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Faculdade de Engenharia de Alimentos

ELENICE CARLA EMIDIO CUNHA

Evaluation and characterization of the bioactive compounds of camu-camu [*Myrciaria dubia* (H.B.K.) Mc Vaugh].

Avaliação e caracterização dos compostos bioativos do camu-camu [*Myrciaria dubia* (H.B.K.) Mc Vaugh].

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AVALIAÇÃO E CARACTERIZAÇÃO DOS COMPOSTOS BIOATIVOS DO CAMU-CAMU [*Myrciaria dubia* (H.B.K.) Mc Vaugh].

Thesis presented to the Faculty of the Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in the area of Food Science.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciência de Alimentos.

Orientadora: Profa Dra Helena Teixeira Godoy

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A ata de defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica da aluna.

"Hoje me sinto mais forte
Mais feliz, quem sabe
Só levo a certeza
De que muito pouco sei
Ou nada sei"

(Almir Sater & Renato Teixeira)

"Noventa por cento do sucesso se baseia simplesmente em insistir"

(*Woody Allen*)

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RESUMO

O camu-camu, fruto nativo da região Amazônica, de mercado ainda pouco explorado, desperta interesse pelo elevado conteúdo de vitamina C e por apresentar uma gama de compostos antioxidantes. Esse estudo teve como objetivo: comparar métodos de extração e análise de vitamina C; caracterizar, identificar e quantificar os compostos fenólicos; avaliar a capacidade antioxidante por desativação de espécies não-fisiológicas e espécies reativas de oxigênio (ROS); determinar a atividade antimicrobiana do camu-camu colhidos em diferentes locais do Brasil e Peru. Para a extração do ácido ascórbico das polpas, foram aplicadas três técnicas de extração, dentre elas a PLE (*Pressurized Liquid Extraction*). Os resultados foram expressos em peso seco (ps), e mostraram que, para o ácido ascórbico, a extração ácida obteve os maiores rendimentos (38.37 g/100 g ps), seguido da PLE (30.04 g/100 g ps) e a maceração (22.66 g/100 g ps). O teor de vitamina C total foi determinado por titulação e por cromatografia líquida de ultra-alta eficiência (UHPLC-DAD), os resultados da titulometria mostraram que o método pode ser útil para análises de rotina, no entanto, a cromatografia permitiu a identificação de L-AA e DHAA. A extração de compostos fenólicos e flavonóides foi otimizada empregando um planejamento centróide simplex. A melhor condição de extração para os compostos da casca e polpa do camu-camu foi uma mistura de etanol e água (80:20, v/v), enquanto que para a semente foi metanol e água (50:50, v/v). As sementes e as polpas apresentaram as maiores concentrações de compostos fenólicos, exceto a polpa comercial. O ácido fenólico *p*-cumárico e outros cinco flavonóides (luteolina, quercetina, rutina, epicatequina, catequina) foram identificados por espectrometria de massas sequencial. Os resultados da capacidade antioxidante *in vitro* frente a radicais $ABTS^{\bullet+}$ e $DPPH^{\bullet}$, capacidade de redução do ferro (FRAP), e por espécies reativas de oxigênio radicalares ($ROO^{\bullet}$ e $O^{\bullet}_2^{-}$), e não radicalares (H_2O e HOCl) foram avaliados, e demonstraram que o camu-camu é eficiente na proteção contra oxidação. Os extratos obtidos das cascas e sementes, principalmente, foram responsáveis pela inibição de *Staphylococcus aureus* (0,02 – 0,62 mg/mL), *Bacillus cereus* (0,04 – 0,62 mg/mL) e *Escherichia coli* (0,04 – 0,31 mg/mL). Portanto, o potencial bioativo do camu-camu e seus subprodutos é um incentivador para a progressão do seu cultivo em diversas regiões, e para a aplicação em indústrias alimentícias, cosméticas e farmacêuticas.

Palavras-chave: *Myrciaria dubia*, vitamina C, compostos fenólicos, espécies reativas de oxigênio (ROS), cromatografia líquida de ultra eficiência (UHPLC).

ABSTRACT

Camu-camu, a native fruit from the Amazon region, whose market opportunities are yet to be explored, arouses interest for the high content of vitamin C and a range of antioxidant compounds. The objective of this study was to compare methods of extraction and analysis of vitamin C; to characterize, identify and quantify the phenolic compounds; to evaluate the antioxidant capacity by scavenging of non-physiological species and reactive oxygen species (ROS); to determine the antimicrobial activity of camu-camu harvested at different locations in Brazil and Peru. For the extraction of ascorbic acid from the pulps, three extraction techniques were applied, among them the PLE (*Pressurized Liquid Extraction*). The results were expressed as dry weight (dw) and it showed that for ascorbic acid, the acid extraction achieved the highest yields (38.37 g/100 g dw), followed by PLE (30.04 g/100 g dw) and maceration (22.66 g/100 g dw). The total vitamin C content was determined by titration and by ultra-high performance liquid chromatography (UHPLC-DAD), the titrimetry result showed that the method may be useful for routine analyzes; however, chromatography allowed the identification of L-AA and DHAA. The extraction of phenolic compounds and flavonoids was optimized using simplex centroid planning. The best extraction condition for the compounds from the camu-camu peel and pulp was a mixture of ethanol and water (80:20, v/v), whereas for the seed it was methanol and water (50:50, v/v). Seeds and pulps presented the highest concentrations of phenolic compounds, except commercial pulp. The *p*-coumaric phenolic acid and five other flavonoids (luteolin, quercetin, rutin, epicatechin, catechin) were identified by sequential mass spectrometry. The results of the *in vitro* antioxidant capacity against radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, iron reduction capacity (FRAP), and by radical reactive oxygen species ($ROO^{\bullet}$ and $O^{\bullet}_2^-$) and of not radical (H_2O and HOCl) were evaluated, and demonstrated that the camu-camu has effective in protecting against oxidation. The extracts obtained from peels and seeds, mainly, were responsible for the inhibition of *Staphylococcus aureus* (0.02 – 0.62 mg/mL), *Bacillus cereus* (0.04 – 0.62 mg/mL) and *Escherichia coli* (0.04 – 0.31 mg/mL). Therefore, the bioactive potential of camu-camu and its by-products is an incentive for the progression of its cultivation in various regions, and for the application in the food, cosmetic and pharmaceutical industries.

Keywords: *Myrciaria dubia*, vitamin C, phenolic compounds, reactive oxygen species (ROS), ultra-high performance liquid chromatography (UHPLC).

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INTRODUÇÃO GERAL

O consumo de frutas tropicais está aumentando no mercado nacional e internacional devido ao crescente reconhecimento de suas propriedades nutricionais e potencial funcional (Costa et al., 2013). Além de nutrientes essenciais, a maioria das frutas contém minerais, fibras, vitaminas e compostos fitoquímicos secundários que estão relacionados à prevenção de doenças cardiovasculares, circulatórias, cancerígenas e neurológicas, além disso, também possuem atividade anti-inflamatória, antialérgica, antitrombótica e antimicrobiana (Rufino et al., 2010; Obon et al., 2011).

A produção mundial de frutas frescas corresponde a 800 milhões de toneladas anuais, e o Brasil é considerado o terceiro maior produtor do mundo, com um total de 41,5 milhões de toneladas, ficando atrás da China e da Índia (FAO, 2015). O cultivo de frutas durante todo o ano é possível graças à ampla extensão territorial e às condições climáticas favoráveis. Além disso, o país se destaca pela extensa biodiversidade de espécies frutíferas nativas e exóticas, sendo muitas espécies ainda desconhecidas e, por isso, poucos frutos encontram-se disponíveis no comércio para consumo ou para desenvolvimento de novas pesquisas (Fujita et al., 2013; Ribeiro et al., 2016).

Nativo da bacia Amazônica, o camu-camu é um fruto cujo habitat natural são as regiões alagadas dos rios e igarapés, onde os caules dos arbustos ficam permanentemente submersos (Nascimento et al., 2013). No entanto, nos últimos anos, pesquisadores vêm tentando melhorar geneticamente suas sementes para permitir o cultivo em regiões secas, aumentando sua comercialização e tornando sua produção mais acessível à população (Nascimento et al., 2013; Pinto et al., 2013; Ribeiro et al., 2016). Atualmente, o camu-camu vem sendo cultivado em terra firme, no interior de São Paulo, na região do Vale do Ribeira e Mirandópolis, onde o cultivo tem se mostrado promissor e economicamente viável (Oliveira, 2013). No exterior, o camu-camu atrai importadores do Japão, Europa e EUA, sendo o Peru o principal exportador (Arévalo, 2007).

O alto conteúdo de vitamina C encontrado no camu-camu (Costa et al., 2013; Chirinos et al., 2010; Grigio et al., 2015; Neves et al., 2015; Ribeiro et al., 2016), eleva o potencial do fruto para ser utilizado como alimento funcional, e para exploração comercial, principalmente para a produção de concentrados e suplementos no mercado de produtos naturais, uma vez que o seu consumo *in natura* é baixo devido à alta acidez. Dessa forma, o camu-camu poderá ser utilizado principalmente como ingrediente, que adicionado a outros

produtos alimentares agregará valor nutricional aos mesmos (Fracassetti et al., 2013; Neves et al., 2015).

Outros fitoquímicos, como os compostos fenólicos, β-caroteno e antocianinas, já foram encontrados no camu-camu. Esses compostos são relatados como promotores da saúde, pois tem ação antioxidante, anti-inflamatória, hipocolesterolêmica e/ou anticarcinogênica (Almeida et al., 2011; De Souza Schmidt Gonçalves et al., 2010). Mas os estudos que relatam as propriedades fitoquímicas do camu-camu ainda são recentes, principalmente dos frutos cultivados em solo seco (Fujita et al., 2013; Ribeiro et al., 2016). Associado ao estudo dos fitoquímicos, técnicas de extração alternativas, como a Extração Líquido Pressurizado (PLE), estão sendo utilizadas com sucesso. As vantagens incluem o alto rendimento de extração e o uso de solventes seguros, como o etanol e a água, ou a mistura de ambos (Herrero et al., 2013).

Diante da crescente descoberta de frutos da região Amazônica com grande potencial nutricional e econômico, o camu-camu vem despertando o interesse da comunidade científica, dos agricultores e consumidores, além das indústrias alimentícias e farmacêuticas. Nesse quesito, a caracterização dos componentes bioativos do fruto e seus subprodutos torna-se importante para incentivar a produção, o consumo e complementar a base de dados científicos.

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OBJETIVOS

OBJETIVO GERAL

Caracterizar, identificar e quantificar os compostos bioativos das cascas, polpas e sementes do camu-camu cultivado em diferentes regiões do Brasil e Peru, além de avaliar a capacidade antioxidante e antimicrobiana desses frutos.

OBJETIVOS ESPECÍFICOS

- Comparar as técnicas: Extração por Líquido Pressurizado (PLE), Extração Ácida e Maceração para extração de ácido ascórbico (L-AA) nas polpas de camu-camu;
- Realizar os ensaios de redução dos extratos para a determinação indireta do ácido dehidroascórbico (DHAA) e quantificação da vitamina C total;
- Adaptar e validar um método cromatográfico para a análise de vitamina C por cromatografia líquida de ultra-alta eficiência com detector de arranjo de diodos (UHPLC-DAD), e comparar os resultados de vitamina C total (L-AA e DHAA) com o método oficial da AOAC (2010);
- Otimizar o solvente extrator, através de um planejamento centróide simplex, utilizando como resposta a concentração dos compostos fenólicos totais e flavonoides totais da casca, polpa e semente do camu-camu.
- Desenvolver e validar um método analítico para a identificação e quantificação dos ácidos fenólicos e flavonóides, por cromatografia líquida de ultra-alta eficiência acoplada à espectrometria de massas (UHPLC-MS/MS);
- Avaliar a capacidade antioxidante *in vitro* frente a radicais $ABTS^{\bullet+}$ e $DPPH^{\bullet}$, capacidade de redução do ferro (FRAP), e capacidade de eliminação de espécies de importância fisiológica radicalares ($ROO^{\bullet}$ e $O^{\bullet}{}_2^-$), e não radicalares (H_2O e HOCl), correlacionando todos os resultados entre si.
- Avaliar a atividade antimicrobiana por meio da Concentração Inibitória Mínima (CIM) e Concentração Bactericida/Fungicida Mínima (CBM/CFM).

Capítulo I. Revisão da Literatura

1. Camu-Camu

O camu-camu [*Myrciaria dubia* (H.B.K.) Mc Vaugh] também conhecido como araçá d'água, araçá de igapó, azedinho, entre outros nomes populares, é uma espécie frutífera nativa das várzeas e lagos da Amazônia brasileira e peruana, pertencente à família *Myrtaceae* (Nascimento et al., 2013; Pinto et al., 2013). A ocorrência desta espécie em toda a Bacia Amazônica abrange diferentes ecossistemas, levando assim à variação genética nas plantas originárias de diferentes regiões, fato que levou o Instituto Nacional de Pesquisas da Amazônia (INPA) a criar um campo experimental, com uma ampla coleção de germoplasma de camu-camu (Yuyama et al., 2011).

De acordo com Arévalo (2002), existem dois tipos de camu-camu, o arbustivo e o arbóreo. Na região amazônica prevalece o arbustivo, que cresce nas margens de rios e lagos de águas escuras, podendo permanecer coberto pela água por até cinco meses, já o arbóreo, pode ser encontrado em zonas de má drenagem. A diferença entre os tipos de cultivo fica evidente na morfologia das plantas, época de colheita, teor de vitamina C e conteúdo mineral dos frutos (Ribeiro et al., 2016). Yuyama et al. (2011) relata que, em solos de terra firme, a adição de nutrientes e água pode ser controlada, fato que não ocorre em ambientes alagados.

O camu-camu é um fruto típico de bosque úmido tropical, caracterizado por temperaturas médias de 26 ºC e precipitação pluvial entre 1600 a 4000 mm, frutificando entre os meses de novembro a março (Oliveira, 2013). Desde 1990, o camu-camu vem sendo cultivado no estado de São Paulo, cujo plantio fora de áreas de várzeas tem se mostrado promissor (Vasquez-Caicedo, 2005). No Vale do Ribeira, por se tratar de uma região com condições edafoclimáticas semelhantes ao da Amazônia, clima quente e úmido, a adaptação da planta foi rápida, produzindo frutos durante o ano inteiro, mas com menores produções nos meses de julho e agosto. O cultivo em Iguape e Registro está em plena expansão (Ribeiro et al., 2002; Yuyama et al., 2011), fato que gera perspectivas promissoras para fins agroindustriais, aumentando a produção, rendimento, e melhoria da qualidade dos frutos e processos tecnológicos para a obtenção de produtos transformados a partir de camu-camu, visando sua exportação para regiões onde o fruto não é cultivado (Rodrigues et al., 2003, Wolff et al., 2016).

No mercado externo, as propriedades do camu-camu despertam interesses econômicos e científicos das indústrias farmacêuticas, apresentando um grande potencial econômico capaz de colocá-lo no mesmo nível de importância de outras frutas tradicionais da região amazônica, como o açaí e cupuaçu (Nascimento et al., 2013; Ribeiro et al., 2016). O Peru é o principal produtor e exportador do fruto para países como Japão, Estados Unidos e para a União Européia (Camere-Colarossi et al., 2016).

Os frutos de camu-camu (Figura 1) apresentam a superfície lisa e brilhante, entre 10 e 30 mm e peso ao redor de 10g. O camu-camu é uma baga globosa, de coloração brilhante róseo a vermelho-escuro, e até púrpura-negro quando maduras (Moraes-de-Souza, 2011). A polpa (Figura 2a) é suculenta e extremamente ácida, e com o amadurecimento, sua cor passa do amarelo esverdeado para rosa, muito provavelmente devido à migração das antocianinas da casca para a polpa (Alves et al., 2002; Rodrigues et al., 2001, Costa et al., 2013). As sementes do fruto (Figura 2b) possuem de 8 mm a 15 mm, são achatadas ou reniformes, e cobertas por uma pequena camada de fibras (Andrade et al., 2006; Grigio et al., 2015). Classificado como fruto não climatérico, o período de colheita do camu-camu pode começar assim que os frutos estejam semi-maduros (verde-vermelho) (Vasquez-Caicedo, 2005).

Figura 1. Frutos de camu-camu após a higienização em laboratório.

Figura 2. Subdivisão em (a) polpa, (b) semente e (c) casca, após despolpamento manual.

O rendimento das partes tissulares dos frutos de camu-camu em peso, segundo relatos da literatura (Yuyama et al., 2002; Vasquez-Caicedo, 2005), estaria entre 17 e 29% para as sementes, e da parte comestível, o pericarpo, formado pelo conjunto polpa e casca estaria entre 71 e 83% (Alves et al., 2002). Apenas a casca, representaria de 15% a 20% do peso total do fruto fresco (Yuyama et al., 2002; Vasquez-Caicedo, 2005).

Devido à elevada acidez da polpa, o camu-camu é mais utilizado no preparo de sucos ou como ingrediente que pode ser misturado a outros produtos como néctares, doces, licores, vinhos, sorvetes, iogurtes ou bebidas isotônicas (Rodrigues et al., 2004). O fruto é uma matéria-prima interessante para uso na indústria de alimentos, de cosméticos, química e farmacológica (Hancock & Viola, 2002). Na indústria de alimentos, pode ser usado principalmente como conservante, uma vez que o ácido ascórbico preserva aromas e sabores, previne o escurecimento enzimático e a descoloração dos pigmentos, aumentando assim, a vida útil dos alimentos (Hancock & Viola, 2002; Rodrigues et al., 2004).

Em termos de composição centesimal das polpas de camu-camu, 94% da polpa é constituída de água, 3,5% de carboidratos, 1,4% de ácido ascórbico (vitamina C) e 1% dos demais componentes (proteínas, lipídios, minerais e fibras) (Genovese et al., 2008; Justi et al., 2000; Maeda et al., 2006; Rufino et al., 2010).

Em relação aos aminoácidos, foi constatado que a serina, valina e leucina passaram de 78,7 para 173,2 $mg.100g^{-1}$ com o amadurecimento do fruto (Zapata & Dufor, 1992). Justi et al. (2000) analisaram o perfil de ácidos graxos e verificaram que a concentração era de 52,5% de poli-insaturados (30% ω3; 13% eicosapentaenoico e 9,5% ω6); 37,5% de saturados e 11,8% do monoinsaturado ácido oleico. Destacaram que o ácido eicosapentaenoico (EPA) é um ácido característico de óleo de peixe e animais marinhos. Para os elementos minerais reconhecidos como nutrientes essenciais, o mais abundante é o potássio (Yuyama et al., 2002). O camu-camu também é rico em flavonóides, que são potentes antioxidantes (Fracassetti et al., 2013; Bataglion et al., 2015), mas a grande importância deste fruto como alimento está relacionada ao elevado teor de vitamina C (Chirinos et al., 2010; Ribeiro et al., 2016). As cascas apresentam 6,18% de fibra alimentar, das quais 5,08% são fibras insolúveis (Yuyama et al., 2002). Os teores de proteína, lipídios e minerais são bastante reduzidos; análises químicas mostram valores de proteínas variando de 0,29% a 1,54% (Maeda et al., 2006; Moraes-de-Souza, 2011; Oliveira, 2013).

2. Vitamina C

A vitamina C é um derivado de hexose sintetizado por vegetais e pela maioria dos animais a partir da glicose e galactose. O ácido L-ascórbico (L-AA) é um composto biologicamente ativo, instável, que se apresenta na forma reduzida e pode ser reversivelmente oxidado a ácido L-dehidroascórbico (DHAA) (Figura 2) (Combs-Jr, 2012; Fracassetti et al., 2013).

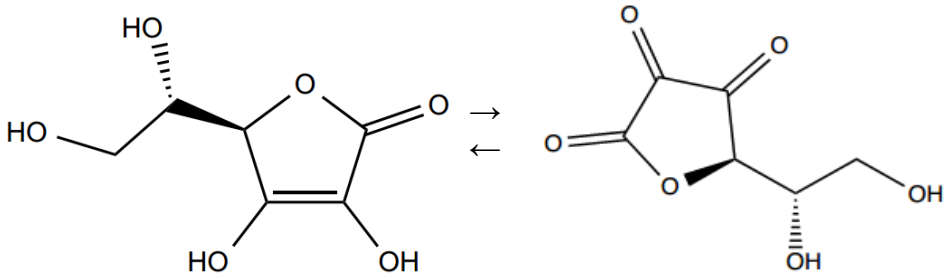


Figura 2. Estruturas químicas dos ácidos L-ascórbico (AA) e L-dehidroascórbico (DHAA)

Embora esteja presente no genoma de humanos e outros mamíferos, o gene responsável pela enzima-flavo L-gulono-1,4-lactona oxidase, envolvido na síntese de ácido ascórbico não é transcrito (Arrigoni & De Tulio, 2002). Portanto, o organismo humano não é capaz de sintetizar L-AA, que deve ser adquirido através da alimentação. Sabe-se que a via endógena de síntese é fonte de espécies reativas de oxigênio (ROS), assim, do ponto de vista evolutivo, a perda da capacidade de sintetizar ácido ascórbico é considerada benéfica e metabolicamente econômica, visto que a dieta é capaz de suprir a deficiência endógena (Steinberg & Rucker, 2013).

A vitamina C é considerada um potente agente redutor (E°'= -170 mV), capaz de reduzir a maioria das ROS fisiologicamente relevantes e regenerar o α-tocoferol, participando do mecanismo protetor contra lipoperoxidação (Halliwell & Gutteridge, 1999). Em meio fisiológico a vitamina C encontra-se predominantemente na forma de monoânion ascorbato (Ames, 2001). Os produtos da oxidação do ascorbato por um elétron e por dois elétrons são, respectivamente, radical ascorbila e ácido dehidroascórbico. O radical ascorbila, é pouco reativo quando comparado a outros radicais livres, o que torna o ácido ascórbico um eficiente

antioxidante, capaz de eliminar espécies altamente reativas e formar um radical de reatividade baixa (Steinberg & Rucker, 2013).

Apesar da grande eficiência antioxidante sobre os radicais livres na fase aquosa, a vitamina C não é capaz de agir nos compartimentos lipofílicos para inibir a peroxidação dos lipídeos (Steinberg & Rucker, 2013). Por outro lado, estudos *in vitro* mostraram que essa vitamina na presença de metais de transição, tais como o ferro, pode atuar como uma molécula pró-oxidante e gerar ROS, como H_2O_2 e $OH^{\cdot}$ (Halliwell & Gutteridge, 1999). Geralmente, esses metais estão disponíveis em quantidades muito limitadas *in vivo* e as propriedades antioxidantes da vitamina C são predominantes *in vivo* (Odin, 1997).

