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Alessandra Piovesana

EXTRAÇÃO, IDENTIFICAÇÃO, QUANTIFICAÇÃO E MICROENCAPSULAMENTO POR ATOMIZAÇÃO E LIOFILIZAÇÃO DE COMPOSTOS BIOATIVOS DOS CÁLICES DE HIBISCO (*Hibiscus sabdariffa L.*)

Porto Alegre
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Orientador: Prof. Dr. Caciano Pelayo Zapata Noreña

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EXTRAÇÃO, IDENTIFICAÇÃO, QUANTIFICAÇÃO E MICROENCAPSULAMENTO POR ATOMIZAÇÃO E LIOFILIZAÇÃO DE COMPOSTOS BIOATIVOS DOS CÁLICES DE HIBISCO (*Hibiscus sabdariffa L.*)

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RESUMO

O interesse pela extração dos compostos bioativos, a partir de fontes naturais, para o emprego na produção de alimentos funcionais tem aumentado, devido, principalmente, à crescente demanda por parte dos consumidores, por produtos mais saudáveis e que possam trazer benefícios à saúde. Dentre as fontes naturais de compostos bioativos, destaca-se o hibisco (*Hibiscus sabdariffa* L.), que é rico em antocianinas, flavonoides, ácidos fenólicos, carotenoides, dentre outros. Entretanto, quando os compostos bioativos são separados de suas matrizes, estes se tornam altamente instáveis frente a diversos fatores ambientais, necessitando serem protegidos. O recobrimento por microencapsulamento é uma alternativa para retardar a degradação desses compostos. Este estudo teve como objetivo a extração, identificação, quantificação e microencapsulamento por atomização e liofilização dos compostos bioativos dos cálices do hibisco. Primeiramente, foi realizada a extração exaustiva total dos carotenoides e compostos fenólicos por meio de solventes orgânicos, para a identificação e quantificação desses compostos. Também foi estudada a extração de antocianinas e demais compostos fenólicos por meio de solvente aquoso acidificado (ácido cítrico 2 %, p/v). A partir do melhor tratamento de extração, o extrato obtido foi microencapsulado mediante atomização e liofilização, empregando goma arábica (GA), goma guar parcialmente hidrolisada (GGPH) e polidextrose (PD) como agentes encapsulantes, na concentração de 10%. Os carotenoides e compostos fenólicos foram identificados e quantificados por HPLC-DAD-MS/MS (cromatografia líquida de alta eficiência com detecção por arranjo de diodos e espectrometria de massa). Vinte e um carotenoides foram encontrados, dos quais, quinze foram identificados. O total de carotenoides nos cálices de hibisco foi de 641,38 ± 23,61 µg/100 g massa fresca, sendo a all-*trans*-luteína e o all-*trans*-β-caroteno os compostos majoritários, representando 49 e 23%, respectivamente. Para os compostos fenólicos, foram encontrados vinte compostos, dos quais, catorze foram identificados. As antocianinas foram os compostos majoritários nos cálices de hibisco, sendo que a delfinidina 3-sambubiosídeo e cianidina 3-sambubiosídeo representaram 41 e 13% do total de compostos fenólicos, respectivamente. Dentre os ácidos fenólicos, os componentes majoritários foram o ácido 3-cafeoilquínico e ácido 5-cafeoilquínico, representando 15 e 13% do total de compostos fenólicos, respectivamente. Para a extração aquosa acidificada, foi utilizado um planejamento experimental fatorial fracionado (2^{4-1}), com quatro fatores: concentração de enzima, temperatura, velocidade de agitação e tempo de extração. A partir da ANOVA, os efeitos principais e de interação foram avaliados, tendo como respostas *Chroma*, antocianinas monoméricas totais (*TMA*), capacidade redutora, ABTS e compostos fenólicos. A partir dos resultados, o melhor tratamento foi: 55 °C, 50 µL de enzima/1000 g extrato, 400 rpm e 4 horas de extração, obtendo-se nessa condição de extração 3,82 mg/g extrato em base seca para *TMA* e 17,59 mg/g de extrato em base seca para compostos fenólicos totais, que resultou em capacidade antioxidante de 7,72 µmol Eq. Trolox/g de extrato em base seca, avaliado por ABTS e de 3,96 mg GAE/g de de extrato em base seca, avaliado pela capacidade redutora. Este extrato foi empregado no estudo de encapsulamento, por atomização (140 ºC) e liofilização (-68 ºC por 24 horas), utilizando GA, GGPH e PD como encapsulantes. Observou-se que o melhor tratamento foi por liofilização empregando GA como encapsulante, resultando em 2,83 mg/g amostra em base seca para *TMA*, capacidade antioxidante de 2,98 mg GAE/g amostra em base seca e 5.67 µmol Eq. Trolox/g amostra em base seca, avaliados por capacidade redutora e ABTS, respectivamente. Entretanto, quando foram avaliadas as propriedades físicas e morfológicas dos pós, as amostras elaboradas por atomização e usando GA e GGPH apresentaram os melhores desempenhos, onde os valores de solubilidade, higroscopicidade e umidade foram de 95,8 e 95,2%, 31,3 e 28,9%, 1,9 e 2,4%, respectivamente. Para a temperatura de transição vítrea (*Tg*), os tratamentos que utilizaram GA e GGPH nos dois métodos de encapsulamento, tiveram os maiores valores de

Tg, variando de 10,9 a 17,4 ºC. Já para os tratamentos que utilizaram a PD como material de parede, os valores foram de (0,7 °C), tanto na atomização como na liofilização. Na microscopia também foi observado um melhor desempenho nas micropartículas atomizadas usando GA e GGPH, as quais mostraram partículas mais esféricas e sem tendência de atração e aderência entre si. Em relação ao diâmetro médio de partícula (D[4, 3]), os tratamentos liofilizados tiveram partículas maiores que os atomizados, variando de 101,7 a 143,1 µm para os liofilizados, e de 5,4 a 7,3 µm para os atomizados. Quanto ao *span*, o qual avalia distribuição de tamanho de partícula, variou de 1,90 a 2,00 para as amostras atomizadas e de 3,06 a 3,19 para as amostras liofilizadas, indicando que houve uma boa uniformidade na distribuição de tamanho de partícula. Conclui-se que o hibisco é uma matriz com ampla composição de compostos bioativos e tem potencial para aplicação em alimentos.

Palavras-chave: hibisco, extração, compostos bioativos, microencapsulamento, atomização, liofilização.

ABSTRACT

The interest in the extraction of bioactive compounds from natural sources, for use in the production of functional foods has increased, mainly due to the growing demand by consumers for healthier products and can bring health benefits. Among the natural sources of bioactive compounds, stands out the hibiscus (*Hibiscus sabdariffa* L.), which is rich in anthocyanins, flavonoids, phenolic acids, carotenoids, among others. However, when the bioactive compounds are separated from their matrix, they become highly unstable against various environmental factors and need to be protected. The coating by microencapsulation is an alternative to slow the degradation of these compounds. This study aimed at the extraction, identification, quantification and microencapsulation by spray drying and freeze drying of bioactive compounds of hibiscus calyces. Firstly, a thorough exhaustive extraction of carotenoids and phenolic compounds by organic solvents was performed for identification and quantification of these compounds. The extraction of anthocyanins was also studied along with other phenolic compounds by an aqueous solvent acidified (2% citric acid, w/v). From the best treatment for extraction, the extract obtained was microencapsulated by spray drying and freeze drying using Arabic gum (GA), partially hydrolyzed guar gum (PHGG) and polydextrose (PD) as encapsulating agents in a concentration of 10%. Carotenoids and phenolic compounds were identified and quantified by HPLC-DAD-MS/MS (high-performance liquid chromatography with diode array detection and mass spectrometry). Twenty-one carotenoids were found, of which fifteen were identified. The total carotenoids in hibiscus calyces was 641.38 ± 23.61 mg/100 g fresh weight, with the all-*trans*-lutein and all-*trans*-β-carotene the major compounds, representing 49 and 23%, respectively. Regarding the phenolic compounds it was found twenty of those, of which fourteen have been identified. Anthocyanins were the main components in the hibiscus calyces, and delphinidin and cyanidin 3-sambubioside 3-sambubioside represented 41 and 13% of total phenolic compounds, respectively. Among the phenolic acids, the major components were the 3-caffeoylquinic acid and 5-caffeoylquinic acid, representing 15 and 13% of total phenolic compounds, respectively. For acidified aqueous extraction, we used a fractional factorial design (2^{4-1}) with four factors: enzyme concentration, temperature, stirring speed and extraction time. From the ANOVA, the main and interaction effects were assessed as answers: *Chroma*, total anthocyanins monomeric (*TMA*), reducing capacity, ABTS and phenolic compounds. From the results, the best treatment was with 55 °C, 50 µL of enzyme/1000 g extract, 400 rpm and 4 hours of extraction, it was obtained in this extraction condition 3.82 mg/g extract on a dry basis for *TMA* and 17.59 mg/g extract on a dry basis for phenolic compounds, which resulted in antioxidant capacity of 7.72 µmol Eq. Trolox/g extract on a dry basis, evaluated by ABTS and 3.96 mg GAE/g extract on a dry basis, assessed by reducing capacity. This extract was used for the encapsulation study, by spray drying (140 °C) and freeze drying (-68 ° C for 24 hours) using GA, PHGG, and PD as encapsulants. It was observed that the best treatment is by freeze drying using GA as encapsulant, resulting in 2.83 mg/g sample on dry basis for *TMA*, antioxidant capacity of 2.98 mg GAE/g sample on dry basis and 5.67 µmol Eq. Trolox/g sample on dry basis, evaluated by reducing capacity and ABTS, respectively. However, when we evaluated the physical and morphological properties of powders, samples prepared by spray drying and using GA and PHGG showed the best performance, and the values for solubility, hygroscopicity and moisture were 95.8 and 95.2%, 31.3 and 28.9%, 1.9 and 2.4%, respectively. For the glass transition temperature (*Tg*), treatments with GA and PHGG on both encapsulation methods had high *Tg* values ranging from 10.9 to 17.4 °C. As for treatments of PD as wall material, the values were (0.7 °C), both the spray drying as in freeze drying. In microscopy was also observed improved performance in spray-dried microparticles using GA and PHGG, which showed more spherical particles and with no tendency to attract and adhere to each other. Regarding the average particle

diameter (D [4, 3]), the freeze-dried treatments had higher spray-dried particles ranging from 101.7 to 143.1 µm for freeze-dried, and 5.4 to 7.3 µm for spray-dried. As the span, which assesses particle size distribution ranged from 1.90 to 2.00 for spray-dried samples and 3.06 to 3.19 for the freeze-dried samples, indicating that there was a good uniformity in the size in the distribution of the size of the particle. It follows that hibiscus is a matrix with broad composition and bioactive compounds have potential for application in foods.

Keywords: hibiscus, extraction, bioactive compounds, microencapsulation, spray drying, freeze drying.

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SUMÁRIO

INTRODUÇÃO

Atualmente, com os recentes avanços nas pesquisas, produtos naturais e alimentos promotores de saúde têm recebido grande atenção de profissionais da área e da população em geral. Com isso, novos conceitos vêm surgindo, como alimentos funcionais, fitonutrientes e nutracêuticos, os quais agem na manutenção do bem-estar, promovendo benefícios à saúde. Alimentos ricos em compostos bioativos estão ligados aos benefícios protetores e promotores de saúde, através da modulação da função imunológica específica para a prevenção de doenças (Pennington, 2002; Bagchi, 2006; Zhao, 2007). Dentre os compostos bioativos, grande destaque tem sido dado aos compostos antioxidantes, que auxiliam na proteção do organismo humano contra o estresse oxidativo, associado à incidência de doenças degenerativas, como câncer, dentre outras (Scalbert e Williamson, 2000).

De acordo com Wong et al. (2014) compostos bioativos com atividade antioxidante têm sido encontrados em altas concentrações em plantas. A extração e a purificação desses compostos bioativos, a partir de fontes naturais, tornaram-se muito importantes e atrativas às indústrias, devido às diversas aplicações desses compostos, como na preparação de aditivos alimentares, produtos nutracêuticos, alimentos funcionais e cosméticos. Esses produtos vem ganhando cada vez mais espaço no mercado em função do aumento da demanda por parte dos consumidores (Cisse et al., 2012).

O hibisco é uma espécie vegetal da família *Malvaceae*, de cultivo anual, proveniente da África Oriental, trazido ao Brasil pelos africanos (Panizza, 1998; Lorenzi e Matos, 2002), sendo reconhecido como alimento funcional nos países da Ásia (China, Japão, Taiwan e Coréia) pela sua rica composição em compostos bioativos (Liu et al., 2005).

O hibisco é uma planta ornamental de flor comestível, encontrada em jardins de muitas casas. Recentemente, seu cultivo vem sendo incentivado em hortas comunitárias no Estado do Rio Grande do Sul, como é o caso da comunidade da Lomba do Pinheiro em Porto Alegre, com o objetivo de incentivar e intensificar o consumo deste produto no dia-a-dia da população em geral, seja na preparação de chás ou na elaboração de picles e geleias.

Alguns estudos vêm mostrando a riqueza do hibisco em compostos bioativos, tais como antocianinas, flavonóides, ácidos fenólicos, dentre outros (Lin et al., 2007; Prenesti et al., 2007), além, dos benefícios que um consumo regular do hibisco pode proporcionar à saúde, apontando diferentes efeitos terapêuticos, tais como hepatoprotetor e antibacteriano (Liu et al., 2006), antioxidante (Ramakrishna et al., 2008), anticolesterol (Lin et al., 2007),

anti-mutagênica (Olvera-García et al., 2008), anti-hipertensivo (Herrera-Arellano et al., 2007), entre outros.

De acordo com Cisse et al. (2012), extratos aquosos dos cálices de hibisco vêm sendo utilizados na produção de bebidas e geleias, além de ser uma fonte de corante natural. Os cálices são a parte mais utilizada e estudada do hibisco, principalmente pela alta concentração em antocianinas (Prenesti et al., 2007; Sanchez-Mendoza et al., 2008).

Além das antocianinas e outros compostos fenólicos que são comumente encontrados nos cálices do hibisco, este estudo aborda, pela primeira vez, o conteúdo e a concentração dos carotenoides nos cálices do hibisco. Os carotenoides fazem parte de um grupo importante de pigmentos naturais, responsáveis por cores de muitas hortaliças, frutas, da gema do ovo, crustáceos e alguns peixes. Além disso, estas cores são uma consequência da presença de ligações duplas conjugadas, o que também lhes confere propriedades antioxidantes (Rodriguez-Amaya et al., 2008; Strati e Oreopoulou, 2011). Esses compostos possuem funções importantes de promoção de saúde e ações como pró-vitamina A, reduzindo o risco de doenças degenerativas e aumentando a atividade do sistema imunológico (Strati e Oreopoulou, 2011).

Contudo, quando os compostos bioativos são separados de suas matrizes, estes se tornam altamente instáveis frente a diversos fatores, como altas temperaturas, presença de luz, oxigênio e enzimas oxidativas que ocorrem durante o processamento e armazenamento. Sendo assim, necessitam ser protegidos e o seu recobrimento por microencapsulamento é uma ótima alternativa a ser aplicada, retardando e/ou prevenindo a sua degradação.

O microencapsulamento tem diversas funções, como proteger os compostos sensíveis, possibilitando a sua incorporação como ingrediente nos alimentos, mascarar ou preservar sabores e aromas, transformar líquidos em ingredientes sólidos para facilitar a manipulação, dentre outras (Desai e Park, 2005). Uma das técnicas mais utilizada para o processo de microencapsulamento na indústria alimentícia é a atomização (*spray drying*), que produz pós de boa qualidade a um baixo custo e é indicada para encapsulamento de compostos sensíveis ao calor, pois o processo de secagem ocorre instantaneamente (Fang e Bhandari, 2011).

Outra técnica de microencapsulamento bastante utilizada é a liofilização, sendo um dos métodos mais eficientes no microencapsulamento de compostos termossensíveis e instáveis, visto que a remoção da água ocorre por sublimação em condições de baixas temperaturas. Em contrapartida, a liofilização é um processo que necessita longos períodos de desidratação (Madene et al., 2006).

Assim, este estudo tem como objetivo identificar e quantificar os compostos fenólicos e carotenoides nos cálices do hibisco, através da extração exaustiva total. Seguido pela otimização da extração aquosa dos compostos fenólicos, e pelo microencapsulamento do extrato aquoso, por meio de atomização e liofilização.

A presente dissertação de mestrado foi desenvolvida principalmente nos Laboratório de Engenharia de Processos em Alimentos e Laboratório de Desidratação do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul.

Este trabalho encontra-se organizado da seguinte forma: no Capítulo 1 está apresentada a revisão bibliográfica abordando os principais pontos do tema proposto. Os Capítulos 2, 3 e 4 apresentam os resultados obtidos, na forma de artigo. No Capítulo 5 é apresentada a discussão geral e, em seguida, as principais conclusões obtidas e as perspectivas para os trabalhos futuros.

OBJETIVO GERAL

Estudar a extração, identificação, quantificação dos compostos bioativos dos cálices do hibisco *(Hibiscus sabdariffa L.)* e microencapsulamento por atomização e liofilização.

OBJETIVOS ESPECÍFICOS

- Realizar a extração exaustiva de compostos fenólicos e carotenoides dos cálices do hibisco por meio de solventes orgânicos e determinar por cromatografia líquida de alta eficiência com detecção por arranjo de diodos e espectrometria de massa (HPLC-DAD-MS/MS);
- Otimizar a extração de antocianinas e demais compostos fenólicos por meio de solvente aquoso acidificado com ácido cítrico (2%, p/v), através de um planejamento fatorial fracionado;
- Realizar o microencapsulamento dos compostos bioativos do extrato aquoso de hibisco, através da atomização e da liofilização, empregando goma arábica, polidextrose e goma guar parcialmente hidrolisada como agentes encapsulantes;
- Avaliar as características físico-químicas e estruturais, bem como, a retenção de antocianinas e capacidade antioxidante das micropartículas obtidas.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1. HIBISCO

O hibisco é um arbusto (Figura 1), pertencente à família botânica *Malvaceae*, de ciclo anual, que alcança de 80 a 140 cm de altura, ereto, ramificado, de caule arroxeado, com folhas verde-arroxeadas, cujas flores solitárias (Figura 2) têm forma de cálice e tonalidade vermelha intensa (Vizzotto e Pereira, 2008). É cultivado em áreas tropicais e subtropicais de ambos os hemisférios (Cisse et al., 2009a), desde o nível do mar até 900 m de altura (Martins, 2003). Nativo dos continentes africano e asiático (Martins, 2003), o hibisco também é conhecido popularmente como vinagreira, rosela, caruru-azedo, azedinha, caruru-da-guiné, azeda-da-guiné, quiabo-azedo, quiabo-róseo, quiabo-roxo, rosélia, groselha, quiabo-de-angola, groselheira (Lorenzi e Matos, 2002). Em outros países, é conhecido como jamaica (Espanha e México), flor da jamaica (México), cardade (Itália), karkade (Arábia), roselle (Inglaterra) ou L'oiselle (França) (Vizzotto e Pereira, 2008; Ojeda et al., 2010).

Figura 1. Planta do hibisco (*Hibiscus sabdariffa* L.).
Fonte: própria.

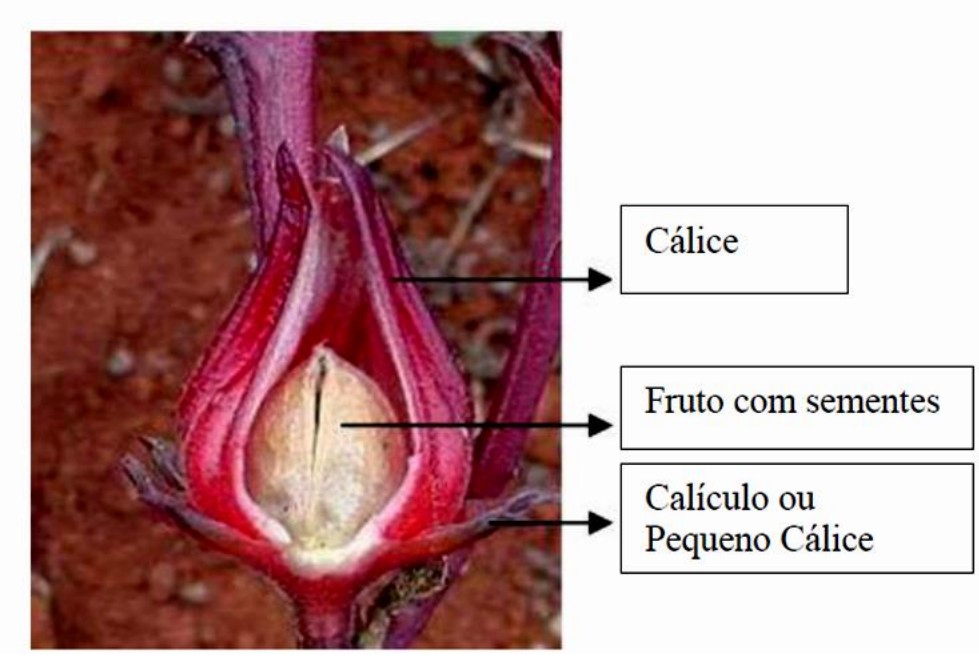

Figura 2. Morfologia do hibisco (*Hibiscus sabdariffa* L.).

Fonte: De Castro et al. (2004).

O hibisco é cultivado com diversas finalidades no mundo, sendo usado tanto para fins decorativos em jardins, como para a elaboração de geleias, bebidas (refrescos) e como fonte de corante natural obtido de suas sementes e pétalas (Lorenzi e Matos, 2002; Cisse et al., 2009a). Na maioria dos casos os cálices são usados para produzir infusões ou chá, de cor avermelhada e sabor ácido adstringente (Lorenzi e Matos, 2002; Aurelio et al., 2008; Cisse et al., 2009a).

Os cálices de hibisco, empregados na indústria alimentícia e farmacêutica, são provenientes principalmente da China, Tailândia e Índia (Aliste, 2006), porém os cálices de melhor qualidade são originários do Sudão, embora em pequenas quantidades (Mendonça, 2011).

Os cálices de hibisco comercializados no Brasil são produzidos na África e Ásia, e importados através de países da Europa, principalmente da Alemanha. Entretanto, estudos indicam que a planta apresenta um bom potencial de adaptação nas condições brasileiras (De Castro et al., 2004; Vizzotto e Pereira, 2008). As primeiras mudas foram introduzidas no Brasil pelos africanos, e atualmente é encontrada em jardins residenciais em diversas regiões do país (Panizza, 1998).

Estudos realizados com hibisco têm demonstrado uma gama de efeitos terapêuticos, como hepatoprotetor, antibacteriano (Liu et al., 2006), antioxidante (Ramakrishna et al., 2008), anticolesterol (Lin et al., 2007), anti-mutagênico (Olvera-García et al., 2008) e anti-hipertensivo (Herrera-Arellano et al., 2007), sendo reconhecido como alimento funcional em países da Ásia (China, Japão, Taiwan e Coréia) (Liu et al., 2005). Os responsáveis por esses efeitos são possivelmente, compostos com capacidade antioxidante, como vitamina C, ácidos fenólicos, flavonoides e antocianinas, presentes no hibisco (Wang et al., 2000; Lin et al., 2007; Prenesti et al., 2007).

O cálice é a parte mais utilizada e estudada do hibisco, principalmente devido às antocianinas presentes (Prenesti et al., 2007; Sanchez-Mendoza et al., 2008), que tem como teores médios 350 mg/kg de matéria seca, onde a delfinidina 3-sambubiosídeo e cianidina 3-sambubiosídeo, representam 71 e 29% do total de antocianinas, respectivamente (Cisse et al., 2009a; Cisse et al., 2011). A Tabela 1 mostra as características físico-químicas dos cálices do hibisco.

Tabela 1. Características físico-químicas dos cálices de *Hibiscus sabdariffa* L.

Componentes	Valores médios
Umidade (g/100 g)	86,3
Proteínas (g/100 g)	6,6
Lipídeos (g/100 g)	2,3
Fibras (g/100 g)	8,8
Cinzas (g /100 g)	5,6
Glicídios (g/100 g)	8,1
Ácido Málico (g/100 g)	1,36
Ácido Ascórbico (mg/100 g)	72,0
Antocianinas (mg /100 g)	350,0

Fonte: Adaptada de Cisse et al. (2009a).

2. ALIMENTOS FUNCIONAIS E COMPOSTOS BIOATIVOS

Conforme Sgarbieri e Pacheco (1999), o conceito de alimento funcional teve origem no Japão, em meados de 1980, a partir da preocupação com a crescente e numerosa população de idosos e prevenção das Doenças Crônicas Não Transmissíveis (DCNT).

Um alimento (ou ingrediente) com propriedade funcional, de acordo com a legislação brasileira vigente, é todo aquele que, além das funções nutricionais básicas, é capaz de produzir também efeitos benéficos à saúde, devendo o seu consumo ser seguro e sem a necessidade de supervisão médica (Brasil, 1999). Sgarbieri e Pacheco (1999) acrescentaram a essa definição a capacidade dos alimentos de retardar o aparecimento de doenças crônicas degenerativas, gerando maior qualidade e expectativa de vida. Também, evidências sugerem que as vantagens de fitoquímicos em frutas e vegetais pode ser ainda maior do que é atualmente reconhecida, porque o estresse oxidativo induzido por radicais livres é envolvido na etiologia de uma grande variedade de doenças crônicas (Liu, 2004).

Conforme Liu (2004), os alimentos podem conter as substâncias ativas responsáveis por ações biológicas e que são chamadas de fitoquímicos ou compostos bioativos. Estes compostos originados nas plantas estão presentes em frutas, legumes, grãos e outros alimentos vegetais, sendo que podem ser classificados como carotenoides, compostos fenólicos, alcaloides, compostos contendo nitrogênio e compostos organossulfurados (Figura 3) (Liu, 2004).

Os compostos fenólicos podem ser pigmentos, que dão a aparência colorida aos alimentos, ou produtos do metabolismo secundário dos vegetais, normalmente derivados de reações de defesa das plantas contra agressões ao meio ambiente (Brand-Williams et al.,1995). Estes compostos agem como antioxidantes, não somente pela sua habilidade em doar hidrogênio ou elétrons, mas também em virtude de seus radicais intermediários estáveis, que impedem a oxidação de vários ingredientes do alimento (Brand-Williams et al., 1995); que estruturalmente possuem um anel aromático com uma ou mais hidroxila (Soares, 2002).

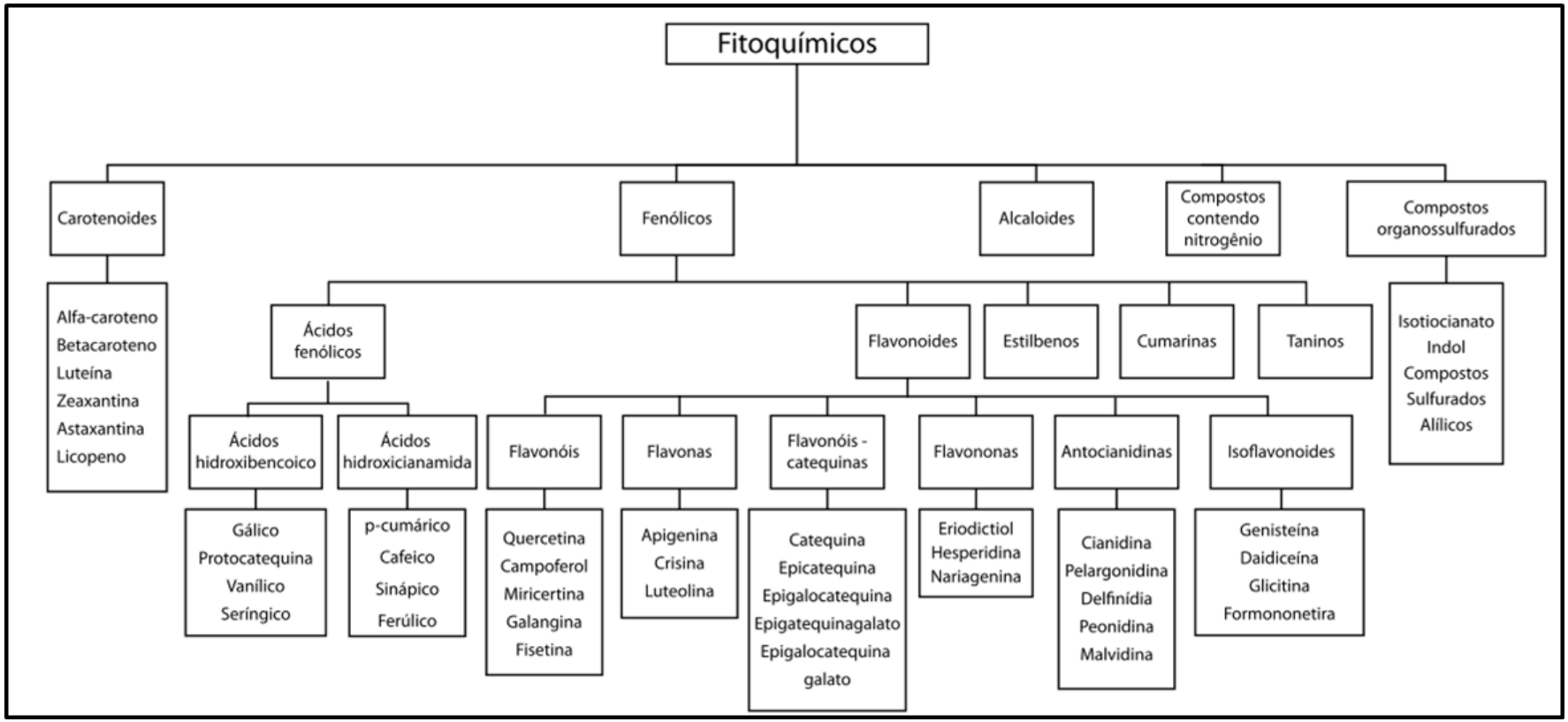

Figura 3. Classificação dos fitoquímicos.

