36.26	1.032
80	36.60	1.032
Ethanol (%)		
50.00	27.85	1.032
99.50	45.01	1.032
Citric acid (%)		
0.0000	23.31	1.032
0.3000	49.54	1.032

Predicted Response for New Design Points Using Model for Xo

Point	Fit	SE Fit	95% CI	95% PI
1	16.0300	2.9201	(9.8396, 22.2204)	(5.3079, 26.7521)
2	14.4800	2.9201	(8.2896, 20.6704)	(3.7579, 25.2021)
3	18.2800	2.9201	(12.0896, 24.4704)	(7.5579, 29.0021)
4	17.3850	2.9201	(11.1946, 23.5754)	(6.6629, 28.1071)
5	30.0600	2.9201	(23.8696, 36.2504)	(19.3379, 40.7821)
6	35.9050	2.9201	(29.7146, 42.0954)	(25.1829, 46.6271)
7	30.7500	2.9201	(24.5596, 36.9404)	(20.0279, 41.4721)
8	23.6250	2.9201	(17.4346, 29.8154)	(12.9029, 34.3471)
9	33.5200	2.9201	(27.3296, 39.7104)	(22.7979, 44.2421)
10	41.4100	2.9201	(35.2196, 47.6004)	(30.6879, 52.1321)
11	41.5150	2.9201	(35.3246, 47.7054)	(30.7929, 52.2371)
12	40.2000	2.9201	(34.0096, 46.3904)	(29.4779, 50.9221)
13	58.1400	2.9201	(51.9496, 64.3304)	(47.4179, 68.8621)
14	60.5250	2.9201	(54.3346, 66.7154)	(49.8029, 71.2471)
15	57.5450	2.9201	(51.3546, 63.7354)	(46.8229, 68.2671)
16	63.4900	2.9201	(57.2996, 69.6804)	(52.7679, 74.2121)
17	16.0300	2.9201	(9.8396, 22.2204)	(5.3079, 26.7521)
18	14.4800	2.9201	(8.2896, 20.6704)	(3.7579, 25.2021)
19	18.2800	2.9201	(12.0896, 24.4704)	(7.5579, 29.0021)
20	17.3850	2.9201	(11.1946, 23.5754)	(6.6629, 28.1071)
21	30.0600	2.9201	(23.8696, 36.2504)	(19.3379, 40.7821)
22	35.9050	2.9201	(29.7146, 42.0954)	(25.1829, 46.6271)
23	30.7500	2.9201	(24.5596, 36.9404)	(20.0279, 41.4721)
24	23.6250	2.9201	(17.4346, 29.8154)	(12.9029, 34.3471)
25	33.5200	2.9201	(27.3296, 39.7104)	(22.7979, 44.2421)
26	41.4100	2.9201	(35.2196, 47.6004)	(30.6879, 52.1321)
27	41.5150	2.9201	(35.3246, 47.7054)	(30.7929, 52.2371)
28	40.2000	2.9201	(34.0096, 46.3904)	(29.4779, 50.9221)
29	58.1400	2.9201	(51.9496, 64.3304)	(47.4179, 68.8621)
30	60.5250	2.9201	(54.3346, 66.7154)	(49.8029, 71.2471)
31	57.5450	2.9201	(51.3546, 63.7354)	(46.8229, 68.2671)
32	63.4900	2.9201	(57.2996, 69.6804)	(52.7679, 74.2121)

Factorial Fit: TA versus Temperature (°C), Pressure (bar), ...

