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LUIZ BRUNO DE SOUSA SABINO

ANTOCIANINAS PRESENTES NOS FRUTOS DO JAMBOLÃO (*Syzygium cumini L.*): ESTUDO DA EXTRAÇÃO E APLICAÇÃO EM SISTEMAS DE LIBERAÇÃO CONTROLADA

FORTALEZA
2019

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Orientador: Professor Dr. Ivanildo José da Silva Júnior
Coorientador: Pesquisador Dr. Edy Sousa de Brito

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Ao meu bom Deus.

À minha amada mãe Neurice.

À minha amada avó Valda.

Ao meu amado Luc.

Dedico.

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“Cada um de nós compõe a sua história e cada ser em si carrega o dom de ser capaz, de ser feliz... “

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RESUMO

As antocianinas são pigmentos vegetais pertencentes a classe dos flavonóides, reconhecidas pelas cores que expressam na natureza e por suas diferentes propriedades biológicas. Em função do grande potencial tecnológico que apresentam, algumas pesquisas têm se concentrado em identificar possíveis fontes vegetais para extração e aplicação destes pigmentos. Neste contexto o objetivo desta pesquisa foi de determinar as melhores condições para a extração das antocianinas presentes nos frutos do jambolão (*Syzygium cumini* L.), aplicar o extrato obtido na formulação de comprimidos a base de carboximetilamido e avaliar a liberação dessas substâncias em meio simulado. A metodologia de superfície de resposta (MSR) foi empregada para otimizar as condições experimentais de obtenção das antocianinas utilizando a extração líquida pressurizada (ELP), extração por ultrassom de banho (EUB) e extração por ultrassom de ponteira (EUP). Um delineamento composto central de face centrada foi utilizado para a otimização dos parâmetros de extração em cada método (concentração de etanol, número de ciclos, tempo, temperatura e potência de ultrassom) em termos de concentração final de antocianinas ($mg.g^{-1}$). A maior recuperação de antocianinas foi obtida a partir do processo realizado com EUP e as condições ótimas foram: potência de ultrassom 300 W, tempo de extração de 7,5 min e concentração de etanol igual a 79,6%. Nestas condições a concentração de antocianinas foi de 60,5 $mg.g^{-1}$, valor semelhante ao predito pelo modelo. As antocianinas delfinidina-3,5-diglucosídeo, petunidina-3,5-diglucosídeo, malvidina-3,5-diglucosídeo foram tentativamente identificadas por UPLC-ESI-QTOF-MS em todos os extratos, o que demonstrou que os métodos não promoveram mudanças qualitativas no perfil das antocianinas extraídas. Nesta pesquisa, o carboximetilamido (CMA) foi sintetizado e usado como excipiente para se avaliar a liberação de antocianinas em fluidos gástrico (FGS) e intestinal (FIS). As análises estruturais demonstraram a forte interação entre os componentes dos comprimidos e a formação de um complexo entre as antocianinas e o CMA. Os perfis de dissolução variaram de acordo com o meio e com a formulação do comprimido. No geral, a liberação das antocianinas foi maior no meio intestinal (FIS), sendo controlada pelo inchamento e erosão da matriz polimérica. Após 9 horas de experimento, simulando o trânsito gastrointestinal, o comprimido C4 (formado pelo complexo antocianinas-CMA) apresentou mais de 90% das antocianinas em dissolução, exibindo maior percentagem de liberação quando comparado aos demais comprimidos. Nestas mesmas condições de dissolução, C4 exibiu forte correlação com o modelo de Korsmeyer-Peppas ($R^2 = 0,98$) e, de acordo com o valor de n (0,41), a liberação das antocianinas deste comprimido foi controlada pelo efeito de difusão. A otimização da formulação C4 permitiu a liberação completa dos pigmentos de forma rápida (30 min) ou prolongada (12 horas), demonstrando que as antocianinas podem ser liberadas em diferentes regiões do trato gastrointestinal. A liberação de 70% das antocianinas resultou na redução de 50% da atividade do radical $ABTS^{\bullet+}$ presente no meio e a atividade antioxidante destas substâncias foi maior no meio intestinal (327μM $Trolox.g^{-1}$) comparado ao gástrico (271 μM $Trolox.g^{-1}$). A interação com o CMA proporcionou maior estabilidade ao extrato antociânico, resultando na formação de um potencial sistema carreador para a entrega gastrintestinal, trazendo mais informações para o estudo destes pigmentos como agentes ativos em diversas formulações destinadas a suplementos e terapias.

Palavras-chave: *Syzygium cumini* L. Extração. Otimização. Carboximetilamido.

ABSTRACT

Anthocyanins are plant pigments belonging to the flavonoids class, recognized by the colours that they express in nature and by their different biological properties. Due to their highlighted technological potential, several researches have focused on identifying possible sources for extraction and application of anthocyanins. In this context the aim of this research was to determine the best conditions for the extraction of the anthocyanins present in jambolan fruit (*Syzygium cumini* L.), to apply the extract in the tablets based on carboxymethyl starch and evaluate the anthocyanins release in simulated medium. The response surface methodology (RSM), was used to optimize the experimental conditions for anthocyanin extraction using pressurized liquid extraction (PLE), bath ultrasound extraction (BUE) and probe ultrasound extraction (PUE). A central face centered design was used to optimize the extraction parameters (ethanol concentration, cycles number, time, temperature and ultrasound potency) in terms of anthocyanin concentration ($mg.g^{-1}$). The highest recovery of anthocyanins was obtained from the process performed with PUE and the optimal conditions were: ultrasound power 300 W, extraction time 7.5 min and ethanol concentration equal to 79.6%. Under these conditions the concentration of anthocyanins was 60.5 $mg.g^{-1}$, that was close to the value predicted by the model. The anthocyanins delphinidin-3,5-diglucoside, petunidine-3,5-diglucoside, malvidine-3,5-diglucoside were tentatively identified by UPLC-ESI-QTOF-MS in all extracts, which demonstrated that the methods did not promoted qualitative changes in jambolan anthocyanin profile. In this research, carboxymethyl starch (CMS) was synthesized and used as excipient to evaluate the release of anthocyanins into gastric (SGF) and intestinal fluids (SIF). The structural analyses demonstrated a strong interaction between the components of the tablets and the formation of a complex between the anthocyanins and the CMS. The dissolution profiles varied according to the medium and to the tablet formulation. In general, the release of anthocyanins was higher in neutral medium (SIF), being controlled by swelling and erosion of the polymer matrix. After 9 hours of assay, simulating the gastrointestinal tract, the C4 (formed by the anthocyanin-CMS complex) tablet presented more than 90% of the anthocyanins in dissolution, showing the higher percentage released when compared to the other tablets. Under the same dissolution conditions, C4 presented a strong correlation with the Korsmeyer-Peppas model (R^2 = 0.98) and, according to the value of n (0.41), the release of anthocyanins from this tablet was controlled by the diffusion effect. The optimization of the C4 formulation allowed the complete release of the pigments either rapidly (30 min) or prolonged (12 hours), demonstrating that anthocyanins can be released in different regions of the gastrointestinal tract. The release of 70% of the anthocyanins resulted in the reduction of 50% of the $ABTS^{\bullet+}$ radical activity present in the medium and the antioxidant activity of these substances was higher in the intestinal environment (327μM $Trolox.g^{-1}$) compared to the gastric (271 μM $Trolox.g^{-1}$). The interaction with the CMA provided greater stability to the anthocyanin extract, resulting in the formation of a potential carrier system for the gastrointestinal delivery, bringing more information to the study of these pigments as active agents in several formulations intended for supplements and therapies.

Keywords: *Syzygium cumini* L; Extraction. Optimization. Carboxymethyl starch.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

$ABTS^{\bullet+}$	[2,2'-azino-bis-(ácido 3-etilbenzotiazolino-6-sulfônico)]
AM	Anthocyanin-mannitol
APVP	Anthocyanin-polyvinylpyrrolidone
BUE	Bath ultrasound extraction
CCD	Central composite design
CCS	Crosscarmellose sodium;
CE	Conventional exraction
CMA	Carboximetilamido
CMS	Carboxymethyl starch
ELP	Extração líquida pressurizada
FTIR	Fourier transform infrared
GIT	Gastrointestinal tract
HPLC	High performance liquid chromatography
MCC	Microcrystalline celulose
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoracetic acid
UAE	Ultrasound-assisted exreaction
PVP	Polyvinylpyrrolidone
SEM	Scanning electron microscopy

SUMÁRIO

1 INTRODUÇÃO

A natureza é fonte de substâncias perfeitamente extraíveis e que podem ser aplicadas em diferentes setores industriais em função das propriedades físico-químicas e biológicas que apresentam. As antocianinas provenientes de diferentes vegetais têm chamado atenção da comunidade científica pelas intensas cores que imprimem na natureza e por sua elevada capacidade antioxidante que resulta em uma pluralidade de efeitos biológicos.

As antocianinas são espécies fenólicas pertencentes a classe dos flavonóides que conferem as diferentes cores a frutas, flores, folhas e raízes e também desempenham funções que vão desde a proteção até à dispersão da unidade vegetal (WALLACE; GIUSTI, 2015). Esses compostos são introduzidos na alimentação humana através de frutas e verduras e também pelo consumo de produtos oriundos do processamento destes alimentos. Estudos têm demostrado que a ingestão de alimentos ricos em antocianinas condiciona o organismo aos diversos efeitos positivos provindos da bioatividade destas substâncias (LIMSITTHICHAIKOON et al., 2015) Neste contexto, frutas e outros vegetais são frequentemente avaliados como possíveis fontes desses pigmentos.

O jambolão (*Syzygium cumini* L.) é um fruto natural da Ásia e bem adaptado a América Latina. No Brasil este fruto é encontrado principalmente na Região Nordeste onde apresenta diferentes sinonímias, sendo também conhecido como jamelão, jambão, ameixa preta e azeitona roxa. O jambolão é uma baga oval, que apresenta como principais características o sabor exótico, resultante da combinação de açúcares, ácidos orgânicos e taninos e a coloração roxa brilhante da epiderme que vai se intensificando ao decorrer da maturação do fruto em função da evidência das antocianinas presentes em sua composição. Muitos trabalhos têm apontado o jambolão como fonte de antocianinas (JAMPANI; NAIK; RAGHAVARAO, 2014; SILVA et al., 2017), demostrando a necessidade de estudos mais profundos quanto a potencial exploração deste fruto em função das diferentes propriedades físicas e biológicas que estes pigmentos apresentam. A extração de antocianinas do jambolão pode representar uma alternativa viável para o aproveitamento do jamboleiro.

A extração é uma das etapas mais importantes para a obtenção de compostos provenientes de vegetais. No estudo da extração de antocianinas, diferentes técnicas têm sido empregadas objetivando a eficiência e segurança do processo. Essas novas técnicas incluem a extração assistida por ultrassom, extração assistida por micro-ondas, extração com fluido sub e supercrítico e extração por solvente pressurizado (KHODDAMI; WILKES; ROBERTS, 2013).

Comparadas com as extrações tradicionais a extração líquida pressurizada (ELP) e a extração assistida por ultrassom apresentam vantagens como melhor repetibilidade, redução do volume de solvente, baixo risco ao operador, controle do processo e maior rendimento da substância de interesse (GOMES et al., 2017).

Na escolha do método mais viável para a extração de biocompostos, faz-se necessário o estudo das variáveis que possam influenciar o processo, bem como a interação entre elas, por estarem diretamente associadas ao rendimento final da substância. Assim, a otimização da extração encontra reforço com a adoção de modelos matemáticos que são usados para descrever o comportamento dos dados experimentais e fazer previsões estatísticas sobre a resposta desejada (BEZERRA et al., 2008; HE et al., 2016). Fatores como a temperatura, pressão, concentração de solvente e tempo de extração são constantemente avaliados em pesquisas que visam otimizar a extração de antocianinas para que se possa alcançar maiores rendimentos (CHEN; ZHAO; YU, 2015; DEMIRDÖVEN; ÖZDOĞAN; ERDOĞAN-TOKATLI, 2015; GOMES et al., 2017). A aplicação industrial dos extratos antociânicos é, portanto, diretamente relacionada ao sucesso do seu processo de extração.

Extratos ricos em antocianinas com elevada atividade antioxidante (FERREIRA; ROSSO; MERCADANTE, 2010), anti-inflamatória (BARANIAK et al., 2015), antimicrobiana (SIDDIQI et al.,2011), cardioprotetora (YOUSUF et al., 2016), antitumoral (LONG et al.,2018) e antidiabética (BALIGA et al., 2011), são fortes candidatos a aplicação em diferentes terapias. No entanto, sabe-se que estas substâncias apresentam elevada instabilidade frente a condições ambientais. Assim, simples operações industriais de processamento ocasionariam a degradação destes pigmentos, trazendo prejuízos ao setor envolvido. Uma alternativa para estabilizar e viabilizar a aplicação das antocianinas é a associação destas moléculas com diferentes polímeros (IDHAM; MUHAMAD; SARMIDI, 2012). Nesta pesquisa o carboximetilamido (CMA) foi sintetizado a partir do amido e associado ao extrato antociânico obtido dos frutos do jambolão. O CMA é um amido aniônico e sua interação com as antocianinas, de carga positiva, resulta em complexo estabilizado por interações eletrostáticas. Este complexo protege as antocianinas das condições ambientais e ainda modula sua liberação em função do pH do meio (WANG et al., 2013). A associação com CMS pode aumentar a viabilidade das antocianinas no organismo humano, considerando-o como sistema. A aplicação industrial das antocianinas tem início a partir da identificação da matéria-prima, extração e estabilização desses pigmentos o que estimula o desenvolvimento de pesquisas no sentindo de se aproveitar o potencial destas moléculas.

2 OBJETIVOS

2.1 Objetivo geral

Determinar as melhores condições para a extração das antocianinas presentes nos frutos do jambolão (*Syzygium cumini* L.), aplicar o extrato obtido na formulação de comprimidos a base de carboximetilamido e avaliar a liberação dessas substâncias em meio simulado.

2.2 Objetivos específicos

- ✓ Selecionar o solvente mais situável para extração das antocianinas do jambolão;
- ✓ Avaliar a extração das antocianinas utilizando a extração líquida pressurizada e ultrassom de banho e ponteira;
- ✓ Otimizar o processo extrativo nos diferentes métodos avaliados por meio da metodologia de superfície de resposta (MSR);
- ✓ Avaliar a influência do método de extração na qualidade do perfil antociânico do jambolão através da identificação por espectrometria de massas (UPLC-ESI-QTOF-MS);
- ✓ Formular comprimidos contendo as antocianinas extraídas do jambolão usando carboximetilamido como excipiente;
- ✓ Caracterizar a estrutura das formulações através de espectroscopia de infravermelho (FTIR) e por microscopia eletrônica de varredura (MEV) e verificar suas propriedades de erosão, retenção de líquido e perda de massa em meio simulado;
- ✓ Simular e otimizar a liberação das antocianinas em meio gástrico e intestinal simulado;
- ✓ Avaliar a atividade antioxidante das antocianinas em meio simulado.

3 REVISÃO DE LITERATURA

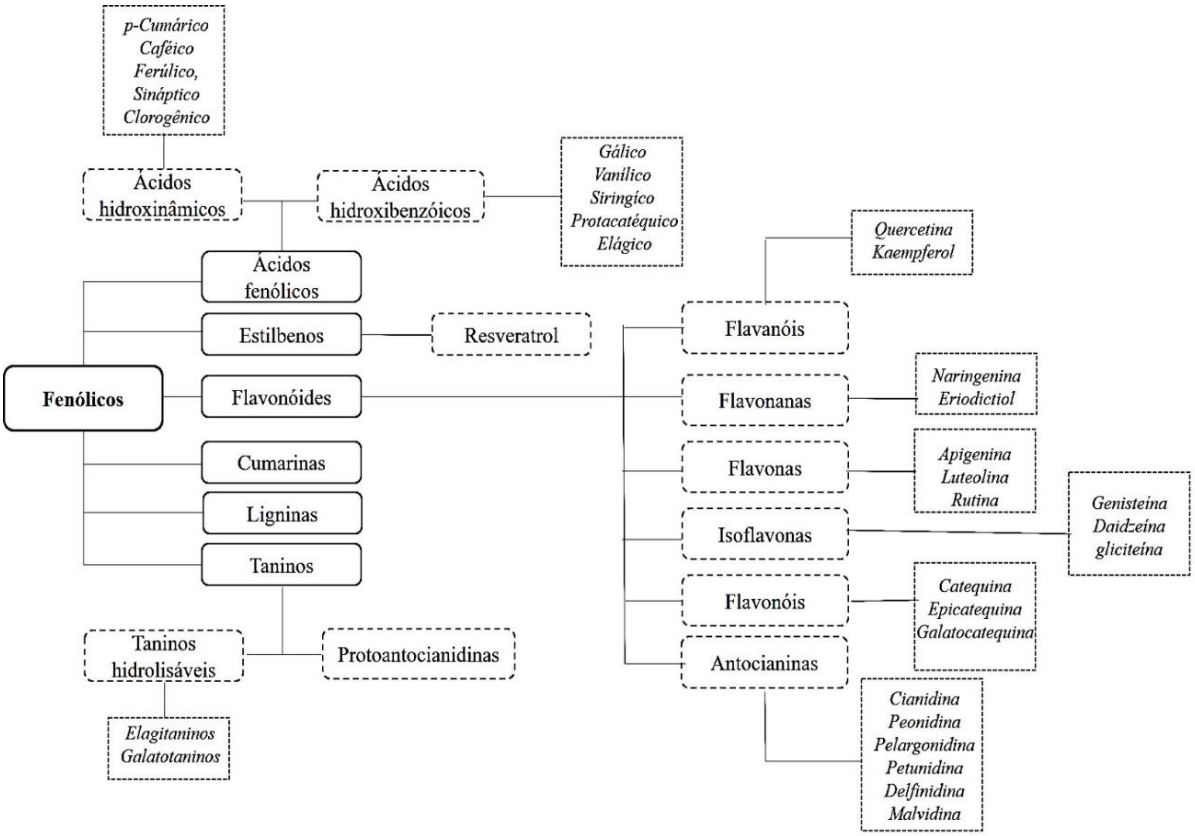
3.1 Compostos fenólicos

Os compostos fenólicos correspondem a um diversificado grupo de fitoquímicos que são oriundos do metabolismo secundário de vegetais, onde desempenham atividades relacionadas ao crescimento, reprodução e defesa (DAI; MUMPER, 2010). São substâncias derivadas da fenilalanina e que ocorrem na natureza conjugados com um ou mais resíduos de açúcar, que por sua vez são ligados a grupos hidroxila. Os fenólicos são um grupo extremamente heterogêneo englobando desde moléculas simples até aquelas com alto grau de polimerização e graças a essa abrangência, frequentemente são identificadas de novas espécies em detrimento das 8000 estruturas já reportadas (VEITCH; GRAYER, 2011).

Devido sua ampla distribuição na natureza, os fenólicos são encontrados em praticamente todos os produtos naturais e são importantes constituintes sensoriais de frutas, folhas, tubérculos, sementes e raízes, conferindo-lhes aromas, cores e sabores característicos (PANDEY; RIZVI, 2009). Os polifenóis são classificados de acordo com número de anéis fenólicos presentes em sua estrutura e também com base nos elementos estruturais que eventualmente interligarão esses anéis. As principais classes fenólicas incluem os ácidos fenólicos, flavonóides, estilbenos e ligninas (SHAHIDI; AMBIGAIPALAN, 2015). A detalhada classificação dos fenólicos bem como suas estruturas mais frequentes na natureza são apresentados na Figura 1.

A introdução dos fenólicos na dieta humana ocorre pela ingestão de vegetais e de produtos oriundos do seu processamento como o chocolate, chás, sucos e vinho tinto, por exemplo. Atualmente grande atenção tem se dado aos compostos fenólicos devido suas propriedades biológicas que têm se mostrado efetivas contra diferentes patologias (DAL MAGRO et al., 2016; SIDDIQI et al., 2011). Em especial, devido ao seu poder antioxidante, os fenólicos vêm sido avaliados como promissores agentes no combate de células cancerígenas. A ação antioxidante de fenólicos pode ser atribuída a sua capacidade de estabilizar radicais livre (como doadores H^+ ou elétrons, por exemplo) e quelar íons catalisadores de processos oxidativos. A ação antioxidante é modulada pela presença de ligações conjugadas dos anéis benzenos, característicos da estrutura fenólica (VEITCH; GRAYER, 2011).

Figura 1 - Divisão dos compostos fenólicos.



Fonte: Adaptado de Shahidi & Ambigaipalan (2015).

3.2 Flavonóides

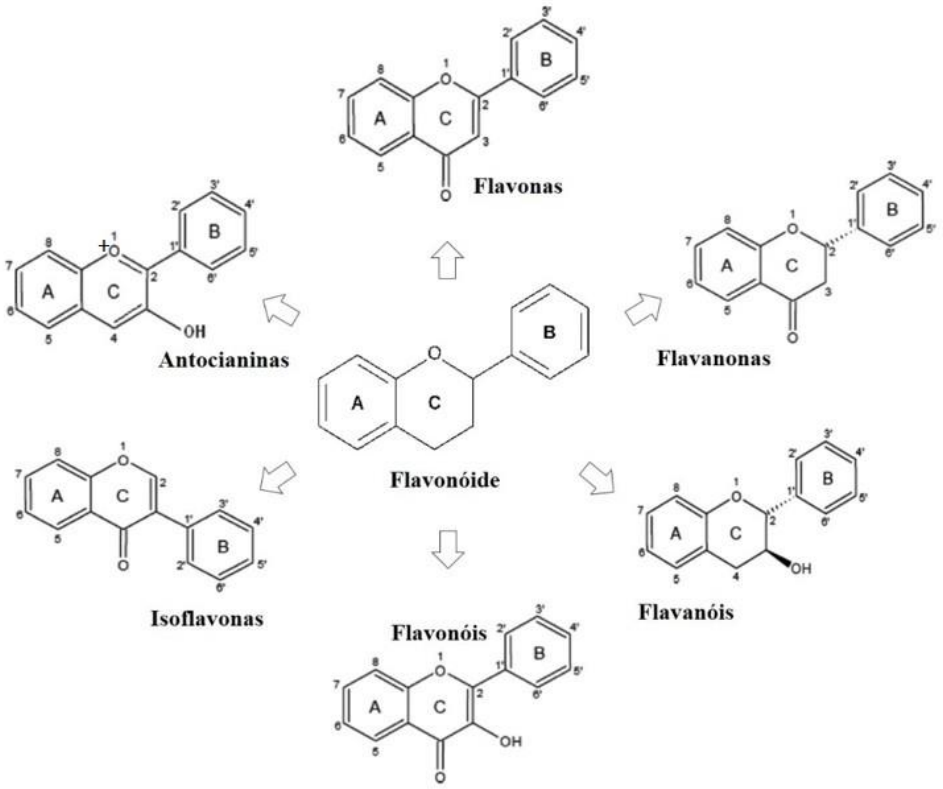
Os flavonóides são compostos caracterizados pelo seu baixo peso molecular e correspondem a classe de fenólicos com maior frequência na natureza, apresentando mais de 4000 espécies quimicamente diferenciáveis já identificadas em diferentes substratos vegetais. São importantes constituintes da dieta humana, presentes em frutas, legumes e bebidas naturais (VEITCH; GRAYER, 2011).

A estrutura dos flavonóides é derivada da fenilalanina e é formada nas vias metabólicas do ácido chíquimico e aceto-malonato. A estrutura básica desses compostos (Figura 2) é o esqueleto difenilpropano (C_6-C_3-C_6) que é estruturado em dois anéis benzeno (A e B) ligados entre si através de um anel de pirano heterocíclico (C). Conforme mostrado na Figura 2, o anel A corresponde a um grupo resorcinol, enquanto o anel B é um originado do catecol. Os flavonóides ocorrem nos vegetais como agliconas ou sob a forma de glicosídeos e/ou derivados metilados e/ou acilados. A quantidade de grupos hidroxilas ligadas a estrutura

da molécula é variável e está associada a vastidão de compostos previamente descritos na literatura (ANGELO; JORGE, 2007).

A classe dos flavonóides é subdividida de acordo com a ligação do anel B ao C e também em função do estado de oxidação e nos grupos funcionais do anel C. Flavonol, flavanol, flavanona, flavona, isoflavona e antocianina são as subclasses de flavonóides e suas respectivas estruturas são ilustradas na Figura 2. As espécies químicas pertencentes a cada subclasse apresentam padrões específicos de substituição do anel A e B relativos a presença de grupos metil e hidroxila, que variam em quantidade e posição (CORCORAN; MCKAY; JEFFREY, 2012; WILLIAMS; GRAYER, 2004)

Figura 2 - Estrutura básica dos flavonóides e sua subdivisão.



Fonte:Elaborado pelo autor.

Os flavonóides são um grupo de substâncias biologicamente ativas apresentando um largo espectro de ação incluindo propriedades antialérgicas, antiviral, anti-inflamatória, antioxidante e vasodilatador, fato que têm impulsionado as pesquisas no sentido de identificar e aplicar essas substâncias (MARCELA; MICHELE; SÔNIA, 2009).

3.3 Antocianinas

As antocianinas (do grego *anthos* = flor e *kianos* = azul) correspondem a mais importante subclasse dos flavonóides e juntamente com as clorofilas, carotenóides e as betalaínas, formam o grupo dos pigmentos vegetais. Na natureza as antocianinas se expressam nas cores observadas em flores, frutos, folhas e tubérculos, que pode variar do azul ao vermelho, incluindo as cores violeta, laranja e rósea (DAL MAGRO et al., 2016). As antocianinas são metabólitos secundários sintetizadas ao longo do amadurecimento vegetal e que além de colorir, irão desempenhar funções de proteção (proteção do aparato fotossintético frente aos altos fluxos de radiação, proteção do DNA, defesa contra parasitas, tolerância do vegetal a estresses como o frio, metais pesados, dessecação e ferimento) e de dispersão do vegetal (estímulo à polinização) (AHMADIANI et al., 2016; WALLACE; GIUSTI, 2015).

Na unidade vegetal, as antocianinas são encontradas nos vacúolos onde podem estar livres ou associadas a compostos orgânicos, íons metálicos e biopolímeros. Geralmente esses pigmentos ocorrem na superfície da planta (epiderme de frutas, pele de leguminosas e pétala de flor, por exemplo) e sua concentração vacuolar é proporcional a intensidade de cor apresentada pela referida parte do vegetal (POURCEL et al., 2010).

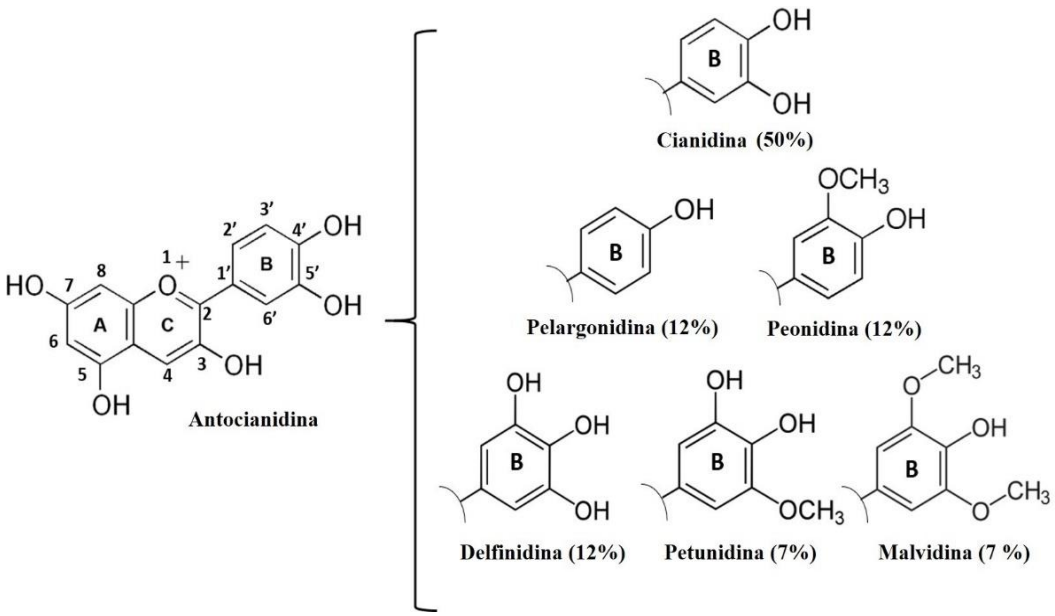
3.3.1 Estruturas

A estrutura precursora das diferentes antocianinas é denominada antocianidina ou aglicona e é mostrada na Figura 3. A estrutura clássica da aglicona é baseada em um esqueleto carbônico costituido por 15 carbonos (C_6-C_3-C_6) e é constituída por dois anéis aromáticos (A e B) ligados entre si através de um anel heterocíclico oxigenado (C), que pode ser do tipo pirano ou pirilo. A elevada oxidação da estrutura pode ser evidenciada pela completa insaturação do anel C e a presença do grupo hidroxila na posição 3. A presença do oxigênio tetravalente de valência livre, também no anel C, resulta na elevada instabilidade destes pigmentos, o que dificulta sua ocorrência natural nas espécies vegetais (KHOO et al., 2017; WELCH; WUB; SIMONB, 2008).

A molécula da antocianina é formada a partir da esterificação de uma antocianidina com uma ou mais porções de açúcar, portanto a forma glicosilada é a expressão comum das antocianinas na natureza. As antocianidinas além de apresentarem uma menor estabilidade, também são menos solúveis em água que as antocianinas, o que leva a crer que a glicosilação proporciona maior estabilidade para estes pigmentos (KONCZAK; ZHANG, 2004). As

antocianidinas mais recorrentes na natureza são a cianidina, delfinidina, malvidina, peonidina, perlargonidina e petunidina, cujas estruturas e frequência são apresentadas na Figura 3.

Figura 3 - Estrutura básica da antocianidina e espécies de antocianinas recorrentes na natureza com as respectivas frequências na natureza.



Fonte: Adaptado de Silva et al., (2016).

A diferenciação estrutural das antocianidinas ocorre devido aos padrões de substituição dos grupos metoxila e hidroxila no anel B (Figura 3) o que irá repercutir diretamente no espectro de coloração de cada uma. A Tabela 1 apresenta os grupos substituintes e a cor expressa por cada antocianidina na natureza. Incrementos no número de grupos hidroxila tendem a tornar a coloração azulada. Na direção contrária, incrementos no número de grupos metoxilas aumentam a intensidade do vermelho. Considerando os demais pigmentos que constituem a planta (isto é, carotenóides, clorofilas, por exemplo) as antocianinas em média correspondem a 80% total dos pigmentos encontrados nas das folhas, 69% nas frutas e 50% nas flores (CASTAÑEDA-OVANDO et al., 2009; CHAVES-SILVA et al., 2018; SILVA et al., 2016; WALLACE; GIUSTI, 2015).

