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FACULDADE DE ENGENHARIA DE ALIMENTOS

RENAN CAMPOS CHISTÉ

AVALIAÇÃO DA EXTRAÇÃO DE COMPOSTOS BIOATIVOS COM PROPRIEDADES ANTIOXIDANTES E CORANTES PRESENTES EM URUCUM E PIQUIÁ

TESE DE DOUTORADO APRESENTADA À FACULDADE DE ENGENHARIA DE ALIMENTOS - UNICAMP - PARA OBTENÇÃO DO TÍTULO DE DOUTOR EM CIÊNCIA DE ALIMENTOS.

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Effect of solvent type on the extractability of bioactive compounds, antioxidant capacity and colour properties of natural annatto extracts

RESUMO GERAL

Os compostos bioativos presentes no reino vegetal possuem importantes funções e ações biológicas, podendo ser considerados promotores da saúde humana. Já é reconhecida a associação entre a ingestão de frutas e vegetais e a diminuição do risco de desenvolvimento de diversas desordens crônico-degenerativas, tais como câncer, inflamações, doenças cardiovasculares, catarata, degeneração macular e outras, sendo os carotenóides e compostos fenólicos alguns dos grupos de compostos bioativos aos quais são atribuídas tais ações.

As sementes e os extratos de urucum (*Bixa orellana L.*) são utilizados como corantes nas indústrias alimentícias, farmacêutica e de cosméticos, devido à presença majoritária do carotenóide bixina. Até então, não havia sido relatada na literatura a composição de compostos fenólicos das sementes de urucum. Dessa forma, foi desenvolvido e validado um método por cromatografia líquida de alta eficiência acoplada aos detectores de arranjo de diodos e espectrômetro de massas (HPLC-DAD-MS/MS) para separar, identificar e quantificar bixina e os compostos fenólicos em semente de urucum. Adicionalmente, foi otimizado um procedimento de extração simultânea desses compostos através da metodologia de superfície de resposta. Além de bixina, conhecida por ser o principal carotenóide em sementes de urucum, a hipolaetina e um derivado de ácido caféico foram identificados, pela primeira vez, como os principais compostos fenólicos. O procedimento otimizado envolveu 15 extrações com acetona:metanol:água (50:40:10, v/v/v) como solvente, razão sólido-líquido de 1:9 (m/v) e 5 min para cada extração em ultrassom. O método cromatográfico proposto foi validado com sucesso para a análise simultânea de compostos fenólicos e bixina em sementes de urucum.

Extratos líquidos de urucum com elevada capacidade antioxidante e potencial de cor foram obtidos a partir da extração de bixina e de compostos fenólicos de sementes de urucum utilizando solventes com diferentes polaridades (água, etanol:água, etanol, etanol:acetato de etila e acetato de etila). Os valores mais elevados de compostos fenólicos totais foram encontrados nos extratos obtidos com água, etanol:água e etanol (0,5 mg equivalentes de ácido gálico/mL), e o valor mais elevado de bixina foi encontrado no extrato obtido com etanol:acetato de etila (5,2 mg/mL), que foi caracterizado como o mais vermelho e o mais vívido (a^* = 40,5, h°=46,1, C^* = 58,4). O extrato obtido com etanol:acetato de etila também apresentou a maior atividade anti-radical livre (4,7 μmol equivalente Trolox/mL) e a maior porcentagem de proteção ao triptofano contra o oxigênio singlete (63,6 %). Por outro lado, acetato de etila e a mistura etanol:água foram os

solventes menos eficazes para a extração de compostos fenólicos e bixina, respectivamente. De acordo com a análise estatística multivariada, etanol:acetato de etila e acetato de etila foram os solventes mais promissores para obtenção de extratos de urucum com ambas as propriedades antioxidantes e de cor.

A partir da extração com diferentes solventes (água, etanol:água, etanol, etanol:acetato de etila e acetato de etila), foram também obtidos extratos liofilizados de urucum, e a capacidade antioxidante na desativação de diferentes espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) foi avaliada. Além disso, os teores de compostos fenólicos e de bixina dos extratos de urucum foram determinados por HPLC-DAD. Todos os extratos de urucum foram capazes de desativar todas as espécies reativas testadas (peróxido de hidrogênio, ácido hipocloroso, oxigênio singlete, radical óxido nítrico, ânion peroxinitrito e radical peroxila), em baixas concentrações na faixa de μg/mL, com exceção do radical superóxido. O extratos de sementes de urucum obtidos com etanol:acetato de etila e acetato de etila, que apresentaram os maiores níveis de hipolaetina e bixina, respectivamente, foram os extratos com a maior capacidade antioxidante. Adicionalmente, o padrão de bixina apresentou os menores valores de IC_{50} na desativação de todas as ROS e RNS testadas.

O piquiá (*Caryocar villosum* (Aubl.) Pers), fruta nativa da região Amazônica, pode ser considerado uma fonte inexplorada de compostos bioativos, uma vez que poucos estudos sobre seus constituintes químicos e fitoquímicos estão disponíveis. Dessa forma, a composição química e fitoquímica da polpa de piquiá foi determinada, incluindo a composição de carotenóides e de compostos fenólicos por HPLC-DAD-MS/MS. De acordo com a composição nutricional, água (52 %) e lipídios (25 %) foram os principais componentes encontrados na polpa e o valor energético total foi 291 kcal/100 g. Sobre os compostos bioativos, a polpa apresentou (base seca) maior teor de compostos fenólicos (236 mg equivalentes de ácido gálico/100 g), flavonóides totais (67 mg equivalentes de catequina/100 g) e taninos totais (60 mg equivalentes de ácido tânico/100 g) em relação ao teor de carotenóides totais (7 mg/100 g) e α-tocoferol (1 mg/100 g). Os principais compostos fenólicos identificados por HPLC-DAD-MS/MS, foram ácido gálico (182 μg/g polpa), seguido por ácido elágico ramnosídeo (107 μg/g polpa) e ácido elágico (104 μg/g polpa). Os principais carotenóides identificados foram all-*trans*-anteraxantina (3 μg/g), all-*trans*-zeaxantina (3 μg/g), all-*trans*-neoxantina (2 μg/g), all-*trans*-violaxantina (1 μg/g) e all-*trans*-β-caroteno (0,7 μg/g). A capacidade anti-radical livre da polpa (3,7 mmol equivalente Trolox/100 g) indica que a polpa pode ser considerada um eficiente sequestrador do radical peroxila.

Foram obtidos também extratos liofilizados de polpa de piquiá a partir da extração com diferentes solventes (água, etanol:água, etanol, etanol:acetato de etila e acetato de etila). Todos os extratos naturais foram caracterizados em relação ao teor de compostos bioativos (compostos fenólicos totais, flavonóides, taninos, carotenóides e tocoferóis). Além disso, a capacidade de desativação do radical peroxila, assim como a porcentagem de proteção contra o oxigênio singlete foi determinada para todos os extratos. Os extratos obtidos com água e a mistura etanol:água apresentaram os maiores teores (base seca) de compostos fenólicos totais (9,2 e 6,3 mg de equivalentes de ácido gálico/g, respectivamente), flavonóides totais (3,8 e 2,5 mg equivalente de catequina/g, respectivamente) e taninos totais (7,6 e 2,4 mg de ácido tânico/g, respectivamente). O extrato obtido com etanol:água também apresentou a maior capacidade de desativação do radical peroxila (ORAC) (0,3 mmol equivalente Trolox/g extrato). Por outro lado, o extrato obtido com etanol, que foi classificado como o de cor mais vívida e amarelo (C^*_{ab} = 13,7 e b^* = 13,3), apresentou o maior teor de carotenóides totais (0,1 mg/g) e maior percentual de proteção contra o oxigênio singlete (10,6 %). Com base nos resultados deste estudo, etanol:água, água e etanol são os solventes mais promissores para obtenção de extratos de piquiá com alto teor de compostos bioativos, proteção contra o oxigênio singlete e capacidade sequestradora do radical peroxila.

Portanto, tais informações são importantes para as indústrias alimentícia, cosmética e farmacêutica, sabendo que o piquiá e o urucum são fontes naturais acessíveis de compostos bioativos para serem usados como potencial matéria-prima para obtenção de extratos contra os danos oxidativos em alimentos ou sistemas biológicos.

SUMMARY

The bioactive compounds found in the plant kingdom have important biological functions and actions and may be considered human health promoters. The association between the intake of fruits and vegetables and the decreased risk of developing several chronic-degenerative disorders is already recognized, being the carotenoids and phenolic compounds, groups of the bioactive compounds responsible for such actions.

The annatto (*Bixa orellana L.*) seeds and extracts are used as colourant in the food, pharmaceutical and cosmetic industries, due to the presence of bixin. As far as we are concerned, no information about the composition of phenolic compounds present in the annatto seeds and extracts was reported. Thus, a method by high performance liquid chromatography coupled to diode array detector and mass spectrometer (HPLC-DAD-MS/MS) was developed and validated for separation, identification and quantification of phenolic compounds and bixin in annatto seeds. Furthermore, using response surface methodology, an optimized procedure for simultaneous extraction of both compound classes was established. In addition to bixin, known to be the main carotenoid in annatto seeds, hypolaetin and a caffeoyl acid derivative were identified as the main phenolic compounds. The optimized procedure involved 15 extractions using acetone:methanol:water (50:40:10, v/v/v) as solvent, a solid–liquid ratio of 1:9 (w/v) and an extraction time of 5 min per extraction in a sonicator. Validation data indicated that the developed HPLC method is suitable for the simultaneous analysis of phenolic compounds and bixin in annatto seeds.

Liquid annatto extracts with high antioxidant capacity and colour potential were obtained from annatto seeds by extraction of phenolic compounds and bixin using solvents with different polarities (water, ethanol:water, ethanol, ethanol:ethyl acetate and ethyl acetate). The highest levels of total phenolic compounds were found in the water, ethanol:water and ethanol extracts (0.5 mg gallic acid equivalent/mL), and the highest level of bixin was found in the ethanol:ethyl acetate extract (5.2 mg/mL), which was characterised as the reddest and the most vivid one (a^* = 40.5, h° = 46.1, C^* = 58.4). The ethanol:ethyl acetate extract also showed the highest antioxidant activity (4.7 μmol Trolox equivalent/mL) and the highest percentage of tryptophan protection against singlet oxygen (63.6 %). On the other hand, ethyl acetate and ethanol:water were the least effective solvents for the extraction of phenolic compounds and bixin, respectively. According to the multivariate statistical analysis, ethanol:ethyl acetate and ethyl acetate were the most promising solvents to obtain annatto extracts with both antioxidant and colour properties.

From the extraction with different solvents (water, ethanol:water, ethanol, ethanol:ethyl acetate and ethyl acetate), freeze-dried annatto extracts were also obtained, and the capacities to scavenge reactive oxygen (ROS) and reactive nitrogen (RNS) species were evaluated. In addition, the levels of phenolic compounds and bixin in the annatto extracts were determined by HPLC-DAD. All annatto extracts were able to scavenge all the tested reactive species (hydrogen peroxide, hypochlorous acid, singlet oxygen, nitric oxide radical, peroxynitrite anion and peroxyl radical) at low concentration in the μg/mL range, with the exception of superoxide radical. The ethanol:ethyl acetate and ethyl acetate extracts of annatto seeds, which presented the highest levels of hypolaetin and bixin, respectively, were the extracts with the highest antioxidant capacity, and the bixin standard presented the lowest IC_{50} values.

Piquia (*Caryocar villosum* (Aubl.) Pers), a native fruit from Amazon region, can be considered an unexploited source of bioactive compounds, since few studies about chemical and phytochemical constituents are available. Thus, the chemical and phytochemical composition of piquia pulp was determinaed, including the composition of carotenoids and phenolic compounds by HPLC-DAD-MS/MS. According to the nutritional composition, water (52 %) and lipids (25 %) were the major components found in the pulp and the total energetic value was 291 Kcal/100 g. Regarding the bioactive compounds, the pulp presented (dry basis) higher content of phenolic compounds (236 mg gallic acid equivalent/100 g), total flavonoids (67 mg catechin equivalent/100 g) and total tannins (60 mg tannic acid equivalent/100 g) than total carotenoids (7 mg/100 g) and α-tochopherol (1 mg/100 g). The major phenolic compounds identified by HPLC-DAD-MS/MS were gallic acid (182 μg/g pulp), followed by ellagic acid rhamnoside (107 μg/g pulp) and ellagic acid (104 μg/g pulp). The main carotenoids identified were all-*trans*-antheraxanthin (3 μg/g), all-*trans*-zeaxanthin (3 μg/g), all-*trans*-neoxanthin (2 μg/g), all-*trans*-violaxanthin (1 μg/g) and all-*trans*-β-carotene (0.7 μg/g). The antioxidant capacity of the pulp (3.7 mmol Trolox/100 g) indicates that the pulp can be considered a good peroxyl radical scavenger.

Freeze-dried extracts of piquia using five solvents with different polarities (water, ethanol:water, ethanol, ethanol:ethyl acetate and ethyl acetate) were also obtained. All natural extracts were characterised in relation to the contents of bioactive compounds (total phenolic compounds, flavonoids, tannins, carotenoids and tocopherols). In addition, the scavenging capacity of all piquia extracts against peroxyl radical, as well as the quenching capacity against singlet oxygen were determined. All the data were used for classification of the piquia extracts applying multivariate statistical analysis. The water and ethanol:water extracts presented the highest levels of total phenolic compounds (9.2 and

6.3 mg gallic acid equivalent/g, respectively), total flavonoids (3.8 and 2.5 mg catechin equivalent/g, respectively) and total tannins (7.6 and 2.4 mg tannic acid/g, respectively). The ethanol:water extract also showed the highest scavenging capacity against peroxyl radical (ORAC) (0.3 mmol Trolox equivalent/g extract). On the other hand, the ethanol extract, which was classified as the most vivid and yellow one (C^*_{ab} = 13.7 and b^* = 13.3), presented the highest level of total carotenoids (0.1 mg/g) and highest percentage of protection against singlet oxygen (10.6 %). Based on the results of this study, ethanol:water mixture, water and ethanol are the most promising solvents to obtain piquia extracts with high contents of bioactive compounds, protection against singlet oxygen and peroxyl radical scavenging capacity.

Therefore, such informations are important for the food, cosmetic and pharmaceutical industries, since piquia and annatto are natural sources of bioactive compounds available for use as a potential raw material to obtain extracts against oxidative damage in foods or biological systems.

INTRODUÇÃO GERAL

Os compostos bioativos, como os carotenóides e compostos fenólicos, possuem importantes funções e ações biológicas, podendo ser considerados promotores da saúde humana (Krinsky, 1994; Van den Berg et al., 2000; Kong et al., 2003). Já é reconhecida a associação entre a ingestão de frutas e vegetais e a diminuição do risco de desenvolvimento de diversas desordens crônico-degenerativas, tais como câncer, inflamações, doenças cardiovasculares, catarata, degeneração macular e outras, sendo os carotenóides e compostos fenólicos alguns dos grupos de compostos bioativos aos quais são atribuídas tais ações (Serdula et al., 1996; Lampe, 1999; Stanner et al., 2004; Huang et al., 2005). Um dos mecanismos responsável pela redução do risco dessas doenças envolve a redução ou inibição de reações de oxidação, por meio da desativação de espécies reativas de oxigênio (ROS) e de nitrogênio (RNS).

Os carotenóides, presentes naturalmente nos alimentos, ou adicionados propositalmente, proporcionam cor aos alimentos, contribuindo no aspecto visual, atributo de fundamental importância na aceitação e escolha de um alimento por seus consumidores (Clydesdale, 1993). Paralelamente, os alimentos podem se beneficiar com a adição destes compostos naturais devido, por exemplo, ao seu potencial antioxidante, prevenindo a oxidação lipídica de alimentos e matérias-primas alimentares, como óleos, carnes, leite e seus derivados, estendendo a sua vida-de-prateleira (Decker et al., 2005).

Os compostos fenólicos, incluindo flavonóides, são conhecidos por inibir a peroxidação lipídica e as lipoxigenases *in vitro*, através da capacidade em seqüestrar radicais livres, tais como o hidroxílico, superóxido e os peroxílicos, os quais reconhecidamente promovem o estresse oxidativo celular (Leong & Shui, 2002; Okonogi, et al., 2007; Tachakittirungrod et al., 2007; Leontowicz et al., 2007). Numerosos estudos mostram que tais compostos, presentes nos alimentos, estão associados com ações anti-carcinogênicas (inibição do câncer de cólon, esôfago, pulmão, fígado, mama e pele), anti-inflamatórias, anti-hepatotóxica, antiviral, antialérgica e antitrombótica (Mazza & Girard, 1998), além de prevenir a oxidação de lipoproteínas de baixa densidade (LDL), diminuindo os riscos da aterosclerose, atuando na prevenção de doenças cardiovasculares e na absorção de produtos tóxicos oriundos da peroxidação lipídica (Chiang et al., 2004; Dubick & Omaye, 2001; Gorelik et al., 2008).

O Brasil é o maior produtor e exportador de sementes e extratos de urucum (*Bixa orellana*), que são utilizados como corantes nas indústrias alimentícias, farmacêutica e de cosméticos (Tocchini & Mercadante, 2001; De Rosso & Mercadante, 2008). Além do

poder corante, a bixina, principal carotenóide responsável pela cor laranja-avermelhada das sementes e extratos de urucum, apresenta atividade antioxidante, como sequestradora de oxigênio singlete e desativadora do estado triplete de sensibilizadores (Di Mascio et al., 1989; Montenegro et al., 2004; Rios et al., 2007). Cardarelli et al. (2008) obtiveram extratos de urucum a partir de solventes com diferentes polaridades, e classificaram tais extratos de acordo com sua propriedade anti-radical livre e poder corante. Entretanto, apesar da reconhecida capacidade antioxidante dos compostos fenólicos, a composição destes compostos em urucum, incluindo os flavonóides, ainda não foi reportada na literatura.

O piquiá (*Caryocar villosum*) é nativo da bacia Amazônica, e os seus frutos apresentam casca acinzentada e polpa alaranjada. Estudos realizados em nosso laboratório mostraram que dentre as 18 frutas tropicais investigadas, o piquiá apresentou os maiores valores de atividade anti-radical livre (TEAC de 25300 mM/100 g), fenóis totais (4623 mg equivalente de ácido gálico/100 g) e flavonóides totais (741 mg equivalente de catequina/100 g), além de apresentar 0,4 mg/100 g de carotenóides totais (Barreto et al., 2009). Entretanto, a composição de compostos fenólicos não foi investigada no estudo anterior e ainda não foi relatado na literatura. A composição de carotenóides presentes na polpa de piquiá foi descrita por Godoy (1993), no entanto, não foi confirmada por técnicas mais avançadas, a exemplo da espectrometria de massas.

Ao contrário de corantes ou outros compostos bioativos obtidos por síntese química que são praticamente puros, extratos naturais podem apresentar, dependendo do tipo de extração e solvente, uma mistura de compostos com características lipofílicas e hidrofílicas, tais como os compostos fenólicos (flavonóides, ácidos fenólicos) e carotenóides. Portanto, o presente trabalho foi conduzido com foco na determinação da composição de compostos fenólicos e carotenóides presentes em urucum e piquiá, assim como para a obtenção de extratos com elevado teor de compostos bioativos a partir da extração com solventes de diferentes polaridades. Adicionalmente, as propriedades antioxidantes e de cor foram avaliadas nos extratos de urucum e piquiá, fontes naturais acessíveis de compostos bioativos, para serem usados como matéria-prima em potencial contra os danos oxidativos em alimentos ou sistemas biológicos.

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CAPÍTULO I

REVISÃO DE LITERATURA

1. Urucum

O urucueiro ou urucuzeiro (*Bixa orellana L.*) (Figura 1) pertence à família BIXACEAE. A planta é originária da América Tropical, possivelmente da flora amazônica, também chamada de *annatto* (inglês), *onoto* (espanhol), *rocou* (francês) e *orleansstrauch* (alemão) (Anselmo et al., 2008). De acordo com as cultivares existentes, o urucueiro apresenta uma grande heterogeneidade em suas características botânicas, tais como tamanho e forma da planta, forma e cor de suas folhas, flores e frutos (Castro et al., 1994).

Figura 1 – (a) árvore de *Bixa orellana*, (b) sementes de urucum dentro das cachopas maduras, (c) sementes de urucum.

O Brasil é o maior produtor e exportador de sementes e extratos de urucum, que são utilizados como corantes nas indústrias alimentícias, farmacêutica e de cosméticos (Tocchini & Mercadante, 2001; De Rosso & Mercadante, 2008). De acordo com o IBGE – Instituto Brasileiro de Geografia e Estatística (2009), a quantidade de semente de urucum produzida no Brasil em 2008 foi de 12.472 toneladas (t), sendo a maior parte oriunda da região norte (5.310 t), seguida do sudeste (3.507 t), nordeste (2.187 t), sul (1.121 t) e centro-oeste (221 t). Dentre os estados brasileiros, Rondônia é o maior produtor de sementes de urucum (23 %), seguido do Pará (18 %) e São Paulo (14 %).

No Brasil, além do amplo emprego na indústria, a preparação comercial contendo 0,20-0,25 % de bixina, conhecida como colorífico, é componente indissociável de inúmeros pratos da culinária brasileira. Este condimento é produzido a partir das sementes de urucum, previamente aquecidas a 70 °C em óleo vegetal, seguido de abrasão com fubá ou farinha de mandioca ou pela mistura destas com urucum em pó, obtido por extração com solventes (Tocchini & Mercadante, 2001; Costa & Chaves, 2005).