Estimated Effects and Coefficients for TA (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		2.930	0.1184	24.75	0.000
Temperature (°C)	0.066	0.033	0.1184	0.28	0.785
Pressure (bar)	0.074	0.037	0.1184	0.31	0.758
Ethanol (%)	-2.496	-1.248	0.1184	-10.54	0.000
Citric acid (%)	-3.391	-1.695	0.1184	-14.32	0.000
Temperature (°C)*Pressure (bar)	0.072	0.036	0.1184	0.30	0.765
Temperature (°C)*Ethanol (%)	0.152	0.076	0.1184	0.64	0.530
Temperature (°C)*Citric acid (%)	0.087	0.043	0.1184	0.37	0.719
Pressure (bar)*Ethanol (%)	0.223	0.112	0.1184	0.94	0.360
Pressure (bar)*Citric acid (%)	-0.179	-0.090	0.1184	-0.76	0.460
Ethanol (%)*Citric acid (%)	1.103	0.552	0.1184	4.66	0.000
Temperature (°C)*Pressure (bar)*Ethanol (%)	-0.062	-0.031	0.1184	-0.26	0.797
Temperature (°C)*Pressure (bar)*Citric acid (%)	-0.179	-0.090	0.1184	-0.76	0.460
Temperature (°C)*Ethanol (%)*Citric acid (%)	-0.267	-0.133	0.1184	-1.13	0.276
Pressure (bar)*Ethanol (%)*Citric acid (%)	-0.136	-0.068	0.1184	-0.57	0.575
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	0.142	0.071	0.1184	0.60	0.557

S = 0.669764 PRESS = 28.7094
R-Sq = 95.54% R-Sq(pred) = 82.16% R-Sq(adj) = 91.36%

Analysis of Variance for TA (coded units)

Source	DF	Seq SS
Main Effects	4	141.875
Temperature (°C)	1	0.034
Pressure (bar)	1	0.044
Ethanol (%)	1	49.825
Citric acid (%)	1	91.971
2-Way Interactions	6	10.677
Temperature (°C)*Pressure (bar)	1	0.041
Temperature (°C)*Ethanol (%)	1	0.185
Temperature (°C)*Citric acid (%)	1	0.060
Pressure (bar)*Ethanol (%)	1	0.398
Pressure (bar)*Citric acid (%)	1	0.257
Ethanol (%)*Citric acid (%)	1	9.735
3-Way Interactions	4	1.005
Temperature (°C)*Pressure (bar)*Ethanol (%)	1	0.031
Temperature (°C)*Pressure (bar)*Citric acid (%)	1	0.257
Temperature (°C)*Ethanol (%)*Citric acid (%)	1	0.570
Pressure (bar)*Ethanol (%)*Citric acid (%)	1	0.147
4-Way Interactions	1	0.161
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	1	0.161
Residual Error	16	7.177
Pure Error	16	7.177
Total	31	160.895

Source	Adj SS	Adj MS
Main Effects	141.875	35.4686
Temperature (°C)	0.034	0.0345
Pressure (bar)	0.044	0.0443
Ethanol (%)	49.825	49.8252
Citric acid (%)	91.971	91.9707
2-Way Interactions	10.677	1.7795
Temperature (°C)*Pressure (bar)	0.041	0.0413
Temperature (°C)*Ethanol (%)	0.185	0.1845

Temperature (°C)*Citric acid (%)		0.060	0.0604
Pressure (bar)*Ethanol (%)		0.398	0.3983
Pressure (bar)*Citric acid (%)		0.257	0.2574
Ethanol (%)*Citric acid (%)		9.735	9.7351
3-Way Interactions		1.005	0.2512
Temperature (°C)*Pressure (bar)*Ethanol (%)		0.031	0.0306
Temperature (°C)*Pressure (bar)*Citric acid (%)		0.257	0.2574
Temperature (°C)*Ethanol (%)*Citric acid (%)		0.570	0.5698
Pressure (bar)*Ethanol (%)*Citric acid (%)		0.147	0.1472
4-Way Interactions		0.161	0.1610
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)		0.161	0.1610
Residual Error		7.177	0.4486
Pure Error		7.177	0.4486
Total			

Source	F	P
Main Effects	79.07	0.000
Temperature (°C)	0.08	0.785
Pressure (bar)	0.10	0.758
Ethanol (%)	111.07	0.000
Citric acid (%)	205.02	0.000
2-Way Interactions	3.97	0.013
Temperature (°C)*Pressure (bar)	0.09	0.765
Temperature (°C)*Ethanol (%)	0.41	0.530
Temperature (°C)*Citric acid (%)	0.13	0.719
Pressure (bar)*Ethanol (%)	0.89	0.360
Pressure (bar)*Citric acid (%)	0.57	0.460
Ethanol (%)*Citric acid (%)	21.70	0.000
3-Way Interactions	0.56	0.695
Temperature (°C)*Pressure (bar)*Ethanol (%)	0.07	0.797
Temperature (°C)*Pressure (bar)*Citric acid (%)	0.57	0.460
Temperature (°C)*Ethanol (%)*Citric acid (%)	1.27	0.276
Pressure (bar)*Ethanol (%)*Citric acid (%)	0.33	0.575
4-Way Interactions	0.36	0.557
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	0.36	0.557
Residual Error		
Pure Error		
Total		