As antocianinas podem ser di ou tri-hidroxi substituídas no anel B. A interação antocianidina-glicosídeo ocorre nos carbonos 3, 5, 7, 3', 5', apresentando maior frequência na posição 3 do anel C com ligações do tipo 3-*O*-glicosídio ou ainda 3,5-*O*-diglicosídio quando ocorre a associação de duas moléculas de açúcar nas referidas posições. Os principais açúcares substituintes são a ramnose, arabinose, galactose, xilose e com maior predominância a glicose (cerca de 90%) (LANDI; TATTINI; GOULD, 2015). A ligação de unidades de açúcar na

posição 4 pode ocorrer eventualmente. As unidades de açúcares podem estar aciladas com grupos alifáticos e/ou aromáticos. Os ácidos malônico (predominante) acético, málico, oxálico, succínico e tartárico são os principais ligantes alinfáticos enquanto que os ácidos hidroxicinâmicos (*p*-cumárico, cafeico, ferúlico, sinápico) e hidroxibenzóicos (*p*-hidroxibenzóico e ácido gálico) são os representantes aromáticos (WELCH; WUB; SIMONB, 2008).

Tabela 1 - Grupos químicos substituintes e espectro de coloração característicos das antocianidinas.

Antocianidina	**Coloração[1]**	**Grupos substituintes[2]**		
		R1	**R2**	**R3**
Cianidina (Cy)	Vermelho	OH	OH	H
Delfinidina (Dp)	Violeta	OH	OH	OH
Malvidina (Mv)	Violeta	OCH_3	OH	OCH_3
Perlagonidina (Pg)	Laranja	H	OH	H
Peonidina (Pn)	Vermelho	OCH_3	OH	H
Petunidina (Pt)	Violeta	OCH_3	OH	OH

Fonte: Adpatado de [1]Mazza e Miniati (1993) e [2]Kerio et al., (2012).

A possibilidade de diferentes ligações e de grupos na estrutura principal da antocianina resulta na diversidade de espécies químicas, número que corresponde a mais de 500 tipos de antocianinas e 23 tipos de antocianidinas (LEE; DURST; WROLSTAD, 2015). Em síntese, as diferenciações observadas para estas substâncias estão relacionadas a fatores como*: a)* a quantidade e a posição das hidroxilas na molécula; *b)* a ocorrência de metilação em uma ou mais hidroxilas presentes; *c)* a natureza, a quantidade e a posição dos açúcares ligados à estrutura da antocianina; e *d)* a natureza e a quantidade de ácidos ligados a esses açúcares (CASTAÑEDA-OVANDO et al., 2009).

3.3.2 Estabilidade de antocianinas

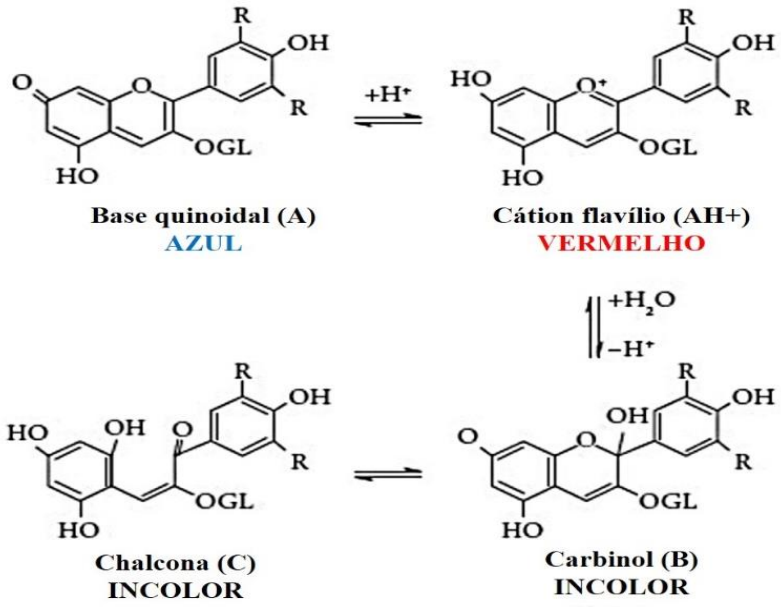
Assim como a maior parte dos pigmentos vegetais, as antocianinas apresentam elevada instabilidade quando expostas a fatores que, na maioria dos casos, são relacionados às condições ambientais. A presença de copigmentos, íons metálicos, luz, temperatura, enzimas, oxigênio e mudanças no pH são exemplos de fatores que influenciam a estabilidade desses pigmentos (WALLACE; GIUSTI, 2015). Por outro lado, a conformação do anel B e a presença de grupos OH- ou -OCH_3, são fatores intrínsecos da estrutura antociânica que reduzem sua estabilidade quando em solução.

A seguir serão abordadas algumas características acerca destes agentes, quanto aos mecanismos relacionados às modificações da estrutura antociânica.

3.3.2.1 Influência do pH do meio

Entre os fatores apresentados anteriormente, o pH é o que apresenta maior influência sobre a estabilidade das antocianinas, fato que se deve a natureza iônica dessas substâncias (KHOO et al., 2017). Em relação ao pH do meio, a discussão inicial se refere as várias formas químicas que as antocianinas podem apresentar em solução: O cátion flavílio (AH^+), base anidra quinoidal (A), pseudo-base carbitol (B) e chalcona (C) que apresentam colorações vermelho, azul, incolor e incolor ou levemente amarelado, respectivamente. Estudos termodinâmicos e cinéticos desenvolvidos inicialmente por McClelland e Gedge (1980), Pina (1998) e Sondheimer (1953) levaram a um esquema usualmente aceito, relativos às diferentes reações (transferência de prótons, isomerização e tautomerização) do cátion flavílio quando as antocianinas estão em diferentes condições de pH. A Figura 4 demonstra esse esquema.

Figura 4 - Modificações das antocianinas em meio aquoso em diferentes faixas de pH.



(AH^+): Cátion flavila; (A): Base quinoidal; (B): Carbinol; (C): Chalcona.
Fonte: Adaptado de Levi et al. (2004).

Em pH ácido (pH ≤ 2,5), a forma dominante das antocianinas é o cátion flavílio (AH^+) que se expressa com coloração vermelha. Um aumento do pH (acima de 3), faz com que

as antocianinas sofram ataque nucleofílico por moléculas de água e como consequência é formado o carbinol (B) e em função da perda prótons a coloração desaparece. O carbitol pode sofrer tautomerismo formando a chalcona (C) que também é incolor. O equilíbrio entre estas duas últimas formas é lento levando horas para ser atingido (Tabela 2). Em valores de pH superiores a 7, as antocianinas são degradadas, dependendo dos seus grupos substituintes (caso das antocianinas aciladas como será discutido adiante) (SILVA et al., 2016).

Tabela 2 - Tipos de equilíbrio químicos das antocianinas em soluções.

Tipo de equilíbrio[1]	**Reação[1]**	**Velocidade da reação[2]**	**Constante de equilíbrio[2]**
Ácido-base	Desprotonação do cátion flavila, formando base Quinoidal	Muito rápida	Ka ' = [HA] / [A][H+]
Hidratação	Hidratação do cátion flavila, formando carbitol	Rápida	Kb ' =[AH+] / [B][H+]
Tautomeria em cadeia	O carbitol tautomerizado para formar chalcona	Lenta	KT = [C] / [B]

Fonte: Adaptado de [1]Lopes et al. (2007) e [2] Bordignon et al. (2009).

A maior parte das antocianinas exibe alta estabilidade em condições ácidas sofrendo degradação em pH elevados. Entretanto, normalmente essas substâncias existem em suas formas incolores (carbinol ou chalcona), considerando por exemplo, o pH neutro de diferentes vegetais (TORSKANGERPOLL; ANDERSEN, 2005). Em todo caso, exceções existem. A petanina (petunidina-3-[6-O(4-O-E-*p*-coumariol-O-α-1ramnopiranosil)-β-d-glicopiranosídeo]-5-*O*-β-*d*-glicopiranosideo) é uma estrutura derivada da peonidiana e que apresenta elevada estabilidade mesmo em faixas de pH acima de 8, o que é resultado de sua estrutura acilada (KHOO et al., 2017). A adição de um ou mais grupos acila na molécula da antocianina resulta na não hidrólise do cátion flavílio, evitando assim a formação do carbinol e possibilitando a formação da base quinoidal (azul). Portanto, a acilação resulta na formação de pigmentos mais estáveis frente às mudanças de pH o que é interessante, principalmente para a indústria alimentícia que pode usufruir das propriedades de cor destes pigmentos nas diferentes faixas de pH que são apresentadas nos sistemas alimentícios. Exemplos de antocianinas aciladas são aquelas encontradas no rabanete, batata vermelha, repolho roxo, cenoura preta e batata-doce roxa (HAMERSKI; REZENDE; SILVA, 2013).

Geralmente as antocianinas estão envolvidas em processos que ocorrem em sistemas líquidos e neste caso o pH assume um papel importante no estudo da estabilidade e consequentemente na aplicação dessas moléculas. Estruturalmente, os substituintes do anel B

(FIGURA 4) e a presença de grupos hidroxilas ou metoxilas ligadas a este anel diminuem a estabilidade da aglicona em meios neutros. Em contraste com as agliconas, os derivados monoglicosídeos e, principalmente, os diglicosídeos são mais estáveis em condições de pH neutro, o que ocorre devido a presença de moléculas de açúcar que irão evitar a degradação dos intermediários instáveis em ácidos fenólicos e aldeídos (FLESCHHUT et al., 2006).

Modificações no pH do meio também promovem alterações na absorção no espectro UV-visível das antocianinas. Segundo Bordignon et al. (2009) o aumento do pH resulta na diminuição do número de ligações duplas conjugadas, que são responsáveis pelo aumento nos máximos de absorção das substâncias devido a protonação do cátion flavílio. A redução dessas ligações faz com que os máximos de absorção das antocianinas se desloquem para comprimentos de onda menores, resultando na perda de sua coloração.

3.3.2.2 Efeito da temperatura

O aumento da temperatura resulta na consecutiva degradação das antocianinas presentes em uma solução ou em um substrato sólido. Essa degradação é fisicamente visível pela formação de compostos complexos de coloração castanho-marrom, cuja síntese é atenuada pela presença de oxigênio (CASTAÑEDA-OVANDO et al., 2009). Antocianinas de estrutura mais simples são mais susceptíveis a degradação pela elevação da temperatura. Em sua pesquisa, Mori et al. (2007) apontaram que o tratamento térmico de vinhos a 35°C reduziu o conteúdo antociânico pra menos da metade ao ser comparado com ensaio realizado a 25°C. Mesmo em pH baixo, temperaturas acima de 35°C podem promover mudanças na coloração das antocianinas em solução (WEST; MAUER, 2013). Por outro lado, um tratamento térmico brando como o branqueamento, pode reduzir a degradação dos pigmentos, uma vez que inativa a polifenol oxidase, enzima comumente encontrada nos tecidos vegetais e responsável pela degradação de fenólicos (CASTAÑEDA-OVANDO et al., 2009).

Como mencionado anteriormente as antocianinas aciladas são mais estáveis frente a variações no pH do meio. De acordo com Lopes et al. (2007) esta afirmativa também se estende para temperatura, uma vez que a metoxilação do grupo acila melhora a integridade estrutural destas moléculas frente a exposição a temperaturas mais elevadas. Esta observação é endossada por Chigurupati et al. (2002) que apontaram que a acilação das antocianinas do repolho roxo, resultaram em uma maior estabilidade na cor do vegetal após o tratamento térmico. A influência da temperatura na estabilidade de antocianinas também foram reportadas por Jiménez et al. (2010) e Lin e Chou (2009) que demostraram que a cinética de degradação

das antocianinas acorre em ordem logarítmica e também que o incremento da temperatura resultou no decréscimo da capacidade antioxidante destas moléculas.

3.3.2.3 Presença de oxigênio e radiações luminosas

O oxigênio está relacionado com a degradação de antocianinas por meio da oxidação direta ou indireta de substâncias presentes no substrato (outros fenólicos, por exemplo), que irão interagir diretamente com estes pigmentos (LOPES et al., 2007). De acordo com Patras et al. (2010) o oxigênio apresenta um efeito ativador importante nas reações oxidativas que são catalisadas por diferentes enzimas como a polifenoloxidase (PPO) e peroxidase (POD). A PPO, na presença de oxigênio, catalisa reações que convertem o ácido clorogênico em seu correspondente *o*-quinona. Este intermediário se condensa com as antocianinas presentes no meio, formando então substâncias complexas de coloração marrom.

As radiações luminosas apresentam dualidade, atuando tanto na síntese como na degradação de antocianinas, ocorrendo este último caso, principalmente durante o armazenamento de produtos que contenham estas moléculas, devido reações fotoquímicas (CASTAÑEDA-OVANDO et al., 2009). No estudo da estabilidade das antocianinas extraídas de *Ranunculus asiaticus* como corantes naturais, Amr e Al-Tamimi (2007) descreveram o efeito das radiações luminosas na redução significativa do conteúdo antociânico. Amostras de milho roxo e batata doce foram submetidos ao tratamento com luz direta durante 10 dias e ao final do experimento a intensa modificação nas coordenadas de cor, indicaram uma redução da tonalidade roxa, resultado atribuído a desestabilização dos pigmentos (CEVALLOS-CASALS; CISNEROS-ZEVALLOS, 2003). Complementando os dados anteriores, de acordo com Contreras-Lopez et al. (2014), a degradação de antocianinas pela aplicação de radiações luminosas segue uma cinética de segunda ordem.

3.3.2.4 Influência da copigmentação

A copigmentação da aglicona é referido como um fenômeno onde a intensidade da coloração das antocianinas é modificada em função de sua interação com flavonóides, alcalóides, aminoácidos, ácidos orgânicos, nucleotídeos, polissacarídeos, metais ou outra antocianina, que são denominados de copigmentos. Esse fenômeno promove a estabilização da cor das folhas, flores e frutos da planta devido a estabilização do carbinol (KHOO et al., 2017). Os copigmentos são normalmente incolores, todavia quando misturados com as antocianinas

produzem um efeito hipercrômico na absorção espectros (região UV-Vis) o que resulta em um incremento da cor (CASTAÑEDA-OVANDO et al., 2009).

Além dos fatores apresentados anteriormente, a literatura faz referência a íons, açúcares, ácido ascórbico e sulfitos como agentes que alteram a estabilidade das antocianinas (CAVALCANTI; SANTOS; MEIRELES, 2011; HAMERSKI; REZENDE; SILVA, 2013; PATRAS et al., 2010).

Em síntese pode-se observar que a estabilidade das antocianinas é modulada principalmente pelas condições ambientais, o que dificulta sua aplicação em possíveis segmentos industriais. Portanto, a minimização, neutralização ou aproveitamento das mudanças estruturais que estas moléculas são passíveis é requerida, baseando-se na possível aplicação de suas propriedades físicas e biológicas.

3.3.3 Propriedades antioxidantes, metabolismo e biodisponibilidade de antocianinas

Além das propriedades cromóforas, as antocianinas também apresentam propriedades bioativas que têm se tornado o foco principal de pesquisas recentes destinadas ao estudo destas moléculas. Assim, os esforços se convergem no sentido de identificar potenciais substratos que eventualmente sejam fontes de antocianinas e avaliar suas propriedades antioxidantes (FERREIRA; ROSSO; MERCADANTE, 2010), anti-inflamatórias (BARANIAK et al., 2015), cardioprotetoras (YOUSUF et al., 2016), antitumorais (LONG et al., 2018) e antidiabéticas (JAYAPRAKASAM et al., 2005), por exemplo.

No geral, as pesquisas têm utilizado diferentes modelos, *in vitro* ou *in vivo*, no sentido de performar a bioatividade de extratos antociânicos e assim têm alcançado inúmeros resultados que endossam o potencial biológico desses pigmentos. Exemplos sobre a ação biológica de antocianinas frente a algumas patologias são apresentados na Tabela 3.

Tabela 3 - Bioatividade de antocianinas frente a diferentes patologias.

Propriedade biológica	**Antocianinas**	**Referência**
Proteção cardiovascular	Padrões glicosídeos de delfinidina, cianidina e malvidina; glicosídeos de pelargonidina extraída do morango;	Rechner e Kroner, (2005); Alvarez-Suarez et al.(2014)
Ação antitumoral	Glicosídeos de cianidina e peonidina extraídos do arroz negro; glicosídeos de cianidina e pelargonidina extraídos da amora e framboesa	Hui et al.(2010); Bowen-Forbes; Zhang; Nair (2010)

Ação antidiabética	Glicosídeos de pelargonidina e malvidina extraídas do jambolão; extrato antociânico obtido do mirtilo	Sharma et al.(2006); Takikawa et al.(2010)
Antiobesidade	Extratos antociânico obtido da soja preta; glicosídeo de cianidina extraído do milho roxo	Kwon et al. (2007); Tsuda et al. (2003)
Antimicrobiana	Glisocídeos e cianidina e delfinidina extraídos do maqui; glicosídeos de cianidina e delfinidina extraídos de diferentes *berries*	Genskowsky et al. (2016); Puupponen-Pimiã et al. (2001)

3.3.3.1 Atividade antioxidante

A maior parte das propriedades biológicas apresentadas pelas antocianinas é derivada da elevada ação antioxidante apresentada por estas moléculas. Diferentes trabalhos já estudaram a atividade antioxidante (AAT) destas substâncias extraídas de diferentes substratos vegetais, como feijão preto (AGUILERA et al., 2016); jambolão (ARUN et al.,2011); arroz negro, batata doce roxa, repolho vermelho, cebola e uva (BOO et al., 2012); cenoura roxa (ERSUS; YURDAGEL, 2007); amora (FERREIRA; ROSSO; MERCADANTE, 2010); cereja (GRIGORAS et al., 2012); e jaboticaba (IBRAHIM SILVA et al., 2013).

A grande notoriedade dada a AAT das antocianinas, deve-se ao fato de que grande parte das doenças da sociedade atual são resultantes da ação deletéria dos radicais livres e espécies reativas de oxigênio, produzidos pelo metabolismo natural de oxigênio ou induzidos por agentes externos. Esses radicais causam agressões contínuas às células e consequentemente aos tecidos, resultando em processos inflamatórios e no desenvolvimento de câncer, por exemplo (RIGO et al., 2002; SILVA et al., 2016). A descoberta de substâncias que possam neutralizar estes radicais é cada vez mais requerida, reforçando o desenvolvimento de pesquisas neste sentido.

A AAT das antocianinas está relacionada à sua habilidade em estabilizar radicais livres pela doação de moléculas de hidrogênio e também inibir enzimas oxidativas como a lipoxigenase e cicloxigenase (BOWEN-FORBES, ZHANG E NAIR, 2010). A deficiência natural de elétrons destes pigmentos faz com que sejam substancialmente reativos e assim como a maioria dos fenólicos, efetivos doadores de hidrogênio. A AAT é, portanto, modulado pela estrutura química das antocianinas. A capacidade de aceitar elétrons desemparelhados de radicais livres varia com a modificação das posições e os tipos de grupos químicos ligados aos

anéis aromáticos das antocianinas. Essa ação também é modulada pelo número, posição e conjugação dos grupos hidroxilas, assim como pela presença de elétrons doadores no anel da estrutura, o que se deve à capacidade que o grupo aromático possui de suportar o desaparecimento de elétrons (KUSKOSKI et al., 2004).

Diferenças entre a capacidade antioxidante de antocianidinas e antocianinas foram apontadas por Stintzing et al. (2002) e se deve a menor estabilidade apresentada pelas antocianidinas, o que as torna estruturas mais reativas e, portanto, com maior AAT que seus glicosídeos. Uma avaliação das antocianidinas (FIGURA 3) revela que a delfinidina, cianidina e petunidina apresentam um poder antioxidante mais pronunciado que as demais, por apresentarem estruturas mais susceptíveis a oxidação em decorrência dos dois grupos hidroxilas substituintes (FLESCHHUT et al., 2006). Kähkönen e Heinonen (2003) avaliaram a atividade antioxidante das agliconas e apontaram que o poder antioxidante destas estruturas é também modulado por seus padrões de glicosilação. Os resultados dessa mesma pesquisa mostraram que delfinidina é a aglicona que apresentou a maior habilidade em estabilizar radicais livres, considerando os diferentes métodos e sistemas avaliados. De acordo com Tamura e Yamagami (1994) a acilação das antocianinas também irá afetar a AAT, resultando em um considerável aumento desta ação, principalmente quando o grupo substituinte é um ácido fenólico.

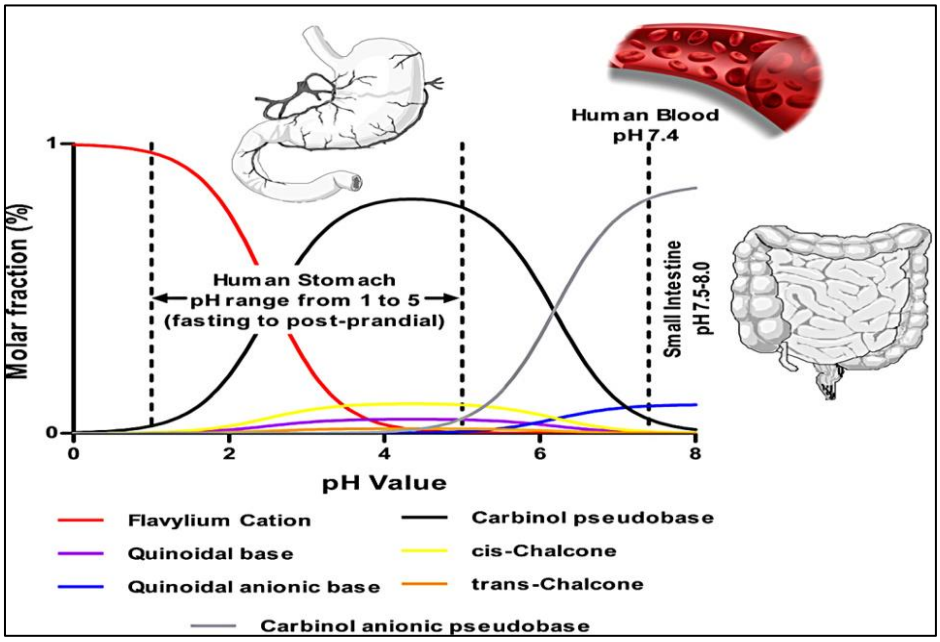
A AAT das antocianinas tem sido avaliada a um longo tempo. Tsuda et al. (1996) avaliou o efeito inibitório de diferentes antocianinas extraídas do feijão (*Phaseolus vulgaris*) na peroxidação lipídica causada pela irradiação de luz UV no tecido de fígado de ratos. Os resultados apontaram que todos os pigmentos exibiram forte atividade antioxidante, reduzindo a formação de malonaldeído. Noda et al. (2000) mostraram que as antocianinas extraídas da casca da berinjela apresentaram AAT semelhante a antioxidantes sintéticos na estabilização de espécies reativas de oxigênio. Os resultados do estudo da AAT das antocianinas extraídas dos frutos do arônia (*Photinia melanocarpa*), madressilva (*Lonicera japônica)* e do abrunheiro (*Prunus spinosa)* apontaram que a AAT dos extratos antociânicos é dependente da concentração destes pigmentos no meio reativo (GABRIELSKA et al.,1999). Por fim, Narayan et al. (1999) determinaram que a peroxidação enzimática e não-enzimática de ácidos graxos poli-insaturados foi significativamente inibida por antocianinas provenientes na cenoura (*Daucus carota* L.) de forma dose-dependente.

3.3.3.2 Metabolismo e biodisponibilidade

É evidente que a extensão dos efeitos benéficos resultantes da ação das antocianinas apresentados em experimentos *in vitro* são dependentes da absorção, metabolismo, distribuição e excreção destes compostos no organismo. As antocianinas são introduzidas na dieta humana através do consumo de frutas, legumes, raízes e bebidas e mesmo que não existam valores de referência para a ingestão diária, sabe-se que uma dieta rica em antocianinas é sinônimo de benrfícios à saúde, apresentando conformidade com a orientação dietética (WALLACE; GIUSTI, 2015).

A biodisponibilidade é definida como a quantidade de um nutriente ingerido que é absorvida e disponível para funções fisiológicas (ETCHEVERRY; GRUSAK; FLEIGE, 2012). Assim, o metabolismo, absorção e consequentemente a bioatividade das antocianinas, podem ser entendidos a partir do estudo de sua biodisponibilidade no organismo humano. Os eventos que marcam o metabolismo destes pigmentos não são perfeitamente esclarecidos, no entanto, sabe-se que a estrutura química, a dose ingerida e a matriz alimentícia são os fatores que irão influenciar a absorção dessas moléculas nos diferentes sítios do organismo (FERNANDES et al., 2014; NORBERTO et al., 2013). Experimentalmente o estudo da biodisponibilidade de antocianinas irá se basear no monitoramento das transformações do cátion flavílio nos diferentes pH do organismo (Figura 5).

Figura 5 - Representação das formas de equilíbrio das antocianinas de acordo com o pH do trato gastrointestinal.



Fonte: Nave et al. (2010).

A passagem das antocianinas pelo trato gastrointestinal (TGI) faz com que esses pigmentos sejam expostos a diferentes condições de pH, o que significa que seu trânsito pelo TGI é marcado pela existência de suas diferentes formas de equilíbrio. É provável que o cátion flavílio seja a forma predominante no estômago devido ao pH ácido, enquanto as outras formas existam mais adiante no TGI. As antocianinas apresentam elevada habilidade em atravessar a mucosa gástrica, sendo portanto, rapidamente absorvidas pelo estômago (PASSAMONTI et al., 2003). Talavéra et al. (2004) detectaram antocianinas na sua forma nativa no estômago, indicando que estas moléculas não são metabolizadas neste órgão. El Mohsen et al. (2006), ao estudarem o metabolismo da perlagonidina, verificaram a presença do ácido *p*-hidroxibenzóico no tecido gástrico de ratos após 2 horas de sua ingestão, o que, entretanto, não foi atribuído ao metabolismo e sim a degradação dessa antocianidina (FERNANDES et al., 2014). O mecanismo real pelo qual as antocianinas são absorvidas pelo estômago permanece desconhecido, entretanto, sua efetiva difusão parece ser controlada pelas bilitranslocases, enzimas transportadoras que se expressam no ambiente gástrico. Em todo caso, seja na sua forma nativa ou metabolizada, após ultrapassarem a mucosa gástrica, as antocianinas alcançam a circulação sistêmica onde estarão disponíveis para exercer suas atividades biológicas (PASSAMONTI et al., 2003).

O conteúdo de antocianinas não absorvido pelo estômago é direcionado para o intestino delgado e neste ambiente irá se apresentar predominantemente na forma carbinol em função do pH neutro. Diferente dos outros fenólicos, as antocianinas são prontamente absorvidas ou metabolizadas neste ambiente e a partir de então lançadas no sistema sanguíneo ou excretadas em sua forma nativa ou em metabólitos (derivados sulfatados ou metilados) na bile ou urina (ICHIYANAGI et al., 2005; MCGHIE et al., 2003; TALAVÉRA et al., 2004). O principal mecanismo relacionado ao transporte das antocianinas no intestino parece estar relacionado ao SGLT1, um transportador de glicose previamente sugerido para outros flavonóides (HOLLMAN et al., 1999). Ao final, as antocianinas remanescentes atingem o colón onde sofrem intensas transformações estruturais que, segundo Woodward et al. (2009), podem ocorrer naturalmente, devido as condições fisiológicas do meio ou devido a atividade da microbiota local, que pode hidrolisar os glicosídeos, convertendo-os em ácido fenólicos.

Os benefícios associados a ingestão das antocianinas parece ser devido a lenta e constante liberação destes compostos do intestino para a corrente sanguínea (FERNANDES et al., 2014). Apesar da sua biodisponibilidade ser considerada baixa, de acordo com DeFuria et al. (2009), as concentrações plasmáticas destes pigmentos parecem ser suficientes para induzir

mudanças na transdução de sinal e expressão gênica *in vivo* e reproduzir seus efeitos biológicos específicos.

3.3.4 Extração de antocianinas

A literatura dispõe de diferentes técnicas para a obtenção de extratos antociânicos. A extração é a primeira etapa na pesquisa de antocianinas e requer algumas particularidades quanto a escolha dos parâmetros (temperatura, tempo e pressão, por exemplo) que envolvem a recuperação destas moléculas, para que não existam modificações em sua estrutura, evitando assim quaisquer prejuízos sensoriais ou funcionais. De uma modo geral as técnicas podem ser divididas em tradicionais e modernas, sendo o primeiro grupo representado principalmente pela maceração dos tecidos vegetais na presença de solventes (ĆUJIĆ et al., 2016).

A extração utilizando solventes é extensivamente usada para extração de diferentes fitoquímicos, incluindo as antocianinas. Este processo pode ser realizado com substratos *in natura* ou que tenham passado por algum tratamento prévio de secagem (por estufa ou liofilização, por exemplo). Geralmente, empregam-se solventes polares como água, etanol, metanol e acetona, devido a polaridade destes pigmentos (MCGHIE; WALTON, 2007). A acidificação do solvente é referenciada como um procedimento importante, por conferir ao extrator uma melhor capacidade em romper as membranas celulares dos tecidos vegetais e dissolver os pigmentos; ademais, um ambiente ácido também irá favorecer a estabilização do cátion flavílio, evitando possíveis modificações na coloração das antocianinas (FRANCIS, 1982). Os ácidos acético, cítrico, clorídrico, fórmico, tartárico, trifluorácetico e propiônico são frequentemente empregados para a acidificação das soluções extratoras, no entanto, essa etapa requer atenção pois uma concentração elevada de ácido poderá resultar na hidrólise da molécula. Assim, convencionou-se que a concentração de ácido não exceda 1% do volume da solução extratora total (CASTAÑEDA-OVANDO et al., 2009; KAPASAKALIDIS; RASTALL; GORDON, 2006).