Atualmente, as perspectivas comerciais para os corantes de urucum são promissoras tanto no mercado interno como no externo, especialmente nos países em

que a indústria de laticínios é bem desenvolvida, uma vez que a bixina é amplamente utilizada para coloração de seus produtos. A bixina é muito usada também como matéria-prima de corantes nas indústrias de panificação, bebidas, condimentos, massas, produtos extrusados, cereais matinais, gelados comestíveis, sobremesas, molhos, condimentos e também na indústria de cosméticos (Castro et al., 1994; Tocchini & Mercadante, 2001; Cardarelli et al., 2008). Ademais, o colorífico, assim como os extratos de urucum, foram relatados na literatura por apresentarem atividade anti-radical livre (Cardarelli et al., 2008), além de atuarem no retardo da oxidação lipídica em carnes de frango grelhadas e armazenadas a -18 °C por 30 dias (Castro et al., 2011) e almôndegas de peixe congeladas (Sancho et al., 2011).

1.1. Carotenóides de urucum

A bixina e a norbixina (Figura 2) são os pigmentos mais importantes encontrados nas sementes de urucum. Pertencem à classe dos carotenóides, sendo que, a norbixina é encontrada em pequenas quantidades na semente, e a bixina constitui o principal pigmento presente perfazendo aproximadamente 80 % do total de carotenóides (Preston & Rickard, 1980).

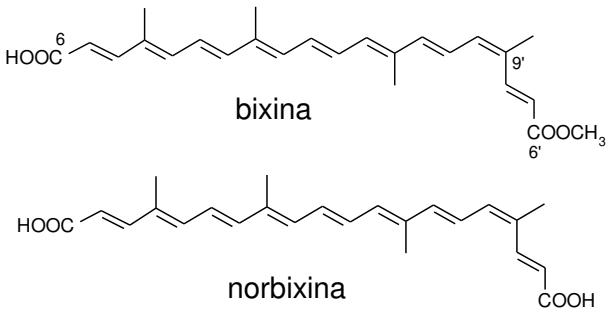

Figura 2 – Estrutura química da bixina ($C_{25}H_{30}O_4$) e da norbixina ($C_{24}H_{28}O_4$).

A bixina (metil (9-*cis*)-hidrogen-6,6'-diapo-Ψ,Ψ-carotenodioato) é também o carotenóide majoritário de colorífico e das preparações lipossolúveis (Mercadante & Pfander, 2001). Foi o primeiro carotenóide natural identificado a possuir configuração *cis* (ou *Z*) como a forma mais estável, diferente do β-caroteno que é encontrado majoritariamente na forma all-*trans* (ou all-*E*). Sendo um diapocarotenóide cuja estrutura é formada por uma cadeia isoprênica de 24 carbonos, contendo um ácido carboxílico e um éster metílico, a bixina é muito diferente dos carotenóides usualmente presentes em

alimentos (cadeia com 40 carbonos), e segundo Cardarelli et al. (2008) apresenta maior afinidade por solventes de média polaridade.

A norbixina (6,6'-diapo-Ψ,Ψ-ácido carotenodióico), produto da saponificação da bixina, é o pigmento principal das preparações hidrossolúveis, sendo encontrada em pequena quantidade nas sementes e nas preparações lipossolúveis (Tocchini & Mercadante, 2001). Sendo também um diapocarotenóide com configuração *cis*, apresenta alta solubilidade em água quando o ácido carboxílico dos dois grupos terminais está na forma de sal de norbixina.

Outros carotenóides presentes em pequenas quantidades foram isolados e identificados de sementes de urucum. O carotenóide 9'*cis*-apo-6'-licopenoato ($C_{33}H_{44}O_2$) foi isolado e identificado por Mercadante et al. (1996), que confirmaram também a presença de fitoeno, fitoflueno, ζ-caroteno e neurosporeno. Outros cinco novos apocarotenóides foram isolados e identificados por Mercadante, Steck & Pfander (1997a): metil 8'apo-β-carotenoato ($C_{31}H_{42}O_2$), metil 7-*cis*,9-*cis*,9'cis-apo-6'licopenoato ($C_{33}H_{44}O_2$), metil 9-*cis*-apo-8'-licopenoato ($C_{31}H_{42}O_2$), metil apo-8'-licopenoato ($C_{31}H_{42}O_2$) e metil apo-6'-licopenoato ($C_{33}H_{44}O_2$). Na continuação do trabalho, o dimetil 9-*cis*-9'-*cis*-6, 6'-diapocaroteno-6-6'-dioato ($C_{26}H_{32}O_4$) e metil 9-*cis*-10'-oxo-6,8'-diapocaroteno-6-dioato ($C_{20}H_{24}O_3$) foram relatados por Mercadante, Steck & Pfander (1997b). Posteriormente, Mercadante, Steck & Pfander (1999) isolaram e identificaram três novos diapocarotenóides esterificados com geranilgeraniol: 6-geranilgeranil-8'-metil-6,8'-diapocaroteno-6,8'dioato ($C_{43}H_{60}O_4$), 6-geranilgeranil-6'-metil-9-*cis*-6,6'-diapocaroteno-6,6'-dioato ($C_{42}H_{62}O_4$) e 6-geranilgeranil-6'-metil-6,6'-diapocaroteno-6,6'-dioato ($C_{45}H_{62}O_4$).

1.2. Compostos fenólicos de urucum

No que diz respeito aos compostos fenólicos, Harborne (1975) relatou o isolamento dos flavonóides 7 glicosil-apigenina, 7 bisulfato-apigenina, 7 glicosil-luteolina, 7 bisulfato-luteolina como majoritários, e a presença da hipolaetina (8 hidróxi-luteolina) como flavonóide minoritário em folhas de urucueiro (Figura 3). No entanto, ainda não foram relatados na literatura informações de identificação e quantificação do teor de compostos fenólicos presentes na semente e/ou extratos de urucum.

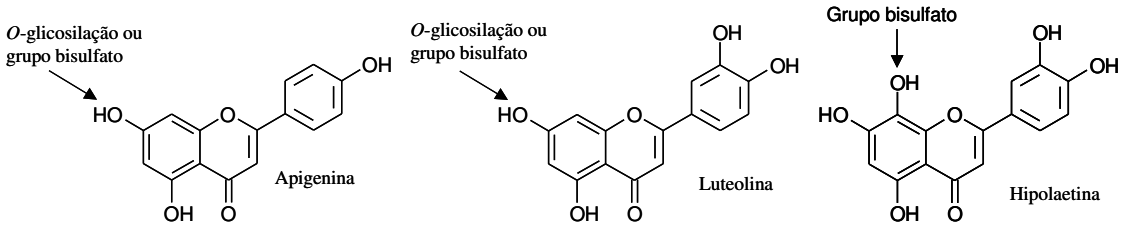

Figura 3 – Estruturas da apigenina ($C_{15}H_{10}O_5$), luteolina ($C_{15}H_{10}O_6$) e hipolaetina ($C_{15}H_{10}O_7$). As posições de *O*-glicosilações e grupos bisulfatos estão indicadas pela seta.

Cardarelli et al. (2008) determinaram o teor de compostos fenólicos totais, por espectrofotometria utilizando o reagente de Folin Ciocalteau, em extratos de urucum de diferentes polaridades encontrando valores de 1,84 (metanol/água), 1,57 (etanol/água), 1,63 (metanol), 1,10 (etanol), 0,95 (acetato de etila) e 0,30 (hexano) mg equivalente de ácido gálico/g de semente.

2. Piquiá

O piquiá (*Caryocar villosum* (Aubl.) Pers) é uma espécie arbórea de grande porte com altura acima de 40–50 m que cresce nas florestas virgens da bacia Amazônica, principalmente na região próxima à foz Amazônica. Pertence à família Caryocaraceae e é também conhecido por pequiá, piquiá verdadeiro, amêndoa-de-espinho (Brasil), *arbre à beurre* (Guiana Francesa), *suari*, *soari* (Suriname), *bats sauari* (Guiana) e *piqui-a*, *pekea* (Malásia) (Clement, 1993). As árvores do gênero *Caryocar* possuem sua principal aplicação na construção de casas e barcos, devido à elevada qualidade de sua madeira.

No entanto, os frutos representam o principal uso popular, fornecendo óleo comestível que pode ser utilizado no preparo de alimentos como substituto da manteiga, em pratos regionais (geralmente com arroz), sabão caseiro e outras aplicações cosméticas (Clement, 1993; Pianovski et al., 2008). O piquiá é consumido da mesma forma que os frutos de outra espécie de mesmo táxon genérico, o pequi (*Caryocar brasiliense* Camb.), o qual, como frutífera, é bem mais conhecida no Brasil, em particular nas regiões Nordeste e Centro-Oeste, onde ocorre espontaneamente (Carvalho & Müller, 2005). Os frutos de *Caryocar villosum* são produzidos de março a maio e possuem formato oblongo-arredondado, aproximadamente de 7-9 cm de diâmetro, com casca marrom-acinzentada e polpa amarelada (Figura 4).

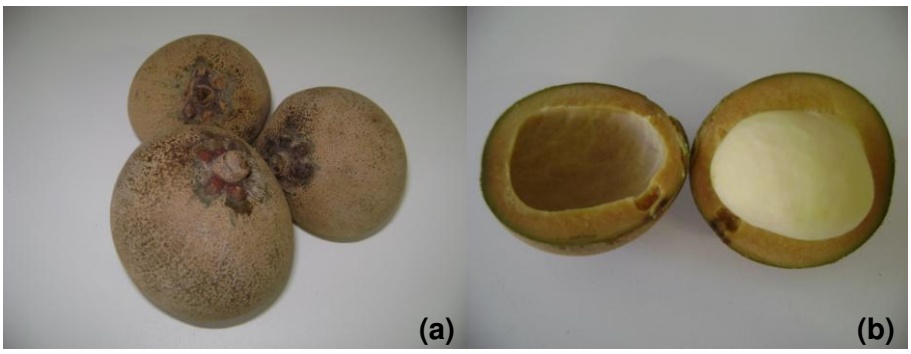

Figura 4 – Frutos de piquiá (a) inteiro e (b) corte longitudinal do fruto.

O mesocarpo de *C. villosum* apresenta 50 % de água, e em matéria seca possui 64 % de óleo, 3 % de carboidratos disponíveis, 83 mg de cálcio/100 g, 52 mg de magnésio/100 g e 41 mg de fósforo/100 g (Marx et al., 1997). Adicionalmente, a polpa de piquiá pode ser considerada também como uma fonte promissora de compostos bioativos, pois apresentou os valores mais elevados de compostos fenólicos (4623 mg eq. de ácido gálico/100 g), flavonóides totais (741 mg equivalente de catequina/100 g) e atividade anti-radical livre (TEAC de 25300 mM/100 g) dentre 18 frutas tropicais (nove da região Amazônica) analisadas por Barreto et al. (2009).

Além disso, Marx et al. (1997) relataram a composição química e os principais componentes da polpa de *Caryocar villosum*, como por exemplo, carboidratos (amido, glucose, sacarose, frutose), aminoácidos livres (asparagina, γ-ácido aminobutírico, alanina, leucina, valina), minerais e elementos traços (cálcio, magnésio, fósforo), matéria insaponificável (esteróis), aminas biogênicas (*O*-fosfoetanolamina, taurina) e compostos voláteis ((*E*)-nerolidol, 2-heptanona, β-bisabolena). Outros fitoquímicos encontrados em *Caryocar villosum* foram relatados na literatura, como as saponinas (triterpenóides com propriedades detergentes e surfactantes), isoladas de extratos metanólicos da casca seca do fruto, polpa e casca do caule, e a toxicidade, atividade antimicrobiana (Magid et al., 2006a), citotóxica e lipolítica (Magid et al., 2006b) também foram encontradas.

2.1. Carotenóides de piquiá

Os carotenóides zeaxantina (majoritário) (Figura 5), β-criptoxantina, β-caroteno, ζ-caroteno, *cis*-β-criptoxantina e α-caroteno (minoritários) foram identificados na polpa de *Caryocar villosum* por Godoy (1993). No entanto, a identificação foi realizada por meio de parâmetros tradicionais (derivatizações químicas) e não por técnicas com maior potencial

de informações, a exemplo da cromatografia líquida acoplada a detectores de arranjo de diodos (HPLC-DAD) e/ou por espectrometria de massas (MS).

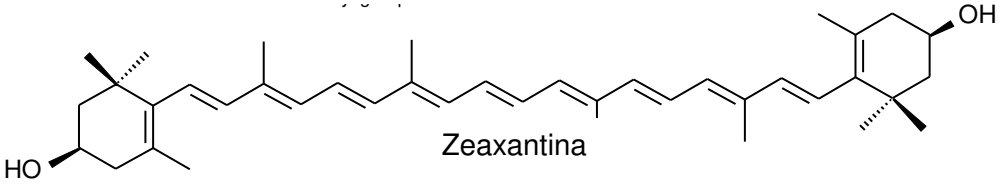

Figura 5 – Estrutura química da zeaxantina ($C_{40}H_{56}O_2$).

Por outro lado, a identificação dos carotenóides de *Caryocar brasiliense* (pequi) foi confirmada por HPLC-DAD e HPLC-MS (Azevedo-Meleiro & Rodriguez-Amaya, 2004), sendo a violaxantina o carotenoide majoritário, seguido de luteína e zeaxantina, e por fim, baixos teores de β-criptoxantina, β-caroteno e neoxantina.

2.2 Compostos fenólicos de piquiá

Os compostos fenólicos da polpa de *Caryocar villosum* ainda não foram relatados na literatura. No entanto, sete novos glicosídeos fenólicos, majoritariamente derivados de ácido gálico e elágico (Figura 6), já foram isolados da casca do caule de *Caryocar villosum* e *Caryocar glabrum*, e a atividade inibitória de tirosinase em cogumelos foram determinadas (Magid et al., 2008).

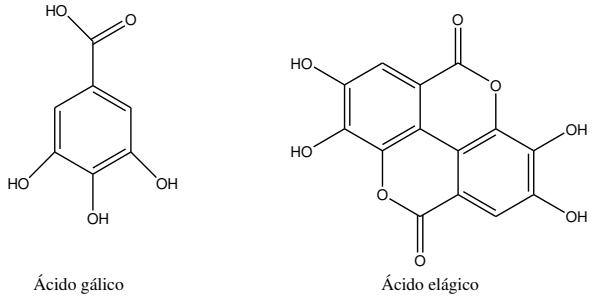

Figura 6 – Estrutura química do ácido gálico ($C_7H_6O_5$) e ácido elágico ($C_{14}H_6O_8$).

Além disso, compostos fenólicos como o ácido *p*-hidroxibenzóico, ácido gálico, ácido quínico, quercetina e quercetina 3-*O*-arabinosídeo foram identificados por espectrometria de massas em extratos etanólicos da casca do fruto de *Caryocar brasiliense*, tendo sido atribuído a estes a elevada atividade antioxidante em ensaios biológicos de peroxidação lipídica quimicamente induzida (Roesler et al., 2008). Em outro estudo mais recente, foram identificados galato de etila, ácido gálico e metil chiquimato por ressonância magnética nuclear (Ascari et al, 2010) em extratos etanólicos também da

casca de *Caryocar brasiliense*, e os autores observaram elevada atividade antioxidante, antimicrobiana e alelopática nos extratos puros, fracionados em misturas ou nos compostos isolados.

3. Otimização da extração de compostos bioativos

A extração de compostos bioativos é essencial para que se possam realizar estudos de composição e capacidade antioxidante. Dessa forma, o isolamento, quantificação e identificação de fitoquímicos em matérias-primas vegetais, e a avaliação de seus potenciais benefícios à saúde tem possibilitado o entendimento do valor profilático ou terapêutico em seres humanos. Do ponto de vista do processo de extração de compostos bioativos em plantas, alguns fatores influenciam na eficácia da extração como, por exemplo, as características do solvente, tempo de extração, temperatura de extração, razão entre massa e volume de solvente e o método de extração utilizado.

A necessidade crescente da otimização de produtos e processos, minimizando custos e tempo, maximizando rendimento, produtividade e qualidade de produtos, dentre outros objetivos, tem levado profissionais de diferentes formações a buscarem técnicas sistemáticas de planejamento de experimentos (Rodrigues e Iemma, 2005). A metodologia do planejamento fatorial, associada à análise estatística de superfície de resposta, é uma ferramenta fundamentada na teoria estatística, que fornece informações seguras sobre o processo, minimizando o empirismo que envolve técnicas de tentativa e erro (Box et al., 1978).

A otimização de processos pode ser realizada através de métodos empíricos ou estatísticos. A tradicional abordagem “um fator por vez” para otimizar processos é lenta e demorada, e as interações entre as diferentes variáveis são ignoradas. Além disso, o procedimento “um fator por vez” pressupõe que as variáveis de processo testadas não interagem entre si, e a resposta do processo será definida como a função direta de um único parâmetro variado. No entanto, a verdadeira resposta resulta da influência na interação entre as variáveis testadas em um processo (Haaland, 1989).

Considerando as limitações da abordagem “um fator por vez”, Box & Wilson (1951) desenvolveram a metodologia de superfície de resposta (RSM) que permite avaliar os efeitos de múltiplos fatores e suas interações sobre uma ou mais respostas. A RSM pode ser efetivamente utilizada para encontrar a combinação de fatores responsáveis pela resposta ótima do processo realizado. Trata-se de um conjunto de técnicas estatísticas e matemáticas amplamente utilizado no desenvolvimento, melhoria e otimização de

diferentes processos químicos, pois requer menor número de experimentos. O delineamento composto central rotacional (DCCR) é a forma mais empregada da RSM e tem sido utilizado por diversos autores na otimização de processos de extração de metabólitos secundários (Liyana-Pathirana & Shahidi, 2005; Silva et al. 2007; Rodrigues et al., 2008; Li et al, 2008; Lu et al. 2008; Qi et al. 2009; Balard et al., 2009).

4. Espécies reativas de oxigênio e de nitrogênio

A oxidação é parte fundamental da vida aeróbica e do metabolismo celular. Assim, as espécies pró-oxidantes são produzidas naturalmente e exercem funções biológicas fundamentais. As espécies reativas de oxigênio (ROS), como o próprio nome indica, são derivadas do oxigênio molecular com atividade redox e maior reatividade e as espécies reativas de nitrogênio (RNS) são derivadas do óxido nítrico ($^{\bullet}NO$) (Gomes et al., 2005; Gomes et al., 2006). No organismo, as ROS e RNS encontram-se envolvidas na produção de energia, fagocitose, regulação do crescimento celular, sinalização intercelular e síntese de substâncias biológicas importantes. Por outro lado, se por alguma razão forem produzidas em excesso, ou se as defesas antioxidantes endógenas funcionarem de forma deficiente, podem provocar oxidações de macromoléculas, como os lípidos, proteínas ou DNA (estresse oxidativo) e as consequentes disfunções biológicas e doenças associadas. (Halliwell & Guteridge 1999; Babior, 2004; Quinn et al., 2004; Valko et al., 2007).

As ROS e RNS são geradas dentro das células pela exposição a agentes endógenos e exógenos. As fontes endógenas podem ser várias, tais como: (1) a cadeia respiratória cuja redução monovalente de uma molécula de oxigênio dá origem a distintas espécies reativas; (2) as células fagocitárias (monócitos, neutrófilos, e macrófagos) que utilizam o sistema de NADPH oxidase, resultando primeiramente na formação do radical ânion superóxido ($O_2^{\bullet -}$), que é um dos principais responsáveis por desencadear a formação das demais espécies reativas de oxigênio e de nitrogênio; (3) a autoxidação de compostos de carbono reduzidos, como aminoácidos, proteínas, lipídios, glicídios e ácidos nucléicos; e (4) a ativação catalítica de diversas enzimas do metabolismo intermediário como a xantina oxidase, aldeído oxidase, monoamino oxidase, ciclooxigenase ou lipoxigenase (Halliwell & Guteridge 1999; Babior, 2004; Quinn et al., 2004). As fontes exógenas de ROS e RNS englobam a exposição a radiações (eletromagnéticas, luz solar, ozônio), a componentes dos cigarros, entre outros (Choe & Min, 2006). O excesso de espécies pró-oxidantes no organismo é combatido por antioxidantes produzidos pelo corpo ou absorvidos da dieta.

A formação de ROS e RNS tem sido amplamente estudada na deterioração oxidativa de produtos alimentares, bem como na patogênese de várias doenças humanas, como aterosclerose, diabetes *mellitus*, inflamações crônicas, doenças neurodegenerativas e certos tipos de câncer (Frankel, 1996; Frankel & German, 2006; Valko et al., 2007).

A ocorrência de espécies reativas de oxigênio em alimentos é inevitável devido à natureza biológica das matrizes alimentícias, podendo causar a diminuição no valor nutricional, além de mudanças físicas e químicas na qualidade dos alimentos durante o armazenamento ou comercialização. As ROS são as principais responsáveis pela iniciação de reações de oxidação nos alimentos, pois podem reagir com lipídios, proteínas, açúcares e vitaminas, produzindo compostos voláteis indesejáveis, degradando ácidos graxos essenciais, aminoácidos e vitaminas e produzindo compostos carcinogênicos (Choe & Min, 2006). As ROS podem mudar as propriedades funcionais das proteínas, lipídios e carboidratos devido à formação de dímeros e trímeros oxidados, tornando o alimento menos aceitável ou ainda inaceitável pelos consumidores (Min & Choe, 2002; Lee et al., 2003).