Fonte: Liu (2004).

3. ATIVIDADE ANTIOXIDANTE

As espécies reativas de oxigênio (ERO) são constituídas por moléculas energeticamente instáveis que adquirem estabilidade ao captar elétrons de moléculas vizinhas, isto é, oxidando-as, e dentro dessas espécies estão os derivados de oxigênio, como o radical superóxido ($O_2^{\bullet -}$) e o radical hidroxila ($HO^{\bullet}$), e derivados sem radicais, como o peróxido de hidrogênio (H_2O_2) (Halliwell, 1996).

Halliwell (1996), menciona que para proteger o organismo destas ERO, existem uma série de sistemas antioxidantes: enzimas específicas que inativam algumas destas ERO, enzimas controladoras da disponibilidade de metais na célula e captadores não protéicos de radicais. Em paralelo, também desenvolvem-se sistemas de regeneração e reparação de macromoléculas, especialmente do DNA, a fim de corrigir possíveis falhas ou sobrecargas nos mecanismos de defesas (Halliwell, 1996).

Conforme Melecchi (2005), as capacidades pró-oxidantes e antioxidantes das células devem ser mantidas em equilíbrio a fim de se evitar o perigo potencial de estresse oxidativo, sendo que quando ocorre aumento das ERO e/ou diminuição da capacidade antioxidante, elas podem lesionar componentes celulares, modificando a estrutura ou função e gerando estresse oxidativo. Isto pode explicar então, a possível associação entre várias doenças e ERO (Melecchi, 2005).

Existe em todo o organismo um sistema de defesa contra a ocorrência do radical livre de oxigênio, que inclui fatores inibidores da formação ou captura dos iniciadores primários do processo de peroxidação dos lipídios, como proteínas ligadas aos íons metálicos ou às enzimas superóxido dismutase (SOD), catalase (CAT) e glutationaperoxidase (GPx) selênio dependentes (Melecchi, 2005). Esses danos exercem papel no envelhecimento e indução de várias doenças degenerativas, tais como doenças cardíacas, catarata, disfunção cognitiva, reumatismo e câncer (Halliwell, 1996; Kaur e Kapoor, 2001; Prior, 2003).

Existem vários métodos para determinar a capacidade antioxidante de um composto, podendo ser expressos de várias formas, dentre eles pode-se mencionar: a remoção de um radical peroxil (ORAC: capacidade de absorção de radicais de oxigênio, TRAP: potencial antioxidante reativo total), a capacidade de redução de metal (FRAP: capacidade antioxidante por redução do ferro, CUPRAC: capacidade antioxidante por redução do cobre), a capacidade de remoção de

radical orgânico (ABTS-2,2'-azino-bis-3-etilbenzotiazolina-6-sulfonato e DPPH - peroxidação do 2,2-difenil-1-picrilhidrazina), a capacidade de eliminação do radical hidroxil (HRSA) e pelo mecanismo baseado na transferência de elétrons (capacidade redutora).

4. COMPOSTOS FENÓLICOS

Os compostos fenólicos são largamente distribuídos na natureza e encontrados principalmente em alimentos de origem vegetal. A maior parte dos compostos fenólicos apresentam-se conjugados com mono e polissacarídeos, através de um ou mais dos grupos fenólicos, e podem também ocorrer como derivados funcionais, como ésteres e metil ésteres flavonoides (Cabrita et al., 2002). Os compostos fenólicos, do ponto de vista químico, são caracterizados por apresentar um núcleo benzênico agrupado a um ou vários grupos hidroxila. Essa característica estrutural dos compostos fenólicos está diretamente ligada a sua atividade antioxidante, sendo influenciada, particularmente, pelo número e posição dos grupos hidroxila e pela natureza das substituições nos anéis aromáticos (Balasundram et al. 2006). Os compostos fenólicos são classificados em compostos flavonoides e não flavonoides (Cabrita et al., 2002).

Os ácidos fenólicos, hidroxibenzóicos e hidroxicinâmicos, e outros derivados fenólicos como os estilbenos fazem parte dos não flavonoides (Flanzy, 2000). Os ácidos fenólicos são compostos simples formados por um anel aromático e os substituintes ligados à sua estrutura, conferindo capacidade de sequestrar espécies reativas, como o radical hidroxila e o oxigênio singleto (Marinova e Yanishlieva, 2003). No hibisco, os ácidos fenólicos são, principalmente, os ácidos hidroxicinâmicos, como o ácido cafeico e o ácido *p*-cumárico, que normalmente, se encontram esterificados ao ácido quínico, formando os ácidos clorogênicos (Figura 4).

Os ácidos clorogênicos são ácidos fenólicos, cuja produção é induzida em resposta às condições adversas de estresse ambiental (Robbins, 2003). O ácido 5-cafeolquínico é o mais comum dos ácidos clorogênicos e o mais conhecido dos fenóis dietéticos biologicamente ativos (Garambone e Rosa, 2007).

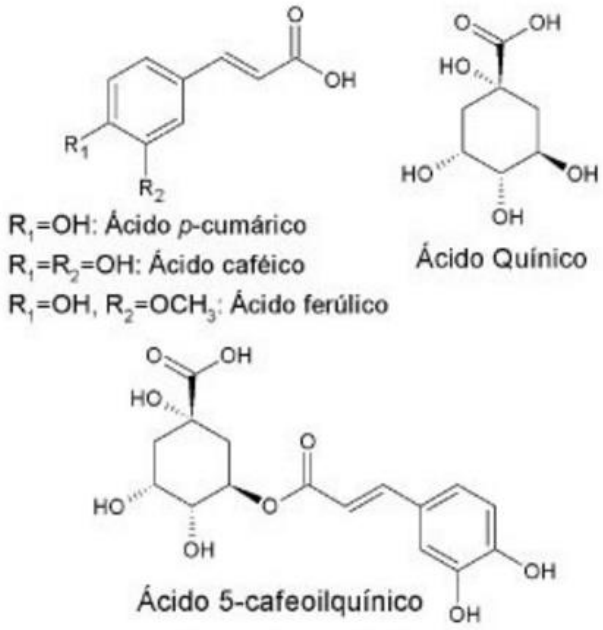

Figura 4. Fórmula estrutural dos ácidos clorogênicos.

Fonte: Oliveira e Bastos (2011).

Os flavonoides possuem uma estrutura marcada pela presença de um esqueleto com 15 átomos de carbono na forma C_6-C_3-C_6, e são divididos em classes dependendo do estado de oxidação do anel central de pirano (Zoecklein et al., 1995). Esta grande família é dividida em inúmeras subclasses, flavonas, flavanonas, isoflavonas, flavonóis, flavanóis e antocianinas, as quais se distinguem entre si pelo grau de oxidação do anel pirano (Balasundram et al., 2006). Em produtos naturais, a maioria das substâncias responsáveis pela coloração pertence à classe dos flavonoides. A Figura 5 apresenta a estrutura química dos principais tipos de flavonóides (Março, 2009).

Os flavonoides podem ser encontrados no estado livre ou polimerizados com outros flavonóides, açúcares e compostos não flavonoides (Cabrita et al., 2002). Os flavonoides são efetivos doadores de hidrogênio. Seu potencial antioxidante é dependente do número e da posição dos grupos de hidrogênio e suas conjugações, e também devido à presença de elétrons nos anéis benzênicos (Cao et al., 1997). Dentre estes, os flavonóis e as antocianidinas são quantitativamente os mais importantes no hibisco.

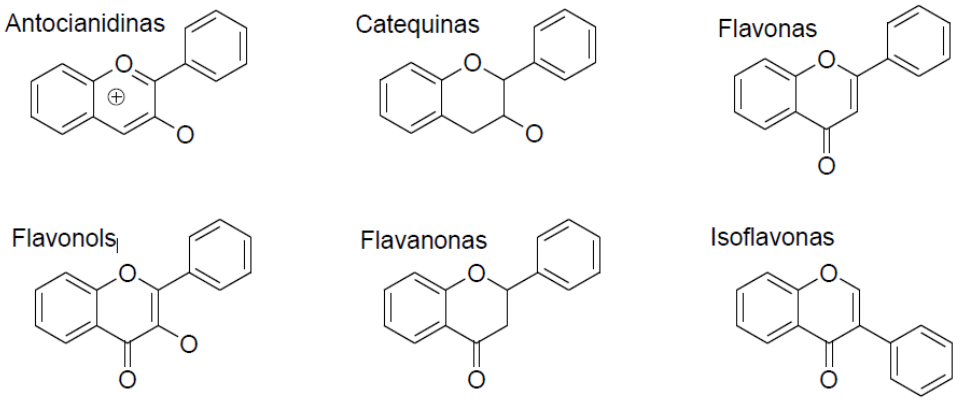

Figura 5. Fórmula estrutural dos principais tipos de flavonoides.
Fonte: Março (2009).

Os flavonóis são caracterizados pela presença de uma insaturação no anel heterocíclico e um grupo hidroxilo na posição 3, sendo os mais importantes: quempferol, quercetina e miricetina. No hibisco encontram-se na forma glicosilada. Os flavonóis são uma série de hidroxiderivados da 3-hidroxiflavona (Flavonol), que constituem uma classe de corantes amarelos de flores, raízes e madeiras. Esses compostos na natureza funcionam como as flavonas, protegendo as plantas dos raios UV, e ainda, funcionam como sinal atrativo para insetos como as abelhas, que conseguem enxergar na faixa extrema do ultravioleta, auxiliando esses insetos na localização do pólen e do néctar. Além de também serem usados para o tingimento de roupas.

Dentro da classe dos flavonoides, as antocianinas e as antocianidinas são importantes por conferir coloração natural a vários alimentos, constituindo um dos maiores grupos de pigmentos hidrossolúveis, sendo responsáveis pela cor vermelha, roxa e azul de muitas frutas, vegetais e flores (Schwartz et al., 2010). As mais comumente encontradas em frutas são: pelargonidina, cianidina, delfinidina, peonidina, petunidina, malvidina e seus derivados (Março, 2009).

As antocianidinas apresentam como estrutura fundamental o cátion *flavilium* (2-fenilbenzopirona), não possuem grupos glicosídeos, diferenciando-se das antocianinas, que possuem uma ou mais hidroxilas ligadas a açúcares (Schwartz et al., 2010), sendo os mais comuns a glicose, xilose, arabinose, ramnose, galactose ou dissacarídeos constituídos por esses açúcares, aos quais podem estar ligados ácidos fenólicos como: *p*-cumárico, caféico, fenílico e vanílico (Schwartz et al., 2010). As variações na estrutura química das antocianidinas (Tabela 2) são principalmente atribuídas ao número e a posição de hidroxilas que a molécula contém e o

grau de metilação destes grupos além da natureza, posição, número e tipo de açúcar e ácido orgânico na molécula (Francis, 1989; Mcghie e Walton, 2007).

Tabela 2. Substituições padrão do cátion *flavilium* formando as principais antocianidinas encontradas na natureza.

Antocianidina	Substituição padrão							Cor
	3	5	6	7	3’	4’	5’	
Pelargonidina	OH	OH	H	OH	H	OH	H	Laranja ou Salmão
Cianidina	OH	OH	H	OH	OH	OH	H	Magenta e Vermelho
Peonidina	OH	OH	H	OH	OCH_3	OH	H	Magenta
Delfinidina	OH	OH	H	OH	OH	OH	OH	Roxo, Lilás ou Azul
Petunidina	OH	OH	H	OH	OCH_3	OH	OH	Roxo
Malvidina	OH	OH	H	OH	OCH_3	OH	OCH_3	Roxo

Fonte: Cavalcanti et al. (2011).

As antocianinas são pigmentos relativamente instáveis, sendo que sua maior estabilidade ocorre em condições ácidas, desse modo, os principais fatores que regem a degradação das antocianinas são pH, temperatura, enzimas como a polifenoloxidase e peroxidase, concentração de oxigênio e ácido ascórbico (Schwartz et al., 2010).

Nijveldt et al. (2001) relataram que as antocianinas podem prevenir danos causados pelos radicais livres através de vários mecanismos, como por exemplo, o carregamento direto do radical livre, e quando oxidadas pelos radicais, estes resultam em um radical menos reativo e mais estável, estabilizando dessa forma as espécies reativas de oxigênio através de sua reação com o componente reativo do radical. De acordo com estes autores, a reação do grupo hidroxila das antocianinas com o radical torna-o inativo e a reação pode ser escrita de acordo com a equação:

$$\text{Antocianina (OH)} + R^{\bullet} \rightarrow \text{Antocianina } (O^{\bullet}) + RH$$

Onde: $R^{\bullet}$ = radical livre; $O^{\bullet}$ = radical livre de oxigênio.

O mecanismo apresentado acima ilustra a importância dos grupos hidroxilas para a capacidade antioxidante elevada das antocianinas, como por exemplo, das delfinidinas e cianidina, que estão presentes nos cálices de hibisco (Kuskoski et al., 2004; Cisse et al., 2011).

De acordo com Wang e Xu (2007), a temperatura elevada é o fator que mais afeta a estabilidade das antocianinas. A exposição do pigmento a temperaturas elevadas proporciona a formação de produtos de coloração marrom, principalmente quando está associado com oxigênio, que também induz e acelera a formação destes compostos escuros (Cavalcanti et al., 2011). Os principais fatores que influenciam a degradação térmica das antocianinas são a sua estrutura, a composição química do meio e o conteúdo de oxigênio (Cisse et al., 2009b).

A luz é outro fator de grande importância na alteração da cor das antocianinas. No entanto, a transformação é mais intensa quando o fator luz é combinado com o efeito do oxigênio (Março, 2009). Porém a estabilidade é aumentada consideravelmente pela presença de compostos como o acetaldeído, aminoácidos e taninos, entretanto esse aumento é atribuído à copigmentação, ou seja, associação entre a antocianina e flavonol (copigmento) por ligações de hidrogênio de modo que o flavonol venha a formar uma estrutura protetora envolvendo a antocianina (Bobbio e Bobbio, 2001).

Segundo Brouillard (1982), a natureza iônica das antocianinas permite que estas mudem de conformação de acordo com o pH do meio, resultando em diferentes cores e tonalidades. Assim, Giusti e Wrolstad (2001) afirmam que em pH inferior a 3, a antocianina existe primeiramente na forma de cátion *flavilium*, com coloração laranja ou vermelha, e com o aumento do pH para 7, a conformação predominante passa a ser a base quinoidal, e a cor muda para azul. Com a alteração do pH para 4,5 ocorre a hidratação do cátion *flavilium*, gerando a pseudobase carbinol, incolor, e assim atinge o equilíbrio com a chalcona que também é incolor. Entretanto, de acordo com Rein (2005), as antocianinas apresentam maior estabilidade em pH ácido (menor que 3). Na Figura 6 estão apresentadas as quatro conformações de antocianinas em equilíbrio em solução aquosa: cátion *flavilium* (AH+), base quinoidal (A), pseudobasecarbinol (B) e a chalcona (C).

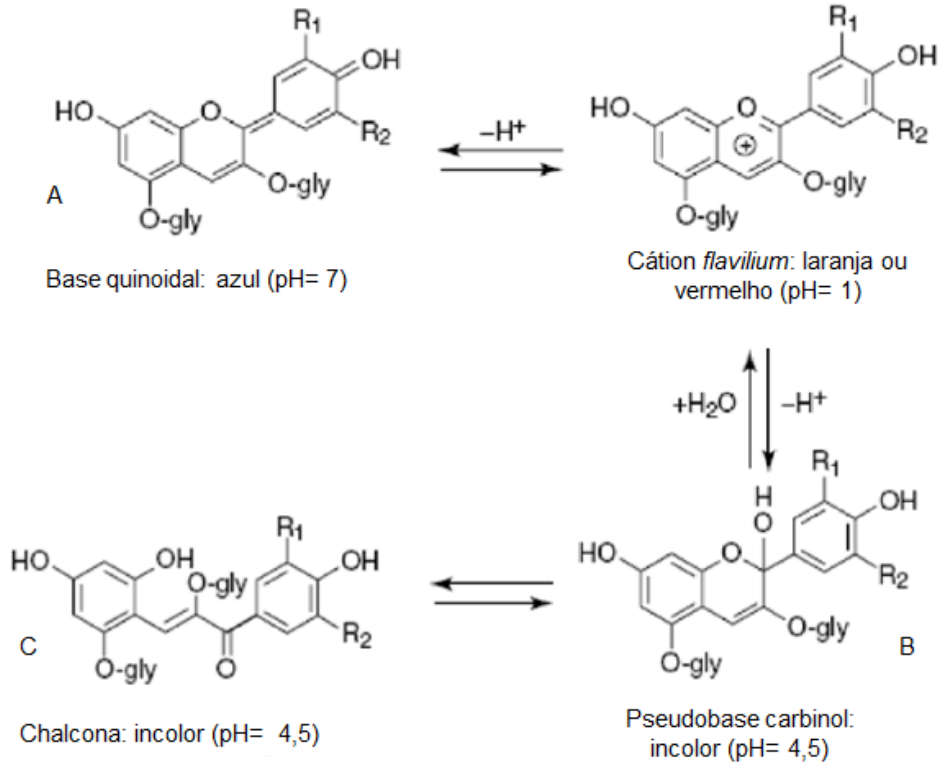

Figura 6. Fórmula estrutural de antocianinas em equilíbrio em solução aquosa.
Fonte: Giusti e Wrolstad (2001).

5. CAROTENOIDES

Os carotenoides fazem parte de um grupo importante de pigmentos naturais, responsáveis por cores de muitas hortaliças, frutas, da gema do ovo, crustáceos e alguns peixes. No entanto, estas cores são uma consequência da presença de ligações duplas conjugadas, o que também lhe conferem propriedades antioxidantes (Rodriguez-Amaya et al., 2008; Strati e Oreopoulou, 2011). Esses compostos possuem funções importantes de promoção de saúde, além de ações como pró-vitamina A e contribuição na prevenção e proteção contra problemas de saúde graves, como doenças cardíacas, doenças degenerativas, câncer e degeneração macular, além de auxiliar no aumento da atividade do sistema imunológico (Fraser e Bramley, 2004; Strati e Oreopoulou, 2011; Rivera e Canela-Garayoa, 2012).

A Figura 7 apresenta estruturas dos principias carotenoides. Esses compostos são reconhecidos pelas suas propriedades antioxidantes, protegendo as células de danos oxidativos provocados por radicais livres e ERO (Shami e Moreira, 2004). Essas moléculas derivadas do

oxigênio, como peróxido de hidrogênio podem ser geradas no citoplasma, nas mitocôndrias ou na membrana, atacando proteínas, lipídios, carboidratos e DNA (Shami e Moreira, 2004).

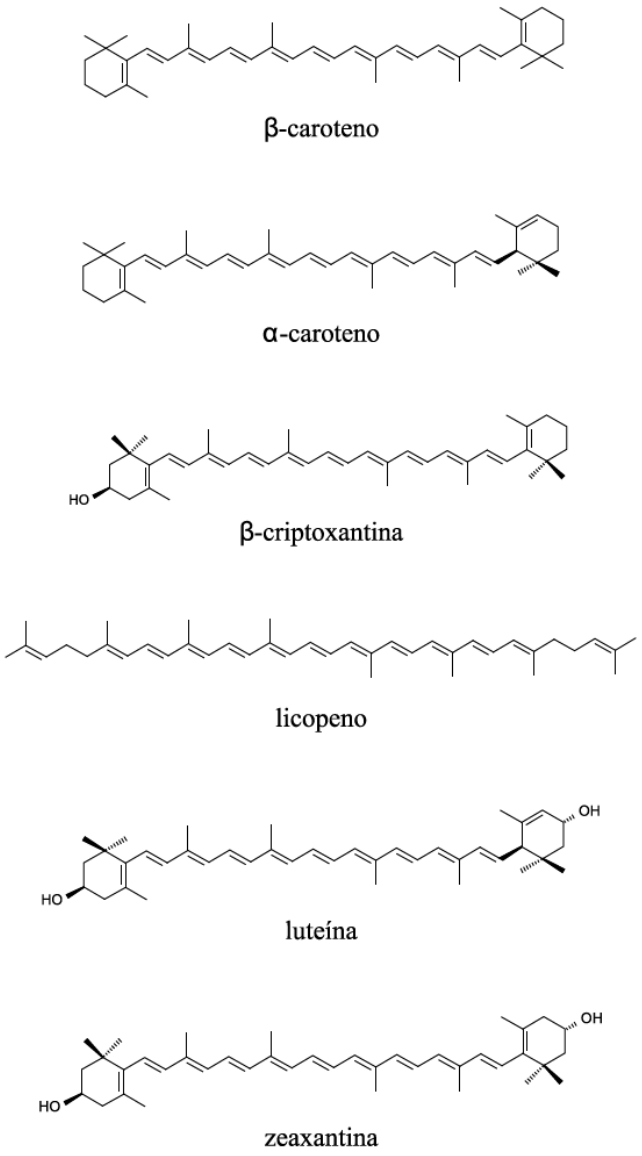

Figura 7. Fórmula estrutural dos carotenoides considerados importantes para a saúde.
Fonte: Rodriguez-Amaya et al. (2008).

Os carotenoides possuem uma estrutura isoprenoide, ou seja, com um número variável de duplas ligações conjugadas que permitem o fácil deslocamento eletrônico nas ligações duplas. Este sistema de ligação carbono-carbono conjugado faz dos carotenoides um eficiente repressor de oxigênio singleto (Hammond e Renzi, 2013).

Os carotenoides são classificados em dois grupos principais, os carotenos, que são hidrocarbonetos, tais como β-caroteno ($C_{40}H_{56}$) e licopeno ($C_{40}H_{56}$) e as xantofilas ($C_{40}H_{56}O_2$), que incluem o oxigênio, o hidrogênio e o carbono. As xantofilas são essencialmente produtos de oxidação dos carotenos e abrange luteína, zeaxantina e β-criptoxantina (Hammond e Renzi, 2013).

6. MICROENCAPSULAMENTO

O encapsulamento é um processo de empacotamento de partículas, como por exemplo, compostos de sabor, pigmentos, acidulantes, nutrientes, enzimas, conservantes (Azeredo, 2005) e compostos bioativos em cápsulas comestíveis. Esta tecnologia tem sido aplicada em diversas áreas, como farmacêutica, cosmética, química, agrícola e alimentícia (Gibbs et al., 1999; Augustin e Hemar, 2009).

As micropartículas resultantes do encapsulamento podem ser classificadas em microcápsulas e microesferas de acordo com a sua estrutura, por isso, o termo encapsulamento tem sido usado em seu sentido mais amplo, englobando tanto a formação de microcápsulas quanto de microesferas (Azeredo, 2005).

As microesferas envolvem a dissolução do núcleo, ou seja, o composto que será encapsulado, em um material de parede, formando uma dispersão, que será posteriormente atomizada em ar aquecido ou liofilizada (Gibbs et al., 1999; De Vos et al., 2010).

No caso de microcápsulas, o material encapsulado é denominado como recheio ou núcleo, e o material que forma a micropartícula, como agente encapsulante, cobertura ou parede (Gibbs et al., 1999). De acordo com esse autor, dependendo das propriedades físico-químicas do núcleo, da composição da parede e da técnica de microencapsulamento usada, diferentes tipos de partículas podem ser obtidas (Figura 8), como: simples esfera rodeada por um revestimento de espessura uniforme; partículas contendo um núcleo de forma irregular; várias partículas de núcleo embutidas numa matriz contínua de material de parede; vários núcleos distintos dentro da mesma cápsula e microcápsulas multicamadas. Estas cápsulas podem ser classificadas por tamanho em três categorias: macrocápsulas (>5000μm), microcápsulas (0,2 – 5000 μm) e nanocápsulas (<0,2 μm) (Azeredo, 2005).

As microcápsulas têm a função de proteger componentes sensíveis dos alimentos, de evitar a perda nutricional, de possibilitar a incorporação de substâncias sensíveis como

ingrediente, de mascarar ou preservar sabores e aromas e de transformar líquidos em ingredientes sólidos para facilitar a manipulação (Desai e Park, 2005).

De acordo com Desai e Park (2005), várias técnicas são empregadas para formar micropartículas, sendo que as principais são: extrusão, liofilização (*freeze drying*), atomização (*spray drying*), coacervação, lipossomas, leito fluidizado, secagem em tambor e inclusão molecular.

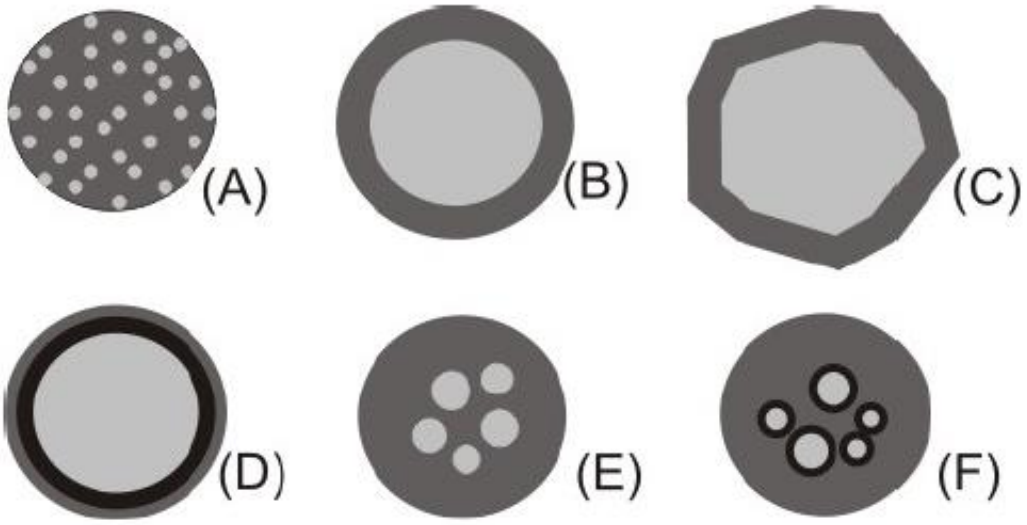

Figura 8. Alguns modelos de micropartículas. (A): matriz (microsfera); (B): microcápsula simples; (C): simples, irregular; (D): duas paredes; (E): vários núcleos; (F): agrupamento de microcápsulas.

Fonte: Gibbs et al. (1999) e Azeredo (2005).

6.1. Atomização (spray drying)

Encapsulamento por *spray drying* tem sido utilizado na indústria alimentícia desde o final dos anos 1950 para o encapsulamento de óleos aromatizantes, evitando sua degradação/oxidação (Desai e Park, 2005). É um método de baixo custo, bastante flexível e que produz partículas de boa qualidade, geralmente do tipo matricial, ou seja, com o núcleo distribuído na forma de micropartículas na matriz seca do material encapsulante (Azeredo, 2005; Desai e Park, 2005; Augustin e Hemar, 2009).

O *spray drying* é uma operação unitária através da qual um produto líquido é atomizado em uma corrente de ar quente, onde instantaneamente se obtém um pó (Gharsallaoui et al., 2007).

O processo básico para a produção de um produto encapsulado por atomização envolve a dissolução do núcleo em uma dispersão do material da matriz, onde a dispersão é atomizada em ar aquecido, para facilitar a rápida remoção da água, sendo que é rapidamente retirada a água das gotas pelo contato com o fluxo de ar quente na câmara de secagem e após, as partículas na forma de pó são separadas do ar de secagem, através de um ciclone, saindo do sistema em temperaturas mais baixas (Figura 9) (Augustin e Hemar, 2009).