Um dos métodos mais empregados no processo de extração de antocianinas foi determinado por Francis (1982) que se baseia na extração sólido-líquido. Neste ensaio o extrato antociânico bruto é obtido através da solubilização da matriz vegetal em metanol acidificado com 1% (v/v) de ácido clorídrico. A escolha do solvente mais situável para a extração de antocianinas é recorrente na literatura (AMR; AL-TAMIMI, 2007; SPIGNO; TRAMELLI; FAVERI, 2007; VEGGI; SANTOS; MEIRELES, 2011). Alguns trabalhos justificam a utilização do metanol como extrator devido sua eficiência na recuperação das antocianinas e

por, aparentemente, estabilizar os pigmentos em dissolução (CASTAÑEDA-OVANDO et al., 2009). Entretanto, devido a pronunciada toxicidade e considerando a possibilidade de aplicação dos extratos para fins alimentícios, muitos estudos buscam substituir o metanol por solventes que possam resultar em uma performance similar ou superior, sem prejuízos ao consumidor. O etanol (RODRIGUES et al., 2015) e acetona (NAYAK et al., 2015; YANG et al., 2017) têm sido constantemente utilizados como solventes para extração das antocianinas mostrando resultados satisfatórios quanto a estabilidade e rendimento destes pigmentos. No entanto, a extração sólido-líquido apresenta aspectos negativos, especialmente quando se trata da possibilidade de extração em larga escala, uma vez que apresenta uma maior resistência a transferência de massa. Além disso a grande quantidade de solvente usado no processo, longo tempo de extração, riscos operacionais e baixa eficiência e rendimento de extração, contribuem para a redução desta técnica (NAYAK et al., 2015; SETYANINGSIH et al., 2016).

Além de ser a etapa inicial a extração pode ser considerada uma das mais críticas do processo de obtenção das antocianinas. Quando se prospecta a aplicação e largas escala, o rendimento é considerado um dos principais fatores de decisão sobre a viabilidade do processo. As pesquisas com fitoquímicos têm evoluído constantemente e assim surgido técnicas que visam maiores rendimentos sem que exista o comprometimento da atividade da molécula-alvo. Sistemas mais modernos e automatizados como o caso da extração com líquido pressurizado (ELP), extração com fluido supercrítico, extração assistida por micro-ondas e sistemas de sonicação (CAI et al., 2016; KANG; KIM; MOON, 2016) estão ganhando espaço na pesquisa de compostos naturais devido a maior eficiência destes métodos comparado aos tradicionais.

3.3.4.1 Extração líquida pressurizada (ELP)

A extração líquida pressurizada (ELP) é uma técnica relativamente nova. Consiste em uma extração automatizada que utiliza temperaturas e pressões elevadas para se obter uma extração em um curto espaço de tempo. O equipamento (Figura 6) permite o emprego de altas temperaturas o que promove uma maior solubilidade da molécula-alvo, melhora a taxa de difusão do solvente na matriz pela redução de sua viscosidade e enfraquece as interações na matriz fenólica. As elevadas pressões permitem que os solventes sejam utilizados acima do seu ponto de ebulição o que reduz o tempo do processo de extração, obtendo-se um rendimento elevado (JENTZER et al., 2015; MUSTAFA; TURNER, 2011). Portanto a eficiência dessa técnica está relacionada a temperatura de operação, pressão, tempo e solvente. Por ser um sistema fechado não há exposição dos extratos a luz, oxigênio e ao calor excessivo, além disso

pode ser considerada uma técnica verde pela redução do consumo de solventes (15-40 mL), redução do tempo de análise (15-20 min), reprodutibilidade e por não ser compatível com solventes demasiadamente tóxicos (HOSSAIN et al., 2011; OKUDA et al., 2009).

Figura 6 - ASE 350 (*Accelerated Solvent Extractor*) utilizado para extração líquida pressurizada.

Fonte: ThermoFisher Scientifc.

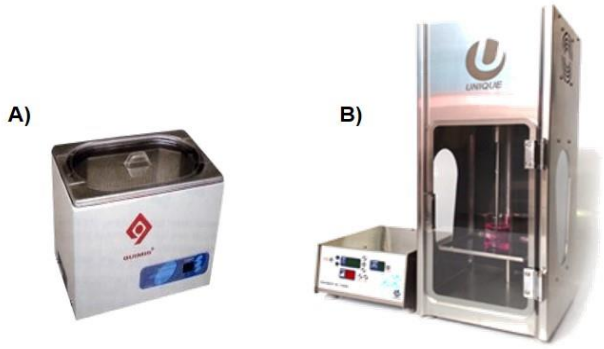
Devido as características de performance e as vantagens apresentadas a ELP já tem sido utilizado para extração de antocianinas e outros fenólicos de diferentes substratos vegetais tais como: da laranja vermelha (NAYAK et al., 2015); aroeira (FEUEREISEN et al., 2017) arroz preto (SETYANINGSIH et al., 2016); maracujá (GOMES et al., 2017); mirtilo (HEFFELS; WEBER; SCHIEBER, 2015) e alecrim, manjericão, orégano, (HOSSAIN et al., 2011).

3.3.4.2 Extração assistida por ultrassom

A extração por ultrassom é uma tecnologia emergente e considerada como um processo alternativo às técnicas convencionais, apresentando inúmeras vantagens que resultam na otimização do processo extrativo de moléculas como proteínas, carotenóides e fenólicos (TIWARI, 2015). Assim como a ELP, a extração assistida por ultrassom é reconhecida como uma técnica verde por reduzir do consumo de energia e tempo, diminuir do número de processos unitários, aumentar o rendimento e não provocar a degradação da molécula-alvo (MANE et al., 2015). O processo de extração por ultrassom também emprega baixas temperaturas e somado com as características anteriores, resulta em menos custos do processo e uma automação de alto nível (TIWARI, 2015).

Existem dois tipos principais de sistemas ultrassônicos usados em escala laboratorial: ultrassom de banho e ultrassom de sonda (Figura 7). Para volumes menores, o sistema de sondagem é considerado mais poderoso. O banho ultrassônico é usado para volumes maiores e tem baixo custo de operação. Ambos sistemas podem ser utilizados em escala industrial

Figura 7 - Ultrassom de banho (A) e ultrassom de sonda (B).



Fonte: Unique (2019).

O principal parâmetro físico do ultrassom é a aplicação de frequências acima do limiar de audição humana (entre 18 kHz e 100 MHz). Uma faixa estendida de ultrassom de 20 kHz a 2 MHz pode resultar na melhoria das reações químicas por efeitos físicos e químicos no sistema por cavitação, causando ruptura de partículas (SANG et al., 2017). A aplicação do ultrassom gera cavitação acústica que é visualizada pela geração de bolhas. A cavitação é a força motriz para os efeitos de extração por ultrassom. Quando as ondas ultrassônicas se propagam no sistema, ocorre a indução de uma série de compressões e rarefações nas moléculas do meio. As mudanças de pressão alternadas causam a formação e o colapso de bolhas no meio líquido. A cavitação acentua a transferência de massa, devido a maior penetração do solvente nos tecidos vegetais e assim resulta em um maior rendimento das sustâncias bioativas.

Visto as inúmeras vantagens apresentadas, algumas pesquisas têm utilizado o ultrassom como a principal técnica para a extração de antocianinas e outros fenólicos. Exemplos são as extrações reportadas para: antocianinas do repolho roxo (DEMIRDÖVEN; ÖZDOĞAN; ERDOĞAN-TOKATLI, 2015); batata doce roxa (FAN et al., 2008); alho (TOMŠIK et al., 2016); amora (YANG et al., 2017); berinjela (DRANCA; OROIAN, 2016); e mirtilo (HE et al., 2016).

3.3.4.3 Otimização da extração de antocianinas

Como reportado anteriormente a eficiência de um processo extrativo é inicialmente mensurada em termos de rendimento da molécula-alvo. No entanto, a viabilidade econômica desses processos também considera fatores inerentes ao processo como o consumo de tempo, solvente e energia. Neste contexto os processos de obtenção de biomoléculas requerem uma otimização de suas variáveis. O termo otimização se refere a melhorar o desempenho de um sistema, processo, ou um produto, para que se possa obter o máximo benefício dele (BEZERRA et al., 2008). O termo otimização é normalmente aplicado nas pesquisas de extração de biocompostos como meio de descobrir as condições nas quais a aplicação de um procedimento possa produzir a melhor resposta possível (ARAUJO; BRERETON, 1996).

Sabe-se que em um sistema/processo as variáveis utilizadas podem influenciar na resposta global. Um exemplo seria os efeitos da concentração do solvente, da temperatura e do tempo de extração num processo descrito para extração de antocianinas. Anteriormente, a otimização dos processos extrativos era realizada através do monitoramento da influência de um fator de cada vez em uma resposta experimental, método conhecido como uma-variável-por-vez. Este método, no entanto, apresenta a grande desvantagem de não incluir efeitos interativos entre as variáveis estudadas, não descrevendo, portanto, os efeitos completos dos parâmetros na resposta (TORBJORN et al., 1998).

Como alternativa a análise univariada, as técnicas multivariadas se tornaram mais utilizadas para a otimização de experimentos e uma das mais relevantes entre essas técnicas é a metodologia de superfície de resposta (MSR). Segundo Bezerra et al. (2008) a metodologia de superfície de resposta corresponde a uma coleção matemática e técnicas estatísticas baseadas no ajuste de uma equação polinomial de dados experimentais, que descrevem o comportamento de um conjunto de dados com o objetivo de fazer previsões estatísticas. Essa técnica, portanto, é aplicada quando a reposta de uma variável depende de um conjunto de outras variáveis. O objetivo é simultaneamente otimizar os níveis dessas variáveis para obter o melhor sistema desempenho (BEZERRA et al., 2008; HE et al., 2016).

Para a aplicação da MSR inicialmente é necessário se definir o modelo experimental mais situável para tratar o conjunto de dados. Neste caso, a literatura dispõe de diferentes matrizes experimentais, como por exemplo os modelos de primeira-ordem ou os quadráticos tipo Box-Behnken, delineamento composto central (DCC) e Doehlert, que são perfeitamente aplicáveis para descrever o comportamento de um conjunto de dados descritos por funções lineares ou quadráticas (HANRAHAN; LU, 2006).

Independente da matriz vegetal e da metodologia utilizada para extração, a MSR tem sido extensivamente empregada no estudo de antocianinas. Chen, Zhao e Yu (2015) avaliaram a influência da concentração de ácido e de etanol e o efeito da temperatura e do tempo na recuperação de compostos fenólicos e antocianinas extraídas da beterraba utilizando ultrassom como técnica. Ao contrário do que se espera, os resultados apontaram que a menor concentração de etanol favoreceu a extração dos compostos. Demirdöven, Ozdoğan e Erdoğan-tokatli (2015) avaliaram o efeito do tempo, concentração de etanol e temperatura na extração de antocianinas do repolho roxo utilizando a extração convencional (líquido-sólido) e ultrassom. A otimização da extração de flavonóides do maracujá foi avaliada por Gomes et al. (2017), utilizando MSR gerada para extração por liquido pressurizado. A otimização da extração de fenólicos de diferentes especiarias foi avaliada por Hossain et al. (2011).

3.3.5 Aplicações de extratos ricos em antocianinas

As antocianinas podem ser perfeitamente utilizadas com diferentes propósitos, baseado em suas propriedades físicas e biológicas. De um modo geral a maior parte das pesquisas relacionados a essas substâncias prospectam sua aplicação no setor alimentício e farmacêutico, onde são fortes candidatos a atuar como corantes naturais, suplementos dietéticos e princípio ativo.

Uma grande preocupação atual é a alimentação adequada e, nesse contexto, percebe-se que o consumidor está cada vez mais consciente quanto à importância de se agregar à dieta produtos naturais e diminuir o consumo de alimentos processados, ricos em aditivos químicos. A cor dos alimentos é um dos atributos sensoriais mais apreciados pelo consumidor podendo ser condicionada pelos pigmentos naturais do alimento ou devido a adição de corantes sintéticos. O único objetivo dos corantes artificiais é conferir cor aos alimentos, uma vez que não apresentam valor nutritivo. Por outro lado, a ingestão de corantes artificiais está associada a casos de alergias e a bioacumulação, o que estaria relacionado ao desenvolvimento de diferentes tipos de câncer. Assim, a tendência é que os consumidores e os produtores de alimentos reduzam o consumo de corantes sintéticos e se voltem ao uso de corantes de origem natural (HAMERSKI; REZENDE; SILVA, 2013).

As antocianinas podem ser perfeitamente utilizadas como corantes naturais devido a sua não-toxicidade e elevada solubilidade, fato que, aliado as reconhecidas propriedades biológicas, tornaria esses pigmentos poderosos ingredientes. Não existem muitas referências a respeito da aplicação de antocianinas na indústria. No entanto, o aditivo alimentar E163 que é

um concentrado antociânico proveniente das cascas de uva, é regularmente usado para colorir bebidas e doces e já é comercializado em alguns países (KHOO et al., 2017).

Já foi mostrado na seção 3.1.3, que as antocianinas apresentam diferentes propriedades biológicas se destacando a ação antioxidante. Extratos antociânicos de várias plantas são largamente utilizados na medicina popular em diferentes países. Como exemplo, cita-se o extrato fenólico obtido dos frutos do jambolão (*Syzygium cumini* L.),matéria-prima avaliada nesta pesquisa, que é tradicionalmente utilizado em países da Ásia no tratamento de diabetes *mellitus* e como auxiliar digestivo (AYYANAR; SUBASH-BABU, 2012). Também são atribuídas às antocianinas desse fruto potente ação no combate a disenteria, obesidade e inflamações na boca (CHAUDHARY; MUKHOPADHYAY, 2012; KUMAR et al., 2008).

A instabilidade das antocianinas é um fator que limita a extensão do seu uso em escalas industriais. Mesmo que esses pigmentos sejam reconhecidos como poderosos antioxidantes não se tem dados ou estudos suficientes que possam atestar sua eficiência como princípio ativo em medicamentos. Atualmente a associação de extratos antociânicos com polímeros, tais como os carboidratos e as proteínas, tem mostrado eficiência na estabilização destes pigmentos. Os diferentes polímeros podem atuar como excipientes no transporte desses pigmentos, protegendo-os dos fatores que resultam em sua degradação, prolongando assim as suas propriedades biológicas, viabilizando então a possível aplicação industrial.

3.3.5.1 Interação de antocianinas com carboidratos

O estudo da interação pigmento-carboidrato foi inicialmente proposta com único interesse de preservar a cor dos pigmentos frente uma possível variação pH do meio, luz e oxigênio em sistemas alimentícios (LEWIS; WALKER; LANCASTER, 1995). Com o passar do tempo, devido a intensificação dos trabalhos que atestavam a bioatividade dessas moléculas, a estabilização passou a ser requerida para manutenção de aspectos funcionais, prezando então pelas propriedades biológicas destas substâncias.

A complexação de antocianinas com carboidratos passou a ser intensivamente estudada. Pectinas, maltodextrina, ciclodextrina, goma arábica (SOUZA et al., 2017), gelatina (AKHAVAN MAHDAVI et al., 2016); quitosana (KAZAN et al., 2017); e amido (WANG et al., 2013) são alguns exemplos de polímeros associados a diferentes extratos antociânicos e que conferiram estabilidade das propriedades físicas e biológica destas substâncias. A encapsulação é uma das técnicas mais utilizadas para associar antocianinas a diferentes polímeros. A encapsulação é definida como uma técnica de captura de substâncias ativas

(centro) dentro de outras substâncias (materiais de parede) para produzir partículas com diâmetros de alguns nanômetros a alguns micrômetros (TAO et al., 2017). A encapsulação além de conferir maior estabilidade também exerce efeitos na solubilidade das substâncias, permitindo a solubilização de componentes hidrofóbicos em matrizes hidrofílicas e vice-versa (JAFARI et al., 2008, LOBATO et al., 2013).

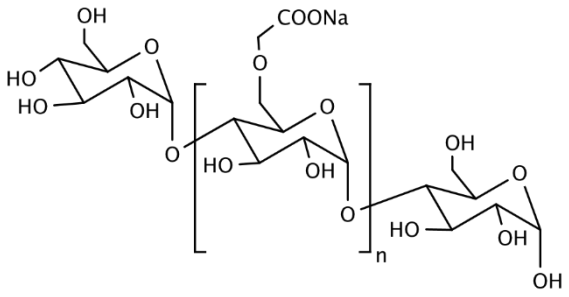
A utilização de antocianinas encapsuladas ou complexadas na forma de géis por exemplo, pode modular a biodisponibilidade destas substâncias, sendo uma alternativa para aumentar a concentração destas substâncias no trato gastrointestinal, o que impulsionaria os efeitos benéficos dessas substâncias (FERNANDES et al., 2018).

3.3.5.1.1 Carboxilmetilamido (CMA)

O amido é um dos carboidratos mais versáteis, renováveis e disponíveis na natureza e é composto por dois polissacarídeos (amilose e amilopectina). Mesmo sendo amplamente utilizado na indústria alimentícia, este polissacarídeo tem seu uso limitado no setor farmacêutico, por exemplo, devido à baixa processabilidade, falta de estabilidade dimensional e propriedades mecânicas irregulares (BARAN; MANO; REIS, 2004). A modificação da estrutura do amido tem sido avaliada como uma alternativa para modular este polissacarídeo de acordo com a intenção da sua aplicação (ASSAAD; MATEESCU, 2010).

O carboximetilamido (CMA) é um amido aniônico modificado através do tratamento com ácido monocloroacético, a fim de introduzir grupos carboxílicos por esterificação (Figura 8). O CMA apresenta a capacidade de formar gel em meio ácido devido a protonação dos grupos carboxílicos presentes em sua superfície. Em contrapartida, esse polímero apresenta elevada hidratação em meios neutros, como no intestino, o que resulta no inchaço de sua matriz e a consecutiva liberação de um agente bioativo que possa estar associado (CALINESCU et al., 2005). Por isso, CMA já foi proposto como um excipiente para liberação controlada de pequenas moléculas e agentes bioativos em formas farmacêuticas sólidas orais (MULHBACHER; ISPAS-SZABO; MATEESCU, 2004). Nesta pesquisa CMA foi avaliado como excipiente para o transporte e liberação das antocianinas extraídas do jambolão. Por apresentarem cargas opostas, antocianinas e CMA formam um complexo, cuja a interação eletrostática pode ser modulada em função do pH do meio, o que pode resultar na liberação controlada desses pigmentos e, consequentemente, na extensão da sua bioviabilidade (WANG et al., 2013).

Figura 8 – Estrutura do carboximetilamido (CMA).



Fonte: Calinescu et al.(2005)

3.3.6 Fontes de antocianinas

As antocianinas podem ser encontradas em diferentes vegetais estando presentes em um número variado de espécies, pertencentes, por exemplo, às famílias *Compositae, Liliaceae, Rhamnaceae*, *Nymphaeaceae, Lobeliaceae, Commelinaceae, Orchidaceae, Leguminosae, Ranunculaceae, Gentianaceae*, entre outras (HAMERSKI et al., 2013). A Tabela 4 apresenta algumas fontes de antocianinas e as respectivas espécies majoritárias.

Tabela 4 - Identificação das antocianinas extraídas de diferentes vegetais.

Fonte	**Antocianina**	**Referência**
Açaí	3-*O*-rutinosil-cianidina	Rosso et al. (2008)
Acerola	3-*O*-ramnosil-cianidina	Rosso et al. (2008)
Aspargo roxo	3-*O*-rutinosil-peonidina	Sakaguchi et al. (2008)
Batata roxa	3-*O*-(cafeoil)-rutinosil-5-*O*-glicosidil-petunidina	Ieri et al. (2011)
Berinjela	3-*O*-glicosil-delfinidina	Azuma et al. (2008)
Cenoura	3-*O*-(xilosil)-glicosil-galactosil-cianidina	Li et al. (2012)
Cereja	3-*O*-rutinosil-cianidina	Grigoras et al. (2012)
Morango	3-*O*-glicosil-pelargonidina	Da Silva et al. (2007)
Repolho roxo	3-*O*-(xilosil)-glicosil-galactosil-cianidina	Assous et al. (2014)

3.3.6.1 O jambolão (Syzygium cumini L.)

O jambolão (*Syzygium cumini* L.) foi o substrato vegetal utilizado nesta pesquisa para a extração e estudo da aplicação das antocianinas. Um capítulo destinado a caracterização deste fruto foi publicado em 2018 no livro "*Exotic Fruits Reference Guide* (ISBN: 978-0-12-803138-4) e é apresentado a seguir.

JAMBOLAN – *Syzygium cumini* L.

Luiz Bruno de Sousa Sabino; Ivanildo José da Silva Júnior; Edy Sousa de Brito

Publicado em: *Exotic Fruits Reference Guide*. 1ª ed. Cambridge: Academic Press, 2018, p. 251-256.

ISBN: 978-0-12-803138-4

3.1.6.1.1 Cultivar origin and botanical aspects

Syzygium cumini (L.) Skeels is a tree belonging to the Myrtaceae Family, originally from India and widely distributed in Asian countries such as Malaysia, Thailand, and the Philippines. The plant was introduced in many countries in the African continent and in Latin America where it easily adapted to the tropical and subtropical climate (GROVER; VATS; RATHI, 2000; MAHMOUD, 2001). Due to its geographic dispersion different synonyms and popular nominations can be used to designate the *S. cumini* species. The main names given to *S. cumini* species are listed in Table 5.

Table 5 - Scientific and popular names used to designate *Syzygium cumini* species.

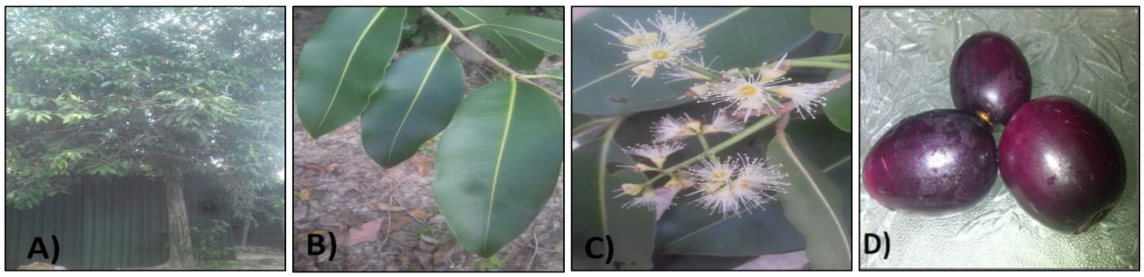
Language[a]	**Names**
Scientific names	*Eugenia jambolana* Lam., *Myrtus cumini* Linn., *Syzygium jambolana* DC., *Syzygium jambolanum* (Lam.) DC., *Eugenia djouant* Perr., *Calyptranthes jambolana* Willd., *Eugenia cumini* (Linn.) Druce., e *Eugenia caryophyllifolia* Lam
Popular names[b]	jambolão, jamun, jamblon, jambolana, jamoon, black plum, blackberry, jamelão, jalão, azeitona-roxa,murta, jambuí, oliva, oliveira, java plum, portuguese plum, malabar plum, purple plum, damson plum, jaman, jambu, jambool, jambhool, jamelong, jamblang, *jiwat*, salam, jambeiro

[b]Popular names including: Indian, English and Portuguese language. Source: [a]Sharma et al. (2006), Grover; Vats e Rathi ,(2000), Timbola et al. (2002), Garcia, Polo e Iha, (2003), Veigas et al. (2007), Ayyanar e Subash-babu (2012);

The *S. cumini* tree (Figure 9A) is broad and densely foliated, reaching a height of 15 m and a crown diameter of 4 m. The immature bark is light brown, while the mature is dark brown and scaly. The leaves (Figure 9B) are 6-12 cm long, leathery, dark green, smooth, shiny and elongate or oblong shape. The flowers (Figure 9C) are small (7-12 mm), numerous, fragrant, white, creamy or greenish. It does not present stems and born in fascicles in the branches of the tree (WARRIER; NAMBIAR; RAMANKUTTY, 1996).

The jambolan fruits (Figure 9D) are berries, fleshy, elliptically shaped and composed of a single dark brown central seed. Its length varies from 1.5 to 3.5 cm and are 2 cm in diameter. The fruits can be found in groups of 4-20 in different parts of the tree canopy (AYYANAR; SUBASH-BABU, 2012; BALIGA et al., 2011).

Figure 9 - **(A)** tree, **(B)** leaves, **(C)** flowers, and **(D)** fruit of *Syzygium cumini* (L.) Skeels.



Source: Elaborated by the author.

3.1.6.1.2 Harvest season

The jambolan harvest begins after 9 - 10 years of planting. The fruits show maturity after flowering, period of 3 - 5 months, depending on the climate of the region and the cultivar (AGRIFARM, 2014; FRUITIPEDIA, 2013). In northern Asia, e.g., flowering begins in March and continues in April and full fruit ripening occurs between June and July, while in Brazil the flowering occurs from September to November and the ripe fruit from December to February (ALBERTON et al., 2001; DANADIO et al., 1998; ROSS, 1999). The jambolan is a non-climacteric fruit and should be harvested at the appropriate stage of maturity, indicated mainly by the complete development of the epicarp coloration. Due to nonuniform maturation time, more than one harvest is necessary (FRUITIPEDIA, 2013). If not harvested, the fruits fall or, due to the attractive colour and aroma, are eaten by bats, squirrels, and monkeys (WARRIER; NAMBIAR; RAMANKUTTY, 1996).

3.1.6.1.3 Fruit physiology and biochemistry

Fruit development takes about 2 months, and it is a process marked by intense changes in its physiological characteristics resulting from the synthesis of new compounds. The remarkable physical change associated with the chemical and biochemical processes that occur in the fruit is the evolution of the colour of its pericarp due to the anthocyanin synthesis.

When immature, the fruit exhibits white pericarp and it turns dark purple or black with maturation. In the physiological maturity, the mesocarp becomes purple, fleshy, juicy and with an exotic flavor resulting from the combination of sweetness, acidity and light astringency. The astringency occurs due tannins and other phenolics present (ALBERTON et al., 2001; BENHERLAL; ARUMUGHAN, 2007; JADHAV; KAMBLE; KADAM, 2009).

3.1.6.1.4 Chemical composition and nutritional value

Jambolan has a complex chemical composition rich in different chemical compounds with nutritional and biological value. In the fruit are found carbohydrates such as sucrose, mannose, glucose, fructose, galactose, and maltose; water soluble vitamins such as thiamine and niacin; the free amino acids: alanine, asparagine, tyrosine, glutamine and cysteine; minerals: sodium, potassium, calcium, phosphorus, iron, and zinc; oxalic and malic acid. The concentration of the compounds is variable depending, e.g., on soil nutrition, climatic conditions and harvesting techniques employed in the cultivation (AYYANAR; SUBASH-BABU, 2012; BALIGA et al., 2011; JADHAV; KAMBLE; KADAM, 2009). The approximate chemical composition of jambolan is present in Table 6.

Different phenolic compounds are present as constituents of the jambolan and these substances are responsible for the colour and flavor characteristic of the fruit. Phenolic acids, flavones, and flavonoids such as gallic acid, myricetin, and anthocyanins, respectively, are examples of phenolic classes present in the fruit composition (FARIA; MARQUES; MERCADANTE, 2011). Anthocyanins are an important subclass of flavonoids responsible for the colouring and different biological properties of jambolan (BALIGA et al., 2011) which makes these substances subject to different studies (ALGARRA et al., 2014; PALLAVI et al., 2012; TSUDA, 2012). In addition to the previously reported components, the fruits may have essential oils such as α-pinene, camphene, β-pinene, myrcene, and cis-ocimene (AYYANAR; SUBASH-BABU, 2012).

Table 6 - Chemical composition of jambolan fruit.

Composition	Content[a]
Moisture	77.2 g/100g
Protein	1.4 g/100g
Fat	0.6 g/100g
Fiber	0.6 g/100g
Carbohydrates	16.6 g/100g
Sucrose	95.5 mg/g
Maltose	210 mg/g
Fructose	57.5 mg/g
Galactose	52.5 mg/g
Vitamins	
β-Carotene	50 mg/100g
Thiamine	0.12 mg/100g
Riboflavin	0.06 mg/100g
Ascorbic acid	30 mg/100g
Minerals	
Na	3.5 mg/100g
K	130 mg/100g
P	18.5 mg/100g
Ca	21.5 mg/100g
Fe	0.15 mg/100g
Mg	49.8 mg/100g
Zn	0.28 mg/100g
Cu	0.07 mg/100g

Source: [a]Noomrio e Dahot (1996).

3.1.6.1.5 Anthocyanins

The profile of anthocyanins that compose the jambolan is marked by the presence of different aglycones linked to a common 3,5-diglycoside. Five of the six aglycones commonly found in foods can be found in this fruit, corresponding to anthocyanins derived from cyanidin, delphinidin, malvidin, peonidin, and petunidin. In general, these anthocyanins have different amounts in plants from different cultivars (AYYANAR; SUBASH-BABU, 2012; FARIA; MARQUES; MERCADANTE, 2011; SARI et al., 2012).

According to Kong et al. (2003) 50% of the anthocyanins present in fruits and vegetables correspond to cyanidin- 3,5-diglycoside, and it is the major anthocyanin in fruits such as strawberry, jabuticaba, blackberry, and jambolan (MAZZA; MINIATI, 1993). Delphinidin, occurring in 12% of the plants and its 3,5-diglycoside form, has already been pointed out as predominant in the jambolan harvested in Brazil, while malvidin glycosides were

reported as predominant in the fruits harvested in India (BRITO et al., 2007; FARIA; MARQUES; MERCADANTE, 2011; KONG et al., 2003; VEIGAS et al., 2007).

Studies have pointed out that the content of anthocyanins found in jambolan was higher than that of vegetables such as grapes and red cabbage, known as sources of these substances (GIUSTI; WROLSTAD, 2003; SARI et al., 2012). In this context, it can be stated that jambolan is a natural source of anthocyanins, which may be an important attribute due to the benefits related to the ingestion of these compounds.

3.1.6.1.6 Biological properties of jambolan

Different studies have indicated that the ingestion of the jambolan presents health beneficial effects due to the pronounced pharmacological effects of extracts obtained from the fruit. Antiviral activities (BHANUPRAKASH et al., 2008), antioxidants (VEIGAS et al., 2007), gastroprotective (CHATURVEDI et al., 2007), hepatoprotective (VEIGAS; SHRIVASTHAVA; NEELWARNE, 2008) and anticancer (BARH, 2008) effects are reported in the literature on the different extracts obtained from jambolan fruit, however, an emphasis has been given to the antidiabetic action of this fruit, which leads to the development of different studies (ARAYNE et al., 2007; GROVER; VATS; RATHI, 2000). These and other biological properties reported to jambolan are related to the various phytochemicals present in its constitution such as anthocyanins and other polyphenols (BALIGA et al., 2011).

3.1.6.1.7 Sensory characteristics

Jambolan is an exotic fruit with a remarkable sensory characteristic. The synthesis of compounds that occur during maturation has a direct impact on the taste, colour, and aroma of the fruit. The flavor is the result of the association of sugars, organic acids, and phenolics, while the aroma is related to the last two compounds. The jambolan does not present a high sweetness and this fact is due to the high concentration of maltose in its composition in contrast to the other sugars. The acidity of the fruits is given by the malic, citric, and mostly by gallic acid. The hydrolysis of these acids, together with phenolics and carotenoids, generate volatile compounds responsible for the aroma (AYYANAR; SUBASH-BABU, 2012). The tannins confer astringency to the pulp of the fruit. The intensity of the astringency can contribute to the development of an unpleasant taste, however in the mature fruit, the association of tannins with sugars and acids reduces the intensity of the astringency and gives to the fruit the exotic flavor.