Cerca de 80-90% da vitamina C consumida é absorvida pelo intestino delgado, por transporte ativo e passivo, com menor absorção na boca e estômago, e o restante é excretado na urina (Halliwell & Gutteridge, 1999). A vitamina C distribui-se amplamente em todos os tecidos do organismo, não é armazenada, mas alguns tecidos como a glândula supra-renal, hipófise e fígado são ricos em L-AA (Steinberg & Rucker, 2013). É um nutriente importante no desempenho de funções biológicas como produção de colágeno, biossíntese de carnitina, conversão do neurotransmissor dopamina a norepinefrina, cicatrização de feridas, fraturas, contusões e sangramentos gengivais, reduz a suscetibilidade às infecções, auxilia a formação de dentes e ossos, aumenta a absorção de ferro, previne o escorbuto, inibe a formação de nitrosaminas, e tem ação antioxidante (Combs-Jr, 2012; Rebecca et al., 2015). Além disso, é importante na absorção de ferro dietético, devido a sua capacidade de reduzir a forma férrica (Fe^{3+}) a ferrosa (Fe^{2+}), propiciando a absorção do ferro não-heme no trato gastrointestinal (Arrigoni & De Tulio, 2002).

Nos alimentos estão presentes as duas formas ativas da vitamina C, ácido L-ascórbico (L-AA) e ácido L-dehidroascórbico (DHAA) e suas proporções tendem a variar com o tempo de armazenamento, já que o DHAA é dependente do tempo de oxidação do L-AA (Neves et al., 2015). Dessa forma, o conteúdo de vitamina C da maioria dos alimentos diminui durante o armazenamento devido aos efeitos agregados de vários processos pelos quais a vitamina pode ser destruída, como a presença de O_2, traços de íons metálicos, calor e pH neutro a alcalino (Rebecca et al., 2015; Steinberg & Rucker, 2013).

As principais fontes são as frutas e vegetais, sendo que o teor de vitamina C pode ser influenciado por vários fatores, como grau de maturação, tratos culturais, condições de plantio, manuseio pré e pós-colheita, estocagem, pH, presença de oxigênio e enzimas (Spínola et al., 2013; Spínola et al., 2014; Tarrago-Trani et al., 2012). Frutos tropicais como o

camu-camu apresentam, em média, de 845 a 3133 mg de vitamina C por 100g do fruto *in natura* (Genovese et al., 2008; Neves et al., 2015), enquanto a acerola, considerada até então a fruta mais rica em vitamina C encontrada no Brasil, apresenta, em média, de 1200 mg por 100g do fruto *in natura* (Assis et al., 2001; Mezadri et al., 2008).

De acordo com a Organização Mundial da Saúde (OMS), a ingestão diária recomendada (RDA) para vitamina C é de 15 a 25 mg para crianças entre 1 e 8 anos; de 45 a 75 mg para jovens entre 9 e 18 anos e de 75 a 90 mg para adultos, respectivamente (FAO/OMS, 2001). Níveis plasmáticos mínimos de vitamina C de 0,2 $mg.L^{-1}$ são suficientes para evitar estresses fisiológicos provenientes de infecções, tabagismo, altitude e temperaturas ambientais extremas (Hemilä, 2011), mas superdosagens de vitamina C (> $3mg.L^{-1}$) não causariam toxicidade, pois ela é usualmente eliminada através da urina e das fezes. Desta forma, os níveis elevados de vitamina C encontrados no camu-camu não representariam risco à saúde (Vasquez-Caicedo, 2005).

3. Extração e Análise da Vitamina C

A extração representa a primeira e mais importante etapa da análise da vitamina C. Devido à sua natureza lábil, o principal objetivo deste procedimento é a estabilização do L-AA, e para prevenir a sua oxidação, as extrações devem ser conduzidas em baixas temperaturas (Nováková et al., 2009). No entanto, sem acidificação do meio, a refrigeração dos extratos é insuficiente para prevenir a degradação da vitamina. Assim, na literatura já foram relatados diferentes tipos de ácidos utilizados para este fim, reduzir o pH do meio, como por exemplo, o ácido metafosfórico, ácido oxálico, ácido acético, ácido tricloroacético, ácido sulfúrico ou ainda estas mesmas soluções combinadas com ácido etilenodiaminotetracético (Rosa et al., 2007).

A extração convencional do L-AA é um procedimento simples, envolvendo a pesagem e homogeneização das amostras em solução aquosa ácida por alguns minutos, e posteriormente faz-se a centrifugação e recolhe-se o sobrenadante. No entanto, envolve um grande número de etapas que pode expor o composto às condições de instabilidade, como a ação do oxigênio, pH, luz e temperatura, tornando-se uma técnica de difícil reprodutibilidade (Nováková et al., 2009).

Nesse quesito, a Extração com Líquido Pressurizado (PLE - *Pressurized Liquid Extraction*), também conhecida como extração acelerada por solvente (ASE), surge como

alternativa de extração pois utiliza ambiente livre de oxigênio e luz, o que tem promovido sua ampla utilização na extração de compostos nutracêuticos (Stalikas, 2007). A técnica utiliza solventes classificados como seguros, a exemplo do etanol e a água ou suas misturas, em volumes significativamente menores, submetidos à alta temperatura e pressão, a fim de extrair determinados compostos de matrizes sólidas ou semi-sólidas em um curto tempo e com a utilização de baixo volume de solvente (Ivanovic et al., 2014, Roseiro et al., 2013). A principal vantagem deste método de extração consiste na utilização de temperaturas acima do ponto de ebulição (80 a 200 °C) dos solventes, os quais são pressurizados normalmente a 10,4 MPa (1500 psi) de forma a serem mantidos no estado líquido. Os altos valores de temperatura aumentam a solubilidade, taxa de difusão e transferência de massa, enquanto os valores de viscosidade e tensão superficial do solvente são menores do que aqueles à temperatura ambiente (Huie, 2002).

Extração com água pressurizada normalmente utiliza temperaturas acima do seu ponto de ebulição normal e, por esta razão, é mais conhecida como extração com água quente pressurizada (PHWE – *Pressurized Hot Water Extraction*) ou extração com água subcrítica (SWE - *Subcritical Water Extraction*) (Herrero et al., 2013). O aumento da temperatura acarreta em diminuição da polaridade aparente da água, e dessa maneira, para a extração de compostos relativamente polares, bons rendimentos podem ser obtidos em torno de 100 °C, enquanto que para compostos menos polares temperaturas acima de 200 °C devem ser utilizadas. Estudos relatam que mesmo sob alta pressão e temperatura, os fitoquímicos são extraídos com sucesso de fontes vegetais (Mustafa & Turner, 2011; Paes et al., 2014). No entanto, não foram encontrados estudos para vitamina C.

Finalmente, entre as metodologias analíticas mais utilizados para a determinação de vitamina C estão: titulometria, método oficial da AOAC (*Association of Official Analytical Chemists*), espectrofotometria (UV/vis), fluorimetria e técnicas de separação, como a eletroforese capilar (EC) e a cromatografia líquida de alta eficiência (CLAE), que é a mais utilizada na determinação de L-AA devido à sua especificidade, sensibilidade e fácil operação (Nováková et al., 2009; Spínola et al., 2012; Tarrago-Trani et al., 2012). Recentemente, a técnica de cromatografia líquida de ultra-alta performance (UHPLC) vem sendo utilizada na determinação de vitamina C, pois apresenta melhor resolução, sensibilidade, corridas ultra-rápidas e uso de baixos volumes solventes (Mazurek & Jamroz, 2015; Spínola et al., 2012; Spínola et al., 2013; Spínola et al., 2014).

A vantagem dos métodos cromatográficos são os vários tipos de detectores que auxiliam na identificação e quantificação dos constituintes da amostra, com elevada sensibilidade e exatidão (Boonpangrak et al., 2016). Tanto o L-AA como o DHAA são compostos importantes da dieta e, consequentemente, existe um interesse crescente na análise simultânea de ambas as moléculas (Nováková et al., 2009). A detecção simultânea, sem recorrer ao método de subtração, é possível graças à detecção por espectrometria de massas. Fenoll et al. (2011) desenvolveram um método sensível e seletivo para determinação dos dois analitos em pimentas, tomates, laranjas e limões. Essa técnica ainda é pouco reportada na literatura, pois apesar da sua alta sensibidade e exatidão, o emprego da espectrometria de massas possui elevado custo de operação, o a que torna inacessível para muitos laboratórios.

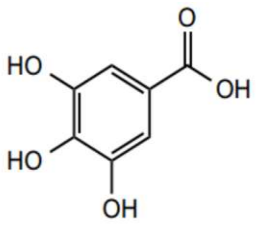
No entanto, a detecção conjunta do L-AA e DHAA ainda é um problema analítico complicado, pois são compostos que possuem propriedades muito diferentes na absorção UV (Nováková et al., 2009). Geralmente, é necessário transformar uma molécula na outra para tornar possível a identificação de ambas usando apenas uma técnica de detecção. O L-AA tem uma forte absorção na zona UV (244 - 265 nm). Já o DHAA apresenta um comprimento de onda de baixa frequência (185 – 210 nm), tornando a sua detecção direta particularmente difícil e susceptível a interferências de uma série de componentes naturais dos alimentos, limitando a escolha de solventes, tampões e reagentes (Steinberg & Rucker, 2013). A redução do DHAA, com auxílio de um agente redutor (ditiotreitol, homocisteína, bromina, L-cisteína, dentre outros), permite a quantificação por diferença entre a vitamina C total após a conversão do conteúdo do L-AA da amostra original. No entanto, a redução também pode conduzir a erros experimentais, uma vez que alguns agentes redutores necessitam que o pH esteja próximo da neutralidade (7,0), e tanto o L-AA como DHAA são instáveis em pH neutro (Spínola et al., 2012).

Embora existam inúmeras metodologias para a determinação de vitamina C (L-AA e DHAA), nenhuma é totalmente satisfatória. Além da instabilidade do composto, a matriz estudada pode apresentar substâncias que influenciam na sua determinação (Mazurek & Jamroz, 2015). Em frutas, principalmente, a ampla diversidade e complexidade, e as mudanças ocorridas nos diferentes estágios de maturação, intensificam a importância da otimização do método de extração na eliminação das substâncias interferentes, como os açúcares e os ácidos orgânicos, assegurando a eficácia da análise cromatográfica (Spínola et al., 2012).

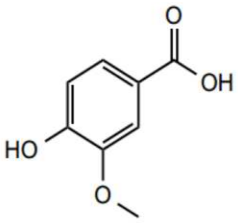
4. Compostos Fenólicos

Os compostos fenólicos são um conjunto de substâncias heterogêneas, derivadas dos metabólitos secundários dos vegetais, que são essenciais para o seu metabolismo e reprodução (Naczk & Shahidi, 2004). São formados em condição de estresse, como infecções, ferimentos, radiações UV, dentre outros. Em alimentos, são responsáveis pela cor, adstringência, aroma e estabilidade oxidativa (Alarcón-Flores et al., 2013).

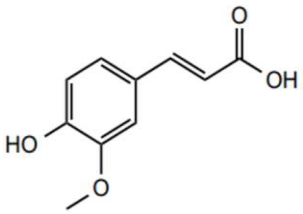
Já foram reportados em torno de 8.000 compostos fenólicos, ou polifenóis, que representam a maior categoria de agentes fitoquímicos amplamente distribuídos no reino vegetal (Crozier et al., 2009). Quimicamente, os compostos fenólicos são definidos como substâncias que possuem anel aromático com um ou mais substituintes hidroxílicos, ou outros grupos funcionais. Os mais importantes são os ácidos fenólicos, que possuem um anel aromático com grupos ligados à sua estrutura, sendo o grupo hidroxila o mais comum, a exemplo do caféico, clorogênico, ferúlico, sináptico, gálico, p-coumárico (Naczk & Shahidi, 2004).



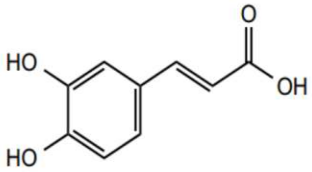
Ácido gálico



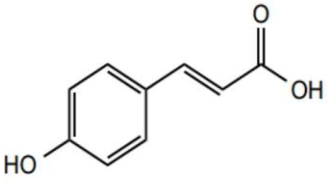
Ácido vanílico



Ácido ferúlico



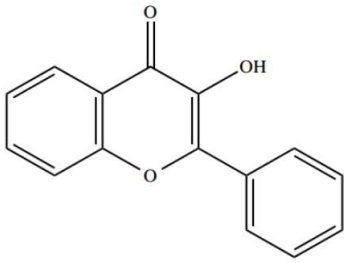
Ácido caféico



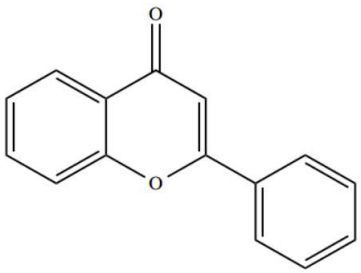
Ácido p-cumárico

Os flavonóides, que ocorrem naturalmente nos alimentos vegetais e são caracterizados estruturalmente como difenipropanos (C_6-C_3-C_6) com 15 átomos de carbono arranjados em 3 anéis, identificados como A, B e C (Volp et al., 2008). A sua estrutura química permite a classificação em flavanonas, flavonas, flavonóis, flavanóis (catequinas),

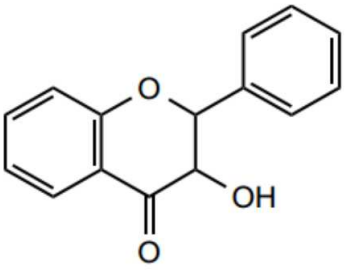
dihidroflavonóis, isoflavonas e antocianinas (Dai & Mumper, 2010; Motilva et al., 2013). As diferenças entre as classes está no número e arranjo dos grupos hidroxila, assim como a natureza e quantidade de alquilações e/ou glicosilações destes grupos.



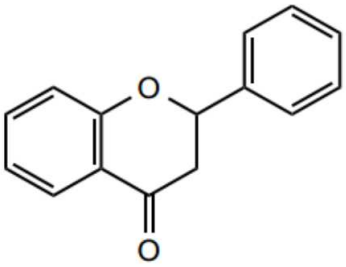
Flavanol



Flavana

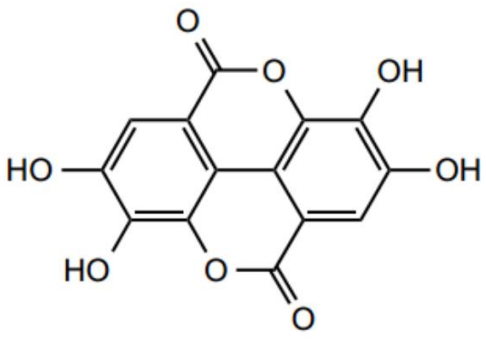


Flavo-3-ol



Flavanona

Por fim, os taninos completam as classes dos principais compostos fenólicos, e são divididos em duas classes: taninos hidrolisáveis, que compreendem polímeros de ácido gálico (galotaninos) ou elágico (elagitaninos), e os taninos condensados (proantocianidinas) (Silva & Silva, 1999). São compostos hidrossolúveis de alto peso molecular com função de precipitação de proteínas e alcalóides (Aron & Kennedy, 2008).



Elagitaninos

A ação antioxidante, conferida por sua estrutura, representa a propriedade mais relevante dos compostos fenólicos. Estudos demonstram seu potencial no combate aos radicais livres, que causam estresse oxidativo e, consequentemente, danos a tecidos e biomoléculas do nosso organismo, exercendo assim, importante papel na prevenção de distúrbios patológicos, tais como a aterosclerose, disfunção cerebral e câncer (Laghari et al., 2011; Del Rio et al., 2010). Nos últimos anos, alguns polifenóis, tais como as catequinas do chá verde e do vinho, as antocianinas dos frutos vermelhos, os flavonóis em destaque na cebola e folhas verdes e as isoflavonas da soja, foram relacionadas ao combate a doenças degenerativas e envelhecimento (Saura-Calixto & Goñi, 2009; Giri et al., 2012).

O primeiro passo após a ingestão de compostos fenólicos presentes na dieta é a liberação dos mesmos de sua matriz. A desglicosilação de flavonóides, a clivagem de proantocianidinas poliméricas e a hidrólise de ácidos fenólicos esterificados são consideradas pré-requisitos para absorção dos mesmos através da barreira intestinal (Manach & Donavan, 2004). Apenas uma pequena parte de compostos fenólicos ingeridos é absorvida pelo intestino delgado pela ação de enzimas presentes nas microvilosidades das células epiteliais do intestino (Donovan et al., 2006). Depois de absorvidos, sofrem metabolização no fígado podendo entrar na corrente sanguínea e serem excretados através da urina, ou ainda, pela circulação enterohepática, onde são excretados pelo fígado como componente da bile de volta para o intestino (Mcbain & Macfarlane, 1997). Os compostos fenólicos que não são absorvidos no intestino delgado vão diretamente para o intestino grosso, onde são degradados pela microflora colônica a compostos mais simples, como ácidos fenólicos, e assim são absorvidos pelo sistema circulatório. Uma vez no intestino grosso, os flavonoides e seus metabólitos podem apresentar benefícios à microbiota colônica por selecionar bactérias probióticas ou inibir a proliferação de células cancerígenas (Del Rio et al., 2010).

O camu-camu é um fruto que apresenta alta concentração de compostos fenólicos totais, com valores entre 1.370 e 24.900 mg de equivalente ácido gálico.$100g^{-1}$ nas cascas e polpas secas, respectivamente, que podem estar relacionados às características sensoriais como amargor e adstringência do fruto em produtos derivados, limitando sua aceitabilidade (Alves et al., 2002; De Souza Schmidt Gonçalves et al., 2010; De Souza Schimith Gonçalves a al., 2014; Neves et al., 2015). A semente e a casca do camu-camu destacam-se no conteúdo de compostos fenólicos, que são significantemente mais abundantes que outras frutas tropicais. No entanto, o método mais utilizado para a quantificação é o de Folin-Ciocalteu, que não é específico, pois está relacionado à capacidade redutora da amostra. Dessa forma, a

presença de ácido ascórbico, açúcares e aminas aromáticas pode interferir na medida e levar à superestimação do teor de compostos fenólicos totais (Chirinos et al., 2010).

Os estudos que fornecem uma caracterização detalhada dos principais compostos fenólicos presentes no camu-camu são recentes. De acordo com Chirinos et al. (2010), independente do estádio de amadurecimento, a família de compostos fenólicos mais importantes é a flavan-3-ol, seguida pelo grupo dos ácidos elágico e gálico. Foram encontrados também: catequina, campeferol, quercetina, rutina, cianidina-3-glicosídeo, delfinidina-3-glicosídeo, ácido elágico (Akter et al., 2011; Fracassetti et al., 2013; De Souza Schmidt Gonçalves et al., 2010). Bataglion et al. (2015) encontraram miricetina, quercetina, luteolina, ácido p-coumarico e ácido gálico. Além disso, comparando os extratos da casca e da semente, o teor de compostos fenólicos no extrato da semente foi maior que no extrato da casca, o que torna interessante a caracterização de constituintes fenólicos nesta matriz (Myoda et al., 2010).

5. Extração e Análise de Compostos Fenólicos

Para obter uma extração eficiente, com altas concentrações dos compostos desejados e com a mínima degradação, a escolha do solvente extrator, juntamente com a técnica de extração utilizada, torna-se de grande importância. O solvente ideal deve ter seletividade máxima, compatibilidade com as propriedades dos materiais extraídos e as maiores capacidades de extração em termos dos coeficientes de substância saturada no solvente (Wijesekera, 1991; Kim & Verpoorte, 2010). O uso de planejamento de misturas para obter rendimentos ótimos, torna-se útil para o estudo sistemático dos efeitos dos solventes puros e também para determinar efeitos sinérgicos e antagônicos entre os solventes nos resultados das extrações de compostos de produtos naturais (Garcia et al., 2010; Lonni et al., 2012; Soares et al., 2012). Nesse contexto, o apelo ambiental pela escolha de solventes "verdes" como água e etanol ganham mais importância em relação aos solventes "não verdes" como metanol, acetona e acetonitrila (Chemat et al., 2012; Handa et al., 2016).

Extrações líquido-líquido e sólido-líquido são os procedimentos mais comumente utilizados nas análises de polifenóis e fenóis simples, devido à facilidade de uso, eficiência e ampla aplicabilidade (Stalikas et al., 2007). Extrações eficientes são obtidas utilizando técnicas tradicionais como a extração exaustiva com Soxhlet, extração assistida por ultrassom, técnicas alternativas como a extração com solvente assistida por micro-ondas,

extração com fluido super-crítico e extração líquida pressurizada (PLE), também conhecida como extração acelerada por solvente (ASE), que tem sido utilizada com sucesso (Roseiro et al., 2013). Nesse caso, uma desvantagem da técnica tradicional em relação à técnica alternativa é o longo tempo de extração. Extrações demoradas podem ocasionar degradação de compostos durante o procedimento, além de exigir maior volume de solvente orgânico, que, em alguns casos, são perigosos para saúde humana e necessitam ser separados dos extratos e adequadamente descartados (Ivanovic et al., 2014; Roseiro et al., 2013).

As técnicas de separação como a cromatografia líquida de alta eficiência (HPLC) e a cromatografia líquida de ultra-alta performance (UHPLC) são extensamente usadas para a separação e quantificação de compostos fenólicos (Motilva et al., 2013). O alto desempenho dessas técnicas associadas à espectrometria de massas tem sido amplamente aceita como a principal ferramenta na separação, identificação, caracterização estrutural e análise quantitativa dos compostos fenólicos (Alarcón-Flores et al., 2013). Nesse sistema, a fonte de ionização por electrospray (ESI) no modo negativo mostrou ser mais eficiente e sensível para caracterização dos ácidos fenólicos e flavonoides (Bataglion et al., 2015). Além disso, o uso de monitoramento seletivo de reações (SRM) na espectrometria de massas sequencial (MSn), utilizando como analisador um triplo quadrupolo (QqQ), tem sido considerado uma ferramenta analítica eficiente, por exibir características excelentes, tais como seletividade, especificidade e sensibilidade (Liu et al., 2010; Bataglion et al., 2015).

6. Antioxidantes e Espécies Reativas de Oxigênio (EROs)

O processo de oxidação está relacionado à obtenção de energia pelos organismos vivos, no qual são produzidas espécies pró-oxidantes importantes para a síntese de substâncias biológicas, fagocitose, regulação do crescimento celular e sinalização intercelular (Almeida et al., 2011).

Os radicais livres e as espécies reativas de oxigênio (ROS), tais como ânion superóxido ($O_2^{\bullet -}$), radical hidroxila ($HO^{\bullet}$), radical peroxila ($ROO^{\bullet}$), bem como derivados de oxigênio não radicalares, como peróxido de hidrogênio (H_2O_2), ácido hipocloroso (HOCl), oxigênio singlete ($^1O_2$) (Halliwell, 2008), são produtos do metabolismo celular liberados durante o processo de redução do oxigênio e utilizados para converter em energia os nutrientes absorvidos dos alimentos. Apesar da similaridade entre as terminologias, é importante distinguir que as EROs incluem as espécies derivadas do oxigênio, tanto

radicalares quanto não radicalares; já os radicais livres são caracterizados por possuírem um elétron desemparelhado em seu último orbital (Halliwell & Gutteridge, 1999).