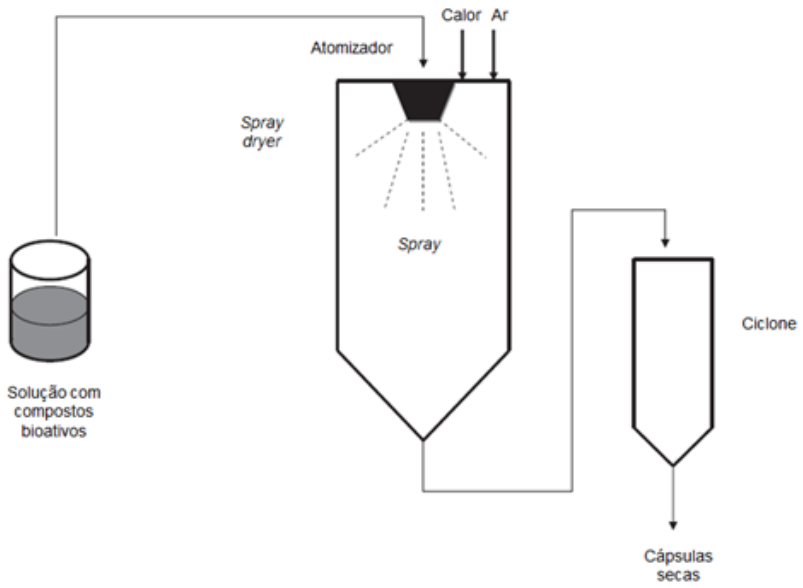

Figura 9. Esquema de secagem por atomização.

Fonte: De Vos et al. (2010).

Diversos estudos mostram que a atomização é um método eficaz no microencapsulamento de compostos fenólicos. Fang e Bhandari (2011) empregaram uma solução de maltodextrina para encapsulamento de suco de *bayberry* (*Myrica rubra* Sieb. et Zucc) utilizando o método de atomização (150 °C), e alcançaram 96% e 94% de retenção de compostos fenólicos totais e antocianinas totais, respectivamente. Jiménez-Aguilar et al. (2011) testaram o microencapsulamento do extrato concentrado de mirtilo (35% de sólidos solúveis) com uma solução de goma Mesquita (17% p/v) na proporção de 67:33 (v/v) e atomizado a 140 e 160 °C, resultando na retenção de 96% das antocianinas monoméricas e 88% da atividade antioxidante.

6.2. Liofilização (freeze drying)

A liofilização é um método de desidratação de um material previamente congelado, sendo que a água é removida por sublimação. Para facilitar a sublimação rápida, o produto congelado é

mantido a pressões muito baixas e o vapor deve ser eliminado por condensação à baixa temperatura. Por consequência disso, é reconhecidamente o melhor método para obtenção de produtos desidratados de alta qualidade, pois o material permanece congelado até estar completamente seco, mantendo assim, sua forma original (Ratti, 2001).

A liofilização é um dos métodos mais eficientes no microencapsulamento de compostos termossensíveis e instáveis em soluções aquosas, visto que a remoção da água ocorre em condições de baixas temperaturas (Madene et al., 2006). Entretanto, é uma técnica de alto custo, e longo tempo de processamento, o que prejudica sua aplicação comercial (Azeredo, 2005; Ratti, 2001).

Alguns estudos aplicaram a liofilização como método de encapsulamento do extrato de hibisco utilizando diferente materiais de parede, sendo eles: trealose e maltodextrina (Duangmal et al., 2008), maltodextrina e goma arábica (Selim et al., 2008) e goma pululana (Gradinaru et al., 2003).

6.3. Material de parede

De acordo com Desai e Park (2005) os materiais de parede (Tabela 3) podem ser selecionados a partir de uma grande variedade de polímeros naturais ou sintéticos, dependendo do material a ser revestido e as características desejadas nas microcápsulas finais. Um material de revestimento ideal, de acordo com Desai e Park (2005), deve apresentar as seguintes características:

1. Boas propriedades reológicas em alta concentração e de fácil manipulação durante o encapsulamento;

2. Capacidade de dispersar ou emulsionar o material ativo e estabilizar a emulsão produzida;

3. Não possuir reatividade com o material a ser encapsulado, tanto durante o processamento e como durante armazenagem prolongada;

4. Ter capacidade de selar e manter o material ativo dentro de sua estrutura durante o processamento ou armazenamento;

5. Facilidade de retirar á água ou solvente durante a atomização;

6. Ser capaz de fornecer máxima proteção para o material ativo contra as condições ambientais (por exemplo, oxigênio, calor, luz, umidade);

7. Promover a liberação controlada de uma substância;

8. Ser barato.

Tabela 3. Materiais de parede utilizados na microencapsulamento de alimentos.

Categoria	Material de revestimento
Carboidratos	Amido, maltodextrina, quitosana, xarope de milho, dextrina, amido modificado, ciclodextrinas, polidextrose, goma guar parcialmente hidrolisada, carboximetilcelulose, metilcelulose, etilcelulose, goma arábica, alginato de sódio, carragena e goma xantana
Lipídeos	Cera, parafina, cera de abelha, diacilglierois, óleos e gorduras
Proteínas	Glúten, caseína, gelatina, albumina e peptídeos

Fonte: Adaptada de Desai e Park (2005).

A escolha de um material de parede para o microencapsulamento é muito importante para a eficiência da encapsulamento e estabilidade da microcápsula, sendo assim, os critérios para a seleção de um material da parede baseiam-se principalmente nas propriedades físico-químicas tais como solubilidade; peso molecular; cristalinidade; difusibilidade; formação de película; propriedade emulsificante e baixa viscosidade (Desai e Park, 2005). Além disso, é fundamental considerar a massa molar do material, uma vez que a adição de agentes encapsulantes de elevada massa molar tendem a aumentar a temperatura de transição vítrea (*Tg*) do produto final, diminuindo sua higroscopicidade e consequentemente aumentando a sua estabilidade (Nayak e Rastogi, 2010; Truong et al., 2005).

6.3.1. Goma arábica

Conforme Ali et al. (2009), a goma arábica é uma fonte nativa de fibra solúvel e apresenta efeito prebiótico, alta tolerância digestiva e impacto benéfico no índice glicêmico do produto final. Contém baixo valor calórico e por não ser cariogênica é considerada apropriada para diversas formulações de alimentos funcionais (bebidas, barras de cereais, produtos extrusados, etc.). Além disso, a goma arábica é um bom material de parede para o encapsulamento através da técnica de *spray drying* (Kanakdande et al., 2007).

Pelas suas propriedades, como capacidade de formação de emulsões, formação de filmes nas interfaces, baixa higroscopicidade, baixa viscosidade, propriedades sensoriais pouco marcadas, a goma arábica é considerada um excelente material encapsulante (Madene et al., 2006).

6.3.2. Goma Guar Parcialmente Hidrolisada

A goma guar é um polissacarídeo solúvel em água, encontrada em sementes de guar (*Cyamopsis tetragonoloba* L.), que é uma planta originária da Índia e Paquistão (Cho e Dreher, 2001). Entretanto, é extremamente viscosa, dificultando a sua utilização como aditivo alimentar uma vez que deve ser adicionada quantidades muito pequenas (Parisi et al., 2005). Dessa forma, a hidrólise da goma guar originou uma goma com melhores características, ou seja, com viscosidade reduzida, podendo ser facilmente adicionada nos alimentos em quantidades superiores à goma guar (Fernandes et al., 2006).

A goma guar parcialmente hidrolisada (GGPH) possui uma cadeia principal constituída por manose e galactose na proporção de 2:1 (Figura10) (Santas et al., 2012). Ela é quase insípida, incolor e inodora, tem aparência de um pó branco e fino, e em solução é transparente e incolor. Ele é ligeiramente doce de sabor, altamente solúvel em água, mas insolúvel em etanol (Kapoor e Juneja, 2009).

A GGPH é uma fibra dietética, considerada prebiótica, possui diversos efeitos benéficos ao organismo humano e atua na redução da diarreia e constipação (Belo et al., 2008; Ustundag et al., 2010), melhora os sintomas da síndrome do intestino irritável (Parisi et al., 2005) e reduz os níveis de colesterol e de glicose no sangue (Tsuda et al., 1998).

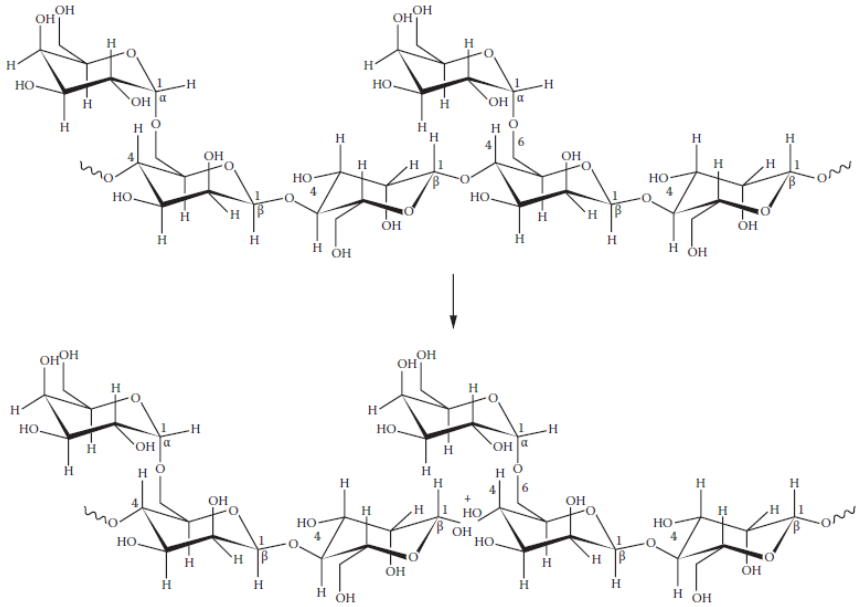

Figura 10. Fórmula estrutural da goma guar intacta e da goma guar parcialmente hidrolisada.

Fonte: Kapoor e Juneja (2009).

6.3.3 Polidextrose

A polidextrose (Figure 11) é um polímero hidrossolúvel, de alta massa molar, constituído por unidades de glicose, unidas por ligações α (1→6), contendo como grupo terminal o sorbitol e/ou ligações monoésteres de ácido cítrico (Gomes et al., 2007). É resistente à ação das enzimas digestivas, à degradação microbiana e fornece baixo valor calórico (aproximadamente 1 kcal/g) (Setser e Racette, 1992; Flood et al., 2004).

É extremamente estável dentro de uma ampla faixa de pH, temperatura, condições de processo e estocagem (Montenegro et al., 2008). Assim, estes atributos conferem propriedades tecnológicas favoráveis à aplicação desse polímero em alimentos, como agente de volume e de melhoramento de textura além de ser um produto não-cariogênico, sendo que é usada principalmente como um substituinte de açúcar e fibra dietética em alimentos; em típicos níveis de utilização, proporciona efeitos fisiológicos semelhantes às de outras fibras dietéticas (Flood et al., 2004).

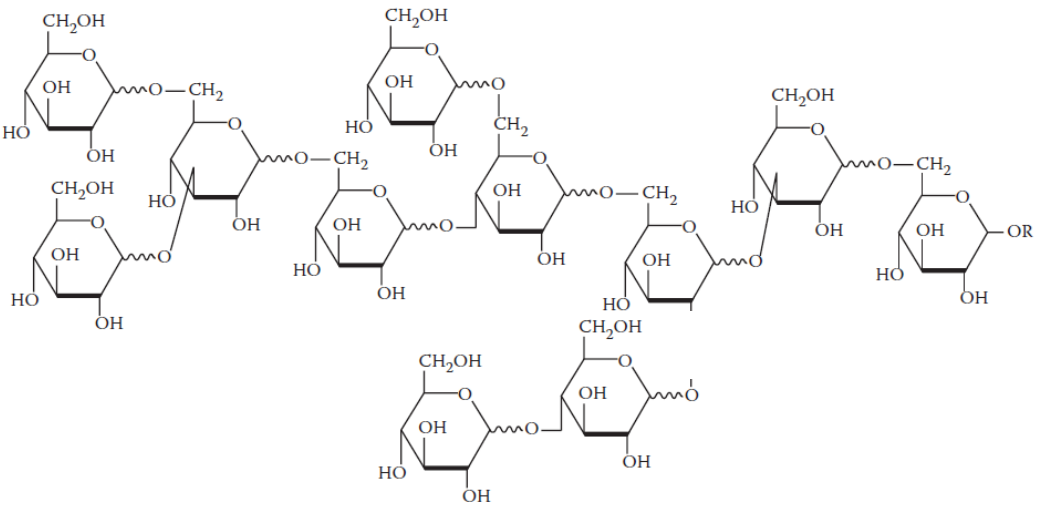

Figura 11. Fórmula estrutural da polidextrose.

Fonte: Stowell (2009).

A polidextrose é reconhecida em muitos países como um ingrediente prebiótico, pois estimula o crescimento de lactobacilos e bifidobactérias no trato intestinal (Paucar-Menacho et al., 2008), bem como pelo seu mecanismo de ação que é semelhante ao de outras fibras solúveis como pectinas, β-glicanas da aveia e a inulina, diminuindo os níveis de colesterol e glicose no sangue (Montenegro et al., 2008).

CAPÍTULO 2

Identification and quantification of carotenoids and phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa* L.) by HPLC-DAD-MS/MS

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Identification and quantification of carotenoids and phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa* L.) by HPLC-DAD-MS/MS

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Abstract

The hibiscus (*Hibiscus sabdariffa* L.) has received increasing interest because it contains high levels of bioactive compounds with remarkable functional properties. The composition of the carotenoids and phenolic compounds from hibiscus calyces was determined by high-performance liquid chromatography coupled to diode array and mass spectrometry detectors (HPLC-DAD-MS/MS). To the best of our knowledge, for the first time a detailed description of the carotenoid composition of hibiscus calyces is reported. Twenty one carotenoids were found and fifteen were identified or tentatively identified. The major carotenoids were all-*trans*-lutein (316.43 ± 19.92 µg/100 g fresh weight.) and all-*trans*-β-carotene (147.76 ± 5.59 µg/100 g f.w.). Twenty phenolic compounds were found and fourteen were identified or tentatively identified. The major phenolic compounds were delphinidin 3-sambubioside (218.17 ± 12.69 mg/100 g f.w.) and 3-caffeoylquinic acid (79.22 ± 7.01 mg/100 g f.w.), representing almost 60% (w/w) of the total phenolic compounds from hibiscus calyces.

Keywords: carotenoids, phenolic compounds, HPLC-DAD-MS/MS, hibiscus calyces.

1. Introduction

Hibiscus sabdariffa L., an edible flower, is also known as roselle, red sorrel or karkade, belonging to the family *Malvaceae*, is an annual herbaceous subshrub. It is an herbaceous plant, cultivated widely in tropical and subtropical areas of both hemispheres. The hibiscus calyces

(outer ring of the fruit) are commonly used in the manufacture of beverages (teas or cold beverages) and jam, as a flavoring agent in the food industry and as an herbal medicine (Cisse, Dornier, Sakho, Ndiaye, Reynes, & Sock, 2009; Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014; Mohamed, Fernández, Pineda, & Aguilar, 2007; 2013).

Some studies have shown several beneficial health effects associated with hibiscus, such as hepatoprotective (Yin, Cao, Xu, Jeney, & Nakao, 2011), antibacterial (Liu, Chen, Wang, Hsu, Yang, & Wang, 2006), antihypertensive (Herrera-Arellano et al., 2007) among others activities. These effects are hypothetically attributed to the antioxidant properties of bioactive compounds, such as phenolic acids, anthocyanins and carotenoids present in hibiscus (Prenesti, Berto, Daniele, & Toso, 2007; Wang, Wang, Lin, Chu, Chou, & Tseng, 2000).

In previous studies, it was reported some phenolic compounds found in hibiscus calyces (*Hibiscus sabdariffa* L.), where the two major anthocyanins were the delphinidin-3-sambubioside and the cyanidin-3-sambubioside (Sindi, Marshall, & Morgan, 2014), as well as also others flavonoids, such as quercetin-3-rutinoside and phenolic acids as caffeoylquinic acids and hibiscus acids (Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011). On the other hand, no reliable data on the carotenoid composition of hibiscus has been previously reported (Wong, Yusof, Ghazali, & Che Man, 2002). The carotenoids are responsible for different colors in food, moreover, the most significant properties attributed to these compounds are the antioxidant capacity and provitamin A activity (Saini, Nile, & Park, 2015). Thus, the objective of this work was to perform a deeply study of the carotenoids and phenolic compounds composition of hibiscus by high performance liquid chromatography coupled to photodiode array detector and mass spectrometry (HPLC-DAD-MS/MS).

2. Materials e methods

2.1. Samples

Agroecological hibiscus (around 15 kg) was obtained from Community Garden – Associação Comunitária da Lomba do Pinheiro, in Porto Alegre, Brazil (GPS coordinates: 30°06'49.4"S and 51°06'30.0"W). The harvest was performed in March 2015. After reception in the laboratory, the flowers were selected and submitted to manual inspection, separating the calyces from the seeds. After that, calyces were cleaned, packed in polyethylene bags, sealed and storage at -18 °C.

2.2. Chemicals

HPLC-grade methanol was obtained from J.T.Baker (Trinidad and Tobago), methyl tert-butyl ether (MTBE) from Tedia (USA) and acetonitrile and formic acid from Panreac (Darmstadt, Germany). The samples and solvents were filtered through Millipore (Massachusetts, USA) membranes (0.22 μm and 0.45 μm) before the HPLC analyses. Standards of all-*trans*-β-carotene, quercetin and 5-caffeoylquinic acid were purchased from Sigma-Aldrich (St. Louis, USA).

2.3. Exhaustive extraction of carotenoids

The carotenoids were exhaustively extracted from hibiscus calyces (5.0 ± 0.5 g) with acetone, transferred to petroleum ether/diethyl ether (1:1, v/v), and saponified with 10% (w/v) methanolic KOH, overnight (~16 h) at room temperature as described by Mariutti, Rodrigues, & Mercadante (2013). After, this the alkali was removed by washing the extract with distilled water, and the solvent was evaporated in a rotary evaporator (T<30 °C). The dried extract was stored in the dark at -18 °C under nitrogen atmosphere until analysis. The extraction procedure was performed in triplicate.

2.4. Extraction of phenolic compounds

The phenolics compounds were exhaustively extracted from hibiscus calyces (4.0 ± 0.5 g) in Falcon tubes with 20 mL of methanol:water (8:2, v/v) acidified (1% HCl) by stirring in a vortex mixer (Q920-A2, Quimis) for 5 minutes at room temperature (22 ± 3 °C), followed by filtration in qualitative filter paper (Rodrigues, Mariutti, & Mercadante, 2013). Then, the filtrated extracts were centrifuged (16R Heraeus Megafuge, Thermo Scientific) at 9.000×g for 5 minutes at 20 ºC and the supernatant was transferred to 100 mL volumetric flask. This procedure was repeated until the extract did not react anymore with the Folin-Ciocalteau reagent, in this case, four times. The supernatants were combined and adjusted to obtain a final volume of 100 mL. The extraction procedure was performed in triplicate.

2.5. HPLC−DAD−MS/MS Analysis

For the chromatographic analysis of the carotenoides and phenolic compounds, it was used a Shimadzu HPLC (Kyoto, Japan) equipped with two pumps, online degasser, column oven, diode array detector and connected in series to a mass spectrometer with an q-TOF analyzer and

electrospray ionization (ESI) source (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany) for carotenoides and phenolic compounds.

Previously to the HPLC analysis, the extracts and the solvents were filtered through Millipore 0.22 and 0.45 μm membranes, respectively, and 20 μL of the extract were injected into the chromatographic system.

The determination of carotenoids and phenolic compounds was performed using the same conditions previously described by Rodrigues, Mariutti, & Mercadante (2013). The carotenoides were separated on a YMC C_{30} column (5 μm, 250 mm×4.6 mm, Waters, Wilmington, DE) using as the mobile phase a linear gradient of a methanol/MTBE mixture from 95:5 (v/v) to 70:30 (v/v) over 30 min, followed by a 50:50 (v/v) ratio for 20 min, and maintaining this proportion for 15 min, at 0.9 mL/min and oven temperature at 29°C. The chromatograms were processed at 450 nm. The column eluate was directly injected into APCI source. The mass spectrometer parameters were set using the same conditions previously described in details by de Rosso & Mercadante (2007). The combination of the parameters such as elution order on the C_{30} column, UV−vis spectral features [maximal absorption wavelength (λ_{max}), spectral fine structure (%III/II), and peak *cis* intensity (%A_B/A_{II})], and MS spectrum characteristics were compared to standard analyzed under the same conditions and data available in the literature (Chisté & Mercadante, 2012; da Silva, Rodrigues, Mercadante, & de Rosso, 2014; Mariutti, Rodrigues, & Mercadante, 2013; Rodrigues, Mariutti, & Mercadante, 2013; Van Breemen, Dong, & Pajkovic, 2012) and considered to the identification of the carotenoids.

The phenolic compounds were separated on a Synergi Hydro-RP C_{18} column (4 μm, 250×4.6 mm, Phenomenex, USA). The run was at a flow rate of 0.7 mL/min, oven temperature at 29 °C, using a mobile phase consisting of water/formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile/formic acid (99.5:0.5, v/v) (solvent B) in a linear gradient from A/B 99:1 to 50:50 in 50 min; then from 50:50 to 1:99 in 5 min. The former ratio (1:99) was maintained for additional 5 min. The column eluate was split to allow only around 0.35 mL/min entering the ESI interface. The UV-vis spectra were obtained between 200 and 800 nm and the chromatograms were processed at 260, 320, 360 and 520 nm. The mass spectra were acquired with a scan range from *m/z* 100 to 700. The MS parameters were set as follows: ESI source in positive and negative ion modes; capillary voltage, 2000 V (positive) or −3000 V (negative); dry gas (N_2) temperature, 310 °C; flow rate, 8 mL/min; nebulizer gas, 2 bar. MS^2 were set in automatic mode applying fragmentation energy of 34 eV. The phenolic compounds were identified on the basis of the

following information: elution order and retention time in the reversed phase column, UV−Vis and MS spectra features compared to standards analyzed under the same conditions, and data available in the literature (Borrás-Linares et al., 2015; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011; Rodrigues & Bragagnolo, 2013; Rodríguez-Medina et al., 2009).

The carotenoids were quantified by HPLC-DAD, using analytical curve with nine-point of all-*trans*-β-carotene (0.12 – 15 μg/mL, R^2 = 0.998). The phenolic compounds were quantified by HPLC-DAD, using analytical curves with nine-point of 5-caffeoylquinic acid (0.50 – 48 μg/mL, R^2 = 0.999), quercetin (0.88 – 57 μg/mL, R^2 = 0.997) and cyanidin (0.10 – 10 μg/mL, R^2 = 0.997).

3. Results and discussion

3.1. Carotenoids

Twenty one carotenoids were separated by HPLC (**Figure 1**) and fifteen carotenoids were identified or tentatively identified based on the combined information obtained from chromatographic elution time, UV-visible features and mass spectra characteristics (**Table 1**). A brief description of the identification of some carotenoids is presented below. The structures of the carotenoids are showed in the **Figure 2**.

Peak 11 was identified as all-*trans*-lutein, based on the absorption characteristics in the UV-Vis similar to that reported in the literature (de Rosso & Mercadante, 2007), due to the protonated molecule $[M + H]^+$ at *m/z* 569. Moreover, other characteristic was observed for this peak, which showed a more intense signal in the MS for the fragment *m/z* at 551 $[M + H - 18]^+$ than the protonated molecule. Due to the presence of a double bond allylic to the hydroxyl group, the fragment *m/z* 551 was more stable than the protonated molecule. In addition, in the MS^2 spectrum the fragments at *m/z* 533 $[M + H – 18 – 18]^+$, *m/z* 495 $[M + H – 18 – 56]^+$, *m/z* 459 $[M + H – 18 – 92]^+$ were found, in agreement with previously studies (de Rosso & Mercadante, 2007).

The carotenes were the last to elute under these conditions, as reported by da Silva, Rodrigues, Mercadante, & de Rosso (2014). **Peaks 18**, **20** and **21** were identified as β-carotene isomers, being them, 13-*cis*-β-carotene, all-*trans*-β-carotene and 9-*cis*-β-carotene, respectively. For identification, it was considered the UV-visible spectra characteristics, chromatographic behavior, co-elution with standards, and mass spectra. The mass spectra of all isomers of β-carotene showed the protonated molecule at *m/z* 537 and a fragment ion in the MS^2 at *m/z* 444 [M

– 92]$^{+}$, corresponding to the loss of the toluene from the polyene chain (de Rosso & Mercadante, 2007).

Peak 14 was identified as phytoene, based on the absorption characteristics in the UV-Vis similar to that reported in the literature (de Rosso & Mercadante, 2007). The mass spectrum showed the molecular ion at *m/z* 545 and the most abundant fragment ion in the MS^2 spectrum at *m/z* 339, corresponding to the cleavage of the closest single bond allylic to the conjugated triene.

The major carotenoids found in hibiscus were all-*trans*-lutein (316 µg/100 g f.w.), all-*trans*-β-carotene (148 µg/100 g f.w.) and phytoene (92 µg/100 g f.w.), representing almost 50, 23 and 15% of the total carotenoid content, respectively. The hibiscus presented a total carotenoid content of 641 µg/100 g f.w. The food classification as good sources of carotenoids was made according to Britton & Khachik (2009), who considered the following content range in µg/100 g: low, 0 – 100; moderate, 100 – 500; high, 500 – 2000; very high > 2000. Thus, hibiscus can be considered a high source of carotenoids. Some studies related that the a diet rich in lutein can reduce the incidence of eye diseases such as age-related macular degeneration, cataract, and retinitis pigmentosa (Olmedilla, Granado, Blanco, & Vaquero, 2003).

Vitamin A activity of a carotenoid is observed when at lest one unsubstituted β-ionone ring bonded to the polyene chain and at least 11 carbons. Thereby, to calculate the vitamin A activity of hibiscus, the following carotenoids were considered: 15-*cis*-β-cryptoxanthin, all-*trans*-α-cryptoxanthin, 13-*cis*-β-carotene, all-*trans*-α-carotene, all-*trans*-β-carotene 9-*cis*-β-carotene (Mariutti, Rodrigues, & Mercadante, 2013). It was followed the NAS-IOM (2001) conversion factor to calculate the vitamin A value by retinol activity equivalent (RAE). The hibiscus presented 13.52 µg RAE/100 g f.w., showing low vitamin A activity when compared with leaf lettuce (*Lactuca sativa*) (184µg RAE/100g), but similar to eggplant (*Solanum melongena*) with value of the 12 µg RAE/100 g (Rodriguez-Amaya, Kimura, Godoy, & Amaya-Farfan, 2008).

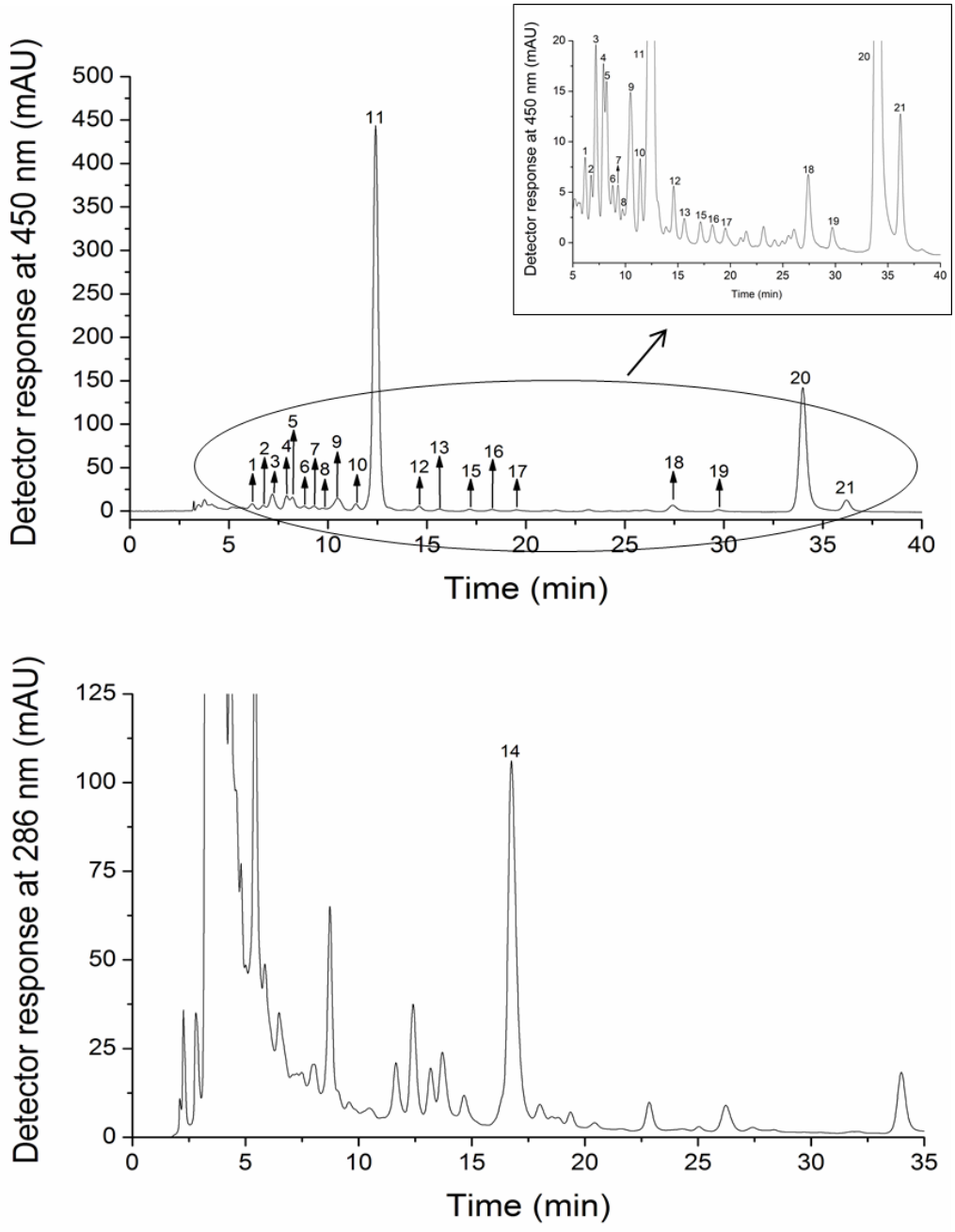


Figure 1. Chromatograms obtained by HPLC−DAD of the carotenoids from hibiscus calyces (*Hibiscus sabdariffa* L.) at 450 nm and 286 nm. Peaks characterization is given in **Table 1**.