4.1. Formação e reatividade das principais ROS e RNS

Há uma inter-relação entre a formação das diferentes ROS e RNS, conforme pode ser observado na Figura 7. O radical ânion superóxido ($O_2^{\bullet -}$) produzido *in vivo*, nomeadamente através da reação entre a enzima NADPH oxidase e o oxigênio ($^3O_2$) durante algum estresse fisiológico (inflamação, por exemplo), é rapidamente convertido a peróxido de hidrogênio (H_2O_2), espontaneamente ou pela enzima superóxido dismutase (SOD) (Cheeseman & Slater, 1993). Apesar de ser uma molécula não-reativa, H_2O_2 interage com íons de metais de transição gerando radical hidroxila ($HO^{\bullet}$). O $HO^{\bullet}$ é uma das espécies reativas mais oxidantes, produzindo danos severos a alvos biológicos em sua proximidade e iniciando a peroxidação lipídica devido a sua conhecida reatividade com ácidos graxos poliinsaturados (AGP), resultando na produção de radicais peroxila ($ROO^{\bullet}$) e outros agentes citotóxicos (Cheeseman & Slater, 1993; Jaeschke, 1995; Chen & Schopfer, 1999). Além disso, a enzima mieloperoxidase (MPO) (H_2O_2 oxidoredutase), que é especificamente encontrada em leucócitos, incluindo neutrófilos e eosinófilos, catalisa a reação entre íons cloreto e H_2O_2 com a formação do ácido hipocloroso (HOCl), um poderoso agente bactericida (Xia & Zweier, 1997). Por outro lado, o oxigênio singlete ($^1O_2$), uma forma altamente reativa de oxigênio, pode ser produzida *in vivo* por enzimas peroxidases (POD) (por exemplo, mieloperoxidase), durante reações catalisadas por

lipoxigenases e também a partir de reações de fotosensibilização através da reação entre o estado triplete excitado de sensibilizadores (a exemplo da riboflavina) e o $^3O_2$ (Di Mascio et al., 1989; Montenegro et al, 2004; Rios et al., 2007).

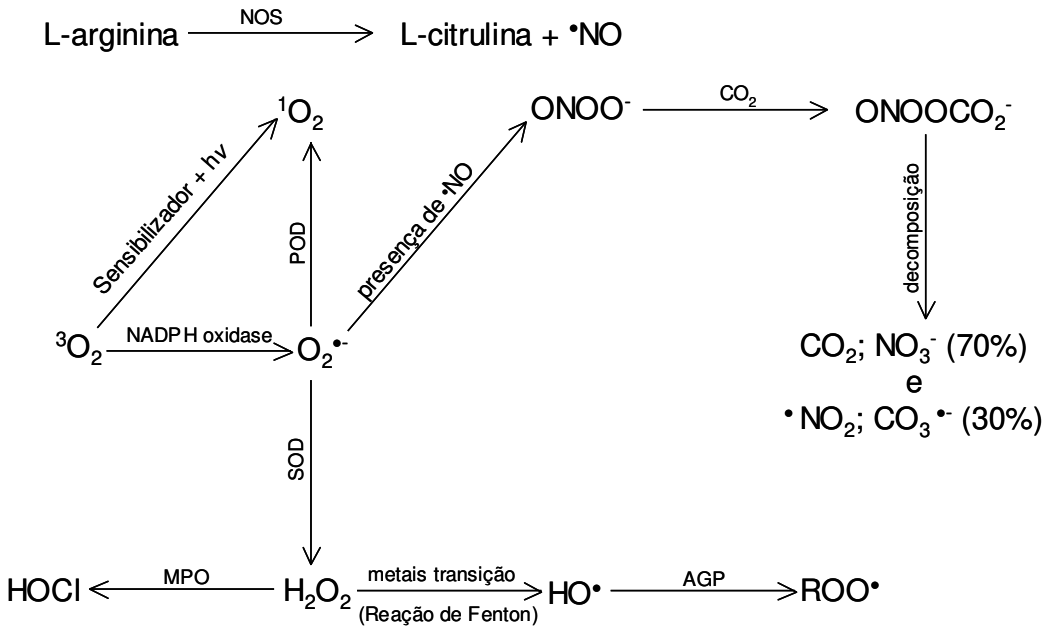

Figura 7. Inter-relações entre as formações de espécies reativas de oxigênio e nitrogênio (Choe & Min, 2006, adaptado).

O óxido nítrico (•NO), uma RNS, é produzido em diferentes tipos de células por uma família de isoenzimas denominadas óxido nítrico sintases (NOS), através da conversão de L-arginina para L-citrulina, e está envolvido em diversas funções fisiológicas (Squadritoa & Pryor, 1998; Freitas et al., 2009). Utilizando novamente o processo inflamatório, como exemplo, durante o mecanismo de defesa contra microorganismos patogênicos, o óxido nítrico é produzido pela NOS existente em macrófagos e neutrófilos ativados (Gomes et al., 2006). Identificado como um fator citotóxico, •NO produzido nessas condições é responsável pela imunidade. Entretanto, a superprodução está envolvida na patogênese de algumas condições fisiológicas, incluindo a inflamação (Kostka, 1995; Nagano, 1999). Além de seus próprios efeitos pró-inflamatórios, •NO pode gerar outras espécies reativas, particularmente o peroxinitrito ($ONOO^-$), através da reação com $O_2^{\bullet-}$ (Miyasaka & Hirata, 1997). O $ONOO^-$ é considerado um oxidante muito efetivo, com reatividade similar a do HO• (Shackelford et al., 2000), responsável por mediar a toxicidade do •NO, e pode causar danos às biomoléculas celulares, levando à morte celular (Priyadarsini et al., 2002). Uma das reações mais rápidas conhecidas para o $ONOO^-$ é a reação com CO_2, resultando na formação do ânion nitrosoperoxicarbonato

($ONOOCO_2^-$). A decomposição do $ONOOCO_2^-$ resulta na formação de CO_2 e NO_3^- (aproximadamente 70 %), assim como do $^{\bullet}NO_2$ e do radical ânion carbonato ($CO_3^{\bullet-}$) (aproximadamente 30 %) (Gomes et al., 2006). Portanto, tendo em conta as elevadas concentrações de CO_2 em condições fisiológicas, é mais provável que muitos dos efeitos do $ONOO^-$ *in vivo* sejam mediados por intermediários reativos resultantes da reação $ONOO^-$ com o CO_2 e não pelo próprio $ONOO^-$ (Jensen, 2003; Squadrito & Prior, 2002).

Apesar das ROS e RNS produzidas pelos fagócitos representarem um mecanismo extremamente importante da defesa do organismo, o seu excesso pode provocar ou agravar danos em sítios inflamatórios.

Quanto à reatividade em meio biológico e alimentício, o $O_2^{\bullet-}$ ao contrário da maioria dos radicais livres é inativo. Em meio aquoso, sua reação principal é a dismutação, na qual uma molécula de H_2O_2 e uma molécula de oxigênio são produzidas. O $O_2^{\bullet-}$ também é uma base fraca cujo ácido conjugado, o radical hidroperóxido ($HOO^{\bullet}$) é mais reativo. O $O_2^{\bullet-}$ participa de certos processos químicos importantes no contexto biológico. O principal deles é auxiliar na produção de radical $HO^{\bullet}$, através da redução de quelatos de Fe^{3+}, formando Fe^{+2}. Dentre os aminoácidos, o único que sofre oxidação pelo radical $O_2^{\bullet-}$ é a cisteína. O radical $O_2^{\bullet-}$ também reage com o $HO^{\bullet}$ produzindo oxigênio singlete (Barreiros et al., 2006).

O radical $HO^{\bullet}$ é o mais deletério ao organismo, pois devido ao seu tempo de vida muito curto dificilmente pode ser sequestrado *in vivo,* é o mais reativo e em teoria, pode oxidar qualquer molécula biológica provavelmente reagindo nas proximidades dos sítios onde foi gerado (Halliwell & Gutteridge, 1999). O $HO^{\bullet}$ frequentemente ataca as moléculas por abstração de hidrogênio ou por adição a insaturações. Nos experimentos de laboratório, o $HO^{\bullet}$ pode facilmente ser sequestrado *in vitro* por várias moléculas, devido a sua alta reatividade. No entanto, para que os resultados *in vitro* se reproduzam *in vivo*, é necessário ministrar alta concentração do antioxidante suficiente para alcançar o local onde o radical $HO^{\bullet}$ está presente e então poder desativá-lo (Barreiros et al., 2006).

O H_2O_2, mais estável que o $HO^{\bullet}$, pode permear membranas e esta propriedade possibilita a ocorrência de reações com alvos biológicos em compartimentos distantes do seu local de formação (Hancok & Desikan, 2001). Devido ao fato da célula possuir Cu^{1+} e Fe^{2+}, ocorre reação do H_2O_2 com estes metais com geração do radical $HO^{\bullet}$ no interior das células (Halliwell, 2000). O H_2O_2 é pouco reativo frente às moléculas orgânicas na ausência de metais de transição, somente oxidando proteínas que apresentem resíduos de metionina ou grupos tióis muito reativos (Barreiros et al., 2006). No entanto, exerce

papel importante no estresse oxidativo por ser capaz de transpor facilmente as membranas celulares.

O $^1O_2$ pode reagir com lipídios da membrana, proteínas, aminoácidos, ácidos nucléicos, carboidratos e tióis (Ryter & Tyrrel, 1998). Além disso, na peroxidação lipídica as ROS oxidam os ácidos graxos poliinsaturados dos fosfolipídios das membranas das células, formando peróxidos lipídicos que podem atingir facilmente alvos mais distantes.

O $^{\bullet}NO$ não é suficientemente reativo para atacar o DNA diretamente, mas pode reagir com $O_2^{\bullet-}$, produzido pelos fagócitos, gerando peroxinitrito. Esse último, por sua vez, pode sofrer reações secundárias, formando agentes capazes de adicionar nitrogênio a aminoácidos aromáticos, a exemplo da tirosina, gerando nitrotirosina e às bases do DNA, em particular a guanina, na qual o produto principal é a 8-nitroguanina (Halliwell & Guteridge 1999; Babior, 2004).

4.2. Ação antioxidante de compostos fenólicos e carotenóides

Segundo a definição de Halliwell & Guteridge (1999), antioxidante é uma substância que, quando se encontra em baixas concentrações comparadas com as do substrato oxidável, previne ou retarda a reação de oxidação. No entanto, não pode reverter o processo oxidativo e nem evitar a rancidez hidrolítica (Rajalakshmi & Narasimhan, 1995).

Os mecanismos através dos quais os antioxidantes em alimentos exercem sua ação podem ser divididos em duas possíveis vias: reações de transferência de um átomo de hidrogênio (*Hydrogen Atom Transfer*, HAT) (Equação 1) ou de transferência de um elétron (*Single Electron Transfer*, SET) (Equação 2). O conhecimento destas duas reações é muito importante para a compreensão e seleção dos métodos utilizados para medir a capacidade antioxidante de compostos bioativos (Prior et al., 2005).

$$R^{\bullet} + AH \rightarrow RH + A^{\bullet} \qquad \text{(Equação 1)}$$

onde $R^{\bullet}$ é o radical livre e AH é o antioxidante. Nesta reação, o novo radical formado é mais estável que o inicial.

(Conjunto de equações 2)

$$R^{\bullet} + AH \rightarrow R^{-} + AH^{\bullet+}$$
$$AH^{\bullet+} + H_2O \leftrightarrow A^{\bullet} + H_3O^{+}$$
$$R^{-} + H_3O^{+} \rightarrow RH + H_2O$$
$$M(III) + AH \rightarrow AH^{+} + M(II)$$

onde $R^{\bullet}$ é o radical livre, AH é o antioxidante e M(III) é um metal com estado de oxidação 3^{+}. Ao contrário da reação HAT, na reação SET o antioxidante transfere um elétron para reduzir o composto, incluindo metais, carbonilas e radicais.

Os compostos fenólicos e os carotenóides podem atuar como antioxidantes primários, reagindo diretamente com os radicais livres – dando lugar a um novo radical menos reativo que o radical livre inicial – ou como antioxidantes secundários regenerando ou potencializando outros sistemas antioxidantes, como certas enzimas (Gorinstein et al., 2000).

Quanto ao modo de ação dos compostos fenólicos, de modo geral, estes compostos e em particular os flavonóides possuem estrutura ideal para a desativação de radicais (Barreiros et al., 2006). A atividade antioxidante dos flavonóides depende da sua estrutura e pode ser determinada por cinco fatores: reatividade como agente doador de H e de elétrons, estabilidade do radical flavanoil formado, reatividade frente a outros antioxidantes, capacidade de quelar metais de transição, solubilidade e interação com as membranas. Os grupos OH podem doar hidrogênio ou um elétron aos radicais $HO^{\bullet}$, $ROO^{\bullet}$ e $ONOO^{-}$, estabilizando-os e transformando o flavonóide, por exemplo, em uma molécula radical relativamente estável (Heim et al., 2002).

Os carotenóides podem capturar $ROO^{\bullet}$ mediante transferência de elétrons ou doação de átomos de hidrogênio, mecanismos estes que levam à formação de uma grande variedade de carotenóides radicais (Choe & Min, 2006). Por outro lado, os carotenóides são reconhecidamente eficientes na desativação do $^1O_2$ (Di Mascio et al., 1989; Montenegro et al, 2004; Rios et al., 2007), conforme demonstrado nas equação 3 a 5. Sua capacidade de desativação depende do número de ligações duplas conjugadas presente na estrutura do carotenóide, e podem apresentar efeitos sinergísticos com outros antioxidantes como a vitamina E ou C (Stahl & Sies, 2005).

$$^1O_2 + Car \xrightarrow{k_r} \text{produtos oxigenados} \qquad \text{(Equação 3)}$$

$$^1O_2 + Car \xrightarrow{k_q} {}^3O_2 + {}^3Car* \qquad \text{(Equação 4)}$$

$$^3Car* \xrightarrow{k_d} Car + calor \qquad \text{(Equação 5)}$$

onde k_r é a constante de desativação química do oxigênio singlete, k_q é a constante de desativação física do oxigênio singlete e k_d é a constante de desativação física do carotenóide.

A desativação do $^1O_2$ por carotenóides ocorre por meio de mecanismos físico e químico, prevalecendo o físico. No mecanismo químico, o $^1O_2$ é desativado com formação

de produtos de oxidação (Equação 3), enquanto no processo físico de desativação não ocorre degradação do carotenóide (Equação 4). Após transferência de energia do $^1O_2$ para o carotenóide, este passa para o estado excitado (Car*) e libera energia na forma de calor (Equação 5), retornando ao estado fundamental para efetuar a desativação de outras moléculas de $^1O_2$. Além disso, os carotenóides são capazes de desativar os sensibilizadores no estado excitado, retornando ao seu estado fundamental.

Em condições normais no organismo, 95 % da desativação do $^1O_2$ é física, restando somente 5 % para reagir quimicamente, o que torna os carotenóides antioxidantes mais efetivos (Palace et al., 1999). Outro aspecto da atividade dos carotenóides diz respeito à polaridade. De fato, em função da posição que o carotenóide ocupa na membrana lipídica (estando orientado para fase aquosa ou para a lipídica), na reação de um mesmo carotenóide com um mesmo radical livre, podem surgir diferentes produtos (El-Agamey et al., 2004). Aqueles que possuem grupos polares nos anéis β são efetivos na prevenção da oxidação das membranas. Essa polaridade os localiza de maneira tal que estão em contato mais próximo com a fase aquosa, reagindo com os radicais que penetram a membrana. Os apolares, tais como o licopeno e o β-caroteno são mais regeneradores do que preventivos, combatendo os radicais formados com mais eficiência no interior da membrana (Woodall et al., 1997).

Com relação à desativação das ROS e de RNS específicas, são encontrados poucos estudos na literatura com dados sobre a capacidade antioxidante de compostos fenólicos. Geralmente estes dados são expressos em valores de IC_{50}, que é a concentração inibitória *in vitro* que reduz 50 % o efeito oxidativo provocado pelas espécies reativas testadas no meio (Tabela 1). No entanto, não foram encontrados dados expressos em valores de IC_{50} mensurando a capacidade antioxidante de carotenóides relacionados à desativação de ROS e RNS, tais como o $O_2^{\bullet -}$, $HO^{\bullet}$, H_2O_2, HOCl, $^1O_2$, $^{\bullet}NO$ e $ONOO^-$.

Tabela 1 - Capacidade antioxidante de compostos fenólicos na desativação de espécies reativas de oxigênio e nitrogênio.

Compostos	IC_{50} (μM)							
							$ONOO^-$	
	$O_2^{\bullet-}$	$HO^\bullet$	H_2O_2	HOCl	$^1O_2$	$^\bullet NO$	Ausência de $NaHCO_3$	Presença de $NaHCO_3$
Quercetina	47	ND	785	1	5	2	0,2	0,4
Luteolina	58	ND	900	7	23	3	0,3	0,6
Apigenina	NF	ND	NA	4	NF	NA	76	21
Crisina	NA	ND	NA	4	NA	NF	188	140
5-Hidroxiflavona	NA	ND	NA	NF	NA	NA	NA	NA
7-Hidroxiflavona	NA	ND	NA	146	NA	NA	NA	NA

$O_2^{\bullet-}$: radical anion superóxido, H_2O_2: peróxido de hidrogênio, HOCl: ácido hipocloroso, $^1O_2$: oxigênio singlete; $^\bullet NO$: radical óxido nítrico, $ONOO^-$: peroxinitrito, NA: Não foi encontrada atividade na faixa de concentração testada, NF: valores não-fornecidos em IC_{50} (μM), ND: não determinado. Fonte: Gomes et al. (2007).

Da mesma forma, também foram encontrados poucos dados na literatura para extratos vegetais (Tabela 2), expressos em valores de IC_{50}, com relação à desativação das mais freqüentes espécies reativas de oxigênio e de nitrogênio.

Tabela 2 – Capacidade antioxidante de extratos vegetais na desativação de espécies reativas de oxigênio e nitrogênio.

Extratos	IC_{50} (μg/mL)							
							$ONOO^-$	
	$O_2^{\bullet-}$	$HO^\bullet$	H_2O_2	HOCl	$^1O_2$	$^\bullet NO$	Ausência de $NaHCO_3$	Presença de $NaHCO_3$
Pedilanthus tithymaloides[a]	143	345	2,5x10³	113	ND	54	44	82
Juglans regia[b]	47	NF	383	ND	ND	2	1	2
Castanea sativa[c]	13	216	410	ND	12	3	1	2
Quercus robur[c]	11	285	251	ND	8	3	1	1
Hypericum androsaemum[d]	33	595	944	ND	28	2	1	1
Eucalyptus globulus[e]	8	104	389	ND	NA	3	1	2
Sesamum indicum[f]	45	456	ND	ND	ND	99	ND	ND

$O_2^{\bullet-}$, radical anion superóxido. H_2O_2, peróxido de hidrogênio. HOCl, ácido hipocloroso. $^1O_2$, oxigênio singlete. $^\bullet NO$, radical óxido nítrico. $ONOO^-$, peroxinitrito. NA, Não foi encontrada atividade na faixa de concentração testada. NF, valores não-fornecidos em IC_{50} (μg/mL). ND, não determinado. [a]Abreu et al. (2006).[b]Almeida et al. (2008a). [c]Almeida et al. (2008b).[d]Almeida et al. (2009a).[e]Almeida et al. (2009b).[f]Visavadiya et al. (2009).

Valores de ORAC (Oxygen Radical Absorbance Capacity), relativos à desativação do $ROO^\bullet$, para padrões de compostos hidrossolúveis, como por exemplo, os compostos fenólicos, são facilmente encontrados na literatura, tais como ácido caféico (4,4), ácido

clorogênico (3,1), quercetina (7,3), genisteína (5,9), rutina (6,0), catequina (6.7), Trolox (1,0), entre outros (Ou et al., 2001, Huang et al., 2002; Gomes et al. 2007), assim como para extratos vegetais (Wu et al., 2004a; Wu et al., 2004b). Valores de ORAC para diversos extratos vegetais em meio lipofílico também são facilmente encontrados na literatura (Wu et al., 2004a; Wu et al., 2004b). No entanto as mesmas informações são escassas para padrões de compostos lipofílicos, como os carotenóides, por exemplo. Bangalore et al. (2005) avaliaram a capacidade antioxidante de diferentes concentrações de licopeno (6,8 a 34,1 μM) adicionadas de β-ciclodextrina (0 a 0,8 %) e resultaram em valores de ORAC na faixa de 0,3 a 0,9 μmol equivalente de Trolox. Huang et al. (2002) encontraram valores de ORAC para os padrões de compostos α-tocoferol (0,5), γ-tocoferol (0,7), δ-tocoferol (1,3), nutrieno (tocotrienol) (0,9), BHT (0,1), entre outros.

Alguns estudos relacionados à capacidade antioxidante de padrões de carotenóides e extratos vegetais ricos em carotenóides foram relatados na literatura abordando o retardo da oxidação lipídica. Haila et al. (1996) estudaram o efeito de luteína, licopeno, corante natural de urucum e as interações destes com γ-tocoferol na formação de hidroperóxidos formados a partir de triacilgliceróis oriundos do processo de autoxidação, concluindo que luteína (30-40 μg/g) e licopeno (20 μg/g) apresentaram efeito pró-oxidante, enquanto o corante natural de urucum (20-60 μg bixina/g) e γ-tocoferol (10 e 15 μg/g) inibiram efetivamente a formação de hidroperóxidos.

Kiokias & Gordon (2003) estudaram o efeito antioxidante do β-caroteno, bixina solúvel em óleo e norbixina solúvel em água na deterioração oxidativa do extrato polar do óleo de oliva virgem. A norbixina (2 mM) foi o único carotenóide que apresentou efeito antioxidante na oxidação lipídica com atividade similar ao δ-tocoferol (0,1 mM). O β-caroteno e a bixina isolados não retardaram a autoxidação; no entanto, foi observado um efeito antioxidante em suas respectivas misturas com o extrato polar do óleo de oliva virgem.

Foi verificado que os extratos de urucum (rico em bixina) foram mais efetivos do que os extratos de *marigold* (rico em luteína) para retardar a oxidação de emulsão do tipo óleo em água, utilizando como iniciador AAPH (2,2′-azobis-amidinopropane dihydrochloride) (Kiokias & Oreopoulou, 2006). Os autores atribuíram esta maior capacidade antioxidante devido à maior polaridade da bixina, que apresenta grupos carboxílicos e ésteres na estrutura.