Embora certo nível de ROS esteja envolvido na regulação de processos fisiológicos, o excesso na produção destes compostos leva à superestimulação de algumas vias intracelulares. Para o funcionamento celular normal, deve haver uma compensação entre a formação de ROS e os níveis de defesas antioxidantes. Se as defesas antioxidantes tornam-se insuficientes frente à excessiva produção de ROS, ocorre o chamado estresse oxidativo. Estresse oxidativo agudo, bem como estresse oxidativo crônico, têm sido relacionados a um grande número de doenças degenerativas, como aterosclerose, diabetes, injúria isquêmica, doenças inflamatórias, câncer, dentre outras (Valko et al., 2007).

Em vista dos prejuízos provenientes do excesso de ROS, as células dispõem de uma variedade de mecanismos de defesa contra os danos causados por estas espécies: as defesas antioxidantes. Os antioxidantes são substâncias que atrasam ou inibem a oxidação de um substrato oxidável de maneira eficaz, mesmo quando presentes em baixas concentrações (Barreiros et al., 2006; Couto & Canniatti-Brazaca, 2010). Eles impedem que o oxigênio se combine com moléculas suscetíveis ou estabilizam a formação de radicais livres e espécies ativas de oxigênio, formando compostos menos reativos (Gottlieb et al, 2009).

Uma alimentação balanceada, rica em frutas e vegetais, representa a principal fonte antioxidantes naturais, dentre os quais estão a vitamina C (ácido ascórbico) e E (tocoferol), os carotenóides e os flavonóides, que têm recebido maior atenção devido à ação benéfica no controle dos danos causados pelos radicais livres ao organismo (Rufino et al., 2010). Devido ao elevado teor de ácido ascórbico e à presença de antocianinas, carotenoides e flavonoides, o mesocarpo do camu-camu (casca e polpa) apresenta notável capacidade antioxidante (Chirinos et al., 2010; Rufino et al., 2010).

Frente à diversidade da estrutura química dos compostos antioxidantes e de seus mecanismos de ação, vários ensaios têm sido desenvolvidos para avaliar a capacidade antioxidante de diferentes amostras (Caetano, 2009). Dessa forma, como não existe um procedimento metodológico universal para avaliar a capacidade antioxidante, torna-se necessário realizar diferentes ensaios, com fundamentos e mecanismos de ação diferentes (Rezaire et al., 2014; Paz et al., 2015).

Baseado nas reações químicas envolvidas, os principais métodos usados para medir a capacidade antioxidante podem ser divididos em duas categorias: (1) métodos de transferência de átomos de hidrogênio, representados pelo ORAC (*Oxygen Radical Absorbance*

Capacity) e (2) métodos de transferência de elétrons pelo método de Folin-Ciocalteu e FRAP (*Ferric Reducing Antioxidant Power*) e sequestro de radicais livres, tais como o $DPPH^{\bullet}$ (2,2-difenil-1-picrilhidrazil) e o $ABTS^{+\bullet}$ (2,2‘-Azino-bis-(3-etilbenzoatiazolina-6-ácido sulfônico). (Huang et al., 2005; Prior et al., 2005)

Alguns estudos da capacidade antioxidante em frutos de camu-camu foram conduzidos utilizando radicais não fisiológicos, tais como $ABTS^{+\bullet}$ e $DPPH^{\bullet}$ (Chirinos et al., 2010; De Souza Schmidt Gonçalves et al., 2010). No entanto, relatos sobre a capacidade de eliminação de espécies de relevância biológica (ROS), são quase inexistentes para o fruto. As ROS possuem propriedades químicas e reatividades diferentes, que vão desde o peróxido de hidrogênio (H_2O_2), que é pouco reativo, ao radical hidroxila ($HO^{\bullet}$), que é altamente reativo (Winterbourn, 2008). Considerando a especificidade de cada espécie reativa, a capacidade de uma determinada matriz para eliminar uma espécie reativas não indica necessariamente que é capaz de eliminar qualquer outra. Isso sugere que a capacidade antioxidante da matriz deve ser avaliada contra diferentes ROS para estabelecer o perfil completo de capacidade antioxidante (Rodrigues et al., 2014).

7. Atividade Antimicrobiana

A resistência dos micro-organismos a antibióticos e antifúngicos produzidos comercialmente têm se tornado uma grande ameaça mundial (González-Zorn & Escudero, 2012). Os principais gêneros bacterianos gram positivos que apresentam resistência aos antimicrobianos são *Staphylococcus* e *Enterococcus* e os principais gêneros/espécies bacterianos (as) gram negativos relatados na literatura são *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas maltophylia*, *Burkholderia cepacea*, *Escherichia coli*, *Klebsiella*, *Proteus*, *Citrobacter*, *Serratia* e *Enterobacter* (Brasil, 2004). As bactérias do gênero *Staphylococcus* são reconhecidas como um dos maiores patógenos responsáveis por uma ampla incidência de infecções, desde moderadas infecções de pele até as mais agressivas bacteremias e septicemias (Kim et al., 2014).

A atividade antimicrobiana de extratos de espécies vegetais e de resíduos gerados pelo processamento de frutas têm sido foco de muitas pesquisas nos últimos anos (Rabelo et al., 2014). Diversas publicações demonstraram que as plantas e os frutos podem diminuir a prevalência da cárie dentária, inibindo o crescimento de patógenos na

cavidade oral, devido ao seu efeito antibacteriano anti-inflamatório e antioxidante (Camere-Colarossi et al., 2016).

Acredita-se que o efeito antimicrobiano de fontes naturais se deve à presença de compostos bioativos, principalmente aos compostos fenólicos (Maier et al., 2009), visto que o mecanismo de ação de certos compostos fenólicos ocorre sobre diferentes estruturas celulares, atuando principalmente na ruptura da membrana celular.

Pesquisadores investigaram os efeitos antimicrobianos para *Staphylococcus aureus, Escherichia coli* e *Saccharomyces Cerevisiae* a partir de diferentes concentrações de extratos obtidos a partir da casca e semente de camu-camu. Os resultados mostraram que houve atividade para *Staphylococcus aureus* a partir do extrato da semente devido aos constituintes lipofílicos (Myoda et al. 2010). Camere-Colarossi et al. (2016) observaram que o extrato metanólico das polpas e sementes possui efeito antimicrobiano contra *Streptococcus mutans* e *Streptococcus sanguinis*, micro-organismos de prevalência na cavidade oral. Mas até o momento, para outros micro-organismos, como *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Candida albicans,* não foram encontrados estudos, o que justifica a investigação dos extratos da casca, polpa e semente para os micro-organismos selecionados.

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Capítulo II

Vitamin C in camu-camu [*Myrciaria dubia* (H.B.K.) McVaugh]: evaluation of extraction and analytical methods

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Abstract

Camu-camu, a typical Amazonian fruit, is known for the high vitamin C content of the peel and pulp. As vitamin C is widely used in the food, pharmaceutical, and cosmetics industries, it is of interest to study new sources, extraction techniques, and analytical methods for the identification and quantification of this compound. Here, evaluation was made of extraction and quantification methods, as well as the differences in vitamin C content according to the origin and part of the camu-camu fruit. The extraction techniques studied were pressurized liquid extraction (PLE), acid extraction, and maceration. The analytical methods evaluated were titrimetry and chromatography. Camu-camu samples were obtained from different regions, and the peel and pulp were studied separately. Acid extraction using acid solvent provided the highest vitamin C yields, while PLE, as a completely clean technique, proved to be a promising alternative for the recovery of ascorbic acid (L-AA). The application of an ultra-high performance liquid chromatography methodology (UHPLC-DAD) enabled the fast identification and quantification of L-AA and dehydroascorbic acid (DHAA), with high resolution, sensitivity, and specificity. The results obtained using the chromatographic and titration methods were not significantly different ($p < 0.05$), indicating that titrimetry is useful for routine analyses. L-AA and DHAA were found in the peel, but only L-AA was found in the pulp. The variation of vitamin C content among the lots could be explained by the edaphoclimatic conditions. The combination of a clean extraction technique and a fast analytical method is a promising approach for the determination of vitamin C in food products.

Keywords: Camu-camu, vitamin C, pressurized liquid extraction (PLE), acid extraction, UHPLC-DAD.

1. Introduction

Vitamin C is one of the most important water-soluble antioxidants present in foods. Both ascorbic acid (L-AA) and its oxidation product, dehydroascorbic acid (DHAA), possess vitamin C activity (Eitenmiller & Landen, 2008; Johnston et al., 2007). They are easily oxidized, especially when exposed to high temperature, oxygen, alkaline pH, traces of metal ions, and some enzymes (Valente et al., 2011). As a nutrient, vitamin C promotes the development and maintenance of the body, acting in the production of collagen, wound healing, iron absorption, reduction of susceptibility to infection, and prevention of mucosal bleeding. The main vitamin C sources are fruits and vegetables (Phillips et al., 2010).

Camu-camu [*Myrciaria dubia* (H.B.K.) McVaugh], also known as "araçá", "caçari", "sarão", and "socoró", is a wild species belonging to the *Myrtaceae* family. It is widely distributed along lake and river banks in the Amazon region, covering parts of Colombia, Venezuela, Peru, and Brazil (Chirinos et al., 2010; Yuyama et al., 2011). In Brazil, seed improvement projects developed by research institutions such as Embrapa Amazônia Oriental and the National Institute of Amazonian Research (INPA) are enabling adaptation of the tree to non-flooded areas (Nascimento et al., 2013). In recent years, this fruit has been the focus of several studies because of its high vitamin C content and antioxidant potential (Costa et al., 2013; Fracassetti et al., 2013; Fujita et al., 2013; Myoda et al., 2010).

The acid techniques widely used for L-AA extraction involve agitation of the sample in acid solution, followed by centrifugation. When used as solvents, acid solutions help to stabilize vitamin C. The most frequently used acids are metaphosphoric acid, oxalic acid, acetic acid, EDTA, and sulfuric acid (Campos et al., 2009; Rosa et al., 2007; Sánchez-Moreno et al., 2003). The use of alternative techniques such as pressurized liquid extraction (PLE), instead of acid extraction, can reduce solvent consumption and increase the extraction rate, due to the combination of high pressure and temperature. Moreover, the use of solvents classified as nontoxic, such as ethanol and water, complies with the requirements of green technology (Herrero et al., 2013; Viganó et al., 2016). PLE has been successfully used to extract heat-sensitive phytochemicals from many plant materials (Machado et al., 2015; Wijngaard et al., 2012).

The simplest and most popular analytical methods for quantification of vitamin C include titrimetric and fluorimetric techniques (Hernadez et al., 2006). Greater specificity and sensitivity, together with easy operation, can be achieved using separation techniques such as

high performance liquid chromatography (HPLC) (Nováková & Vlcková, 2009; Spínola et al., 2012; Tarrago-Trani et al., 2012). More recently, ultra-high performance liquid chromatography (UHPLC) has been used for the determination of vitamin C, offering shorter analysis time, increased sensitivity, efficient separation and resolution, and use of less solvent (Mazurek & Jmroz, 2015; Spínola et al., 2012; 2013; 2014). Another important advantage of chromatographic methods is the availability of various types of detectors, enabling identification and quantification of the sample constituents with high sensitivity and accuracy (Eitenmiller & Landen, 2008; Quirós et al., 2009). These methods can be used to differentiate vitamin C from other compounds, employing indirect determination with UV-Vis detectors or simultaneous determination of different species with mass detectors. Both L-AA and DHAA are important components of the human diet, so consequently there is interest in the simultaneous analysis of both molecules (Nováková & Vlcková, 2009).

The objective of this study was evaluate the techniques of extraction made of PLE, acid extraction, and extraction by maceration for the L-AA in camu-camu. Apply to the UHPLC-DAD methodology for the determination of L-AA and DHAA, and to compare with the official method (AOAC, 2007). Besides to evaluate the differences in the vitamin C contents of fruits harvested in different regions.

2. Materials and Methods

2.1. Reagents and other materials

The following analytical or HPLC grade reagents and standards were used: L-ascorbic acid 99% (Mallinckrodt, USA), dithiothreitol (DTT) (Sigma Aldrich, Germany), metaphosphoric acid (Panreac, USA), Tris buffer (Synth), ethylenediaminetetraacetic acid disodium salt - EDTA (Chemco), oxalic acid (Nuclear), acetic acid (Synth), formic acid (Emsure), HPLC grade sulfuric acid (J.T. Baker), and HPLC grade acetonitrile (J.T. Baker). Ultra-pure water was obtained from a Milli-Q system (Millipore, USA). Before injection, the samples were filtered using polyethylene 0.20 μm porosity Millex HV filter units (Millipore, Brazil).

2.2. Sample preparation

Five different camu-camu samples, in lots of approximately 2.5 kg, were analyzed: two lots of fruits of Iguape-SP in Brazil (IG), two lots of Sete Barras-SP in Brazil (SB) and one lot of Tarapoto in the Amazonian region of Peru (P). The fruits obtained in Brazil were collected at the ideal maturation stage for the harvest, determined by the coloration of the reddish-green peel, between the months of April and September of 2015. The fruits of Peru, also harvested at the ideal stage, were collected in January 2016.

In addition, camu-camu peels (E) were donated by Embrapa Amazônia Oriental, located in Belem, Para, Brazil. Commercial pulps (C) freshly processed were donated by Pulps St. Peter Indústria, Comercio e Distribuidora Ltda, located in Castanhal, Para, Brazil.

The mature camu-camu fruits were manually peeled to separate the pulp from the peel, after which the samples were lyophilized, frozen, and stored protected from light, under vaccum, at -18 °C. Moisture contents were determined gravimetrically, with drying in an oven at 105 °C, until constant weight (AOAC, 2010).

2.3. Camu-camu extraction

2.3.1. Pressurized liquid extraction

The dynamic PLE method employed distilled water as solvent, with approximately 1.0 g of sample. A temperature of 70 °C was selected since this resulted in a higher L-AA yield in preliminary tests. The pressure was kept constant at 10.0 ± 0.5 MPa. The solvent flow rate was 3.0 mL/min. The extraction time was 120 min, defined in preliminary tests to determine the start of the diffusion-controlled phase of the extraction. The extracts were collected in glass flasks and aliquots were injected into the chromatographic system. All the extraction assays were carried out in duplicate.

Figure 1 shows the apparatus used for PLE, which was described by Viganó et al. (2016). The solvent was pumped from the reservoir using an HPLC pump (PU-2080, JASCO, Tokyo, Japan) and passed through a coil in a heating bath (MA-126, Marconi, Brazil) in order to reach the process temperature. Next, it entered a jacketed 10.9 cm^3 extraction column (Autic, Brazil) and flowed through the sample bed, where the extractable compounds were dissolved, and which the extract was collected after depressurization by passage through a micrometer valve (model 10VRMM2812, Autoclave Engineers, USA).

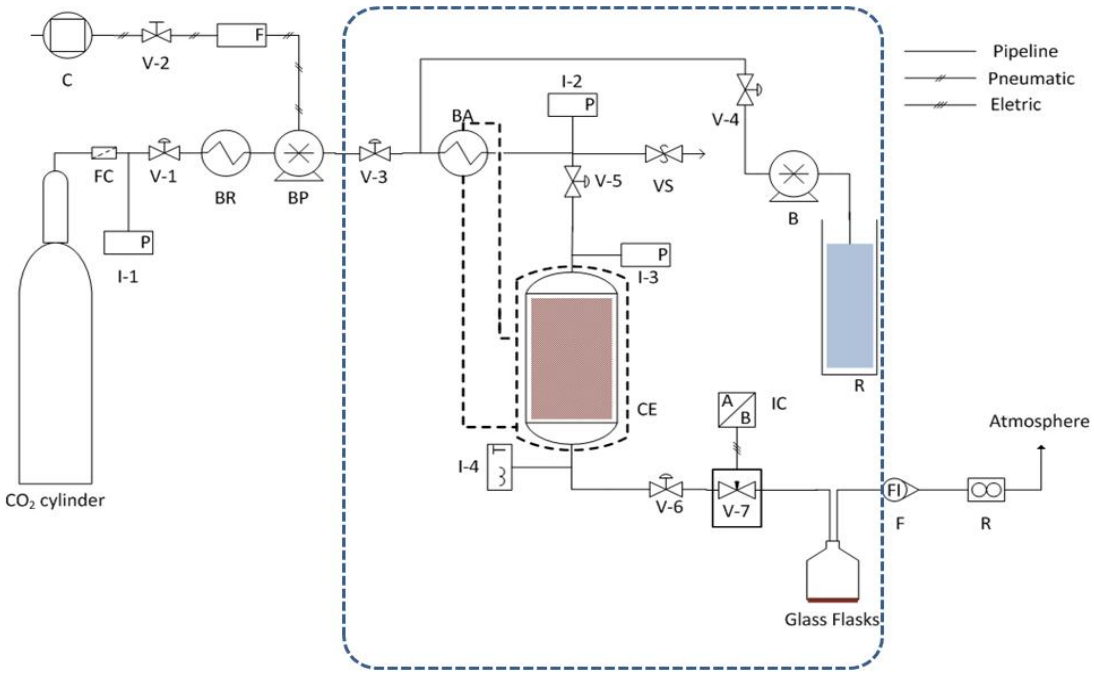

Figure 1. Flowchart of the PLE extraction apparatus (Viganó et al., 2016); V-1 to V-6: block valves; V-7: micrometer valve; VS: safety valve; C: compressor; F: air filter; FC: CO_2 filter; BR: refrigeration bath; BP: CO_2 pump; BA: heating bath; CE: jacketed extraction column; R: solvent reservoir; B: HPLC pump; I-1, I-2, and I-3: pressure indicators; I-4: temperature indicators; IC: temperature controller; F: flow meter; R: total flow meter.

2.3.2. Acid extraction

The following acidic aqueous solutions were tested as stabilizers in the acid extraction: 5% metaphosphoric acid (MPA) (Klimczak & Gliszczynska-Swiglo, 2015); 3% MPA, 8% acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA) (Spínola et al., 2012); 0.1% oxalic acid (Couto & Canniatti-Brazaca, 2010); 0.05 M high purity sulfuric acid (Rosa et al., 2007); and 3% MPA and 8% acetic acid (Sánchez-Moreno et al., 2003).

The extraction procedure was adapted from Rosa et al. (2007) and Sánchez-Moreno et al. (2003). Lyophilized peel or pulp (250 mg) was weighed into a 50 mL centrifuge tube and 15 mL of the aqueous solution containing the selected acids was added. The tubes were shaken for 2 min in vortex and centrifuged for 10 min in a refrigerated centrifuge at 4 °C and 3000 g. The supernatant was collected and re-extracted with 5 mL of acidic aqueous solution. The final volume was adjusted to 25 mL. The assays were performed in triplicate.

Maceration was performed under conditions similar to those for PLE. Erlenmeyer flasks containing the sample and the solvent were placed in a bath and heated statically at 70 °C for 120 min, at atmospheric pressure and oxygen (O_2). The assays were performed in triplicate.

All the extracts were filtered through polyvinylidene difluoride (PVDF) membranes (0.22 μm) before injection into the chromatographic system. The procedures were performed at 25 ± 2 °C, protected from light.

2.4. DHAA reduction

DHAA was determined by reduction to L-AA using the DTT (D-thiothreitol) reducing agent, according to the methodology of Campos et al. (2009), with some modifications. In order to improve the efficiency of the reducing agent, 0.7 mL of Tris buffer (pH 8.0) was added to 1 mL of extract, raising the pH of the solution to 5.5. A 0.3 mL volume of DTT (20 mg/mL) was then added, resulting in a final volume of 2 mL. The reaction was allowed to proceed for 120 min at room temperature, protected from light. A 0.5 mL volume of sulfuric acid (0.4 M) was then added to reduce the pH of the solution to approximately pH 2, followed by filtration of the extracts through PVDF membranes (0.22 μm) and immediate injection into the chromatographic system.

2.5. Chromatographic conditions

Quantification of the L-AA + DHAA contents of the samples was used on the method described by Spínola et al. (2012), using a Waters ACQUITY ultra-high performance liquid chromatography (UHPLC) system equipped with a binary pump, autosampler, and diode array detector (DAD) operated at a wavelength of 245 nm. Separation was performed using a Kinetex column (100 mm × 2.1 mm, 1.7 μm) (Phenomenex) kept at 25 °C. The isocratic mobile phase was composed of 0.1% (v/v) formic acid, at a flow rate of 0.25 mL/min, and the injection volume was 1 μL.

L-AA was identified by comparison of the retention times for the standard and the sample, and by the UV spectrum. Quantification of L-AA was achieved using external standards. Calibration curves were constructed using L-AA standard solutions prepared in 0.05 M sulfuric acid, at eight concentrations in the range from 10 to 150 μg/mL. The DHAA

content was determined indirectly, by subtraction of the L-AA content from the total ascorbic acid content (DHAA + L-AA).

2.6. Validation of the chromatographic method

The chromatographic method was validated according to the norms established by the Brazilian National Health Surveillance Agency (ANVISA, 2003).

LODs and LOQs were determined in solvent, as the lowest concentration level that yielded a signal-to-noise (S/N) ratio in the extracts of 3 and 10, respectively. Linearity (between 10 and 150 μg/mL) was evaluated in 8 points each, using model fitting analysis of variance (ANOVA, 95% probability level). Precision was evaluated under repeatability (with 10 measurements throughout the day) and reproducibility (with 10 measurements on three different days), and expressed in terms of relative standard deviations (RSD%).

Recovery studies were carried in peel, through the addition of aliquots of the standards in the concentratios of 100 μg mL^{-1} and 150 μg mL^{-1} and extracted with acid solution. Before the extraction procedure, this mixture permaneced for 30 min for proper interactin between the analyte and matrix.

2.7. Titrimetric method

The results were validated by comparing the L-AA concentrations obtained using the chromatographic method with those obtained using the official AOAC titrimetric method (AOAC, 2007). The lyophilized samples were extracted with 2 mL of extractant solution (5% MPA) and titrated with 0.05% 2,6-dichlorophenol-indophenol solution in sodium bicarbonate, until the persistence of a rosy color for more than 5s. The indophenol solution was standardized daily with the L-AA solution. All the determinations were repeated three times. The results were expressed as g ascorbic acid per 100 g of dry weight (dw).

2.8. Statistical analysis

Data analysis was performed using Systat v. 10 statistical software (SPSS Inc., USA). The results were evaluated statistically by unidirectional analysis of variance (ANOVA) and Tukey's test (5% confidence level).

3. Results and Discussion

3.1. Effect of the extraction solvent and extraction technique

Figure 2 shows the yields of L-AA extracted from the camu-camu pulp by acid extraction using different acidic aqueous solutions. Acids offer protection against oxidation by withdrawing metal ions from the medium by chelation, preventing the action of copper (II) or iron (III) ions, and by protein precipitation, which inactivates oxidase enzymes (Rosa et al., 2007).