As previously reported (see “Fruit Physiology and Biochemistry” and “Chemical Composition and Nutritional Value” sections) the typical jambolan colour is conferred by the different anthocyanins present. Cultivation issues will influence the quality of the aglycone and consequently the different shades presented by the fruits. Besides, the colouring anthocyanins will contribute to the development of the flavor due to its catabolism in the respiratory processes of the fruit.

3.1.6.1.8 Harvest and postharvest conservation

Jambolan is considered a perishable fruit especially for the fragility of its pulp and epicarp that offers little protection against physical damages or infectious agents. However, special care in harvesting and post harvesting is essential to extend the shelf-life of the fruit.

Under environmental conditions the shelf-life of jambolan is 2 days. Refrigerated storage and moisture control are postharvest practices that are commonly applied to vegetables and may be allied to reduce perishability due to the reduction of catabolic reactions resulting from respiration. Temperatures of 8 -10 °C and an atmosphere with relative humidity of 85% - 95% showed positive effects extending the shelf-life of jambolan to three weeks. The use of modified atmospheres reduces gas exchanges and slows down degradation processes. Silva et al. 2017) indicated that polyvinyl chloride films associated with storage at 5 °C kept jambolan quality for 10 days, preventing the incidence of rot in this period. In general, a high waste percentage is still seen in the harvest of the jambolan mainly due to the lack of investments and appreciation (LAGO; GOMES; SILVA, 2006).

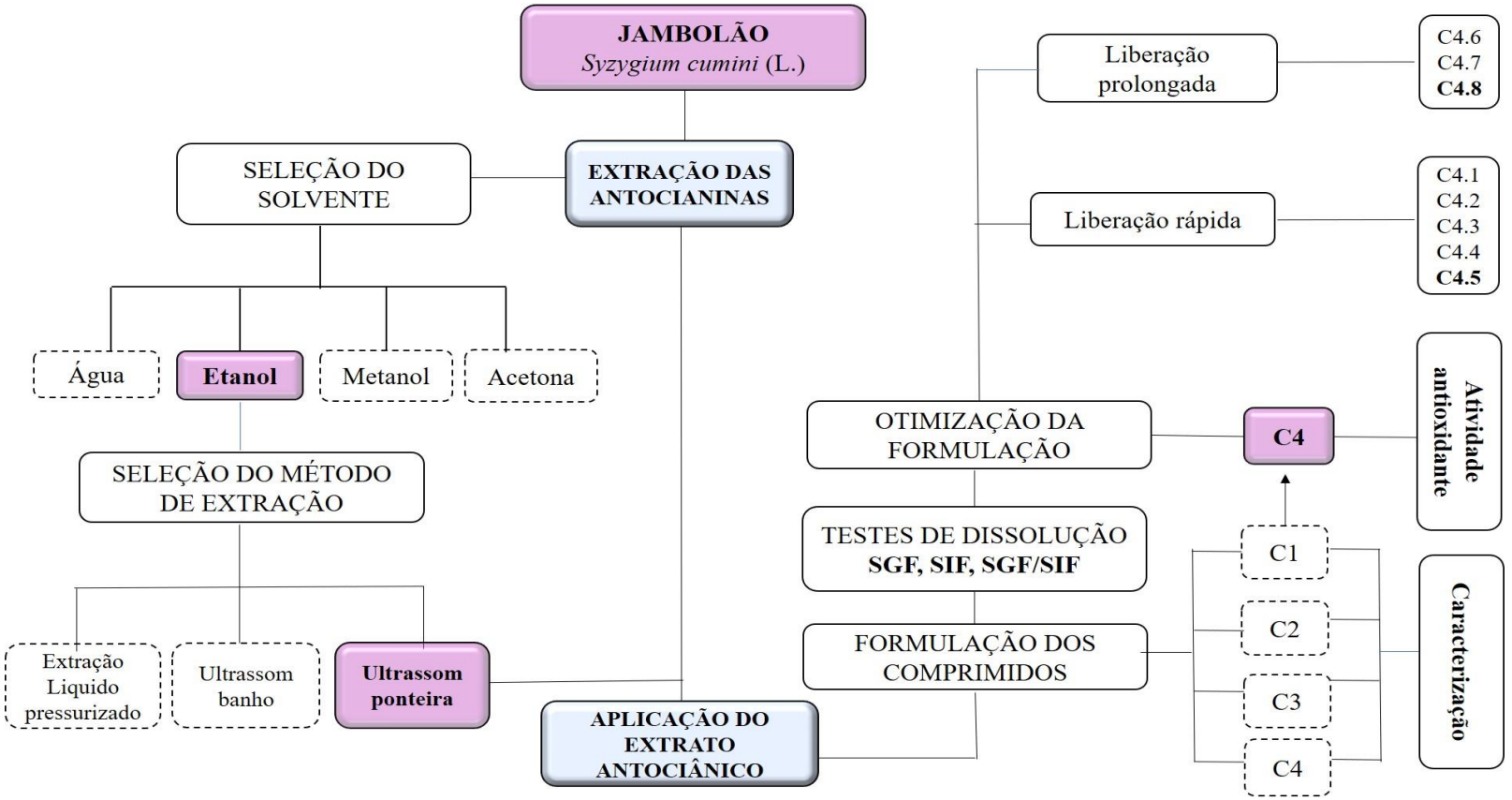
3.1.6.1.9 Industrial application and potential industrial application

Both the rind and the flesh of jambolan is a consumed, resulting in approximately 80% of the total of the fruit being edible (SHAJIB et al., 2013). In general, the final destination is different in each location. In Brazil, e.g., the tree has a domestic origin and the fruit is consumed almost *in natura* and there is no evidence as to the use of the fruit as an industrial raw material. In Asia the fruit is commonly processed and found in the form of juices, pulps, and jellies. In parts of India, jambolan pulp is used to make wine and the immature fruit serves as a base for the manufacture of vinegar, which according to local culture is a potent antidiuretic (BANERJEE; DASGUPTA; DE, 2005). Dried fruits are considered delicacies, finding a market in the United States and in several European countries (VEIGAS et al., 2007).

Considering the nutritional and biological aspects previously reported, there is a great potential for the industrialization of jambolan both in the food and pharmaceutical sectors. However, what is observed is that, independently of the producing region, the lack of investments in technologies for the postharvest handling of the jambolan results in high rates of waste, not stimulating the cultivation and commercialization of the jambolan.

4 ORGANIZAÇÃO DO PROCESSO EXPERIMENTAL

Figura 10 – Organização experimental relativo ao desenvolvimento da Tese.



***SGF:** Fluido gástrico simulado; **SIF:** Fluido intestinal simulado; **SGF/SIF**: Trânsito gastrointestinal simulado.
Fonte: Elaborado pelo o autor

5 ETAPAS

5.1 Otimização da extração líquida pressurizada e métodos de ultrassom para recuperação de antocianinas presentes nos frutos do jambolão (*Syzygium cumini* L.)

OPTIMIZATION OF PRESSURIZED LIQUID EXTRACTION AND ULTRASOUND METHODS FOR RECOVERY OF ANTHOCYANINS PRESENT IN JAMBOLAN FRUIT (*Syzygium cumini* L.)

Luiz Bruno de Sousa Sabino; Elenilson Godoy Alves Filho; Fabiano André Narciso Fernandes; Edy Sousa de Brito, Ivanildo José da Silva Júnior

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ABSTRACT

Response surface methodology was employed to optimize the experimental conditions for pressurized liquid extraction (PLE), bath ultrasound extraction (BUE) and probe ultrasound extraction (PUE) of anthocyanins extracted from jambolan fruits (*Syzygium cumini* L.). A face-centered central composite designed was used for the optimization of extraction parameters in terms of anthocyanin concentration. Among the evaluated methods the highest anthocyanin recovery was obtained from the experiment carried out using PUE. Results pointed that the optimal conditions obtained by RSM for PUE anthocyanin extraction from jambolan were as follows: ultrasound power density 5000 W/L, time extraction 7.5 min and 79.6% of ethanol. In the optimal conditions the concentration of anthocyanins was 60.5 $mg.g^{-1}$, which was well matched with the predicted value. The anthocyanins delphinidin-3,5-diglucoside, petunidin-3,5-diglucoside, malvidin-3,5-diglucoside were identified by UPLC-ESI-QTOF-MS in all extracts, which demonstrate that even under different conditions the methods did not promote qualitative changes in the anthocyanins profile from jambolan fruits.

Keywords: *Syzygium cumini*. Extraction. Response surface. P|ressurized liquid extraction. Ultrasound extraction.

1 INTRODUCTION

Jambolan is the fruit of the *Syzygium cumini* L. (Syn. *Eugenia jambolana* Lam.; *Eugenia cumini* Druce) trees, widely distributed in Asia and Latina America (BALIGA et al., 2011). The fruit is recognized by its medicinal properties (e.g. diuretic, antidiabetic, antioxidant and antiproliferative actions), which is related to phenolic composition, including phenolic acids, flavones, flavonoids, and anthocyanins (AQIL et al., 2012; FARIA; MARQUES; MERCADANTE, 2011).

Anthocyanins play an important role in physical and sensorial properties of jambolan. They are responsible for its intense purple color and remarkable exotic flavor (AYYANAR; SUBASH-BABU, 2012). Chemically, anthocyanins are glycosylated polyhydroxy and polymethoxyderivatives of 2-phenylbenzopyrylium (flavylium) salts. In nature, anthocyanins are responsible for the red, orange, blue or purple colors in several fruits, vegetables and flowers (WU et al., 2006). Previous reports showed that the anthocyanin profile in jambolan is marked by the presence of different diglucosides aglycones deriving from cyanidin-3,5-diglucosides, delphinidin-3,5-diglucosides, malvidin-3,5-diglucosides, peonidine-3,5-diglucosides and petunidine-3,5-diglucosides (AYYANAR; SUBASH-BABU, 2012; FARIA; MARQUES; MERCADANTE, 2011; SARI et al., 2012).

Previous studies reported that the anthocyanins content in jambolan is higher than in fruits recognized as sources of this substances, such as acerola, camu-camu, jaboticaba and red cabbage (JAMPANI; NAIK; RAGHAVARAO, 2014; WALLACE; GIUSTI, 2015). The regular consumption of jambolan was associated to health benefits assured by its high anthocyanins content, which includes cardioprotective, vision improvement, antidiabetic, antineurodegenerative (WICZKOWSKI; SZAWARA-NOWAK; TOPOLSKA, 2013), antioxidant, gastroprotective, hepatoprotective (VEIGAS et al., 2007), induction of insulin production in isolated pancreatic cells (JAYAPRAKASAM et al., 2005) and reduction in inflammatory responses (TALL et al., 2004).

Anthocyanins are water-soluble and non-toxic pigments, becoming promising candidates for industrial application as food colorants or in the pharmaceutical industry for tablet/capsule coatings, syrups, health concentrates and as supplements (RIGO et al., 2002). However, to anthocyanin exploration, at first, is important to determinate the most suitable method for its obtainment considering all the factors that can affect its yield, composition and quality. In this regarding, several extraction methods and combinations of different parameters

(e.g. solvent, temperature and pressure) were regularly tested to reach the maximum anthocyanin recovery under feasible conditions (CASTAÑEDA-OVANDO et al., 2009).

The conventional extraction (CE), employed to different plant matrices, is based on solid/liquid contact, generally performed by agitation or centrifugation, which occurs under atmospheric pressures to obtain the stable flavylium cation. However, it exhibits limitations and disadvantages such as a high solvent consumption and time-consuming making necessary the use of alternatives to reduce the extraction drawbacks (MANE et al., 2015). Compared to CE, ultrasound-assisted extraction (UAE) is a technique with advantages including reduced processing time and solvent volume, increased yields and production of non-denatured products (CHEMAT et al., 2017). The UAE has an effective mass transfer and solvent penetration through the disruption of plant cell walls via acoustical cavitation. Pressurized liquid extraction (PLE) is a technique currently used in the extraction of bioactive compounds being suitable for thermolabile compounds as anthocyanins. In PLE the process occurs in a temperature above the solvent boiling point, promoting a high extraction kinetics and a reduction of solvent consumption (GOMES et al., 2017; SETYANINGSIH et al., 2016).

Therefore, considering the importance to find potential sources of anthocyanins as well as determinate optimal processes to increase the extraction, this study aimed to optimize the extraction of anthocyanins from jambolan by the application of pressurized liquid extraction (PLE) and ultrasound techniques (bath and probe), defining the yield efficiency of the method. To our knowledge, this is the first investigation to evaluate the effect of different extractions methods in recovery and in the quality of anthocyanins from jambolan fruit.

2 MATERIAL AND METHODS

2.1 Chemicals

Ethanol (99%), acetone, methanol, trifluoracetic acid were obtained from Sigma Aldrich (Saint Louis, USA). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Pennsylvania, USA). Water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA). All the other chemicals were analytical grade.

2.2 Plant material

The jambolan fruits were obtained from a domestic organic cultivar in Cascavel city (Ceara state, Brazil). The seeds were manually removed and the edible portion (peel and pulp) were freeze dried in a Labconco Freeze Dry-5 dryer (Labconco, MO) at -50 °C under pressure of 0.6 Pa and vacuum for 48 h. After drying, the samples were milled to obtain a fine powder. Jambolan powder was stored under refrigeration (-5 °C) in polyethylene bowls free from humidity and oxygen until the analysis.

2.3 Anthocyanin extraction

2.3.1 Solvent selection

Aqueous solutions of ethanol, methanol, acetone (80% v/v), and water acidified with 0.1% of trifluoroacetic acid (TFA) were prepared to perform the anthocyanin extraction. An amount of 4 g of the powder was added in a falcon tube and dispersed in 24 mL of the selected solvent. The extraction was performed in a Rotina 380R centrifuge (Hettich, USA) at 2540 *g* /25 °C for 20 min. The procedure was repeated for three times until the residue became colorlessness. The extracts were pulled together and concentrated in a R-215 rotavapor (Bunch, Flawil, SWI) under reduced pressure. The procedure was repeated three times for each solution and the concentrated extract was stored at -5 °C until analysis. The anthocyanins extracts resulted from each solvent were freeze dried and the ratio between the mass obtained after drying and the mass of fruit used at the beginning of extraction was used to calculate the individual yield (%).

2.3.2 Pressurized liquid extraction (PLE)

The pressurized liquid extraction (PLE) was carried out in an ASE 350 system Dionex (Sunnyvale, CA, USA). The samples (4 g) were placed in 100 mL stainless steel cells (Dionex, Sunnyvale, CA, USA) containing a cellulose filter at the bottom to avoid the presence of suspended particles in the collection vial. The automatic process was carried out at a pressure of 11,721 kPa, time rinsing of 5 min, and 10 min of extraction time at each cycle and purge with a flow of nitrogen (200 s). The static extraction was performed under conditions obtained from the experimental design (Table 7). Acidified ethanol solutions (TFA, 0.1% v/v) were used

as solvent, and the temperature and number of static cycles were controlled via equipment panel. After the extraction, the samples were concentrated in a R-215 rotavapor (Bunch, Flawil, SWI) and kept frozen (-5 °C) until the analysis.

Table 7 - Experimental values and coded levels of the independent variables used for the face centred, central composite design (CCD).

Method	**Code units**	**Factor**	**Levels**		
			-1	0	+1
PLE	X_1	Temperature (°C)	80	90	100
	X_2	Ethanol (%)	60	70	80
	X_3	Cycle	1	2	3
BUE	X_1	Temperature (°C)	25	30	40
	X_2	Ethanol (%)	60	70	80
	X_3	Time (min)	20	30	40
PUE	X_1	Ultrasound power density (W/L)	1650	5000	8350
	X_2	Ethanol (%)	60	70	80
	X_3	Time (min)	5	10	15

PLE: Pressurized liquid extraction; BUE: Bath ultrasound extraction; PUE: Probe ultrasound extraction.
Source: Elaborated by the author.

2.3.3 Bath ultrasound extraction (BUE)

An amount of 4 g of jambolan powder and 60 mL of solvent were added to a 250 mL beaker and subjected to acoustic waves using an ultrasonic bath (Unique model USC 1400, Indaiatuba, Brazil) operating at 40 kHz, nominal power of 135 W, filled with 1.2 L of water and effective ultrasonic power density of 112.5 W/L. The effective ultrasound power density was measured using the calorimetric method (LÖNING; HORST; HOFFMANN, 2002)(LÖNING; HORST; HOFFMANN, 2002). Only one beaker was sonicated in each run. The beaker with the sample was placed over the ultrasonic transducer to guarantee the same sonication conditions in each run. The initial temperature of the water and sample in the ultrasonic bath was 30ºC and raised by 2 - 3 º C at the end of each sonication period.

Ethanol solutions were used as extraction solvent, and time and temperature were controlled according to the values presented in Table 7. After extraction, the supernatant was filtered using a cellulose filter and the solvent was eliminated employing the same conditions described in previous section. The extracts were stored in vials at -5 °C until the analysis.

2.3.4 Probe ultrasound extraction (PUE)

A probe ultrasound (Ultronique model QR500, Brazil) operating at 20 kHz, coupled with a 13 mm titanium tip, was used to perform the extraction. Sample (4 g) and solvent (60 mL) were mixed in a 250 mL jacketed beaker and subjected to ultrasound. The effective ultrasound power density applied to the samples was 8300 W/L, which was measured using the calorimetric method (LÖNING; HORST; HOFFMANN, 2002). The temperature was controlled using a thermostatic water bath to produce a continuous flow of cold water through the beaker jacket. The temperature of the bath was set to maintain the temperature of the extraction mixture at 30 ± 1 °C. After extraction, the samples were filtered, and the solvent was removed as previously described. The extracts were stored at -5 °C until analysis. The sample:solvent ratio was maintained constant at 1:15 w/v.

2.3.5 Conventional extraction (CE)

An amount of 4 g of jambolan powder was mixed with 60 mL aqueous ethanol solutions (60, 70 and 80% v/v acidified with TFA acid 0.1% v/v) and anthocyanins were extracted using a Rotina centrifuge (Hettich, USA) with the conditions described in the earlier section (see solvent selection at section *2.3.1*). The extracts obtained were concentrated similarly to described for the previous methods, kept in dark flasks and stored at -5 °C until the analysis.

2.4 Experimental design and optimization

The extraction efficiency is related to the interaction of several parameters and their effects may be either independent or interactive (HE et al., 2016). For this purpose, a face centered, central composite design (CCD) was employed to evaluate the parameters: temperature (°C), extraction cycles, percentage of solvent (%v/v), time (min), and ultrasound power (W). Levels, factors and the coded variables are presented in Table 7. The complete design consisted of 18 experiments including four replicates of the central point (Table 8). The experimental results were fitted with a second order polynomial equation (Equation 1), where Y represent the predicted variable while B_0, *Bi*, *Bii* and *Bij* are the constant, linear coefficient, quadratic, and the cross-product coefficient of the model, respectively. *Xi* and *Xj* are the independent variables while *k* equals to the number of the tested factors.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j \quad \text{(Equation 1)}$$

The statistical analysis was performed using Software Statistica version 10 (Stat Soft Inc., Oklahoma, USA). The analysis of variance (ANOVA) and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at $p \leqslant 0.05$. Adequacy of the developed models was tested by performing coefficient of determination (R^2), adjusted coefficient of determination (Adj R^2) and lack of fit significance. After the regression coefficients were then used to build the surfaces plots to predict the relationship between the independent variables and response (BEZERRA et al., 2008).

2.5 Total monomeric anthocyanins

The anthocyanin content in jambolan powder was measured using the pH differential method (LEE; DURST; WROLSTAD, 2015). The extracts were solubilized in potassium chloride (KCl) buffer (pH 1.0) and sodium acetate ($NaC_2H_3O_2$) buffer (pH 4.5), and read in an L5S UV-visible spectrophotometer (Shanghai Analytical Instrument, China) using a quartz cuvette (1 mL) at 520 and 700 nm. Deionized water was used as blank. The total anthocyanins was expressed as cyanidin-3-O-glucoside equivalent according to Equation 2, where A: $(A_{520} - A_{700})pH_{1.0} - (A_{520} - A_{700})pH_{4.5}$; Mw: molecular weight of cyanindin-diglucoside (449.2 g/mol); DF = dilution factor; Ma: extinction coefficient (26,900 mol/L.cm); L = path length (1 cm); m = weight of jambolan powder; V= the total volume (mL) of solvent extractor.

$$\text{Anthocyanin concentration } \left(\frac{\text{mg}}{\text{g}}\right) = \frac{\text{A} \times \text{Mw} \times \text{DF} \times \text{V} \times 1000}{Ma \times \text{L} \times \text{m}} \quad \text{(Equation 2)}$$

2.6 Identification and relative contribution of anthocyanin

The identification of the anthocyanins was performed using an UPLC-ESI-QTOF-MS (Waters, USA). A C_{18} column (150 mm × 2.1, 1.7 µm) Waters (USA) was used at a flow rate of 0.4 mL.min^{-1} at 40 °C and injection volume of 5µL. The eluent was the combination of acidified water (0.1% of formic acid) and acidified acetonitrile (0.1% formic acid). The gradient

varied linearly from 5 to 95% acetonitrile (v/v) in 15 min. The mass spectra were acquired using positive electrospray ionization and at 80 V fragmentation tension for a 100–1000 amu mass range. Drying gas pressure of 241 kPa, spray gas pressure of 275 kPa, drying gas temperature of 370 °C, capillary tensions of 3500 V and spray shield tension of 600 V were used. The LC system was coupled to the MS detector with a 50% split. The compounds were tentatively identified based on mass spectrometry data for molecular ions.

The relative contribution of the peak area was calculated based on total ion abundance from the peaks in the samples, since the relative amplitude of the peaks measures provides the relative abundance of the isotopic forms in the chromatograms. Therefore, the normalized mean in the base peak intensity (BPI) at the retention times 2.70, 2.90 and 3.15 min were determined.

2.7 Statistical analysis

All the experiments were performed in Software Statistica version 10. The experiments were carried out in triplicate and the data were described as means ± SD (standard deviation). The results were evaluated using the variance ANOVA single factor analysis with significant level of 0.05. Tukey test was employed to mean comparison.

3 RESULTS AND DISCUSSION

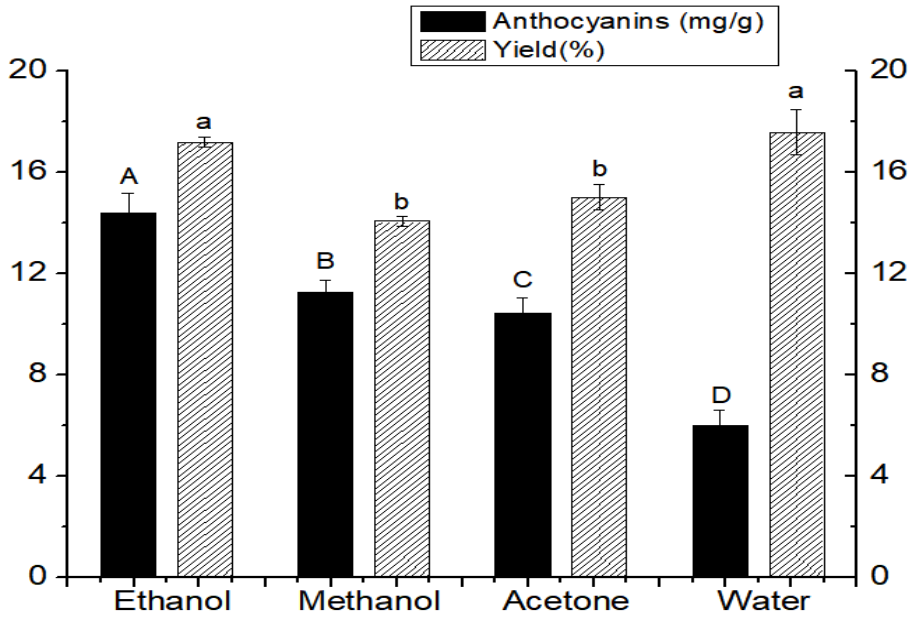
3.1 Solvent selection

The selection of the most suitable solvent was the initial step to study the extraction and characterization of phytochemicals from jambolan, since it determines the amount and type of phenolic compounds extracted (HE et al., 2016; SPIGNO; TRAMELLI; FAVERI, 2007). In this research, the solvents methanol, ethanol, acetone, and water were evaluated for anthocyanin extraction from jambolan fruits, and the results are presented in Figure 11. Total anthocyanin (TA) content ranged from 6.0 to 14.4 $mg.g^{-1}$. The acidified ethanol showed the best extraction capacity ($p \leqslant 0.05$), extracting 58.2% more anthocyanin than the acidified water. The TA extracted employing methanol (11.25 $mg.g^{-1}$) and acetone (10.43 $mg.g^{-1}$) were statistically similar at 95% of confidence level ($p \leqslant 0.05$).

The yield of anthocyanins extraction ranged from 14.1 to 17.6% (Figure 11). The extraction yields using water and ethanol were 17.6% and 17.2% respectively, which were not

statistically different between them ($p \leqslant 0.05$). On the other hand, these yields were higher than the ones obtained using methanol (14.1%) and acetone (15.0%). It is known that different solvents applied to extract anthocyanins are based in polarity, and methanol is widely used by allegation to promote a higher recovery and stabilization of organic compounds (KAPASAKALIDIS; RASTALL; GORDON, 2006). However, according to literature other solvents can result in an anthocyanin extraction similar or higher than methanol as observed in present study (LAPORNIK; PROŠEK; WONDRA, 2005; WANG et al., 2016). A closely observation suggests that the efficiency of an extraction could be related to the plant matrix (species, tissue and physical characteristics, for example), which shows the importance of studies related to most appropriate solvent for extraction (WANG et al., 2016).

Figure 11 - Concentration ($mg.g^{-1}$) and yield (%) of anthocyanin extracted from jambolan fruit using acidified ethanol, methanol, acetone and water as solvents.



Means with the same letter in the same bar are not statistically different by the Tukey test ($p < 0.05$). Means with the same letter in the same bar are not statistically different by the Tukey test ($p < 0.05$).
Source: Elaborated by the author.

The present study defined ethanol as the most efficient and suitable solvent for the extraction of anthocyanins in jambolan, since it presented an extraction capacity 22% higher than methanol. In addition, the use of methanol as solvent is not appropriate to food and pharmaceutics processing due to its toxicity. Furthermore, ethanol is categorized as GRAS (Generally Recognized as Safe) for use in food processing (PILNA et al., 2015).

3.2 Extractions of anthocyanins from jambolan

This study evaluated three extraction techniques to obtain anthocyanins from jambolan using acidified ethanol as solvent: PLE, BUE and PUE. Table 8 describes the results obtained by each method. The content of anthocyanin obtained by the PLE method ranged from 10.0 to 45.0 $mg.g^{-1}$ corresponding to the treatments 6 and 12, respectively. The highest anthocyanin content was obtained at 90 °C and maximal ethanol concentration (80% v/v) after 2 extraction cycles. The lowest anthocyanin content was attained in experiment 6, which was carried out at 100 °C with the lowest amount of ethanol (60% v/v) after 3 extraction cycles.

For the BUE method, the influence of temperature, extraction time, and percentage of ethanol were evaluated. The highest temperature (40 °C) and mean values of time and percentage of solvent (30 min and 70% v/v) correspondent to experiment 10, resulted in the lowest anthocyanin content (16.5 $mg.g^{-1}$), while at the same percentage of solvent, temperature at 30 °C and extraction time of 40 min the highest content of anthocyanin was obtained: 55.0 $mg.g^{-1}$ in experiment 12.

Among the three methods, the highest content of anthocyanin (60.5 $mg.g^{-1}$) was obtained in experiment 14 using the PUE method, which is resultant from the combination of the lowest ultrasound power level (5000 W/L), mid-point level of extraction time of 10 min, and high amount of ethanol (80% v/v). The extracts obtained by conventional extraction (CE) at 60, 70 and 80% (v/v) of solvent presented concentrations equal to 11.9, 16.5 and 19.6 $mg.g^{-1}$, respectively.

The interactions among the independent and dependent variables (X_1, X_2 and X_3) in the models were evaluated by R^2 value, F-value and p-value (Table 9). The coefficient of determination (R^2) for all the extractions were higher than 0.99%, which means that the experimental data was correctly explained (higher that 99%). The adjusted R^2 value indicated that the non-significant terms are not included in the models for anthocyanin extraction employing PLE (Adj R^2 = 0.97), BUE (Adj R^2 = 0.99), and PUE (Adj R^2 = 0.99). The appropriate fit of the models can be checked by the significance and non-significance of p-values and lack of fit. The coefficient of variation (CV) is a dimensionless value that allowed the verification of the degree of dispersion of the data. The CV values for the methods varying from 5.21 to 10.5% indicated the low dispersion of the data that implies in a good reproducibility of the models (SILVA et al., 2011). Therefore, the models showed adequacy to predict the content of anthocyanin, which were fitted following the second-order polynomial

equations (Equations 3 - 5) in order to describe the relationship between the codified extraction factors in each process.

Table 8 - Face centred, central composite design (CCD) experimental design with the independent variables and experimental data for the responses.

Treat.	PLE					BUE					PUE				
	Independent Variables			Dependet Variable		Independent Variables			Dependet Variable		Independent Variables			Dependet Variable	
	T (°C)	EtOH (%)	Cycle	Ant. ($mg.g^{-1}$)		T (°C)	t (min)	EtOH (%)	Ant. ($mg.g^{-1}$)		P (W/L)	t (min)	EtOH (%)	Ant. ($mg.g^{-1}$)	
				Observ.*	Pred.**				Observ.	Pred.				Observ.	Pred.
1	80	60	1	16.5	17.0	25	20	60	35.5	34.3	1650	5	60	20.3	20.7
2	80	60	3	13.6	13.1	25	20	80	32.2	34.8	1650	5	80	55.7	59.8
3	80	80	1	40.9	40.3	25	40	60	33.6	32.3	1650	15	60	15.2	14.8
4	80	80	3	29.4	31.1	25	40	80	35.7	35.9	1650	15	80	35.7	36.5
5	100	60	1	11.4	9.6	40	20	60	30.6	29.1	8350	5	60	26.3	25.7
6	100	60	3	10.0	8.2	40	20	80	28.2	27.8	8350	5	80	49.7	50.3
7	100	80	1	42.2	42.6	40	40	60	29.7	30.7	8350	15	60	37.3	37.7
8	100	80	3	36.4	35.8	40	40	80	33.5	32.4	8350	15	80	45.1	44.9
9	80	70	2	18.8	17.6	25	30	70	22.3	21.8	1650	10	70	23.5	22.8
10	100	70	2	14.6	16.2	40	30	70	16.5	17.4	8350	10	70	29.8	29.5
11	90	60	2	20.8	21.5	30	20	70	52.1	53.5	5000	5	70	38.7	38.5
12	90	80	2	45.0	46.9	30	40	70	55.0	54.2	5000	15	70	33.6	32.8
13	90	70	1	20.3	19.5	30	30	60	40.6	41.8	5000	10	60	38.8	38.8
14	90	70	3	13.0	14.2	30	30	80	42.0	43.3	5000	10	80	60.5	62.0
15	90	70	2	23.1	21.6	30	30	70	42.8	41.9	5000	10	70	37.5	37.9
16	90	70	2	22.0	21.6	30	30	70	41.7	41.9	5000	10	70	36.8	37.9
17	90	70	2	23.6	21.6	30	30	70	41.0	41.9	5000	10	70	37.5	37.9
18	90	70	2	21.4	21.6	30	30	70	41.1	41.9	5000	10	70	38.1	37.9

PLE: Pressurized liquid extraction; BUE: Bath ultrasound extraction; PUE: Probe ultrasound extraction; Ant.: Total anthocyanins ($mg.g^{-1}$) *Observed values for the experiment; **Predicted values obtained for the model; Means with the same letter in the same row are not statistically different by the Tukey test ($p \leqslant 0.05$).