Dessa forma, o estudo da composição do urucum e do piquiá, assim como a obtenção de extratos vegetais com potencial antioxidante, a partir dessas fontes acessíveis de compostos bioativos, devem ser realizados com o intuito de fornecer uma

importante base de conhecimento para as indústrias alimentícia, cosmética e farmacêutica no desenvolvimento de produtos em potencial contra os danos oxidativos em alimentos ou sistemas biológicos.

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CAPÍTULO II

Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds

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Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds

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ABSTRACT

This study was designed to identify and quantify the carotenoids and phenolic compounds from annatto seeds using high performance liquid chromatography coupled to diode array and mass spectrometer detectors (HPLC–DAD-MS/MS). Furthermore, using response surface methodology, an optimized procedure for simultaneous extraction of these compounds was established. In addition to bixin, known to be the main carotenoid in annatto seeds, hypolaetin and a caffeoyl acid derivative were identified as the main phenolic compounds. The optimized procedure involved 15 extractions using acetone:methanol:water (50:40:10, v/v/v) as solvent, a solid–liquid ratio of 1:9 (m/v) and an extraction time of 5 min. Validation data indicated that the HPLC method proposed provided good linearity, sensitivity, procedure accuracy, system precision and suggested its suitability for the simultaneous analysis of phenolic compounds and carotenoids in annatto seeds.

1. Introduction

Brazil is one of the major producers and exporters of the annatto seeds and extracts. These are used as coloring agents in the food, pharmaceutical and cosmetic industries [1] and are known for their antioxidant capacity, which is due to carotenoids and phenolic compounds.

Bixin (methyl (9-*cis*)-hydrogen-6,6′-diapo-Ψ,Ψ-carotenedioate) (Fig. 1), accounting some 80% of the carotenoids present [2], is responsible for the reddish-orange color of the annatto seeds and their extracts. Its antioxidant capacity results from its ability to quench singlet oxygen, deactivate the excited triplet state of sensitizers and scavenge free radicals [3–5].

Although the seeds are reported to contain phenolic compounds [6], no information about the composition of phenolic compounds present in the seeds and extracts were found. According to Harbone [7], the leaves were found to contain the flavones apigenin 7-glucoside, apigenin 7-bisulphate, luteolin 7-glucoside, luteolin 7-bisulphate as the major compounds, and the flavonoid hypolaetin 8-bisulphate as the minor one (Fig. 1).

Both carotenoids and phenolic compounds are considered to promote human health, since they are responsible for critical biological functions [8,9]. These compounds are associated with a decrease in the risk of development of inflammations and cataracts, as well as various chronic degenerative diseases, such as cancer, cardiovascular diseases and macular degeneration [10–12]. In general, the reduction in the risk of these diseases involves the inhibition of oxidative reactions via the quenching of singlet oxygen and scavenging of free radicals.

Response surface methodology (RSM), introduced by Box and Wilson [13], is useful for the evaluation of the effects of multiple factors and their interactions and can be effectively used to find the combinations of these factors, which will produce an optimal response. One of the main advantages of this methodology is that it generally requires fewer experiments than would be necessary for a traditional full factorial design, yet provides statistically valid results. The central composite rotational design (CCRD) is the most popular form of RSM and it has been utilized to optimize the extraction process of secondary metabolites [14–16].

The objective of this study was the development and validation of a simple and reliable method for simultaneous separation, identification and quantification of the main carotenoids and phenolic compounds in annatto seeds and extracts using high performance liquid chromatography coupled to diode array and mass spectrometer detectors (HPLC–DAD-MS/MS). Moreover, the conditions for

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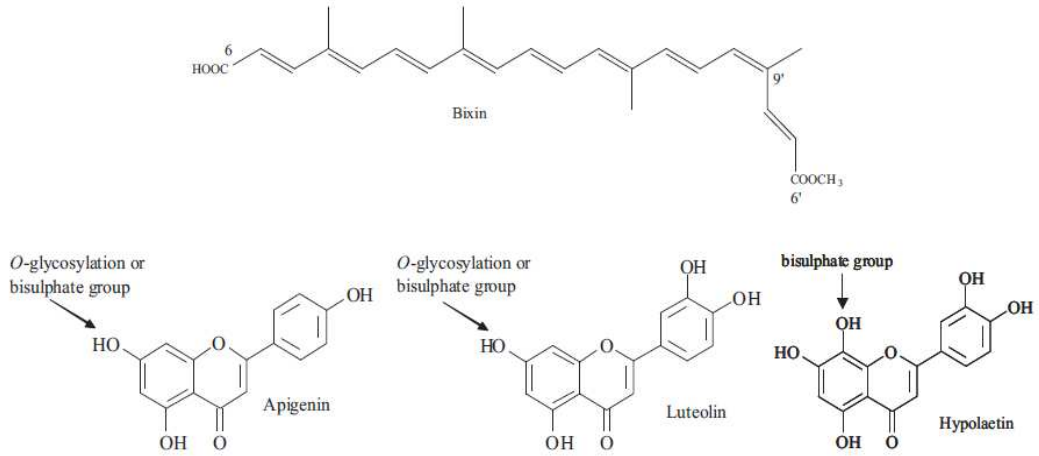

Fig. 1. Structures of bixin ($C_{25}H_{30}O_4$), apigenin, luteolin and hypolaetin with common *O*-glycosylation and bisulphate positions indicated with an arrow.

simultaneous extraction of compounds from both carotenoids and phenolic classes were optimized by response surface methodology.

2. Materials and methods

2.1. Materials

In August 2008, annatto seeds were obtained from the local market in Campinas, São Paulo, Brazil. The seeds were portioned (500 g), vacuum packed and stored under light-free conditions at room temperature until analysis. The acetone and methanol (P.A.) used on the extraction procedure were obtained from Synth (São Paulo, Brazil). Methanol and acetonitrile of chromatographic grade were obtained from J.T. Baker (Phillipsburg, USA) and ultrapure water was obtained from the Millipore system (Billerica, USA). Formic acid was purchased from Merck (Darmstadt, Germany). The standards quercetin and rutin were purchased from Sigma–Aldrich Co. (St. Louis, USA), and gallic acid from Extrasynthèse (Lyon Nord, France). The bixin standard was isolated in our laboratory [17] and re-crystallized to achieve 98% purity, as determined by HPLC–DAD.

2.2. Extraction procedure

The phenolic compounds and carotenoids were extracted from about 1 g of annatto seeds using different ratio values of methanol:water (8:2, v/v) in acetone solution at 25 ± 1 °C using ultrasound equipment (Unique, São Paulo, Brazil). The proportion of methanol:water (8:2) in acetone varied from 10 to 90%, and the solid (g)–liquid (mL) ratio from 1:1 to 1:9 (m/v); extraction time and number of extractions were also investigated (from 5 to 25 min and from 5 to 25 extractions, respectively). After extraction, the extracts were stored at −36 °C until analysis.

2.2.1. Experimental design

The optimal conditions for the extraction of bixin and phenolic compounds from annatto seeds were determined by response surface methodology (RSM) and analyzed using Statistica® 6.0 software [18]. A central composite rotational design (CCRD) with two levels and four factors (solvent composition, number of extractions, solid–liquid ratio and extraction time) was used (Table 1). The acetone is the standard solvent used for quantitative determination of bixin content in annatto seeds [19] and methanol:water solution (8:2, v/v) is the standard solvent for the quantitative extraction of phenolic compounds [20]. However, in this study they were used combined in order to verify the improvement of the simultaneous extraction of bixin and phenolic compounds. The responses of the experimental design were carotenoid content (quantified as bixin) and phenolic compounds content. Regression coefficients were determined for the experimental data by fitting to a quadratic model (Eq. (1)):

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} X_i X_j \quad (i = 1-3, j = 1-3) \tag{1}$$

where Y the predicted response; b_0 a constant; b_i the linear coefficient; b_{ii} the quadratic coefficient; and b_{ij} the interaction coefficient of variables; i and j, and X_i and X_j are independent variables; and k the number of tested variables. The adequacy of the model was determined by evaluating lack of fit, coefficient of determination (R^2) and Fisher test value (F-value) obtained from the analysis of variance (ANOVA) generated by the software. Statistical significance of the results of the model and variables was determined at 5% and 10% ($\alpha = 0.05$ and $\alpha = 0.10$). The quadratic model equation shown above is used to build the response surfaces, with three-dimensional response surface plots and contour plots generated by maintaining one of the response variables at its optimal level and plotting it against two independent variables. The levels of the independent variables used in the analysis are given in Table 2. The CCRD consisted of a 2^4 factorial design plus 8 axial points and 4 repetitions at the central point (Table 2) totalizing 28 extractions.

2.2.2. Verification of the model obtained by RSM

The optimal conditions for the simultaneous extraction of bixin and total phenolic compounds were determined by the response surface models. Experiments were conducted in triplicate under

Table 1
Central composite rotational design: independent variable levels (original and coded).

Independent variable		Level				
		−2	−1	0	+1	+2
Acetone in methanol/water 8:2 (Acetone:X)	X_1	10%	30%	50%	70%	90%
Number of extractions	X_2	5	10	15	20	25
Solid–liquid ratio (1:X)	X_3	1	3	5	7	9
Time (min)	X_4	5	10	15	20	25

Table 2
Central composite rotational design and experimental responses of bixin and total phenolic contents.

Experiment	Independent variable levels				Response	
	X_1	X_2	X_3	X_4	Bixin (mg/g)	TPC (mg/g)
1	−1	−1	−1	−1	9.7	1.7
2	1	−1	−1	−1	7.6	2.4
3	−1	1	−1	−1	4.0	2.4
4	1	1	−1	−1	3.5	2.1
5	−1	−1	1	−1	12.0	2.7
6	1	−1	1	−1	9.4	2.4
7	−1	1	1	−1	13.6	2.4
8	1	1	1	−1	11.0	2.5
9	−1	−1	−1	1	12.3	2.1
10	1	−1	−1	1	9.4	1.7
11	−1	1	−1	1	12.7	2.1
12	1	1	−1	1	12.0	2.3
13	−1	−1	1	1	9.6	2.2
14	1	−1	1	1	10.6	3.1
15	−1	1	1	1	10.2	2.7
16	1	1	1	1	11.5	2.6
17	−2	0	0	0	10.9	2.1
18	2	0	0	0	9.2	2.3
19	0	−2	0	0	10.3	1.5
20	0	2	0	0	9.8	3.0
21	0	0	−2	0	11.4	1.3
22	0	0	2	0	13.4	2.7
23	0	0	0	−2	10.7	2.4
24	0	0	0	2	11.2	2.0
25 (CP)	0	0	0	0	11.0	2.4
26 (CP)	0	0	0	0	11.0	2.1
27 (CP)	0	0	0	0	11.7	2.7
28 (CP)	0	0	0	0	12.8	2.6

X_1 = acetone in methanol/water (%), X_2 = number of extractions, X_3 = solid–liquid ratio (m/v), X_4 = time (min). CP: central point; TPC: total phenolic compounds.

the optimal conditions and the results were compared with the predicted values to check the reliability of the predictive extraction model.

2.2.3. Bixin quantification

The bixin concentration of the seeds and of the 28 extracts was determined using an adaptation of the methodology described by FAO/WHO [19]. For the seeds, 1 g was weighed and the pigment thoroughly extracted with acetone until the seeds were colorless. Aliquots (0.1 mL) of the 28 extracts were evaporated under N_2 flow and re-suspended to 10 mL with acetone. Absorbance was measured with a UV-visible spectrophotometer (Agilent, Santa Clara, USA) at 487 nm and the bixin concentration was calculated according to the Lambert–Beer law, using $E^{1\%}_{1\,cm} = 3090$ [19].

2.2.4. Total phenolic compounds quantification

For the annatto seeds, 5 g were weighed and extracted five times with methanol:water (8:2, v/v) in ultrasound equipment (Unique model, São Paulo, Brazil) for 10 min at 25 °C. After extraction, the extracts were evaporated under vacuum ($T < 40$ °C), transferred with 5 mL of methanol to 25 mL volumetric flask and filled with distilled water. For the 28 extracts obtained by RSM, aliquots (2.5 mL) were evaporated under N_2 flow, re-suspended in 2.5 mL of methanol, transferred to 10 mL volumetric flask and filled with distilled water. These extracts were then put in the freezer for 20 min before centrifugation at $290 \times g$ for 20 min. The total phenolic content of the seeds and extracts was determined using the Folin-Ciocalteu colorimetric method [20], and was expressed as milligrams of gallic acid equivalent (GAE) per mL of extract. All measurements were performed in triplicate.

2.3. HPLC–DAD-MS/MS analysis

Simultaneous extraction of bixin and phenolic compounds under the optimal conditions, established by RSM, produced an extract which was subjected to solvent evaporating under N_2 flow, dissolved in methanol and injected into a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with a 20 μL loop, and diode array detector (DAD) (SPD-M20A) connected in series to a mass spectrometer (MS/MS) from Bruker Daltonics (Esquire 4000 model, Bremen, Germany), with an electrospray ionization source (ESI) and an ion-trap analyzer. The compounds were separated on a C_{18} Luna column (5 μm, 250 mm × 4.6 mm, Phenomenex) at 0.9 mL/min of flow, column temperature at 29 °C, with a mobile phase consisting of water:formic acid (98:2, v/v) (solvent A) and methanol:formic acid (98:2, v/v) (solvent B) in gradient from A:B 70:30 to 40:60 in 15 min; then from 40:60 to 20:80 in 10 min; and finally from 20:80 to 5:95 in 10 min. This latter ratio (5:95) was maintained for an additional 10 min. The column eluate was split to allow only 0.15 mL/min to enter the ESI interface. The spectra were obtained between 200 and 600 nm with the chromatograms processed at 320 nm (phenolic compounds) and 459 nm (bixin). The mass spectra were acquired with a scan range from 100 to 800 m/z; the MS parameters were set as follows: ESI source in positive ion mode; capillary voltage: 1500 V, end plate offset: −500 V, capillary exit: 120 V, skimmer 1: −10 V, skimmer 2: −5 V, dry gas (N_2) temperature: 325 °C; flow rate: 8 L/min; nebulizer: 30 psi; MS/MS fragmentation energy: 1.4 V. The bixin and phenolic compounds were identified by comparison of elution order in the reverse phase column and retention time of the peaks in relation to standards, and UV-visible and mass spectra features. Bixin and phenolic compounds were quantified by comparison to external standards using seven-point calibration curves based on standard solutions (measurements in duplicate), with concentrations varying from 0.5 to 12.0 μg/mL for bixin, 0.6 to 12.5 μg/mL for rutin, and 0.4 to 10 μg/mL for quercetin.

2.4. Validation of the HPLC–DAD method

The results were validated to show compliance with international requirements for analytical methods for the quality control of pharmaceuticals [21] using measures of linearity, limits of detection (LOD) and quantification (LOQ), recovery and repeatability. Linearity was revealed by the coefficient of determination (R^2) of the seven-point calibration curves of the standard solutions. The recovery analysis for bixin and phenolic compounds was conducted simultaneously using two levels of addition (5.7 and 10 μg/mL) for bixin, rutin and quercetin standards, with 6 replicates for each level. Repeatability was evaluated based on the values of relative standard deviation (RSD %) in relation to values of the standards injected (bixin, rutin and quercetin).

3. Results and discussion

3.1. Validation of HPLC–DAD method

This is the first study to fully validate a HPLC method to be applied for the simultaneous analysis of secondary metabolites from *Bixa orellana* (annatto) seeds. These validation parameters included linearity, selectivity, procedure accuracy, system precision, recovery and stability. For the target compounds, linear regression analyses were performed by using external calibration curves. The parameters for the calibration curves (slope, intercept, relative standard deviation of slope, relative standard deviation of intercept and correlation coefficient) are shown in Table 3.

Table 3
Statistical analysis for external calibration curves of rutin, quercetin and bixin.

Compound	Linearity range (μg/mL)	Slope (*a*)	Intercept (*b*)	R^2	LOD (μg/mL)	LOQ (μg/mL)
Rutin	0.6–12.5	41,655 (1.6%)	2586 (24.0%)	0.9996	0.0049	0.0151
Quercetin	0.4–10.0	98,726 (1.1%)	19,681 (24.4%)	0.9994	0.0019	0.0058
Bixin	0.5–12.0	195,141 (5.0%)	45,697 (43.8%)	0.9958	0.1850	0.5630

Experimental conditions described in Section 2. LOD: limit of detection; LOQ: limit of quantification. For each curve, the equation is $y = ax + b$, where y is the peak area, x the concentration of the analyte (μg/mL), a the slope, b the intercept and R^2 the coefficient of correlation. Relative standard deviation (RSD) values are given in parentheses.

Table 4
Recovery values for rutin, quercetin and bixin added to extracts of annatto seeds.

Compound	Level 1				Level 2			
	Original (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)	Original (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)
Rutin	nd	5.7	5.5 ± 0.21	96.3 (3.7)	nd	10	9.7 ± 0.14	94.3 (2.9)
Quercetin	nd	5.7	5.9 ± 0.16	103.2 (2.8)	nd	10	10.1 ± 0.22	102.4 (4.3)
Bixin	188.3 ± 0.06	5.7	193.9 ± 0.54	95.2 (2.0)	215.9 ± 0.2	10	226.7 ± 0.65	105.2 (0.7)

nd: not detected. Mean ± standard deviation for the amount found, $n = 6$. Mean (relative standard deviation) for recovery, $n = 6$.

Excellent linearity was found for all of the analytes in the peak areas for the concentrations tested. The LOD and LOQ values were experimentally verified by the LOD and LOQ concentrations of the standard solutions of rutin, quercetin and bixin injected. These results indicate that the proposed HPLC method is sufficiently selective to quantify bixin and phenolic compounds in extracts of annatto seeds.

The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of known quantities of standard reference compounds to the sample weighed of annatto seeds and analyzed using the optimal conditions. The percentage of recovery obtained by comparing the results from the original samples and the fortified samples are reported in Table 4. Since the recovery rates obtained were close to 100% in almost all cases, this method can be considered accurate.

The precision of the chromatographic system was tested by performing intra and inter-day multiple injections of a solution containing the standards of rutin, quercetin and bixin, and then checking the RSD of retention times and peak areas. Six injections were performed each day in two different days. The intra and inter-day RSD values for both retention times and peak areas (Table 5) indicate the high precision of the chromatographic system.

Stability was tested with the standard solutions. These were stored for two months in amber glass flasks under air at −36 °C prior to analysis. The analytes in solution did not show any appreciable change in chromatographic profile over the two-month period, showing a standard deviation of the concentration of 0.3 for rutin, 0.3 for quercetin and 1.4 for bixin. No degradation products were detected by HPLC–DAD.

The validation data indicated that the proposed HPLC method provides good linearity, sensitivity, procedure accuracy, system precision, as well as highlighting its suitability for the simultaneous analysis of phenolic compounds and bixin in annatto seeds.

3.2. Characteristics of annatto seeds

The commercial seeds of annatto used in this study presented 14.1 ± 1.9 mg bixin/g and 1.7 ± 0.05 mg GAE/g of phenolic compounds, both on wet basis, measured spectrophotometrically. Although the total phenolic compounds levels for annatto seeds were not found in the literature, the average concentration of bixin is reported to vary from 12 to 23 mg/g, depending on environmental factors such as temperature, illumination, rainfall, soil, and cultivar [22,23].

3.3. Optimization of simultaneous extraction of bixin and phenolic compounds using RSM

The concentration obtained at the RSM central point for bixin and total phenolic compounds revealed little variation (RSD = 7.2% and 11.2%, respectively) indicating a good repeatability of the extraction procedure. Table 2 shows the variation in bixin content (from 3.5 to 13.6 mg/g of seeds) and total phenolic content (from 1.3 to 3.1 mg GAE/g of seeds). The bixin determined, at the central point conditions, accounted for 77–90% of the bixin in the raw seeds. On the other hand, the total phenolic content of the extracts obtained by RSM showed that the extraction seems to be more efficient than that from the seeds (only methanol:water (8:2, v/v) without acetone), since their values exceeded the original value in more than 90% of the experiments. This efficiency can be attributed to the use of the acetone combined with methanol:water solution, as previously observed [24].

The efficiency of solvents in the extraction of bixin and total phenolic compounds was studied by Cardarelli et al. [6], who found that the polarity of the solvents is crucial and that bixin has greater affinity for solvents with medium polarity. In relation to the total phenolic compounds, minimum values were obtained with hexane (0.30 mg GAE/g) and maximum with

Table 5
Intra and inter-day precision values for retention times (t_R) and peak area obtained with extracts of annatto seeds.

Compound	Intra-day precision (n = 6, mean)								Inter-day precision (n = 12, mean)			
	Day 1				Day 2							
	t_R (min)	RSD (%)	Area (mAU)	RSD (%)	t_R (min)	RSD (%)	Area (mAU)	RSD (%)	t_R (min)	RSD (%)	Area (mAU)	RSD (%)
Rutin	12.4 ± 0.1	0.4	12,387 ± 210	1.7	12.4 ± 0.3	2.4	12,487 ± 299	2.4	12.4 ± 0.1	0.4	12,437 ± 870	7.0
Quercetin	18.1 ± 0.0	<0.1	39,105 ± 860	2.2	18.1 ± 0.1	0.5	38,095 ± 647	1.7	18.1 ± 0.1	0.3	38,600 ± 1582	4.1
Bixin	38.6 ± 0.1	0.1	3,737,612 ± 85,965	2.3	38.6 ± 0.1	0.2	3,827,653 ± 72,725	1.9	38.6 ± 0.1	0.1	3,782,633 ± 139,957	3.7

Experimental conditions described in Section 2. Mean ± standard deviation; RSD: relative standard deviation.

Table 6
Chromatographic and spectroscopic characteristics of compounds isolated from extracts of annatto seeds.