Unusual Observations for TA

Obs	StdOrder	TA	Fit	SE Fit	Residual	St Resid
1	1	7.90000	6.92500	0.47359	0.97500	2.06R
17	17	5.95000	6.92500	0.47359	-0.97500	-2.06R

R denotes an observation with a large standardized residual.

Estimated Coefficients for TA using data in uncoded units

Term	Coef
Constant	14.8470
Temperature (°C)	-0.0910107
Pressure (bar)	-0.0571599
Ethanol (%)	-0.130741
Citric acid (%)	-42.7467
Temperature (°C)*Pressure (bar)	0.00096291
Temperature (°C)*Ethanol (%)	0.00102132
Temperature (°C)*Citric acid (%)	0.423556
Pressure (bar)*Ethanol (%)	0.000653199
Pressure (bar)*Citric acid (%)	0.228204
Ethanol (%)*Citric acid (%)	0.382379
Temperature (°C)*Pressure (bar)*Ethanol (%)	-9.14703E-06
Temperature (°C)*Pressure (bar)*Citric acid (%)	-0.00450271

Temperature (°C)*Ethanol (%)* Citric acid (%)	-0.00451927
Pressure (bar)*Ethanol (%)* Citric acid (%)	-0.00251964
Temperature (°C)*Pressure (bar)* Ethanol (%)*Citric acid (%)	4.24617E-05

Least Squares Means for TA

	Mean	SE Mean
Temperature (°C)		
30	2.898	0.1674
60	2.963	0.1674
Pressure (bar)		
20	2.893	0.1674
80	2.967	0.1674
Ethanol (%)		
50.00	4.178	0.1674
99.50	1.683	0.1674
Citric acid (%)		
0.0000	4.626	0.1674
0.3000	1.235	0.1674

Predicted Response for New Design Points Using Model for TA

Point	Fit	SE Fit	95% CI	95% PI
1	6.92500	0.47359	(5.92102, 7.92898)	(5.18606, 8.66394)
2	6.03000	0.47359	(5.02602, 7.03398)	(4.29106, 7.76894)
3	6.36500	0.47359	(5.36102, 7.36898)	(4.62606, 8.10394)
4	6.38000	0.47359	(5.37602, 7.38398)	(4.64106, 8.11894)
5	2.34500	0.47359	(1.34102, 3.34898)	(0.60606, 4.08394)
6	2.69500	0.47359	(1.69102, 3.69898)	(0.95606, 4.43394)
7	2.91000	0.47359	(1.90602, 3.91398)	(1.17106, 4.64894)
8	3.35500	0.47359	(2.35102, 4.35898)	(1.61606, 5.09394)
9	1.80000	0.47359	(0.79602, 2.80398)	(0.06106, 3.53894)
10	2.25500	0.47359	(1.25102, 3.25898)	(0.51606, 3.99394)
11	1.79500	0.47359	(0.79102, 2.79898)	(0.05606, 3.53394)
12	1.87500	0.47359	(0.87102, 2.87898)	(0.13606, 3.61394)
13	0.51500	0.47359	(-0.48898, 1.51898)	(-1.22394, 2.25394)
14	0.58000	0.47359	(-0.42398, 1.58398)	(-1.15894, 2.31894)
15	0.52500	0.47359	(-0.47898, 1.52898)	(-1.21394, 2.26394)
16	0.53500	0.47359	(-0.46898, 1.53898)	(-1.20394, 2.27394)
17	6.92500	0.47359	(5.92102, 7.92898)	(5.18606, 8.66394)
18	6.03000	0.47359	(5.02602, 7.03398)	(4.29106, 7.76894)
19	6.36500	0.47359	(5.36102, 7.36898)	(4.62606, 8.10394)
20	6.38000	0.47359	(5.37602, 7.38398)	(4.64106, 8.11894)
21	2.34500	0.47359	(1.34102, 3.34898)	(0.60606, 4.08394)
22	2.69500	0.47359	(1.69102, 3.69898)	(0.95606, 4.43394)
23	2.91000	0.47359	(1.90602, 3.91398)	(1.17106, 4.64894)
24	3.35500	0.47359	(2.35102, 4.35898)	(1.61606, 5.09394)
25	1.80000	0.47359	(0.79602, 2.80398)	(0.06106, 3.53894)
26	2.25500	0.47359	(1.25102, 3.25898)	(0.51606, 3.99394)
27	1.79500	0.47359	(0.79102, 2.79898)	(0.05606, 3.53394)
28	1.87500	0.47359	(0.87102, 2.87898)	(0.13606, 3.61394)
29	0.51500	0.47359	(-0.48898, 1.51898)	(-1.22394, 2.25394)
30	0.58000	0.47359	(-0.42398, 1.58398)	(-1.15894, 2.31894)
31	0.52500	0.47359	(-0.47898, 1.52898)	(-1.21394, 2.26394)
32	0.53500	0.47359	(-0.46898, 1.53898)	(-1.20394, 2.27394)