Source: Elaborated by the author.

Table 9 - ANOVA for response surface polynomial model of all independent variables.

Source	PLE					BUE					PUE				
	SS	DF	MS	*F*-VALUE	*p*-VALUE	SS	DF	MS	*F*-VALUE	*p*-VALUE	SS	DF	MS	*F*-VALUE	*p*-VALUE
Model	2394.84	8	299.35	22.33	< 0.0001[a]	1729.65	8	216.2	14.9	0.000242[a]	2759.24	8	111.60	15.52	0.0002[a]
X_1	2,37	1	2.37	3.35	0.1647[ns]	43.6	1	43.6	66.9	0.004[a]	111.60	1	111.60	363.94	0.0003[a]
X_2	1606.85	1	1606.85	2269.73	0.00002[a]	11.77	1	11.77	18.06	0.024[b]	79.65	1	79.65	260.0	0.0005[a]
X_3	83.47	1	83.47	117.93	0.0017[a]	0.294	1	0.294	0.451	0.5500[ns]	1338.80	1	1338.80	4366.5	<0.0001[a]
X_1X_2	37.01	1	37.01	52.28	0.0055[a]	0.455	1	0.455	0.700	0.4650[ns]	159.70	1	159.70	520.42	0.0001[a]
X_1X_3	6.01	1	6.01	8.47	0.0618[ns]	0.631	1	0.631	1.00	0.400[ns]	106.34	1	106.34	346.84	0.0003[a]
X_2X_3	20.91	1	20.91	29.54	0.0122[b]	17.05	1	17.05	26.17	0.014[b]	152.13	1	152.13	496.18	0.0001[a]
X_1^2	71.52	1	71.56	101.02	0.0021[a]	1197.0	1	1197.0	1839.70	0.00003[a]	376.20	1	376.20	1227.0	<0.0001[a]
X_2^2	494.0	1	494.0	697.64	0.0001[a]	456.7	1	456.7	701.07	0.00012[a]	14.34	1	14.34	46.80	0.0064[a]
X_3^3	37.01	1	37.01	102,81	0.002[a]	0.054	1	0.054	0.083	0.800[ns]	420.70	1	420.70	1372.01	<0.0001[a]
Lack of fit	19,64	5	3.93	5.55	0.095[ns]	7.25	5	1.45	2.23	0.2712[ns]	5.163	5	1.033	3.37	0.1732[ns]
Pure error	2,12	3	0.708			1.96	3	0.652			0.920	3	0.307		
Cor. Total	2274,16	17	133.77			1599.0	17				2559.3	17			
R^2	0,9904					0.9942					0.99762				
Adj R^2	0,9797					0.99					0.995				
CV (%)	9,95					10.5					5.21				

SS – sum of squares; DF – degree of freedom; MS – mean squares; CV – coefficient of variation; ADJ R^2: Adjusted R^2.
[a] Means significance ($p < 0.01$); [b] Means significance ($p < 0.05$); [ns] Means not significance ($p > 0.05$).
Source: Elaborated by the author.

3.2.1 Anthocyanin extraction using the PLE method

Table 9 presents all the variables with significant effects on total anthocyanin. Equation 3 was used to build the three-dimensional response surface plots (Figure 12A-C). The Pareto Chart, presented in Figure 13, illustrates the relationship among the PLE operating conditions on extraction of anthocyanins from the jambolan. Ethanol (X_2), extraction cycles (X_3), the cross product between temperature and ethanol (X_1X_2), ethanol and cycles (X_2X_3) and all the quadratics interactions X_{12}, X_{22} and X_{32} were the most significant factors on PLE method for extraction of anthocyanin from jambolan fruit (Figure 13). It was possible to check that the amount of ethanol (X_2) was the most significant variable for anthocyanin extraction with $p < 0.0001$, while its interaction with number of cycles (X_2X_3) presented the lowest influence at $p < 0.05$ ($p = 0.0122$). The quadratic term X_{22} presented highly significance ($p = 0.0001$) followed by X_{12} ($p = 0.0021$) and X_{32} ($p = 0.002$). The linear term of temperature (X_1) and its interaction with the extractions cycles (X_1X_3) were non-significant ($p > 0.05$).

An increase on anthocyanin content was noted when the amount of ethanol increased from 60 to 80% at any temperature (Figure 12A). The high effect of ethanol in the extraction can be attributed to the increased polarity in media, which promotes higher solubilization of the anthocyanins according to the ‘‘like dissolves like” principle (TABARAKI; HEIDARIZADI; BENVIDI, 2012). Temperature is the factor that affects the extraction efficiency of PLE promoting a higher diffusion of the phytochemicals in plant tissue and, consequently, increases their recovery (GALVAN et al., 2012). However, in this research the temperature presented low significance because the high pressure used in PLE method (11.721 kPa) prevented the degradation of anthocyanins by heat due to the increase in the stability of the covalent bonds within molecules (HOSSAIN et al., 2011).

The significant interaction between temperature and ethanol affected positively the extraction as showed in Pareto chart (Figure 13), suggesting a synergistic action among these variables on anthocyanin recovery. This fact may be explained by the reduction in solvent viscosity promoted by the rise of temperature increasing the mass transfer (AVHAD; RATHOD, 2015). Figure 12B shows that the anthocyanin content increased with the increase of extraction cycles until close to the midpoint of the three-dimensional response plot, when the anthocyanin content reached the highest amount, decreasing after 2 cycles. The destructive behaviour resulted for the increase in temperatures levels was evidenced in Fig 12B, especially above 97°C. That result is expected, once high temperatures leading to the anthocyanins

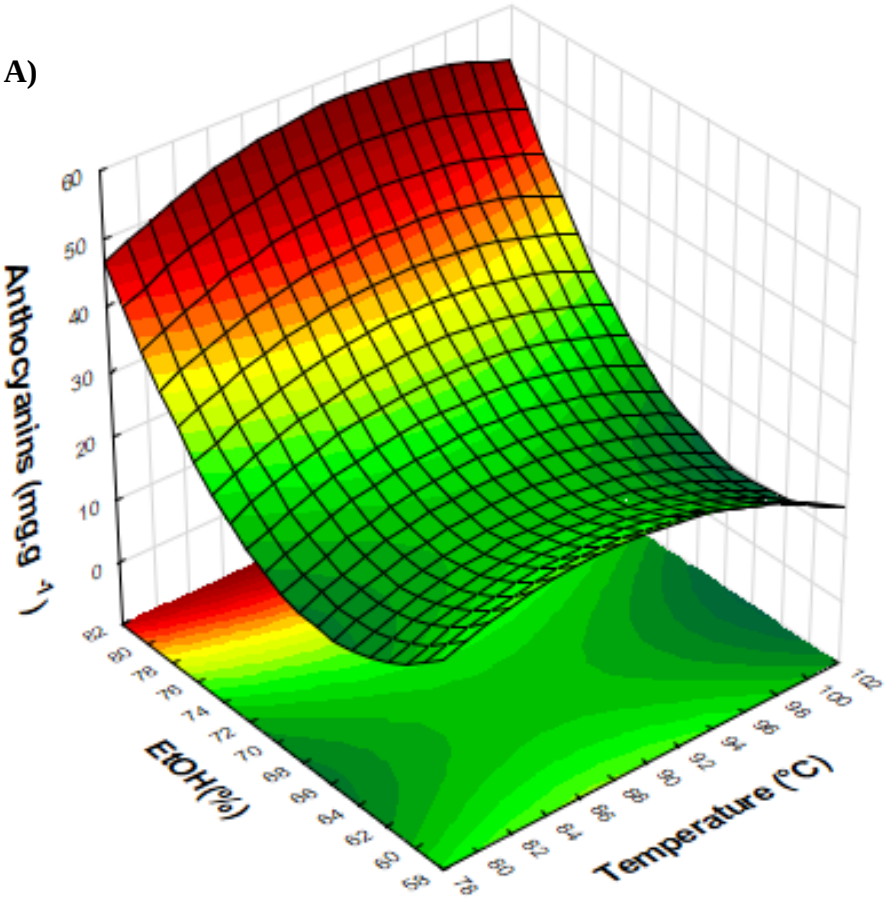
hydrolysis. The Pareto Chart reinforce the impact of temperature, presenting the negative values of its linear and quadratic effects (Figure 13).

Therefore, the optimum conditions for maximizing the extraction of anthocyanins from jambolan fruits using the PLE method was predicted applying the desirability function, as follows: temperature of 90 °C, 80% of ethanol, and number of extraction cycles equal to 1, resulting in an extract with anthocyanin content of 50 $mg.g^{-1}$. In these conditions, the anthocyanin content was 47.05 $mg.g^{-1}$, which represented a relative error of 6% compared with the value predicted by the model.

Figure 12 - Response surface for interactions between the independent variables on extraction of anthocyanins from jambolan using the PLE method. **(A)** Anthocyanin concentration vs ethanol concentration and temperature; **(B)** Anthocyanin concentration vs static cycles and temperature; **(C)** Anthocyanin concentration vs static cycles and ethanol concentration.

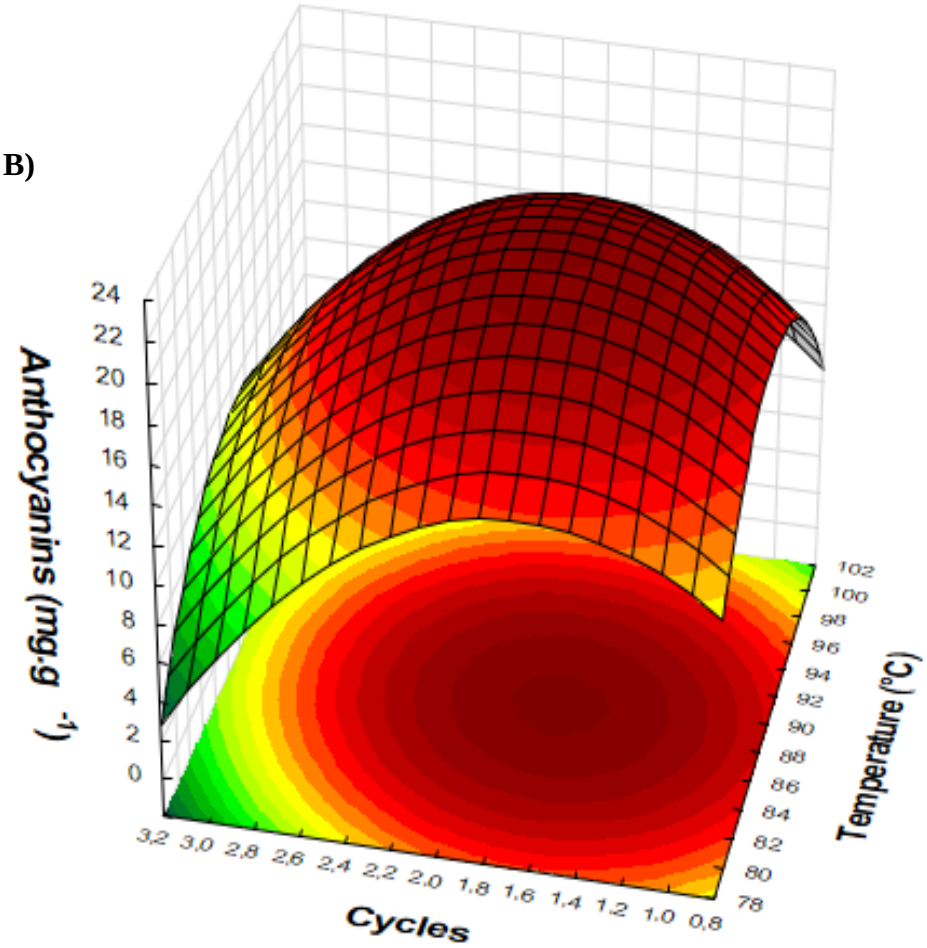
$$Y_1(mg.g^{-1}) = 21.84 + 12.7X_2 - 2.9X_3 + 2.15X_1X_2 - 1.62X_2X_3 - 5.14X_1^2 + 13.5X_2^2 - 5.18X_3^2$$

(Equation 3)

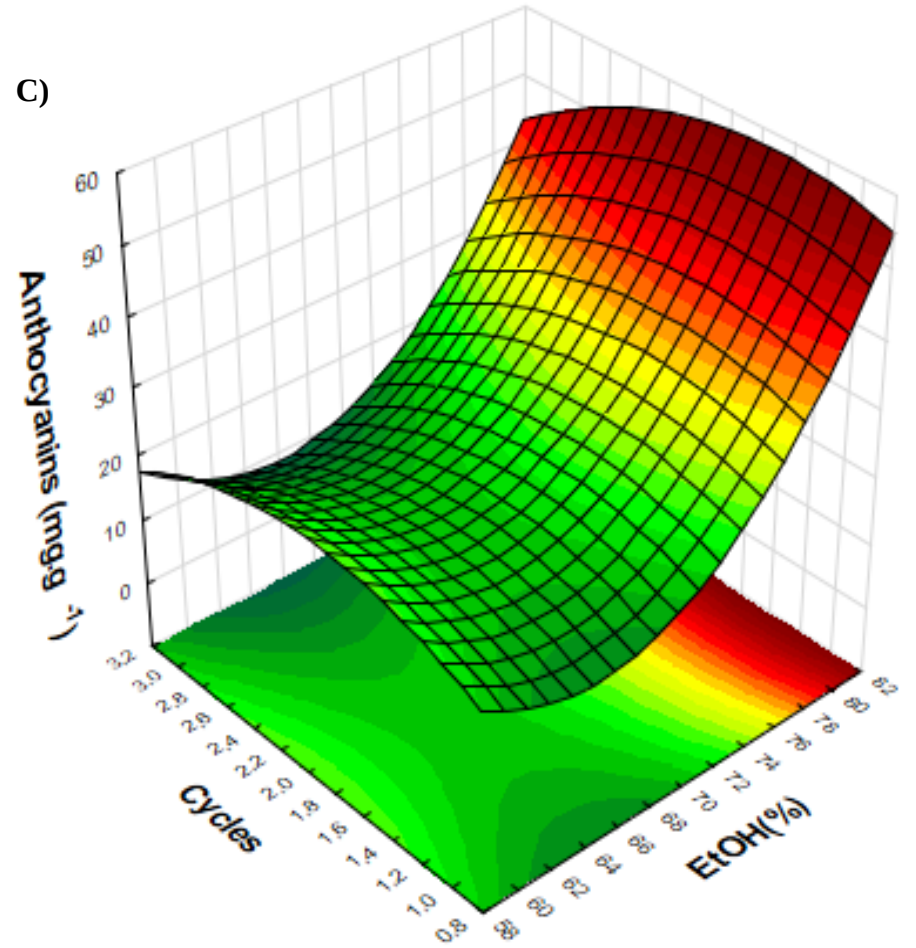


.

B)

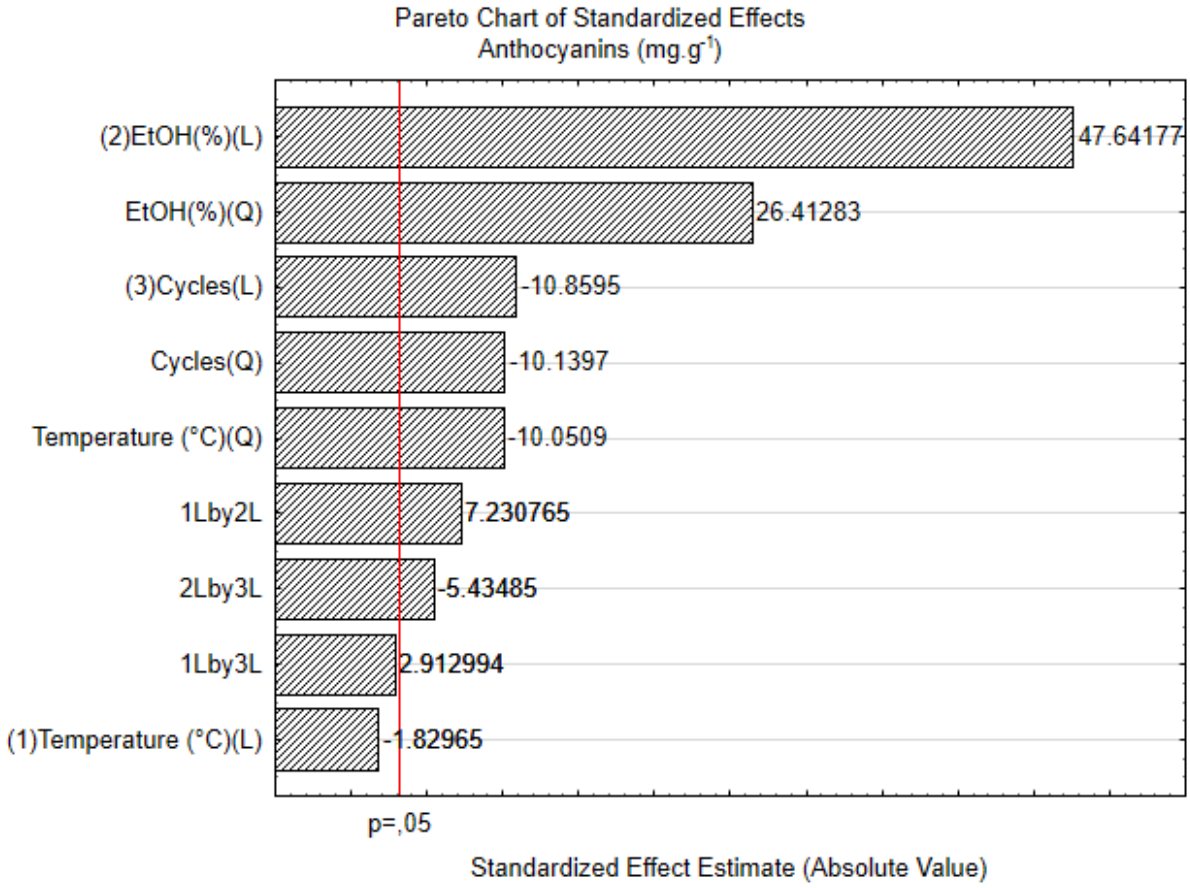


C)



Source: Elaborated by the author.

Figure 13 – Pareto Chart of the evaluated effects in anthocyanin extraction using PLE method.



Source: Elaborated by the author.

3.2.2 Anthocyanin extraction using the BUE method

Temperature (X_1), extraction time (X_2), and amount of ethanol (X_3) were the independent variables evaluated in the BUE method. Equation 4 was graphically represented by the three-dimensional response surface plots (Figure 14A-C). Figure 14A illustrates the response surface plot that represent the effect on anthocyanin extraction varying the temperature and time, as well as the mutual effect with constant percentage of ethanol at 60%. The Pareto chart, illustrated at Figure 15, presented the effects of the variables and their interactions.

The anthocyanin extraction was negatively influenced by the linear ($p = 0.004$) and quadratic effect ($p = 0.00003$) of temperature (Figure 15). Temperatures above 36 °C presented a negative effect on anthocyanin recovery, significantly decreasing its content at 43 °C. On the other hand, the higher anthocyanin content was observed at 32 °C. Regarding to time, the Pareto chart demonstrated that it an increase in the values of this parameter resulted in a positive

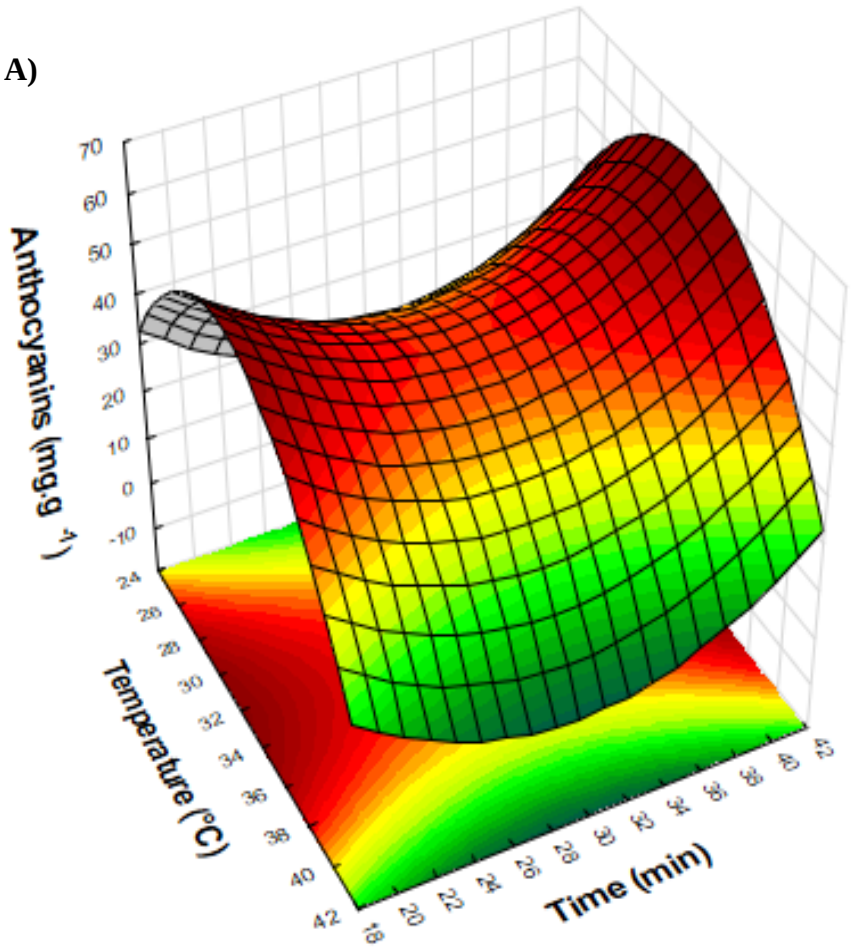
anthocyanin recovery, which was favored near to 40 min (Figure 14B-C). Usually anthocyanin recovery tends to increase along the extraction time until a point where it decreases substantially (RAMIĆ et al., 2015), which may explain the positive and negative influence from the linear and quadratic effect of time, as previously reported (PRAKASH MARAN et al., 2017). However, in our study only positive influence on anthocyanin extraction was observed, differing from previous data (CHEN; ZHAO; YU, 2015b; DEMIRDÖVEN; ÖZDOĞAN; ERDOĞAN-TOKATLI, 2015). The result can be explained considering the time ranges, which may be considered low with no destructive effects on anthocyanins.

It is possible to check from Pareto chart (Figure 15) that the extraction time had influence on anthocyanin recovery using the BUE method compared to the others evaluated terms. Time is a critical variable due to the exposition of vegetal substrate to the cavitation micro-bubbles generated in the ultrasound process. Cavitation micro-bubbles promote a continuous damage in plant tissues, breaking the cells increasing the release and solubilization of anthocyanins in the solvent. However, prolonged cavitation process tends to denature the anthocyanin due to their intense collapse (VILKHU et al., 2008).

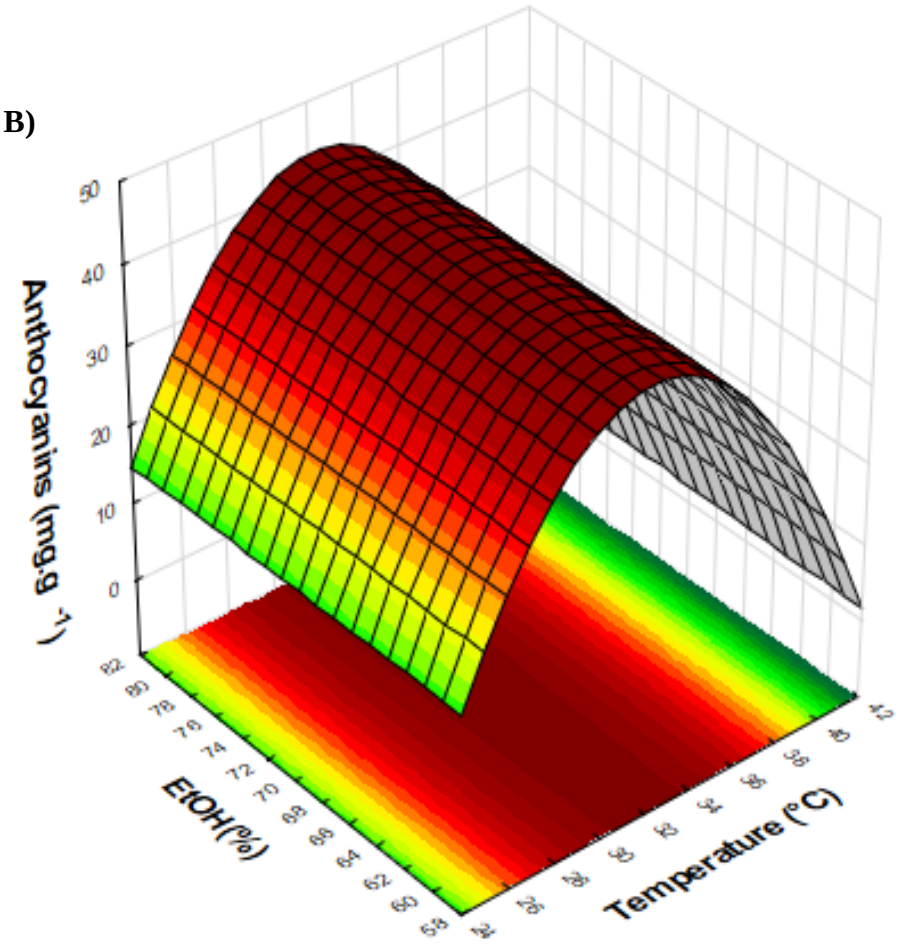
The optimized operational conditions for the BUE method that predicted an anthocyanin recovery of 57.0 $mg.g^{-1}$ were: 32.5 °C; 40 min; 80% v/v of ethanol. The experimental reproduction of these conditions resulted in an extract with content of anthocyanins equal to 54.2 $mg.g^{-1}$. The relative difference between the experimental and predicted values was 5.2%.

Figure 14 - Response surface for interactions between the independent variables on extraction of anthocyanins from jambolan using the BUE method. **(A)** Anthocyanin concentration vs temperature and time; **(B)** Anthocyanin concentration vs ethanol concentration and temperature; **(C)** Anthocyanin concentration vs ethanol concentration and time.

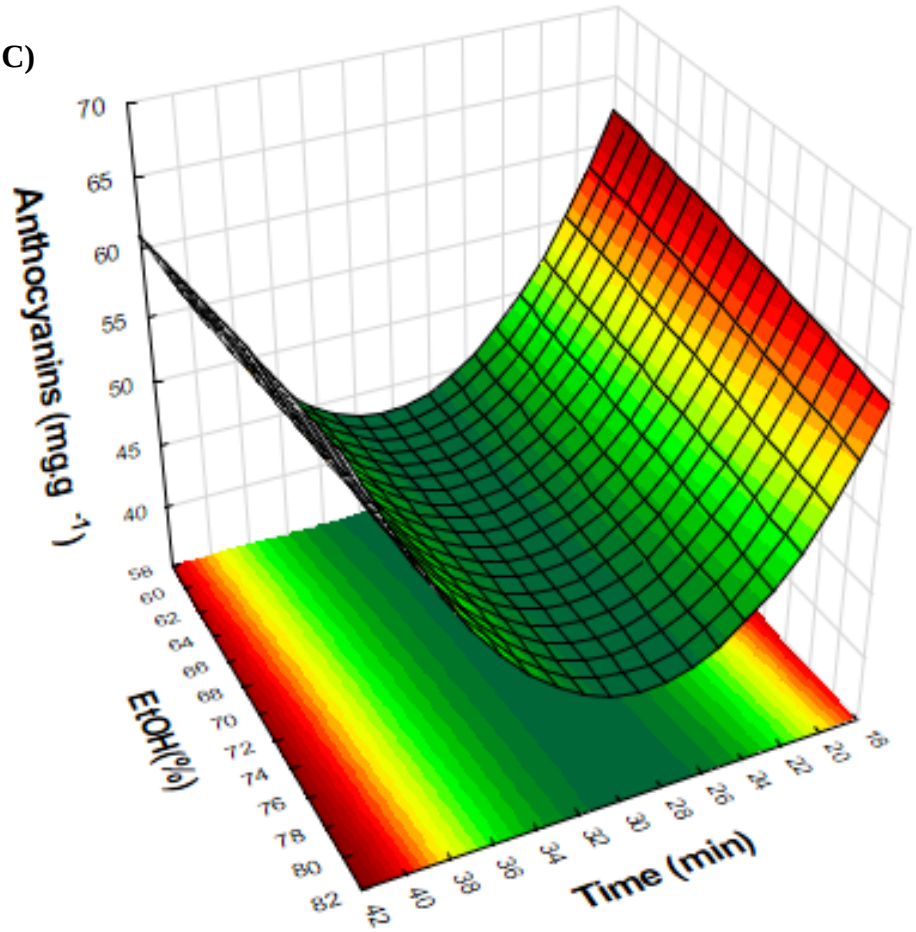
$$Y_2(mg.g^{-1}) = 43.52 - 2.09X_1 - 1.09X_2 + 1.46X_2X_3 - 24.02X_1^2 + 13.0X_2^2 \quad \text{(Equation 4)}$$



B)

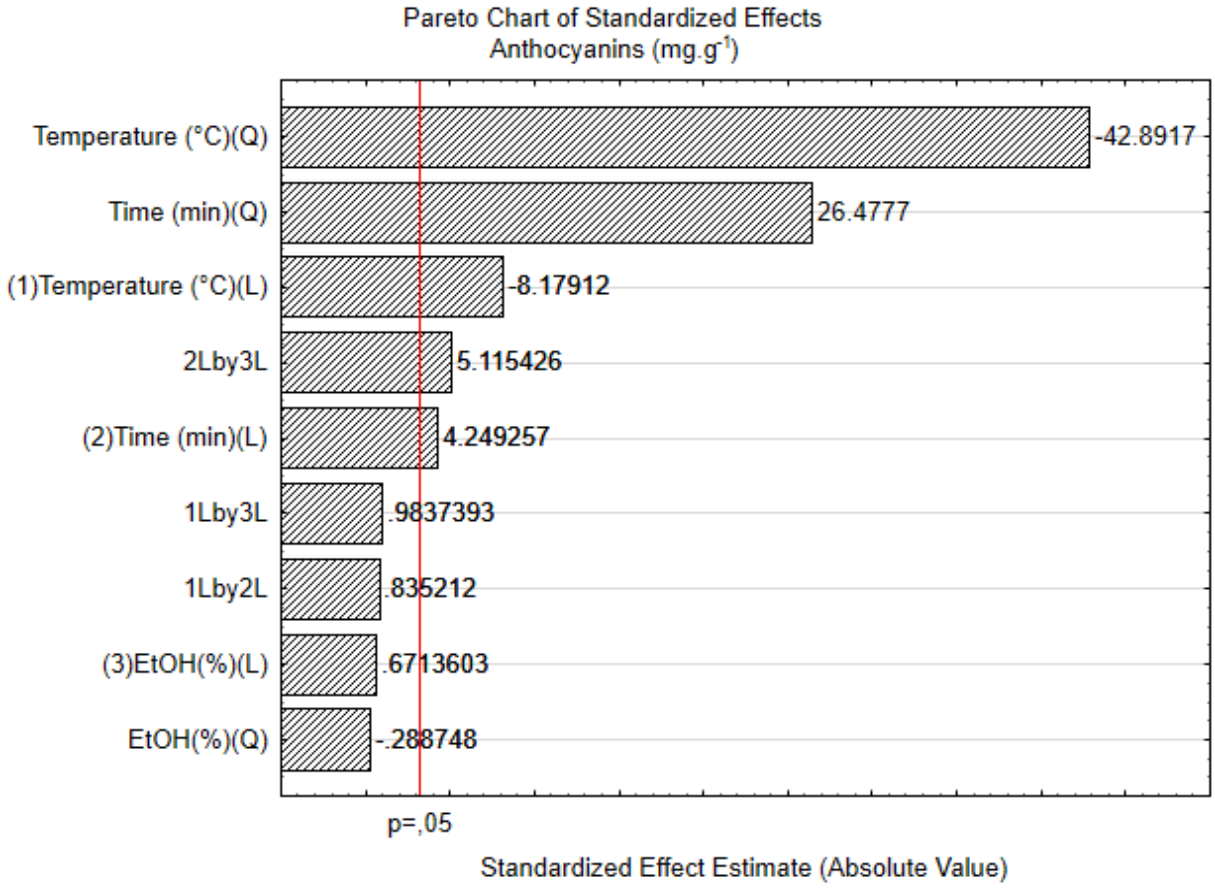


C)



Source: Elaborated by the author.