Peaks[a]	Compound	Concentration (μg/g)[b]	t_R range (min)	λ_{max} (nm)[c]	$[M+H]^+$ (m/z)	MS^2 (+) (m/z)[d]	$[M-H]^-$ (m/z)	MS^2 (−) (m/z)[d]
1	Hypolaetin	316.8 ± 5.6	13.5–13.8	321	303	**285 [M+H-18]$^+$**, 275 [M+H-28]$^+$, 257 [M+H-18-28]$^+$, 231 [M+H-72]$^+$	301	283 [M−H-18]$^-$, **257 [M−H-18-26]$^-$**, 229, 185
2	Caffeoyl acid derivative	228.6 ± 5.8	15.1–15.6	316	nd	365 [M+H-18]$^{+e}$, 347 [M+H-18-18]$^{+f}$, 305^f [M+H-18-18-42]$^{+f}$, **203 [M+H-18-162]$^{+f}$**, 185 [M+H-18-180]$^{+f}$	381	337 [M−H-44]$^-$, 313$^-$, 247, **201 [M−H-180]$^-$**, 179, 135
3	Bixin	21.353.5 ± 325.7	38.5–38.8	430, 459, 487	395	377 [M+H-18]$^+$, 363 [M+H-32]$^+$, **335 [M+H-32-28]$^+$**, 317 [M+H-32-28-18]$^+$, 282, 260, 209, 157	393	361 [M−H-32]$^-$, **349 [M−H-44]$^-$**, 317 [M−H-32-44]$^-$

[a] Numbered according to the chromatogram shown in Fig. 2.
[b] Peaks 1 and 2 were quantified as equivalent to quercetin and peak 3 was quantified as bixin (five replicates for all compounds).
[c] Solvent: gradient of 2% formic acid in water and methanol with 2% formic acid.
[d] In the MS^2, the most abundant ion is shown in boldface.
[e] In-source detected fragment. nd: not detected.
[f] MS/MS from the fragment with 365u.

methanol:water (1.84 mg GAE/g), while intermediate levels of total phenolic compounds were obtained using ethanol and ethyl acetate [6].

The model proposed for bixin extraction (Eq. (2)) fits the experimental data, with $R^2 = 0.80$ (data available in Tables 1 and 2 of the Supplementary data). This indicates that the model can be used to predict responses, and represents adequately the effect of the independent variables:

$$\text{Bixin (mg/g)} = 11.62 - 0.53X_1^2 - 0.53X_2^2 + 0.93X_3 + 0.68X_4 + 0.71X_2X_3 + 1.06X_2X_4 - 1.73X_3X_4 \quad (2)$$

The model showed that for bixin extraction, the solid–liquid ratio and extraction time effects were significant ($p < 0.05$) and positive, i.e., there was an increase in the bixin content extracted as the solid–liquid ratio and the extraction time increased. On the other hand, an increase in the percentage of methanol:water in the acetone led to a decrease in bixin content extracted. This result was expected, since the official method published by the FAO/WHO [19] recommends acetone alone as the solvent for the extraction of bixin.

According to the response surface model (shown in Fig. 1 of Supplementary data) the best condition for bixin extraction was 15 extractions with 50% methanol:water (8:2, v/v) in acetone, solid–liquid ratio of 1:9 and extraction time of 5 min.

For the total phenolic compounds extraction, the effect of the number of extractions and the solid–liquid ratio were found to be significant ($p < 0.10$) and positive, i.e., the extraction efficiency improving at higher solid–liquid ratios and with the numbers of extraction. The results of the ANOVA (data available in Tables 1 and 2 of Supplementary data) reveal a relatively low coefficient of determination ($R^2 = 0.51$), so the model proposed (total phenolic (mg GAE/g) = $2.45 + 0.14X_2 + 0.28X_3$) is unable to explain adequately the behavior of the experimental data. However, the optimal conditions for the bixin extraction also gave a high yield of phenolic compounds. The response surface were plotted using the coefficients of the two significant linear variables to illustrate the tendency of the process; the optimum point for the phenolic compound extraction suggest a solid–liquid ratio close to 1:9 (available in Fig. 2 of Supplementary data).

3.4. Validation of RSM model for simultaneous extraction of bixin and phenolic compounds

The experimental validation of the models proposed for the simultaneous extraction of bixin and the phenolic compounds were performed, in triplicate, at the optimal conditions (50% of methanol:water (8:2, v/v) in acetone, 15 extractions, a solid–liquid ratio of 1:9 (m/v) and 5 min extraction). The bixin content was found to be 11.1 ± 0.3 mg/g (RSD = 3.2%) and the phenolic compounds was 1.9 ± 0.08 mg/g (RSD = 4.3%). There were no significant differences ($p < 0.05$) between the results of the optimization experiments and those predicted by the models, confirming the good prediction capacity of the models for the extraction of bixin and phenolic compounds from annatto seeds.

3.5. Application of the validated HPLC–DAD-MS/MS to annatto extracts

Fig. 2 presents the chromatogram of the extract obtained at the optimal conditions established by RSM. Two different compositions of the mobile phase were tested, one consisting of acetonitrile and

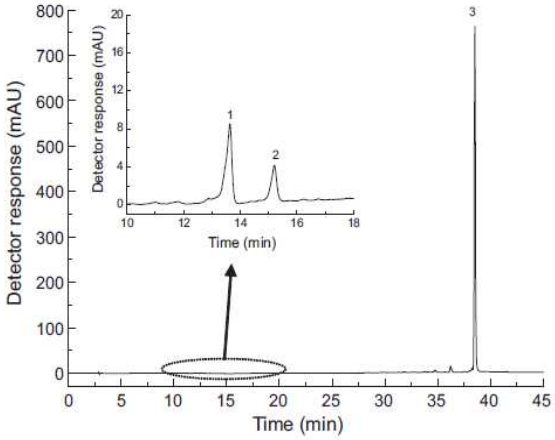

Fig. 2. Chromatogram obtained by HPLC–DAD of phenolic compounds and bixin from annatto seed extracts. Chromatographic conditions: see text. Processed at 459 nm; figure inset processed at 320 nm (peak characterization is given in Table 6).

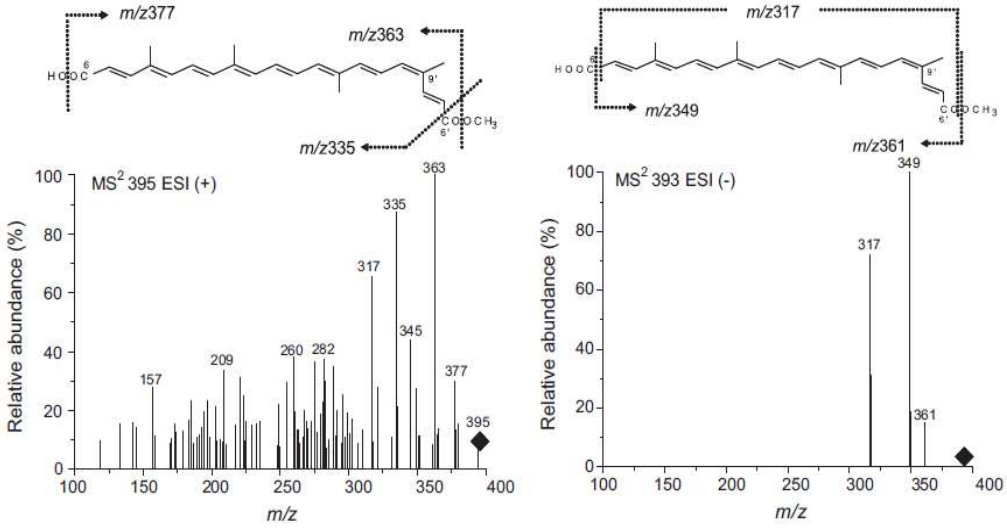

Fig. 3. MS^2 spectra of bixin obtained on ion trap instrument equipped with ESI source (positive and negative ion mode) showing main fragmentation pattern.

water both with 2% formic acid (data not shown) and the other of methanol and water both with 2% formic acid. The applied chromatographic elution method (see Section 2) with the two organic solvents led to the separation of 2 phenolic compounds, as well as carotenoids, especially bixin. The discussion focuses on the identification of the two phenolic compounds and the carotenoid bixin.

Peak 1 was tentatively identified as a pentahydroxyflavone, MW 302, based on UV-visible and MS spectra features (Table 6). In the positive ion mode, peak 1 revealed the presence of the protonated molecule $[M+H]^+$ at m/z 303, and the MS/MS showed consecutive losses of H_2O (m/z 285), CO (m/z 275), CO_2 (m/z 209), H_2O+CO (m/z 257), and $CO+CO_2$ (m/z 231) from the protonated molecule. The molecular weight was confirmed by the negative ion mode spectrum, with the deprotonated molecule ($[M-H]^-$) at m/z 301 and the MS/MS showing neutral losses of H_2O at m/z 283, and of $H_2O+C_2H_2$ at m/z 257. Such fragmentations are typically observed for flavones and hydroxyflavones [25–27]. Since Harbone [7] reported the presence of hypolaetin derivatives in annatto leaves, peak 1 was identified as hypolaetin (Fig. 1).

Peak 2 was tentatively identified as a caffeoyl acid derivative, MW 382, with a deprotonated molecule $[M-H]^-$ at m/z 381 in the negative ion mode, whereas in the positive ion mode the protonated molecular ion was not observed, but a strong in-source fragmentation was observed at m/z 365 $[M+H-18]^+$. According to Table 6, the identification was confirmed by the fragments obtained from the ion at m/z 365, such as neutral losses of H_2O (m/z 347), of $H_2O+C_2H_2O$ (m/z 305), of caffeoyl moiety (m/z 203), and of caffeic acid (m/z 185), all in the positive ion mode. Moreover, in the negative ion mode, the MS/MS showed neutral losses of CO_2 (m/z 337) and caffeic acid (m/z 201) from the deprotonated molecule (m/z 381). The specific structure of the compound eluted as peak 2 could not be determined. However, these fragmentations are typically observed for caffeoyl acid derivatives [28–30].

Peak 3 was identified as bixin (MW 394), the major carotenoid present in annatto seeds. In the positive ion mode (Table 6), peak 3 showed the protonated molecule at m/z 395 and MS^2 showing losses of H_2O (m/z 377), CH_3OH (m/z 363), CH_3OH+CO (m/z 335) and $CH_3OH+CO+H_2O$ (m/z 317) (Fig. 3). The negative ion mode spectrum confirmed the assignment of the molecular weight, with the deprotonated molecule ($[M-H]^-$) at m/z 393 and MS/MS showing losses of CH_3OH (m/z 361), $COO^\bullet$ (m/z 349) and $CH_3OH+COO^\bullet$ (m/z 317) (Fig. 3). This fragmentation pattern for bixin has already been reported in the literature [31–33]. The identification of peak 3 as bixin was also confirmed by co-elution with bixin standard (98% purity), and by the UV-visible features (%III/II = 33 and $\%AB/A_{II}$ = 10%), in accordance with many studies [5,17,34].

4. Conclusions

In summary, the proposed method for the simultaneous extraction, separation, identification and quantification of phenolic compounds and bixin were successfully validated. The optimized procedure for simultaneous extraction of these compounds by response surface methodology provided the best conditions to extract phenolic compounds and bixin from annatto seeds. Additionally, hypolaetin and caffeoyl acid derivative were identified for the first time in annatto seeds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.094.

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Supplementary Material

Simultaneous extraction and analysis by high performance liquid chromatography coupled to diode array and mass spectrometric detectors of bixin and phenolic compounds from annatto seeds

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Supplemental Table 1. Regression coefficients of predicted quadratic polynomial models for the simultaneous extraction responses of levels of bixin and phenolic compounds from annatto seeds.

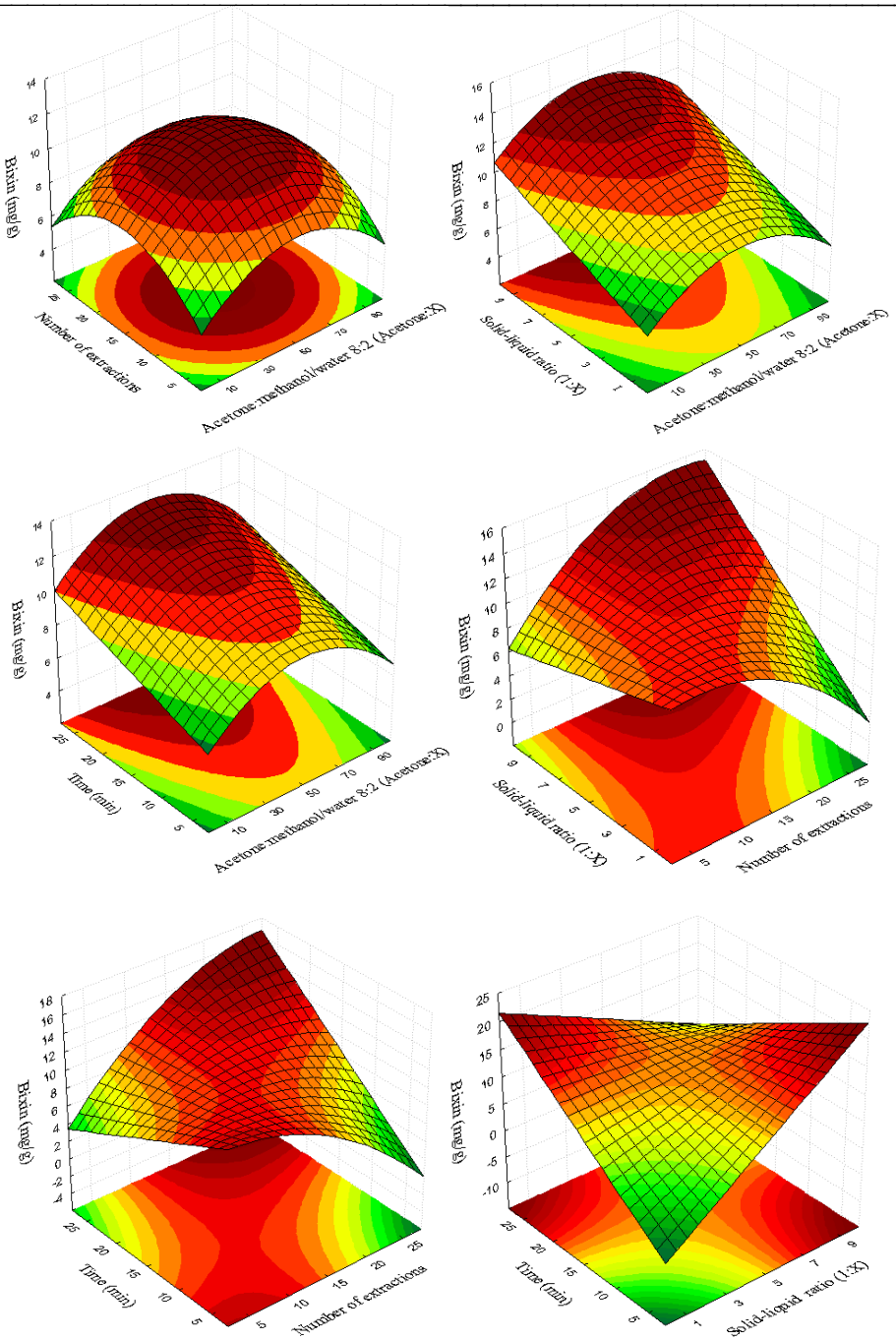
	Bixin	Total phenolic compounds
Mean	11.62*	2.45*
X_1 (L)	-0.43**	0.05
X_1 (Q)	-0.53*	-0.03
X_2 (L)	-0.14	0.14**
X_2 (Q)	-0.53*	-0.02
X_3 (L)	0.93*	0.28*
X_3 (Q)	0.06	-0.09
X_4 (L)	0.68*	-0.03
X_4 (Q)	-0.29	-0.03
X_1X_2	0.26	-0.05
X_1X_3	0.32	0.02
X_1X_4	-0.07	0.04
X_2X_3	0.71*	-0.07
X_2X_4	1.06*	0.01
X_3X_4	-1.73*	0.06

X_1 = acetone in methanol/water (%), X_2 = number of extractions, X_3 = solid-liquid ratio (m/v), X_4 = time (min), X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 are interactions between the four variables. (L): linear, (Q): quadratic. *Statistically significant ($p<0.05$). ** Statistically significant ($p<0.10$).

Supplemental Table 2. ANOVA for the effect of amount of methanol/water (8:2, v/v) in acetone, number of extractions, solid-liquid ratio and time on the contents of bixin and total phenolic compounds using a quadratic response surface model.

	Variation source	Sum of squares	Degrees of freedom	Mean square	F_{value}	R^2
Bixin	Regression	116.5	7	16.6	11.9	0.8063
	Residues	27.9	20	1.4		
	Total	144.5				
TPC	Regression	2.3	2	1.1	13.1	0.5113
	Residues	2.2	25	0.1		
	Total	4.6				

TPC: total phenolic compounds. $F_{0.05;\,7;\,20} = 2.51$; $F_{0.1;\,2;\,25} = 2.53$.



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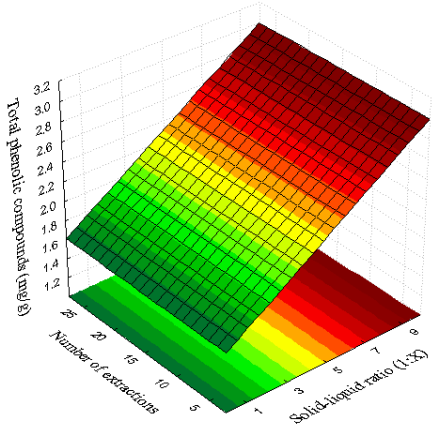
Supplemental Figure 1. Response surfaces obtained for bixin (mg/g) from annatto seed extracts by the predictive model of the central rotational design 2^4.



Supplemental Figure 2. Response surface obtained for total phenolic compounds (mg/g) from annatto seed extracts by the predictive model of the central rotational design 2^4.

CAPÍTULO III

Effect of solvent type on the extractability of bioactive compounds, antioxidant capacity and colour properties of natural annatto extracts

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Original article

Effect of solvent type on the extractability of bioactive compounds, antioxidant capacity and colour properties of natural annatto extracts

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Summary This study was conducted to obtain annatto extracts with both high antioxidant capacity and colour potential using solvents of different polarities (water, ethanol/water, ethanol, ethanol/ethyl acetate and ethyl acetate). The highest levels of total phenolic compounds were found in the water, ethanol/water and ethanol extracts (0.5 mg GAE mL^{-1}), and the highest level of bixin was found in the ethanol/ethyl acetate extract (5.2 mg mL^{-1}), which was characterised as the reddest and the most vivid one (a^* = 40.5, h° = 46.1, C^* = 58.4). The ethanol/ethyl acetate extract also showed the highest antioxidant activity (4.7 μM TEAC mL^{-1}) and the highest percentage of tryptophan protection against singlet oxygen (63.6%). On the other hand, ethyl acetate and ethanol/water were the least effective solvents for the extraction of phenolic compounds and bixin, respectively. According to the multivariate statistical analysis, ethanol/ethyl acetate and ethyl acetate were the most promising solvents to obtain annatto extracts with both antioxidant and colour properties.

Keywords *Bixa orellana*, bixin, colour, hierarchical cluster analysis, phenolic compounds, principal components analysis.

Introduction

Annatto is a natural colouring agent obtained from the outer coats of the seeds of the tropical shrub *Bixa orellana*. Annatto and its extracts are designated collectively as E160b and permitted as a food additive all around the world (Scotter, 2009). Brazil is one of the largest producers and exporters of seeds and annatto preparations (Balaswamy *et al*., 2006), which are used in the food, pharmaceutical and cosmetic industries.

Bixin (methyl (9-*cis*)-hydrogen-6,6′-diapo-Ψ,Ψ-carotenedioate) is the main carotenoid responsible for the orange-red colour in the seeds and extracts of annatto (Fig. 1), representing approximately 80% of the total carotenoids (Preston & Rickard, 1980). Besides the colourant property, bixin is known to be a very efficient quencher of singlet oxygen ($^1O_2$) and triplet state of sensitisers (Montenegro *et al*., 2004; Rios *et al*., 2007). In photosensitised reactions, photons are absorbed by a sensitiser (S), resulting in a long-lived, energy-rich state(s) (typically triplet state) of the sensitiser ($^3S^*$) (eqn 1). The $^3S^*$ can react directly with molecular oxygen ($^3O_2$) giving singlet molecular oxygen ($^1O_2$) (eqn 2). Both reactive species ($^3S^*$ and $^1O_2$) can be efficiently quenched by carotenoids (Bix, bixin in this case) because of an efficient energy-transfer process, resulting in the carotenoid triplet state ($^3Bix^*$) (eqns 3 and 4). Once formed, $^3Bix^*$ returns harmlessly to its ground state (Bix) with the liberation of heat (eqn 5). In general, the reactive quenching reaction between $^1O_2$ and carotenoids, yielding oxidation products, is several orders of magnitude smaller than the physical quenching process (eqn 4). The bleaching of all-*trans* carotenoids by $^1O_2$-mediated oxidation is only commonly observed after prolonged photosensitisation periods.

$$S \xrightarrow{hv} {}^3S^* \quad (1)$$

$${}^3S^* + {}^3O_2 \rightarrow S + {}^1O_2 \quad (2)$$

$${}^3S^* + Bix \rightarrow S + {}^3Bix^* \quad (3)$$

$${}^1O_2 + Bix \rightarrow {}^3O_2 + {}^3Bix^* \quad (4)$$

$${}^3Bix^* \rightarrow Bix \quad (5)$$

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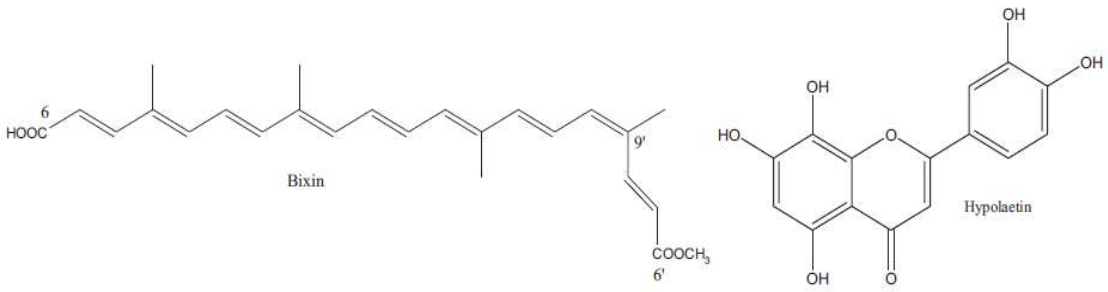

Figure 1 Chemical structure of bixin ($C_{25}H_{30}O_4$) and hypolaetin ($C_{15}H_{10}O_7$).