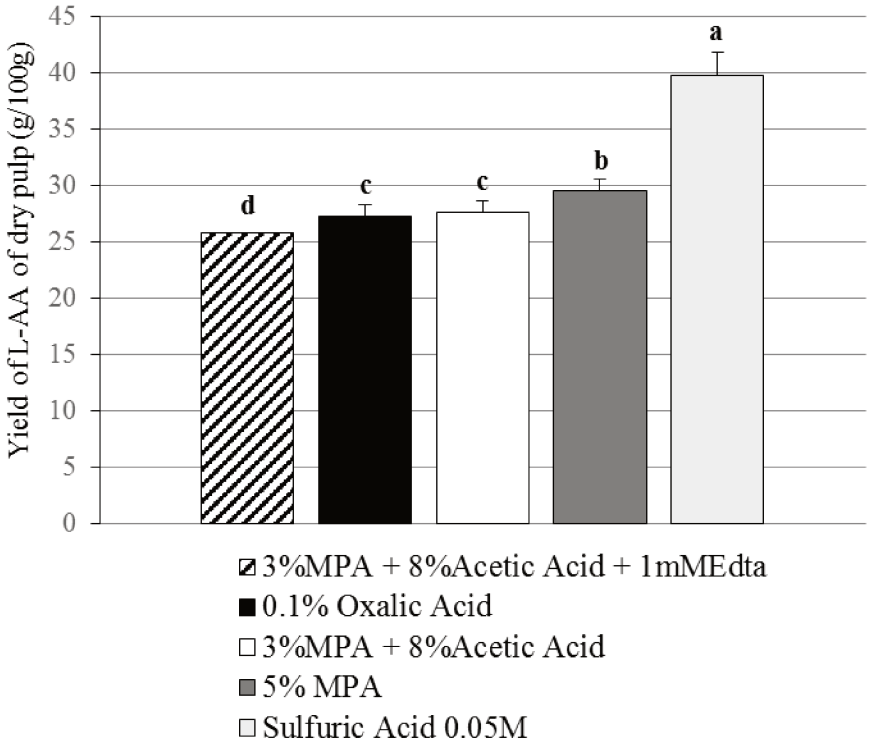

Figure 2. Yields of L-AA obtained from dry pulp of camu-camu from the Amazon region by acid extraction using different acidic solvents. The same letters above the columns indicate no significant difference ($p < 0.05$) by the Tukey test ($n = 6$). EDTA (ethylenediaminetetraacetic acid); MPA (metaphosphoric acid).

Solvents that normally limit the oxidation of L-AA to less than 5% include metaphosphoric acid (3-6%, m/v), acetic acid, 0.05 M sulfuric acid, and 0.005 M EDTA (Eitenmiller & Landen, 2008; Spínola et al., 2012). In this study, the highest extraction of L-AA (39.86 g/100 g of dry pulp) was achieved using high purity 0.05 M sulfuric acid. In

addition to providing greater stability, this medium has also been found to increase the sensitivity and reproducibility of the method (Rosa et al., 2007). Therefore, 0.05 M sulfuric acid was selected as the extractant solution for the acid extraction.

Comparison of the results obtained for the different techniques (Table 1) showed that the acid extraction provided the highest yield of L-AA, with exception of sample SB. The pulps not present DHAA content. The stability conferred by the acid in the solvent could explain the higher ascorbic acid content obtained using the acid extraction, since the presence of H^+ hinders its ionization and prevents irreversible hydrolysis to 2,3-diketogulonic acid (Eitenmiller & Landen, 2008).

Table 1. L-AA content of camu-camu pulp, expressed as g/100 g dry weigth (dw), obtained using different extraction techniques.

Pulp sample	Lot	PLE	Acid	Maceration
		g/100 g dw	g/100 g dw	g/100 g dw
		L-AA	L-AA	L-AA
IG	1	24.91±0.50^{b}	29.26±0.88^{a}	20.92±0.46^{c}
	2	18.43±1.66^{a}	18.96±0.76^{a}	12.23±1.66^{b}
SB	1	22.26±1.01^{b}	31.71±0.74^{a}	14.46±0.01^{c}
	2	30.01±0.18^{a}	27.95±1.04^{b}	20.75±0.39^{c}
P	1	30.04±0.35^{b}	38.37±2.13^{a}	22.62±0.44^{c}
C	1	18.90±1.60^{a}	18.05±0.66^{ab}	15.46±0.28^{b}

The same letters in a line (n = 3) indicate no significant difference ($p < 0.05$).
IG = Iguape; SB = Sete Barras; P = Peru; C = Commercial.
nd: not detected.

Although with lower yields, on average 88% in relation acid extraction, PLE can be considered an alternative extraction technique for ascorbic acid, since it use only water as solvent, so the analyst was not exposed to toxic solvents and there was no toxic waste generation (Mustafa & Turner, 2011). The fact that PLE obtained a high yield without the use of stabilizing agents can be explained by the high temperature, which favored the interruption of the molecular interactions of Van der Waals, hydrogen bonds and dipole-dipole interactions between the plant matrix and the extractable compounds. Thus, there was a reduction in the activation energy required for desorption, facilitation of solvent penetration in the matrix and rapid dissolution of the solutes (Viganó et al., 2016; Wijngaard et al., 2012).

Maceration extraction carried out under similar conditions as PLE, but at atmospheric pressure and with a different flow rate, resulted in the lowest L-AA yields, in the range 12.23-22.66 g/100 g dw. In this case, greater exposure to oxygen without the protection of acid could have contributed to the low extraction yield and poor ascorbic acid stability (Hossein & Azami, 2014; Spínola et al., 2013).

Dehydroascorbic acid (DHAA) was not detected in the fruit pulps analyzed. The presence of this compound in raw food is indicative of L-AA oxidation, which can occur in the presence of oxygen and metallic ions (Fe^{3+}, Ag^{+}, and Cu^{2+}), at alkaline pH and when exposed to high temperature, as well as due to enzymatic reactions (Davey & Montagu, 2000; Nováková et al., 2008; Phillips et al., 2010).

3.2 Figures of merit of the chromatographic method

The chromatographic conditions were first optimized in order to minimize the L-AA analysis time. The retention time was 1.15 ± 0.05 min, with a total run time of 4 min. The viability of the UHPLC-DAD method for analysis of L-AA was evaluated considering the following parameters: LOD, LOQ, linearity, precision and recovery.

The limits of detection and quantification were 0.01 and 0.08 μg/mL, respectively. The method was linear in the range from 10 to 150 μg/mL ($R^2 = 0.9858$). Analysis of variance revealed that the regression was significant in this range, and that the mathematical model showed no lack of fit ($p < 0.05$), demonstrating that the method was suitable for this analysis. The intra-day precision (calculated as the relative standard deviation) was 3.92% (n = 10), while the inter-day precision was 4.78% (n = 30). The retention time of ascorbic acid varied by less than 0.07% (n = 10).

The recoveries of L-AA for the two levels 100 ppm and 150 ppm were 88.96% ± 4.65 and 90.89% ± 5.88. Therefore, the proposed method could be considered satisfactory in terms of the parameters evaluated.

3.3 Comparison of analytical methods: chromatographic and titrimetric

Table 2 presents the vitamin C results obtained for the camu-camu peel and pulp extracts, comparing the chromatographic with titulometric method.

Table 2. Comparison of analytical methods for determination of total vitamin C (g/100g dw) in camu-camu peel and pulp, using acid extraction.

Origin	Lot	Sample	Analysis Methods			
			Titration	UPLC		
			g/100 g dw	g/100 g dw		
				L-AA	DHAA	L-AA+DHAA
IG	1	Peel	10.28 ± 0.19[a]	8.65 ± 0.15	1.56 ± 0.21	10.21 ± 0.19[ab]
	1	Pulp	19.82 ± 1.52[b]	29.26 ± 0.88	nd	29.26 ± 0.88[a]
SB	1	Peel	10.75 ± 0.50[a]	8.32 ± 0.48	1.91 ± 0.18	10.23 ± 0.44[a]
	1	Pulp	25.20 ± 3.46[b]	31.71 ± 0.74	nd	31.71 ± 0.74[a]
IG	2	Peel	7.16 ± 0.31[a]	5.98 ± 0.33	0.62 ± 0.34	6.60 ± 0.28[a]
	2	Pulp	23.34 ± 0.60[a]	18.96 ± 0.76	nd	18.96 ± 0.76[b]
SB	2	Peel	8.31 ± 0.24[a]	7.26 ± 0.07	0.94 ± 0.07	8.20 ± 0.02[a]
	2	Pulp	37.52 ± 2.09[a]	27.95 ± 1.04	nd	27.95 ± 1.04[b]
P	1	Peel	11.59 ± 0.99[a]	8.62 ± 0.31	1.79 ± 0.17	10.42 ± 0.41[a]
	1	Pulp	39.03 ± 2.93[a]	38.37 ± 2.13	nd	38.37 ± 2.13[a]
E	1	Peel	17.19 ± 1.87[a]	13.56 ± 0.42	2.16 ± 0.10	15.72 ± 0.41[a]
C	1	Pulp	20.71 ± 2.86[a]	18.05 ± 0.66	nd	18.05 ± 0.66[a]

The same letters in a line (n = 3), indicate no significant difference ($p < 0.05$).
IG = Iguape; SB = Sete Barras; P = Peru; E = Embrapa; C = Commercial.
nd = not detected

Figure 3 shows the chromatogram obtained from camu-camu peel extract for total vitamin C, with ascorbic acid and dehydroascorbic acid, after reduction.

In the case of the camu-camu peel, the differences between the methods were not statistically significant ($p < 0.05$), while analyses of the pulp resulted in differences for only three samples. Titration is still the main routine method used in laboratories, due to its greater simplicity and lower cost, compared to techniques such as chromatography (Eitenmiller & Landen, 2008). However, disadvantages of titration include exposure to light and oxygen during the analysis, which can lead to L-AA degradation. Other problems are the interference at the turning point of the indicator, caused by color samples and low specificity, due to the presence of other reducing substances that may reduce indophenol, such as sugars and organic acids, and cause an overestimation of the L-AA content (Quirós et al., 2009).

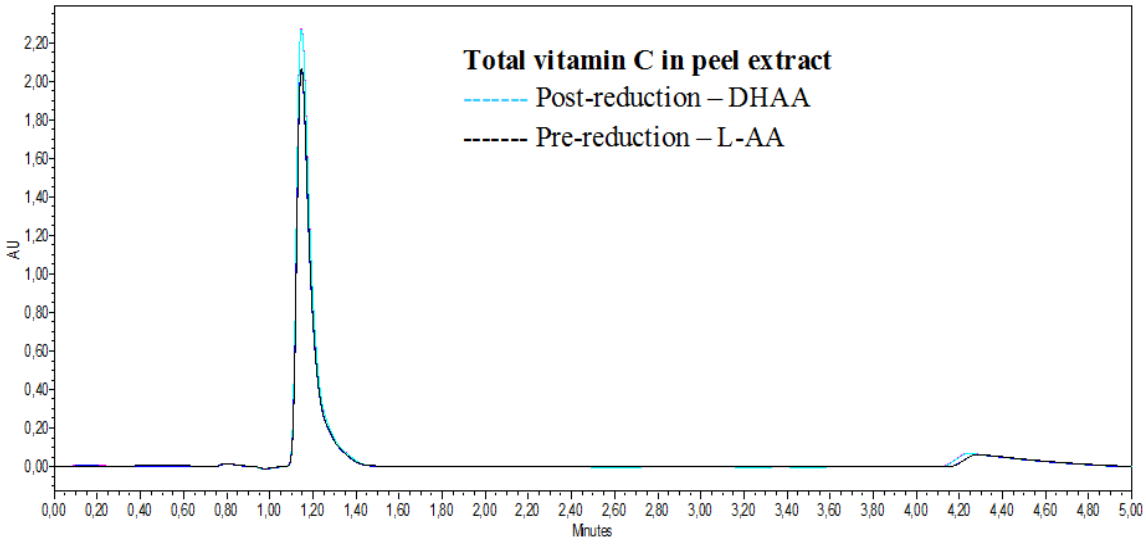

Figure 3. Chromatograms of total vitamin C in peel (P) extract (diluted 1:10) obtained using acid extraction with acidic aqueous solution, before and after reduction with DTT.

The chromatographic method permitted detection of the forms of vitamin C L-AA and DHAA, with high sensitivity and specificity, distinguishing them from other reducing agents present in the samples. This ability is advantageous, since post-harvest conditions influence the formation of DHAA, with factors such as storage time, water activity, presence of oxygen, light, and high temperature promoting the conversion of L-AA to DHAA.

UHPLC is the latest advancement in chromatographic separation techniques and is based on the same principles as high performance liquid chromatography (HPLC). However, UHPLC provides higher efficiency and resolution, shorter analysis times, and greater sensitivity (Spínola et al., 2012). Klimczak & Gliszczynska-Swiglo (2015) compare UHPLC and HPLC methods for AA determination, and using UHPLC method, the total time (1.56 min) of analysis was 2.5 times shorter than with HPLC (4.01 min) and solvent consumption decreased from 12 to 1.2 mL/min.

3.4. Influence of fruit origin on the content of vitamin C (L-AA + DHAA)

The vitamin C (L-AA + DHAA) contents of lots of fruit obtained from different regions are shown in Table 3. The highest L-AA content, 1914.66 mg/100 g fresh pulp (fp), was found in the camu-camu pulp from Peru. Previous studies have reported contents in the range from 845 to 3133 mg/100g of fp (Genovese et al., 2008; Neves et al., 2015), while

values higher than 6000 mg/100g of fp have been found for specific genotypes (Yuyama et al., 2002).

Table 3. Comparison of vitamin C contents of camu-camu fruits (fresh sample, fs) from different regions and lots, using acid extraction and quantification by UHPLC-DAD.

Origin	Lot	Moisture (%)	Peel (mg/100 g fs)		
			L-AA	DHAA	L-AA+DHAA
IG	1	89.68 ± 0.85	892.68 ± 15	160.99 ± 20	1053.67 ± 19[a]
	2	91.32 ± 0.09	616.10 ± 33	63.98 ± 34	680.08 ± 28[c]
SB	1	90.7 ± 0.21	858.62 ± 48	196.08 ± 17	1054.7 ± 44[b]
	2	91 ± 0.11	749.23 ± 6	95.97 ± 7	845.2 ± 2[c]
P	1	90.19 ± 0.07	889.59 ± 31	184.72 ± 17	1074.31 ± 40[a]
			Pulp (mg/100 g fs)		
IG	1	94.81 ± 0.61	1518.08 ± 88	nd	1518.08 ± 88[b]
	2	92.56 ± 0.34	1409.88 ± 75	nd	1409.88 ± 75[b]
SB	1	93.72 ± 0.71	1755.26 ± 104	nd	1755.26 ± 104[a]
	2	94.12 ± 0.21	1863.96 ± 74	nd	1863.96 ± 74[a]
P	1	95.01 ± 0.41	1914.66 ± 213	nd	1914.66 ± 213[a]
C	1	92.81 ± 0.22	1297.07 ± 66	nd	1297.07 ± 66[c]

The same letters in a line (n = 3), indicate no significant difference ($p < 0.05$).
IG = Iguape; SB = Sete Barras; P = Peru; C = Commercial.
nd: not detected.

The commercial pulp presented lower vitamin C content, compared to the collected fruits. An important point to note is that the processing of fruits to obtain commercial pulps can lead to degradation of this compound.

Evaluation of the influence of the region of origin showed that the L-AA contents of the fruit pulps were higher for samples from regions liable to flooding. Ribeiro et al. (2016) found a difference in the L-AA contents of camu-camu fruits from regions with dry soil (13756.79 mg/100 g dw) and flooded soil (11821.29 mg/100 g dw). It was suggested that the time of harvest and soil irrigation could have been the main factors that affected the ascorbic acid content, because ascorbic acid is synthesized from the carbohydrates produced during photosynthesis. Another possible explanation was related to solar irradiation, since the vitamin C content was higher in months with greater insolation. Chirinos et al. (2010) reported that several factors can contribute to such differences, including climatic variations during plant development, soil management techniques, stage of maturation at harvest, genetic variations, post-harvest handling, and storage and processing conditions, in addition to

the method used for vitamin analysis. In the present study, the camu-camu from the Amazon region was harvested in the summer, whereas in the other studies, samples were obtained in the spring and autumn, when exposure to solar irradiation was lower, leading to lower L-AA contents.

The content of dehydroascorbic acid represented from 10.4 to 22.9% of the total vitamin C (L-AA + DHAA) present in the peels, but DHAA was not found in the pulps. Despite being considered the first product of oxidative degradation of ascorbic acid, DHAA can present activity of up to about 80% of the L-AA activity. These findings were in agreement with other studies that have found DHAA contributions of between 10 and 20% to total vitamin C in fruits and vegetables (Borowski et al., 2008).

4. Conclusions

Acid extraction provided the greatest extraction and stability of L-AA. However, pressurized liquid extraction (PLE), which is a completely clean technique, was shown to be a viable alternative for obtaining extracts rich in vitamin C. Chromatography was shown to be an important tool for the analysis of ascorbic and dehydroascorbic acid in food matrices. In addition, the method used was satisfactory for the determination of total vitamin C, as demonstrated by the figures of merit (LOD, LOQ, precision, selectivity and sensitivity). Samples of camu-camu showed high levels of vitamin C, especially in the pulps. The vitamin C content differed according to region of origin and associated climatic conditions.

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Capítulo III

A method for extraction, identification and quantification of phenolic compounds in camu-camu fruit using an ultra-high liquid chromatograph coupled with mass spectrometry in tandem.

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Abstract

The phenolic compounds in camu-camu fruit have received considerable interest due to its possible relations to promote human health. An analytical method for determination of phenolic acids and flavonoid compounds in camu-camu fruit, employing solid-liquid extraction and ultra-high performance liquid chromatograph coupled with tandem mass spectrometry (UHPLC-MS/MS) was developed. The effects of different solvents and their mixtures on the sample preparation was determined by a statistical design. The binary mixture ethanol:water 80:20 (v/v) showed higher extraction of the compounds from peels and pulps, and the best condition for seeds was methanol:water 50:50 (v/v). The phenolic acid (*p*-coumaric acid), flavonols (rutin and quercetin), flavones (luteolin), and flavans ((+)-catechin and (-)-epicatechin) were detected and quantified. The major compound found in camu-camu fruit was the catechin, ranged from 138.5 ± 19.6 to 2988.91 ± 536.44 mg $100g^{-1}$ dw. The *p*-coumaric acid, the only compound of the quantified phenolic acid class, ranged from 7.45 ± 0.31 to 117.92 ± 9.25 mg $100g^{-1}$ dw to peel, pulps and seeds.

Keywords: *Myrciaria dubia*, solvents extraction, phenolics compounds, UHPLC-MS/MS.

1. Introduction

The consumption of fruits and vegetables is recommended in dietary guidelines worldwide, and the intake of fruits which are rich in nutrients and phytochemicals, like camu-camu fruit, provide significant health benefits due to their high levels of polyphenols, antioxidants, vitamins, minerals, and fibers, that can prevent diseases and disorders associated with oxidative stress (Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, & Campos, 2010; Rufino, Alves, de Brito, Pérez-Jiménez, Saura-Calixto, & Mancini-Filho, 2010; Yahia, 2010).

Research on phytochemicals and health has been a focus of interest in the last decade due to their antioxidant activity and free radical-scavenging ability (Tomas-Barberan & Andres-Lacueva, 2012). In particular the phenolic compounds, that are one of the most extensive groups of phytochemicals broadly distributed in plants, appear to be involved in several beneficial effects in human health such as cardiovascular and cerebrovascular diseases, liver disease, inflammation, cancer, and AIDS (Liu, Zhao, Zhong, & Jiang, 2012).

They are classified into four families: flavonoids, phenolic acids, lignans and stilbenes. The major classes of flavonoids include flavonols, flavones, flavanones, flavanols, anthocyanidins and isoflavones (Terao & Murota, 2008). Different types of polyphenols such as anthocyanins (cyanidin-3-glucoside and delphinidin-3-glucoside), quercetin, quercitrin, rutin, myricetin, naringenin, catechin, kaempferol, ellagic acid and eriodictyol are found in camu-camu fruits (Akter, Oh, Eun, & Ahmed, 2011; Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, & Campos, 2010; Reynertson, Yang, Jiang, Basile, & Kennelly, 2008; Rufino, Alves, de Brito, Pérez-Jiménez, Saura-Calixto, & Mancini-Filho, 2010).

Extraction of these compounds is influenced by various factors, including the type of solvent (Michiels, Kevers, Pincemail, Defraigne, & Dommes, 2012). In order to obtain a complete extraction and the largest concentrations of desired products the ideal solvent should have maximum selectivity, compatibility with the properties of the extracted materials and the largest extraction capacities in terms of their saturated substance coefficients in the solvent (Kim & Verpoorte, 2010). Methanol (or ethanol) and water are the acid solvents for extraction of phenolic acids and flavonoid compounds in fruits (Bataglion, da Silva, Eberlin, & Koolen, 2014; Fracassetti, Costa, Moulay, & Tomas-Barberan, 2013). A comprehensive investigation of the effect of mixed solvent systems of methanol/water and ethanol/water may improve the efficiency and economize the use of organic solvents and time (Naczk & Shahidi, 2004).

Analytical methods employed in analysis of phenolic acids and flavonoids were summarized in recently published review articles (Bataglion, da Silva, Eberlin, & Koolen,

2014; Fracassetti, Costa, Moulay, & Tomas-Barberan, 2013). A variety of analytical methods including spectrophotometric methods for assessment of total content of phenolic compounds (Folin-Ciocalteu assay), HPLC, GC-MS, EC and LC-MS, have been developed for the identification and quantification of phenolic acids and flavonoids in fruits (Bataglion, da Silva, Eberlin, & Koolen, 2015). Compared to the above mentioned methods, the development of multiresidue method using UHPLC-MS/MS is a powerful analytical technique to rapidly quantify multi-components in complex matrices, such as fruit seed, peel and pulp, due to its rapid separation power, great sensitivity and high specificity (Fang, Wang, Hao, Li, & Guo, 2015; Pandey, Chandra, Kumar, Dutt, & Sharma, 2016).

Based on this scenario, the aim of this study was to investigate, through a statistical design simplex-centroid, the best solvent extractor for phenolic acids and flavonoid compounds in camu-camu fruit. The effects of ethanol, methanol and water on the extraction polyphenols was evaluated and the responses given in total phenolic content (TPC) and total flavonoid content (TFC). For the identification and quantification of these compounds, it was developed and validated an ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) method, for the determination of the compounds in peel, pulp and seed of camu-camu fruits.

2. Materials and Methods

2.1. Chemicals

Standards of *p*-coumaric acid 98%, (+)-catechin 98%, (-)-epicatechin 98%, rutin 94%, luteolin 98%, quercetin 98%, 3,4-hydroxybenzoic acid 99% used as internal standard (IS), were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol was from J.T. Baker (Mexico City, D.F., Mexico), acetic acid, and water was purified by a Milli-Q gradient system (Millipore, Milford, MA, USA). For sample extractions were used ethanol and methanol PA (Synth, Brasil), and all the other chemicals used were of a analytical grade.

Stock solutions of each standard compound (1000 µg mL^{-1}) were prepared and stored in ethanol at -80ºC. An intermediate solution containing all standard compounds (100 µg mL^{-1}) was prepared in ethanol and dilutions from this solution were done at 6 different levels for calibration curves (concentrations of 0.25 – 5 µg mL^{-1}) of standards and method

validation. A stock solution of internal standard (IS) was prepared and stored in ethanol and dilutions were done to reach a final concentration of 1 μg mL^{-1}.