Table 1. Chromatographic, UV−Vis, and Mass Spectroscopy Characteristics of carotenoids content in the hibiscus calyces (*Hibiscus sabdariffa*L.), obtained by HPLC−DAD−MS/MS.

peak[a]	carotenoid[b]	concentration (µg/100 g fresh weight)[c]	t_R (min)[d]	λ_{max} (nm)[e]	% III/II	% A_B/II	$[M + H]^+$ (*m/z*)	MS/MS (*m/z*)
1	not identified 1	4.09 ± 0.29	6.2	332, 398, 419, 444	50	78	601	583, 547, 490
2	not identified 2	1.95 ± 0.11	6.7	328, 397, 420, 446	77	68	601	583, 565, 545, 490
3	not identified 3	10.36 ± 0.79	7.2	374, 399, 421, 447	88	0	551 (source)	533, 495
4	not identified 4	4.95 ± 0.26	7.9	398, 421, 447	79	0	601	583, 565
5	13-*cis*-violaxanthin	2.40 ± 0.84	8.2	328, 412, 435, 463	75	19	601	583, 565, 491
6	all-*trans*-neochrome	1.43 ± 0.35	8.8	398, 421, 446	88	0	601	583, 565, 221
7	mixture 1	3.25 ± 0.06	9.3	326, 423, 444, 469	14	56	601	583, 565, 490
8	mixture 2	1.03 ± 0.03	9.7	381, 401, 425	115	0	601	583, 565, 491, 221
9	13-*cis*-lutein	13.44 ± 0.52	10.5	330, 421, 441, 467	17	57	569	551, 533, 495, 477, 459
10	13'-*cis*-lutein	4.63 ± 0.25	11.4	326, 411, 437, 464	43	79	569	551, 533, 495, 459, 477
11	all-*trans*-lutein	316.43 ± 19.92	12.4	418, 444, 471	62	0	569	551, 533, 495
12	all-*trans*-zeaxanthin	4.70 ± 0.35	14.6	423, 449, 474	26	0	569	n.d.[f]
13	9-*cis*-lutein	2.39 ± 0.17	15.6	330, 412, 438, 467	56	n.c.[g]	569	551, 533, 495, 477
14	Phytoene	92.07 ± 3.94	16.7	271, 281, 294	0	0	545	489, 435, 339
15	15-*cis*-β-cryptoxanthin	2.29 ± 0.15	17.1	330, 418, 445, 472	58	60	553	535, 461
16	9'-*cis*-lutein	2.27 ± 0.13	18.3	330, 418, 438, 467	n.c.	n.c.	551 (source)	551
17	all-*trans*-α-cryptoxanthin	2.10 ± 0.13	19.5	402, 445, 471	60	0	553	535, 495
18	13-*cis*-β-carotene	7.97 ± 0.22	27.4	336, 412, 444, 468	11	47	537	481, 444,413, 400
19	all-*trans*-α-carotene	2.87 ± 0.13	29.7	413, 445, 472	71	0	537	481, 444
20	all-*trans*-β-carotene	147.76 ± 5.59	34.0	421, 451, 476	25	0	537	444, 413, 400
21	9-*cis*-β-carotene	12.99 ± 0.70	36.2	315, 418, 446, 471	33	n.c.	537	444, 413, 400
Total carotenoids (µg/100 g fresh weight)[c]		641.38 ± 23.61						
Vitamina A value (µg RAE/100 g fresh weight)[h]		13.52 ± 0.42						

[a]Numbered according to the chromatograms shown in Figure 1. [b]Tentative identification based on UV−vis and mass spectra as well as relative HPLC retention times and published data. [c]The peaks were quantified (n = 3) as being equivalent all-*trans*-β-carotene. [d]Retention time on the C_{30} column. [e]Linear gradient of methanol/MTBE. [f]n.d.: not detected. [g]n.c.: not calculated. [h]RAE: retinol activity equivalent.

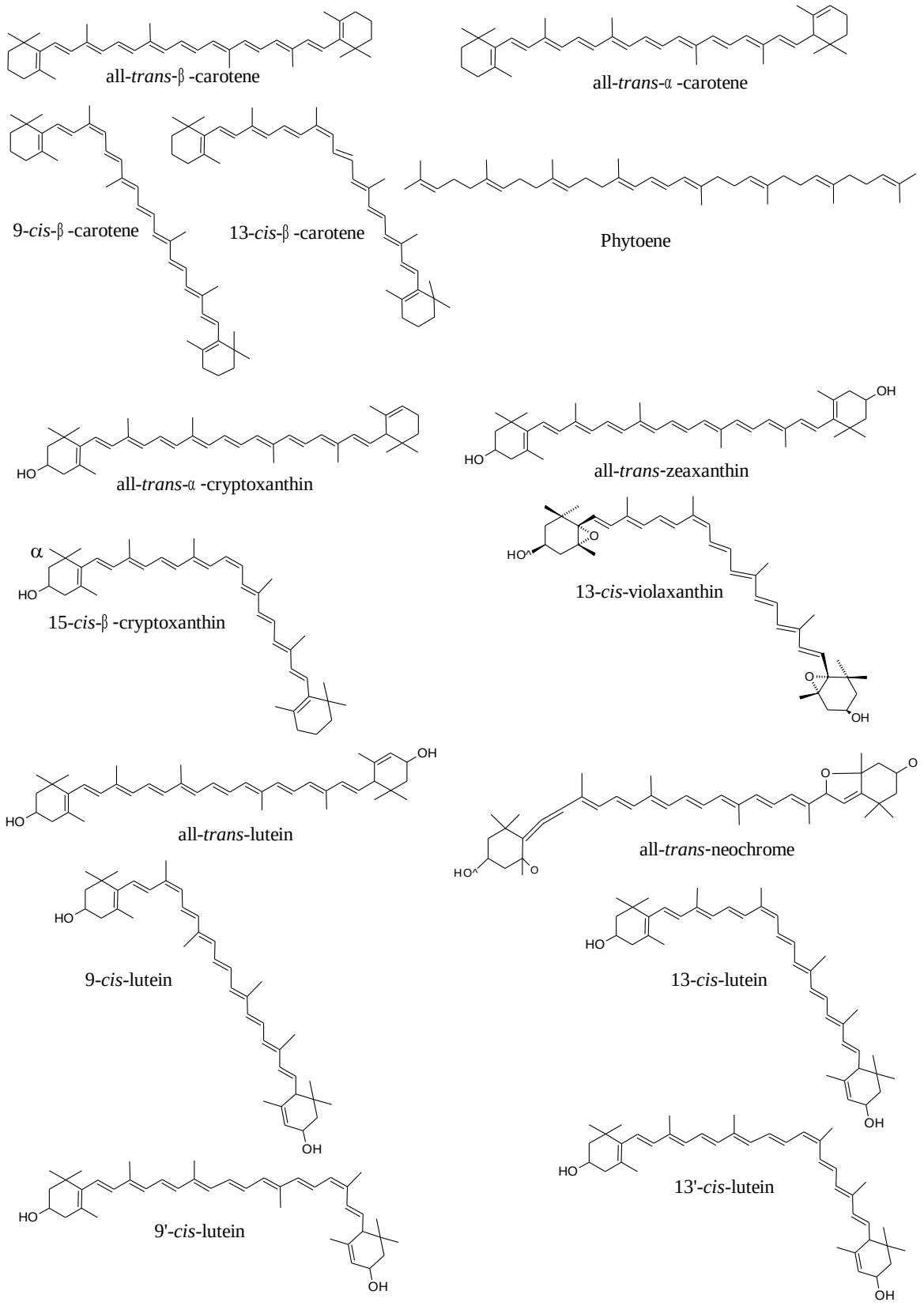


Figure 2. Structures of the carotenoids detected in hibiscus calyces (*Hibiscus sabdariffa* L.).

3.2. Phenolic compounds

Regarding the profile of phenolic compounds, previous studies by HPLC-DAD-MS/MS have already done the characterization of this matrix grown in other regions of the world such as Senegal (Rodríguez-Medina, et al., 2009), Mexico (Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011) and Spain (Borrás-Linares, et al., 2015), but none of the studies performed total exhaustive extraction. Until now, there were no characterization studies of phenolic compounds of hibiscus grown in Brazil.

Twenty phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa* L.) were separated and fourteen were identified (**Table 2** and **Figure 3**). The structures of the phenolic compounds are showed in the **Figure 4**.

Peak 1 (t_R = 5.9 min, λ_{max} = 261) was identified as hydroxycitric acid, based on UV-vis spectrum, deprotonated molecule $[M–H]^-$ at *m/z* 207 and mass fragments (MS^2) at *m/z* 189 [M – H – 18] and 127 [M – H – 48], corresponding to the loss of a molecule of water and carbon dioxide, respectively (Borrás-Linares, et al., 2015). Moreover, **peak 1** showed MS spectrum and MS^2 fragmentation patterns similar to data previously reported in the literature (Borrás-Linares, et al., 2015).

Peak 2 (t_R = 6.6 min, λ_{max} = 261) was identified as hibiscus acid, based on UV-vis spectrum, deprotonated molecule $[M–H]^-$ at *m/z* 189 and mass fragments (MS^2) at *m/z* 127, deriving from the typical losses of water and CO_2 from the main ion (Borrás-Linares, et al., 2015; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011). It is a lactone of the previous compound.

Peak 3 (t_R = 17.5 min, λ_{max} = 325), **peak 7** (t_R = 21.1 min, λ_{max} = 326) and **peak 8** (t_R = 21.6 min, λ_{max} = 323) were identified as caffeoylquinic acid, showing a deprotonated molecule $[M – H]^-$ at *m/z* 353, indicating that these compounds are chlorogenic acids (CQA) isomers. These isomers were differentiated based on UV-vis spectrum, retention time, MS^2 and compared to commercial standards. The peaks 3 and 7 were identified as 3-caffeoylquinic acid and 5-caffeoylquinic acid, respectively, showing the same MS^2 base peak at *m/z* 191 [quinic acid – H]. They could be distinguished by comparing the relative intensity of the secondary ion from caffeoyl moiety [caffeic acid – H] at *m/z* 179, which has a higher intensity at the 3-caffeoylquinic acid than 5-caffeoylquinic acid (Rodrigues & Bragagnolo, 2013). Regarding the peak 8, it was identified as 4-caffeoylquinic, presenting a MS^2 base peak at *m/z* 173 [quinic acid – H – H_2O]

due to the dehydration of the quinic acid ion, a feature of cinnamoyl group bonded to the quinic acid moiety at the position 4 (Rodrigues & Bragagnolo, 2013).

Peak 5 (t_R = 20.3 min, λ_{max} = 310) and **peak 12** (t_R = 24.8 min, λ_{max} = 311) were identified as *p*-coumaroylquinic acid (p-CoQA), showing a deprotonated molecule $[M - H]^-$ at *m/z* 337, indicating that these compounds are p-CoQA isomers. The peak 5 and peak 12 were identified as 3-*p*-coumaroylquinic acid and 5-*p*-coumaroylquinic acid, respectively. As previously reported in the literature, this compounds can be distinguished by base peak, the 3-*p*-coumaroylquinic acid showed higher intensity at *m/z* 163 [*p*-coumaric acid – H], while the 5-*p*-coumaroylquinic acid showed higher intensity at *m/z* 191 [quinic acid – H] (Rodrigues & Bragagnolo, 2013).

Peak 4 (t_R = 18.9 min, λ_{max} = 525) was identified as delphinidin-3-sambubioside, based on UV-vis spectrum, protonated molecule $[M+H]^+$ at *m/z* 597 and mass fragments (MS^2) at *m/z* 303 $[M + H - 194]^+$, corresponding to the loss of one sambubiose molecule (Borrás-Linares, et al., 2015; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011; Rodríguez-Medina, et al., 2009). The detection of a mass fragment at *m/z* 303 in the MS^2 spectrum indicated the presence of a Delphinidin molecule.

Peak 17 (t_R = 27.0 min, λ_{max} = 329) was identified as 5-O-caffeoylshikimic acid, based on UV-vis spectrum, deprotonated molecule $[M - H]^-$ at *m/z* 335 and mass fragments (MS^2) at *m/z* 161, corresponding the deprotonated dihydroxycinnamic acid less a molecule of water $[179 - H_2O]^-$, and *m/z* 135, deriving from a decarboxylation of the deprotonated dihydroxycinnamic acid $[179 - CO_2]^-$ (Rodríguez-Medina, et al., 2009).

A typical chromatogram of the anthocyanins at 520 nm from hibiscus was processed, as shown in Figure 2. Two hibiscus anthocyanins were identified, as follows:

Peak 6 (t_R = 20.6 min, λ_{max} = 518) was identified as cyanidin-3-sambubioside, based on UV-vis spectrum, protonated molecule $[M + H]^+$ at *m/z* 581 and mass fragments (MS^2) at *m/z* 287 $[M + H - 194]^+$, corresponding to the loss of one sambubiose molecule (Borrás-Linares, et al., 2015; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011; Rodríguez-Medina, et al., 2009). The detection of a mass fragment at *m/z* 287 in the MS^2 spectrum indicated the presence of a Cyanidin molecule.

Regarding flavonoids identified of the hibiscus can be highlighted the derivatives of quercetin, kaempferol and myricetin, as follows:

Peak 11 (t_R = 24.3 min, λ_{max} = 349) was identified as myricetin 3-sambubioside, based on UV-vis spectrum, deprotonated molecule $[M - H]^-$ at *m/z* 611 and mass fragments (MS^2) at *m/z*

317 $[M - H - 294]^-$, corresponding to the loss of sugar (sambubiose) moiety. The detection of a mass fragment at *m/z* 317 in the MS^2 spectrum indicated the presence of a Myricetin molecule.

Peak 16 (t_R = 26.4 min, λ_{max} = 354), **peak 18** (t_R = 27.7 min, λ_{max} = 354) and **peak 19** (t_R = 29.2 min, λ_{max} =353) were identified as quercetin derivatives, showing deprotonated molecules $[M - H]^-$ at *m/z* 595, 609 and 463, respectively. These compounds presented both the same mass fragments at *m/z* 300 and 301, corresponding to the loss of sugar moieties, sambubiose $[M - H - 194]^-$, rutinose $[M - H - 308]^-$, and glucose $[M - H - 161]^-$, respectively (Kahle, Kraus, & Richling, 2005; Rodríguez-Medina, et al., 2009). The detection of a mass fragment at *m/z* 301 in the MS^2 spectrum indicated the presence of a Quercetin molecule.

Peak 20 (t_R = 29.9 min, λ_{max} = 345) was identified as kaempferol 3-O-rutinoside, based on UV-vis spectrum, deprotonated molecule $[M - H]^-$ at *m/z* 593 and mass fragments (MS^2) at *m/z* 285 $[M - H - 308]^-$, corresponding to the loss of a rutinose moiety (Borrás-Linares, et al., 2015; Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011). The detection of a mass fragment at *m/z* 285 in the MS^2 spectrum indicated the presence of a Kaempferol molecule.

Regarding the hibiscus acid derivatives detected (hydroxycitric acid and hibiscus acid), their concentrations were not estimated because the values were below the quantification detection limit of the analytical curves.

For the total phenolic acids, its concentration was 194.50 mg/100 g fresh weight (f.w.), highlighting the 3-caffeoylquinic acid and 5-caffeoylquinic acid representing approximately 40% and 35% of the total phenolic acids, respectively. Although the chlorogenic acids (CGA) concentration found in hibiscus is significant, it is low when compared to found in coffee (around 10%), the main source of CGA of the human diet (Rodrigues & Bragagnolo, 2013). Several studies have attributed to CGA the antibacterial and anti-inflammatory activities (dos Santos, Almeida, Lopes, & de Souza, 2006). Besides, the consumption of CGA has been associated with a number of health benefits such as reduction of the relative risk of type 2 diabetes, cardiovascular disease, and Alzheimer's disease (Lindsay et al., 2002; Ranheim & Halvorsen, 2005).

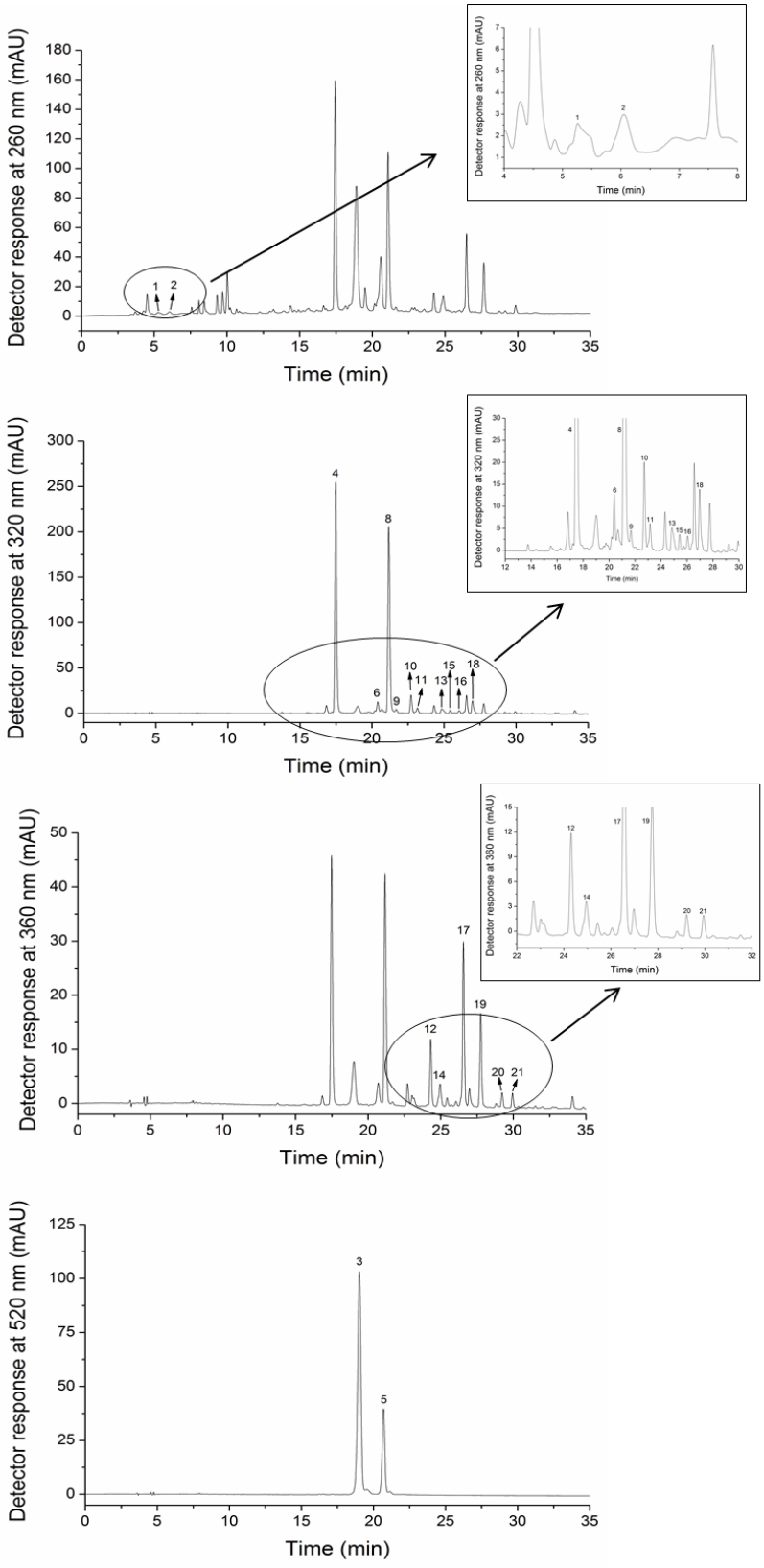
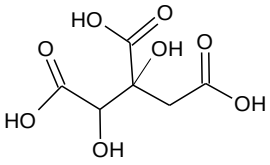


Figure 3. Chromatograms obtained by HPLC−DAD of the phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa* L.) at 260 nm, 320 nm, 360 nm and 520 nm. Peaks characterization is given in **Table 2**.

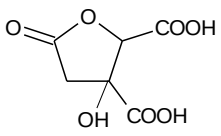
Table 2. Chromatographic, UV−Vis, Mass Spectroscopy Characteristics and quantification of the phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa*L.), obtained by HPLC−DAD−MS/MS.

peak[a]	phenolic compound[b]	concentration(mg/100 g fresh weight)	t_R (min)[c]	λ_{max}(nm)[d]	$[M+H]^+$	MS^2 (+)	$[M - H]^-$	MS^2 (-)
1	hydroxycitric acid	n.q.[e]	5.9	261	n.d.[f]	n.d.	207	127, 115
2	hibiscus acid	n.q.	6.6	261	n.d.	n.d.	189	127
3	3-caffeoylquinic acid[1]	79.22 ± 7.01	17.5	325	n.d.	n.d.	353	191, 179, 135
4	delphinidin 3-sambubioside[2]	218.17 ± 12.69	18.9	280, 525	597	303	n.d.	n.d.
5	3-*p*-coumaroylquinic acid[1]	6.89 ± 0.54	20.3	310	339	147, 119	337	191, 163, 119
6	cyanidin 3-sambubioside[2]	70.42 ± 5.26	20.6	280, 518	581	287	n.d.	n.d.
7	5-caffeoylquinic acid[1]	68.35 ± 0.19	21.1	326	355	163	353	191, 179, 135
8	4-caffeoylquinic acid[1]	1.78 ± 0.07	21.6	323	355	163	353	191, 179, 173, 135
9	not identified 1[1]	14.45 ± 0.12	22.6	325	n.d.	n.d.	n.d.	n.d.
10	not identified 2[1]	2.25 ± 0.10	23.1	308	n.d.	n.d.	353	161, 135, 127
11	myricetin 3-sambubioside[3]	3.74 ± 0.17	24.3	349	613	319	611	317
12	5-*p*-coumaroylquinic acid[1]	4.55 ± 0.24	24.8	311	339	147 (source)	337	191, 173, 163, 119
13	not identified 3[3]	2.55 ± 0.08	25.0	354	n.d.	n.d.	755	301
14	not identified 4[1]	3.22 ± 0.05	25.4	326	n.d.	n.d.	n.d.	n.d.
15	not identified 5[1]	2.34 ± 0.01	26.0	326	n.d.	n.d.	n.d.	n.d.
16	quercetin 3-sambubioside	8.88 ± 0.72	26.4	354	597	303	595	301
17	5-O-caffeoylshikimic acid[1]	11.43 ± 0.87	27.0	292, 329	n.d.	n.d.	335	161, 135
18	quercetin 3-rutinoside[3]	7.77 ± 0.28	27.7	354	611	303	609	301
19	quercetin 3-glucoside[3]	1.49 ± 0.04	29.2	353	n.d.	n.d.	463	301
20	kaempferol 3-O-rutinoside[3]	1.56 ± 0.02	29.9	345	n.d.	n.d.	593	285
Total phenolic acids (mg/100 g fresh weight)		194.50 ± 6.40						
Total anthocyanins (mg/100 g fresh weight)		288.58 ± 17.95						
Total flavonoids (mg/100 g fresh weight)		25.99 ±1.04						
Total phenolics (mg/100 g fresh weight)		509.07 ± 25.39						

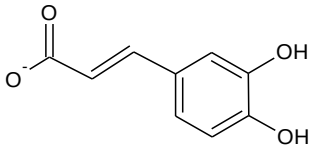
[a]Numbered according to the chromatograms shown in Figure 2. [b]Tentative identification based on UV−vis and mass spectra as well as relative HPLC retention times and published data. [c]Retention time on the C_{18} column (5 μm). [d]Linear gradient of water/acetonitrile acidified with formic acid/acetonitrile acidified with 0.5% of formic acid. [e] n. q.: not quantified. [f]n. d.: not detected. The peaks were quantified (n = 3) as being equivalent 5-caffeoylquinic acid[1], cyanidin[2], quercetin[3].



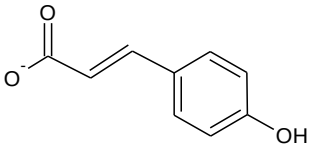
hydroxycitric acid



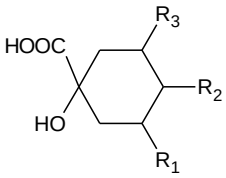
hibiscus acid



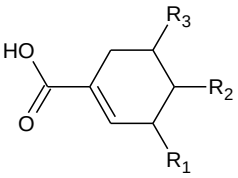
caffeoyl



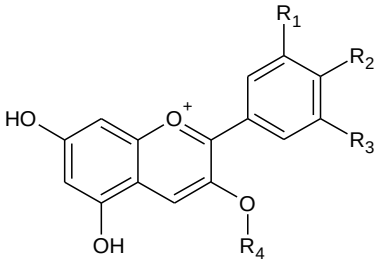
p-coumaroyl



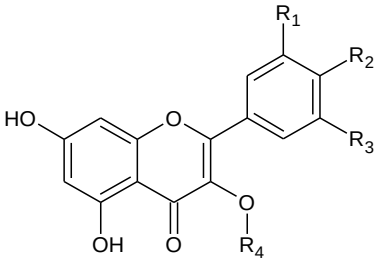
Compounds	**R_1**	**R_2**	**R_3**
quinic acid	OH	OH	OH
3-caffeoylquinic acid	caffeoyl	OH	OH
4-caffeoylquinic acid	OH	caffeoyl	OH
5-caffeoylquinic acid	OH	OH	caffeoyl
3-p-coumaroylquinic acid	p-coumaroyl	OH	OH
5-p-coumaroylquinic acid	OH	OH	p-coumaroyl



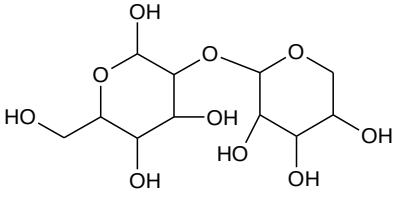
Compound	**R_1**	**R_2**	**R_3**
5-O-caffeoylshikimic acid	OH	OH	caffeoyl



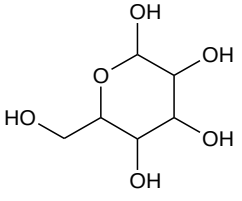
Compounds	**R_1**	**R_2**	**R_3**	**R_4**
delphinidin 3-sambubioside	OH	OH	OH	sambubioside
cyanidin 3-sambubioside	OH	OH	H	sambubioside



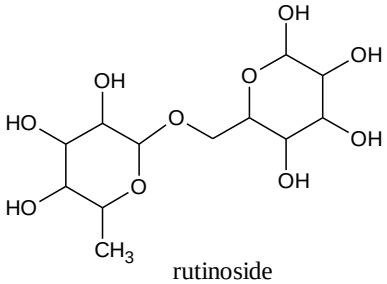
Compounds	**R_1**	**R_2**	**R_3**	**R_4**
myricetin 3-sambubioside	OH	OH	OH	sambubioside
quercetin 3-sambubioside	OH	OH	H	sambubioside
quercetin 3-rutinoside	OH	OH	H	rutinoside
quercetin 3-glucoside	OH	OH	H	glucoside
kaempferol 3-O-rutinoside	H	OH	OH	rutinoside



sambubioside



glucoside



rutinoside

Figure 4. Structures of the phenolic compounds detected in hibiscus calyces (*Hibiscus sabdariffa* L.).