Besides the presence of carotenoids, annatto seed extracts also contain phenolic compounds, namely hypolaetin (Fig. 1), as the major compound, and a caffeoyl acid derivative, as the minor one (Chisté *et al.*, 2011a). The levels of phenolic compounds in annatto seed extracts were 20–30 times lower than the bixin contents (Chisté *et al.*, 2011b). Therefore, annatto extracts can be used as an easy accessible source of natural antioxidants, because they showed an efficient scavenger capacity against the reactive oxygen and nitrogen species (Chisté *et al.*, 2011b). Carotenoids and phenolic compounds have been associated with the reduction in the risks of various chronic degenerative disorders, such as cancer, inflammations, cardiovascular diseases, cataracts and macular degeneration (Krinsky, 1994; Serdula *et al.*, 1996; Huang *et al.*, 2005). In general, these risk-preventing effects are claimed to be related to the inhibition of oxidation reactions. In parallel, the food can benefit from the addition of these natural compounds, because of their potential antioxidant, preventing the oxidation of foods and raw foods, such as oils, meat, milk and dairy products, extending its shelf life (Decker *et al.*, 2005).

In addition, natural extracts may show, depending on the type of extraction and solvent employed, other compounds with beneficial actions to human health, in opposite to the colouring agents obtained by chemical synthesis with a degree of high purity. Our research group produced annatto extracts with different solvents (methanol, ethanol, ethyl acetate and hexane) and performed the classification based on their antioxidant (TEAC assay) and colourant properties (Cardarelli *et al.*, 2008). Ethanol and ethyl acetate are regarded as presenting more applicability for food use considering the good efficiency in the extraction of bixin and phenolic compounds (Cardarelli *et al.*, 2008) and lower toxicity and risk to human health (FDA, 1997). Because the main food industry application of annatto preparations is directed to impart colour to several products, such as butter, cheese, bakery products, oils, ice creams, sausages, cereals and extruded products (Rios & Mercadante, 2004), which may possess natural photosensitisers in its composition, the protection against singlet oxygen is an important issue in such products. Therefore, the aim of this study was to obtain the extracts from annatto seeds with both high antioxidant properties, including protection against singlet oxygen, and colour parameters by the extraction using solvents with different polarities, all allowed to be applied in the food industries.

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2′azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)), potassium persulphate and methylene blue (MB) were purchased from Sigma-Aldrich (Steinheim, Germany). L-Tryptophan was purchased from Fisher Scientific (Pittsburgh, PA, USA), and Folin-Ciocalteau reagent, from Dinamica (São Paulo, Brazil). Dichloromethane, methanol, ethanol, ethyl acetate, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate (NaH_2PO_4) and potassium phosphate (KH_2PO_4) were all of analytical grade and purchased from Synth (São Paulo, Brazil). The gallic acid standard was purchased from Extrasynthèse (Lyon Nord, France).

Sample

In August 2008, annatto seeds were obtained from the local market in Campinas, São Paulo, Brazil (22°50′S; 47°00′W). The seeds were portioned (500 g), vacuum-packed and stored under light-free conditions at room temperature until analysis.

Preparation of annatto extracts

Triplicate extractions were performed in a completely randomised design, using the following solvents: water, ethanol/water (1:1, v/v), ethanol, ethanol/ethyl acetate (1:1, v/v) and ethyl acetate. These solvents were chosen considering the permissibility of residues in the extracts

after evaporation, according to the Commission Directive 95/45/EC. (1995) from European Communities. The intact annatto seeds were weighed (20 g), and the solvents were added in the mass/solvent ratio of 1:2 (m/v) and stirred on an orbital shaker MA 140/CFT (Marconi, São Paulo, Brazil) at 168 r.p.m. for 15 h at room temperature (25 °C). After the extraction time, only the liquid extract was transferred to a volumetric flask and filled with the respective solvent to 50 mL. Each liquid annatto extract was transferred to ambar flasks, sealed under N_2 flow and stored at −36 ºC until analysis.

Bixin quantification

In a previous study, bixin separated by HPLC comprised from 80% to 96% of the total peak area of the carotenoids in annatto extracts (Chisté *et al.*, 2011b). Thus, bixin concentration in seeds and annatto extracts was determined using the methodology described by FAO/WHO (1982). Because bixin is located on the outer coats of the seeds, the exhaustive extraction was performed by washing the intact seeds (1 g) with dichloromethane until the seeds were colourless, the extracts were pooled, and the volume was made up to 100 mL with the same solvent. For the extracts obtained with solvents of different polarities, aliquots of 0.1 mL were evaporated under N_2 flow and resuspended in 10 mL of dichloromethane. Absorbance was measured with an UV–Visible spectrophotometer (Agilent, Santa Clara, CA, USA) at 487 nm, and bixin concentration was calculated according to the Lambert–Beer's law, using $E^{1\%}_{1\,cm} = 2826$ (FAO/WHO, 1982). All measurements were taken in triplicate.

Total phenolic compounds quantification

For the annatto seeds, 5 g of intact seeds was weighed and extracted five times with methanol/water (8:2, v/v) in ultrasound equipment (Unique model, São Paulo, Brazil) for 10 min at 25 °C. After extraction, the extract was evaporated under vacuum ($T < 40$ °C), dissolved with 5 mL of methanol, transferred to 25-mL volumetric flask and filled with distilled water. For the extracts obtained according to item 2.3, an aliquot (2.5 mL) was evaporated under N_2 flow, resuspended in 2.5 mL of methanol, transferred to 10-mL volumetric flask and filled with distilled water. These extracts were then kept in the freezer for 20 min before centrifugation at 290 ***g*** for 20 min. The total phenolic compound content from seeds and extracts was determined using the Folin–Ciocalteau colorimetric method (Singleton *et al.*, 1999), with an UV–visible spectrophotometer at 750 nm, and the results were expressed as milligrams of gallic acid equivalent (GAE) per mL of extract or g of seeds. All measurements were taken in triplicate.

Colour measurements

Colour measurements in annatto extracts were taken using a spectrocolorimeter Color Quest XE (HunterLab, Reston, VA, USA) using reflectance with specular excluded, equipped with the light source D65 and observation angle of 10°. Using the values obtained in the CIELAB system, L^* (lightness), the chromatic coordinates a^* (red-green component) and b^* (yellow-blue component), and the values of C^* (chroma) and $h°$ (hue angle) were calculated according to eqns 6 and 7. All measurements were taken in triplicate with the crude extract.

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2} \tag{6}$$

$$h° = \arctan\left(\frac{b^*}{a^*}\right) \tag{7}$$

In vitro antioxidant capacity determination

Scavenging capacity

The scavenging capacity against $ABTS^{\cdot+}$ radical was determined according to the method described by Re *et al.* (1999). The radical cation $ABTS^{\cdot+}$ (7 mM solution) was formed by chemical reaction with potassium persulphate (2.45 mM). In a cuvette, 1980 μL of radical cation $ABTS^{\cdot+}$ solution and an aliquot of 20 μL of the different annatto extracts or of the Trolox standard solution were added. The absorbance was determined at 734 nm after 10 min using an UV–visible spectrophotometer. The results were analysed through the inhibition percentage curves as a function of extracts or Trolox standard solution (0.3–1.5 mM) concentration, and the free radical scavenging capacity was expressed as Trolox equivalent antioxidant activity (TEAC). All measurements were taken in triplicate.

Percentage of protection against singlet oxygen ($^1O_2$)

The percentage of protection against $^1O_2$ was evaluated according to methods described by Montenegro *et al.* (2004) with adaptations. The reactions were performed using 950 μL of L-tryptophan (TRP) (100 μM) as the actinometer and 950 μL of MB (10 μM) as sensitiser, both solutions prepared in phosphate-buffered saline (pH 7.2). In addition, an aliquot (100 μL) of the different annatto extracts was evaporated under N_2 flow, resuspended in 100 μL of ethanol/ethyl acetate (1:1, v/v) and added to the solution under moderate agitation, air atmosphere and temperature set at 25 ± 1 °C. The blank was maintained under the same conditions, replacing 100 μL of annatto extract by 100 μL of ethanol/ethyl acetate (1:1, v/v). The excitation source used was a 150-W filament lamp coupled

with red and orange cut-off filters to avoid direct excitation of the carotenoids. The excitation light (> 620 nm) was focused into the sample cell at right angle, and the intensity decay of fluorescence emission of TRP (excitation at 276 nm and emission at 356 nm) was monitored during 20 min using a fluorescence spectrophotometer (Varian Cary Eclipse, Santa Clara, CA, USA). Kinetics data obtained from the intensity decay of TRP fluorescence emission were fitted to a first-order reaction (eqn 8), and the rate constants were calculated (eqn 9). The protection percentage that annatto extracts (EXT) offered to the actinometer (TRP) was calculated through eqn 10.

$$Y = Y_{\infty} + A.\exp^{(-k.x)} \tag{8}$$

$$k = \frac{\ln 2}{t_{1/2}} \tag{9}$$

$$\text{Protection } (\%) = \frac{k_{obs}^{TRP} - k_{obs}^{TRP+EXT}}{k_{obs}^{TRP}} \times 100 \tag{10}$$

where Y is the intensity of TRP fluorescence; Y_{∞} is the intensity of TRP fluorescence at infinite time; A is pre-exponential factor; k is pseudo-first-order rate constant; x is reaction time; $t_{1/2}$ is half-life (min); k_{obs}^{TRP} is the observed pseudo-first-order rate constant fitted to TRP decay curve (obtained in the blank experiment); and $k_{obs}^{TRP+EXT}$ is the observed pseudo-first-order rate constant fitted to TRP decay curve in the presence of annatto extract (EXT).

Stability of annatto extracts

After extraction, all annatto extracts were stored at −36 °C, and to verify the stability of bixin and phenolic compounds in solution, the contents of these compounds were evaluated during 8 months. For this purpose, two extracts with opposite polarities were selected: the least polar one – ethyl acetate (EtOAc) – and the most polar one – water (H_2O). The quantification of bixin and phenolic compounds was performed, in triplicate, according to the methodologies previously described (FAO/WHO, 1982; Singleton *et al.*, 1999).

Statistical analysis

The results (mean ± SD) of bixin, phenolic compounds, antioxidant capacity and colour parameters were analysed with the Statistica 6.0 software (Statsoft Inc., 2001) using nonlinear regression analysis, analysis of variance (ANOVA) and Tukey test ($P < 0.05$). In addition, two multivariate exploratory techniques, principal components analysis (PCA) and hierarchical cluster analysis (HCA), were applied for characterisation of the extracts, using the Statistica 6.0 software package. For PCA, colour parameters (L^* and C^*) and bixin and phenolic compounds were the active variables used in the derivation of the principal components; the supplementary variables (antioxidant capacity responses from two methodologies and the chemical characteristics of the solvents) were projected onto the factor space. The hierarchical tree was obtained considering the active variables of PCA and the extracts were joined by unweighted pair-group average as the linkage rule, considering the Euclidian distances as the coefficient of similarity.

Results and discussion

Characteristics of annatto seeds

The commercial annatto seeds used in this study presented 14.9 ± 0.5 mg bixin g^{-1} of seeds and 1.7 ± 0.1 mg GAE g^{-1} of seeds for total phenolic compounds, both in wet basis (moisture of 10.2 ± 0.1%). The average concentration of bixin is reported to vary from 12 to 23 mg g^{-1} seeds, depending on edaphic–climatic (such as temperature, illumination, rainfall and soil) and genetic (cultivar) factors (Shuhama *et al.*, 2003). Cardarelli *et al.* (2008) and Costa & Chaves (2005) found lower levels of bixin (7.5 and 11.8 mg g^{-1}) in annatto seeds collected in Campinas and Teresina (Piauí, Brazil), respectively. However, Balaswamy *et al.* (2006) reported values varying from 10 to 17 mg bixin g^{-1} in annatto seeds from India, after applying different extraction procedures. The phenolic compound level detected in the annatto seeds was similar to that reported by Chisté *et al.* (2011a,b).

Stability of annatto extracts under storage

The levels of bixin and total phenolic compounds in the solutions tested (ethyl acetate and water extracts) were not statistically different ($P < 0.05$) during storage for eight months at −36 °C (Fig. 2), showing that these compounds are highly stable in solution in both polar and nonpolar solvents. Balaswamy *et al.* (2006) observed 15% loss of bixin in annatto seeds stored during 360 days at 30 ºC. The same authors also reported that the stability was higher when bixin was added to an oil solution and stored in the dark at lower temperature (5–8 °C).

Characteristics of annatto extracts obtained by different solvents

According to Table 1, the extracts obtained with water, ethanol/water mixture and ethanol presented the highest values of total phenolic compounds with no statistical difference ($P < 0.05$) between them. Considering the

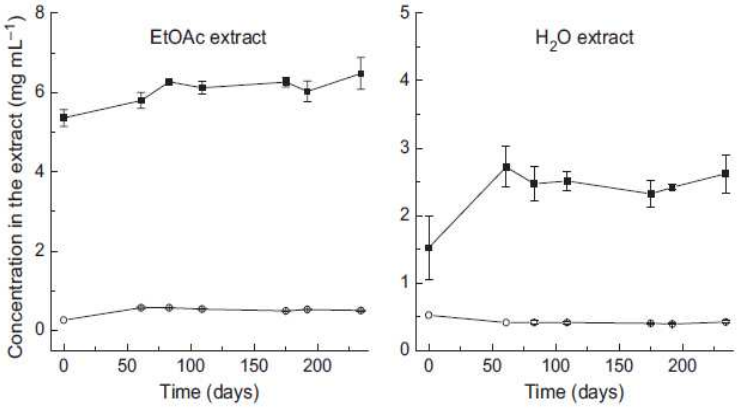

Figure 2 Stability of bixin (■) and total phenolic compounds (○) in annatto extract solutions stored at −36 °C.

content of total phenolic compounds found in annatto seeds (1.7 mg GAE g^{-1}), these solvents showed the highest efficiency in the extraction of phenolic compounds (69–77%), whilst the lowest yield was obtained with ethyl acetate (39%).

In relation to the bixin contents, the extracts obtained with ethanol, ethanol/ethyl acetate mixture and ethyl acetate showed the highest values, with no statistical difference ($P < 0.05$) between them. Based on the bixin level found in annatto seeds (14.9 mg bixin g^{-1}), these solvents showed the highest yield of bixin (77–87%), whilst ethanol/water mixture presented the lowest efficiency (4%). The structure of bixin with 25 carbons and an acid and methyl ester end-groups is more polar than the carotenoids usually found in foods (chain with 40 carbons) and showed more affinity for medium polar solvents (Cardarelli *et al.*, 2008).

The characteristics of the solvents used in this study, such as solvent polarity index (SPI) and the solvent selectivity triangle, described by Snyder *et al.* (1993) (Table 2), were considered to explain the extraction efficiency of phenolic compounds and bixin from annatto seeds. Considering the results of Tables 1 and 2, in general, the extraction of bioactive compounds in annatto seeds seems to be dependent on the polarity of the solvent applied, i.e. the higher the polarity of solvent, the higher the extraction of phenolic compounds (more polar compounds) and, on the other hand, the lower the polarity, the higher the extraction of bixin (less polar compound). The selectivity triangle, in which organic solvents are classified according to their ability to interact with the solute either as a dipole (dipolarity, π*), as a proton acceptor (acidity, α), or as a proton donor (basicity, β), (Table 2), also explained

Table 1 Total phenolic compounds (TPC), bixin, CIELAB parameters, free radical scavenging as Trolox equivalent antioxidant activity (TEAC) and protection percentage against singlet oxygen of annatto extracts obtained with solvents of different polarities

	Annatto extracts				
Characteristics	**H_2O**	**EtOH/H_2O**	**EtOH**	**EtOH/EtOAc**	**EtOAc**
Bioactive compounds					
TPC (mg GAE mL^{-1})	0.47 ± 0.05^{ab}	0.46 ± 0.04^{ab}	0.52 ± 0.05^{a}	0.36 ± 0.03^{bc}	0.26 ± 0.06^{c}
Bixin (mg mL^{-1})	1.84 ± 0.29^{b}	0.23 ± 0.02^{c}	4.63 ± 0.35^{a}	5.20 ± 0.42^{a}	4.64 ± 0.74^{a}
Colour parameters (CIELAB)					
*L**	28.90 ± 0.05^{a}	20.72 ± 0.81^{b}	28.56 ± 1.43^{a}	27.93 ± 0.44^{a}	30.08 ± 1.63^{a}
*a**	36.83 ± 1.00^{a}	24.24 ± 1.54^{b}	36.41 ± 3.09^{a}	40.49 ± 0.95^{a}	38.67 ± 3.16^{a}
*b**	37.17 ± 1.83^{ab}	26.81 ± 3.30^{b}	37.74 ± 2.35^{ab}	42.12 ± 1.87^{a}	37.90 ± 8.86^{ab}
h°	43.83 ± 3.57^{a}	47.70 ± 1.83^{a}	46.06 ± 0.76^{a}	46.11 ± 0.73^{a}	43.09 ± 3.87^{a}
*C**	52.33 ± 1.75^{a}	36.13 ± 3.41^{b}	52.45 ± 3.38^{a}	58.43 ± 1.73^{a}	54.26 ± 7.26^{a}
Antioxidant capacity					
TEAC (μM of Trolox equivalent mL^{-1} of extract)	3.63 ± 0.08^{b}	3.18 ± 0.19^{b}	4.71 ± 0.16^{a}	4.74 ± 0.05^{a}	4.63 ± 0.03^{a}
Protection against singlet oxygen ($^1O_2$) (%)	20.12 ± 0.91^{c}	8.53 ± 0.51^{d}	45.14 ± 2.45^{b}	63.62 ± 2.22^{a}	49.31 ± 2.71^{b}

Mean ± SD (three independent experiments).

Means with the same superscript letters at the same line do not present statistical difference ($P < 0.05$).

H_2O, distilled water; EtOH/H_2O, ethanol/water (1:1, v/v); EtOH, ethanol; EtOH/EtOAc, ethanol/ ethyl acetate (1:1, v/v); EtOAc, ethyl acetate; GAE, gallic acid equivalent.

Table 2 Characteristics of solvents used for the preparation of annatto extracts

	Characteristics			
		Normalised selectivity factors†		
Solvents	SPI*	π*	α	β
H_2O	9.0	0.45	0.43	0.48
EtOH/H_2O	7.1	0.35	0.41	0.42
EtOH	5.2	0.25	0.39	0.36
EtOH/EtOAc	4.7	0.40	0.19	0.41
EtOAc	4.3	0.55	0.00	0.45

*SPI, solvent polarity index (Snyder, 1974).

†π*, dipolarity, α, acidity and β, basicity (Snyder *et al.*, 1993).

The normalised selectivity factors for the mixtures EtOH/H_2O and EtOH/EtOAc were calculated considering the solvent proportion and the respective values for pure solvents.

H_2O, distilled water; EtOH/H_2O, ethanol/water (1:1, v/v); EtOH, ethanol; EtOH/EtOAc, ethanol/ethyl acetate (1:1, v/v); EtOAc, ethyl acetate.

the yield obtained for the different compounds with the different solvents. The solvents that presented predominant property of acidity (water, ethanol/water and ethanol) showed to be more efficient for the extraction of phenolic compounds. However, none of the solvent characteristics alone explained the bixin yield found in all different extracts. The fact that the amount of bixin in the water extract was higher than that found in the ethanol/water mixture indicated that predominant dipolar property might play an important role in the extraction of bixin with water-based solvents. Because the dipolarity value of water is higher than that of the ethanol/water mixture, water was able to extract higher amounts of bixin than the ethanol/water mixture.

According to the colour parameters (Table 1), the similar values of $h°$ (43.1–47.7) located all annatto extracts in the CIELAB space corresponding to the orange colour. In addition, no statistical difference ($P < 0.05$) was verified for L^* and a^* values in all extracts, excepting for the ethanol/water mixture. The ethanol/water extract, which presented the lowest bixin content, was the darkest ($L^* = 20.7$), the least red ($a^* = 24.2$) and vivid ($C^* = 36.1$). On the other hand, the extract obtained with ethanol/ethyl acetate mixture, which showed the highest bixin level, was characterised by a high intensity of red ($a^* = 40.5$; $h° = 46.1$) and vivid colour ($C^* = 58.4$).

In relation to the TEAC assay, all extracts showed the same kinetic profile, with fast absorbance decay of $ABTS^{\cdot+}$ after 1 min, followed by no significant changes in absorbance after 10 min. The TEAC values of ethanol, ethanol/ethyl acetate and ethyl acetate annatto extracts were higher, with no statistical difference ($P < 0.05$) between them, as compared to those found in water and ethanol/water ones (Table 1). Because the bixin contents in ethanol, ethanol/ethyl acetate and ethyl acetate extracts were from 9 to 15 times greater than the contents of total phenolic compounds, bixin most likely contributes with greater influence than phenolic compounds to the antioxidant capacity of annatto extracts. Because ABTS assay is based on a single electron transfer reaction between the tested antioxidant and the formed radical (Huang *et al.*, 2005), the carotenoid bixin is able to scavenge the radical cation $ABTS^{\cdot+}$ most probably due to the presence of resonant electrons in its many conjugated double bonds (eleven in the bixin structure). The TEAC values of annatto extracts found in the present study were higher than those previously reported for annatto seed extracts (Cardarelli *et al.*, 2008) for EtOH/H_2O (0.80), EtOH (0.53) and EtOAc (0.41), expressed in μM of Trolox equivalent mL^{-1} of extract.