2.2. Samples tratament

Fruits of camu-camu were harvested, in lots of 2.5 kg, from rural properties the cities of Iguape - SP the cities of Iguape - SP (IG) and Sete Barras - SP (SB), in the state of São Paulo, Brazil, and in the city of Tarapoto in Peru (P). Others camu-camu fruits processed and lyophilized from Pará, Brazil, being a sample of peels from Embrapa Amazônia Oriental (E), and a sample of commercial pulp from St. Petersburg located in Castanhal (C).

The ripeness state of each fruit was carefully checked by the coloration of the reddish-green peel, where only samples with the proper state to be consumed were used (ripe fruits). The fruits were sanitized, manually depulped for separating the peels, pulps and seeds. All parts were independently frozen, lyophilized, vacuum-sealed (oxygen-free) and stored protected from light in metallized packaged at -18°C, until analysis.

2.3. Extraction of the phenolic acids and flavonoid compounds from fruit using solid-liquid extraction (SLE)

The extractor solvent optimization was performed for peel, pulp and seed using a simplex-centroid mixture design involving three solvents: methanol, ethanol and water. The extraction procedure was adapted from Fracassetti, Costa, Moulay, & Tomas-Barberan (2013). The samples were weighted (300 mg) in a 50 mL test tube, then 20 mL of the solvent mixture was added, shaken for 2 min in vortex, and submitted to ultrasound bath for 15 min, at 28ºC, constant temperature. After the mixture was taken to the refrigerated centrifuge (4 ºC at 4000g) for 10 min. A volume of the extract (9 mL) was separated and dried on a rotary evaporator at 60 °C to dryness, and reconstituted with 1 mL of the corresponding solvent extractor mixture (water, ethanol and/or methanol). The concentrated extract was stored in amber flasks at -80 °C. All the experiments were carried out in random order and done in triplicate.

2.4. Total phenolic compounds (TPC) and total flavonoid compounds (TFC)

The total phenolics and total flavonoids were determined by spectrophotometric methods, according to Singleton & Rossi (1965) was adapted by Scherer & Godoy (2014). For TPC, the reaction medium consisted of a 25 µL aliquot of the sample extract, 125 µL of Folin-Ciocalteau, and 100µL mL of sodium carbonate (7.5% w/v). The mixture was kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 760 nm and the results were expressed as mg of gallic acid equivalents (GAE)/g dry weight of sample, based on a curve calibration of gallic acid ranging from 10 to 78 µg mL^{-1}.

The TFC was determined according to Zhishen, Mengcheng & Jianming (1999), with some modifications. Briefly, 100 µL of diluted sample (1:4) was mixed with 30 µL of sodium nitrite (2.5% w/v). This solution was mixed well and allowed to react for 5 min and then 30 µL of 10% aluminum chloride solution was added to the test tubes and the solution was allowed to react for 5 min more. Then, 200 µL of a 1 mol/L NaOH solution and 240 µL of water was added to the tubes and the absorbance was read at a wavelength of 510 nm. The absorbance was read at a wavelength of 510 nm. The results were expressed as mg of catechin (CTE)/g dry weight (dw) of sample, based on a curve calibration of catechin ranging from 20 to 200 µg mL^{-1}.

2.5. UHPLC-MS/MS

Analyses of phenolic compounds and flavonoids were performed using an UHPLC–MS/MS (Waters ACQUITY UPLC™) consisted of a ultra-high performance liquid chromatography system coupled to a mass spectrometer in tandem, equipped with an electrospray ionization (ESI) source. The chromatographic separation was performed using a Kinetex column (100 mm × 2.1 mm, 1.7 µm) (Phenomenex®) kept at 40 °C, using a quaternary solvent manager (Acquity H-Class, Waters). Solvents A, B and C were respectively water with 0.1% of acetic acid, methanol and acetonitrile. The gradient elution was as follows: 0–2 min: A:B, 95:5 (V:V); 2.01–3.49 min: 45:55, A:B (V:V); 3.5–4.59 min: 40:10:50, A:B:C (V:V:V); 5 min: 40:45:15, A:B:C, (V:V:V); 5.01–6.50 min: 95:5, A:B (V:V); at a flow rate of 0.4 mL min^{-1}. The autosampler temperature was maintained at 10 °C and the injection volume was 1 µL.

For MS detection, the ESI source was operated in negative ion mode, nitrogen was used as desolvation gas and argon was used as the collision gas at a pressure of 3.7 x 10^{-3} mbar.

The conditions were as follows: capillary voltage of 2.60 kV, desolvation gas temperature of 700 ºC, source temperature of 150 ºC, desolvation gas flow rate of 1000 L h^{-1}, and cone gas flow of 50 L h^{-1}. The dwell time for transition was 20 ms. The list the optimal collision energy (CE), the cone voltage (CV), and the ions chosen for quantitation and confirmation are presented in Table 1.

Table 1. Mass spectrometry parameters for SRM transitions.

Phytochemicals	**Family**	**Precursor ion *(m/z)* a**	**Cone voltage (kV)**	**Product ion *(m/z)* b**	**Collision energy (CE)**
Catechin	Flavans	289	33 25	245 123	14 20
Epicatechin	Flavans	289	35 20	245 109	17 28
p-coumaric acid	Phenolic acids	163	22 25	119 93	15 28
Luteolin	Flavones	285	22 50	133 30	30 27
Rutin	Flavonols	609	60	300 271	40
Quercetin	Flavonols	301	25 45	151 121	18 26
3,4-dihydroxibenzoic acid	Phenolic acids	153	32	109	16

[a] First transition used for quantitation.

[b] Second transition used for identification

These parameters were optimized by infusion into the mass spectrometer of a standard solution of the analytes in methanol (1.0 mg L^{-1}), at a flow rate of 20 µL min^{-1}. Data were acquired and processed by MassLynx (version 4.1, Waters).

2.6. Method validation

The extraction method was validated through 10 replicates for the mixture of solvents with the best yield, which were also used to assess the precision of the extraction. The UHPLC-MS/MS method was validated considering the following parameters: limit of detection (LOD), limit of quantification (LOQ), linearity, matrix effects, inter- and intra-day precision, recovery and stability of the standard solution.

LODs and LOQs were determined in solvent, as the lowest concentration level that yielded a signal-to-noise (S/N) ratio in the extracts of 3 and 10, respectively. The linearity of the method was evaluated in 6 points each, with ranging from 0.25 to 5 μg mL^{-1} in solvent used to prepare the calibration curve. The slopes obtained for calibration curves of the same concentration in solvent (ethanol:water) and matrix extracts (mix of the peel, pulp and seed) were employed to estimate the matrix effects using the equation: Matrix Effect (%) = [(matrix slope – solvent slope)/solvent slope] x 100. Precision was evaluated under repeatability and reproducibility conditions, and expressed in terms of relative standard deviations (RSD%). Recovery studies were carried in mix of peel, pulp and seed, through the addition of aliquots of the standards in the concentratios of 0.25 μg mL^{-1} (LOQ) and 1 μg mL^{-1} and extracted with the ethanol:water (80:20). Before the extraction procedure, this mixture permaneced for 30 min for proper interactin between the analyte and matrix. The stability of the working solution was evaluated by comparing freshly prepared solutions and stored solutions at -80 °C for 4 weeks.

2.7. Statistical analysis

The mathematical models and ANOVA regression results were performed using Statistica 10.0 software (StatSoft, Tulsa, OK, USA). All analyses were run in triplicate and results were expressed as mean ± standard deviation (SD). Differences among means were first analyzed by ANOVA test and then Tukey test ($p < 0.05$).

3. Results and Discussions

3.1. Solvent effect on the content of total phenolics compounds and total flavonoids

The content total phenolic and flavonoid compounds present in the peels, pulp and seeds, determined from the simplex-centroid design is given in Table 2, and the results were expressed in mg g^{-1} dw.

The peels and pulps presented similar optimal extraction regions for the compounds studied. For the flavonoids, the optimum extraction region was ample, as can be seen in Figure 1. Two solvent proportions included in this range were tested, ethanol: water (60:20 v/v) and (80:20 v/v). The results showed better extraction yields for ethanol: water proportions 80:20

(v/v), with 8.07 mg CTE g^{-1} dw for peel, and 1.91 mg CTE g^{-1} dw for pulp. While the mixture 60:40 (v/v) presented 6.96 mg of CTE g^{-1} dw for peel and 1 mg of CTE g^{-1}dw for pulp.

For the phenolic compounds, the best yields was found in the proportions ethanol: water (50:50). However, the 80:20 (v/v) proportions was tested and no presented significant difference ($p < 0.05$) between them, choosing to use the same extractor solution for all.

For the seeds, the binary mixture methanol: water (50:50 v/v) showed higher extraction yield (i.e. 129.63 mg.GAE/g of dw) from the phenolic and flavonoid compounds.

In general, phenolic extracts are always a mixture of different classes of the soluble compounds in the solvent system used (Naczk & Shahidi, 2004). The binary mixture ethanol:water is considered by many researchers, a green extraction solvent (Chemat, Vian, & Cravotto, 2012; Handa, de Lima, Guelfi, Georgetti, & Ida, 2016).

Table 2. Mixture design solvent proportions and concentration of total phenolic compounds and flavonoids of camu-camu measured at 760 and 520 nm, respectively.

Experim.	Variables			Concentration (mg.GAE/g dw and mg.CTE/g dw)					
	Ethanol	Methanol	Water	Phenolics Compounds			Flavonoids		
	(A)	(B)	(C)	Peel	Pulp	Seed	Peel	Pulp	Seed
1	30	0	0	43.51	64.41	55.49	6.57	1.87	11.94
2	0	30	0	37.71	74.28	83.40	7.40	2.05	22.26
3	0	0	30	33.68	38.13	60.53	3.63	1.26	16.82
4	15	15	0	32.29	55.08	59.06	7.40	1.77	22.19
5	15	0	15	68.95	81.55	98.53	8.10	1.96	20.57
6	0	15	15	49.34	79.03	129.63	6.27	1.15	29.96
7	10	10	10	59.55	64.06	112.33	6.24	1.78	30.56
8	10	10	10	57.77	74.93	137.41	6.52	1.75	29.85
9	10	10	10	59.79	81.21	121.19	6.49	1.53	26.63
10	20	5	5	53.63	56.85	127.45	6.49	1.77	26.36
11	5	20	5	52.27	42.80	86.94	5.82	1.57	28.40
12	5	5	20	61.75	53.99	105.79	5.97	1.69	25.65

GAE - gallic acid equivalents
CTE – catechin equivalents

The generated regression coefficients of the model of the phenolic compounds and flavonoid concentration responses are shown in Table 3. The quadratic model for the peel and full cubic for the seed were significant at the 95% confidence level, without lack of fit for the total phenolic compounds. For flavonoids, the special cubic model for the peel, quadratic for the pulp and seed were adequate to explain the results.

Table 3. Analysis of variance of the values for content of phenolic and flavonoid compounds in the experimental conditions.

			Coefficients (error)												
	Response	**Model indicated**	**Intercept**	**A**	**B**	**C**	**AB**	**AC**	**BC**	**ABC**	**AB (A-B)**	**AC (A-C)**	**BC (B-C)**	**Significance Regression (if p <0.05)**	**Adjustment of the Model (if p > 0.05)**
Phenolics	Peel	Quadratic	-	42.16	38.30	33.89		133.41					70.47	0.0003	0.9088
	Pulp	-	-												
	Seed	Full Cubic	-	55.72	83.62	60.76							231.59	0.0338	0.8692
Flavonoids	Peel	Special Cubic	-	6.46	7.2	3.76		12.03		-36.21				0.0386	0.7981
	Pulp	Quadratic	-	1.83	2.01	1.31		1.58	-1.64					0.0241	0.6978
	Seed	Quadratic	-	12.11	22.42	16.99							42.34	0.0031	0.8692

[A]Ethanol, [B]Methanol, [C]Water

Fig. 1 shows the response surface of the effect of the solvent compositions on the total phenolic and flavonoid compounds of camu-camu extracts. The results show that the ideal extraction region included mixtures of binary and ternary solvents. The definition of the solvent extractor was based on the greater extraction of both classes of compounds from the binary mixtures.

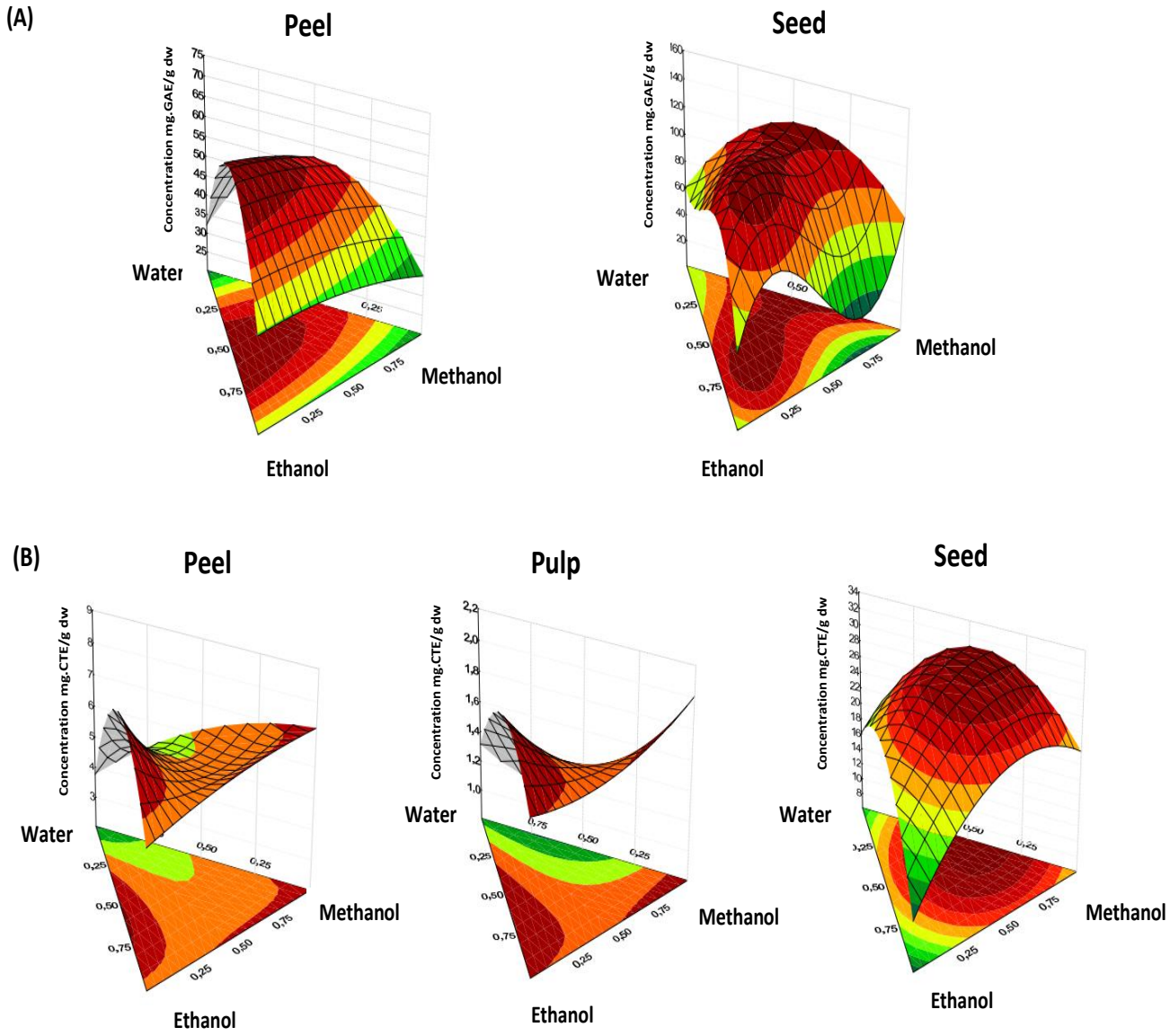

Figure 1. Response surface model for the total phenolic compounds concentration (A) in peel and seed; and (B) response surface model for the flavonoid concentration in peel, pulp and seed.

3.2. Method development and validation

A selective, accurate and fast UHPLC-MS/MS method was developed and validated for simultaneous identification and quantification of six phenolic compounds in camu-camu fruit. The selection of phenolic compounds was made according to previous reports in the literature on the characterization of these compounds in camu-camu extracts. For identification and confirmation, the retention time of each analyte, one transition ion for quantification and two for confirmation was used. The deprotonated molecules ($[M-H]^-$) for all compounds were determined and their MS conditions optimized by infusion into the mass spectrometer, using a standard solution of the analytes in water: ethanol 50:50 (v/v), at the flow rate of 20 μL min^{-1}.

Figure 2 shows the spectrogram of a standard solution obtained after the optimization of the method, with the separation of (+)- catechin and (-)- epicatechin isomers.

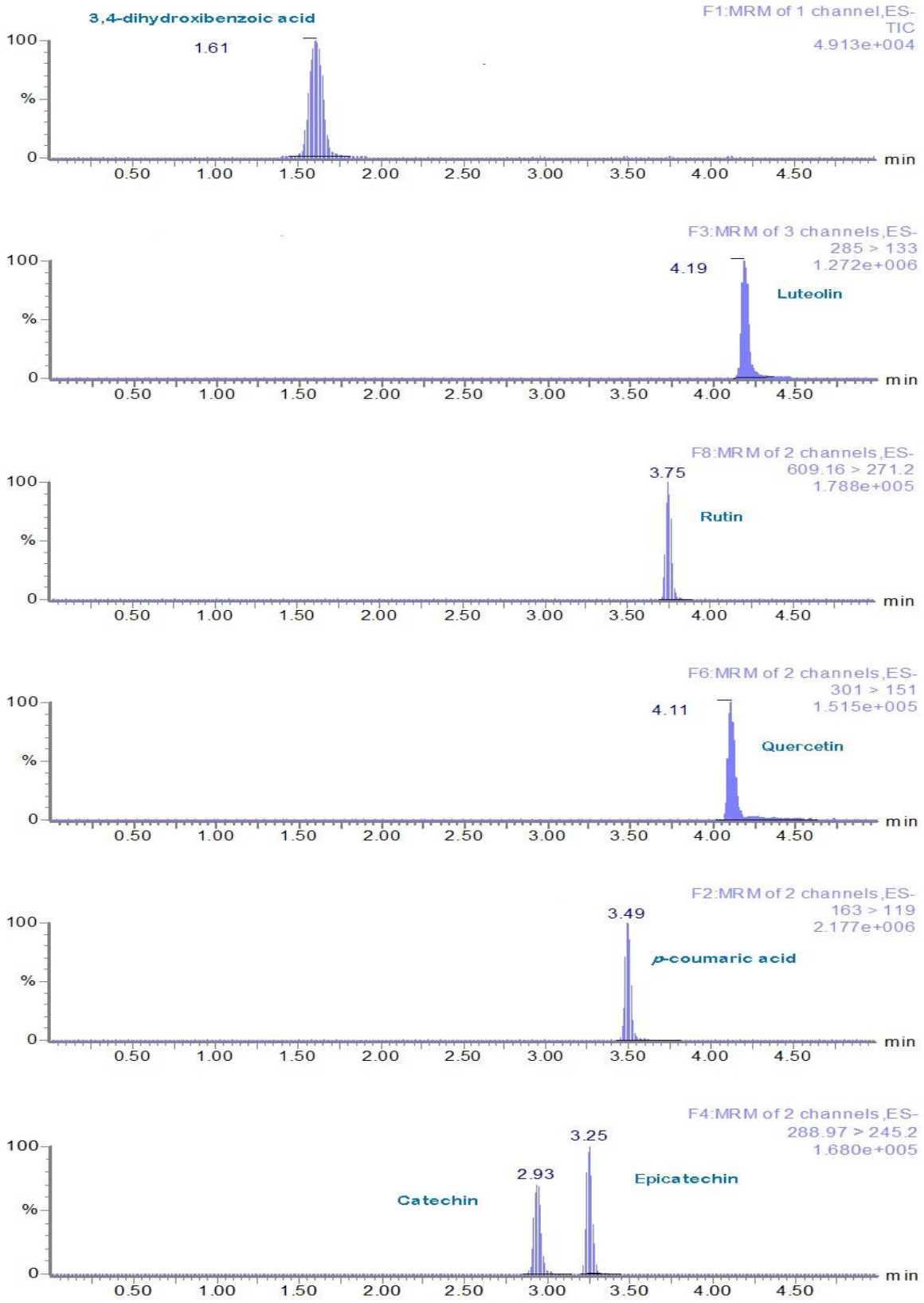

Figure 2. UHPLC–MS/MS spectrograms obtained from standards of phenolics compounds at 1 mg L^{-1}.

Table 4 shows the results of method validation for each analyte. The highest analytical sensibility was achived using the proposed method with limits of detection (LOD) and of quantification (LOQ) of 0.003 µg mL^{-1} and of quantification (LOQ) 0.006 µg mL^{-1}, for the *p*-coumaric acid and luteolin. Bataglion, da Silva, Eberlin, & Koolen (2015) reported a LOD of 0.038 ng mL^{-1} 0.042 ng mL^{-1} for camu-camu extracts using chromatography coupled to mass spectrometry system. Adequate linearity was achieved for solvent calibrations with determinations coefficients (R^2) exceeding 0,9891 in all cases. In addition, analysis of variance (ANOVA) demonstrated high significance of the regression ($p < 0.01$) and no significance of the lack of fit ($p > 0.999$) in the studied range for solvent calibrations. The slopes obtained using the equation: Matrix Effect (%) = [(matrix slope – solvent slope)/solvent slope] x 100, showed a low signal suppression (ME = - 16,66%), indicating a negligible effect of the matrix on anlysis. As a great result, mean recoveries between 81.17 to 111% in 250 ng mL^{-1} and from 83.67 to 104% in 1000 ng mL^{-1} were obtaneid for the selected compounds. Recovery was considered acceptable, above 80%. The precision results (repeatability) evaluated in ten independent replicates at each spiked level was analyzed on the same day, with (RSD%) values lower than 2.1%. Under reproducibility conditions, a total of ten independent replicates was analyzed on two different days, totaling twelve replicates for each spiked level, whose RSD values lower than 3.5%. The working solution were considered stable, about 90-105% of the initial concentration was found.

Table 4. Validation parameters of the developed method to determine phenolic compounds in camu-camu fruit.

Analytes	**Retention time**	**Regression equation**	**r^2**	**Linear range (µg mL^{-1})**	**LOD (µg mL^{-1})**	**LOQ (µg mL^{-1})**	**RSD (%)**	
							Intra-day (n = 10)	**Inter-day (n = 10)**
Catechin	2.91	0.1705x – 0.0007	0.9976	0.25 - 5	0.025	0.05	1.8	2.5
Epicatechin	3.21	0.2314x – 0.0006	0.9934	0.25 - 5	0.01	0.02	2.1	3.1
p-coumaric	3.45	2.8855x – 0.0418	0.9936	0.25 - 5	0.003	0.006	1.2	2.4
Luteolin	4.17	1.2661x – 0.3649	0.9891	0.25 - 3	0.003	0.006	1.6	1.9
Rutin	3.72	2.0972x + 0.0984	0.994	0.25 - 5	0.007	0.014	1.4	3.5
Quercetin	4.09	0.2446x – 0.0401	0.9909	0.25 - 5	0.125	0.25	1.9	2.3

3.3. Phenolic compound profile

The profile and the content of the phenolic acids and flavonoids (expressed in mg $100g^{-1}$ dry weight, dw) determined by UHPLC-MS/MS is shown in Table 5. A total of six compounds were identified by comparing the retention times and MS/MS data with isolated reference compounds and information available in literature (Bataglion, da Silva, Eberlin, & Koolen, 2015).