The total anthocyanins in hibiscus calyces was 288.58 mg/100 g f.w. Among the anthocyanins of hibiscus, delphinidin3-sambubioside was the main anthocyanin, being approximately 75.6% of the total anthocyanins, followed by cyanidin3-sambubioside (24.4%), which it is in agreement with the results obtained by Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall (2011) and Christian, Nair, & Jackson (2006). Two anthocyanin-glycosides based on cyanidin and delphinidin, respectively, were also found in the edible flower marigold (*Tagetes erecta*), having a total of 33 mg/g (dry basis) (Navarro-González, González-Barrio, García-Valverde, Bautista-Ortín, & Periago, 2015).

The non-anthocyanic flavonoids concentration found in the hibiscus was 25.99 mg/100 g f.w. Among the flavonoids identified and quantified of the hibiscus, it was found the derivatives of quercetin, kaempferol and myricetin, agreeing with the results obtained by Borras-Linares et al. (2015), highlighting the quercetin derivatives, quercetin3-sambubioside and quercetin3-rutinoside, as the main flavonoids of the hibiscus. The quercetin is one of the most effective antioxidant flavonoids (Bonilla, Mayen, Merida, & Medina, 1999). These compounds were approximately 38 and 33% of the total flavonoids content of the hibiscus, respectively, followed in descending order myricetin 3-sambubioside (16%), kaempferol 3-O-rutinoside (6.6%), and quercetin 3-glucoside (6.4%). Several studies have demonstrated an inverse relation between rich diets in flavonoids and risk of cardiovascular diseases and the regulation of cancer promoter genes (Lin, Lin, Chen, Lin, Chou, & Wang, 2007; McKay, Chen, Saltzman, & Blumberg, 2010; Odigie, Ettarh, & Adigun, 2003).

4. Conclusion

A detailed quantitative composition of carotenoids and phenolic compounds was successfully determined by HPLC-DAD-MS/MS for the first time. In this study we have shown that the hibiscus calyces can be considered as a food rich in lutein, chlorogenic acids and anthocyanins (delphinidin 3-sambubioside).

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CAPÍTULO 3

Aqueous extraction of phenolic compounds from hibiscus calyces (*Hibiscus sabdariffa* L.)

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Abstract

The separation of phenolic compounds by aqueous extraction from hibiscus calyces, a edible flower, was studied. The experiments were conducted by a fractional factorial design. Four factors were evaluated: enzyme concentration (Novozym 33095), extraction temperature, stirring speed and extraction time. The extracts produced were subjected to analysis of color (*L**, *a**, *b** and *Chroma*), total monomeric anthocyanins (*TMA*), antioxidant capacity (reducing capacity and ABTS) and identification and quantification of phenolic compounds determined by high-performance liquid chromatography coupled to diode array and mass spectrometry detectors (HPLC-DAD-MS/MS). Fourteen phenolic compounds were identified. The anthocyanins found in hibiscus extract were delphinidin 3-sambubioside and cyanidin 3-sambubioside. Temperature and stirring speed evaluated in factorial design were significant in all treatments. The results showed that the best conditions to hibiscus calyces extract was enzyme concentration of 50 µL/1000 g hibiscus extract, 400 rpm of stirring speed at 55 ºC by 4 hours of extraction.

Keywords: extraction, hibiscus calyces, phenolic compounds, antioxidant activity, HPLC-DAD-MS/MS.

1. Introduction

Hibiscus sabdariffa L. is a native plant from Africa and Asia, belonging to *Malvaceae* family. Depicted as a bushy and branched plant, with a height of up to 2.5 m, of purplish stem with green-purple leaves, whose solitary flowers consisting of 5 valves that have a shape of calyx with intense red hue (Sindi, Marshall, & Morgan,

2014); growing in tropical and subtropical areas of both hemispheres of the world (Cisse et al., 2009). The hibiscus is known by several names such as vinegar plant (Brazil), jamaica (Spain and Mexico), cardade (Italy), karkade (Arabia), roselle (England) or L'oiselle (France) (Ojeda et al., 2010).

Hibiscus calyx is widely used to make infusions or teas, as well as in the preparation of jellies and/or as natural dye source (Lorenzi & Matos, 2008; Mahadevan, Shivali, & Kamboj, 2009). In traditional medicine, the hibiscus is used in the form of tea to treat several disorders such as constipation, cancer, heart disease, urinary tract infections, diabetes, high blood pressure and hepatic disorders (Ajay, Chai, Mustafa, Gilani, & Mustafa, 2007; Ali, Wabel, & Blunden, 2005; McKay, Chen, Saltzman, & Blumberg, 2010; Mozaffari-Khosravi, Jalali-Khanabadi, Afkhami-Ardekani, & Fatehi, 2009).

Nowadays, natural antioxidants from plant sources have had special importance, especially compounds, such as, polyphenols and flavonoids (anthocyanins) (Sindi, Marshall, & Morgan, 2014). Several studies have reported on the antioxidant capacity of hibiscus (Farombi & Fakoya, 2005; Oboh & Rocha, 2008; Ramakrishna, Jayaprakasha, Jena, & Singh, 2008), as well as activity anti-inflammatory (Fakeye, Pal, Bawankule, & Khanuja, 2008), antimicrobial (Jaroni & Ravishankar, 2012; Kang, Seok, Kim, Eun, & Oh, 2007), anticolesterol (Lin et al., 2007), hepatoprotective (Liu et al., 2006; Yin, Cao, Xu, Jeney, & Nakao, 2011), anticancer (Lin, Huang, Huang, Chen, & Wang, 2005; Olvera-García et al., 2008), antihypertensive (Herrera-Arellano et al., 2007), cardioprotective (Alarcón-Alonso et al., 2012; Carvajal-Zarrabal et al., 2009; Fernández-Arroyo et al., 2012; Hopkins, Lamm, Funk, & Ritenbaugh, 2013), anti-adipogenic (Herranz-López et al., 2012), immunomodulatory (Fakeye, Pal, Bawankule, & Khanuja, 2008) and diuretic activity (Alarcón-Alonso et al., 2012). The compounds responsible for these effects are phenolic and anthocyanins present in hibiscus (Fernández-Arroyo et al., 2011; Prenesti, Berto, Daniele, & Toso, 2007; Wang et al., 2000).

The extraction of bioactive compounds from natural sources has become important due to their use as phytochemicals in the preparation of food supplements, nutraceuticals or functional food ingredients, food additives, pharmaceutical and cosmetic products (Cisse et al., 2012). However, the extraction of phenolic compounds from the fruits and vegetable sources depends on several factors (Saikia, Mahnot, & Mahanta, 2015), such as the solvent type, stirring speed, solid and solvent ratio,

extraction time and temperature (Fernández-Agulló, Freire, & González-Álvarez, 2015; Luthria, Mukhopadhyay, & Kwansa, 2006; Nepote, Grosso, & Guzmán, 2005).

The anthocyanins are highly appreciated in the food industry for their coloring properties, which can give several hues in food from red to violet (Lauro & Francis, 2000; Prodanov, Domínguez, Blázquez, Salinas, & Alonso, 2005; Reyes & Cisneros-Zevallos, 2007). Many edible plants, including the hibiscus calyces, are sources of anthocyanins, representing the largest group of water-soluble pigments in the plant (Cisse et al., 2009; Du & Francis, 1973; Wong, Yusof, Ghazali, & Man, 2002). According to Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich , aqueous extracts from the calyces of hibiscus contain two main anthocyanins: delphinidin 3-sambubioside and cyanidin 3-sambubioside, besides other phenolic compounds as phenolic acids and organic acid (hydroxycitric acid and hibiscus acid).

In this context, the aim of this work was the extraction and identification of the major phenolic compounds from hibiscus calyces, obtained by different conditions, using acidulated water as a solvent.

2. Materials and Methods

2.1. Raw material

Agroecological hibiscus was obtained from Community Garden – Associação Comunitária da Lomba do Pinheiro, in Porto Alegre. The harvest was performed in March 2015. After received in the laboratory, the flowers were selected and submitted to manual inspection, separating the calyces from the seeds. After that, calyces were cleaned and then packed in polyethylene bags, sealed and storage at -18 °C.

2.2. Chemicals

Enzyme complex of pectinase, hemicellulase and cellulase (Novozym 33095), as described by Dal Magro et al. (2016) was donated by Novozymes (Spain). HPLC-grade methanol was supplied by J.T.Baker (Trinidad y Tobago), acetonitrile and formic acid from Panreac (Darmstadt, Germany). Millipore (Massachusetts, USA) membranes (0.22 μm and 0.45 μm) were used before the HPLC analyses. The ABTS (2,2'azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid), trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), standards caffeic acid, coumaric acid, ferulic acid, quercetin and

cyanidin were purchased from Sigma-Aldrich (St. Louis, USA). All reagents were analytical grade.

2.3. Aqueous extraction

In order to do the enzymatic inactivation of peroxidase and polyphenoloxidase, the calyces were submitted to steam blanching for 4 min by autoclave at 100 ºC and atmospheric pressure, and immediately, cooled for 3 min in an ice bath (Fante & Noreña, 2012). Then, acidified water with citric acid (2% w/v) was added to the calyces (1:5), and the mixture was mixed and triturated in a blender (Britânia).

During the extraction procedure, the agitation was realized in a mechanical stirrer (RW20, IKA) with a rod stirrer (R1342, propeller diameter: 50 mm, shaft diameter: 8 mm, shaft length: 350 mm). The temperature was controlled by a water bath (Laborota 4000, Heidoplph). After the extraction process, the extracts were centrifuged at 10000 × *g* (CR21GIII, Hitachi Koki) for 10 min and filtered with Whatman filter paper Nº 01, to separate the water insoluble solids.

2.4. Experimental design and statistical analysis

For this study, it was utilized a 2^{4-1} fractional factorial design (**Table 1**), resulting in eight treatments. Four independent factors at two levels: were studied enzyme (x_1) (0; 50 µL/1000 g extract), extraction temperature (x_2) (35; 55 °C), stirring speed (x_3) (200; 400 rpm) and extraction time (x_4) (3; 5 h).

The variables x_i were coded as X_i, according to the equation:

$$X_i = (x_i - \bar{x_i}) / \Delta x_i \qquad (1)$$

Where $\bar{x_i}$ is the mean value of each independent variable, and Δx_i is the step change value. The levels of the independent variables in coded (-1, +1) and real values are shown in **Table 1**.

The following model was fitted to the data (Equation 1) through regression analysis:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j \qquad (2)$$

Where Y is the response variable, β_0, β_i, and β_{ij} are coefficients of the term independent, the linear and interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j.

Table 1. Treatments defined by fractional factorial experimental design with range of coded and real values to aqueous extraction of hibiscus calyces.

Treatment	X_1 Enzyme (µL/1000 g)	X_2 Temperature (ºC)	X_3 Stirring speed (rpm)	X_4 Time of extraction (h)
T1	-1 (0)	-1 (35)	-1 (200)	-1 (3)
T2	+1 (50)	-1 (35)	-1 (200)	+1 (5)
T3	-1 (0)	+1 (55)	-1 (200)	+1 (5)
T4	+1 (50)	+1 (55)	-1 (200)	-1 (3)
T5	-1 (0)	-1 (35)	+1 (400)	+1 (5)
T6	+1 (50)	-1 (35)	+1 (400)	-1 (3)
T7	-1 (0)	+1 (55)	+1 (400)	-1 (3)
T8	+1 (50)	+1 (55)	+1 (400)	+1 (5)

The analysis of *Chroma*, *TMA* (total monomeric anthocyanins), reducing capacity, ABTS and phenolic compounds were the responses variables. The resulting models were used to plot response surfaces. Data were subjected to ANOVA, and the treatments to Tukey's multiple comparison tests, using the software SAS 9.3. The experiments were performed in triplicates, and the results were expressed as the mean ± standard deviation (SD).

2.5. Colorimetric analysis

The measured of color of the extracts was made using a colorimeter (CR400/410, Minolta Co. Ltd., Osaka, Japan), according to the CIELAB (L^*, a^*, b^*) system, where L^* indicates lightness (0 = black and 100 = white), a^* and b^* are coordinates for green ($-a^*$)/red ($+a^*$), and blue ($-b^*$)/yellow ($+b^*$). Before measurement, the instrument was calibrated using white ceramic plate. *Chroma* ($C^* = [a^{*2} + b^{*2}]^{1/2}$) was calculated, which indicates color's purity or saturation.

2.6. Spectrophotometric analysis

All readings were performed in spectrophotometer (Genesys S10, Thermo Scientific).

2.6.1. Total monomeric anthocyanins (*TMA*)

The total anthocyanin content was determined by method of differential pH (Lee, Durst, & Wrolstad, 2005). All absorbance readings were made with distilled water as a blank. The extracts were mixed with buffer solutions pH 1.0 and 4.5. The absorbance was measured in spectrophotometer at 520 and 700 nm. The concentrations are expressed in mg of delphinidin 3-sambubioside (MW = 577 g/mol, ε = 26.000 L/mol) per g extract on dry basis (Cisse, Vaillant, Acosta, Dhuique-Mayer, & Dornier, 2009).

2.6.2. Reducing capacity

The reducing capacity was determined by the Folin-Ciocalteu method proposed by Singleton and Rossi (1965). Firstly, the extracts were diluted in distilled water to adjust the results in the standard curve of gallic acid, following, it was added the Folin-Ciocalteu reagent and the calcium carbonate solution (20 % w/v). After 2 h of reaction in the dark, the absorbance was measured on the spectrophotometer at a wavelength of 765 nm and the results were expressed in mg of gallic acid (GAE) per g extract on dry basis.

2.6.3. Antioxidant capacity (ABTS)

Antioxidant capacity was evaluated following the methodology described by Re et al. (1999). ABTS radical was produced by reacting 7 mM ABTS (2,2'azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid) stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in a dark room at room temperature (± 20 ºC) for 16 h before using. The ABTS radical solution was diluted with ethanol until absorbance of 0.70 (± 0.02) at 750 nm. The hibiscus extract was diluted in water distillated to produce between 20 – 80 % inhibition of the initial ABTS absorbance. An aliquot of 300 µL of the sample was added to 2.7 mL of ABTS radical solution and the decrease of absorbance at 750 nm was recorded during 6 min in a spectrophotometer. The results were calculated based on a calibration curve of Trolox (1.5 – 20 µM), and Trolox-equivalent antioxidant capacity (TEAC) values were expressed as µmol of Trolox per g extract on dry basis.

2.7. Phenolic compounds (HPLC-DAD-MS/MS)

The protocol described by Rodrigues, Mariutti & Mercadante (2013) for

determination of the phenolic compounds was utilized. Chromatographic analyses were performed using a Shimadzu HPLC (Kyoto, Japan) equipped with two pumps, on-line degasser, column oven, connected in series to a diode array detector and a mass spectrometer with an Q-TOF analyzer and electrospray ionization (ESI) source (Bruker Daltonics, model micrOTOF-QIII, Bremen, Germany).

Previously to the HPLC analysis, hibiscus calyces extracts samples were filtered through 0.22 μm cellulose acetate membrane filter (Millipore, Massachusetts, USA) and 20 μL were directly injected into the chromatographic system. The phenolic compounds were separated in a C_{18} Atlantis T3-RP column (5 μm, 250 × 4.6 mm, Dublin, Ireland) for identification, and to improve the peak separation was used a Synergi Hydro-RP C_{18} column (4 μm, 250 × 4.6 mm, Phenomenex, USA) for quantification. The flow rate used was of 0.7 mL/min, oven temperature at 29 °C, using a mobile phase consisting of water/formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile/formic acid (99.5:0.5, v/v) (solvent B) in a linear gradient from A/B 99:1 to 50:50 in 50 min; then from 50:50 to 1:99 in 5 min. The former ratio (1:99) was maintained for additional 5 min. The column eluate was split to allow only around 0.35 mL/min entering the ESI interface. The UV-vis spectra were obtained from 200 to 800 nm and the chromatograms were processed at 260, 320, 360 and 520 nm.

The mass spectra were obtained with a scan range from m/z 100 to 700. The MS parameters were set as follows: ESI source in positive and negative ion modes; capillary voltage, 2000 V (positive) or −3000 V (negative); end plate offset, −500 V; dry gas (N_2) temperature, 310 °C; flow rate, 8 mL/min; nebulizer gas, 2 bar. MS^2 were set in automatic mode applying fragmentation energy of 34 V. The phenolic compounds were identified on the basis of the following information: elution order and retention time in the reversed phase column, UV−Vis and MS spectra features compared to standards analyzed under the same conditions, and data available in the literature.

The phenolic compounds were quantified by HPLC-DAD, using analytical curves with nine-point of caffeoylquinic acid, ferulic acid, quercetin and cyanidin. Data analysis were very well fixed to linear models ($r^2 > 0.99$).

3. Results and discussion

3.1. Effect of the variables on the responses

The linear and interaction effects of the variables enzyme concentration (X_1), temperature (X_2), stirring speed (X_3) and time of extraction (X_4) are shown in **Table 2**.

The equations that describe the behavior in the extraction of the *Chroma* (Y_1), TMA (Y_2), reducing capacity (Y_3), ABTS (Y_4) and phenolic compounds (Y_5) in relation to the responses variables are presented in the Equations below.

$$Y_1 = 34.7675 - 2.29X_2 - 1.3225X_3 \quad (3)$$

$$Y_2 = 3.40375 + 0.07375X_1 + 0.19125X_2 + 0.13125X_3 + 0.05875X_2X_3 \quad (4)$$

$$Y_3 = 3.74 + 0.2275X_2 + 0.1025X_3 \quad (5)$$

$$Y_4 = 6.6975 + 0.565X_2 + 0.2575X_3 + 0.1775X_4 - 0.135X_1X_3 + 0.18X_2X_3 \quad (6)$$

$$Y_5 = 14.07875 + 0.68625X_1 + 2.03125X_2 + 0.90625X_3 \quad (7)$$

Where X_1, X_2, X_3, and X_4 are the coded values for enzyme, temperature, stirring speed and time, respectively. The significance of each coefficient and the coefficients of determination are shown in **Table 2**. For better understanding, contour lines plots for *Chroma*, *TMA*, reducing capacity, ABTS and phenolic compounds were generated in accordance with Equations 3 to 7.

Table 2. *P*-values for the effect of the explanatory variables on the different responses.

Source[a]	Color (*Chroma*)	*TMA*[b]	Reducing capacity	ABTS	Phenolic compounds
X_1	n.s.[c]	0.021*	n.s.	n.s	0.023*
X_2	0.005*	0.001*	<0.001*	0.003*	0.001*
X_3	0.023*	0.004*	0.009*	0.006*	0.011*
X_4	n.s.	n.s.	n.s.	0.009*	n.s.
X_1X_2	n.s.	n.s.	n.s.	n.s.	n.s.
X_1X_3	n.s.	n.s.	n.s.	0.0118*	n.s.
X_1X_4	n.s.	n.s.	n.s.	n.s.	n.s.
X_2X_3	n.s.	0.0371*	n.s.	0.0088*	n.s.
X_2X_4	n.s.	n.s.	n.s.	n.s.	n.s.
X_3X_4	n.s.	n.s.	n.s.	n.s.	n.s.
R square	0.96	0.99	0.97	0.99	0.99

[a] X_1: enzyme; X_2: temperature; X_3: stirring speed; X_4: time.
[b] Total monomeric anthocyanins (*TMA*).
[c] n.s.: not significant.
* Significant (*P*-value < 0.05 at the 95% confidence interval).

For *Chroma* and reducing capacity only the linear effects of the temperature and stirring speed were significant (Equation 3 and 5, **Figure 1a** and **1b**, respectively). For phenolic compounds the linear effects of the enzyme, temperature and stirring speed were significant (Equation 7 and **Figure 1c**). Three linear effects were significant for *TMA* (X_1, X_2 and X_3) and ABTS (X_2, X_3 and X_4). Furthermore, the interaction effect of X_2X_3 was significant for *TMA*, while X_1X_3 and X_2X_3 were significant for ABTS (Equation 4 and 6, **Figure 2a**, **2b** and **2c**, respectively).

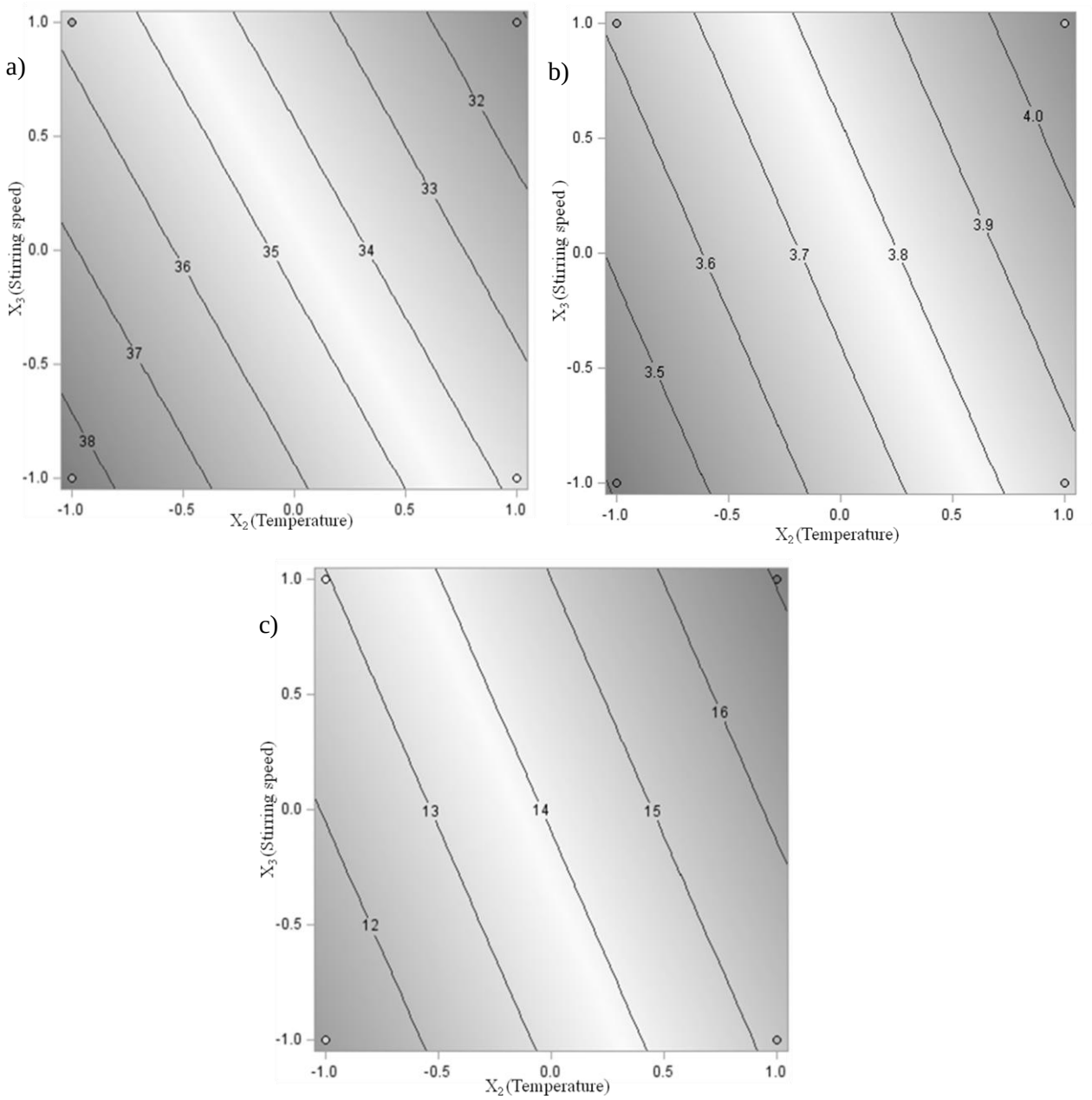

Figure 1. Contour plots for *Chroma* (Y_1) (a), reducing capacity (Y_3) (b) and phenolic compounds (Y_5) (c), where the variable X_1 (enzyme) was fixed in 0.5, for stirring speed versus temperature in the hibiscus calyces extraction.

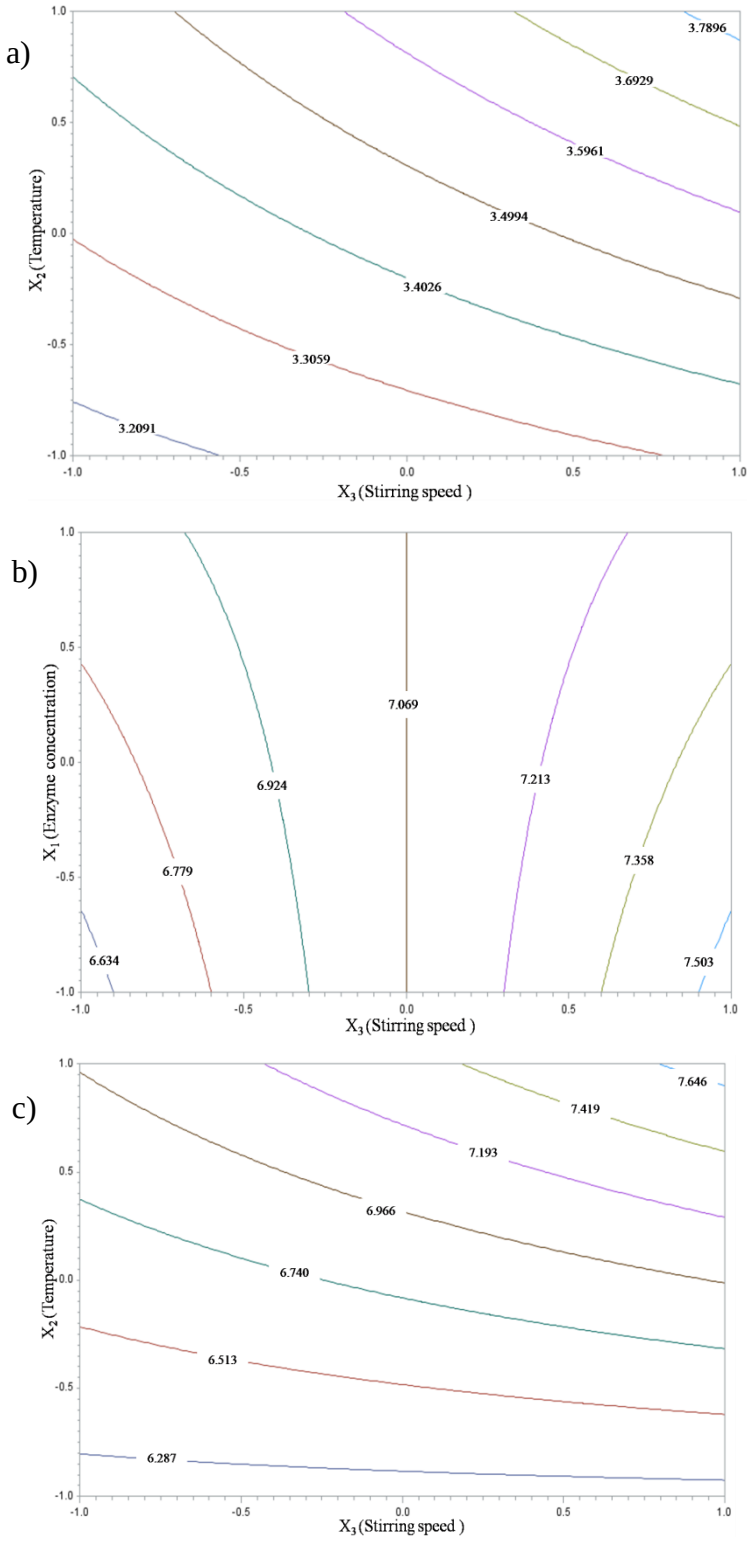

Figure 2. Contour plots for *TMA* (Y_2) (a), where the variable X_1 was fixed in 0.5 for temperature versus stirring speed, and for ABTS (Y_4) for X_1X_3 (b), where the variables X_2 and X_4 were fixed in 0.5 and X_2X_3 (c), where the variables X_1 and X_4 were fixed in 0.5, for enzyme concentration versus stirring speed and temperature versus stirring speed in the hibiscus calyces extraction. Where: X_1: enzyme; X_2: temperature; X_3: stirring speed; X_4: time.

Chroma values increased when temperature and stirring speed decreased (**Figure 1a**). On the other hand, in the **Figure 1b** and **1c**, it was observed that both reducing capacity and phenolic content increased with stirring speed and temperature. In **Figure 2a** and **2c**, for anthocyanins (*TMA*) and ABTS, it can be observed a typical behavior of interaction between factors, where the better extraction conditions were achieved when for these two variables (stirring speed and temperature) were used in their higher levels. In addition, it was observed in the interaction between enzyme concentration and stirring speed (**Figure 2b**) that lower levels of enzyme concentration and higher levels of stirring speed resulted in the higher antioxidant capacity values (ABTS). However, the model for ABTS allows observed that the increase in the temperature, stirring speed and time extraction provided an increase in the ABTS values. The effect of the temperature was the most significant (Equation 6), having positive interaction with the stirring speed (**Figure 2b**).