Figure 15 – Pareto Chart of the evaluated effects in anthocyanin extraction using BUE method.



Source: Elaborated by the author.

3.2.3 Anthocyanin extraction using the PUE method

Ultrasound power density *(X_1)*, time *(X_2)*, and percentage of ethanol *(X_3)* were the parameters evaluated in PUE method, and their effect on anthocyanin recovery is present in Table 9. Equation 6 was used to build the three-dimensional response surface plots (Figure 16A-C). Figure 16A shows the response surface plot representing the effect of varying the ultrasonic power and extraction time, with the synergistic effect on anthocyanin extraction keeping the amount of ethanol constant at 60%. A higher extraction of anthocyanins is favored by an increase of the ultrasonic power density from the lowest level (1650 W/L) to the mid-level (5000 W/L) when the extraction reached its highest value. On other hand, when ultrasound power was continuous increased to the higher level, a progressive degradation of anthocyanins was observed, especially after 7500 W/L. It was possible to check in Pareto chart, described at Figure 17, that both time parameters affected negatively the extraction process promoting a

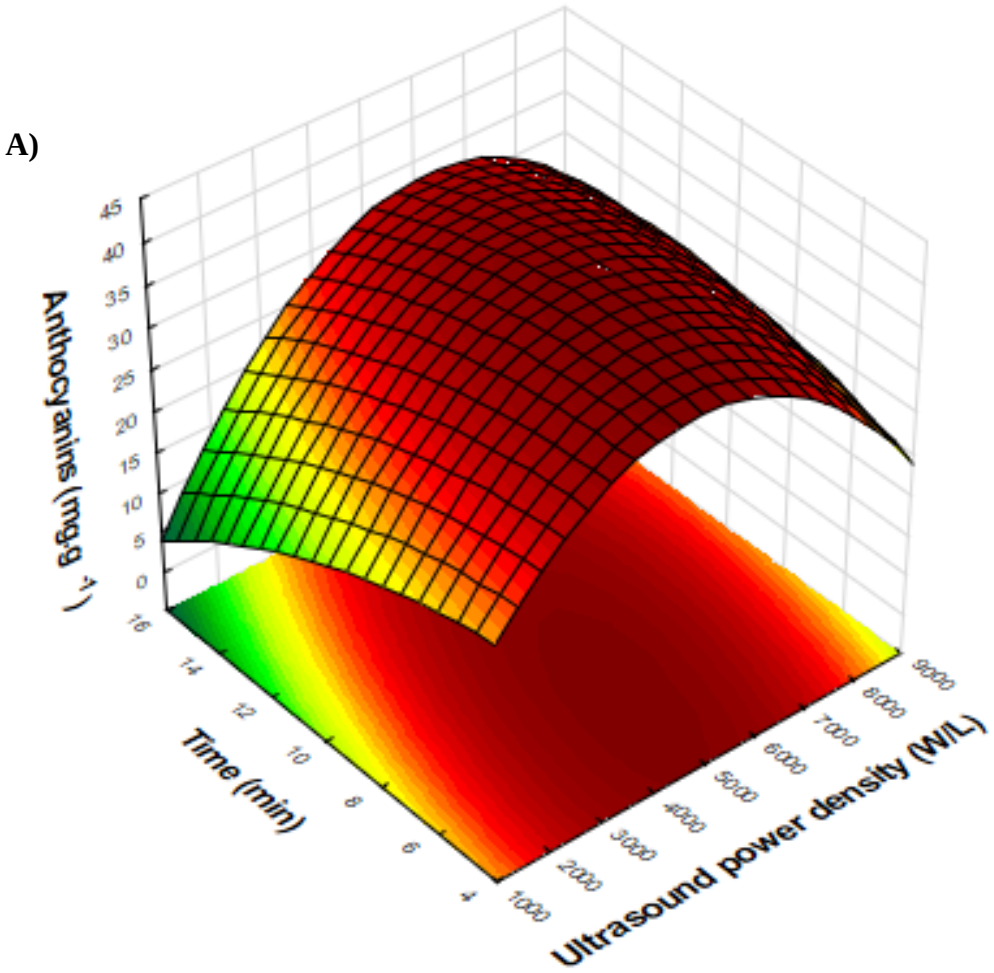
massive reduction of anthocyanin content after 14 min (Figure 16C). Ultrasound power is an important variable in the PUE method, and this study demonstrated that the solvent polarity is the most significant variable for anthocyanin extraction (Figure 16B-C).

In PUE method, the formation of cavitation bubbles in the region around the probe allows a higher intensity and efficient sonication process resulting in fast extraction with minimal ultrasonic energy loss. Differently, the BUE method presents a non-uniform distribution of ultrasonic waves, which promotes an unevenly spread and a low ultrasound intensity resulting in requirement of additional time for complete extraction. Therefore, as expected, the extraction time presented different effect between PUE and BUE due the cavitation uniformity and intensity from each method.

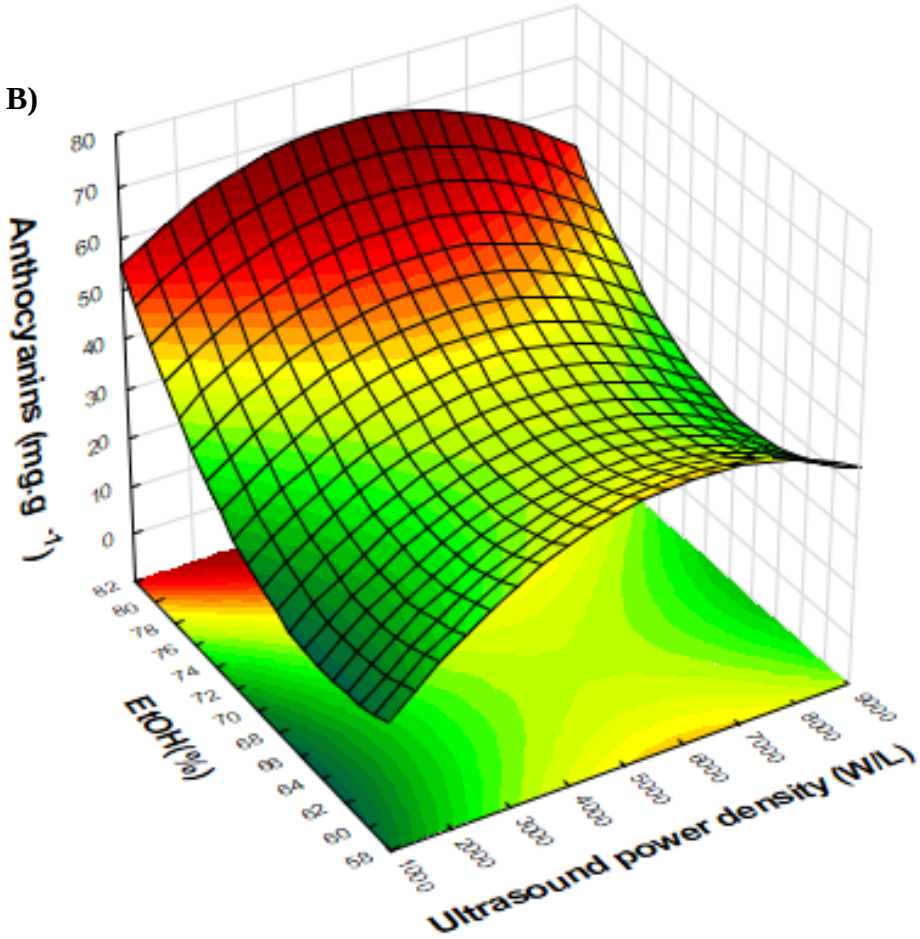
The optimization of the response surface for anthocyanin recovery resulted in the following operating conditions: ultrasound power density of 5000 W/L, 7.5 min of extraction time, and ethanol concentration of 79.6% v/v. In the optimized conditions, the experimental content of anthocyanin was 60.5 $mg.g^{-1}$, which was close to the value predicted by the model (62.0 $mg.g^{-1}$) with a relative error less than 5%.

Figure 16 - Response surface for interactions between the independent variables on extraction of anthocyanins from jambolan using the PUE method. **(A)** Anthocyanin concentration vs time and ultrasound power; **(B)** Anthocyanin concentration vs ethanol concentration and ultrasound power; **(C)** Anthocyanin concentration *vs* time and ethanol concentration.

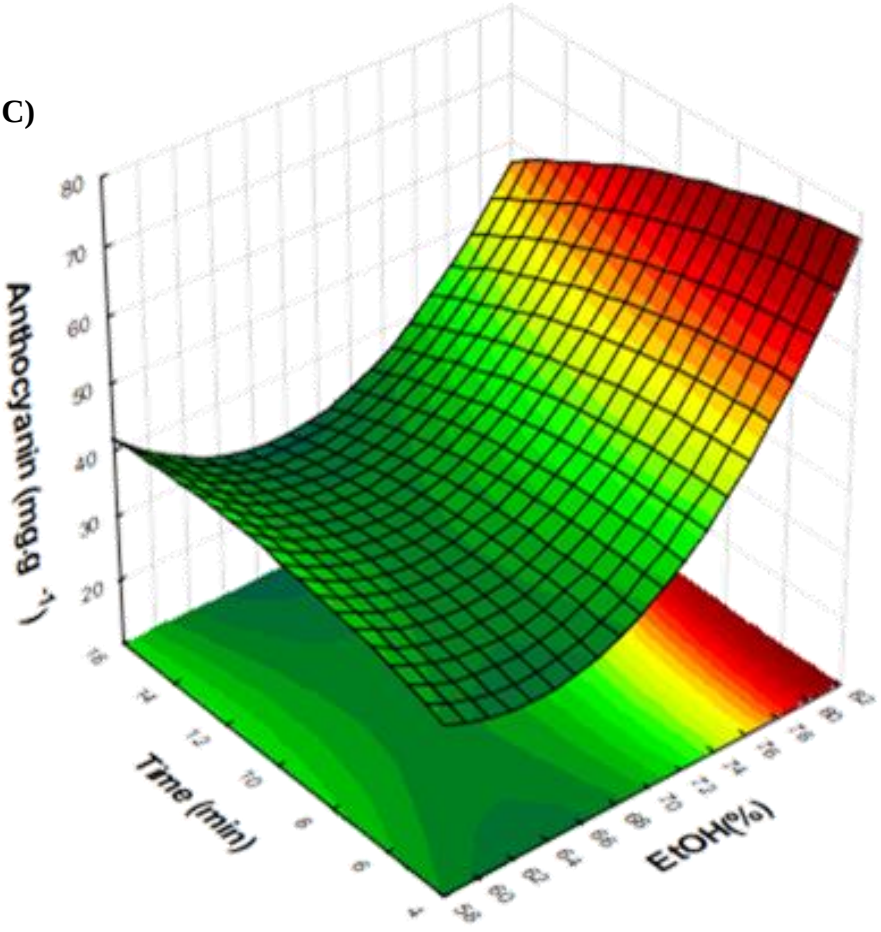
$$Y_3(mg.g^{-1}) = 37.94 + 3.341 - 2.82X_2 + 1.57X_3 + 4.46X_1X_2 - 3.65X_1X_3 - 4.36X_2X_3 - 11.8X_1^2 - 2.30X_2^2 + 12.46X_3^2 \quad \text{(Equation 5)}$$



B)

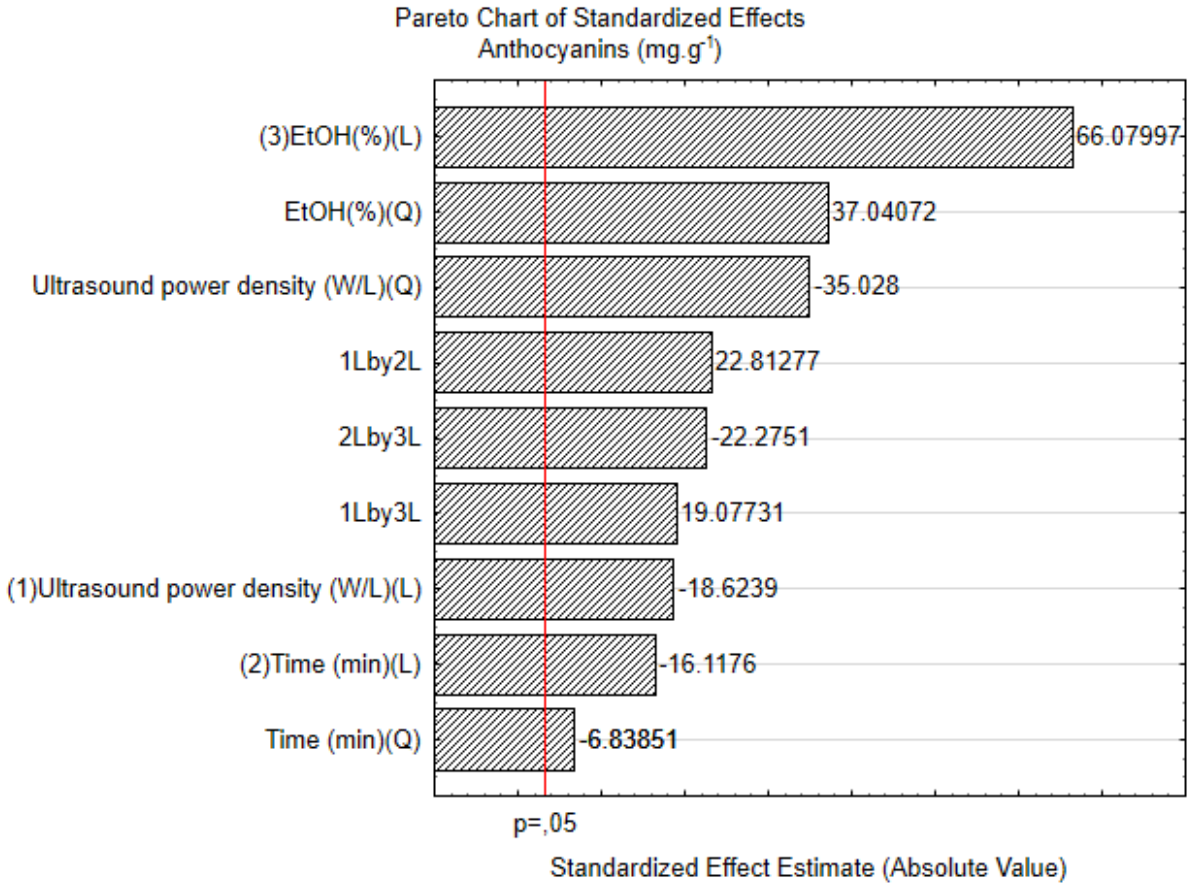


C)



Source:Elaborated by the author.

Figure 17 - Pareto chart of the evaluated effects in anthocyanin extraction using PUE method.



Source: Elaborated by the author.

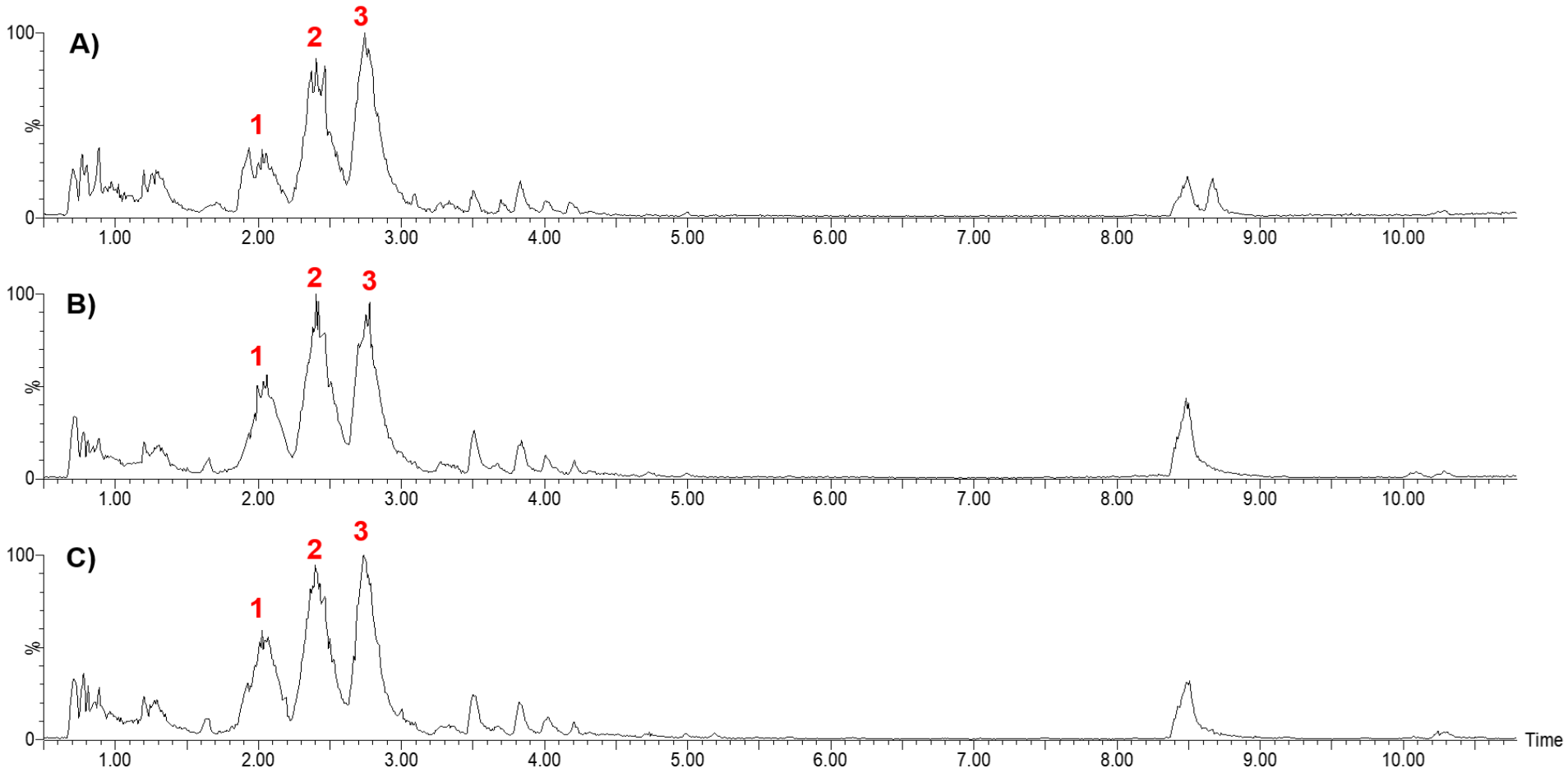
3.3. Identification and relative contribution of anthocyanins

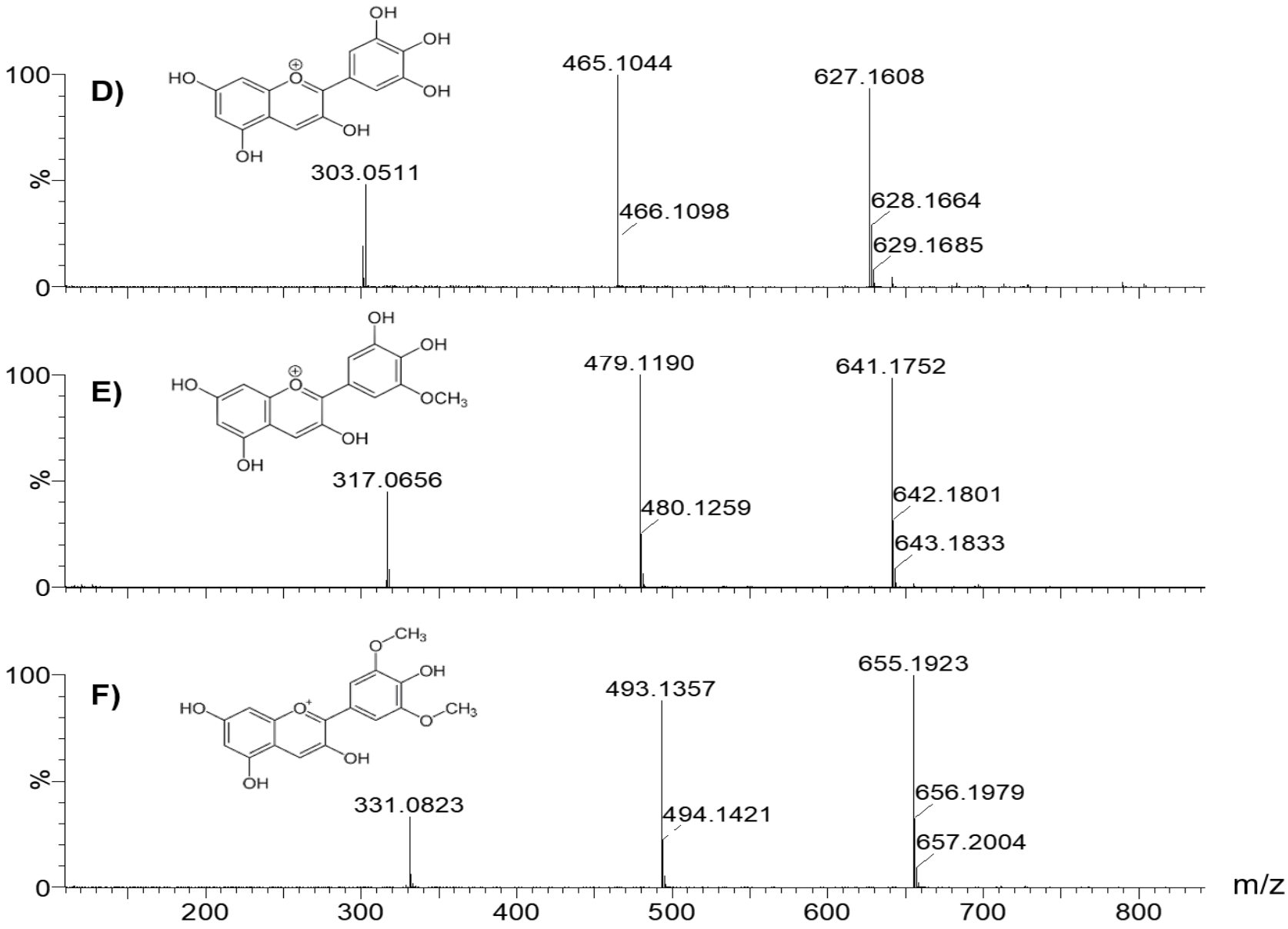
In order to detail the presence of the anthocyanins, the jambolan extracts with highest concentrations (from experiment 12 of the PLE method, and 11 of both ultrasound methods) were analyzed by UPLC-ESI-QTOF-MS. Figure 18A-C present the spectra of each method resulted from the injection of the crude extracts. Three major anthocyanins were identified in all samples as diglucosides of (1) delphinidin, (2) petunidin, and (3) malvidin, based on *m/z* values and fragmentation profiles, as shown in Figure 18D-F, Table 10, and also corroborated by previous works (BRITO et al., 2007; VEIGAS et al., 2007). The results pointed that the quality of anthocyanins extracted were independent of the method used, however, the relative contribution showed differences in the proportion of anthocyanins extracted by each method. In the PLE, the peaks 1, 2 and 3 represented, respectively, 19.01, 24.30 and 27.03% of the total peak area (based on total ion abundance) reveled at 520nm. That extraction method

presented the lowest proportion values for all anthocyanins, being statistically differing compared to ultrasound methods ($p < 0.05$). The only difference observed between ultrasound methods was related to the proportion of malvidin (peak 3) that was significative higher in PUE than BUE. The other anthocyanins did not present statistical difference ($p < 0.05$). The three anthocyanins found in *S. cumini* extracts already were reported by other authors and delphinidin, petunidin and malvidin-3,5-diglucoside presented concentrations of 23-45%, 32-35%, and 15-38%, respectively (BRITO et al., 2007; FARIA; MARQUES; MERCADANTE, 2011; LI et al., 2009; VEIGAS et al., 2007). However, the results related to the type of anthocyanin extracted for each method, did not shown statistical difference ($p < 0.05$), which is means that these three anthocyanins are present in the same proportions in the fruits.

Two ions are observed in a typical ESI positive MS: The protonated molecular ion $[M + H]^+$ and a fragment ion $[M + H - X]^+$ resulting from loss of sugar moiety. The anthocyanins have a natural residual positive charge, one observes a true molecular ion [M]+ and a fragment ion $[M - X]^+$ which is of the underivatised aglycone. The X represent the difference between the molecular ion and fragment and its value indicates the nature of the sugar molecule (ABDEL-AAL, YOUNG; RABALSKI, 2006). The compound represented by the peak 1 produced ions at m/z 627.1607, m/z 465.1050 and m/z 303.0466. Compound 2 revelated ions at m/z 641.1752, m/z 479.1205 and m/z 317.0611317 and the compound 3 at m/z 655.1924, m/z 493.1368 and m/z 331.0772627 (Figure 18D-E and Table 10). The results point that the aglycones are delphinidin (m/z 303), petunidin (m/z 317) and malvidin (m/z 331) for peaks 1, 2 and 3, respectively. The differences of m/z 162 and m/z 324 from the aglycone in the two fragments (presented in all three compounds) revealed the presence of two glucose [M^+ - 162 Da] molecules (VEIGAS et al., 2007).

Figure 18 - The UPLC-ESI-QTOF-MS spectra of the *S. cumini* anthocyanins obtained from **(A)** pressurized liquid extraction, **(B)** bath ultrasound extraction **(C)** probe ultrasound extraction, **(D)** delphinidin- diglucoside, **(E)** petunidin-diglucoside and **(F)** malvidin-diglucoside.





Source: Elaborated by the author.

Table 10 - Peaks assignment and relative contribution of each anthocyanin present in jambolan extract, with $[M-H]^+$ ion observed and produced ions (MS/MS*).*

Peak	Compound	Retention time (min)	Molecular ion (*m/z*)	Fragment ion (*m/z*)	Relative anthocyanin contribution (% Area)		
					PLE	**BUE**	**PUE**
	Anthocyanins						
1	Delphinidin-3,5-diglucoside	2.70	627.1607	465.1050 /303.0466	19.01 ± 6.7^{bA}	36.07 ± 4.65^{aA}	44.90 ± 7.73^{aA}
2	Petunidin-3,5-diglucoside	2.90	641.1752	479.1205 /317.0611	24.30 ± 3.9^{bA}	35.65 ± 1.15^{aA}	40.13 ± 3.95^{aA}
3	Malvidin-3,5-diglucoside	3.15	655.1924	493.1368 /331.0772	27.03 ± 4.4^{cA}	32.83 ± 1.25^{bA}	40.23 ± 3.23^{aA}

Means with same letter in the lines are not statistically different by the Tukey test ($p < 0.05$).
Means with same letter in the columns are not statistically different by the Tukey test ($p < 0.05$).
Source: Elaborated by the author.

4 CONCLUSIONS

The Pressurized Liquid Extraction (PLE) method presented the lowest recovery of anthocyanins compared to Bath Ultrasound Extraction (BUE) and Probe Ultrasound Extraction (PUE). Between the methods were not noticed quality difference in anthocyanins profile being found delphinidin-3,5-diglucoside, petunidin-3,5-diglucosid, malvidin-3,5-diglucoside in all extracts. However, these compounds were found in a higher proportion in PUE and BUE than PLE. Due the fast and efficient extraction, this research suggests the use of PUE for obtaining jambolan extracts rich in anthocyanins could be potentially used in different industrial sectors due it knew biochemical and physical properties, which may reduce the costs of extraction process.

Acknowledgment

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5.2 Estudo da liberação das antocianinas extraídas do jambolão (*Syzygium cumini* L.) a partir de comprimidos monolíticos a base de carboximetilamido.