Factorial Fit: AY versus Temperature (°C), Pressure (bar), ...

Estimated Effects and Coefficients for AY (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		6.740	0.1583	42.59	0.000
Temperature (°C)	0.306	0.153	0.1583	0.97	0.349
Pressure (bar)	0.344	0.172	0.1583	1.09	0.293
Ethanol (%)	-3.002	-1.501	0.1583	-9.48	0.000
Citric acid (%)	-3.699	-1.850	0.1583	-11.69	0.000
Temperature (°C)*Pressure (bar)	-0.589	-0.295	0.1583	-1.86	0.081
Temperature (°C)*Ethanol (%)	0.227	0.113	0.1583	0.72	0.484
Temperature (°C)*Citric acid (%)	0.642	0.321	0.1583	2.03	0.060
Pressure (bar)*Ethanol (%)	-0.227	-0.113	0.1583	-0.72	0.484
Pressure (bar)*Citric acid (%)	-0.442	-0.221	0.1583	-1.40	0.182
Ethanol (%)*Citric acid (%)	-0.946	-0.473	0.1583	-2.99	0.009
Temperature (°C)*Pressure (bar)*Ethanol (%)	-0.253	-0.127	0.1583	-0.80	0.436
Temperature (°C)*Pressure (bar)*Citric acid (%)	-0.151	-0.075	0.1583	-0.48	0.641
Temperature (°C)*Ethanol (%)*Citric acid (%)	-0.842	-0.421	0.1583	-2.66	0.017
Pressure (bar)*Ethanol (%)*Citric acid (%)	0.342	0.171	0.1583	1.08	0.296
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	0.951	0.475	0.1583	3.00	0.008

S = 0.895239 PRESS = 51.293
R-Sq = 94.33% R-Sq(pred) = 77.33% R-Sq(adj) = 89.02%

Analysis of Variance for AY (coded units)

Source	DF	Seq SS
Main Effects	4	183.269
Temperature (°C)	1	0.747
Pressure (bar)	1	0.949
Ethanol (%)	1	72.090
Citric acid (%)	1	109.483
2-Way Interactions	6	15.614
Temperature (°C)*Pressure (bar)	1	2.779
Temperature (°C)*Ethanol (%)	1	0.412
Temperature (°C)*Citric acid (%)	1	3.296
Pressure (bar)*Ethanol (%)	1	0.412
Pressure (bar)*Citric acid (%)	1	1.562
Ethanol (%)*Citric acid (%)	1	7.154
3-Way Interactions	4	7.299
Temperature (°C)*Pressure (bar)*Ethanol (%)	1	0.513
Temperature (°C)*Pressure (bar)*Citric acid (%)	1	0.182
Temperature (°C)*Ethanol (%)*Citric acid (%)	1	5.670
Pressure (bar)*Ethanol (%)*Citric acid (%)	1	0.935
4-Way Interactions	1	7.230
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	1	7.230
Residual Error	16	12.823
Pure Error	16	12.823
Total	31	226.235