3.2. Colorimetric analysis

Table 3 shows the color parameters for different extraction conditions. *L** values were significantly lower (darker color) when either higher levels of temperature or stirring speed were used. This may be due to the formation of some dark compounds during the extraction process. The higher values of *a**, *b** and *Chroma* were obtained at 35 ºC. The results also showed that they are located in the first quadrant of the hue circle, indicating a tendency to yellowness and redness. The best extracts with red color may be related to the extraction of anthocyanins (Ramirez-Rodrigues, Plaza, Azeredo, Balaban, & Marshall, 2011).

3.3. Total monomeric anthocyanin (*TMA*)

The mean values of *TMA* varied from 3.07 to 3.82 mg/g extract on dry basis for the hibiscus calyces extracts (**Figure 3a**). These values are in agreement with Christian and Jackson (2009) that reached mean values from 1.8 to 3.5 mg/g extract on dry basis for *TMA* using organic solvents in the extraction. Thus, when used to ideal conditions in hibiscus aqueous extraction process, it is possible to achieve similar levels of total monomeric anthocyanins compared with extraction with organic solvents.

Table 3. Color parameters of the hibiscus calyces extracts prepared following the treatments defined by fractional factorial experimental design showed in **Table 1**.

Treatments	*L**	*a**	*b**	*Chroma**
T1	35.03 ± 0.68^{a}	33.40 ± 0.69^{a}	19.88 ± 0.64^{a}	38.87 ± 0.92^{a}
T2	32.61 ± 0.67^{ab}	32.67 ± 0.78^{ab}	17.79 ± 0.81^{ab}	37.20 ± 1.07^{ab}
T3	31.14 ± 0.69^{bc}	29.82 ± 0.59^{cde}	15.24 ± 0.14^{cd}	33.49 ± 0.59^{cde}
T4	30.66 ± 0.21^{bc}	30.56 ± 0.16^{bcd}	16.64 ± 0.19^{bc}	34.80 ± 0.05^{bcd}
T5	31.22 ± 0.09^{bc}	31.09 ± 0.09^{abc}	16.26 ± 0.15^{bc}	35.08 ± 0.15^{bc}
T6	31.16 ± 0.92^{bc}	32.63 ± 0.95^{ab}	17.61 ± 0.99^{abc}	37.08 ± 1.30^{ab}
T7	29.59 ± 0.79^{c}	27.44 ± 0.74^{e}	12.91 ± 0.73^{d}	30.32 ± 0.98^{e}
T8	28.81 ± 0.99^{c}	28.24 ± 1.03^{de}	13.49 ± 0.52^{d}	31.30 ± 1.16^{de}

Means followed by the same letters in the same columns do not differ by Tukey test at 5% probability.

The model (Equation 3) permitted to evaluate the effects of the factors. The increase in the stirring speed and the temperature increased the *TMA* concentrations and, this effect was the most significant (regression coefficient of 0.19125) (**Table 6**), but as consequence of the interaction between these two main effects, they must not be analyzed alone, but together. The highest anthocyanins extractions were obtained for T8 and T7, because they did not show significant difference (**Figure 3a**).

3.4. Phenolic compounds

Fourteen phenolic compounds were identified by HPLC-DAD-MS/MS as described in section 2.7, based on the combined information obtained from chromatographic elution on C_{18} column, UV-vis and mass spectra characteristics (**Figure 4** and **Table 4**). The MS^2 fragments characteristic of the chemical structure and functional groups allowed the confirmation of the assigned protonated and deprotonated molecules. The concentration on dry basis of the different the phenolic compounds obtained by different treatments are shown in **Table 5**.

The total phenolic acids varied from 4165 to 6929 μg/g extract on dry basis, being the 3-caffeoylquinic acid the main phenolic acid of the hibiscus extract (2089 to 3568 μg/g extract on dry basis), followed, in descending order, by 5-caffeoylquinic acid (1726 to 2751 μg/g extract on dry basis), 3-*p*-Coumaroylquinic acid (163 to 310 μg/g extract on dry basis), 5-p-coumaroylquinic acid (72 to 127 μg/g extract on dry basis), 4-caffeoylquinic acid (63 to 107 μg/g extract on dry basis), 5-O-Caffeoylshikimic acid (40

to 59 μg/g extract on dry basis) and Ferulic acid derived (10 to 25 μg/g extract on dry basis). The T8 and T7 extractions provided the highest values of total phenolic acid (6929 and 6751 μg/g extract on dry basis, respectively), in these treatments can be observed that the use of both the higher levels of temperature and stirring speed, showing the importance of these variables for the extraction of phenolic acids (Equation 6, **Figure 1c**). Several studies reported the importance of phenolic acid in antioxidant activity, where they were associated with good vasodilatory and antimicrobial activity (Borrás-Linares et al., 2015; Fernández-Arroyo, et al., 2012; Mudnic et al., 2010).

The content of total anthocyanins quantified by HPLC ranged from 5456 to 10084 μg/g extract on dry basis. The highest content for total anthocyanins was also obtained by T8 and T7 treatments, 10084 and 9395 μg/g extract on dry basis, respectively. These results were much higher than those found by Salazar-González et al. (2012) who reported 2147 μg/g of total anthocyanins in hibiscus aqueous extract.

Regarding the anthocyanins of hibiscus extract, it was identified and quantified two anthocyanins, highlighting the Delphinidin 3-sambubioside (up to 7516 μg/g extract on dry basis) as the main anthocyanin of the hibiscus, followed by Cyanidin-3-sambubioside (up to 2568 μg/g extract on dry basis), which is in agreement with the results obtained by Ramirez-Rodrigues et al. (2011) and Christian et al. (2006), who reported delphinidin and cyanidin sambubiosides as the anthocyanins in hibiscus aqueous extracts. Anthocyanins are flavonoids widely distributed in nature and are responsible for most of the blue, violet and all shades of red, present in flowers and fruits (Muñoz-Espada, Wood, Bordelon, & Watkins, 2004). They constitute a large percentage of phenolic compounds from hibiscus, representing the most important compounds of the phenolic profile in this flower, with potential applications as a functional and/or as a natural food colorant. Moreover, the anthocyanins have a variety of bioactivity properties, such as antioxidant, cardioprotective, anticancer, anti-inflammation, anti aging and antimicrobial activities (Ali, Wabel, & Blunden, 2005; Farombi & Fakoya, 2005; Fernández-Arroyo et al., 2012; Fernández-Arroyo et al., 2011; Liu et al., 2006; McKay, Chen, Saltzman, & Blumberg, 2010; Villanueva-Carvajal, Dominguez-Lopez, Bernal-Martínez, & Díaz-Bandera, 2013).

For total flavonoids found in the hibiscus extracts, the values ranged from 349 to 598 μg/g extract on dry basis. The highest concentrations were achieved by T3 and T8 (518 and 581 μg/g extract on dry basis, respectively) while the lowest concentrations were obtained by T1 (349 μg/g extract on dry basis). Among the main flavonoids

identified and quantified on the hibiscus calyces extracts, it can be highlighted the derivatives of quercetin, kaempferol and myricetin, in accordance to the results reported by Borrás-Linares et al. (2015). Many studies have demonstrated an inverse relation between rich diets of flavonoids and risk of cardiovascular diseases and the regulation of cancer promoter genes (McKay, Chen, Saltzman, & Blumberg, 2010; Odigie, Ettarh, & Adigun, 2003).

In general, T8 provided the highest extraction of phenolic compounds (17595 μg/g extract on dry basis), followed by T7 (16656 μg/g extract on dry basis), showing a positive effect mainly of the temperature and stirring speed in the extraction of phenolic compounds. According to Pinelo et al. (2006), who studied the profile of the phenolic compounds in grapes, the phenolic compounds can be linked or entangled in the polysaccharides of the cell walls, being confined in the cell vacuoles, or it may be associated with cell nuclei through different chemical bindings or unions of physical nature depending on the composition and disposition of both phenols and polysaccharides.

Thus, it can be concluded that treatments in which high temperatures combined with high stirring speed provided greater degradation of hibiscus cells, increasing the release of intracellular components and of phenolic compounds bound to the cell wall polysaccharides. These results are in accordance to Cabrera et al. (2009), who showed that the temperature acts on the cell wall degradation, aiding to the extraction of intracellular compounds. Other studies also emphasized the importance among temperature, time and stirring on the extraction of phenolic compounds (Luthria, Mukhopadhyay, & Kwansa, 2006; Nepote, Grosso, & Guzmán, 2005; Wong, Yusof, Ghazali, & Che Man, 2003).

3.4. Antioxidant capacity

Antioxidant capacity was analyzed by reducing capacity and ABTS. The results ranged from 3.41 to 4.16 mg GAE/g extract on dry basis and 5.77 to 7.72 μmol Eq. Trolox/g extract on dry basis, respectively, these results were expressed for gram of hibiscus on dry basis (**Figure 3b** and **3c**). The highest antioxidant capacity measured by ABTS were obtained by T7 and T8 (**Figure 3c**). In addition, for reducing capacity, these treatments are statistically equal to the treatments that used also high levels of temperatures (T3 and T4) (**Figure 3b**). These results are also in agreement with Wong et al. (2003) and Lima et al. (2014), who observed that the antioxidant capacity values

increased with the increase of temperature, due to the higher mass transfer the higher separation of antioxidant phenolic compounds.

Thus, the highest antioxidant capacities were obtained by treatments that provided the highest extraction of phenolic compounds. It is important to highlight that several studies have been correlated the antioxidant activity with the phenolic content (Moreno-Montoro, Olalla-Herrera, Gimenez-Martinez, Navarro-Alarcon, & Rufián-Henares, 2015; Prenesti, Berto, Daniele, & Toso, 2007; Sánchez-Mendoza, Domínguez-López, Navarro-Galindo, & López-Sandoval, 2008).

Table 6 shows the Pearson correlation coefficients between the color parameters, phenolic acids, total phenolics, anthocyanins, total monomeric, flavonoids, antioxidant capacity and ABTS in several extractions. The highest correlation coefficients were observed between ABTS (0.90) with phenolic acids, total phenolics and anthocyanins. Reducing capacity assays exhibited high positive correlation with phenolic acids (0.90), total phenolics (0.87) and anthocyanins (0.86). In the other hand, both parameters *a** or *b** were also highly correlated, but showed negative relationship (ranged between -0.98 and -0.84 and -0.95 and 0.84, respectively) with phenolic acids, total phenolics, anthocyanins, total monomeric, flavonoids, ABTS and reducing capacity.

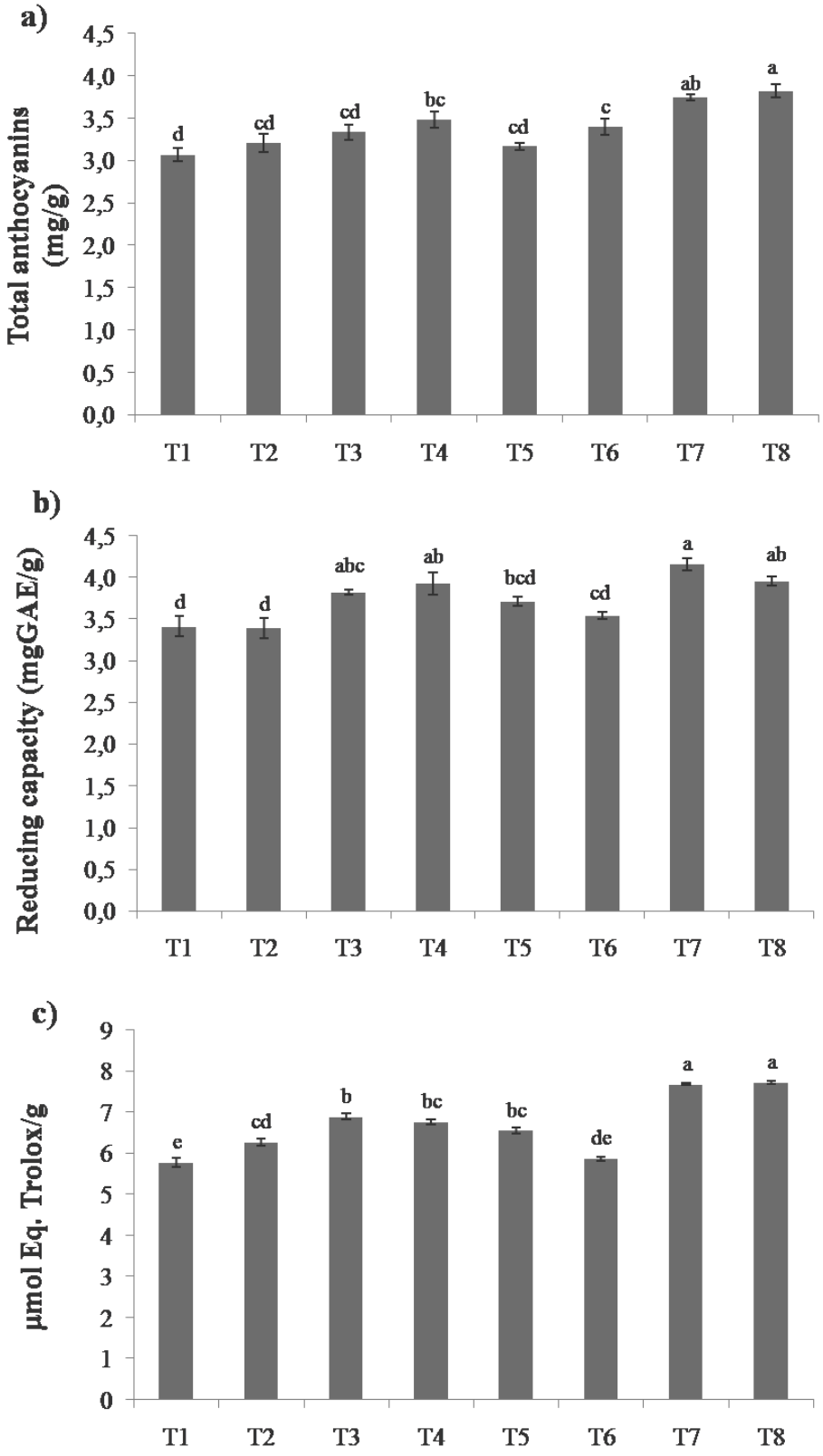

Figure 3. Determination of total monomeric anthocyanins (*TMA*) (a), reducing capacity (b) and ABTS (c) for the hibiscus calyces extracts prepared following the fractional factorial experimental design showed in **Table 1**.

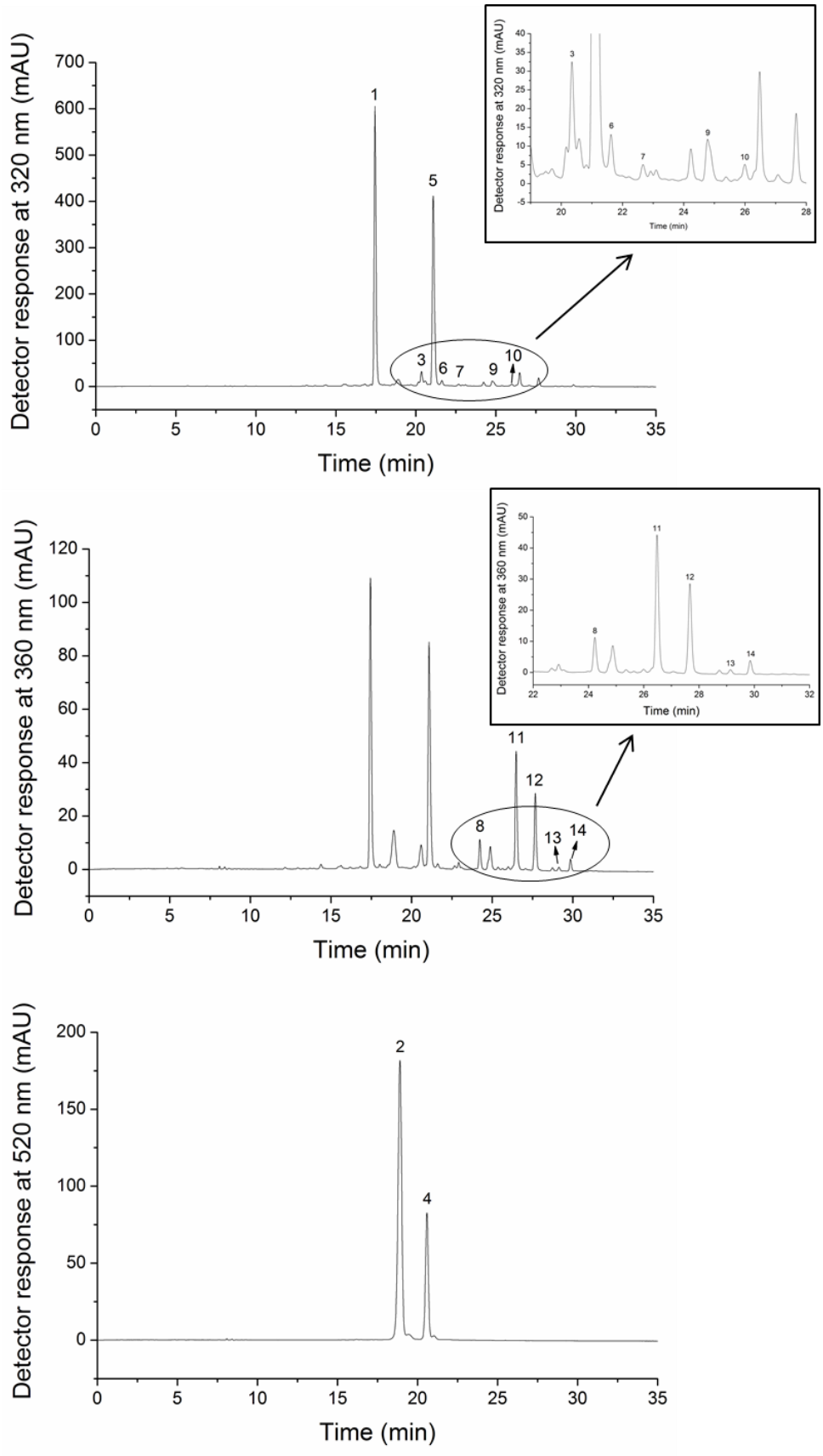

Figure 4. Chromatograms obtained by HPLC−DAD of the phenolic compounds from hibiscus calyces extract. Peak characterization is given in **Table 4**.

Table 4. Chromatographic, UV−Vis, and Mass Spectroscopy characteristics of phenolic compounds in the hibiscus calyces extracts, obtained by HPLC−DAD−MS/MS.

Peak[a]	**Compound**[b]	**Rt**[c] **(min)**	**$\lambda_{máx}$ (nm)**[d]	**$[M+H]^+$ or $[M]^+$**	**MS^2 (+)**	**$[M-H]^-$**	**MS^2 (-)**
1	3-caffeoylquinic acid	17.4	325	n.d.[e]	n.d.	353	191, 135, 179, 161
2	Delphinidin 3-sambubioside	18.9	280, 525	597	303	n.d.	n.d.
3	3-*p*-coumaroylquinic acid	20.3	309	339	147	337	119, 163, 191
4	Cyanidin 3-sambubioside	20.6	280, 518	581	287	n.d.	n.d.
5	5-caffeoylquinic acid	21.1	325	355	163	353	191
6	4-caffeoylquinic acid	21.6	325	355	163	353	191, 135
7	Ferulic acid derived	22.7	291 (sh) f, 321	n.d.	n.d.	353	193, 161, 134, 127
8	Myricetin 3- sambubioside	24.2	349	613	319	611	316, 317
9	5-*p*-coumaroylquinic acid	24.8	310	339	147 (source)	337	191, 173, 163, 119
11	5-O-caffeoylshikimic acid	26.0	292 (sh), 329	n.d.	n.d.	335	161, 135, 191
10	Quercetin 3-sambubioside	26.4	351	597	303	595	300, 301
12	Quercetin 3-rutinoside	27.6	356	611	303	609	300, 301
13	Quercetin 3-glucoside	29.1	342	n.d.	n.d.	463	300, 301
14	Kaempferol 3-O-rutinoside	29.8	342	n.d.	n.d.	593	285

[a]Numbered according to the chromatograms shown in **Figure 4**.
[b]Tentative identification based on UV−vis and mass spectra as well as relative HPLC retention times and published data.
[c]Retention time on the C_{18} column.
[d]Solvent, linear gradient of water and acetonitrile both with 0.5% formic acid.
[e]Not detected (n.d.).
[f] shoulder (*sh)*.

Table 5. Phenolic compounds concentration in the hibiscus calyces extracts prepared following the fractional factorial experimental design showed in **Table 1**.

Peaks	Phenolic compounds	Concentration (µg/g extract on dry basis) [1]							
		T1	T2	T3	T4	T5	T6	T7	T8
1	3-caffeoylquinic acid[1]	2088.86	2560.28	2979.78	3211.35	2632.31	2775.98	3486.63	3568.43
2	Delphinidin 3-sambubioside[2]	4035.49	5304.83	5945.75	6602.71	5129.33	5409.26	6937.87	7515.74
3	3-p-Coumaroylquinic acid[1]	162.90	202.45	233.85	271.31	201.02	212.47	295.41	310.36
4	Cyanidin 3-sambubioside[2]	1420.70	1786.52	2012.17	2185.27	1745.77	1950.48	2457.71	2568.34
5	5-caffeoylquinic acid[1]	1726.27	2047.86	2537.48	2609.59	2096.17	2203.34	2678.89	2750.79
6	4-caffeoylquinic acid[1]	63.21	74.14	84.47	95.92	80.31	80.83	96.96	107.08
7	Ferulic acid derived[3]	12.28	19.62	25.06	21.52	10.08	11.69	21.06	17.78
8	Myricetin 3-sambubioside [4]	51.39	63.73	82.83	72.34.	50.68	59.92	65.93	87.12
9	5-p-Coumaroylquinic acid[1]	72.31	88.70	100.97	107.09	83.75	93.49	126.55	126.88
10	5-O-Caffeoylshikimic acid[3]	39.70	40.82	58.95	46.67	43.05	42.17	46.36	47.96
11	Quercetin 3-sambubioside[4]	152.61	179.82	219.04	230.75	184.14	205.37	235.55	262.83
12	Quercetin 3-rutinoside[4]	110.23	125.71	151.83	160.42	123.88	129.34	164.11	183.36
13	Quercetin 3-glucoside[4]	13.06	13.63	16.58	14.34	14.42	13.64	14.03	17.83
14	Kaempferol 3-O-rutinoside[4]	21.94	25.44	27.51	27.55	24.51	24.77	29.32	30.33
	Total phenolic acids[5]	4165 ± 89^{g}	5033 ± 69^{f}	6020 ± 36^{d}	6363 ± 134^{c}	5146 ± 33^{f}	5419 ± 5^{e}	6751 ± 13^{b}	6929 ± 114^{a}
	Total anthocyanins[6]	5456 ± 65^{h}	7091 ± 86^{f}	7957 ± 110^{d}	8787 ± 101^{c}	6875 ± 63^{g}	7359 ± 141^{e}	9395 ± 48^{b}	10084 ± 93^{a}
	Total flavonoids[7]	349 ± 5^{d}	408 ± 27^{c}	518 ± 13^{b}	505 ± 26^{b}	397 ± 11^{c}	433 ± 7^{c}	509 ± 19^{b}	581 ± 23^{a}
	Total phenolic compounds[8]	9970 ± 160^{g}	12533 ± 183^{f}	14476 ± 159^{d}	15657 ± 262^{c}	12419 ± 108^{f}	13213 ± 143^{e}	16656 ± 81^{b}	17595 ± 231^{a}

Different letters in the same line indicate significant differences ($p < 0.05$). The peaks were quantified as equivalent of 5-caffeoylquinic acid[1], cyanidin[2], ferulic acid[3] and quercetin[4].
[5]Total phenolic acids include 3-caffeoylquinic acid, 3-p-Coumaroylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, Ferulic acid derived, 5-p-Coumaroylquinic acid and 5-O-Caffeoylshikimic acid. [6]Total anthocyanins include Delphinidin 3-sambubioside and Cyanidin 3-sambubioside. [7]Total flavonoids include Myricetin 3-sambubioside, Quercetin 3-sambubioside, Quercetin 3-rutinoside, Quercetin 3-glucoside and Kaempferol 3-O-rutinoside. [8]Total phenolic compounds include all compounds.

Table 6. Coefficients of Pearson correlation between the color parameters (*L**, *a**, *b**, *Chroma*), phenolic acids (Phen acids), anthocyanins (Antho), flavonoides (Flavo), total phenolics (T phen), reducing capacity (RC), total monomeric anthocyanins (*TMA*) and ABTS.

	L*	***a****	***b****	***Chroma***	**Phen acids**	**Antho**	**Flavo**	**T phen**	**RC**	***TMA***	**ABTS**
L*	1.000000										
a*	0.848285	1.000000									
b*	0.907974	0.975271	1.000000								
Chroma	0.876854	0.996396	0.990474	1.000000							
Phen acids	-0.936975	-0.908969	-0.906757	-0.914115	1.000000						
Antho	-0.932805	-0.885753	-0.890416	-0.893005	0.988382	1.000000					
Flavo	-0.869714	-0.849719	-0.844246	-0.853037	0.959407	0.949849	1.000000				
T phen	-0.936293	-0.896569	-0.898357	-0.902934	0.995673	0.998190	0.958156	1.000000			
RC	-0.829489	-0.951541	-0.896897	-0.937079	0.906409	0.860719	0.812782	0.879747	1.000000		
TMA	-0.878837	-0.856228	-0.853784	-0.859443	0.933257	0.955702	0.878204	0.949132	0.823791	1.000000	
ABTS	-0.836731	-0.980115	-0.959244	-0.977718	0.900057	0.901612	0.857023	0.903031	0.901137	0.853789	1.000000

All coefficient were significant ($p < 0.05$).

4. Conclusion

Currently, the consumers looking to buy products with healthy characteristics and that can prevent some disease. Food industry has been concerned to with developing new products with these characteristics.

In this work, it was observed that the treatments that used the higher levels of temperature and stirring speed were able of improving the extraction of phenolic compounds in the hibiscus. The best extract was considered the treatment eight, that obtained a aqueous extract with higher concentrations of total phenolic acids (6.93 mg/g extract on dry basis), total anthocyanins (10.08 mg/g extract on dry basis), total flavonoids (0.58 mg/g extract on dry basis) and total phenolic compound (17.59 mg/g extract on dry basis). The use of acidulated water as solvent has showed promising when compared with other studies that use organic solvent extraction to be used in the food industry, as for example, for production of microcapsules or natural colorant.

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CAPÍTULO 4

Microencapsulation of bioactive compounds from hibiscus calyces (*Hibiscus sabdariffa* L.) by spray drying and freeze drying using gum Arabic, polydextrose, and partially hydrolyzed guar gum as encapsulating agents

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Abstract

Hibiscus calyces extract was microencapsulated by spray drying or freeze drying using gum Arabic (GA), partially hydrolyzed guar gum (PHGG) or polydextrose (PD) at 10% as encapsulating agents. The retention of anthocyanins ranged from 59.8 to 64.6% and from 66.4 to 74.3% and for antioxidant activity, from 66.54 to 71.71 and 69.90 to 73.26% for spray-dried and for freeze-dried powders, respectively. The best result obtained, for the anthocyanins content (2.83 mg delphinidin 3-sambubioside/g sample on dry basis), reducing capacity (2.98 mg GAE/g sample on dry basis) and ABTS (5.67 μmol Trolox Equivalent/g sample on dry basis), was for the powder produced by freeze drying using GA as encapsulating. Regarding physical powders properties, samples produced by spray drying using GA, followed of PHGG had the best results, with values of 95.80 and 95.20%, 31.33 and 28.87%, 17.43 and 10.96 ºC for solubility, hygroscopicity and *Tg*, respectively. Microscopy analysis also indicated that powders produced by spray drying using GA and PHGG had the best structures, showing particles of spherical shape and without agglomeration. The relative span values calculated from particle size distribution data, ranged from 1.90 to 2.00 for spray-dried samples and from 3.06 to 3.19 for freeze-dried samples, indicating that there was a wide distribution of particle size.

Keywords: Spray drying, freeze drying, microencapsulation, anthocyanins, antioxidant capacity, encapsulating agents.

1. Introduction

Nowadays, natural products and health-promoting foods have received great attention of professionals in the food area and the population in general. This way, there is an increase demand for functional foods and nutraceuticals products that assist in maintenance and improvement of health and in modulating of specific immune functions in order to prevent diseases (Bagchi, 2006; Zhao, 2007).

Hibiscus (*Hibiscus sabdariffa* L.), also known as roselle or karkade or red sorrel, is an annual tropical herbaceous sub shrub (Borrás-Linares et al., 2015) and is one of the most common flowering plants grown worldwide (Ahmed & Abozed, 2015). The calyx (part of the outer ring of the flower) is commonly used in beverages and foods such as teas, jams, and jellies (Ahmed & Abozed, 2015; Cisse et al., 2009).