Regarding the percentage of protection against singlet oxygen, illustrated in Fig. 3a, all annatto extracts presented an excellent fit to the first-order reaction ($R^2 = 0.99$) for the fluorescence intensity decay of

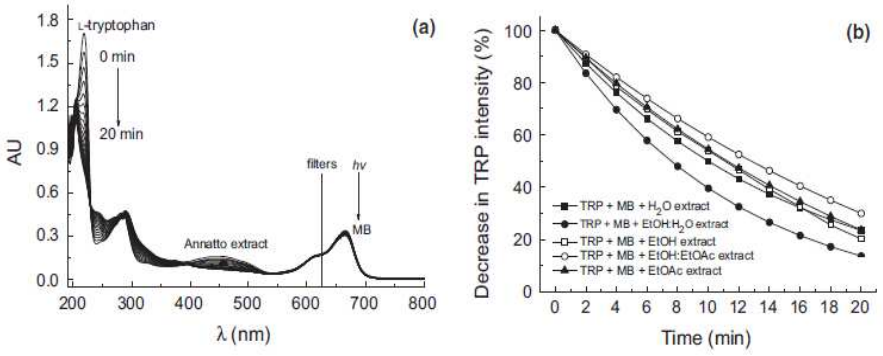

Figure 3 (a) Illustration of the experimental system for the measurement of protection against singlet oxygen, accompanied by a spectrophotometer and (b) fluorescence intensity decay of tryptophan (TRP) in the presence of methylene blue (MB) and annatto extracts.

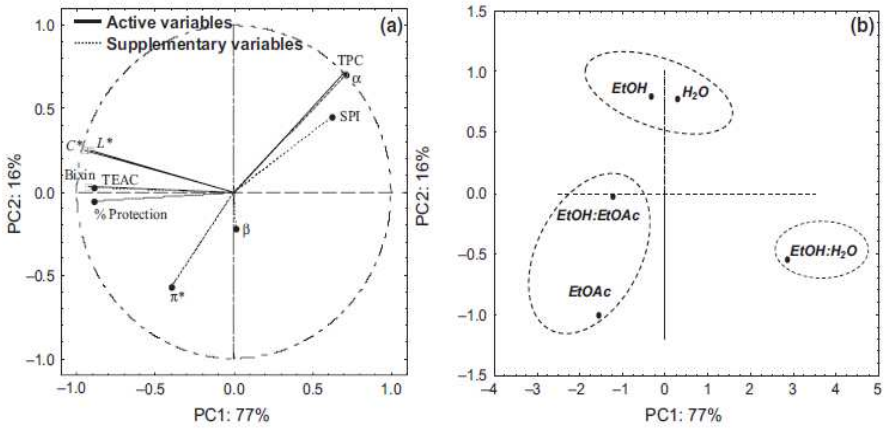

Figure 4 PCA plot of composition, colour and antioxidant properties of the different annatto extracts. (a) Variable projection and (b) scatterplot for the cases with suggested drawn grouping ellipses.

tryptophan, in the presence of the sensitiser and annatto extracts, as shown in Fig. 3b. The extract obtained with ethanol/ethyl acetate showed the highest percentage of protection (63.6%), i.e. this extract delayed more efficiently the degradation of tryptophan by the singlet oxygen generated by the photosensitisation of MB. As expected, the highest value of protection was associated with the highest level of bixin in the annatto extract. Consequently, the half-life of tryptophan was superior in the ethanol/ethyl acetate mixture extract (22.6 min), followed by the ethyl acetate (17.7 min), ethanol (12.4 min), water (10.8 min) and ethanol/water (8.2 min) extracts.

It is well known that bixin is an efficient quencher of both excited triplet state of sensitiser and singlet oxygen (Montenegro *et al.*, 2004; Rios *et al.*, 2007). Rios *et al.* (2007) showed that bixin is a very efficient $^1O_2$ quencher in fluid solutions because of an efficient energy-transfer process and confirmed that the energy level of bixin triplet state is lower than that of $^1O_2$ (18 and 22.5 kcal mol^{-1}, respectively).

Classification of annatto extracts by multivariate statistical analysis

In the PCA analysis, the first two components accounted for 93% of the explained variance. A positive correlation between bixin contents and the colour parameters L^* and C^* ($r = 0.75$ and $r = 0.87$, respectively) was observed. Bixin contents in annatto extracts were also positively correlated with TEAC values ($r = 0.99$) and percentage of protection ($r = 0.98$), whilst the contents of phenolic compounds did not show any positive correlation with both antioxidant capacity methods ($r = -0.56$ and $r = -0.62$, respectively) (Fig. 4a). Considering the correlations obtained by the PCA analysis, the bixin content presented the largest contribution to the antioxidant capacity in annatto extracts.

The phenolic compounds presented positive correlation with the acidity property ($r = 0.99$) and with a less extend to the SPI ($r = 0.69$), i.e. the higher the acidity and the SPI values, the higher the yield of total phenolic compounds in the extract. The polarity ($r = -0.80$), acidity ($r = -0.63$) and basicity ($r = -0.37$) properties of the solvents showed negative correlation with bixin yield, whilst dipolar value showed a weak positive correlation ($r = 0.08$). Thus, the extraction of bixin seems to be more efficient with solvents with both low values of SPI and of acidity property.

Therefore, the annatto extracts were divided into three groups (Fig. 4b): one group was formed by the extracts containing ethyl acetate (EtOAc); another, the

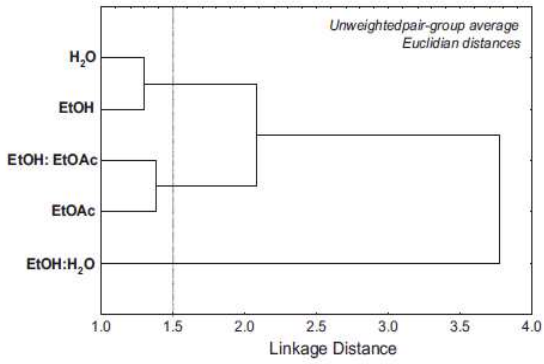

Figure 5 Dendrogram obtained by hierarchical cluster analysis analysis, considering the active variables of PCA for the different annatto extracts.

extracts containing only ethanol (EtOH) or water (H_2O); and the last one, by the extracts containing ethanol/water (EtOH:H_2O). Based on the PCA analysis, Figure 4b clearly shows the localisation of the extracts according to the solvent efficiency in the yield of colour (bixin and colour parameters) as PC1 and antioxidant capacity of the extracts (TEAC and percentage of protection against $^1O_2$) as PC2. The annatto extracts obtained with ethyl acetate were characterised by the highest percentage of protection against the singlet oxygen, highest TEAC values and the best colour intensity, probably, again, due to the presence of high levels of bixin.

Figure 5 shows the dendrogram obtained when HCA was applied taking into consideration the the active variables of PCA. The tree diagram provides evidence for three groups, which can be observed in PCA plot (Fig. 4b).

Conclusion

The extracts obtained with ethyl acetate were characterised by the greatest values of protection against singlet oxygen, free radical scavenger capacity and colour intensity. In annatto extracts, these properties were all closely related to the bixin contents, but not to the phenolic compounds levels. Colour is considered to be the main attribute influencing the acceptance and preference of a food product (Clydesdale, 1993), and antioxidant capacity is related to the prevention of some chronic diseases (Krinsky, 1994; Serdula *et al.*, 1996; Huang *et al.*, 2005); thus, the solvents containing ethyl acetate can accomplish both requirements. This information is important to the industry because annatto is a known accessible source of natural colourants.

Acknowledgment

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CAPÍTULO IV

In vitro scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species

Renan Campos Chisté, Adriana Zerlotti Mercadante, Ana Gomes, Eduarda Fernandes, José Luís Fontes da Costa Lima & Neura Bragagnolo

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In vitro scavenging capacity of annatto seed extracts against reactive oxygen and nitrogen species

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ABSTRACT

Bixa orellana L. (annatto), from Bixaceae family, is a native plant of tropical America, which accumulates several carotenoids (including bixin and norbixin), terpenoids, tocotrienols and flavonoids with potential antioxidant activity. In the present study, the *in vitro* scavenging capacity of annatto seed extracts against reactive oxygen species (ROS) and reactive nitrogen species (RNS) was evaluated and compared to the bixin standard. Annatto extracts were obtained using solvents with different polarities and their phenolic compounds and bixin levels were determined by high performance liquid chromatography coupled to diode array detector. All annatto extracts were able to scavenge all the reactive species tested at the low μg/mL range, with the exception of superoxide radical. The ethanol:ethyl acetate and ethyl acetate extracts of annatto seeds, which presented the highest levels of hypolaetin and bixin, respectively, were the extracts with the highest antioxidant capacity, although bixin standard presented the lowest IC_{50} values.

1. Introduction

Bixa orellana L. (annatto), from Bixaceae family, is a native plant of tropical America, which accumulates several carotenoid derivatives (including bixin and norbixin), terpenoids, tocotrienols and flavonoids in the seeds and leaves (Rodrigues et al., 2007). Brazil is one of the largest producers and exporters of seeds and annatto extracts, for the use as natural colouring agents in foods, pharmaceutical and cosmetics industries.

Bixin (methyl (9-*cis*)-hydrogen-6,6′-diapo-Ψ,Ψ-carotenedioate) is the main carotenoid responsible for the orange-red colour in the seeds and extracts of annatto (Fig. 1), representing approximately 80% of the total carotenoids (Preston & Rickard, 1980). Besides the colorant property, bixin is known to be a very efficient quencher of singlet oxygen ($^1O_2$) and triplet state of sensitizers (Montenegro, Rios, Mercadante, Nazareno, & Borsarelli, 2004; Rios, Mercadante, & Borsarelli, 2007). The main phenolic compounds identified in the seeds and/or annatto extracts were hypolaetin and a caffeoyl acid derivative (Chisté, Yamashita, Gozzo & Mercadante, 2011).

Carotenoids and phenolic compounds belong to the group of bioactive compounds to which has been attributed the ability of reducing the risks of various chronic degenerative disorders, such as cancer, inflammations, cardiovascular diseases, cataracts, and macular degeneration (Serdula et al., 1996). In general, these risk-preventing effects are claimed to be related to the inhibition of oxidation reactions.

Information about the scavenging capacity of stable radicals not found in physiological conditions, such as $ABTS^{\cdot+}$, is available in the literature for annatto extracts (Cardarelli, Benassi, & Mercadante, 2008). However, no data related to scavenging capacity of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were found in the literature. Thus, the aim of this study was to evaluate the scavenging capacities of annatto extracts obtained with solvents with different polarities for the ROS superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\cdot}$), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and the RNS nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$) in order to understand their antioxidant capacity. In addition, the amounts of phenolic compounds and bixin in annatto extracts were determined by high performance liquid chromatography coupled to diode array detector (HPLC-DAD).

2. Materials and methods

2.1. Chemicals

The solvents ethanol, ethyl acetate, dichloromethane and methanol (P.A.) were obtained from Synth (São Paulo, Brazil). Methanol

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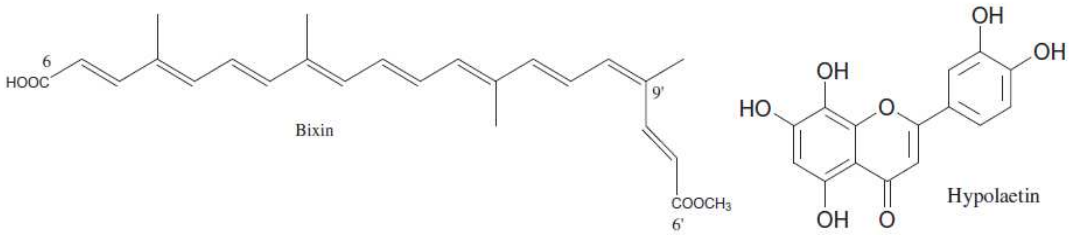

Fig. 1. Structure of bixin (carotenoid) and hypolaetin (phenolic compound) from annatto seeds and extracts.

of chromatographic grade was obtained from J.T. Baker (Phillipsburg, USA) and ultrapure water from the Millipore system (Billerica, USA). Acid formic was purchased from Merck (Darmstadt, Germany) and Folin–Ciocalteau from Dinâmica (São Paulo, Brazil). The bixin standard was isolated in our laboratory and recrystallized (Rios & Mercadante, 2004) to achieve 98% purity degree, determined by HPLC-DAD. Dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, sodium hypochlorite solution, with 4% available chlorine, diethylenetriaminepentaacetic acid (DTPA), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), lucigenin and quercetin were obtained from Sigma–Aldrich (St. Louis, USA). α,α′-Azodiisobutyramidine dihydrochloride (AAPH), histidine and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt was obtained from Aldrich (Milwaukee, USA).

2.2. Annatto seed samples

Annatto seeds were obtained in August 2008, in the local trade market of Campinas, São Paulo State, Brazil. The seeds were divided in eight shares of 500 g, vacuum packed and stored at room temperature, protected from light, until analysis.

2.3. Preparation of annatto extracts

Extractions were performed on different days, in triplicate, using the following solvents: ethanol, ethanol:water (1:1, v/v), ethanol:ethyl acetate (1:1, v/v), ethyl acetate and water. These solvents were chosen considering the permissibility of residue in the extracts after evaporation, according to the Commission Directive 95/45/EC from European Communities (Commission Directive 95/45/EC, 1995). The annatto seeds were weighed (20 g) and the solvents were added at 1:2 ratio (mass:solvent), stirred on an orbital shaker MA 140/CFT (Marconi, São Paulo, Brazil) at 168 rpm for 15 h at room temperature. After the seeds decanted in the bottom of the erlenmeyer the extracts were transferred to 50 mL volumetric flasks and filled with the respective solvent, followed by lyophilisation (Liobras, São Paulo, Brazil). The freeze-dried extracts were transferred to ambar flasks, sealed under N_2 flow and stored at −36 °C until analysis.

2.4. Bixin quantification

The spectrophotometric quantification of bixin was performed in the seeds according to the methodology described by FAO/WHO (1982) with adaptations. For the seeds, 1 g was weighed and the pigment was thoroughly extracted with dichloromethane until colourless. Absorbance was measured with a UV–Visible spectrophotometer (Agilent, Santa Clara, CA) at 470 nm and bixin concentration was calculated according to the Lambert–Beer law, using $E^{1\%}_{1\,cm} = 2826$ (FAO/WHO, 1982). The measurements were performed in triplicate.

2.5. Total phenolic compounds quantification

The total phenolic compounds were determined in the annatto seeds according to Singleton, Orthofer and Lamuela-Ravento's (1999). For the annatto seeds, 5 g were weighed and extracted five times with methanol:water (8:2, v/v) in ultrasound equipment (Unique model, São Paulo, Brazil). After extraction, the extracts were evaporated under vacuum ($T < 40$ °C), transferred to a volumetric flask and filled with distilled water. The extracts were then put in the freezer for 20 min before centrifugation at 290*g* for 20 min. The total phenolic compounds content of the seeds was determined using the Folin–Ciocalteau colorimetric method (1999), and was expressed as milligrams of gallic acid equivalent (GAE) per mL of extract. All measurements were performed in triplicate.

2.6. Bixin and phenolic compounds analysis by HPLC-DAD

The freeze-dried extracts were dissolved in methanol and injected in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (LC-20AD), degasser unit (DGU-20A5), Rheodyne injection valve with a 20 μL loop, and a photodiode array detector (DAD) (SPD-M20A). All compounds were separated on a C_{18} Luna column (5 μm, 250 × 4.6 mm, Phenomenex, CA) using as mobile phase a gradient of water:formic acid (98:2, v/v) (solvent A) and methanol:formic acid (98:2, v/v) (solvent B) from 70 A:30 B to 40:60 in 15 min; from 40:60 to 20:80 in 10 min; from 20:80 to 5:95 in 10 min; the latter proportion being maintained for a further 10 min, at 0.9 mL/min and column temperature set at 29 °C, according to Chisté et al. (2011). The spectra were obtained between 200 and 600 nm and the chromatograms were processed at 320 nm (phenolic compounds) and 459 nm (bixin). The quantification of phenolic compounds and bixin was carried out using, respectively, external calibration curves for quercetin (0.4–10 μg/mL) and bixin (0.5–12.0 μg/mL) with seven concentration levels (duplicate).

2.7. ROS and RNS scavenging assays

A microplate reader (Synergy HT, Biotek, Vermont, USA), for fluorescence, UV/vis and luminescence measurements, equipped with a thermostat, was used for all the assays. Each ROS and RNS scavenging assay corresponds to four experiments, performed in triplicate. The IC_{50} values were calculated from the curves of percentage of inhibition using the GraphPad Prism 5 software. Quercetin was used as positive control in the scavenging assays of $O_2^{\cdot-}$, H_2O_2, HOCl, $^1O_2$, ·NO, $ONOO^-$ and its values were similar to those reported by Gomes et al. (2007).

2.7.1. Superoxide radical scavenging assay

The $O_2^{\cdot-}$ was generated by the NADH/PMS system and the $O_2^{\cdot-}$ scavenging capacity was determined by monitoring the effect of the tested extracts and bixin standard on the $O_2^{\cdot-}$-induced reduction of NBT at 560 nm for 2 min (Gomes et al., 2007). The assay was performed at room temperature. The reaction mixtures in the sample wells contained the following reactants at the indicated final concentrations (in a final volume of 300 μL): NADH (166 μM), NBT (43 μM), the tested annatto extracts and bixin standard at five concentrations, all dissolved in DMSO, and PMS (2.7 μM). NADH, NBT, and PMS were dissolved in 19 mM phosphate buffer, pH 7.4. No direct effect was observed between DMSO and $O_2^{\cdot-}$ in the present assay conditions. The effects were expressed as the inhibition, in percentage, of the NBT reduction to diformazan.

2.7.2. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity was measured by monitoring the effect of the tested extracts on the H_2O_2-induced oxidation of lucigenin (Gomes et al., 2007). Reaction mixtures contained the following reagents at final concentrations (final volume of 250 μL): 50 mM Tris–HCl buffer, pH 7.4, lucigenin (0.8 mM), dissolved in the buffer solution, annatto extracts and bixin standard at five concentrations, completely dissolved in DMSO, and 1% H_2O_2. The assays were performed at 37 °C. The chemiluminescence signal was detected in the microplate reader immediately after the plate introduction. The results were expressed as the inhibition, in percentage, of the H_2O_2-induced oxidation of lucigenin.

2.7.3. Hypochlorous acid scavenging assay

The HOCl scavenging capacity was measured by monitoring the effect of the extracts on HOCl-induced oxidation of DHR to rhodamine 123, as previously described (Gomes et al., 2007). HOCl was prepared by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with dropwise addition of 10% H_2SO_4. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} and the proper dilution made in a 100 mM phosphate buffer at pH 7.4. A 2.89 mM stock solution of DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR were diluted in the phosphate buffer from the stock solution immediately before the determinations and placed on ice, in the dark. Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 μL): 100 mM phosphate buffer solution at pH 7.4, annatto extracts and bixin standard at five different concentrations dissolved in DMSO, DHR (5 μM), and HOCl (5 μM). The fluorimetric assays were performed at 37 °C in the microplate reader, at the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm. The fluorescence signal was measured immediately after the plate introduction. The results were expressed as the inhibition, in percentage, of HOCl-induced oxidation of DHR.

2.7.4. Singlet oxygen scavenging assay

The $^1O_2$ scavenging capacity was measured by monitoring the effect of the tested annatto extracts and bixin standard on the oxidation of non-fluorescent DHR to fluorescent rhodamine 123 by this ROS, as previously described (Gomes et al., 2007). $^1O_2$ was generated by the thermal decomposition of a previously synthesised water-soluble endoperoxide $NDPO_2$ (disodium 3,3′-(1,4-naphthalene) bispropionate). $NDPO_2$ working solutions, diluted in 100 mM phosphate buffer, pH 7.4, were prepared immediately before each assay. A 2.89 mM stock solution of DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR were diluted in the phosphate buffer from the stock solution immediately before the determinations and placed on ice, in the dark. Histidine solutions in phosphate buffer were daily prepared. Reaction mixtures contained the following reactants at the indicated final concentrations (in a final volume of 250 μL): histidine (10 mM), annatto extracts and bixin standard at five different concentrations dissolved in DMSO, DHR (50 μM), and $NDPO_2$ (1 mM). The fluorimetric assays were performed at 37 °C in the microplate reader, using the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm. The fluorescence was measured after 30 min incubation period. The results were expressed as the inhibition, in percentage, of $^1O_2$-induced oxidation of DHR.

2.7.5. Peroxyl radical scavenging assay

The $ROO^{\cdot}$ scavenging capacity was measured by monitoring the effect of the tested extracts on the fluorescence decay resulting from $ROO^{\cdot}$-induced oxidation of fluorescein and expressed as the "Oxygen Radical Absorbance Capacity" (ORAC) (Ou, Hampsch-Woodill, & Prior, 2001). $ROO^{\cdot}$ was generated by thermodecomposition of AAPH. Reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (in a final volume of 200 μL): fluorescein (61 nM), annatto extracts and bixin standard in five concentrations dissolved in acetone and subsequently diluted in 75 mM phosphate buffer, pH 7.4, and AAPH (19 mM). Working solutions of fluorescein were diluted in 75 mM phosphate buffer, pH 7.4, to 1/5000-fold, from a 1.53 mg/mL stock solution, which was previously prepared and kept at ≈4 °C. AAPH was dissolved in phosphate buffer. The mixture was preincubated in the microplate reader at 37 °C during 15 min. The fluorescence signal was monitored every minute at the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm until the total decay of fluorescence. Trolox was used as standard control and the results were expressed as ORAC values, calculated according to Gomes et al. (2007). Briefly, the net protection provided by the antioxidant extracts or standard was calculated using the difference between the area under the fluorescence decay curve in the presence of the sample ($AUC_{antioxidant}$) and in its absence (AUC_{blank}). Regression equations between net AUC and the concentration of the sample were calculated for all the annatto extracts, bixin standard and Trolox. ORAC values were calculated by using the standard curve of each assay. Final results were expressed in μmol Trolox equivalents/mg extract.