Source	Adj SS	Adj MS
Main Effects	183.269	45.817
Temperature (°C)	0.747	0.747
Pressure (bar)	0.949	0.949
Ethanol (%)	72.090	72.090
Citric acid (%)	109.483	109.483
2-Way Interactions	15.614	2.602
Temperature (°C)*Pressure (bar)	2.779	2.779
Temperature (°C)*Ethanol (%)	0.412	0.412

Temperature (°C)*Citric acid (%)	3.296	3.296
Pressure (bar)*Ethanol (%)	0.412	0.412
Pressure (bar)*Citric acid (%)	1.562	1.562
Ethanol (%)*Citric acid (%)	7.154	7.154
3-Way Interactions	7.299	1.825
Temperature (°C)*Pressure (bar)*Ethanol (%)	0.513	0.513
Temperature (°C)*Pressure (bar)*Citric acid (%)	0.182	0.182
Temperature (°C)*Ethanol (%)*Citric acid (%)	5.670	5.670
Pressure (bar)*Ethanol (%)*Citric acid (%)	0.935	0.935
4-Way Interactions	7.230	7.230
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	7.230	7.230
Residual Error	12.823	0.801
Pure Error	12.823	0.801
Total		

Source	F	P
Main Effects	57.17	0.000
Temperature (°C)	0.93	0.349
Pressure (bar)	1.18	0.293
Ethanol (%)	89.95	0.000
Citric acid (%)	136.61	0.000
2-Way Interactions	3.25	0.028
Temperature (°C)*Pressure (bar)	3.47	0.081
Temperature (°C)*Ethanol (%)	0.51	0.484
Temperature (°C)*Citric acid (%)	4.11	0.060
Pressure (bar)*Ethanol (%)	0.51	0.484
Pressure (bar)*Citric acid (%)	1.95	0.182
Ethanol (%)*Citric acid (%)	8.93	0.009
3-Way Interactions	2.28	0.106
Temperature (°C)*Pressure (bar)*Ethanol (%)	0.64	0.436
Temperature (°C)*Pressure (bar)*Citric acid (%)	0.23	0.641
Temperature (°C)*Ethanol (%)*Citric acid (%)	7.07	0.017
Pressure (bar)*Ethanol (%)*Citric acid (%)	1.17	0.296
4-Way Interactions	9.02	0.008
Temperature (°C)*Pressure (bar)*Ethanol (%)*Citric acid (%)	9.02	0.008
Residual Error		
Pure Error		
Total		

Unusual Observations for AY

Obs	StdOrder	AY	Fit	SE Fit	Residual	St Resid
3	3	12.0600	10.6150	0.6330	1.4450	2.28R
19	19	9.1700	10.6150	0.6330	-1.4450	-2.28R

R denotes an observation with a large standardized residual.

Estimated Coefficients for AY using data in uncoded units

Term	Coef
Constant	22.9460
Temperature (°C)	-0.296404
Pressure (bar)	-0.118108
Ethanol (%)	-0.208754
Citric acid (%)	-76.8270
Temperature (°C)*Pressure (bar)	0.00355202
Temperature (°C)*Ethanol (%)	0.00414141
Temperature (°C)*Citric acid (%)	1.82682
Pressure (bar)*Ethanol (%)	0.00204882
Pressure (bar)*Citric acid (%)	0.843412
Ethanol (%)*Citric acid (%)	0.776207
Temperature (°C)*Pressure (bar)*Ethanol (%)	-5.40404E-05
Temperature (°C)*Pressure (bar)*Citric acid (%)	-0.0223830