Aqueous extracts from the calyces of hibiscus contain two main anthocyanins: delphinidin 3-sambubioside and cyanidin 3-sambubioside, besides other phenolic compounds as phenolic acids and organic acid (hydroxycitric acid and hibiscus acid) (Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014; Gradinaru, Biliaderis, Kallithraka, Kefalas, & Garcia-Viguera, 2003).

Currently, more than 80% of food active compounds and more than 30% of drugs are produced from bioactive natural products (Ren et al., 2013). When these compounds are isolated from natural sources, they might be directly added into the food. Oftentimes, delivery system encapsulated can be required to ensure the effectiveness of these compounds, avoiding the destruction and oxidation due to exposure to some environmental factors (light, oxygen, temperature and enzymatic activity) (De Vos, Faas, Spasojevic, & Sikkema, 2010; Fang & Bhandari, 2012; Saikia, Mahnot, & Mahanta, 2015). In this sense, microencapsulation technique, such as spray drying and freeze drying are used to encapsulate bioactive compounds (Paini et al., 2015; Saikia, Mahnot, & Mahanta, 2015).

Spray drying has been largely used for microencapsulation of polyphenols and other heat labile compounds (Cal & Sollohub, 2010). Several studies reported freeze drying as an efficient way to microencapsulation method for heat-sensitive compounds such as anthocyanins (Jafari, Mahdavi-Khazaei, & Hemmati-Kakhki, 2016). However, this method has the disadvantage of extensive time for the drying process, and also it has high costs (Her, Song, Lee, & Lee, 2010). According to Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel (2007), several types of encapsulating agents have been used in food, such as the polysaccharides (maltodextrins, starches, corn syrups and gum

Arabic), proteins (casein, gelatin, milk serum, soy and wheat) and lipids (stearic acid, mono- and diglycerides). Gum Arabic (GA) is one of the most common encapsulating agents used in microencapsulation to be a fiber highly soluble, low viscosity, good emulsifying, and film forming properties, however, problems as variability in quality, uncertainty supply and price increased, it has led to look for alternatives that might replace it totally or partially (Sarkar, Gupta, Variyar, Sharma, & Singhal, 2013). The partially hydrolyzed guar gum (PHGG) and polydextrose (PD) are encapsulating agents that have not been well studied yet in foods. The PHGG is a polymer highly soluble in water and has desirable properties as tasteless, odorless, colorless and low viscosity (Kapoor & Juneja, 2009). PD is also a water-soluble polymer, odorless, colorless and stable within a wide range of pH and temperature (Kapoor & Juneja, 2009; Mitchell, 1996). These three encapsulating agents have prebiotic properties (Cho & Samuel, 2009).

The aim of this study was to use gum Arabic (GA), partially hydrolyzed guar gum (PHGG) and polydextrose (PD) as encapsulating agents on the microencapsulation by spray and freeze drying of the aqueous hibiscus calyces extract.

2. Materials and methods

2.1. Materials

The hibiscus was obtained from farmer which uses agroecological practices for sustainable agriculture. The samples were selected and the calyces separated of the seed, cleaned and then packed in polyethylene bags, sealed and frozen at -18 °C until before using.

Gum arabic (Instantgum BA, Nexira Brasil Com Ltda., Brasil), polydextrose (MasterSense Ing Alim Ltda., Brasil) and partially hydrolyzed guar gum (Sunfiber R, R & S Blumos, Com. Prod. Alimentícios Ltda., Brasil) were used as encapsulating agents. ABTS (2,2'azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, USA). All reagents were analytical grade.

2.2. Extraction process

The calyces were submitted to blanching by 4 min in autoclave at 100 ºC and atmospheric pressure, in order to enzymatic inactivation, and immediately cooled for 3 min in an ice bath (Fante & Noreña, 2012). Then, acidified water with citric acid (2%

w/v) was added in the ratio1:5 (hibiscus:acidified water, w/w) and ground in a blender (Britânia). Next, the hibiscus calyces extracts were submitted to agitation (400 rpm) in mechanical stirrer (RW20, Ika) with rod stirrer (R1342) (propeller diameter: 50 mm, shaft diameter: 8 mm, shaft length: 350 mm) at 55 ºC controlled by the water bath (LABOROTA 4000, Heidoplph). In this stage, commercial enzyme complex of pectinase, hemicellulase and cellulase (Novozym 33095) was added into the extract according to the manufacturer's recommendation with aim improve the extraction of bioactive compounds. After, the hibiscus calyces extracts were centrifuged at 10000×*g* (Hitachi) for 10 min and filtered with Whatman filter paper No. 01, for separating the solid residues.

2.3. Preparation of microencapsulated powders

Three dispersions were prepared from the hibiscus calyces extract containing encapsulating agents: 10% gum Arabic (GA) (w/v), 10% partially hydrolyzed guar gum (PHGG) (w/v) and 10% polydextrose (PD) (w/v). These dispersions were prepared at 6500 rpm by 5 min using an Ultra-Turrax (T25, Ika) and then were encapsulated by spray drying or freeze drying.

Spray drying process was performed in a laboratory scale equipment (Mini Spray Dryer LM MSDI 1.0 Labmaq, Brazil) using a dual pneumatic atomizing nozzles, with a 1.0 mm diameter nozzle, with the following conditions: dispersion flow rate of 0.60 L/h, and air temperature, pressure and flow rate of 140± 2 ºC, 3.5 kg-f/cm^2, and 40.5 L/h, respectively.

For freeze drying process, the three dispersions were previously frozen in a ultra-freezer (Liotop UFR30) at -68 ºC for 24 h and then placed in a freeze dryer (Liotop L101) at -57 ºC, at vacuum pressure, less than 20 μmHg for 48 h. Finally, the dried samples were crushed with mortar and pestle.

Spray-dried and freeze-dried samples were stopped in both polyethylene bag and aluminum foil, sealed and placed in a desiccator containing silica for further analyses.

2.4. Colorimetric analysis

Color measurement of the powders was done in a colorimeter (CR400/410, Minolta, Osaka, Japan), according to the CIELAB (*L**, *a**, *b**) system, where *L** indicates lightness (0 = black and 100 = white), *a** and *b** are coordinates for green (-*a**)/red (+*a**), and blue (-*b**)/yellow (+*b**). Before measuring, the instrument was

calibrated using white ceramic plate. Hue angle ($H^* = \tan^{-1} b^*/a^*$) was calculated, which indicates the color of the sample (0º or360º = red, 90º = yellow, 180º = green, and 270º = blue), while *Chroma* ($C^* = [a^{*2} + b^{*2}]^{1/2}$) indicates color's purity or saturation.

2.5. Total monomeric anthocyanins

The total monomeric anthocyanins content (*TMA*) was determined by differential pH method (Lee, Durst, & Wrolstad, 2005). All absorbance readings were made with distilled water as blank. The extracts were mixed with buffer solutions pH 1.0 and 4.5 and the absorbances were measured in spectrophotometer (Genesys S10, Thermo Scientific) at 520 and 700 nm. The *TMA* (expressed in delphinidin 3-sambubioside equivalents, mg/g on dry basis) was calculated using the equation (1):

$$TMA = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \qquad (1)$$

Where: $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$; MW (molecular weight) = 577 g/mol for delphinidin 3-sambubioside; DF = dilution factor; l = 1 (pathlength in cm); ε = 26.000 molar extinction coefficient, in L/mol/cm, for delphinidin 3-sambubioside (Cisse et al., 2012); and 10^3 = factor for conversion from g to mg.

2.6. Reducing capacity

The reducing capacity was determined for the Folin-Ciocalteu method proposed by Singleton & Rossi (1965). A mixture of 1 mL of diluted sample, 0.5 mL of Folin-Ciocalteu reagent and 2.5 mL of sodium carbonate buffer were placed in assay tube. After 2 h of reaction in the dark, the absorbance was measured at 765 nm and the results were expressed in mg of gallic acid per g on dry basis by standard curve.

2.7. ABTS

Antioxidant capacity was estimated using ABTS method, following the methodology described by Re et al. (1999). ABTS radical was produced by reacting 7 mM ABTS (2,2'azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid) stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in dark at room temperature (±20 ºC) for 16 h before using. The ABTS radical solution was diluted with ethanol (~99.5% of purity) and, the extract and the powders with phosphate buffer (pH 7) until the absorbance to reach 0.70 (± 0.02) at 734 nm. The hibiscus calyces extract

was diluted to produce between 20–80% of inhibition of the initial ABTS absorbance. An aliquot of 300 μL of the sample was added to 2.7 mL of ABTS radical solution and the decrease of absorbance at 734 nm was recorded during 6 min in a spectrophotometer. The results were calculated based on a calibration curve of Trolox (1.5–20 μM). TEAC (Trolox-equivalent antioxidant capacity) values were expressed as μmol of Trolox equivalent/g sample on dry basis.

2.8. Hydroxyl radical-scavenging activity (HRSA)

The antioxidant capacity performed using the method of the HRSA was done according to Meng, Fang, Qin, Zhuang, & Zhang (2012), in which, 100 μL of $FeSO_4$ (0.02 M), 45 μL of H_2O_2 (0.15%), and 1 mL of salicylic acid (8 mM) were mixed with 4 ml of distilled water in that order. Next, 1 mL of sample diluted in distilled water was added to this mixture and reacted for 30 min at 37 ºC. The absorbance was measured in spectrophotometer at 593 nm. The result was expressed as percentage of scavenging, and calculated according with the following equation:

$$Scavenging\ activity\ (\%) = [1 - (A_{sample}/A_{control})] \times 100\% \quad (2)$$

Where: A_{sample} =absorbance of the sample; $A_{control}$ = absorbance of the control.

2.9. Determination of the physical properties of the microencapsulated powders

The moisture content of the samples was done from the weight loss after heating the sample at 105 ºC determined by AOAC method 925.10 (AOAC, 1990). The measurement of water activity (A_w) was done directly in electronic meter (3TE-DECAGON, Aqualab, Pullman, USA) according to AOAC method 978.18 (AOAC, 1990).

The solubility was determined according to the method described by Cano-Chauca, Stringheta, Ramos, & Cal-Vidal (2005), with modifications. For that, 1 g powder was suspended in 100 mL distilled water and mixed under magnetic stirring (Lab Disc, Ika) for 5 min. Then, the solution was centrifuged at 3000×g (THERMO 16R, Thermo Scientific) for 15 min and an aliquot of 25 mL of the supernatant was transferred to a 50 mL beaker and oven-dried at 105 ºC until constant weight. The solubility (%) was calculated by weight difference of the powder before and after its drying in the oven, according to equation 3:

$$Solubility\ (\%) = \frac{P_a - P_b}{0.25} \times 100 \qquad (3)$$

Where: P_a (g) is the mass of the beaker plus sample after dried, and P_b (g) is the initial mass of the beaker.

The hygroscopicity was determined according with Tonon, Brabet, Pallet, Brat, & Hubinger (2009), where 1 g sample was placed in an airtight glass container with saturated NaCl (relative humidity of 75%), and stored in an incubator chamber (411/FDP Ethik Technology, Brazil) at 25 ºC. After 1 week, samples were weighed every 24 h until equilibrium was reached. The hygroscopicity was expressed as percentage (%) or 1 g of adsorbed moisture per 100 g dry solids (g/100 g) using the following equation (Caparino et al., 2012):

$$Higroscopicty\ (\%) = \frac{\Delta m\ /(M + M_i)}{1 + \Delta m/M_i} \qquad (4)$$

Where: Δm (g) is the weight increase of the powder after the equilibrium was reached; M is the initial mass of the powder; and M_i is the free water content in the powder before exposure to moist air environment.

The glass transition temperature (*Tg*) of the samples was determined by differential scanning calorimetry (DSC) (DSC Q2000, TA Instruments, New Castle, DE). Enough amount of sample was used to fill the bottom (~5 mg) and placed in hermetic aluminum pans (Tzero, TA Instruments), and one empty aluminum pan was used as reference. The purge gas was realized with ultra-pure nitrogen (flow 50 mL/min). The sample was first cooled to -20 ºC, and then scanned from -20 ºC to 120 ºC at a heating rate of 40 ºC/ min. The *Tg* values were calculated using the software TA Universal Analysis 5.5.3.

2.10. Particle morphology and size distribution

The morphology of the microparticles was analyzed using scanning electron microscope (JSM 6060, Jeol Ltd., Japan). Samples were placed in stubs with double-sided tape carbon for fixation, metallized with gold, and after this in the microscope operating at a voltage of 5 kV and 300 × magnification for the freeze-dried powders, and 2000 × for spray-dried powders.

The particle size distribution was measured using a laser light diffraction instrument (CILAS 1180, Compagnie Industrielle de Lasers, France). The samples were suspended in isopropyl alcohol under constant stirring and sonicated using ultrasound during 30 s. The average diameter (D[4, 3]) and the equivalent volume diameters at

10% (d_{10}), 50% (d_{50}), and 90% (d_{90}) cumulative volume were determined by The Particle Expert software. The particle size distribution in the powder (span) was calculated using the equation: Span = $[(d_{90}- d_{10})/d_{50}]$ (Fernandes, Borges, & Botrel, 2014).

3. Results and discussion

3.1. Colorimetric analysis

The results of colorimetric analysis (*L**, *a**, *b**, *Chroma* and *Hue*) are showed in **Table 1**. The lower values of *L** (54 to 56) were to the treatments using freeze drying, and the higher values (72 to 76) for spray-dried treatments, showing that the freeze-dried treatments resulted in samples darker. However, all treatments were statistically different ($p<0.05$). The parameter *a** values ranged from 30 to 48, while the parameter *b** ranged from 1 to 9, showing that all data were located in the first quadrant (+ *a**, + *b**), indicating a tendency to redness color. These values are attributed to anthocyanins content, which are responsible for the red color (Jiménez-Aguilar et al., 2011). For these two parameters all treatments were statistically different, however the freeze-dried samples were higher than the spray-dried samples. The freeze-dried samples also showed higher values for the *Chroma*, that indicates that these samples had higher color purity or saturation, which indicated smaller losses of anthocyanins compounds. The *Hue* values were close to zero, which confirmed the tendency of the samples to the red color (*Hue* = 0 or 360), with slightly higher values for freeze-dried microparticles.

Table 1. Color parameters of the hibiscus calyces extract microencapsulated with gum arabic (GA), partially hydrolyzed guar gum (PHGG) and polydextrose (PD) by spray drying and freeze drying.

Treatments[1]	*L**	*a**	*b**	*Chroma*	*Hue*
T1	75.96±0.14^{a}	30.80±0.18^{f}	1.20±0.03^{f}	30.82±0.18^{f}	0.39±0.00^{f}
T2	74.20±0.02^{b}	33.97±0.07^{e}	1.85±0.03^{e}	34.02±0.07^{e}	0.43±0.00^{e}
T3	72.62±0.01^{c}	38.55±0.02^{d}	3.92±0.04^{d}	38.75±0.01^{d}	0.49±0.00^{d}
T4	56.27±0.01^{d}	42.20±0.03^{c}	4.39±0.03^{c}	42.42±0.03^{c}	0.64±0.00^{c}
T5	56.09±0.04^{e}	42.98±0.04^{b}	5.04±0.04^{b}	43.27±0.04^{b}	0.65±0.00^{b}
T6	54.52±0.01^{f}	48.01±0.03^{a}	9.25±0.04^{a}	48.89±0.03^{a}	0.72±0.00^{a}

Values are means ± standard deviation (SD). Mean values with different letters in the same column are significantly different ($p<0.05$). [1]T1: spray-dried, with 10% GA; T2: spray-dried, with 10% PHGG; T3: spray-dried, with 10% PD; T4: freeze-dried, with 10% GA; T5: freeze-dried, with 10% PHGG; T6: freeze-dried, with 10% PD.

3.2. Total monomeric anthocyanins and antioxidant capacity

The results of total monomeric anthocyanins (*TMA*) and antioxidant capacity including reducing capacity, ABTS and HRSA in the powders produced are showed in the **Table 2**. The anthocyanins content ranged from 2.28 to 2.83 mg/g on dry basis in the microparticles, which decreased significantly when compared with the extract that was 3.81 mg/g. During spray drying, the retention percentages ranged from 59.8 to 64.6% and this decrease was a consequence of the anthocyanins instability to light, high temperature and oxygen (Tonon, Brabet, Pallet, Brat, & Hubinger, 2009). The freeze-dried treatments showed higher retention of anthocyanins than spray-dried, from 66.4 to 74.3%. Kuck & Noreña (2016) suggested that after freeze drying process might occur degradation of the compounds during grinding of the samples due to exposure to oxygen and light resulting in oxidation reactions. On the other hand, powders produced with PHGG and PD did not differ significantly between each other, however these treatments were statistically lower (p<0.05) than the powders produced with GA.

Table 2. Total monomeric anthocyanins (*TMA*) and antioxidant activity (by reducing capacity, ABTS and HRSA) of the hibiscus calyces aqueous extract microencapsulated with gum Arabic(GA), partially hydrolyzed guar gum (PHGG), and polydextrose (PD) by spray drying and freeze-drying.

Treatments[1]	*TMA**	Reducing capacity**	ABTS***	HRSA****
Extract	3.81 ± 0.06^{a}	3.95 ± 0.04^{a}	7.74 ± 0.03^{a}	80.38± 0.35
T1	2.46 ± 0.04^{d}	2.67 ± 0.10^{c}	5.55 ± 0.02^{c}	90.60 ± 0.22^{a}
T2	2.31 ± 0.05^{e}	2.46 ± 0.07^{d}	5.16 ± 0.07^{e}	90.61 ± 0.06^{a}
T3	2.28 ± 0.01^{e}	2.40 ± 0.01^{d}	5.15 ± 0.01^{e}	90.22 ± 0.13^{ab}
T4	2.83 ± 0.01^{b}	2.98 ± 0.02^{b}	5.67 ± 0.05^{b}	88.72 ± 0.27^{c}
T5	2.59 ± 0.08^{c}	2.72 ± 0.07^{c}	5.47 ± 0.03^{d}	89.82 ± 0.28^{b}
T6	2.53 ± 0.02^{c}	2.70 ± 0.02^{c}	5.41 ± 0.11^{d}	89.95 ± 0.10^{b}

Values are means ± SD. Different letters within the same column indicate significant differences (p< 0.05). *mg delphinidin 3-sambubioside/g sample on dry basis, **mg GAE/g sample on dry basis, ***μmol Trolox Equivalent/g sample on dry basis, ****% of scavenging activity. The weight of the encapsulating agents was discounted. [1]T1: spray-dried, with 10% GA; T2: spray-dried, with 10% PHGG; T3: spray-dried, with 10% PD; T4: freeze-dried, with 10% GA; T5: freeze-dried, with 10% PHGG; T6: freeze-dried, with 10% PD.

The reducing capacity in the microparticles ranged from 2.40 to 2.98 mg GAE/g sample on dry basis, and they were lower than the extract (3.95 mg GAE/g). Freeze-

dried samples and spray-dried treatment utilizing GA had the higher values of reducing (p>0.05). For ABTS method, the samples using GA showed the higher values.

From the data, it can be verified that when the concentration of anthocyanins decreased in all treatments, the antioxidant capacities by reducing capacity and ABTS also diminished. Daniel, Huerta, Sosa, & Mendoza (2012) found that during fixed bed drying of hibiscus, the content of anthocyanin was also related to antioxidant activity.

The HRSA values showed that spray-dried treatments had higher values than the freeze-dried treatment (p<0.05). Kuck & Noreña (2016) found values between 73.5 to 84.7% in grape skin microparticles encapsulated with10% of GA, PHGG or PD.

3.3. Physical properties of the microencapsulated powders

The **Figure 1** shows the moisture content, water activity, solubility, hygroscopicity and *Tg* of hibiscus calyces extract microencapsulated with GA, PHGG and PD by spray drying and freeze drying.

The moisture content (**Figure 1A**) ranged from 1.94 to 6.40%, in which the lower moisture content were for spray drying samples (p<0.05). The water activity values (**Figure 1B**) ranged from 0.137 to 0.339, which indicate that all powers had high stability. The lower values in water activity also occurred with treatments using spray drying (p<0.05).

All powder samples were very soluble (**Figure 1C**), with values ranging from 91.2 to 97.7%. These results are in accordance with values reported by Rigon & Noreña (2016) that used gum Arabic and polydextrose in blackberry extract, and Kuck & Noreña (2016) that also utilized the same encapsulating agent in grape skin phenolic extract. However, freeze-dried powders solubilities produced with PHGG and PD were significantly less.

The hygroscopicity (**Figure 1D**) of the microparticles ranged from 28.9 to 44.7%. In general, the hygroscopicity of the spray drying samples was lower. The hygroscopicity is related with water adsorption from the particles and the relative humidity in air from the environment, being that the higher relative humidity, the higher the hygroscopicity.

The stability of the microparticles during storage is directly related with the *Tg*. The *Tg* is defined as the temperature at which an amorphous system changes from a glassy to a rubbery state and vice versa (Mrad, Bonazzi, Courtois, Kechaou, & Mihoubi, 2013). Beristain, Azuara, & Vernon-Carter (2002) mention that higher *Tg* values in

materials results in more stable particles during processing and storage. *Tg* is also considered an important indicator of the onset of the deterioration mechanisms and alteration in the quality of the foodstuffs (Khalloufi, El-Maslouhi, & Ratti, 2000). The *Tg* of the powders produced (**Figure 1E**) ranged from 0.67 to 17.43 ºC. The powders produced with GA utilizing spray drying and freeze drying showed the higher values (17.43 and 17.16 ºC, respectively), following by the treatments using PHGG for spray-dried and freeze-dried (10.96 and 13.53 ºC, respectively). The microparticles produced using PD as encapsulating agent obtained the smaller *Tg*, with values of 0.67 ºC for both drying methods.

According to Fernandes, Borges, & Botrel (2014), several factors affect in the glass transition, such as temperature, moisture content, the molecular weight and the chemical structure of the material utilized. Thus, as a consequence of adding carrier material, the final *Tg* is also related to the molecular weight of polymers presents in this material (the *Tg* increases with increasing molecular weight). The molecular weight of the materials used is less than 5000 Da (Mitchell, 1996) for PD, in average of 20,000 Da for PHGG (Kapoor & Juneja, 2009) and in average 350,000 Da for GA (Al-Assaf, Phillips, & Williams, 2005).

3.4.Particle morphology and size distribution

On scanning electron microscopy of the microparticles showed in the **Figure 2**, it is possible to observe that in spray-dried samples (**Figure 2A** and **2B**) the majority of particles had shape almost spherical, of different sizes, without aggregation, except for treatment 3 (**Figure 2C**), which utilized polidextrose that presented irregular forms and aggregation between the microparticles. According to Kuck & Noreña (2016), aggregation occur due to the tendency of polydextrose to bind water and to form bridge between molecules. The structural characteristics of freeze-dried microparticles (**Figure 2D**, **2E** and **2F**) were very different than spray-dried microparticles, presenting various sizes, irregular shapes, like ice crystal or broken glass. Structural characteristics of the freeze-dried microparticles is provided by the frozen surface, where the sublimation takes place, being that the removal of water results in a porous structure without shrinkage (Aguilera & Stanley, 1999).

The particle size distribution (span) and the average diameter (D[4, 3]) of the six treatments are presented in **Table 3**. Jafari, Assadpoor, He, & Bhandari (2008) describes several factors that are relevant to determination the diameter of

microparticles, as the method used and the drying conditions, the properties of the material, the concentration and viscosity of the encapsulated material.

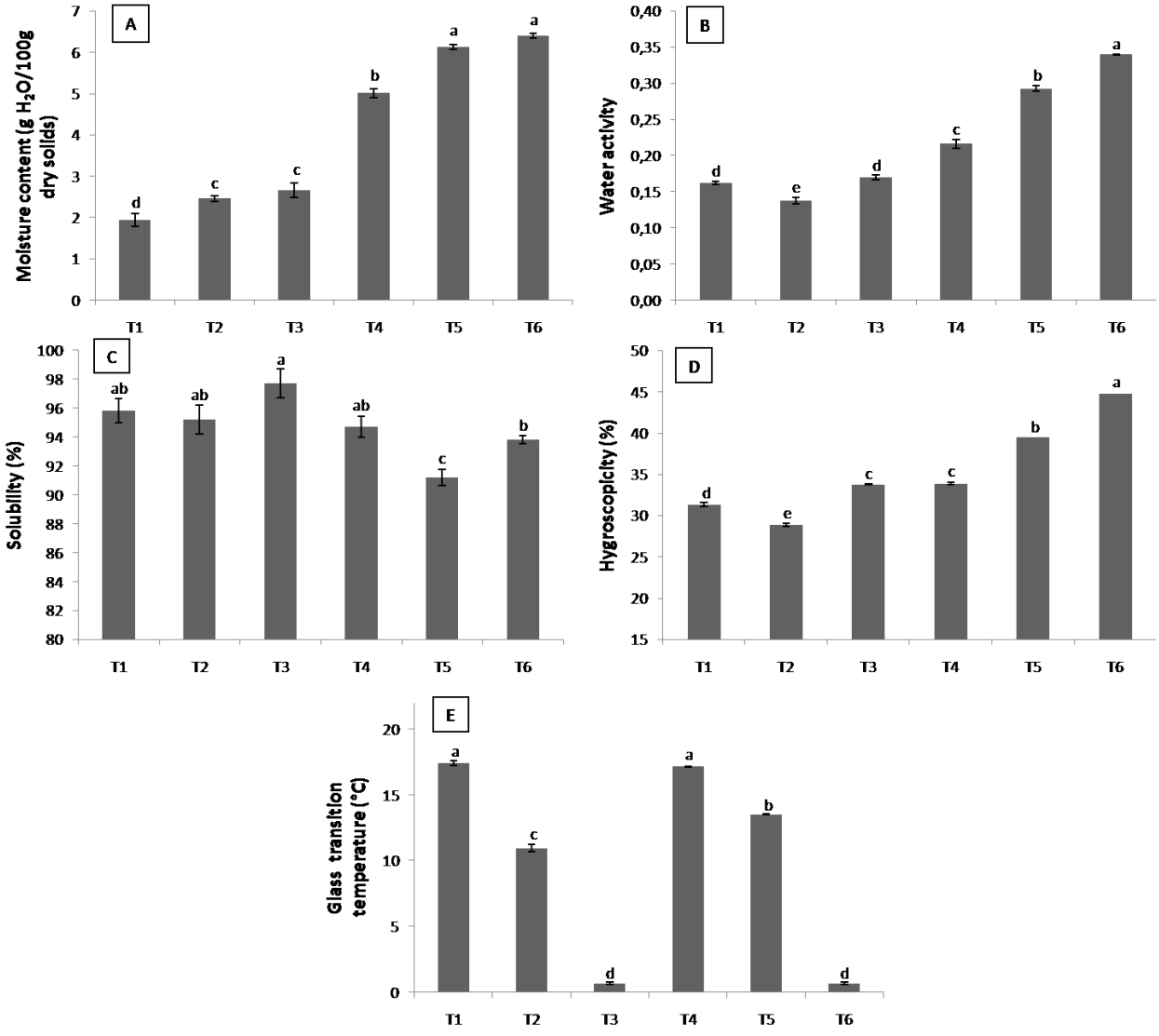

Figure 1. Moisture content (A), water activity (B), solubility (C), hygroscopicity (D) and glass transition temperature (E) of hibiscus calyces extract microencapsulated with gum Arabic (GA), partially hydrolyzed guar gum (PHGG), and polydextrose (PD) by spray drying and freeze drying. T1: spray-dried, with 10% GA; T2: spray-dried, with 10% PHGG; T3: spray-dried, with 10% PD; T4: freeze-dried, with 10% GA; T5: freeze-dried, with 10% PHGG; T6: freeze-dried, with 10% PD.