RELEASE STUDY OF THE ANTHOCYANINS EXTRACTED FROM JAMBOLAN (*Syzygium cumini* L.) FROM MONOLITHIC TABLETS BASED ON CARBOXYMETHYL STARCH

ABSTRACT

The association between anthocyanins-rich extracts and polymers has been studied as an effort to protect and ensure the delivery of these substances in different systems without pronounced losses in their bioactivity. In our research carboxymethyl starch (CMS) was synthesized and used as excipient for anthocyanins delivery, aiming to develop, characterize and evaluate monolithic CMS based-tablets formulations for the release of anthocyanins (ACNs) in simulated fluids. ACNs from jambolan fruit were used as active agent in different CMS-based tablet formulations. The ACNs dissolutions profiles were obtained in simulated gastric (SGF) and intestinal (SIF) medium in the appropriated time. The structural analysis showed a strong interaction between tablet ingredients, mainly due the ACNs and CMS interaction, supported by the FTIR and SEM structural analysis. The dissolution profiles varied as the dissolution medium and tablet formulation and in general, the ACNs release increased in neutral medium (SIF) where was controlled by the matrix swelling and erosion. The results indicated that the interaction with CMS provided a higher stability to ACNs extract, resulting in a formation of a potential carrier system for ACNs gastrointestinal delivery. This research presented important information about the use of CMS as excipient for ACNs delivery and good references to prospect their future application.

Key-words: Anthocyanins. *Syzygium cumini*. Carboxymethyl starch. Monolithic tablets. Delivery.

1 INTRODUCTION

In recent years, there has been a crescent interest in exploring plant biocompounds due the evidence that their regular ingestion results in benefits to health. Anthocyanins are to the most distributed class of water-soluble pigments found in nature, being responsible for the blue, purple, red and pink shades of several vegetal species including flowers, fruits and leaves. Chemically anthocyanins are polyhydroxyl and polymethoxyl derivates of 2-phenylbenzopyrylium or flavylium salts and until the moment, more than 500 anthocyanins *O*-glycosylated structures were identified (CHAVES-SILVA et al., 2018).

Anthocyanins (ACNs) present different biological properties and are included in the list of natural compounds known to work as powerful antioxidants (GALVANO et al., 2004; LONG et al., 2018). *In vivo* and *in vitro* tests have shown that anthocyanins-rich extracts presented antioxidant, anti-inflammatory, antitumor, anti-atherosclerotic and chemopreventive activities (DEL BÒ et al., 2010; FERNANDES et al., 2018). These properties are related to the ACNs natural electron deficiency, resulting in an elevated reactivity and ability to act as proton donor, stabilizing reactive oxygen species (ROS). Due the higher content in fruits, vegetables and beverage, the typical U.S. diets contain a range of 12.5−215.0 mg of anthocyanins/day. However, the only dietary reference of ACNs has currently defined by China, that proposed the level of 50 mg/day for these compounds (WANG et al., 2013). In this research we used the ACNs extracted from jambolan fruit (*Syzygium cumini* L.), a tropical fruit related as source of these compounds (FARIA; MARQUES; MERCADANTE, 2011).

In a regular diet, the ACNs concentration must be sufficiently high to supply all their pharmacological benefits once several studies claim that these molecules are poorly absorbed in the gastrointestinal tract (GIT) even shortly after their ingestion (CZANK et al., 2013; FERNANDES et al., 2014; MCGHIE; WALTON, 2007). It is known that in humans, ACNs metabolism follows different pathways being rapidly absorbed through the stomach and small intestine involving the action of specific enzymes such as bilitranslocases (FERNANDES et al., 2014; MUELLER et al., 2017; PASSAMONTI et al., 2003). However, several factors can affect ACNs bioavibility in the human body. In the colon, these pigments are exposed to local microbiota and degraded to parental forms as such the phenolics acids and aldehydes, losing their activity (KEPPLER; HUMPF, 2005). The neutral intestine pH also affects the stability and consequentely the ACNs bioavailability (WANG et al., 2013). The use of different

polymers has been investigated in an attempt to protect ACNs and boost their functionality in biological systems (FERNANDES et al., 2018a; JAFARI; MAHDAVI-KHAZAEI; HEMMATI-KAKHKI, 2016).

Due the hydrophilic characteristics, ACNs are compatible with a water-based gel formulation as gum, maltodextrin and starches, association, forming polar solid matrices (IDHAM; MUHAMAD; SARMIDI, 2012). Starch is a natural, biodegradable and cheap polymer that presents interesting characteristics to industry (e.g. stability to heat and pH variations). However, due to the poor processability, dimensional stability and mechanical properties, the use of this polymer in pharmaceutical fields is limited (BARAN; MANO; REIS, 2004; LU; XIAO; XU, 2009; WANG et al., 2013). On other hand, starch properties can be modulated by physical, enzymatic and chemical modifications which improve its use for the formulation of starch-based products such as tablets and capsules (ASSAAD; MATEESCU, 2010). Carboxymethyl starch (CMS) is an anionic starch resulted by introduction of carboxylic groups in its native structure through etherification reaction with monochloroacetic acid (BLEMUR et al., 2016) and was been proposed as a novel pH sensitive excipient for bioactive agents delivery (BROUILLET; BATAILLE; CARTILIER, 2008; CALINESCU et al., 2005). In this research CMS was used as excipient to ACNs delivery. Previous studies pointed that CMS tablets in gastric and intestinal simulated fluids facilitates the swelling of the matrix and the release of the active compounds loaded (ASSAAD; MATEESCU, 2010; LANDI; TATTINI; GOULD, 2015; MULHBACHER; ISPAS-SZABO; MATEESCU, 2004).

Considering the CMS as a promisor excipient for biocompounds delivery and the efforts to ensure the ACNs integrity in the human organism, this work aimed to formulate and characterize CMS monolithic based-tablets for ACNs delivery. We evaluated the ACNs release behaviour in simulated gastric and intestine medium, optimizing the formulations for ensure a programmable release time. To the best of our knowledge this is the first study to evaluate the use of CMS as excipient to delivery ACNs extracted from jambolan, bringing reliable information to prospect their use in diet supplement or in natural therapies.

2 MATERIAL AND METHODS

2.1 Chemicals

The ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt (purity 98%), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) 97% absolute ethanol, D- mannitol, trifluoracetic acid (TFA, 99%), microcrystalline cellulose (MCC), gum arabic, sodium chloroacetate (SCA, 98%) were obtained from Sigma Aldrich (Saint Louis, MO, USA). High amylose corn starch (Hylon VII) was provided by Ingredion (Westchester, IN, USA). Sodium croscarmellose was purchase from Blanver (São Paulo, SP, Brazil). Explotab® and Prosolv® were obtained from JRS pharma (Patterson, NY, USA). Polyvinylpyrrolidone (International Specialty Products, USA). The other chemicals used in this research were analytical grade.

2.2 Plant material and anthocyanins (ACNs) extraction

Jambolan fruit was obtained from a domestic pomace localized in Cascavel, Ceara, Brazil. The seeds were manually removed and the edible portion (around to 30% of fruit) was squeezed and freeze dried in Freeze Dry-5 dryer (Labconco, MO, USA) at -50 °C under pressure from 5 mtorr (9.67×10^{-5} psi) and vacuum. After the material was crushed until podwer form and storaged under refrigeration (-5 °C) and oxygen free.

A QR500 probe ultrasound extractor (Ultronique, Brazil, 20 kHz) coupled with a 13 mm titanium tip was used to perform ACNs extraction. Sample and solvent were mixed in a 250 mL jacketed beaker and subjected to ultrasound. The temperature was controlled with a thermostatic water bath (30.0 °C ± 0.1) through the beaker jacket. The ultrasound power (300 W) and extraction time (7.5 min) were controlled. After extraction, the extract was filtered and the solvent removed in a R-215 rotavapor (Bunch, Flawil, SWI) at 40 °C. In this process, the fruit:solvent proportion was 1:15 w/v and aqueous ethanol solution (80% v/v) acidified with TFA (0.1% v/v) was used as solvent.

2.3 Synthesis of carboxymethyl starch (CMS)

Carboxymethyl starch was prepared following as described by (ASSAAD; MATEESCU, 2010) with slight modifications. Initially 150 g high amylose corn starch was dispersed in 50 mL of pure water in a beaker (2 L) under continuous vertical stirring (Servodyne Mixer, 50000-40, IL, USA) and temperature constant (50 °C) to ensure the polymer solubilization. Then 500 mL of 2.0 M of NaOH were added to the beaker and stirring was maintained (30 min) for starch gelatinization. CMS occurred by adding sodium chloroacetate until the alkaline (0.9 M NaOH) mixture. After 1 hour of reaction, 550 mL of distillated water were added to reduce the temperature and the reaction was stopped by addition of 120 mL of acetic acid (pH, 3.0). The synthesized CMS was precipitated using aqueous methanol and washed repeatedly with 2 L of methanol (70 %). CMS was washed with 1L of pure methanol/acetone, dried at 35 ◦C for 24 h and, after, sieved on a 300 μm screen. The powder was storage in plastic bottle and free of humidity.

2.4 Tablet preparation

For the tablets formulations a half of the ACNs concentrated extract was freeze-dried and the other was kept in liquid form. The samples were stored at -20 °C until analysis.

2.4.1 Preparation of anthocyanins powders

For tablets elaboration the ACNs were directly associated with CMS or before with mannitol or polyvinylpyrrolidone (PVP), that were used as bulking agents for C2 and C3 tablets formulation, respectively. For the ACNs-mannitol (AM) preparation, 60 mL of aqueous mannitol (20% w/v) were gently homogenized with 40 mL of anthocyanin concentrated extract. The ACNs-PVP (APVP) was obtained similarly, but using PVP solution (20 % w/v). The ACNs extract were also mixed with CMS powder in the proportion of 1:2 (% w/v). The AM, APVP and ACNs-CMS mixtures were kept under constant stirring until complete homogeneous. The mixtures were frozen (-80° C), freeze-dried, crushed and sieved on a 300 μm screen. The samples were stored in dark glass bottle kept at -20 °C free from humidity and light.

2.4.2 Formulations

Four formulations named as C1, C2, C3 and C4 were prepared (Figure 19). The detailed information of each tablet composition is present in Table 11. C1 tablet was elaborated from the directly mixed of the dried ACNs extract which CMS in the same proportion (% w/w). For C2 and C3 tablets, CMS was mixed with AM and APVP powders, respectively, in the same proportions (% w/w). The C4 tablet corresponded to the ACNs-CMS powder resulted from the freeze-drying of the ACNs extract with CMS mentioned in 2.4.1 (Table 11).

The dissolutions profiles showed that C4 tablet was the most suitable to perform ACNs release, so its formulation was optimized to evaluate the programmable release. The formulations C 4.1, C4.2, C 4.3, C 4.4 and C 4.5 were developed to perform the fast release (30 min in SGF) while C 4.6. C 4.7 and C 4.8 to evaluate the sustained release (12 hrs in SGF/SIF). Detailed information about the composition of each tablet formulation are present in Table 11.

Figure 19 - Characteristics of the CMS-based monolithic tablets formulations elaborated to evaluate the ACNs delivery. C1, C2, C3 and C4 correspond to tablets functionalized with ACNs while C represent the unloaded CMS tablet

Source: Elaborated by the author.

Table 11 - Detailed composition of the monolithic tablets.

Samples		Components (% w/w)									
		CMS	ACNs	AM	APVP	ACNs-CMS*	CCS	Prosolv	Gum arabic	Explotab	MCC
	C1	50	50	-	-	-	-	-	-	-	-
	C2	50	-	50	-	-	-	-	-	-	-
	C3	50	-	-	50	-	-	-	-	-	
	C4	-	-	-	-	100	-	-	-	-	-
Fast release	C 4.1	-	-	-	-	90	10	-	-	-	-
	C 4.2	-	-	-	-	67	6	27	-	-	-
	C 4.3	-	-	-	-	67	6	-	27	-	-
	C 4.4	-	-	-	-	47	-	-	35	18	-
	C 4.5	-	-	-	-	47	-	-	-	18	35
Sustained release	C 4.6	-	-	-	-	47	18	-	-	-	35
	C 4.7	-	-	-	-	55	10	-	-	-	35
	C 4.8	-	-	-	-	80	10	-	-	-	10

CMS: Carboxymethyl starch; AM: Anthocyanins-mannitol powder;APVP: Anthocyann-polyvynilpirrolydone powder; CCS: Crosscarmellose sodium; MCC: Microcrystalline cellulose; *ACNs-CMS: Complex obtained from mixture and freeze-drier of CMS with ACNs (% 1:2 w/w).
Source: Elaborated by the author.

2.4.3 Tablets forming

Tablets of 500 mg were prepared with CMS only or combined with other ingredients. Before the forming, the components of each formulation were gently mixed in a porcelain mortar and sieved (300 µm screen) to ensure the tablets complete homogeneity. The tablets were formed by direct compression of a properly homogenous mixture of the components of each formulation on a Carver hydraulic press (Wabash, IN, USA) using flat-faced punches of 12 mm diameter and employing a pressure of 2.5 tonnes.

2.5 Anthocyanins dissolution assay

Dissolution tests were performed in pepsin-free simulated gastric fluid (SGF, pH 1.2) and pancreatin-free simulated intestinal fluid (SIF, pH 6.8) prepared following USP methods (US PHARMACOPEIA, 2010). *In vitro* dissolution tests were carried out in a G25 Controlled Environment Incubator Shaker (Alt, Boston, USA) at 100 rpm and 37 °C. The dissolution of C1, C2, C3 and C4 tablets was followed: *(i)* in SGF until complete release, *(ii)* in SIF until complete release, and (*iii*) in SGF for 2 hrs and then in SIF up to ACNs complete release. Aliquots of 2 mL at each time were collected and then measure at 280 nm in a Lambda 750 Spectrophotometer (Parkin-Elmer, Massachusetts, USA) for SIF or SGF medium. The quantity of ACNs dissolute in 50 mL of the medium was determinate with calibration curves prepared in SGF (R^2 = 0.9982) and SIF (R^2 = 0.9973) using purified ACNs extract as standard with concentrations ranging from 0.125 to 4 $mg.mL^{-1}$.

To study the ACNs release kinetics from CMS-based matrix tablets, the release data in SGF, SIF and SGF/SIF were fitted to the well-known to Korsmeyer–Peppas equation (Equation 6), often employed to describe the release behaviour from polymeric systems when the mechanism is not well known or when more than one type of release phenomenon is involved (KORSMEYER et al., 1983).

$$\frac{M_t}{M_\infty} = kt^n \quad \text{(Equation 6)}$$

where Mt/M∞ corresponds to the fraction of anthocyanins released at the time; *k* is a kinetic constant relating to the properties of the matrix, the properties of the anthocyanins, and the geometric characteristics of the dosage form; *n* is the release exponent characteristic of the

anthocyanins release. The value of *n* was used to evaluate the ACNs release mechanism. Values of n = 0.45 indicates a diffusion-controlled release (Fickian diffusion) ; n = 0.89 corresponding to a swelling-controlled release (case-II transport); values between 0.45 and 0.89 refer to anomalous diffusion or Non Fickian-diffusion, corresponding to the combination of both phenomena (WEI et al., 2009). Values of n > 0.89 have been observed, which has been attributed as Super Case II (or erosion controlled) according to literature (KORSMEYER et al., 1983). To obtain the values of k and *n* and the correlation coefficient (R^2) the log (M_t/M_∞) was plotted against log(t). The portion of the profiles where M_t/M_∞ is ≤ 70% was only used.

2.5 Samples characterization

2.5.1 Scanning electron microscopy (SEM)

The evaluation of the morphology of native starch (Hylon VII), CMS, and ACNs-CMS complex (C4) in a Hitachi S-3400N microscope (Oxford instruments, High Wycombe, UK) with SE and BSE detectors for observation and an Xact C3400. Samples fragments were fixed in stubs adhered to carbon conductor to obtain the images with different magnifications.

2.5.2 Fourier transform infrared spectroscopy (FTIR)

The samples were evaluated by Fourier Transform Infrared (FTIR) spectroscopy (Nicolet 4700, Madison, WI, USA). The spectra were recorded from 4000 to 400 cm^{-1} at 2 cm^{-1} resolution and with a total of 32 scans.

2.5.3 Erosion and fluid uptake

To evaluate the erosion and fluid uptake (SGF or SIF) unloaded CMS tablets were evaluated in the same conditions as described in dissolution tests (100 rpm and 37 °C). Initially the tablets (500 mg) were incubated for 2 h in SGF or in SIF. After the tablets were removed from solution medium, dried with paper to eliminate the water excess, and weighed before (wet tablets) and after freeze-drying (dried tablets). Erosion and fluid uptake were expressed in percentage and calculated by the equations 7 and 8 as described by Calinescu et al. (2007):

$$Erosion\ (\%) = \frac{(W_i - W_d)}{W_i} \times 100 \text{ (Equation 7)}$$

$$Fluid\ uptake\ (\%) = \frac{(W_w - W_d)}{W_d} \times 100 \text{ (Equation 8)}$$

where W_i is the initial weight of the tablet, W_w is the weight of wet tablet and W_d is the weight of dried tablet.

The tablet mass percentage remaining after erosion was calculated from the following Equation 9.

$$\%\ Remaining = 100 - Erosion\ (\%) \text{ (Equation 9)}$$

2.6 Antioxidant capacity

The ability to reduce 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation was evaluated for the ACNs released from C4 tablet prepared according to the recommended by Konan, Tien e Mateescu (2016). Aliquots of 50 µL were collected during the dissolution test (in SGF and SIF) and mixed with 950 µL of $ABTS^{\bullet+}$ solution and the absorbances were read after 1 min of reaction at 734 nm. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) µM of Trolox.g^{-1}. The decrease of the initial absorbance of the $ABTS^{\bullet+}$ (0.700 nm) was evaluated and plotted against of the percentage of ACNs released in the referred medium.

2.7 Statistic analysis

All the experiments were performed in Software OriginPro version 8. The experiments were carried out in triplicate and and error bars represent the standard error for three measurements of each data.

3 RESULTS AND DISCUSSION

3.1 Samples characterization

3.1.1 FTIR analysis

The FTIR spectra of native starch and carboxymethyl starch (CMS) is present in Figure 20A. The introduction of new characteristical bands resulted in several changes on native starch structure after its carboxymethylation. The CMS presented three new bands at 1417, 1600 and 1701 cm^{-1}. The two first bands were attributed, respectively, to the symmetrical and asymmetrical stretching vibration of carboxylate groups (COO-). These bands overlapped the sign of hydroxyl groups (OH-) in native starch spectrum (1640 cm^{-1}) which disappeared in CMS spectrum. The new band at 1701 cm^{-1} was related to the introduction of carboxylic groups (COOH) in CMS structure (ASSAAD; MATEESCU, 2010; BLEMUR et al., 2016). Together the assignments indicating that the carboxymethylation of the native starch has occurred successfully.

To evaluate the behaviour of CMS matrix in dissolution medium, the tablets were analysed by FTIR after incubation during 2 hrs in SGF (pH 1.2) and in SIF (pH 6.8) and after the incubation during 2 hrs in SGF and after additionally 6 hrs in SIF. In SGF was noted a that the bands at 1600 and 1417 cm^{-1} (Figure 18B) decreased due the protonation of carboxylate to carboxylic acid groups characterized by the introduction of a new absorption band at 1724 cm^{-1}. On the other hand, after the incubation in SIF the sign at 1724 cm^{-1} disappeared (Figure 20B) as consequence of the deprotonation of carboxylic acid groups to carboxylate. The previous CMS signs recurrence absorbing in the bands at 1592 and 1417 cm^{-1}.

Figure 20C-D shows the infrared spectra for the anthocyanins (ACNs), mannitol, PVP, anthocyanin-mannitol (AM) and anthocyanin-PVP (APVP). The FTIR spectra of mannitol and PVP shown specifics signs of these compounds. Mannitol spectrum (Figure 20C) exhibited a strong sign at 3300 cm^{-1} related to the OH stretching in hydrogen bonds. The sign at 2952 cm^{-1} was attributed C-H vibrations and the bands near from 1100 cm^{-1} (1411, 1281.1 and 1077 cm^{-1}) belonged to C-O and C-C stretching. In PVP spectrum (Figure 20D) its signature was registered at the signs 1648 cm^{-1} and 1290 cm^{-1} related to C=O and C-N vibrations, respectively (DHUMALE et al., 2012; LIN et al., 2016).

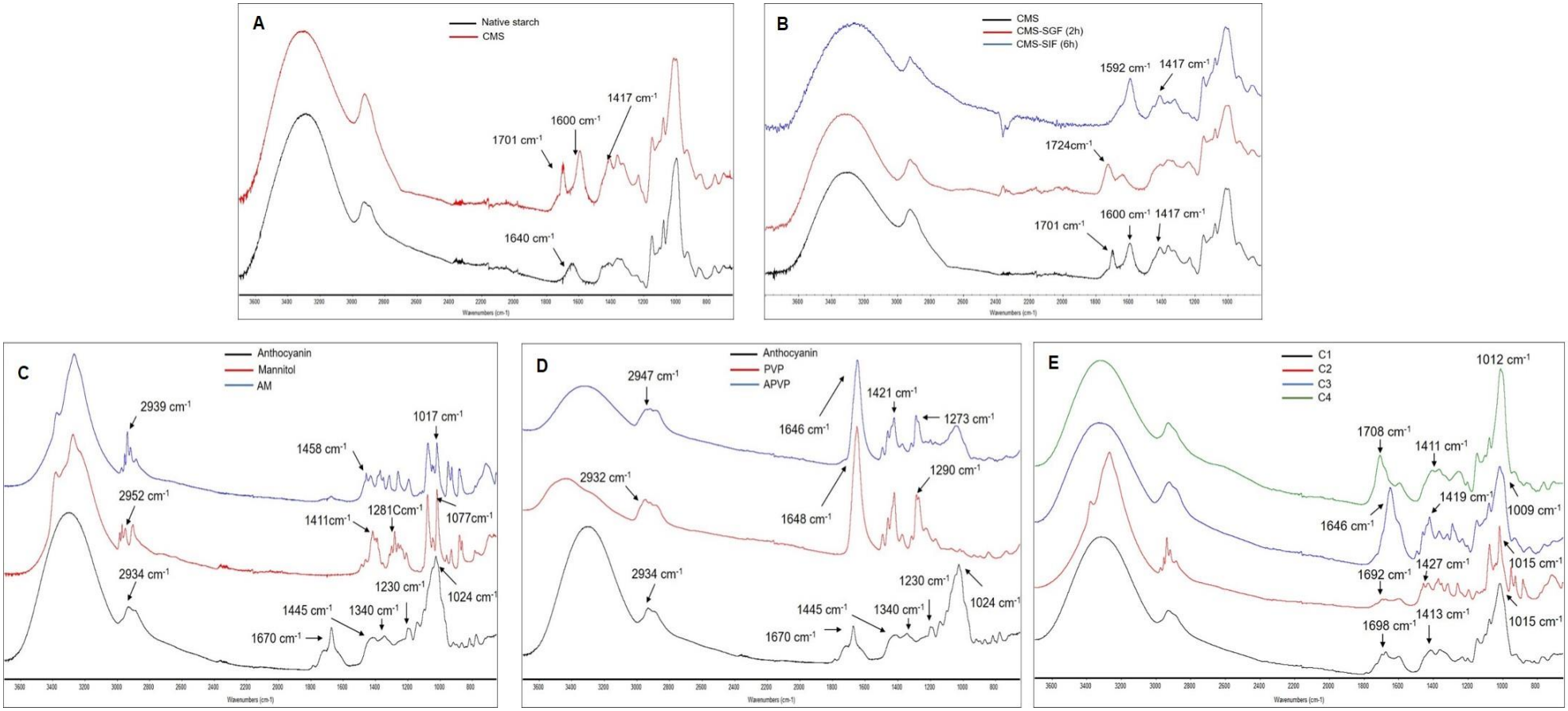
The FTIR spectra of the ACNs showed several bands characteristic of the chemical structure of these molecules. Bands at 3303 cm^{-1} are characteristics of the OH groups. Benzene groups vibrations were assigned between 692.3 - 916.9 cm^{-1} and sign at 2934 cm^{-1} is specific to the C-H stretching of benzene ring. A strong absorption at 1024 cm^{-1} is assigned to aromatic ring C-H deformation, as well as bands at 1670 and 1455 cm^{-1} to vibration of the carbonyl group (C=O) of the benzopyran aromatic ring and the stretching vibration (C=C) of the aromatic ring, respectively. A band at 1230 cm^{-1} refers to the stretching of pyran rings that is the signature of flavonoid compounds, while the sing at 1340 cm^{-1} corresponded to C-O angular deformations of phenols (GE et al., 2018; LIMSITTHICHAIKOON et al., 2015; PEREIRA; ARRUDA; STEFAN, 2015).

In AM FTIR spectrum (FIGURE 20C) the hydrogen group increased its sign intensity but was remained in the band 3300 cm^{-1}. The C-H assignments was shifted to 2936.7 $cm^{-1.}$ The peaks related to C=C and C-H stretching became more sharper, less intense and with new assigns (1458 and 1017.7 cm^{-1}, respectively). The vibration of C=O (1670 cm^{-1}) decreased substantially. In the APVP (Figure 18D) the sign of the C-H group shifted to 2947 cm^{-1}, while the C=O was overlapped at the band at 1646 cm^{-1} due its interaction of C=O groups of ACNs and APVP. The C=C peak shifted to 1421 cm^{-1} and the PVP characteristic peak for C-N became 1273.2 cm^{-1} due it interaction whit ACNs pyran rings (LABORDE et al., 2006).

Figure 20E presents the FTIR spectrum of the tablets. Signs nearby from 1650 cm^{-1} (C1: 1698 cm^{-1}; C2: 1692 cm^{-1}; C3 cm^{-1}: 1646 cm^{-1}; C4: 1708.6 cm^{-1}) were resulted from the interaction of OH- and C=O with COO- groups from ACNs and CMS, respectively. The characteristic peak of ACNs, at 1230 cm^{-1}, corresponding to stretching of pyran rings disappeared, while the assignment of aromatic ring C-H deformation (at 1024 cm^{-1}) changed in all tablet formulations spectra, especially on C4 where became more intense at 1012 cm^{-1} (Figure 20E). C4 spectra showed more intense changes in the new signs, probably resulted of the freeze-drier process which allowed the greater interaction of ACNs and CMS. In general, the clear alteration in the region between 1000-1800 cm^{-1}, verified in all tablets spectra, indicated the presence of aromatic rings with ortho substitution of ACNs and consecutively the association between the tablets components (PEREIRA; ARRUDA; STEFAN, 2015).

The FTIR data seems to confirm the interactions between the components of the monolithic tablets especially verified in intense change of the ACNs structure after their association with mannitol, PVP and CMS.

Figure 20 - FTIR spectra of **(A)** native starch and CMS; **(B)** CMS tablets incubated in SGF (pH 1.2) for 2 h followed by SIF (pH 6.8) for additional 6 h, **(C)** anthocyanin extract, mannitol and the complex anthocyanin-mannitol (AM), **(D)** anthocyanin extract, polyvinylpyrrolidone and the complex anthocyanin-polyvinylpyrrolidone (APVP), and **(E)** tablets formulations C1, C2, C3 and C4. The individual FTIR spectra is indicated by the specific colours in the figure legend.



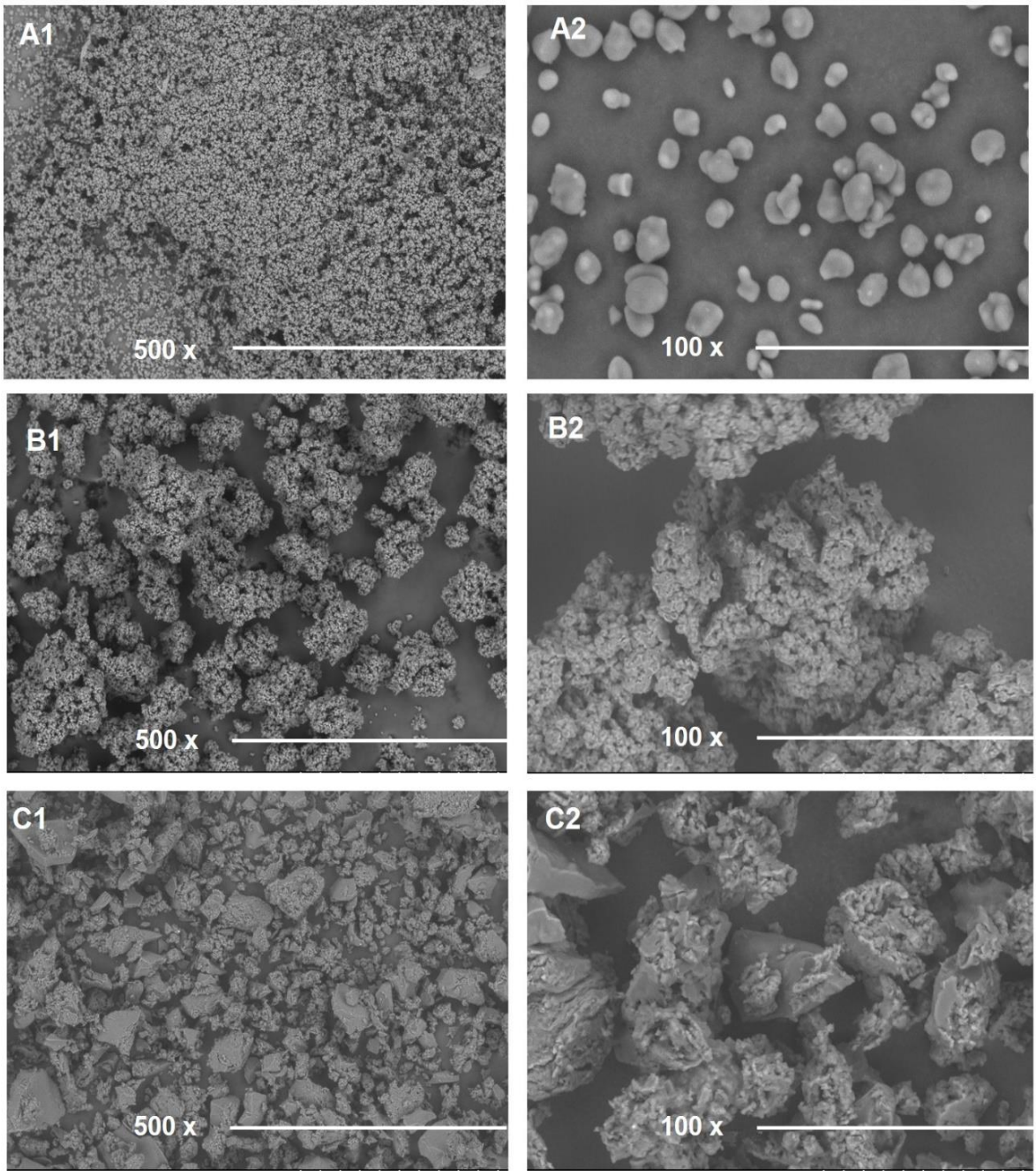
Source: Elaborated by the author.