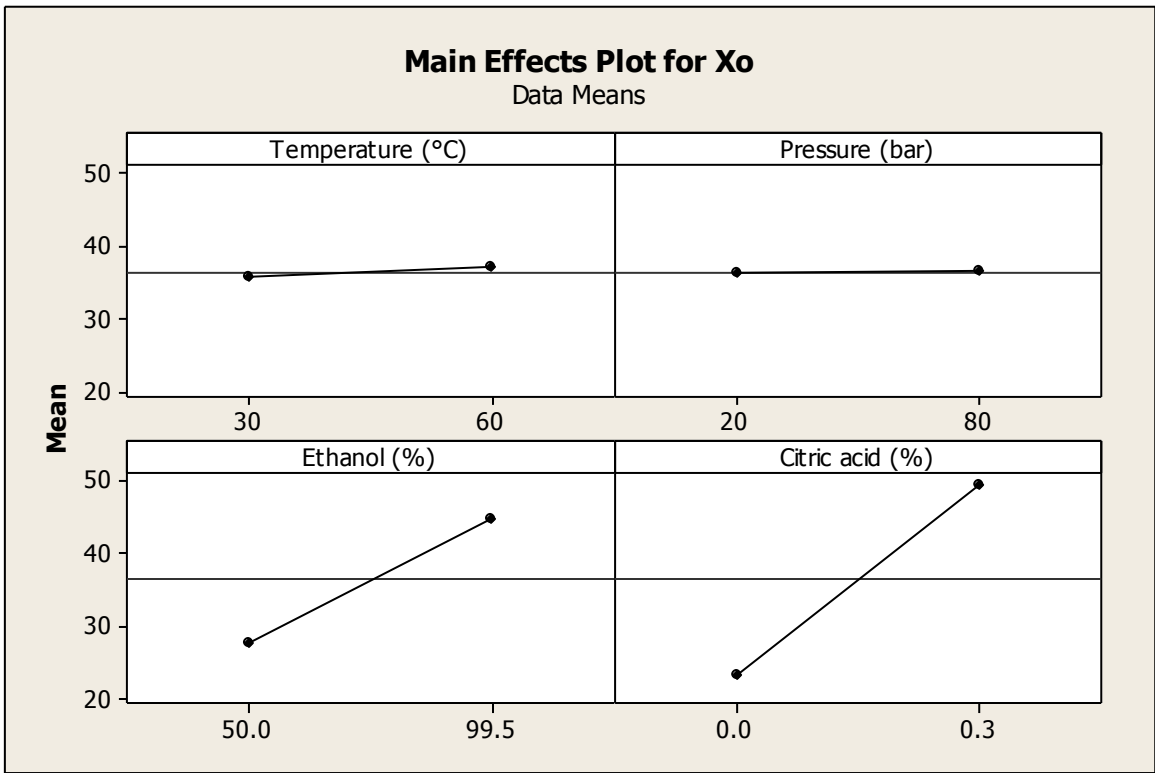
Temperature (°C)*Ethanol (%)* Citric acid (%)	-0.0217845
Pressure (bar)*Ethanol (%)* Citric acid (%)	-0.0112682
Temperature (°C)*Pressure (bar)* Ethanol (%)*Citric acid (%)	0.000284512