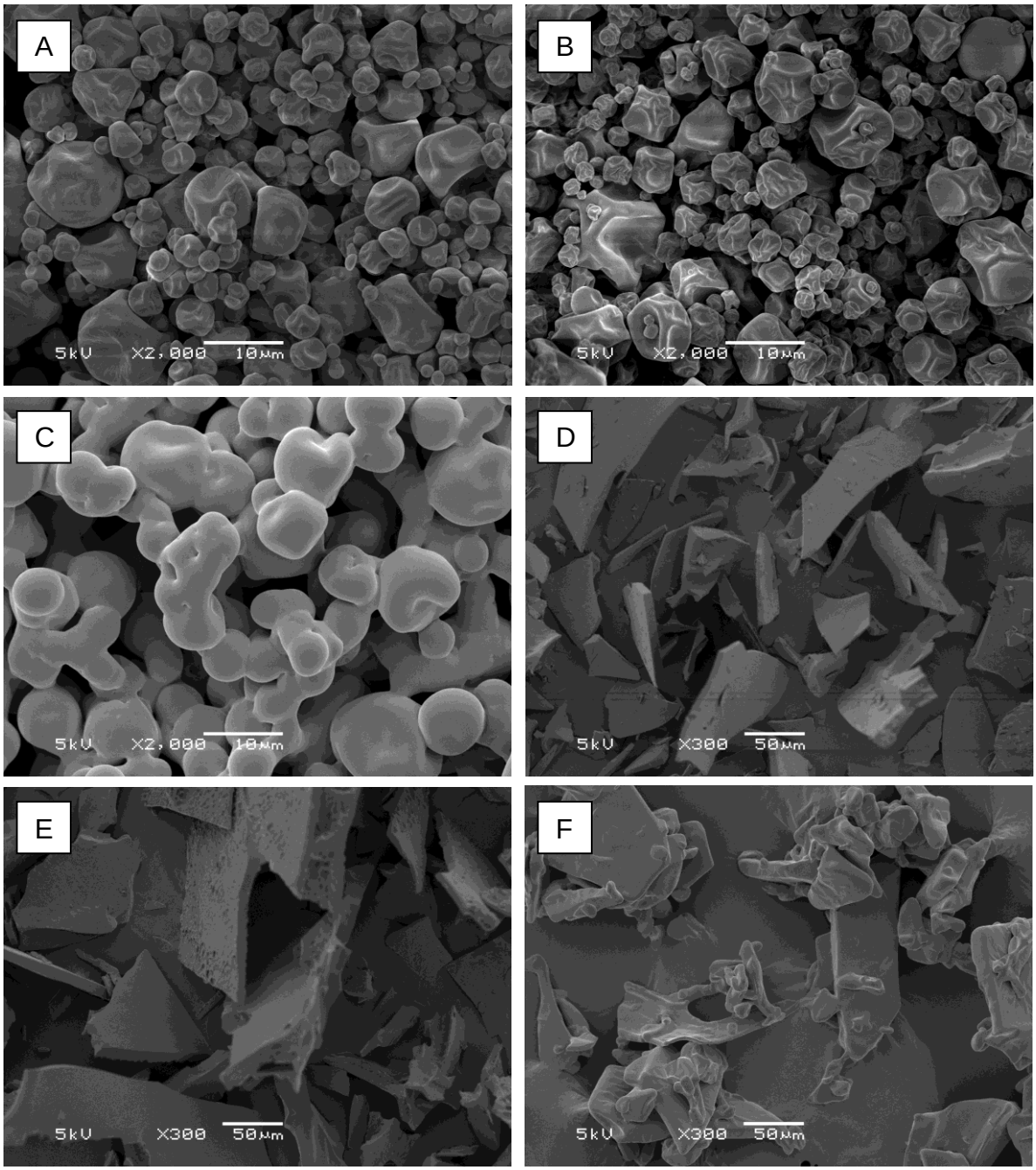

Figure 2. Scanning electron microscopy images of the microparticles developed of hibiscus calyces extract using as encapsulating agents: gum Arabic (GA), polydextrose (PD) and partially hydrolyzed guar gum (PHGG) by spray drying and freeze drying. A: T1 (spray-dried, with 10% GA); B: T2 (spray-dried, with 10% PHGG); C: T3 (spray-dried, with 10% PD); D: T4 (freeze-dried, with 10% GA); E: T5 (freeze-dried, with 10% PHGG) and F: T6 (freeze-dried, with 10% PD).

The spray-dried microparticles showed the smaller average diameters (D[4, 3]) (5.4– 7.3 µm) while the higher average diameters (D[4, 3]) (101.7 – 143.1 µm) were observed for the freeze dried microparticles. This is consequence to grinding of the freeze-dried samples after drying. In addition, the size particle of the freeze-dried microparticles were higher because low temperatures are used in the freeze-drying process and the absence of forces for breaking up the frozen sample into droplets or to alter their surface topology during the drying by evaporation (Chen, Chi, & Xu, 2012). Man, Irwandi, & Abdullah (1999) reported that the particle diameter of the spray-dried product ranges from 1 to 15 µm and the freeze-dried products can reach 300 µm.

The relative span values (**Table 3**) ranged from 1.90 to 2.00 for spray-dried samples and from 3.06 to 3.19 for freeze-dried samples. According to Noreña, Bayarri, & Costell (2015), the relative span is used to verify the uniformity and homogeneity in the distribution of the particles, and when higher than 1, this value indicates that there is a wide distribution of particle sizes.

Table 3. Average diameter and particle size distribution (Span) of the hibiscus calyces extract microencapsulated with gum arabic (GA), polydextrose (PD) and partially hydrolyzed guar gum (PHGG) by spray drying and freeze drying.

Treatments[1]	Average diameter (D[4, 3]) (µm)	Span
T1	5.43	1.99
T2	6.10	2.00
T3	7.26	1.90
T4	101.73	3.06
T5	143.08	3.19
T6	132.50	3.16

[1]T1: spray-dried, with 10% GA; T2: spray-dried, with 10% PHGG; T3: spray-dried, with 10% PD; T4: freeze-dried, with 10% GA; T5: freeze-dried, with 10% PHGG; T6: freeze-dried, with 10% PD.

When all treatments were evaluated together, the microparticles produced by freeze drying using GA as encapsulating agent showed the best functional properties. However, when physicals properties and structural characteristics were evaluated, the microparticles produced by spray-dried using GA, followed of PHGG presented better characteristics.

4. Conclusions

The microencapsulation of hibiscus calyces extract by spray drying and freeze drying, using GA, PHGG and PD as encapsulating agents were successful. The best results obtained for anthocyanins content, reducing capacity and ABTS were to powder produced by freeze drying using GA as encapsulating. However, due to importance of physical properties in powders, such as solubility, hygroscopicity and *Tg*, samples produced by spray drying using GA, followed of PHGG had the better results. The microscopy analysis also indicated that powder produced by spray drying using GA and PHGG had the better structures, showing particles of spherical shape and without agglomeration. The microparticles produced might have applications in the food industry, either as colorant or for incorporating in functional foods, however more studies will be needed for those applications.

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CAPÍTULO 5

DISCUSSÃO GERAL

Os resultados obtidos foram apresentados na forma de três artigos científicos. Inicialmente, foram identificados e quantificados os compostos bioativos presentes nos cálices do hibisco: carotenoides e compostos fenólicos, por meio de cromatografia líquida de alta eficiência com detecção por arranjo de diodos e espectrometria de massas em sequência (HPLC-DAD-MS/MS). Na sequência, foram estudadas as condições de extração aquosa em meio ácido (ácido cítrico 2%, p/v) dos compostos bioativos do hibisco através de um planejamento experimental, onde foram avaliados os efeitos da adição de enzima (complexo enzimático de pectinase, hemicelulase e celulase), temperatura, velocidade de agitação e o tempo de extração. Também foi estudado o microencapsulamento do extrato resultante do melhor tratamento obtido na etapa anterior, onde foram empregados os métodos de encapsulamento por atomização e por liofilização, utilizando a goma arábica (GA), goma guar parcialmente hidrolisada (GGPH) e a polidextrose (PD) como encapsulantes.

Existem vários métodos para determinar a capacidade antioxidante de um composto, dentre eles pode-se mencionar: a remoção de um radical peroxil (ORAC: capacidade de absorção de radicais de oxigênio, TRAP: potencial antioxidante reativo total), a capacidade de redução de metal (FRAP: capacidade antioxidante por redução do ferro, CUPRAC: capacidade antioxidante por redução do cobre), a capacidade de remoção de radical orgânico (ABTS- 2,2'-azino-bis-3-etilbenzotiazolina-6-sulfonato e DPPH - peroxidação do 2,2-difenil-1-picrilhidrazina), a capacidade de eliminação do radical hidroxil (HRSA) e pelo mecanismo baseado na transferência de elétrons (capacidade redutora). Neste estudo, a capacidade antioxidante foi avaliada, nos extratos aquosos e nas micropartículas, pelos métodos de ABTS e capacidade redutora (Artigo 2, Capítulo 3) e ABTS, capacidade redutora e HRSA (Artigo 3, Capítulo 4), respectivamente.

Para a identificação e quantificação dos carotenoides e compostos fenólicos, foram obtidos dois extratos de hibisco por meio de extração exaustiva: um extrato para carotenoides, utilizando acetona (100%) como solvente de extração, e um outro extrato com metanol/água (8:2 v/v) acidificado (1% HCl) para compostos fenólicos. Após, os extratos foram filtrados em membranas de politetrafluoretileno, para o extrato de

carotenoides, e polietersulfona, para o extrato de compostos fenólicos (0,22 µm) e injetados (20 µL) no HPLC-DAD-MS/MS. A seguir, foi realizada a identificação de cada composto, através de tempos de retenção de cada composto, características espectrais UV-vis e fragmentos gerados no espectrômetro de massas através da comparação com dados reportados na literatura, tanto para carotenoides (Chiste e Mercadante, 2012; Van Breemen et al., 2012; Mariutti et al., 2013; Rodrigues et al., 2013; Da Silva et al., 2014), como para compostos fenólicos (Rodriguez-Medina et al., 2009; Ramirez-Rodrigues et al., 2011; Rodrigues e Bragagnolo, 2013; Borras-Linares et al., 2015).

Este estudo traz como novidade a composição de carotenoides presentes nos cálices do hibisco, no qual foram encontrados vinte e um carotenoides, destes, quinze foram identificados por HPLC-DAD-MS/MS. Esses carotenoides foram quantificados em equivalentes de β-caroteno (Tabela 1 e Figura 1 do Capítulo 2). O total de carotenoides encontrado no cálice de hibisco foi de 641,38±23,61 µg/100 g massa fresca, cujos compostos majoritários foram a all-*trans*-luteína e o all-*trans*-β-caroteno com 316,43±19,92 e 147,76±5,59 µg/100 g massa fresca, representando 49 e 23%, respectivamente.

Em relação ao perfil de compostos fenólicos, estudos anteriores realizaram a caracterização dessa matriz cultivadas em outras regiões do mundo, como Senegal (Rodriguez-Medina et al., 2009), México (Ramirez-Rodrigues et al., 2011) e Espanha (Borras-Linares et al., 2015), porém em nenhum deles foi realizada a extração exaustiva total. Até então não havia estudos de caracterização de compostos fenólicos do hibisco cultivado no Brasil. Este trabalho destaca-se pelas maiores concentrações de compostos fenólicos encontradas (509,07±25,39 mg/100 g massa fresca), sendo 60% maiores que Ramirez-Rodrigues et al. (2011).

Para os compostos fenólicos, foram encontrados vinte compostos, dos quais, catorze foram identificados. A quantificação foi realizada em equivalentes de ácido 5-cafeoilquínico, quercitina e cianidina (Tabela 2 e Figura 2 do Capítulo 2). As antocianinas foram os compostos majoritários nos cálices de hibisco, sendo elas a delfinidina 3-sambubiosídeo e cianidina 3-sambubiosídeo, com 218,17±12,69 e 70,42±5,26 mg/100 g massa fresca, representando 41 e 13% do total de compostos fenólicos, respectivamente. Entre os ácidos fenólicos, os componentes majoritários foram o ácido 3-cafeoilquínico e ácido 5-cafeoilquínico, com 79,22±7,01 e 68,35±0,19 mg/100g massa fresca, representando 15 e 13% do total de compostos fenólicos,

respectivamente. As antocianinas e os ácidos fenólicos são relatados por diversos estudos, como compostos fitoquímicos com possíveis benefícios, como capacidade antioxidante, cardioprotetora, antiinflamatória, anticancerígena e atividade antimicrobiana (Ali et al., 2005; Liu et al., 2006; Mckay et al., 2010), mostrando assim a importância do consumo de alimentos preparados com hibisco.

Posteriormente, foi realizado o estudo de extração com o objetivo de definir as melhores condições de obtenção dos compostos bioativos dos cálices de hibisco, utilizando solvente aquoso acidificado com ácido cítrico (2%, p/v). Nos trabalhos de separação e purificação de extratos naturais para a utilização em alimentos, deve-se ter o cuidado na escolha do solvente de extração para que o mesmo não contamine o produto, ressaltando também a importância da utilização do solvente aquoso comparado aos demais solventes, contribuindo assim, para preservação do meio ambiente, pois não há geração de resíduos poluentes. O ácido cítrico foi empregado com o intuito de diminuir o pH do meio (~2,2), visto a maior estabilidade dos compostos fenólicos nessa condição. A natureza iônica das antocianinas permite que estas mudem de conformação de acordo com o pH do meio, resultando em diferentes cores e tonalidades (Brouillard, 1982). De acordo com Rein (2005), as antocianinas apresentam maior estabilidade em pH ácido (abaixo de 3,0), as quais podem ter quatro conformações diferentes: base quinoidal, cátion *flavilium*, pseudobase carbinol e chalcona (Brouillard et al., 2010).

O planejamento experimental utilizado foi um fatorial fracionado (2^{4-1}), com plano de resolução IV, com quatro fatores: concentração de enzima (X_1), temperatura (X_2), velocidade de agitação (X_3) e tempo de extração (X_4), resultando em oito tratamentos (Tabela 1 do Capítulo 3). As variáveis respostas foram *Chroma* (Y_1), antocianinas monoméricas totais (*TMA*) (Y_2), capacidade redutora (Y_3), ABTS (Y_4) e compostos fenólicos (Y_5).

A partir das ANOVAS, que se encontram no Apêndice A, os efeitos principais e de interação foram avaliados. As equações que descrevem o comportamento na extração quanto ao *Chroma* (Y_1), antocianinas monoméricas totais (*TMA*) (Y_2), capacidade redutora (Y_3), ABTS (Y_4) e compostos fenólicos (Y_5) em relação às variáveis respostas são apresentadas nas equações abaixo:

$$Y_1 = 34.7675 - 2.29X_2 - 1.3225X_3$$

$$Y_2 = 3.40375 + 0.07375X_1 + 0.19125X_2 + 0.13125X_3 + 0.05875X_2X_3$$

$$Y_3 = 3.74 + 0.2275X_2 + 0.1025X_3$$

$$Y_4 = 6.6975 + 0.565X_2 + 0.2575X_3 + 0.1775X_4 - 0.135X_1X_3 + 0.18X_2X_3$$

$$Y_5 = 14.07875 + 0.68625X_1 + 2.03125X_2 + 0.90625X_3$$

Onde X_1, X_2, X_3, e X_4 são as variáveis codificadas para enzima, temperatura, velocidade de agitação e tempo, respectivamente. Para melhor compreensão, os gráficos delinhas de contorno foram gerados a partir dessas equações (Figuras 1 e 2 do Capítulo 3).

Para o *Chroma* (Y_1), os efeitos principais temperatura (X_2) e velocidade de agitação (X_3) foram significativos (Figura 1a do Capítulo 3), sendo que os maiores valores de *Chroma* foram obtidos quando as duas variáveis foram utilizadas nos seus menores níveis, que corresponde a 35 ºC e 200 rpm de velocidade de agitação.

Para *TMA* (Y_2), os efeitos significativos foram concentração de enzima (X_1), temperatura (X_2) e velocidade de agitação (X_3) e a interação $X_2 \times X_3$. A partir dos resultados obtidos, verifica-se que os valores de *TMA* aumentam com a adição de enzima. A maior parte das antocianinas em matrizes vegetais se encontram acumuladas no interior das células vegetais, assim a ação dessas enzimas proporcionou uma maior degradação da parede celular liberando mais facilmente os compostos intracelulares (Cabrera et al., 2009; Dal Magro et al., 2016). No efeito de interação entre a temperatura e a velocidade de agitação, a partir da superfície de resposta (Figura 2a do Capítulo 3) foi observado que seus maiores níveis (55 ºC e 400 rpm, respectivamente) alcançaram maiores teores de *TMA*.

Para capacidade redutora (Y_3), apenas os efeitos principais da temperatura (X_2) e velocidade de agitação (X_3) foram significativas (Figura 1b do Capítulo 3), sendo maiores os valores de capacidade redutora quando aumenta a temperatura e/ou a velocidade de agitação.

Em relação ao ABTS (Y_4) foi observado significância dos efeitos principais temperatura (X_2), velocidade de agitação (X_3), tempo de extração (X_4) e dos efeitos de interação entre concentração de enzima e velocidade de agitação ($X_1 \times X_3$) e entre temperatura e velocidade de agitação ($X_2 \times X_3$). A partir dos resultados, verifica-se que os valores de ABTS aumentam com o tempo de extração. Como existem interações entre a concentração de enzima e a velocidade de agitação e desta com a temperatura, elas não podem ser discutidas isoladamente, porém em conjunto. Assim, a partir das Figuras 2b e

2c do Capítulo 3, que representam as superfícies de resposta, pode-se observar que utilizar maior velocidade de agitação (400 rpm) e maior temperatura (55 ºC), resulta na obtenção dos maiores valores de capacidade antioxidante (ABTS).

Para os compostos fenólicos (Y_5), o efeitos principais de concentração de enzima (X_1), temperatura (X_2) e velocidade de agitação (X_3) foram significativos (Figura 1c do Capítulo 3), sendo que com o aumento dos níveis de qualquer uma dessas variáveis resultaram em maiores extrações de compostos fenólicos. No caso das enzimas, o emprego de complexos enzimáticos tais como pectinase, hemicelulase e celulase promovem a hidrólise de polissacarídeos estruturais de vegetais, auxiliando na extração dos compostos fenólicos ligados a estes polissacarídeos (Romero-Cascales et al., 2012).

A correlação de Pearson (Tabela 6 do Capítulo 3) também foi usada para explicar a relação que existem entre as variáveis de respostas estudadas, sendo que a maior correlação foi 0,90 para o ABTS com os ácidos fenólicos, antocianinas e compostos fenólicos totais, ou seja, quanto maior eram os valores para ácidos fenólicos, antocianinas e compostos fenólicos totais, maior a capacidade antioxidante medida por ABTS (Prenesti et al., 2007). A capacidade redutora também exibiu alta correlação positiva com ácidos fenólicos (0,90), compostos fenólicos totais (0,87) e antocianinas (0,86). Os parâmetros *a** e *b**, também foram altamente correlacionados, porém de forma inversa (variando de -0,98 a -0,84) com os ácidos fenólicos, fenólicos totais, antocianinas monoméricas totais (*TMA*), flavonoides, ABTS e capacidade redutora.

Dos resultados obtidos, apresentados na Figura 3 e Tabela 5 do Capítulo 3, pode-se concluir que o tratamento número oito (55 °C, 50 µL de enzima/1000 g extrato, 400 rpme tempo de extração de 4 horas) alcançou a melhor condição de extração de antocianinas (*TMA*) (3,82 mg/g extrato em base seca) e compostos fenólicos totais (17.59 mg/g extrato em base seca), que resultou também em uma melhor capacidade antioxidante, tanto no ABTS (7,72 µmol Eq. Trolox/g extrato em base seca) como na capacidade redutora (3,96 mg GAE/g extrato em base seca).

O extrato obtido na condição do tratamento antes mencionado foi empregado no estudo do microencapsulamento através de atomização (140 ºC) e liofilização (-68 ºC por 24 horas). A concentração de agente encapsulante empregada foi de 10% (p/v), para goma arábica (GA), goma guar parcialmente hidrolisada (GGPH) e polidextrose (PD). Desse modo, foram estudados seis tratamentos, três atomizados e três liofilizados.

Os valores das análises colorimétricas (*L**, *a**, *b**, *Chroma* e *Hue*) são apresentados na Tabela 1 do Capítulo 4. Os menores valores de *L** foram encontrados

para os tratamentos liofilizados, que apresentaram valores de 54 a 56, enquanto que os atomizados tiveram valores entre 72 e 76. Esses valores indicam que as amostras liofilizadas foram mais escuras. Os valores para todos os pós produzidos em relação ao parâmetro *a** variaram de 30 a 48 e no parâmetro *b** de 1 a 9, ficando no primeiro quadrante do círculo cromático (+ *a**, + *b**), que vai do amarelo ao vermelho. Esses resultados indicaram que as amostras tiveram cores com tendência ao vermelho, o que é atribuído à presença de antocianinas (Jiménez-Aguilar et al., 2011). Os valores para *Chroma* foram maiores nos pós obtidos por liofilização, o que mostra que houve maior pureza ou saturação de cor. Esse melhor desempenho nas amostras liofilizadas pode ter ocorrido pela ausência de exposição à alta temperatura durante a sublimação, visto que a temperatura elevada é o fator que mais afeta a estabilidade das antocianinas (Wang e Xu, 2007), assim como a oxidação, que está associada ao oxigênio, que induz e acelera a formação de compostos escuros (coloração marrom) (Cavalcanti et al., 2011). Os valores para o ângulo *Hue* foram próximos de 0 ou 360º para todos os pós produzidos, tanto por atomização como por liofilização, o que confirma a tendência à cor vermelha nas micropartículas de pó de hibisco.

As amostras obtidas por atomização com GA e com GGPH, em geral, apresentaram as melhores características físicas como pós, quanto à solubilidade, higroscopicidade e umidade (Figura 1 do Capítulo 4) com valores de 95,8 e 95,2%, 31,3 e 28,9%, 1,9 e 2,4%, respectivamente.

Para a temperatura de transição vítrea (*Tg*), os tratamentos que utilizaram GA e GGPH nos dois métodos de encapsulamento, tiveram os maiores valores de *Tg*, variando de 10,96 a 17,43 ºC. Para os tratamentos que utilizaram a PD como material de parede, apresentaram valores inferiores (0,67 °C), tanto na atomização como na liofilização. A adição de agentes encapsulantes de elevada massa molar tendem a aumentar a *Tg* do produto final, diminuir sua higroscopicidade e assim, aumentar a sua estabilidade (Nayak e Rastogi, 2010). Pelos maiores valores de *Tg*, sugere-se que a GA e a GGPH desempenharam melhor essa função.

As micrografias, geradas na microscopia eletrônica de varredura, podem ser observadas na Figura 2 do Capítulo 4. Foi possível visualizar a morfologia das micropartículas, as quais mostraram formato mais esférico e homogêneo nas amostras atomizadas, já as liofilizadas mostraram formato semelhante ao de “vidro quebrado”, além de serem mais irregulares. E ainda, os pós que foram produzidos com a PD como

agente encapsulante, apresentou tendência à forte atração e aderência entre si, tanto no atomizado como no liofilizado.

O diâmetro médio de partícula (D[4, 3]) e *span* são mostrados na Tabela 3 do Capítulo 4. Para o diâmetro médio de partícula (D[4, 3]), observou-se que os tratamentos liofilizados tiveram partículas maiores que os atomizados, variando de 101,73 a 143,08 µm para os liofilizados, e de 5,43 a 7,26 µm para os atomizados. O maior tamanho de partícula nas amostras liofilizadas é consequência da moagem realizada após a liofilização. Quanto ao *span*, o qual avalia distribuição de tamanho de partícula, variou de 1,90 a 2,00 para as amostras atomizadas e de 3,06 a 3,19 para as amostras liofilizadas. De acordo com Noreña et al. (2015), estes valores indicam que houve uma boa uniformidade na distribuição de tamanho de partícula, pois estão acima de 1.

Com relação às antocianinas e a capacidade antioxidante avaliada pelos métodos de ABTS e capacidade redutora (Tabela 2 do Capítulo 4), o tratamento liofilizado com GA teve um melhor desempenho com 74,3% de retenção de antocianinas, 73,3% e 75,4% de retenção no ABTS e capacidade redutora, respectivamente. Já para o método de HRSA, todas as amostras atomizadas tiveram melhor desempenho, com valores variando de 90,2 a 90,6% de atividade sequestrante.

Quando se comparam todos os resultados obtidos dos tratamentos usados, pode-se concluir que apesar do pó produzido por liofilização usando GA ter apresentado o maior conteúdo de antocianinas e maior capacidade antioxidante, avaliada por ABTS e capacidade redutora, os pós obtidos por atomização com GA e seguidos daqueles produzidos com GGPH foram os que tiveram melhores características físicas e estruturais.

CONCLUSÃO

- O cálice do hibisco pode ser considerado uma matriz vegetal com ampla variedade de compostos bioativos, no qual se encontram carotenoides e compostos fenólicos;
- A all-*trans*-luteína e o all-*trans*-β-caroteno foram os carotenoides majoritários no cálice de hibisco, com 316,43 ± 19,92 e 147,76 ± 5,59 µg/100 g massa fresca, respectivamente, do total de carotenoides;
- As antocianinas delfinidina 3-sambubiosídeo e cianidina 3-sambubiosídeo, com 218,17 ± 12,69 e 70.42 ± 5.26 mg/100 g massa fresca, respectivamente, e os ácidos fenólicos ácido 3-cafeoilquínico e ácido 5-cafeoilquínico, com 79,22 ± 7,01 e 68,35 ± 0,19 g massa fresca, respectivamente, foram os compostos fenólicos majoritários no cálice de hibisco;
- A melhor condição de extração aquosa acidificada foi obtida a 55 °C, com velocidade de agitação de 400 rpm, com 50 µL de enzima/1000 g extrato e 4 horas de extração;
- As micropartículas do extrato de hibisco produzidas por atomização e GA como encapsulante, seguido pelas produzidas com GGPH como encapsulante, mostraram melhores propriedades físicas e morfológicas como pós, com valores de 95,8 e 95,2%, 31,3 e 28,9%, 17,4 e 10,9 ºC, 0,16 e 0,14, 1,94 e 2,45%, para solubilidade, higroscopicidade, *Tg*, A_w e umidade, respectivamente, além de terem formato esférico e não apresentarem tendência à atração e aderência entre si;
- As micropartículas produzidas por liofilização apresentaram maior retenção de antocianinas e capacidade antioxidante.

PERSPECTIVAS

Realizar estudos de purificação no extrato aquoso para selecionar apenas os compostos de interesse.

Realizar maiores estudos *in vitro,* como por exemplo, pelo método ORAC, sobre a capacidade antioxidante da matriz hibisco (cálice) e das micropartículas obtidas por atomização empregando a goma arábica (GA) e a goma guar parcialmente hidrolisada (GGPH) como encapsulante.

Testar diferentes temperaturas de atomização, vazão de alimentação e concentrações dos agentes encapsulantes.

As micropartículas do extrato de hibisco são promissoras, podendo ser empregadas na indústria alimentícia como antioxidante ou corante, por essa razão, sugere-se estudar a aplicação em alimentos, como iogurtes, bebidas lácteas, gelatinas, e a estabilidade das micropartículas.

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APÊNDICE A

Nas tabelas a seguir as variáveis codificadas representam: concentração de enzima (X_1), temperatura (X_2), velocidade de agitação (X_3) e tempo de extração (X_4). Nas ANOVAS somente são apresentados os efeitos significativos para os experimentos de extração de compostos bioativos usando água acidificada como solvente.

Tabela A1. Análise de variância para Cor (*Chroma*).

Fonte de variação	GL	SQ	QM	F	Pr
X_2	1	41,9528	41,9528	54,95	0,0051
X_3	1	13,9920	13,9920	18,33	0,0234
Modelo	4	59,3393	14,8348	19,43	0,0175
Erro experimental	3	2,2904	0,7635		
Total	7	61,6297			

Tabela A2. Análise de variância para Antocianinas Monoméricas Totais(*TMA*).

Fonte de variação	GL	SQ	QM	F	Pr
X_1	1	0,0435	0,0435	20,28	0,0205
X_2	1	0,2926	0,2926	136,36	0,0013
X_3	1	0,1378	0,1378	64,22	0,0041
$X_2 X_3$	1	0,0276	0,0276	12,87	0,0371
Modelo	4	0,5015	0,1254	58,43	0,0035
Erro experimental	3	0,0064	0,0021		
Total	7	0,5080			

Tabela A3. Análise de variância paracapacidade redutora.

Fonte de variação	GL	SQ	QM	F	Pr
X_2	1	0,4140	0,4140	110,05	0,0005
X_3	1	0,0840	0,0840	22,34	0,0091
Modelo	3	0,5245	0,1748	46,47	0,0014
Erro experimental	4	0,0150	0,0038		
Total	7	0,5396			

Tabela A4. Análise de variância para ABTS.

Fonte de variação	GL	SQ	QM	F	Pr
X_2	1	2,5538	2,5538	51076,0	0,0028
X_3	1	0,5304	0,5304	10609,0	0,0062
X_4	1	0,2520	0,2520	5041,00	0,0090
X_1X_3	1	0,1458	0,1458	2916,00	0,0118
X_2X_3	1	0,2592	0,2592	5184,00	0,0088
Modelo	6	3,7463	0,6244	12487,7	0.0068
Erro experimental	1	0,0000	0,0000		
Total	7	3,7463			

Tabela A5. Análise de variância para compostos fenólicos.

Fonte de variação	GL	SQ	QM	F	Pr
X_1	1	3,7675	3,7675	18,52	0,0231
X_2	1	33,0078	33,0078	162,22	0,0010
X_3	1	6,5703	6,5703	32,29	0,0108
Modelo	4	43,8506	10,9627	53,88	0,0040
Erro experimental	3	0,6104	0,2035		
Total	7	44,4611			