P-coumaric acid, was the only phenolic acid present in the peels, pulps and seeds of the camu-camu fruit, in high concentrations, with levels ranging from 7.45 ± 0.31 to 117.92 ± 9.25 mg $100g^{-1}$ dw. The lowest concentration found in this study is fifteen times greater that found by Bataglion, da Silva, Eberlin, & Koolen (2015) in camu-camu pulps (0.51 ± 0.06 mg $100g^{-1}$ dw). Another phenolic acid reported by Azevêdo, Fujita, Oliveira, Genovese, & Correia (2014) on camu-camu residues was the siringic acid. This phenolic acid has proved to be an efficient in vivo antidiabetic agent, in addition to other health-relevant attributes (Muthukumaran, Srinivasan, Venkatesan, Ramachandran, & Muruganathan, 2013).

Of the class of flavonoids, the main compound found in the peels, pulps and seeds was catechin, ranging from 138.5 ± 19.6 to 2988.91 ± 536.44 mg $100g^{-1}$ dw. Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, & Campos (2010) found catechin contents of 1.3, 1.7 and 2.2 mg $100g^{-1}$ dw for green, reddish-green and red camu-camu fruits, respectively Azevêdo, Fujita, Oliveira, Genovese, & Correia (2014) found 12.4 ± 1.0 mg $100g^{-1}$ of the catechin in camu-camu residues. The luteolin was found in the peel "E" (29.83 ± 0.07 mg $100g^{-1}$ dw) and in the pulp "P" (77.22 ± 0.13 mg $100g^{-1}$ dw); rutin (1.05 ± 0.13 to 116.29 ± 16.43 mg $100g^{-1}$ dw); quercetin (4.3 ± 0.18 to 26.45 ± 0.83 mg $100g^{-1}$ dw); epicatechin (6.53 ± 0.23 to 418.57 ± 36.16 mg $100g^{-1}$ dw) were also quantifieds. Bataglion, da Silva, Eberlin, & Koolen (2015) found 2.93 ± 0.11 mg $100g^{-1}$ dw quercetin and 0.31 ± 0.02 mg $100g^{-1}$ dw luteolin. De Souza Schmidt Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese (2014) found 84 ± 11 quercetin (mg 100 g^{-1} dw).

The chemical composition of a fruit is known to be highly dependent on the growing and processing conditions. The differences found can be explained by the fact that the amount and type of phenolic compounds can vary according to the geographic region and period of the year and that during processing parts of the fruit (De Souza Schmidt Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese, 2014).

The high structural diversity found in flavonoids occurs due to the small chemical modifications occurring in the basic structure by means of hydroxylation, methylation, acylation, glycosylation, hydrogenation, malonylations and sulfation (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). In literature, camu-camu is described as a fruit rich in flavonoids such as quercetin, rutin and catechin (De Souza Schmidt Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese, 2014; Johnston, Sharp, Clifford, & Morgan, 2005). Among bioactive compounds, flavonoids are the most investigated class of polyphenols due to their antioxidant and anti-inflammatory activities already described. De Souza Schmidt Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese (2014) reported that camu-camu extracts were able to correct the dyslipidemia and lipid peroxidation in plasma associated with diabetes, in animal model. Fujita, Sarkar, Wu, Kennelly, Shetty, & Genovese (2015) reported wich high antioxidant activity in camu-camu fruit provided protection against microvascular complications, as well as against some bacterial borne infections.

Significant differences ($p > 0.05$) were observed among fruit matrices harvested at different locations (Table 5). Among the peels, the "E" sample presented the highest concentration for all evaluated compounds. In the evaluation of the pulps, there was a great variation in the contents among samples; however, sample "C" was the one that presented the lowest total concentration of the phenolic content. The same variation was observed for the seeds, in which a large part of the compounds differed statistically from each other. The lowest concentration was found in the IG pulp. In general, the content of phenolic compounds follows the order: peels < pulps < seeds. The high concentration of flavonoids in fruit pulp highlights the importance of its use as a functional food in the diet.

Table 5. Phenolic compounds content in peel, pulp and seed camu-camu extracts (mg $100g^{-1}$ dw).

Samples		Phenolic compounds (mg $100g^{-1}$ dw)						Total
		Luteolin	Rutin	Quercetin	*p*-coumaric	Epicatechin	Catechin	
Peel	IG	ND	15.94 ± 1.81^{b}	4.30 ± 0.18^{d}	27.34 ± 0.95^{b}	12.77 ± 0.60^{b}	528.65 ± 40.81^{a}	589.02 ± 44.42^{Bd}
	SB	ND	24.07 ± 1.86^{b}	5.80 ± 0.31^{c}	23.23 ± 1.26^{b}	7.33 ± 0.77^{b}	434.18 ± 22.74^{b}	494.63 ± 26.99^{Cd}
	P	ND	21.08 ± 0.1^{b}	7.24 ± 0.37^{b}	23.70 ± 3.04^{b}	14.78 ± 0.15^{b}	138.5 ± 19.6^{c}	205.4 ± 84.8^{De}
	E	29.83 ± 0.07^{b}	116.29 ± 16.43^{a}	10.48 ± 0.91^{a}	32.69 ± 0.91^{a}	55.51 ± 7.82^{a}	606.81 ± 37.68^{a}	821.81 ± 64.0^{Ad}
Pulp	IG	ND	1.05 ± 0.13^{d}	10.73 ± 1.10^{b}	22.76 ± 2.34^{a}	292.83 ± 29.95^{a}	2269.30 ± 309.88^{a}	$2596.69 \pm 344.78^{Aabc}$
	SB	ND	8.50 ± 0.28^{a}	8.55 ± 0.36^{b}	7.45 ± 0.31^{b}	122.32 ± 2.32^{c}	1924.43 ± 275.85^{a}	2071.26 ± 279.74^{Ac}
	P	77.22 ± 0.13^{a}	4.17 ± 0.34^{c}	10.93 ± 1.36^{b}	23.89 ± 2.91^{a}	205.83 ± 14.44^{b}	2230.96 ± 434.94^{a}	2475.81 ± 454.86^{Abc}
	C	ND	5.86 ± 0.12^{b}	26.45 ± 0.83^{a}	20.76 ± 0.86^{a}	6.53 ± 0.23^{d}	1108.49 ± 10.63^{b}	1168.11 ± 12.72^{Ad}
Seed	IG	ND	16.21 ± 3.52^{a}	7.99 ± 0.89^{b}	23.59 ± 2.61^{c}	52.45 ± 2.68^{b}	1909.90 ± 340.16^{b}	2010.17 ± 350.1^{Bc}
	SB	ND	20.10 ± 1.27^{a}	8.80 ± 0.85^{b}	45.35 ± 3.94^{b}	77.07 ± 7.476^{b}	2988.91 ± 536.44^{a}	3140.25 ± 550.30^{Aab}
	P	ND	16.52 ± 0.81^{a}	19.97 ± 0.87^{a}	117.92 ± 9.25^{a}	418.57 ± 36.16^{a}	2747.79 ± 180.24^{ab}	3320.79 ± 229.06^{Aa}

The results were expressed as mean ± standard deviation (n = 3).

Means followed by the same letter in the line and upper case in the column for each sample class does not presented significant difference among each other at $p < 0.05$ by the Tukey test (n = 3).

LOD µg mL^{-1}: (Luteolin 0.003; Rutin 0.007; Quercetin 0.125; *p*-coumaric acid 0.003; Epicatechin 0.01; Catechin 0.025)

4. Conclusion

The results showed that the solvent ethanol:water 80:20 (v/v), considered clean, presented the highest concentration of compounds for the peels and pulps. Six phenolic compounds were simultaneously identified and quantified by UHPLC-MS/MS: catechin, rutin, epicatechin, luteolin, quercetin and p-coumaric acid. A rapid methodology was developed with MS detection, with LOD (0.003 µg mL^{-1}), LOQ (0.006 µg mL^{-1}), and recovery (80%). Comparing with the different parts of camu-camu, seed and pulp presented the highest concentrations for all the phenolics found, except for the commercial pulp, which may have its profile altered by the processing, since they are unstable compounds. Thus, the use of camu-camu pulp, for consumption or as a functional ingredient, to enrich food products can contribute significantly as a source of bioactive compounds for the diet.

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Capítulo IV

Free-radical scavenging capacity and antimicrobial properties of peels, pulps and seeds of camu-camu [*Myrciaria dubia* (H.B.K.) Mc Vaugh].

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Abstract

The camu-camu is a rich fruit in antioxidant compounds essential for protecting human health against oxidative damage and diseases caused by microorganisms. This work was conducted with the purpose of determining the total phenolic compounds (TPC) and total flavonoids (TFC) of peels, pulps and seeds, and correlate with their antioxidant capacity *in vitro* by different methods, such as 3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), ferric reducing antioxidant power (FRAP), and scavenging capacities against peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and anion superoxide ($O2^{\bullet-}$). Besides, this work entended to evaluate the antimicrobial activity of these extracts against bacterias and yeast. The results of the Principal Component Analysis (PCA) showed that TPC presented a higher amount in the pulps and positive correlation with the ABTS, DPPH, FRAP methods and scavenging capacity against $ROO^{\bullet}$, H_2O_2, HOCl. In addition, the TFC had better correlation with the scavenging capacity against $O_2^{\bullet-}$. The extracts obtained from peels and seeds, mainly, were responsible for the inhibition of *Staphylococcus aureus* (0.02 – 0.62 mg/mL), *Bacillus cereus* (0.04 – 0.62 mg/mL) and *Escherichia coli* (0.04 – 0.31 mg/mL).This in vitro study suggests that camu-camu may provide protection against oxidative stress and bacterial diseases.

Keywords: Bioactive compounds, reactive oxygen species (ROS), antimicrobial activity.

1. Introduction

Recent studies[1,2,3] suggest that the ingestion of camu-camu [*Myrciaria dubia* (H. B. K.) McVough], a fruit native to the Amazon rainforest belonging to the Myrtaceae family, rich in vitamin C and polyphenols, may represent a natural source of bioactive compounds, increasing plasma antioxidant capacity, reducing plasmatic lipid levels, triacylglycerol, and inflammatory diseases associated to complications of type 2 diabetes. In addition, the fruit presents an antimicrobial effect.[4]

Different methodologies have been employed to evaluate antioxidant capacity *in vitro*. In fruits, the most cited methods in the literature are based on the ferric reducing antioxidant power (FRAP), and deactivation of non-biological radicals such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$)[5,6,7,8]. These radicals are not present in the biological systems and have different properties as compared to the reactive oxygen species (ROS) produced in the human body.

In the biological systems, the production of ROS is important to maintain homeostasis, but the excess in the production of these compounds leads to the overstimulation of some intracellular ways. For normal cellular functioning, there must be a balance between the formation of ROS and levels of antioxidant defenses. If antioxidant defenses become insufficient in the face of excessive ROS production, oxidative stress occurs, which is usually associated to the appearance of several diseases. [9-11] For the camu-camu, there are some data in the literature concerning the scavenging capacity against species of the physiological importance as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxyl radicals ($ROO^{\bullet}$).[7]

Thus, the objective of this study was to determinated the total phenolic compounds (TPC) and total flavonoids (TFC), and correlate with antioxidant capacity *in vitro* by ABTS, DPPH, FRAP methods and scavenging capacities of peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and anion superoxide ($O_2^{\bullet-}$) applying multivariate statistical analysis. Besides to evaluated the antimicrobial activity of these extracts against bacterias gram-negative and gram-positive.

2. Materials and methods

2.1. Samples

The camu-camu fruits, in lots of 2.5 kg, were harvested from two rural properties, located in the cities of Iguape (IG) and Sete Barras (SB), in Sao Paulo State, Brazil, in two lots (April and September 2015). A single lot (2.5 kg), of camu-camu fruits was harvested in the city of Tarapoto in Peru (P), in January 2016. Following each harvest, the fruits were placed inside sealed plastic bags and transpoted in ice-boxes to the laboratory at the same day they were harvested. At the laboratory, the fruits were sanitized, manually depulped to separate the peels, pulps and seeds. All parts were lyophilized and packaged, vacuum-sealed (oxygen-free) and stored in freezer (-18°C) until analysis. The lyophilized seeds were ground in an analytical mill (IKA® model A-11).

Other camu-camu fruits freshly processed and lyophilized from Para, Brazil, being a sample of peels from Embrapa Amazônia Oriental (E), and a sample commercial pulps from St. Petersburg, located in Castanhal (C) were donated to this work.

2.2. Physicochemical characterization

Total soluble solids of the peels and pulps were determined by refractometry (model R2i300, Reichert, USA), according to AOAC[12], and the results were expressed as ºBrix. The titratable acidity (TA) was determined by diluting each sample in distilled water and tittering with NaOH to pH 8.1 and the result was expressed in equivalents of citric acid (AOAC, 2010). The pH was evaluated using a potentiometer (Digimed, São Paulo, Brazil). The maturation index *("Ratio"*) was calculated by the relation between the soluble solids contents of ºBrix and A (acidity).

2.3. Bioactive compounds determination

A Flumear Omega (BMG, Labtech®) microplate reader for fluorescence, UV/vis and luminescence measurements equipped with a thermostat was used for all antioxidant capacity assays with non-physiological and physiological radicals.

2.3.1. Total phenolic content (TPC)

The extraction of phenolics compounds was performed as follows: Samples were extracted in a solvent mixture, ethanol:water 80:20 (20mL), for peels and pulps, and methanol:water 50:50 (15mL), for seeds. The extracts were shaken for 2 min with a vortex, and remained for 15 to 20 min in an ultrasonic bath, at 10° C (optimized method in chapter III). Then, they were centrifuged, filtered and the volume adjusted to 25 mL in a volumetric flask. The total phenolics were determined by spectrophotometric method, using the Folin-Ciocalteu reagent, according to Singleton *et al.*[13], adapted to a microplate reader by Scherer & Godoy (2014). The reaction medium consisted of a 25 µL aliquot of the sample extract, 125 µL of Folin-Ciocalteau, and 100µL mL of sodium carbonate (7.5% w/v). The mixture was kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 760 nm and the results were expressed as mg of gallic acid equivalent (GAE)/g dry weight of sample, based on a curve calibration of gallic acid ranging from 10 to 78 µg mL^{-1}.

2.3.2. Total flavonoids content (TFC)

The TFC was determined according to Zhishen. Mengcheng and Jianming,[14] with some modifications. Briefly, 100 µL of diluted sample (1:4) was mixed with 30 µL of sodium nitrite (2.5% w/v). This solution was mixed well and allowed to react for 5 min and then 30 µL of 10% aluminum chloride solution was added to the test tubes, and the solution was allowed to react for 5 min more. Then, 200 µL of a 1 mol/L NaOH solution and 240 µL of water was added to the tubes and the absorbance was read at 510 nm. The results were expressed as mg of catechin equivalent (CTE)/g dry weight of sample, based on a curve calibration of catechin ranging from 20 to 200 µg mL^{-1}.

2.4. Measurement of antioxidant activity

2.4.1. Free radical-scavenging ability by the use of ABTS radical cation (ABTS assay)

For ABTS (2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) assay, the procedure followed the method of Re *et al.*[15] with few modifications. The ABTS radical ($ABTS^{\bullet+}$) was generated by reaction of 5 mL ABTS stock solution with 5 mL potassium

persulfate solution, resulting in a final solution of ABTS 7 mM and 2.45 mM final concentration of potassium persulfate solution, kept in the dark for 16h at 22 ± 1°C). $ABTS^{\bullet+}$ solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 was obtained at 734 nm. Fruit extracts (10 μL) or reference substances (Trolox), were allowed to react with 9.99 mL of the resulting blue-green ABTS radical solution in a dark condition. Results were expressed in mM Trolox eq/g dry weight of sample.

2.4.2. Free radical-scavenging ability by the use of DPPH cation (DPPH assay)

The DPPH (1,1-diphenyl-2-picrylhydrazil) assay was carried out according to the procedure described by Mensor *et al.*,[16] with some modifications. DPPH (0.3 mM) was prepared in ethanol in order to obtain an absorbance of 0.7 ± 0.02 units at 518 nm. Fruit extracts (180 μL) or controls (Trolox) were allowed to react with 70 μL of DPPH radical solution for 30 min in dark and the decrease in absorbance from the resulting solution was monitored. The reduction in the absorbance at 518 nm was recorded and the $DPPH^{\bullet}$ scavenging activity was expressed as mM Trolox eq/g dry weight of sample.

2.4.3. Ferric reducing antioxidant power (FRAP assay)

Ferric reducing antioxidant power (FRAP) assay was performed following the method of Benzie and Strain,[17] with minor modifications. FRAP reagent was prepared by adding 10 volumes of 300 mM acetate buffer, 1 volume of 10 mM 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) in 40 mM HCl and 1 volume of 20 mM ferric chloride. A mixture was formed with an 80 μL aliquot of extract and 1920 μL of the FRAP reagent, remaining in a water bath at 37°C (15 min) before reading. Absorbance was measured at 595 nm and the results were expressed as mM Trolox eq/g dry weight of sample.

2.5. ROS scavenging assays

Ascorbic acid was used as positive control in the scavenging assays of H_2O_2, HOCl and $O_2^{\bullet-}$, due to its predominance in the sample. Each ROS scavenging assay corresponds to four independent experiments carried out, in duplicate, using the sample extract at five different concentrations. Except for peroxyl radical scavenging capacity, the

results are presented as percent of inhibition (IC_{50} values) calculated by non-linear regression analysis using the GraphPad Prism 5.03 software (Graph Pad Software Inc., San Diego, CA).

2.5.1. Peroxyl radical scavenging assay (ORAC)

Method was carried out according to Dávalos *et al.*[18] with some modifications. A volume of 20 µL of the extract was mixed with 120 µL fluoresceine and incubated for 15 min at 37 ºC in the microplate before the injection of 60 µL AAPH solution. The plate was incubated for a minimum of 30 min at 37 °C. Fluorescence was read for 80 min (excitation = 485 nm; emission = 520 nm). The antioxidant capacity was expressed as mM Trolox eq/g dry weight of sample.

2.5.2. $O_2^{\bullet-}$ scavenging assay

The non-enzymatic system NADH/PMS/O_2 was used to generate $O_2^{\bullet-}$, which promotes the reduction of NBT into a purple coloured diformazan compound.[19] The reaction mixtures in the sample wells contained NADH (166 µM). NBT (43.3 µM), aliquots of each extract (0.3125 µg mL^{-1} – 400 µg mL^{-1}) and the positive control at five concentrations, and PMS (2.7 µM), NADH, NBT, and PMS were dissolved in 19 mM phosphate buffer, pH 7.4. Thus, the capacity of each extract and the positive control to inhibit the reduction of NBT was monitored spectrophotometrically at 560 nm for 2 min at 37°C.[20] Ascorbic acid was used as a positive control (37.5 µg mL^{-1} – 1000 µg mL^{-1}).

2.5.3. H_2O_2 scavenging assay

The effect of camu-camu extract and the positive control (ascorbic acid) against the H_2O_2-induced oxidation of lucigenin was monitored by chemiluminescence at 37 °C and the signal was detected immediately after the introduction of the plate in the reader.[19] The assay was carried out in reaction mixtures containing the following reagents at the indicated final concentrations (final volume of 250 µL): 50 mM Tris–HCl buffer, pH 7.4, lucigenin (0.8 mM), dissolved in the buffer solution, aliquots of each extract (62.5 µg mL^{-1} – 1000 µg mL^{-1}) and the positive control at five concentrations and H_2O_2 (1%), Ascorbic acid was used as a positive control (37.5 µg mL^{-1} – 1000 µg mL^{-1}).

2.5.4. HOCl scavenging assay

The HOCl scavenging capacity was measured by monitoring the effect of the camu-camu extracts and standards on HOCl-induced oxidation of DHR to rhodamine 123 (Rezk *et al.* [21]adapted to a microplate reader according to Gomes *et al.* [22]). The HOCl was prepared using a 1% NaOCl solution (w/v), dropwise addition of 10% H_2SO_4 to pH 6.2 and the concentration of HOCl was determined by spectrophotometry at 235 nm and molar absorptivity of 100 $M^{-1}.cm^{-1}$. The assay was carried out in reaction mixtures containing 100 mM phosphate buffer solution at pH 7.4. aliquots of each extract and the positive control at five concentrations. DHR (5 µM) and HOCl (5 µM). The HOCl scavenging property of the extracts (0.9375 $\mu g.mL^{-1}$ – 30 $\mu g.mL^{-1}$) was expressed in terms of IC_{50} which represents the concentration of freeze-dried sample needed to achieve 50% scavenging. Ascorbic acid was used as positive control (0.3 $\mu g.mL^{-1}$ – 156 $\mu g.mL^{-1}$).

2.6. Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal/Fungicidal Concentration (MBC/MFC)

MIC tests were run in triplicate according to CLSI[23] using tissue culture microplates (96 wells) containing 100 µL of Müller-Hinton broth for bacteria and RPMI for *Candida albicans*. The extracts were tested against *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC 11775, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 13388, *Bacilus cereus* and *Candida albicans* ATCC 10231.

The lyophilized extracts of camu-camu (peels, pulp and seed) were diluted with Müller-Hinton broth (DIFCO®) at the initial concentrations of 10, 20 and 50 mg/mL and 100 µL were transferred to the first well. Serial dilutions were then performed to obtain the subsequent concentrations. Standardized inoculum (100 µL) was added to all wells, and the plates tested for bacteria were incubated at 36°C for 24 hours and at 36°C for 48 hours for *Candida albicans* under aerobic conditions. They were then evaluated and MIC was defined as the lowest concentration of the extracts that inhibited growth. Based on the MIC results 10 µL of the cells suspension from the wells showing no visible microbial growth and from the wells above were them subcultured in Petri dishes containing Sabouraud Dextrose Agar medium (SDA- Merck®) for *Candida albicans* and Nutrient Agar (DIFCO®) media for bacteria. The plates were at the same way for MIC. The MBC/MFC was defined as the lowest

sample concentration showing no cell growth on the inoculated agar surface. The tests were performed in triplicate.

2.7. Experimental design and data analysis

The IC_{50} values were calculated from the curves of inhibition percentage using the GraphPad Prism 6 software. Data of the antioxidant capacity were analyzed with ANOVA and Tukey's test on significant differences in means. The Statistic software program was employed, and the significance level between mean values was set at 5% ($p < 0.05$). Principal Component Analysis (PCA) was performed using the Pirouette 3.11 program, the data was previously auto-scaled. All data were adquired in triplicate, and the results were presented as mean values with their corresponding standard deviations.

3. Results and Discussion

3.1. Physicochemical characterization

Table 1 show the average of three determinations and the standard deviation. The moisture content found for the peels was from 89.68 to 90.7%, pulps from 92.81 to 95.01% and seeds from 55.53 to 58.87%.