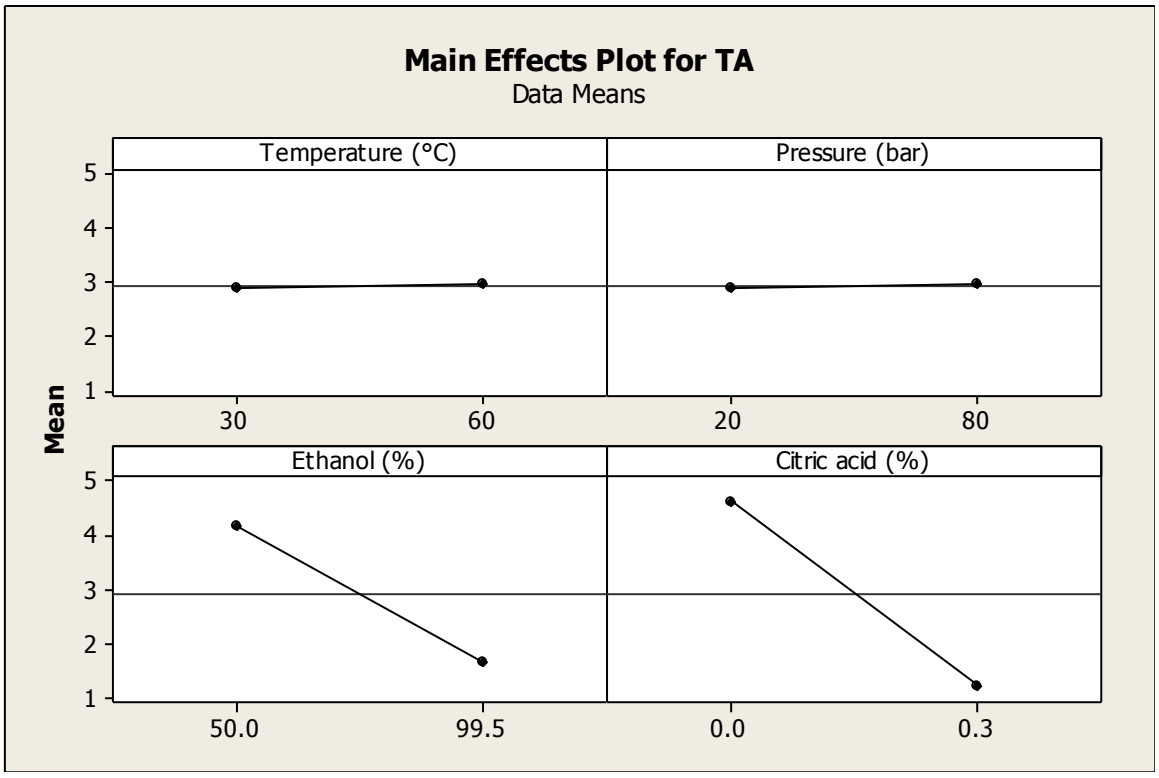
Least Squares Means for AY

	Mean	SE Mean
Temperature (°C)		
30	6.587	0.2238
60	6.893	0.2238
Pressure (bar)		
20	6.567	0.2238
80	6.912	0.2238
Ethanol (%)		
50.00	8.241	0.2238
99.50	5.239	0.2238
Citric acid (%)		
0.0000	8.589	0.2238
0.3000	4.890	0.2238

Predicted Response for New Design Points Using Model for AY

Point	Fit	SE Fit	95% CI	95% PI
1	10.0250	0.6330	(8.6830, 11.3670)	(7.7007, 12.3493)
2	7.8550	0.6330	(6.5130, 9.1970)	(5.5307, 10.1793)
3	10.6150	0.6330	(9.2730, 11.9570)	(8.2907, 12.9393)
4	9.9750	0.6330	(8.6330, 11.3170)	(7.6507, 12.2993)
5	6.2650	0.6330	(4.9230, 7.6070)	(3.9407, 8.5893)
6	8.6400	0.6330	(7.2980, 9.9820)	(6.3157, 10.9643)
7	8.1250	0.6330	(6.7830, 9.4670)	(5.8007, 10.4493)
8	7.2150	0.6330	(5.8730, 8.5570)	(4.8907, 9.5393)
9	5.4700	0.6330	(4.1280, 6.8120)	(3.1457, 7.7943)
10	8.4700	0.6330	(7.1280, 9.8120)	(6.1457, 10.7943)
11	6.6950	0.6330	(5.3530, 8.0370)	(4.3707, 9.0193)
12	6.8200	0.6330	(5.4780, 8.1620)	(4.4957, 9.1443)
13	2.7200	0.6330	(1.3780, 4.0620)	(0.3957, 5.0443)
14	3.0950	0.6330	(1.7530, 4.4370)	(0.7707, 5.4193)
15	2.7800	0.6330	(1.4380, 4.1220)	(0.4557, 5.1043)
16	3.0700	0.6330	(1.7280, 4.4120)	(0.7457, 5.3943)
17	10.0250	0.6330	(8.6830, 11.3670)	(7.7007, 12.3493)
18	7.8550	0.6330	(6.5130, 9.1970)	(5.5307, 10.1793)
19	10.6150	0.6330	(9.2730, 11.9570)	(8.2907, 12.9393)
20	9.9750	0.6330	(8.6330, 11.3170)	(7.6507, 12.2993)
21	6.2650	0.6330	(4.9230, 7.6070)	(3.9407, 8.5893)
22	8.6400	0.6330	(7.2980, 9.9820)	(6.3157, 10.9643)
23	8.1250	0.6330	(6.7830, 9.4670)	(5.8007, 10.4493)
24	7.2150	0.6330	(5.8730, 8.5570)	(4.8907, 9.5393)
25	5.4700	0.6330	(4.1280, 6.8120)	(3.1457, 7.7943)
26	8.4700	0.6330	(7.1280, 9.8120)	(6.1457, 10.7943)
27	6.6950	0.6330	(5.3530, 8.0370)	(4.3707, 9.0193)
28	6.8200	0.6330	(5.4780, 8.1620)	(4.4957, 9.1443)
29	2.7200	0.6330	(1.3780, 4.0620)	(0.3957, 5.0443)
30	3.0950	0.6330	(1.7530, 4.4370)	(0.7707, 5.4193)
31	2.7800	0.6330	(1.4380, 4.1220)	(0.4557, 5.1043)
32	3.0700	0.6330	(1.7280, 4.4120)	(0.7457, 5.3943)