3.1.2 Scanning electron microscopy (SEM)

The electromicrographs of native starch, CMS and the ACNs-CMS complex are exhibited in Figure 21. The morphology of native starch was characterized by granules with irregular sizes, rounded in shape and with smooth surface. Hyllon VII presents in its constitution a higher quantity of amylose and its structure is characterized by a double helix-B form, stabilized by hydrogen bonds between the hydroxyl groups of glucopyranose units (FRICIU et al., 2013). Differently from native starch, CMS micrographs (Fig. 21, B1 and B2) demonstrated granules with irregular shape and a pronounced agglomeration of smaller particles. The wrinkled surface presented by CMS may was resulting of the structural reorganization promoted by the interaction between hydrogen and hydroxyl groups (LEMIEUX; GOSSELIN; MATEESCU, 2010).

The structural characteristics from the association of ACNs and CMS are shown in the Fig 21C. The external morphology of the powder obtained after the freeze-drying process presented different from those exhibited for native starch and CMS indicating that structural changes occurred. The SEM micrographs exhibited a structure similar to broken glass with different sizes and shapes typical of freeze-dried materials (KUCK; PELAYO; NOREÑA, 2016; YAMASHITA et al., 2017). That glassy structure revels a strong association between ACNs and CMS, also observed in FTIR analysis, which may explain the stabilization of these pigments (YAMASHITA et al., 2017).

Figure 21 - Scanning electron microscopy micrographs of **(A)** native starch – Hyllon VII, **(B)** CMS, and **(C)** ACNs-CMS complex at magnifications of 500 x (1) and 100 x (2).

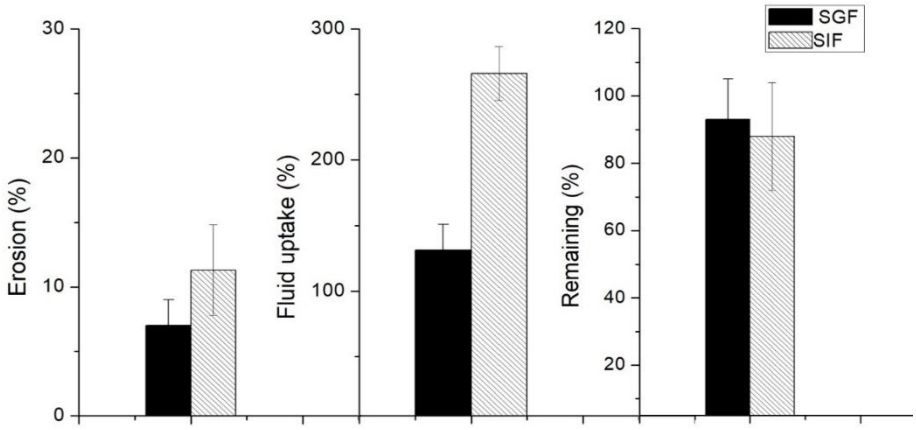


Source: Elaborated by the author.

3.1.3 Erosion and fluid uptake

Erosion and fluid uptake studies were carried out in unloaded CMS tablets to evaluate primarily the effect of CMS-fluid interaction that will enable the release of ACNs in simulated medium. CMS tablets were kept incubated for 2 h in SGF (pH = 1.2) or SIF (pH = 6.8) to simulate the retention time in the stomach or intestine, respectively, and evaluate the effect of pH medium on tablets erosion and fluid uptake. As shown in Figure 22, the CMS tablets presented greater percentage of fluid uptake and erosion in SIF than in SGF medium. The percentage of CMS remaining in the matrix, provides the idea of the polymer amount dissolved in the fluids. After 2 hours of dissolution in SGF and SIF, the tablets presented 93 and 88% of mass remaining, respectively, that is, the mass loss no exceeded 20% of tablet weight in both cases.

Figure 22 - Erosion, fluid uptake and mass remaining by CMS unloaded tablets (500 mg, n=3) after 2 h in SGF or 2 h in SIF (37 ◦C, 100 rpm).



SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid.
Source: Elaborated by the author.

The differences on the fluid uptake and tablets erosion, between SGF and SIF, are attributed to different matrix hydration degrees resulted by the change in the pH of these medium (SRIAMORNSAK; THIRAWONG; WEERAPOL, 2007). The high hydrogen ion concentration in acid medium, contributes to the protonation of CMS carboxylate groups which will ensure the network stabilization of the matrix by hydrogen bonds and result in the lower fluid uptake and tablet erosion in SGF. On the other hand, in SIF, the CMS deprotonation result in a higher hydration of the COO^- groups and, consequently, in a higher fluid absorption and

tablet erosion (ASSAAD; MATEESCU, 2010). These results are also supported by the FTIR previous data.

Once the mechanism of release is dependent on the swelling characteristics of polymeric matrices the results obtained in this section can explain the behaviour of CMS matrix on the ACNs release study at different pH examined ahead.

3.2. In vitro anthocyanins release study

3.2.1 Study of ACNs release from the CMS-based tablets in simulated medium

The behaviour of ACNs release from C1, C2, C3 and C4 tablets were evaluated in SGF (pH =1.2) or SIF (pH = 6.8) to mimic the gastric and intestinal residence, respectively. The dissolution profiles at 37 °C were presented in Figure 23. ACNs release was influenced by the pH of the medium. From the dissolution profile presented after SGF incubation (Figure 23A), we noticed that at the first 2 hrs were released more than 30% of the ACNs loaded in all tablets. However, the rate release decreased after 5 hrs and remained stable at 7 hrs of dissolution. At the end, the amount of ACNs released in this medium were 81.55; 84.2; 80.2 and 85% for the tablets C1, C2, C3 and C4, respectively. Figure 24 presented the physical characteristics of each tablet after the dissolution. According to Wang et al. (2013) the electrostatic interactions between ACNs and charged polymers are modulate by the pH changing. The stabilization of ACNs-CMS interactions was indicated by the remaining coloration belonging to the ACNs amount unreleased (average of 18%) after 9 hrs of dissolution. Visually, it was verified that the tablets kept their shape and did not swelling, however, C2 presented a considerable loss of mass, which can be attributed to the increase of its erosion caused by mannitol content (Figure 24).

In SIF dissolution medium, the ACNs releasing presented a different pattern than presented in SGF, being characterized by a slower initial release in the first hour of experiment (Figure 23B). However, from 4 to 8.5 hrs of dissolution, the ACNs releasing was progressively increased in the C1, C2 and C4 tablet. That result is reasonable, once in the neutral medium, CMS matrix presents a higher hydration, becoming it more permeable; as result, the ACNs was easily diffused through the gel-layer, reaching a greater percentage released (LAMOUDI; CHAUMEIL; DAOUD, 2016). The C4 tablet presented the highest percentage of ACNs released, similarly to the observed in SGF dissolution. At 9 hrs the tablets were unstructured as

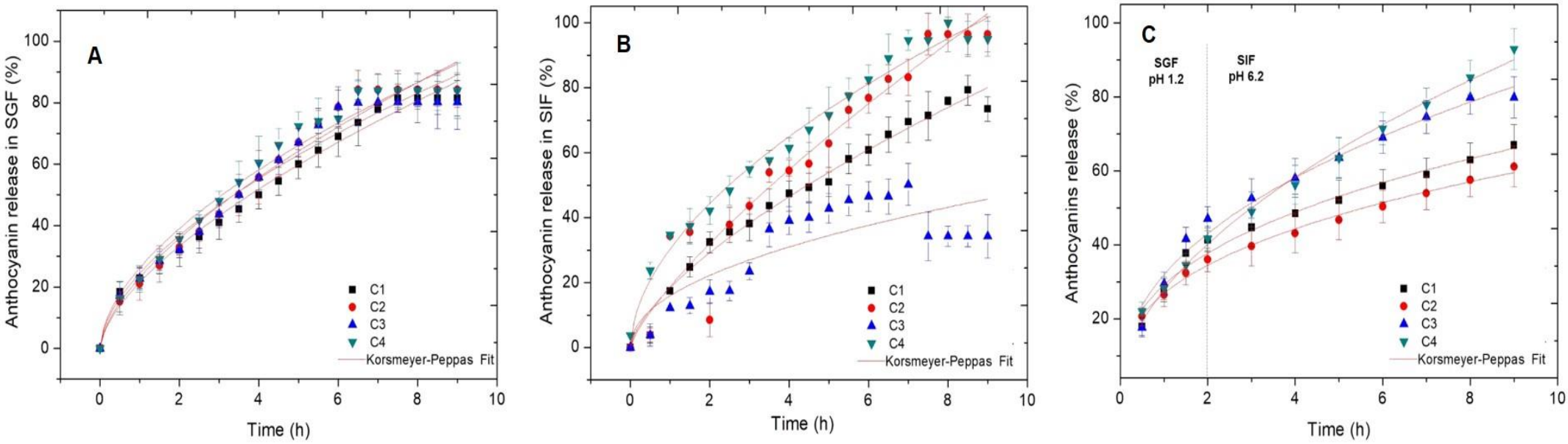
the result of the higher hydration and erosion of CMS matrix in SIF as shown in Fig 23B. At this point, C1 tablet has been completely disintegrated; C2 and C3 showed a larger mass loss, possibly related to the mannitol and PVP present in their respective composition, which may enhance the tablets erosion; At last, C4 tablet exhibited a pronounced swelling but maintained its structure. It is interest to note that the reduced pigmentation shown by the tablets, has matched with the amount of ACNs dissolute in SIF medium, that were higher than in SGF. However, the longer exposition of ACNs in neutral medium resulted in their degradation, indeed verified at 9 hrs of dissolution of all tablet (Figure 23B) (CASTAÑEDA-OVANDO et al., 2009; FLORES et al., 2015).

In order to simulate the transit from stomach to intestine the tablets were first incubated in SGF for 2 hrs and then transferred to a fresh SIF medium until complete dissolution (approximately 7 hrs). The dissolution profiles were obtained at 37 °C and are shown in Figure 23C. As expected, the initial 2 hrs of dissolution followed the pattern previously presented in Figure 23A, and at that point, the percentage release ranging from 36 to 47%. After the incubation in SGF the tablets were separated and changed to intestinal-mimic medium. Was verified that increase of pH from 1.2 to 6.8 was followed by the increase of the ACNs release in the medium, noted since the first hour until the end of incubation (Figure 23C). As previously described, the pH can tune the interaction between ACNs and CMS. According with literature that affinity is mainly determinate by the weakly charged specie, in our case by ACNs charge (LI et al., 2010). Due SIF pH conditions, the positive charges of ACNs are reduced, decreasing so their binding affinity with CMS which led to the effective dissolution in the medium (WANG et al., 2013). No pronounced ACNs degradation was verified in the SGF/SIF dissolution, but C2 presented a percentage released lower than expected (61.2%). The results together pointed that due the combination of swelling and erosion, the ACNs can be progressively release from the tablets for at least 9h of test.

In our research mannitol and PVP were evaluated as bulking agents in C2 and C3 tablets formulations. These adjuvants are commonly used for drugs formulations and can act in the protection od active agents during the drugs processing or increase their release, for example (MADERUELO; ZARZUELO; LANAO, 2011). However, the presence of these compounds did not influence the ACNs release, considering that the amount released from C2 and C3 never exceeded 80% in SGF/SIG (Figure 23C). From the dissolution profiles was clearly noted that C4 tablet presented the greater stability, releasing more than 90% payload in the simulated

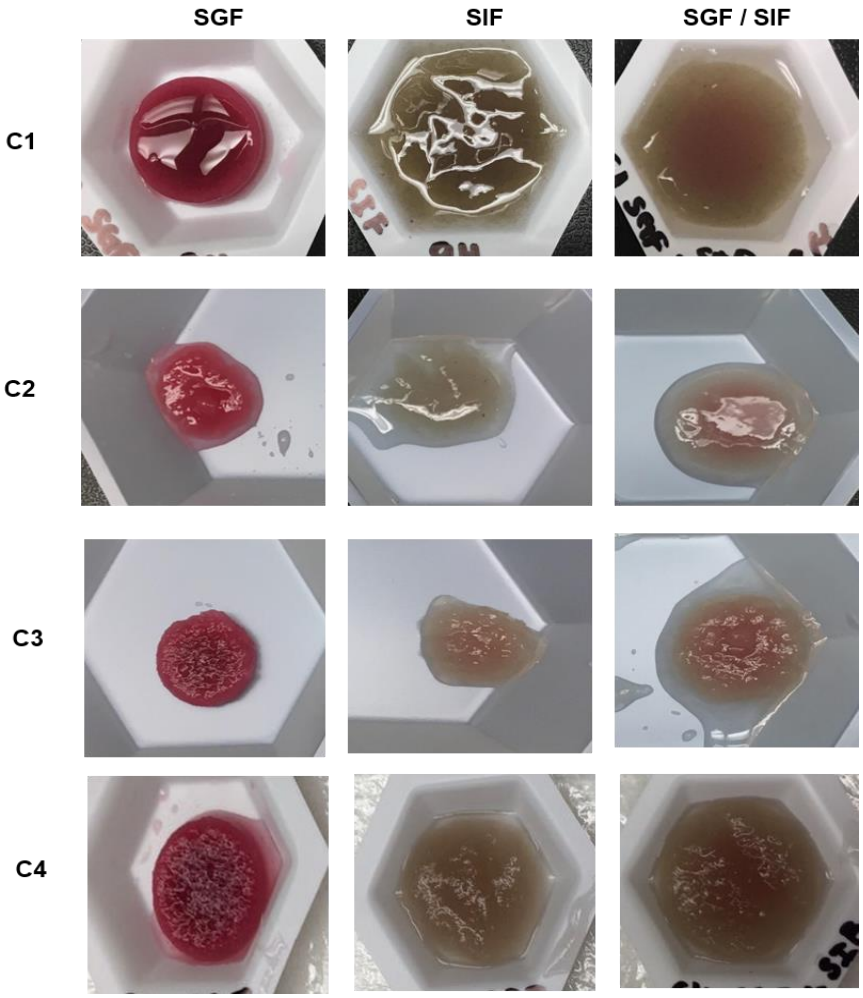
gastrointestinal medium, being the most suitable to perform the ACNs delivery for a prolongated time.

Figure 23 - Release kinetic of ACNs dissolution from the CMS based-tablets (500 mg, n = 3) incubated in: **(A)** SGF (pH 1.2) for 9 hrs, **(B)** SIF (pH 6.8) for 9 hrs, and **(C)** in SGF for 2 hrs and then transferred in SIF until complete dissolution at 37 °C.



Source: Elaborated by the author.

Figure 24 - Physical and structural characteristics of the tablets after dissolution in SGF (pH 1.2), SIF (pH 6.8), and in SGF/SIF for 9 hrs at 37°C.



Source: Elaborated by the author.

3.2.2 Modelling of the anthocyanins release

The ACNs release data were fitted to the Korsmeyer–Peppas model and the parameters, from for the dissolution in SGF, SIF and SGF/SIF, are shown in Table 12. The dissolution data are used to determine the mechanism of ACNs release for the model in the case $Mt/M\infty \leq 70\%$. In general the mechanism of drug release from matrices as CMS (i.e. swellable polymers) is complex and not completely understood, thus in some systems the release can occurs by either purely diffusion or erosion controlled, while most systems exhibit a combination of these (GLAESSL et al., 2010; LAMOUDI; CHAUMEIL; DAOUD, 2016). In our study, considering the correlations coefficient (R^2) higher or equal to 0.90, some dissolution

data showed a good fit with Korsmeyer–Peppas equation suggesting a combinate effect of diffusion and erosion mechanisms for ACNs release (KORSMEYER et al., 1983).

The data obtained to SGF medium revealed a high correlations coefficient ($R^2 \geq 0.95$) for the tablets in acid medium. The formulations presented n values in the range of 0.5 - 0.87, corresponding to an anomalous (non-Fickian) diffusion mechanism (i.e. $0.45 < n < 0.89$), which suggests that both diffusion of the ACNs in the hydrated matrix and its own erosion modulate the release (LAMOUDI; CHAUMEIL; DAOUD, 2016). In this medium the values of the log of the kinetic constant (k) was found to vary slightly according to the tablet formulation.

In the SIF, on the other hand, the model did not show a good fit into Korsmeyer–Peppas equation for the tablets, probably due to the extensive swelling of CMS in neutral medium. Exception for C1 ($R^2 = 0.95$) and C4 ($R^2 = 0.90$). The values of n were larger than 0.89 for a C1, C2 and C4, corresponding to a 'Super case II' transport, in which ACNs release, apparently is mainly due the polymer swelling (SRIAMORNSAK; THIRAWONG; WEERAPOL, 2007; WEI et al., 2006). We noticed that the values of the log (k) were slightly higher in SGF than SIF that may be attributed to ACNs slower initial diffusion in neutral medium due the CMS gel layer forming. That results also indicated that release of ACNs from the tablets has pH sensitivity and decreases with increasing pH (WEI et al., 2009).

The data obtained from SGF/SIF dissolution presenting values of R^2 greater or equal to 0.95 for all tablets. The values of n were ranged from 0.41 to 0.80 and the values of log (k) were similar than presented for the SGF profile. C2 and C4 tablets presented n values closely from 0.45 indicating that release mechanism was determinate by Fickian diffusion, that is, the ACNs release from the both tablets were controlled by the diffusion. The n value of C1 and C3 tablets are indicative of an anomalous (non-Fickian) diffusion release, however in C1 ($n = 0.50$) the diffusion seems to be the primary mechanism of release, differently from C3 ($n = 0.80$) in which is the erosion. According to Table 12 the dissolution data obtained from all tablets in SGF and SGF/SIF can be well described by the Korsmeyer–Peppas model ($R^2 \geq 0.95$ for all data set), specially C4 which has presented the high correlation in both cases.

Table 12 - Modelling of ACNs release from CMS-based tablets.

Samples	Korsmeyer-Peppas model											
	SGF				SIF				SGF/SIF			
	n	log(k)	R^2	Mechanism	n	log(k)	R^2	Mechanism	n	log(k)	R^2	Mechanism
C1	0.5	1.47	0.97	Anomalous	0.94	1.06	0.95	Super II case	0.51	1.47	0.96	Anomalous
C2	0.75	1.44	0.95	Anomalous	0.99	1.14	0.80	Super II case	0.41	1.40	0.95	Fickian diffusion
C3	0.87	1.50	0.97	Anomalous	0.62	1.07	0.80	Anomalous	0.80	1.50	0.97	Anomalous
C4	0.74	1.45	0.99	Anomalous	1.13	1.1	0.90	Super II case	0.46	1.44	0.98	Fickian diffusion

SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid; SGF/SIF: Simulated gastrointestinal transit.
Source: Elaborated by the author.

3.2.3 Formulation optimization

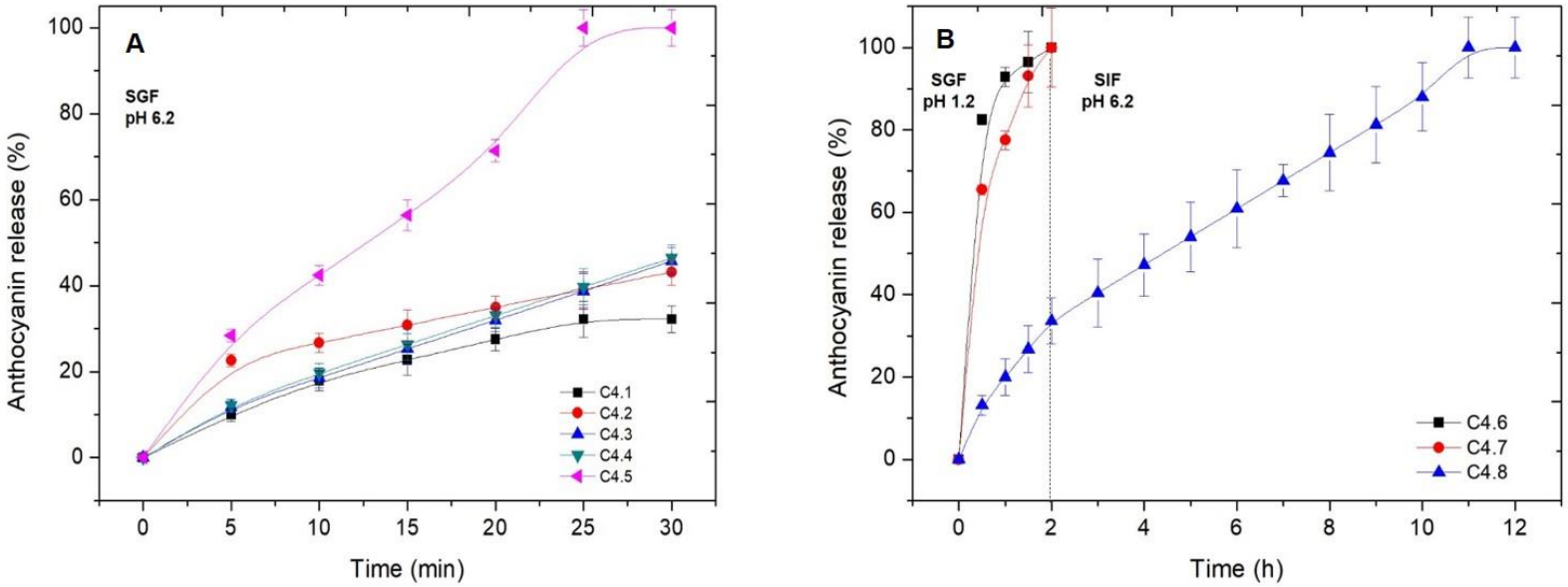
Considering the previous results, the formulation of C4 tablet was chosen and optimized to evaluated the programmable ACNs release from the modifications in its composition to reach a desired release time. Thus, from C4 were derived the formulations C4.1, C4.2, C4.3, C4.4 and C4.5 which presented in their formulations different disintegrating agents (Table 12) do perform the fast release. The percentage release was evaluated in stomach-mimic medium during 30 min and the profiles are shown in Figure 25A. From the dissolution data it was observed that C4.5 tablet, containing Explotab® and microcrystalline cellulose (MCC), presented totally disintegration in approximately 25 min of dissolution, releasing 100% of its ACNs content. Otherwise the other formulations did not completely disintegrate and the amount released no exceeded 50% payload. The fitted proportions of both disintegrating agents resulted in completely ACNs released in 30 min of dissolution. Explotab® is a commercial low substituted carboxymethyl starch that can promote a rapid fluid uptake, leading to a substantially increase in the volume of granules and the consecutive tablet disintegration (SHARMA; SONAWANE, 2017). On another hand, MCC resulted in a highly hygroscopic of the tablet, due the increase of the porosity which will lead to a good disintegration and consecutive ACNs release (YASSIN et al., 2015). The C4.5 tablet can be classified as fast or immediate dosage form, once 100% of labelled amount of ACNs were dissolves within 30 min of dissolution (KAUR; MEHARA, 2016).

Aiming to prolong the ACNs release, simulating an intestinal-targeted delivery, more three formulations were developed from C4. Thus, C4.6, C4.7 and C4.8 tablets were kept on dissolution during 12 hrs in SGF (2 hrs) and SIF (10 hrs). The dissolutions profiles are exhibited in the Figure 25B. All formulations presented in their composition crosscarmellose sodium (CCS) and MCC but in different proportions (Table 12). For C4.6 and C4.7 tablets a bursting release was noted in 30 min and after 2 hrs these tablets released 100% of their ACNs content, demonstrating that was not been suitable to intestinal delivery of ACNs, once released all content in gastric simulated medium in 2 hrs of assay. Otherwise, we noticed a progressive released of ACNs from the C4.8 tablet until 12 hrs of dissolution. A greater amount of C4 (80% w/w of the tablet) and a less amount of croscarmellose sodium (CCS) and MCC (both 10% w/w of the tablet) make up C4.8 tablet. CCS is described as a cross-linked polymer of

carboxymethylcellulose and once in dissolution promotes an increase on the swelling of the tablets, leading to the drug release (ZHAO; AUGSBURGER, 2006).

In this research the combination of swelling and erosion promoted by CCS and MCC, led to the progressive release of ACNs during the dissolution covering the gastric and eventually an intestinal transit where the ACNs were completely released. According to Fernandes et al. (2014) the health benefits associated with ACNs ingestion can be explained by their a slow and continuous through the gut into the bloodstream. From the C4.8 tablet dissolution profile we conclude that this dosage form is suitable to delivery of these molecules on the intestine, boosting their bioavailability and extend possible therapeutic effects.

Figure 25 - Dissolution profile of the ACNs released from (A) the tablets formulated for the fast release in SGF for 30 min at 37°C, and (B) for the tablets formulated to sustained release in SGF for 2 hrs and then transferred in SIF for 10 hrs at 37 °C.

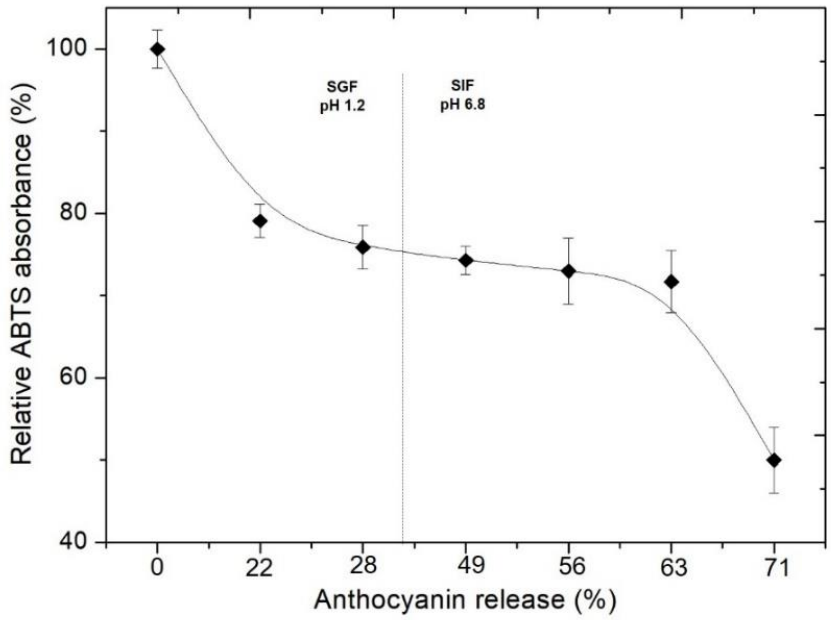


Source: Elaborated by the author.

3.4 Antioxidant capacity of the ACNs released in simulated medium

The ability in reducing the free radical $ABTS^{\bullet+}$ was evaluated for the ACNs released from C4 tablet in SGF/SIG medium and the results are shown in Figure 26. As expected, was observed that an increase in the ACNs concentration in the medium resulted in a decrease of the free-radical concentration. From Figure 26 we verified that 50% of the $ABTS^{\bullet+}$ were reduced when the released of ACNs reached approximately 71%. ACNs are substances easily oxidise and are reported to promote the stabilization of reactive free radicals by the donation of a free electron or hydrogen atoms (CASTAÑEDA-OVANDO et al., 2009).

Figure 26 - Scavenging effect of anthocyanins released form dosage form in simulated dissolution medium.



Source: Elaborated by the author.

The ACNs metabolism is not complete understood, but appears that these substances are quickly absorbed both by through the gastric wall and in small intestine, reaching the bloodstream and being available to exert their biological activities (FERNANDES et al., 2012; NORBERTO et al., 2013). However, ACNs present a low bioavibilty in the plasm after the metabolism process (KHOO et al., 2017) with reduce their potential benefits in the organism. On another hand, after 7 hours we observed an antioxidant capacity of 327μM Trolox.g^{-1} in intestinal medium and 271μM Trolox.g^{-1} in gastric medium, for the ACNs released

from C4 tablet. The results mean that the continuous release of ACNS in the simulate medium can boost their bioavibility. The difference between the simulated medium was reasonable to expect once the percentage released in SIF was 10% higher than that found for the SGF at this time.

4 CONCLUSIONS

Anthocyanins extracts was easily combinate with CMS to obtain different tablets formulations. The ACNs were released from the CMS matrix due a combination of swelling and their erosion, that occurred mainly in neutral conditions presented by SIF. The presence of adjuvants in the formulations did not present influence on ACNs release rate, on another hand, the C4 tablet, composed only by ACNs and CMS, presented the most stable dissolution profile, releasing more the 90% of the payload in SGF/SIF medium after 9 hrs, being therefore suitable for ACNs targeted delivery. The Korsmeyer–Peppas model was good fitted with the ACNs release for all tablets evaluated in SGF or in SGF/SFI, presenting value R^2 higher than 0.90. However, the evaluation of the exponent n was revealed the ACNs release mechanism has varied mainly due the dissolution medium. The dosage-forms derivate from C4 table were able to release of 100% of their ACNs content in 30 min or sustain the release for more than 11 hrs of dissolution, which means that modification in the tablet formulation allowed the ACNs released in different sites of the gastrointestinal tract. The CMS is suitable as excipient for ACNs delivery, promoting stabilization and protection of these substances, which will impact in the increase of their bioactivity in different systems. This paper presents useful information for the future studies related to the use of CMS as carrier of ACNs, prospecting an extension of the application of these and other natural substances in function of their biological properties

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6 CONCLUSÕES

As ideias que fundamentaram a execução desta pesquisa foram a avalição de uma possível nova fonte vegetal para a extração de antocianinas, de um método situável para sua extração e a aplicação final destes pigmentos.

O jambolão é um fruto amplamente disponível no Nordeste brasileiro e apresenta grande potencial para estudos tecnológicos em função da sua composição química. Os resultados apresentados nesta pesquisa, em adição aos já existentes na literatura, reforçam e prospectam uma maior participação deste fruto na pesquisa de antocianinas e outros fitoquímicos de interesse industrial.

A partir do estudo sistemático e otimização dos parâmetros que regem os métodos de extração avaliados nesta pesquisa, desenvolveu-se modelos ajustados que puderam explicar e predizer a extração das antocianinas do jambolão. Entre os métodos avaliados o ultrassom de ponteira foi aquele que resultou em uma maior recuperação dos pigmentos, no entanto, diferenças qualitativas não foram observadas no perfil antociânico dos extratos obtidos em cada método.

O carboximetilamido se mostrou um excipiente adequado para o transporte e liberação de antocianinas em função de suas propriedades de hidratação e formação de gel, o que controlou a liberação dos pigmentos nos meios que se propuseram a simular o ambiente gástrico e intestinal. As formas de liberação rápida e prolongada foram desenvolvidas com êxito e podem resultar na liberação de antocianinas em diferentes zonas no trato gastrointestinal de acordo com a ação desejada.

Os resultados apresentados nesta Tese são úteis para estudos futuros que venham avaliar a potencial das antocianinas como princípio ativo na elaboração de suplementos, fármacos ou ingredientes na indústria alimentícia, por exemplo.

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