Table 1. Phisicoquimic characterization of peel, pulp and seeds of camu-camu fruits.

Sample		**Components**				
		Moisture (%)	**pH**	**Soluble solids (ºBrix)**	**Acidity**	**Ratio (Brix/Acidity)**
Peel	IG	89.68 ± 0.85	2.62 ± 0.03	3.56 ± 0.60	1.32 ± 0.02	2.69
	SB	90.70 ± 0.21	2.38 ± 0.04	2.76 ± 0.15	1.13 ± 0.02	2.44
	P	90.19 ± 0.07	3.54 ± 0.01	1.4 ± 0.1	0.38 ± 0.01	3.68
Pulp	IG	94.81 ± 0.61	2.31 ± 0.01	5.76 ± 0.05	2.37 ± 0.09	2.43
	SB	93.72 ± 0.71	2.23 ± 0.01	6.15 ± 0.05	2.70 ± 0.01	2.27
	P	95.01 ± 0.41	3.47 ± 0.02	6.1 ± 0.1	1.82 ± 0.03	3.35
	C	92.81 ± 0.22	2.99 ± 0.23	5.23 ± 0.09	2.12 ± 0.07	2.46
Seed	IG	58.87 ± 1.67	5.24 ± 0.09	-	0.95 ± 0.02	-
	SB	57.53 ± 2.66	4.78 ± 0.09	-	0.99 ± 0.06	-
	P	55.53 ± 0.66	5.67 ± 0.06	-	0.92 ± 0.04	-

Values are expressed as means ± SD (n=3). Acidity results expressed as mg citric acid/100g sample. IG = Iguape; SB = Sete Barras; P = Peru; E = Embrapa; C = Commercial.

According to the results of the freshs peel and pulp, camu-camu is a fruit with high acidity and low sugar content. These factors discourage their consumption in natura, justifying the search for products with better sensorial acceptance, however, incresead fruit preservation, prevent rapid deterioration and fermentation, providing better post-harvest conditions.[24,25]

The samples cultivated in dry soil regions in Brazil (IG and SB) and the commercial pulp (P) presented similar characteristics regarding the content of pH, ºbrix and acidity, differing from the cultivated sample in regions of flooded soil in Peru (P), which presented higher pH and acidity. As reported by Yuyama *et al.*[26], the highest levels of °brix in fruits cultivated in Brazil may be related to the type of management, since in soil on dry land the addition of nutrients and water can be controlled, which did not occur in flooded environments.

The low pH and high acidity observed for camu-camu fruit are similar to those for lemon. The pH values found in this study are similar to those reported by Akter *et al.*[27], Zanatta *et al.*[28], Fujita *et al.*[24] and Freitas *et al.*[25] found pH values varying between 1.44 ± 0.01 and 2.06 ± 0.01 for different camu-camu genotypes harvested in the Brazilian Amazon region. Neves *et al.*[29] found pH levels between 3.12 to 3.34 and high acidity of 1.96 a 3.12.

In relation to the fruit ripening index, represented by the ratio of soluble solids to titratable total acidity, it was observed that one of the samples showed a more advanced stage of maturation. These changes may occur due to the increase in carbohydrate levels as the fruits mature. The results showed that the pulp (P) showed a more advanced stage of maturation in relation to the other samples. The ratio stands out as the best practice or criterion for evaluating fruit flavor. [4, 30]

3.2. Bioactive compounds: total phenolics and total flavonoids

The total phenolic compounds are in Figure 1 (A), expressed in dry weight (g). The highest amounts of polyphenols were detected in the in natura pulp, with higher levels in SB and P (343.9 ± 30.71 and 357.30 ± 13.85 mg.GAE/g, respectly). The frozen commercial pulp (C) presented levels of 175.67 ± 13.14 mg.GAE/ g, this value is seven times higher than found by De Souza Schmidt Gonçalves *et al.*[1] for frozen pulp (24.900 mg GAE/100g). The peel (E) with higher phenolic content presented about 134.61 ± 5.29 mg.GAE/ g and in the seeds, the highest content was found in the IG sample (115.12 ± 9.62 mg.GAE /g).

The content of phenolic compounds reported for camu-camu fruits is higher than those of acerola, araçá, cambuci, tucumã, uxi and fruits of the same genus, such as *Myrciaria vexato* and *Myrciaria cauliflora*.[6,8,31,32] However, the results found in this study are even greater than the authors who also evaluated the potential of camu-camu.[8,32,33,34] According to Prior *et al*[35]. and Singleton *et al.*[13] high levels of ascorbic acid present in camu-camu can interfere with the same oxidation/ reduction reaction that detects phenolics. This corresponds to the high levels of ascorbic acid found in the same fruits by our research group, especially in pulps.

Figure 1 (B) shows the results for total flavonoids. Fruits and vegetables are the main dietary source of flavonoids, and their potential health benefits are associated with powerful hydrogen-donating and also their reducing properties, which contribute to redox regulation in cells.[36] In this study, the highest contents of the flavonoid were reported in the seeds (35.68 ± 1.99 and 27.61 ± 4.83 mg. CTE /g dw) and peels (12.31 ± 0.54 and 11.35 ± 0.88 mg. CTE /g dw) of the samples collected in Brazil (IG and SB).

Significant diferences ($p < 0.05$) were observed between total phenolic and flavonoid levels of peels, pulps and seeds grown in different regions. The variations measured can be attributed to several factors including cultivar and harvest or storage time, geographical origin, also extraction methods and solvents used. [37]

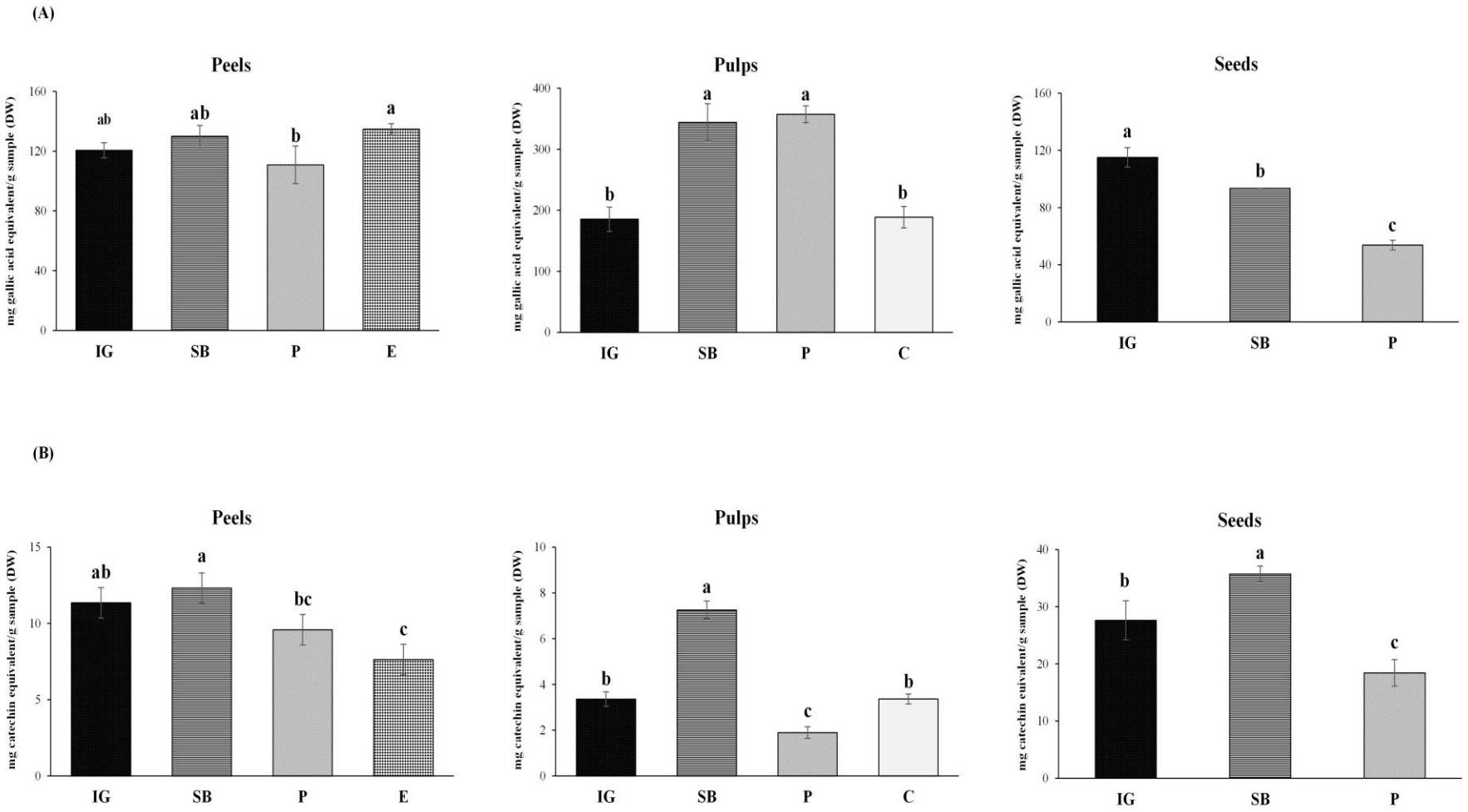

Figure 1. (A) Results of analysis total phenolic content (TPC) and (B) results of total flavonoids content (TFC) for peels, pulps and seeds of the different samples harvested in Brazil and Peru.

3.3. Antioxidant capacity for non fisiologic and fisiologic radicals

It is believed that the phenolic compounds and ascorbic acid contents are the main responsible for the high antioxidant activity of camu-camu fruits.[6,33] The results presented in Table 2 are consistent with the vitamin C determinations performed by our group, which shows higher antioxidant activity of the pulps due to the high content of vitamin C.

The bioactive components of the sample contribute distinctly in each specific method of antioxidant activities, even using the same matrix. The differences found can be explained by the fact that the amount and type of compounds can vary according to the geographic region, and period of the year, and that during processing parts of the fruit. [38,39] In this study, the highest activities were found in the cam-camu pulps, the values reported by ABTS method (1831.1 to 8665.93 mmol Trolox Eq/g), DPPH (514.58 to 1308.25 mmol Trolox Eq/g) and FRAP (1171.59 to 1564.17 mmol Trolox Eq/g), were superior to those of the peels and seeds; however, these samples also presented higher antioxidant potential than those reported by other autors for camu-camu fruits. Fracassetti *et al.*[5], analyzing camu-camu by DPPH method, found 1036.4 ± 211.2 µmol Trolox Eq/g in camu-camu flour and 510.5 ± 156.1 µmol de Trolox Eq/g in dry pulp. De Souza Schmidt Gonçalves *et al.* [1] for the same method, reported 1679 µmol de Trolox Eq/g for frozen pulp.

ABTS and DPPH are methods that form radicals of non-physiological origin and are based on the same reaction principle: single electron transfer.[40,41] The main limitation of these methods is related to the measurement of the specific oxidant-reducing power, not necessarily equivalent to the antioxidant capacity. Besides that, this radicals are not present in the biological system and have different properties as compared to the ROS produced in the human body to maintain homeostasis. [9]

The scavenging capacity towards peroxyl radical (ROO•) of the pulps ranged from 262.04 to 551.84 mmol Trolox Eq/g dw, and were higher than those from the peels (395.68 to 418.20 mmol Trolox Eq/g dw) and seeds (215.16 to 346.01 mmol Trolox Eq/g dw). This antioxidant capacity was higher than reported for camu-camu by Fracassetti *et al.* [5], De Souza Schmidt Gonçalves *et al.* [42] and Neves *et al.* [29] for the same methods. As well, for other fruits, like acerola (3458 - 5883 µmol Trolox Eq/100g dw), uxi (116.5 µmol Trolox Eq/100g dw), açaí (602.5 µmol Trolox Eq/100g dw), murici (132.6 µmol Trolox Eq/100g dw), strawberry (153.6 µmol Trolox Eq/g dw) and blackberry (204 µmol Trolox Eq/g dw).[8,29]

Table 2. Antioxidant capacity in Trolox (ET) and IC_{50} of camu-camu peels, pulps and seeds from Brazil and Peru.

		Camu-camu extracts					
Assay	**Sample**	**IG**	**SB**	**P**	**E**	**C**	
ABTS (mM Trolox/g dw)	Peel	1038.96 ± 22.45^{bB}	1093.03 ± 71.10^{bB}	1007.96 ± 77.32^{bB}	1326.72 ± 32.88^{a}	-	
	Pulp	1831.10 ± 121.14^{cA}	8665.93 ± 1075.63^{aA}	3531.35 ± 223.10^{bA}	-	5050.30 ± 337.35^{b}	
	Seed	779.10 ± 76.34^{aB}	662.67 ± 76.33^{aC}	365.79 ± 28.70^{bC}	-	-	
DPPH (mM Trolox/g dw)	Peel	295.57 ± 2.63^{bcC}	292.90 ± 4.78^{cC}	297.35 ± 4.63^{bB}	534.47 ± 2.77^{a}		
	Pulp	538.16 ± 20.48^{bA}	584.53 ± 18.48^{bA}	1308.25 ± 4.82^{aA}	-	514.58 ± 4.85^{b}	
	Seed	466.16 ± 35.68^{aB}	386.41 ± 0.77^{aB}	241.60 ± 12.07^{bC}	-	-	
FRAP (mM Trolox/g dw)	Peel	896.54 ± 58.14^{bB}	908.41 ± 54.77^{bB}	904.03 ± 118.61^{bA}	1337.06 ± 147.74^{a}	-	
	Pulp	1435.43 ± 71.40^{aA}	1514.07 ± 167.73^{aA}	1171.59 ± 191.06^{aA}	-	1564.17 ± 173.55^{a}	
	Seed	1603.85 ± 204.21^{aA}	737.78 ± 22.40^{bB}	866.90 ± 72.54^{bA}	-	-	
$ROO^{\bullet}$ (mM Trolox/g dw)	Peel	413.68 ± 33.79^{aA}	412.72 ± 5.79^{aB}	418.20 ± 13.25^{aAB}	395.68 ± 7.47^{a}	-	-
	Pulp	262.04 ± 22.90^{bB}	551.84 ± 15.96^{aA}	522.47 ± 81.68^{aA}	-	330.61 ± 11.29^{b}	-
	Seed	332.80 ± 26.48^{aAB}	346.01 ± 0.88^{aC}	215.16 ± 34.87^{bB}	-	-	-
							Pos. Control (Vitamin C)
HOCl IC 50 ($\mu g\ mL^{-1}$)	Peel	4.65 ± 0.27^{bB}	5.62 ± 0.33^{bC}	4.77 ± 0.12^{bC}	3.75 ± 0.09^{a}	-	3.13 ± 0.22^{a}
	Pulp	2.57 ± 0.29^{cA}	1.25 ± 0.11^{aA}	1.90 ± 0.04^{bA}	-	2.58 ± 0.18^{cA}	3.13 ± 0.22^{d}
	Seed	5.18 ± 0.54^{bB}	5.26 ± 0.22^{bC}	6.69 ± 0.29^{cD}	-	-	3.13 ± 0.22^{a}
H_2O_2 IC 50 ($\mu g\ mL^{-1}$)	Peel	462.46 ± 8.43^{bC}	716.66 ± 16.09^{cC}	771.2 ± 17.01^{dC}	811.86 ± 18.30^{e}	-	143.93 ± 11.56^{a}
	Pulp	280.26 ± 15.31^{cdB}	226.1 ± 8.28^{bB}	244.03 ± 14.95^{bcB}	-	286.36 ± 18.86^{d}	143.93 ± 11.56^{a}
	Seed	428.66 ± 17.68^{bC}	736.4 ± 16.10^{cC}	750.13 ± 13.52^{cC}	-	-	143.93 ± 11.56^{a}
$O_2^{\bullet -}$ IC 50 ($\mu g\ mL^{-1}$)	Peel	179.86 ± 10.92^{aC}	142.16 ± 9.41^{aB}	130.1 ± 12.52^{aB}	245.56 ± 2.28^{c}	-	290.46 ± 16.13^{d}
	Pulp	75.29 ± 3.75^{aB}	185.93 ± 4.80^{cC}	107.63 ± 2.97^{bB}	-	60.38 ± 5.58^{a}	290.46 ± 16.13^{d}
	Seed	34.25 ± 2.71^{aA}	58.80 ± 5.85^{bA}	45.16 ± 2.74^{abA}	-	-	290.46 ± 16.13^{c}

Means followed by the same letter in the line (n = 3) and upper case in the column for each sample class does not show significant difference among each other at $p < 0.05$ by the Tukey test.

With regard to the scavenging capacity against H_2O_2, the pulps of camu-camu had the highest efficiency, with values ranging from 244.03 to 286.36 μg.mL^{-1}. The peels and seed showed IC_{50} at 462.46 μg.mL^{-1} and 428.66 μg.mL^{-1}, respectively. The values found were higher than those reported by Ribeiro *et al.*[19] for the pulp of *Psidium cattleianum* (378 μg.mL^{-1}), commonly known as strawberry guava, belonging to *Myrtaceae* Family, the same family of camu-camu, while Ribeiro *et al.*[43] reported a value similar to that found in this study for leaves of *V. cauliflora species.* Berto *et al.* [44] reported for seed extract of *C. bracteosa* an IC_{50} of 426 ug.mL^{-1}, peels extract (894 ug.mL^{-1}), while the pulp extract could decrease the oxidizing effect of H_2O_2 only by 29%, at the highest tested concentration (1000 ug.mL^{-1}). According to Vissotto *et al.*[45], the lemon presented highest efficiency compared to camu-camu, with the lowest IC_{50} values 143 μg.mL^{-1}. In general, the IC_{50} values to scavenge H_2O_2 are higher in comparison with other ROS (Figure 2) and sometimes the IC_{50} can not be achieved in the maximum concentration tested.[20,46] Extracts capable of eliminating hydrogen peroxide, such as camu-camu for example, have high potential to be used as food protectors.

The results for the HOCl suggest a high elimination potential for pulp extracts, with IC_{50} ranging from 1.25 to 2.57 μg.mL^{-1}, followed by peels extracts (IC_{50} of 3.75 to 5.62 μg.mL^{-1}) and seed (IC_{50} from 5.18 to 6.69 μg.mL^{-1}). When compared to the extracts of *V. cauliflora* (2.6 and 3.6 μg.mL^{-1})[43], *Caryocar villosum* pulp (3.6 to 299 μg.mL^{-1})[20], and *Solanum sessiliflorum* pulp (13 μg.mL^{-1})[47], the extracts of the camu-camu pulp demonstrated a greater capacity to scavenge HOCl. Besides that, camu-camu pulp was even more efficient than ascorbic acid. The HOCl is the strongest oxidant produced by neutrophils and is considered as a potent pro-inflammatory agent, and can also react with $O_2^{\bullet-}$ to generate another microbicidal species $^{\bullet}OH$. [27]

Seed extracts presented high elimination capacity against $O_2^{\bullet-}$ with IC_{50} ranging from 34.25 to 58.8 μg.mL^{-1}, while the peels and pulps extracts showed values between 130.1 to 179.86 μg.mL^{-1}, and 60.38 to 185.93 μg.mL^{-1}, respectively. In this assay all extracts were more efficient than ascorbic acid in the elimination of ROS. Berto *et al.* [48] found greater activity against $O_2^{\bullet-}$ for extracts of *C. bracteosa*, with an IC_{50} of 11.5 μg.mL^{-1}. Ribeiro *et al.* [49] reported for *V. cauliflora* extract scavenged $O_2^{\bullet-}$ at concentrations in the range of 10.3 to 53.8 μg.mL^{-1}.

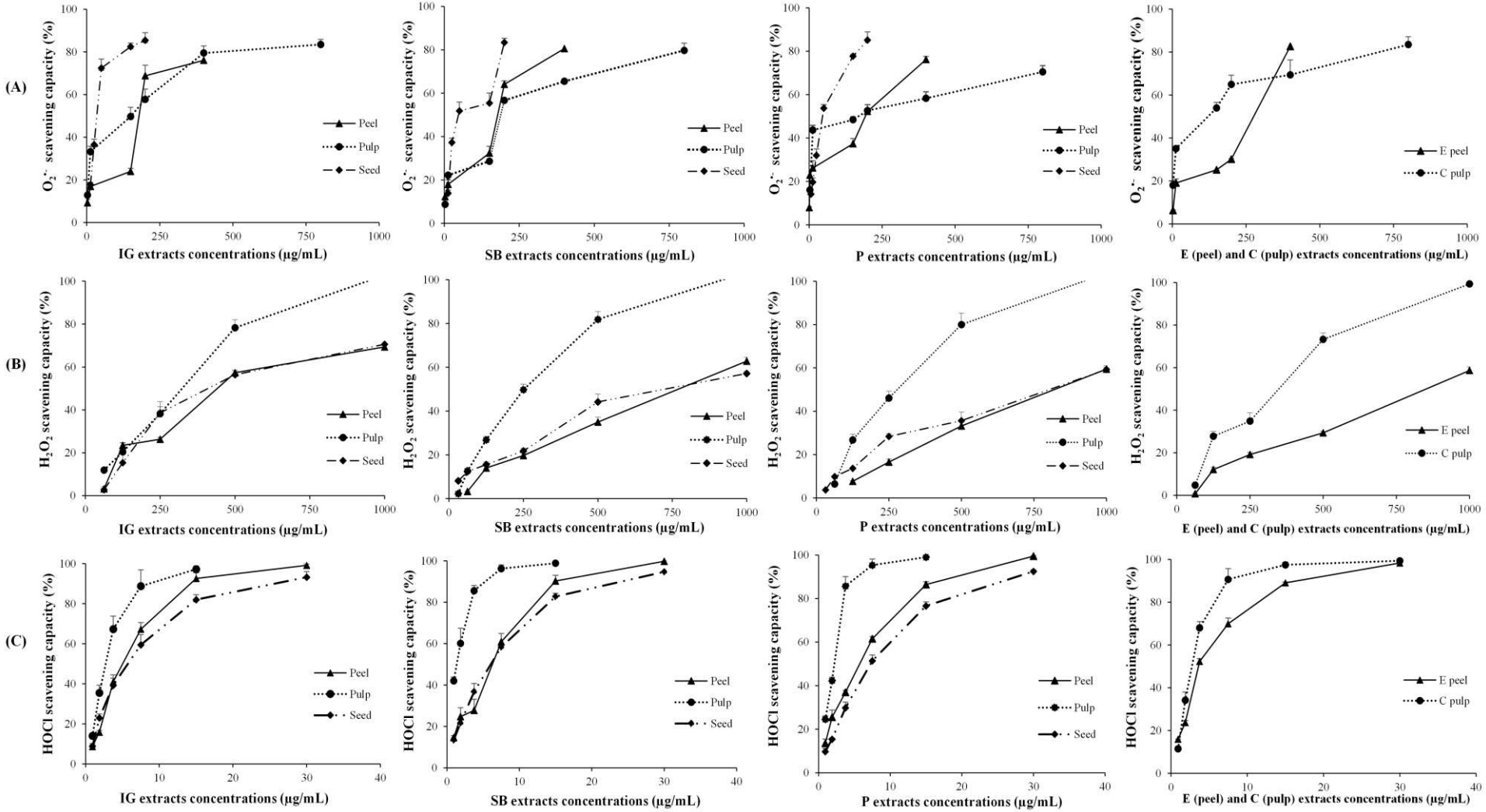

Figure 2. Scavenging capacities of the peel, pulp and seed extracts of camu-camu fruits against (a) superoxide radical ($O_2^{\bullet-}$), (b) hydrogen peroxide (H_2O_2), (c) hypochlorous acid (HOCl). Eachpoint shows the standard error of the mean bars and represents the values from four experiments.