Main Effects Plot for Xo
Data Means
Temperature (°C)
Pressure (bar)
Ethanol (%)
Citric acid (%)
Mean
50
40
30
20
30
60
20
80
50.0
99.5
0.0
0.3


Main Effects Plot for TA
Data Means
Temperature (°C)
Pressure (bar)
Ethanol (%)
Citric acid (%)
Mean
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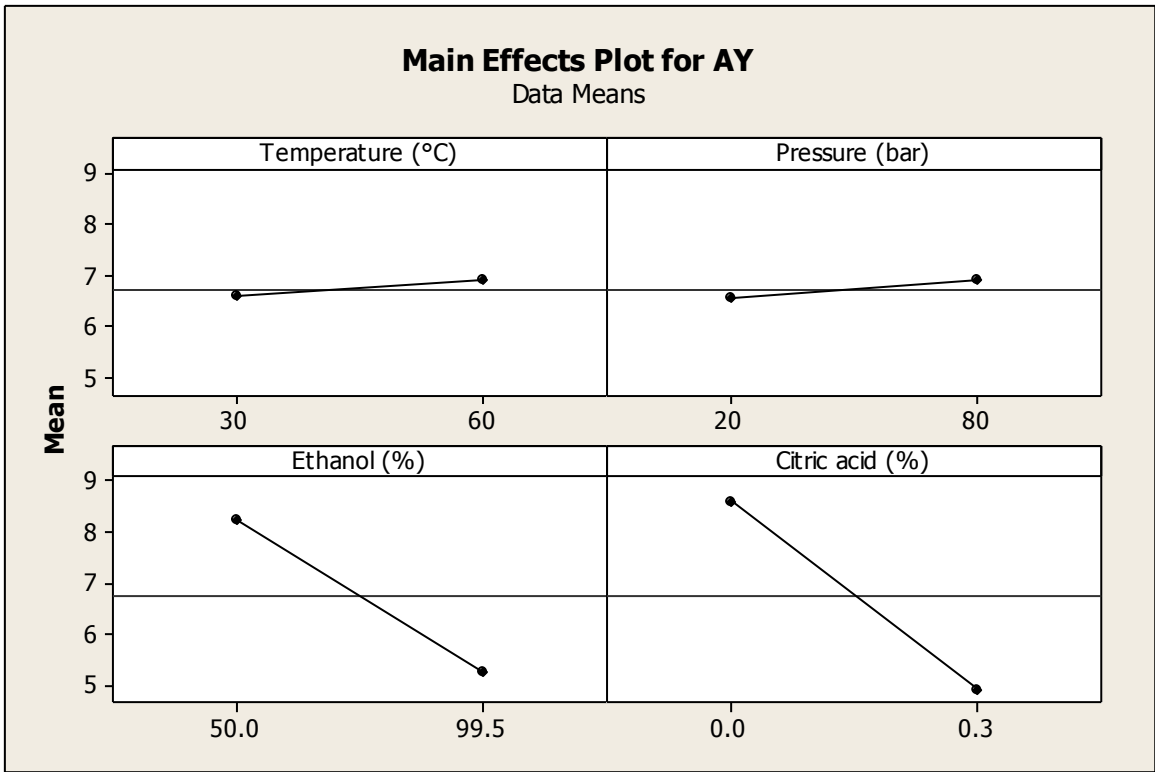
4
3
2
1
30
60
20
80
50.0
99.5
0.0
0.3


Main Effects Plot for AY
Data Means
Temperature (°C)
Pressure (bar)
Ethanol (%)
Citric acid (%)
Mean
9
8
7
6
5
30
60
20
80
50.0
99.5
0.0
0.3