Summarily, the results suggest that camu-camu fruits have higher scavenging capacity of species of physiological relevance than other native Brazilian fruits. Thus, could be a viable option for maintaining a balanced immune response and antioxidant improving anti-inflammatory processes. In addition, it can be asserted that there is a direct relationship between total phenolic content and antioxidant capacity of the pulp and peel of each fruit analyzed here.

To determine possible relationships between the bioactive compounds of the samples and the antioxidant capacity against the different methods used, the main component analysis (PCA) and the results presented in Figure 3 were applied. Together, PC_1 and PC_2 explained 91.12% of the variation observed. PC1, with 53.79% of the variation, promoted the separation into five groups: from pulps C, SB and P, which presented higher phenolic contents and antioxidant capacity for ABTS, DPPH and FRAP and for $ROO^{\bullet}$, H_2O_2 and HOCl.

The group of IG pulps, which also presented high antioxidant capacity. The group of all the peels, which presented higher values of antioxidant capacity by the $ROO^{\bullet}$ method. The group of seeds SB and P, and finally, the group formed by the seeds IG, which are related to the higher levels of flavonoids and antioxidant capacity by the superoxide method. Applying the principal component analysis in different in vitro methods to determine the antioxidant capacity of some phenolic compounds. Hossain *et al.* [50] found significant correlations between the DPPH, FRAP, ORAC and ABTS methods. Many studies have used PCA as a tool to correlate results obtained from in vitro methods to determine the antioxidant potential of several matrices.[51,52]

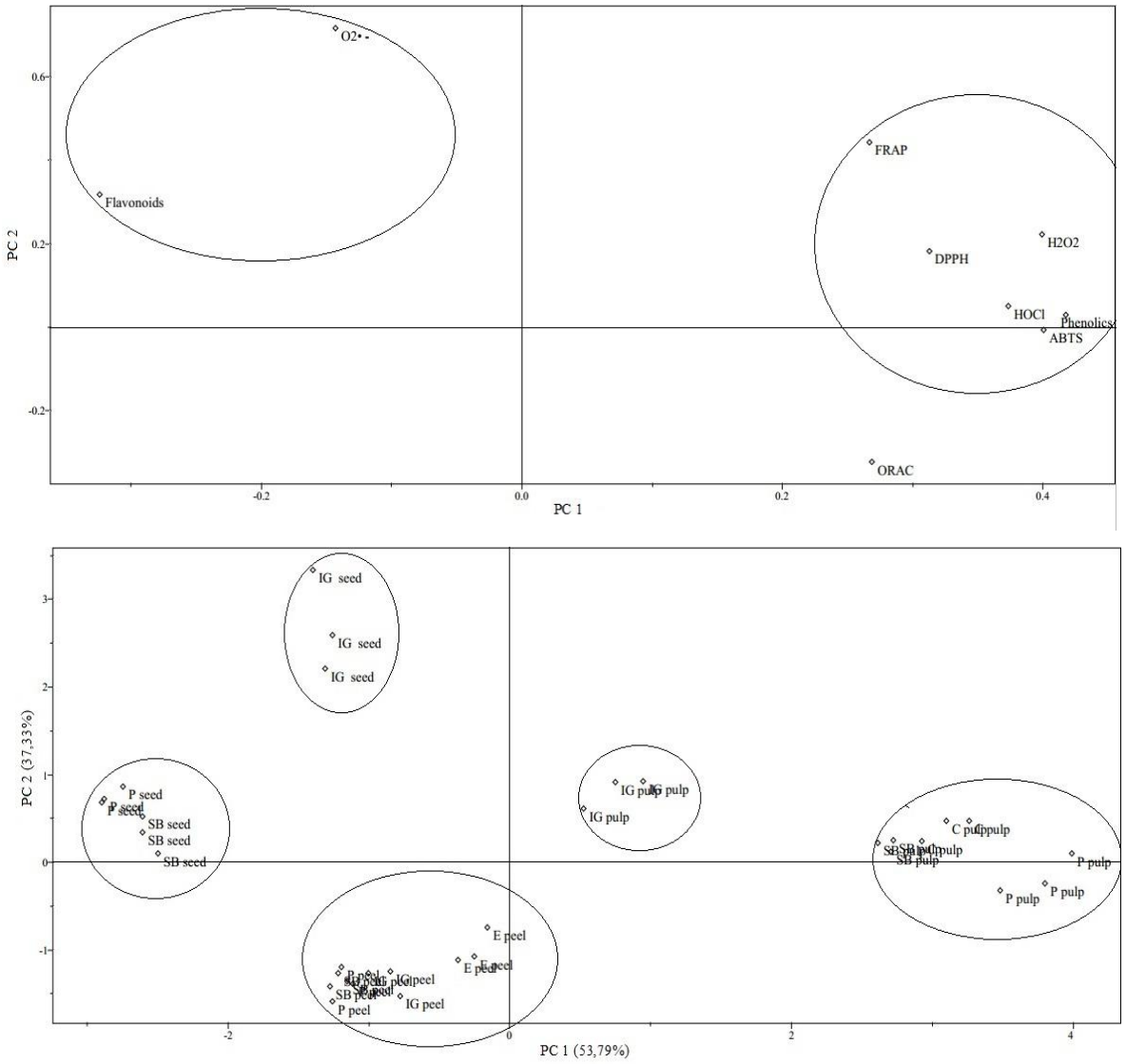

Figure 3. Graphs of scores for total phenolic compounds, flavonoid and antioxidant capacity of the camu-camu peels, pulp and seed. (A). PC for all samples; (B) PC for the methods correlated with all samples.

3.4. Antimicrobial activity

Table 3 shows the results for the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicide Concentration (MBC/MFC) of the camu-camu extracts obtained from the peels, pulps and seeds. These matrices showed inhibitory effect (MIC) for *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*, In addition, the seeds of Brazilian fruits (IG and SB) and the pulp of Peruvian fruits

(P) showed inhibitory activity for *Escherichia coli*. However, no sample had activity against *Candida Albicans*.

Table 3. Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration/Minimum Fungicide (MBC/MFC) in mg/mL of the samples.

Sample		Microorganisms											
		Escherichia coli ATCC 11775		*Salmonella choleraesuis* ATCC 10708		*Pseudomonas aeruginosa* ATCC 13388		*Staphylococcus aureus* ATCC 6538		*Bacillus cereus*		*Candida albicans* ATCC 10231	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
IG	Peel	> 10.0	> 10.0	10.0	> 10.0	> 10.0	> 10.0	0.02	> 10.0	0.04	> 10.0	NA	NA
	Pulp	> 20.0	> 20.0	2.5	> 20.0	5.0	>20.0	1.25	> 20.0	1.25	> 20.0	NA	NA
	Seed	0.04	10.0	0.62	10.0	1.25	> 10.0	0.02	> 10.0	0.08	> 10.0	NA	NA
SB	Peel	> 10.0	> 10.0	1.25	> 10.0	5.0	> 10.0	0.02	> 10.0	0.08	> 10.0	NA	NA
	Pulp	> 50.0	> 50.0	1.56	> 50.0	6.25	> 50.0	3.12	12.5	3.12	> 50.0	NA	NA
	Seed	0.04	>10.0	1.25	>10.0	1.25	> 10.0	0.04	> 10.0	0.08	> 10.0	NA	NA
P	Peel	> 10.0	> 10.0	1.25	> 10.0	5.0	> 10.0	0.08	> 10.0	0.15	10.0	NA	NA
	Pulp	> 20.0	> 20.0	2.5	> 20.0	5.0	> 20.0	2.50	> 20.0	2.50	> 20.0	NA	NA
	Seed	0.04	10.0	1.25	10.0	2.50	> 10.0	0.02	10.0	0.08	10.0	NA	NA
E	Peel	> 10.0	> 10.0	1.25	> 10.0	5.0	> 10.0	0.62	10.0	0.62	> 10.0	NA	NA
C	Pulp	0.31	>20.0	2.5	>20.0	2.5	> 10.0	0.31	10.0	0.16	> 10.0	NA	NA

NA = No Activity

Duarte *et al.* [53] classified MICs for plant essential oils as low at the limit of 0.5 mg/mL, moderate inhibition between 0.6 and 0.9 mg/ mL, and high inhibition of MICs above 1.0 mg/ mL. In this study, the peel and seed extracts from the IG, SB and P samples showed high inhibition of *Staphylococcus aureus* (ranging from 0.02 to 0.08 mg/mL) and *Bacillus cereus* (0.4 to 0.15 mg/mL). And only the pulp (C) showed high inhibition for both microorganisms (0.31 and 0.16 mg/mL, respectively). Inhibition of the peel (E) was moderate (0.62 mg/mL). In a previous papers[54,24, 31] we reported that the seed and peel extract of camu-camu fruit showed antimicrobial activity against *Staphylococcus aureus* due to lipophilic constituents.

For *Escherichia coli*, the seeds showed high inhibition (0.04 mg/mL) and pulp (C) promoted inhibition from 0.31 mg/mL. Between moderate and low the inhibitory activities of the extracts were found for *Salmonella choleraesuis* and *Pseudomonas aeruginosa*.

According to Da Silva *et al.* [55] reported that Gram-negative bacteria have more complex cell membranes (peptidoglycan, periplasm, and lipopolysaccharide) that make penetration difficult for antimicrobial agents, and that, the fact that the partial hydrophobicity

of phenolics and flavonoids allows them to destabilize the lipopolysaccharide cell wall of Gram-negative bacteria. [56, 57]

The results of MBC ranged from 10 mg/mL to 50 mg/mL. This variation can be attributed to fruit pH and the content of the phenolic compounds. The low pH and the presence of phenolic compounds and flavonoids may result in conditions that are adverse for foodborne pathogens.[58,59] Camu-camu fruits presents higher contents of flavonoids. [5, 27, 32, 33, 42, 60] The results of this study showed that the highest antimicrobial activity was correlated to the samples that presented higher polyphenolic content.

4. Conclusion

The profile of phenolic compounds and flavonoids of extracts obtained from camu-camu influenced their high antioxidant activity, against oxygen reactive species of physiological and non-physiological relevance. However, it is recognized that the high vitamin C content of this fruit also contributes significantly to these results, especially in pulps, which were most effective against almost all ROS. The behavior of the bioactive compounds varied according to the region of culture. In addition, the extracts obtained from different parts of the fruit were responsible for the inhibition of gram-positive and negative bacteria. Thus it was concluded that the Amazon native fruit as a promising source of bioactive compounds with high antioxidant and antimicrobial properties may be exhibiting great potential against oxidative stress, inflammatory and bacterial diseases.

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DISCUSSÃO GERAL

Vários fatores afetam a qualidade da vida moderna, de forma que a população deve ter consciência da importância de alimentos contendo componentes que auxiliam a promoção da saúde e melhoram o estado nutricional. Neste aspecto, as frutas se destacam, pois são amplamente consumidas por suas características organolépticas e por apresentarem em sua composição compostos biologicamente ativos (Jimenez et al., 2014). As recomendações nutricionais sugerem que o consumo de cinco porções diárias de frutas e verduras é ideal e necessário para obtenção dos micronutrientes necessários e para a promoção uma alimentação saudável (Heimendiger et al., 1996).

O camu-camu é um fruto cujo habitat varia desde solos férteis da várzea do Peru, onde há influência direta dos Andes, até solos paupérrimos da praia de areia branca do Rio Negro. As plantas conseguem adaptar-se conforme seu habitat, em solos férteis, as raízes são curtas e ficam próximas ao caule principal; por outro lado, em solo pobre como areia branca, o sistema radicular pode estender-se três vezes ou mais a sua altura. Esse fato pode influenciar diretamente na produção do fruto, onde a planta bem nutrida produz todos os anos, e as plantas em solos pobres produzem a cada dois ou três anos (Yuyama et al., 2011). A árvore frutifica entre os meses de novembro a março, e é considerada a fruta como maior teor de vitamina C já estudada (Penn, 2006).

Não foram encontrados estudos na literatura sobre a utilização da técnica de PLE para extração de vitamina C em matrizes vegetais. No presente estudo, os rendimentos de extração do L-AA utilizando a água como solvente corresponderam, em média, a 88% do rendimento obtido pela extração convencional, no qual utiliza-se uma solução ácida para extração e estabilização do composto. Este alto rendimento pode ser explicado pela alta temperatura, que favoreceu a interrupção das interações moleculares de *van der waals*, ligações de hidrogênio e interações dipolo-dipolo entre a matriz da planta e os compostos extraíveis (Viganó et al., 2016; Wijngaard et al., 2012). A utilização de técnicas "verdes" em alimentos representa menos uso de solventes tóxicos em matrizes alimentares, e isso implica em menor geração de resíduos, além de ser uma técnica segura para os manipuladores durante a análise. Já em relação à maceração, o baixo rendimento de extração era esperado devido à exposição do extrato ao oxigênio por um período prolongado, sem auxílio de um ácido para extração e estabilização (Spínola et al., 2013).

Em relação aos métodos de determinação de vitamina C total, quando foi comparada a titulação com o método cromatográfico, não houve diferenças estatisticamente

significantes ($p < 0,05$) para a maioria das amostras. Na literatura alguns estudos já haviam reportado a equivalência dos resultados entre os métodos (Hernadez et al., 2006; Tarrago-Trani et al., 2012). O método titulométrico, considerado o método oficial da AOAC para análise da vitamina, ainda é muito utilizado pela maioria nos laboratórios devido ao baixo custo dos materiais necessários, no entanto, suas desvantagens incluem o não reconhecimento do ponto de viragem em amostras coloridas por analistas não treinados, e analiticamente, a presença de interferentes que podem reduzir o indofenol e causar uma superestimação do teor de L-AA, e a exposição da amostra à fatores degradantes durante a análise, como a luz e oxigênio (Quirós et al., 2009). Já o método cromatográfico, garante a detecção das duas formas de vitamina C (L-AA e DHAA) com alta sensibilidade e especificidade, distinguindo-os de outros agentes redutores presentes nas amostras e reduzindo a exposição a fatores ambientais degradantes (Spínola et al., 2012).

Os frutos *in natura* analisados neste estudo foram provenientes das cidades de Iguape e Sete Barras, localizadas no Vale do Ribeira, estado de São Paulo. E da cidade de Tarapoto, localizada no Peru. Além disso, foram cedidas cascas liofilizadas de frutos cultivados pela Embrapa Amazônia Oriental, e polpas comerciais recém-processadas cedidas pela indústria São Pedro®, ambas localizadas em Belém, estado do Pará. No geral, o teor de vitamina C total (L-AA e DHAA) encontrado nos frutos variaram de 680 a 1074 mg/100g de cascas *in natura*, e de 1297 a 1915 mg/100g para as polpas in natura. Na literatura, os resultados de vitamina C total reportados para as polpas variam de 845 a 7355 mg/100 g de polpa *in natura* (Yuyama et al., 2002, Genovese et al., 2008; Neves et al., 2015), e em polpas liofilizadas chegaram a 13756.79 mg/100 g.

Os frutos analisados no presente estudo, a média de peso apresentada foi de 9,2 g, e o rendimento das partes tissulares: casca (1,47g – 16%); polpa (5,06g – 55%); e semente (2,67g – 29%). O teor médio de ácido ascórbico das polpas analisadas corresponde a ± 1620 mg/100g. Considerando que um adulto deve ingerir entre 75 e 90 mg de vitamina C por dia para atingir as recomendações nutricionais, o consumo da polpa *in natura* de 1 camu-camu (± 82 mg/100g) seria suficiente para suprir as necessidades da vitamina C.

Quanto à localização, observou-se que a região de origem influenciou nos teores de L-AA das cascas e polpas, e que os teores foram maiores para as regiões sujeitas a inundações, como é o caso das amostras peruanas, colhidas na região amazônica. Esse resultado contradiz Ribeiro et al. (2016), que encontraram maiores teores de L-AA de frutos camu-camu de regiões com solo seco (13.756,79 mg/100g ps) quando comparado ao solo inundado (11.821,29 mg/100g ps). Chirinos et al. (2010) relataram que vários fatores podem

contribuir para tais diferenças, incluindo variações climáticas durante o desenvolvimento da planta, técnicas de manejo do solo, estágio de maturação na colheita, variações genéticas, manuseio pós-colheita e condições de armazenamento e processamento, além do método utilizado para análise de vitaminas.

Em relação aos compostos fenólicos e flavonóides, as condições de extração foram otimizadas por meio de técnicas multivariadas, utilizando como solventes água, etanol e metanol, e suas respectivas combinações (Lopes et al., 2011; Handa et al., 2016), para as polpas e os subprodutos (casca e semente). A melhor condição de extração para ambos os compostos nas polpas e cascas do camu-camu foi a mistura de etanol e água (80:20 v/v), enquanto que para a semente foi metanol e água (50:50 v/v). A identificação e quantificação dos compostos fenólicos e flavonóides foi realizada através da técnica de separação por cromatografia líquida de ultra-alta eficiência acoplada ao espectrômetro de massas, com fonte de ionização por eletrospray (ESI^-), e como analisador um triplo quadrupolo no modo de monitoramento seletivo de reações (SRM), o que torna o método adequado, por exibir características como seletividade e especificidade.

A catequina foi o principal flavonóide encontrado no camu-camu (casca, polpa e semente), variando de 138,5 a 2988 mg/100g pf, o que corresponde a 67 a 95% dos flavonoides totais presentes nos extratos hidrofílicos. Chirinos et al. (2010) encontraram para o camu-camu, conteúdos de catequina de 1,3, 1,7 e 2,2 mg/100g em frutos verdes, semi-maduros e maduros, respectivamente. Nos extratos também estão presentes outros flavonoides como a luteolina, rutina, quercetina e epicatequina. O ácido *p*-coumarico foi o único ácido fenólico encontrado no camu-camu (casca, polpa e semente), com níveis variando de 7,45 a 117,92 mg/100g pf. Vale ressaltar que foram extraídos apenas os compostos fenólicos na forma livre, que representam a menor fração, já que a maior parte destes compostos encontra-se na forma de complexos ligados a polímeros da parede celular (carboidratos e proteínas) dos vegetais, por meio de ligação, éster e glicosídicas. (Chen & Zuo, 2007; Ahmad et al., 2016).

Os extratos hidrofílicos das variedades de camu-camu analisadas destacaram-se pela elevada capacidade antioxidante para radicais não fisiológicos $ABTS^{+\bullet}$ e $DPPH^{\bullet}$, capacidade de redução de ferro (FRAP), desativação de radicais de origem fisiológica $ROO^{\bullet}$ e $O_2^{\bullet-}$, e das espécies reativas moleculares H_2O_2 e HOCl. Acredita-se que o alto teor compostos bioativos presentes nos frutos contribuem para a sua alta capacidade antioxidante, como é o caso das polpas de camu-camu, que de acordo com os resultados deste estudo, apresentaram as maiores concentrações de vitamina C, fenólicos e flavonoides. E possivelmente são capazes de desativar outras EROs e espécies reativas de nitrogênio (ERN) através da

transferência de elétrons e da presença de ligações duplas (Horstman et al., 2002; Anouar et al., 2009).

Resumidamente, os resultados sugerem que os frutos de camu-camu apresentam maior capacidade de eliminação para espécies radicais de relevância fisiológica quando comparado a outras frutas brasileiras. Embora a vitamina C seja considerada o maior contribuinte na atividade antioxidante, grande parte dos autores sugerem que os compostos fenólicos são os maiores responsáveis pela atividade antioxidante em frutos (Heim et al., 2002; Sun et al., 2002). No entanto, para frutos como o camu-camu, que apresentam elevado teor de vitamina C, a maior contribuição para a atividade antioxidante total dos frutos se deve à composição da vitamina.

Quanto à atividade antimicrobiana, determinada pela concentração inibitória mínima (MIC) e pela concentração bactericida/fungicida mínima (MBC/MFC), os resultados mostraram que os extratos etanólicos de camu-camu obtidos à partir das cascas e polpas, e metanólicos obtidos das sementes apresentaram efeito inibitório para *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* e *Bacillus cereus* e para *Escherichia coli*. No entanto, nenhuma amostra teve atividade contra o fungo *Candida Albicans*. Os extratos de casca e semente das amostras IG, SB e P mostraram alta inibição de *Staphylococcus aureus* (variando de 0,02 a 0,08 mg/mL) e *Bacillus cereus* (0,4 a 0,15 mg/mL). A importância desse amplo espectro de proteção bacteriano atribuído ao fruto está relacionada à bioconservação dos alimentos. A bioconservação é um sistema amplamente aceito e reconhecido como um procedimento natural, capaz de prover a extensão da vida útil e satisfatória segurança microbiológica de alimentos (Fiorentini et al., 2001; Ristori, Pereira & Gelli, 2002). Portanto, o camu-camu pode ser indicado como uma boa opção para a substituição de aditivos químicos em alimentos.

CONCLUSÃO GERAL

A aplicação de uma técnica alternativa, para extração de ácido ascórbico, alcançou rendimentos semelhantes à extração ácida, muito utilizada para extração do composto. A extração com líquido pressurizado (PLE), técnica aplicada com sucesso no estudo de fitoquímicos como antocianinas, também pode ser utilizada para ácido ascórbico.

O alto conteúdo de compostos bioativos encontrados do camu-camu contribuíram diretamente para a sua elevada capacidade antioxidante e antimicrobiana. Os extratos das cascas, polpas e sementes foram capazes de eliminar espécies reativas de importância fisiológica, que em excesso são prejudiciais à saúde do indivíduo. Estudo *in vivo*, são necessários para comprovar sua atuação biológica.

Os teores dos compostos determinados variaram entre as regiões analisadas. A diversidade climática, o solo (seco e inundado) e o tipo de cultivo, são relatados como os principais responsáveis por essa variação.

Por fim, a utilização do camu-camu e seus subprodutos (cascas e sementes) é interessante para a indústria de alimentos, tanto para aplicação como conservantes naturais em alimentos ou como ingrediente funcionais para o enriquecimento de produtos alimentares.

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ANEXO

UNICAMP

Profa Dra Míriam Dupas Hubinger,

Coordenadora de Pós-Graduação – FEA

Prezada Dra Míriam,

Informamos que não foi possível a obtenção do cadastro do Patrimônio Genético visto que o sistema PATGEN/SISGEN encontra-se em funcionamento a pouco tempo e ainda não foram repassadas orientações suficientes pela Pró-Reitoria de Pesquisa a respeito da forma correta de preenchimento do cadastro. Por esse motivo, solicitamos dar entrada ao processo de defesa da aluna Elenice Carla Emídio Cunha sem a anexação desse documento. Esperamos que até a data de defesa tenhamos informações suficientes para regularizarmos o cadastro no sistema PATGN/SISGEN

Campinas, 27 de novembro de 2017

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