2.7.6. Nitric oxide scavenging assay

The $^{\cdot}NO$ scavenging capacity was measured by monitoring the effect of the annatto extracts and bixin on $^{\cdot}NO$-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T) (Gomes et al., 2007). $^{\cdot}NO$ was generated by decomposition of NOC-5. A stock solution of 2.76 mM DAF-2 in DMSO was purged with nitrogen and stored at -20 °C. Working solutions of DAF-2, diluted with phosphate buffer solution (KH_2PO_4 50 mM, pH 7.4) to 1/368-fold from the stock solution, were placed on ice, in the dark, immediately before the analysis. The reaction mixtures in the sample wells contained the following reagents at the indicated final concentrations (final volume of 300 μL): DAF-2 (5 μM), annatto extracts and bixin standard at five concentrations, dissolved in DMSO, and NOC-5 (10 μM). The fluorescence signal was detected in the microplate reader after a 30 min period at 37 °C incubation with emission wavelength at 528 ± 20 nm and excitation at 485 ± 20 nm. The results were expressed as the percentage of inhibition of $^{\cdot}NO$-induced oxidation of DAF-2.

2.7.7. Peroxynitrite scavenging assay

The $ONOO^-$ scavenging capacity was measured by monitoring the effect of the tested annatto extracts and bixin standard on $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123 (Gomes et al., 2007). $ONOO^-$ was synthesised as previously described by 123 Gomes et al. (2007). A stock solution

of 2.89 mM DHR in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR, properly diluted with the buffer solution (90 mM NaCl, 50 mM Na_3PO_4, and 5 mM KCl, pH 7.4), were placed on ice, in the dark, immediately before the determinations. In the beginning of the experiments, 100 μM DTPA was added to the buffer. Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 μL): DHR (5 μM), annatto extracts and bixin standard at five different concentrations, dissolved in DMSO, and $ONOO^-$ (600 nM). The assays were performed at 37 °C. The fluorescence signal was detected in the microplate reader after 2 min incubation period at emission wavelength at 528 ± 20 nm and excitation at 485 ± 20 nm. In a parallel set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$ in order to simulate the physiological CO_2 concentrations. This evaluation is important because, under physiological conditions, the reaction between $ONOO^-$ and bicarbonate is predominant, with a very fast rate constant ($k = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$) (Whiteman, Ketsawatsakul, & Halliwell, 2002). The results were expressed as the inhibition, in percentage, of $ONOO^-$-induced oxidation of DHR.

3. Results and discussion

3.1. Characteristics of annatto seeds

The annatto seeds used in this study presented 14 ± 2 mg/g of bixin and 1.7 ± 0.1 mg GAE/g of total phenolic compounds, both in wet basis, as determined by spectrophotometric assay. The main phenolic compound found in annatto seeds and extracts was pentahydroxyflavone, also known as hypolaetin, along with a caffeoyl acid derivative (Chisté et al., 2011). The phenolic compounds level for annatto seeds, in this study, was very similar to those found by Chisté et al. (2011), and the average concentration of bixin is reported to range from 12 to 23 mg/g, depending on environmental factors such as temperature, illumination, rainfall, soil, and cultivar (Shuhama, Aguiar, Oliveira, & Freitas, 2003).

3.2. Bixin and phenolic compounds of freeze-dried extracts from annatto seeds

The HPLC analysis of the freeze-dried extracts allowed the separation of 2 phenolic compounds, some minor carotenoids and bixin, the major one (Fig. 2). The identification of hypolaetin (peak 1), caffeoyl acid derivative (peak 2) and bixin (peak 3) was previously carried out in our laboratory (Chisté et al., 2011). According to

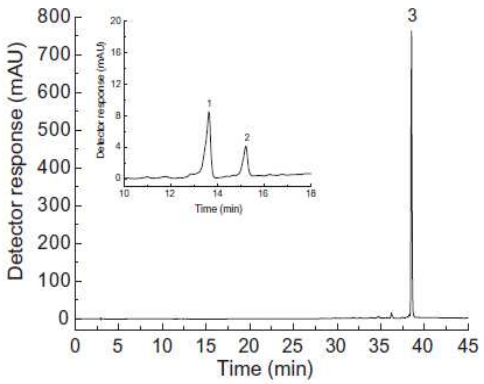

Fig. 2. Chromatogram obtained by HPLC–DAD of the ethanol:ethyl acetate extract (EtOH:EtOAc) from annatto seeds. Chromatographic conditions: see text. Processed at 459 nm and figure inset at 320 nm. Peaks: (1) hypolaetin, (2) caffeoyl acid derivative and (3) bixin.

Table 1
Concentration obtained by HPLC of the antioxidant compounds from freeze-dried extracts from annatto seeds.

Extracts	Concentration (mg/g)		
	Hypolaetin	Caffeoyl acid derivative	Bixin
H_2O	3.71 ± 0.06[c]	2.62 ± 0.03[b]	119.88 ± 2.55[c]
EtOH:H_2O	4.30 ± 0.01[b]	2.57 ± 0.01[b]	15.06 ± 0.18[d]
EtOH	6.47 ± 0.03[a]	4.70 ± 0.03[a]	266.06 ± 0.95[b]
EtOH:EtOAc	6.50 ± 0.01[a]	4.81 ± 0.11[a]	273.04 ± 1.35[b]
EtOAc	6.41 ± 0.02[a]	4.86 ± 0.09[a]	311.35 ± 2.95[a]

H_2O = distilled water; EtOH:H_2O = ethanol:water; EtOH = ethanol; EtOH:EtOAc = ethanol:ethyl acetate; EtOAc = ethyl acetate. Mean ± standard error (triplicate), in dry basis. Means with the same superscript letters at the same column do not present statistical difference ($p < 0.05$).

Table 1, the levels of hypolaetin, caffeoyl acid derivative and bixin of annatto seeds were dependent on the solvent polarity. The ethanol:ethyl acetate mixture extract showed the highest values of hypolaetin (6.50 ± 0.01 mg/g), and the water extract presented the lowest one (3.71 ± 0.06 mg/g). The highest amount of caffeoyl acid derivative (4.86 ± 0.09 mg/g) was detected in the ethyl acetate extract and the lowest one in the ethanol:water extract (2.57 ± 0.01 mg/g). The bixin peak comprised 96% of the total area of the carotenoids separated in the ethanol extract, 94% in the water and ethyl acetate extract, 93% in the ethanol:ethyl acetate extract and 80% in the ethanol:water extract. The bixin concentration was significantly higher in the ethyl acetate annatto extract (311 ± 3 mg/g) than those found in the other solvent extracts (Table 1).

3.3. Scavenging of ROS and RNS by annatto extracts

The annatto extract presented a remarkable capacity to scavenge all the ROS and RNS with the exception of $O_2^{\cdot-}$. All the IC_{50} values were found at the μg/mL level (Table 2). Following a broader definition of antioxidant, suggested by Halliwell and co-workers (Halliwell, Murcia, Chirico, & Aruoma, 1995), as any substance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate, all the extracts show antioxidant activity.

The five extracts, as well as bixin standard, scavenged the ROS and RNS in a concentration-dependent manner (Figs. 3 and 4). In general, the ethanol:ethyl acetate mixture and ethyl acetate extracts showed the best scavenging activity results for all the ROS and RNS, followed by the ethanol extract, most probably due to the presence of higher levels of bixin and phenolic compounds (Table 1) as compared to the water containing extracts. These two extracts (ethanol:ethyl acetate mixture and ethyl acetate) only showed lower activity than bixin standard. The ethanol:water mixture extract was able to scavenge all the tested reactive species; however, the scavenging capacity of this extract (which had the lowest bixin content) was lower in all cases when compared to the other extracts.

None of the extracts prevented the $O_2^{\cdot-}$-dependent reduction of NBT in the tested conditions, with the exception of bixin standard that showed a concentration dependent inhibition of the $O_2^{\cdot-}$-induced reduction of NBT (Fig. 3) with an efficient scavenging effect. In fact, bixin (IC_{50} of 26 μg/mL) showed higher $O_2^{\cdot-}$ scavenging capacity than Trolox (IC_{50} of 452 μg/mL), but lower activity than quercetin, used as positive control (IC_{50} of 18 μg/mL) and than ascorbic acid (IC_{50} of 0.028 μg/mL) (Almeida, Fernandes, Lima, Costa, & Bahia, 2008a, 2008b).

The water extract presented a good capacity in the quenching of $^1O_2$, with the same IC_{50} value obtained for ethanol:ethyl acetate and ethyl acetate extract (3 μg/mL), which showed the best values

Table 2
Superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitric oxide ($^{\cdot}NO$), peroxynitrite ($ONOO^-$), peroxyl radical ($ROO^{\cdot}$) scavenging and singlet oxygen ($^1O_2$) quenching activities of annatto extracts and bixin standard.

Extracts/carotenoid	IC_{50} (μg/mL)						$ORAC_{ROO}$(μmol Trolox equivalents/mg extract)
	$O_2^{\cdot-}$	H_2O_2	HOCl	$^1O_2$	$^{\cdot}NO$	$ONOO^-$	
						Absence of $NaHCO_3$	Presence of 25 mM $NaHCO_3$
H_2O	NA	26.0 ± 2.0	1.0 ± 0.3	3.0 ± 0.3	9.0 ± 0.4	3.0 ± 1.0	4.0 ± 1.0	0.11 ± 0.02
EtOH:H_2O	NA	47.0 ± 9.0	3.0 ± 0.8	9.0 ± 3.0	9.0 ± 1.0	7.0 ± 2.0	8.0 ± 1.0	0.01 ± 0.02
EtOH	NA	11.0 ± 1.0	1.0 ± 0.4	19.0 ± 2.0	9.0 ± 1.0	2.0 ± 1.0	4.0 ± 1.0	0.14 ± 0.03
EtOH:EtOAc	NA	12.0 ± 2.0	0.3 ± 0.0	3.0 ± 0.3	8.0 ± 1.0	2.0 ± 0.3	2.0 ± 0.2	0.08 ± 0.00
EtOAc	NA	11.0 ± 1.0	1.0 ± 0.4	3.0 ± 0.2	7.0 ± 1.0	3.0 ± 0.2	2.0 ± 0.2	0.03 ± 0.01
Bixin standard	26.0 ± 5.7	3.0 ± 0.6	0.3 ± 0.1	1.0 ± 0.1	3.0 ± 0.8	1.0 ± 0.0	0.4 ± 0.0	0.19 ± 0.02

IC_{50} = inhibitory concentration, *in vitro*, to decrease in 50% the amount of reactive species in the tested media (mean ± standard error). ORAC = oxygen radical absorbance capacity (mean ± standard error). NA = no activity was found within the assayed concentration range (15–25 μg/mL). H_2O = distilled water; EtOH:H_2O = ethanol:water, EtOH = ethanol; EtOH:EtOAc = ethanol:ethyl acetate; EtOAc = ethyl acetate.

for bixin and phenolic compounds contents (Fig. 3). Quercetin showed an IC_{50} of 0.5 μg/mL. The IC_{50} values of these extracts showed better efficiency as $^1O_2$ quenchers than extracts from *Juglans regia* leaves (Almeida et al., 2008a), *Castanea sativa*, *Quercus robur* (Almeida et al., 2008b), *Hypericum androsaemum* (Almeida, Fernandes, Lima, Costa, & Bahia, 2009) and *Eucalyptus globulus* leaf water extract (Almeida, Fernandes, Lima, Valentão, et al., 2009). As expected, bixin showed lower IC_{50} than all annatto extracts. Model studies on the photosensitized isomerisation of bixin show that while bixin in the ground electronic state is stable to thermal isomerization, energy transfer via photosensitization gives rise to the higher energy triplet state precursor, which readily isomerizes to the *trans*-isomer (Montenegro et al., 2004). Rios et al. (2007) showed that bixin, is a very efficient $^1O_2$ quencher in fluid solutions due to an efficient energy-transfer process, and confirmed that the triplet state energy level for bixin is lower than that of $^1O_2$ (18 kcal mol^{-1} and 22.5 kcal mol^{-1}, respectively).

The ethanolic extract, which possesses the highest levels of phenolic compounds, presented higher $ROO^{\cdot}$ scavenging capacity as compared to the other extracts, although lower than the bixin standard (Table 2). Wu et al. (2004) used the ratio of ORAC values and total phenolic (TP) (ORAC/TP) to rank the raw food samples of the United States into four groups (0–5, 5–10, 10–15 and >15). Most ratio values of fruits and vegetables were close to 10, indicating a strong positive linear correlation between TP and antioxidant capacity. The highest antioxidant capacity tended to have high ORAC/TP ratio. Considering the sum of the contents of hypolaetin and caffeoyl acid derivative (PC) for each annatto extract (Table 1), the highest ORAC/PC ratio values were found for the water (ORAC/PC = 17) and ethanol (ORAC/PC = 13) extracts, which ranked into the fourth (>15) and third group (10–15), respectively. The ethanol:ethyl acetate was ranked into the second group (5–10) with ORAC/PC ratio of 7.0, and the ethanol:water and ethyl acetate extract into the first group (0–5) with ORAC/PC ratios of 1.4 and 2.6, respectively. In contrast to most fruits and vegetables (Wu et al., 2004), the great ratio values found for annatto extracts are probably due to the high contents of bixin in the extracts, 30–40 times higher than the levels of phenolic compounds. This hypothesis can be confirmed by the high ORAC values found for bixin.

The scavenging capacities for H_2O_2 were within the same range for the ethanol, ethanol:ethyl acetate and ethyl acetate extracts, probably due to the presence of high levels of bixin since bixin showed even lower IC_{50} value. The IC_{50} values for all the tested extracts in this study showed better capacity than other extracts found in the literature, such as medicinal tincture from *J. regia* leaf extract (Almeida et al., 2008a), *C. sativa* and *Q. robur* (Almeida et al., 2008b), *H. androsaemum* (Almeida, Fernandes, Lima, Costa, et al., 2009), *E. globulus* (Almeida, Fernandes, Lima, Valentão, et al., 2009) and *Pedilanthus tithymaloides* (Abreu et al., 2006). The H_2O_2 scavenging capacity of all annatto extracts analysed in this study was also better than that of quercetin (245 μg/mL), tested in the same conditions, ascorbic acid (200 μg/mL) (Abreu et al., 2006; Almeida et al., 2008a, 2008b; Almeida, Fernandes, Lima, Costa, et al., 2009; Almeida, Fernandes, Lima, Valentão, et al., 2009).

All IC_{50} values for the scavenging capacity of HOCl were very similar, and only ethanol:ethyl acetate extract showed low value as bixin standard. It was also observed that bixin can be considered an excellent scavenger of HOCl, since lipoic acid, an essential cofactor for mitochondrial enzymes and a naturally occurring antioxidant, presented an IC_{50} value of 1.2 μg/mL (Abreu et al., 2006), showing lower efficiency than bixin standard. However, the IC_{50} obtained for quercetin was even lower (0.07 μg/mL).

Regarding to $^{\cdot}NO$ scavenging property, all annatto extracts presented lower efficiency than other plant extracts reported in the literature (Abreu et al., 2006; Almeida et al., 2008a, 2008b; Almeida, Fernandes, Lima, Costa, et al., 2009; Almeida, Fernandes, Lima,

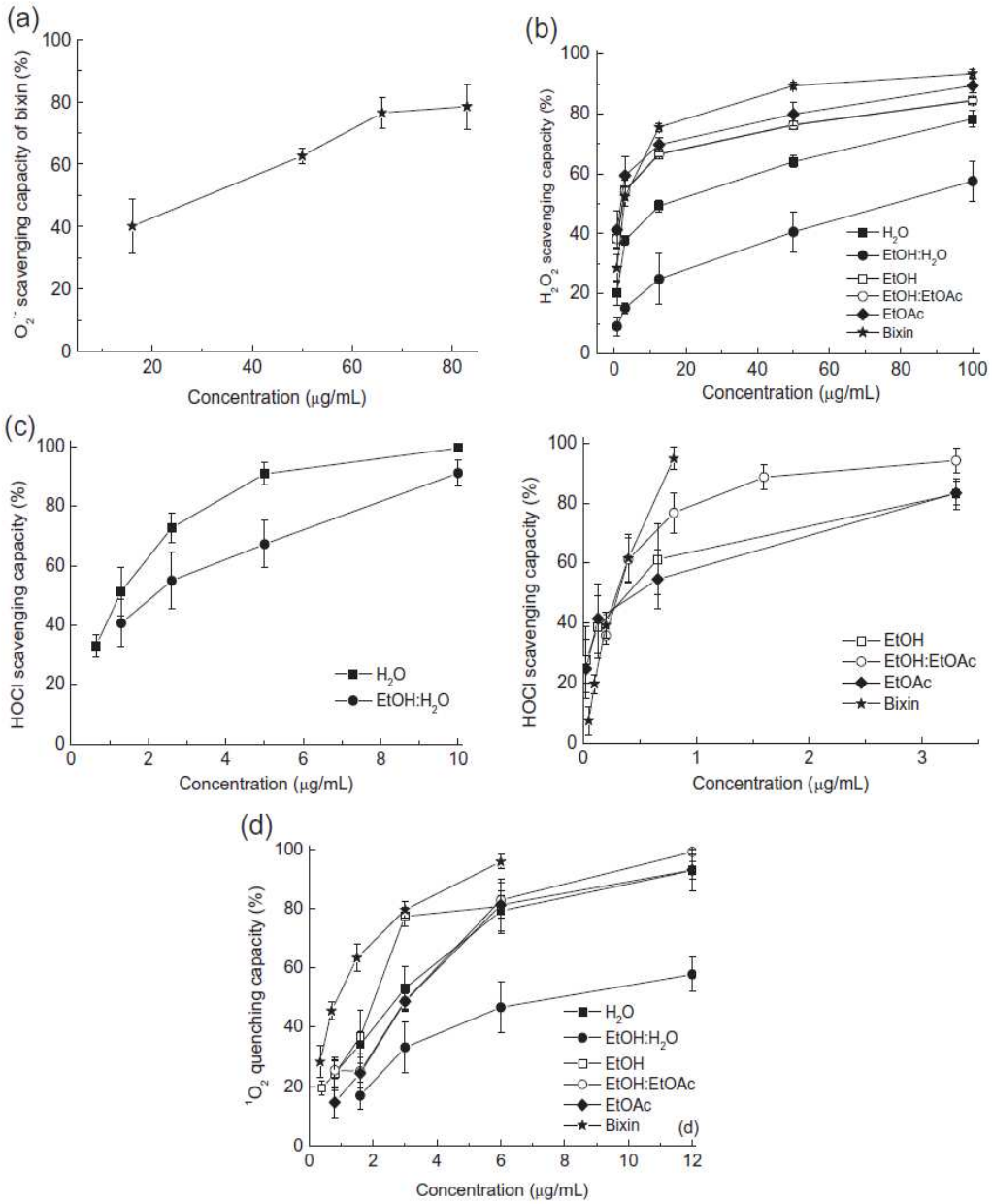

Fig. 3. (a) Superoxide radical ($O_2^{\cdot-}$) scavenging capacity of the bixin standard. (b) hydrogen peroxide (H_2O_2), (c) hypochlorous acid (HOCl) scavenging capacities and (d) singlet oxygen ($^1O_2$) quenching activity of the annatto extracts. Each point shows the standard error bars and represents the values obtained from four experiments, in five concentrations, performed in triplicate.

Valentão, et al., 2009). The IC_{50} values were closer for all the extracts and only bixin standard presented as high capacity to scavenge ·NO as other extracts, such as *J. regia* (Almeida et al., 2008a), *C. sativa*, *Q. robur* (Almeida et al., 2008b), *Hypericum androsaemu* (Almeida, Fernandes, Lima, Costa, et al., 2009), *E. globulus* (Almeida, Fernandes, Lima, Valentão, et al., 2009) and *P. tithymaloides* (Abreu et al., 2006). The ·NO scavenging capacities for all annatto extracts were lower than quercetin (0.07 μg/mL) and rutin (0.86 μg/mL) (Abreu et al., 2006; Almeida, Fernandes, Lima, Valentão, et al., 2009). In relation to $ONOO^-$ scavenging capacity, all the extracts presented very similar IC_{50} values, either in the absence or presence of $NaHCO_3$, and only bixin standard presented good capacity for $ONOO^-$ scavenging as compared to the same extracts cited above for ·NO (Almeida et al., 2008a). The ethanol:water extract, which presented the lowest bixin content, showed the lowest scavenging capacity with and without $NaHCO_3$. The $ONOO^-$ scavenging capacities of all annatto extracts were lower than those of quercetin (IC_{50}s of 0.20 μg/mL and 0.13 μg/mL, with and without $NaHCO_3$, respectively). The annatto extract is also expected to be an effective scavenger for $\cdot NO_2$ and $CO_3^{\cdot-}$ since the scavenging effectiveness is maintained in the presence of HCO_3^-. It has been reported that physiological concentrations of CO_2 can modulate $ONOO^-$ reactivity due to the fast reaction between these two compounds, yielding $\cdot NO_2$ and $CO_3^{\cdot-}$, which are the main responsible radicals for the nitration and oxidation reactions that are usually observed *in vivo*. Thus, a scavenger can directly trap $ONOO^-$ only if it reacts faster with the later than does CO_2. On the

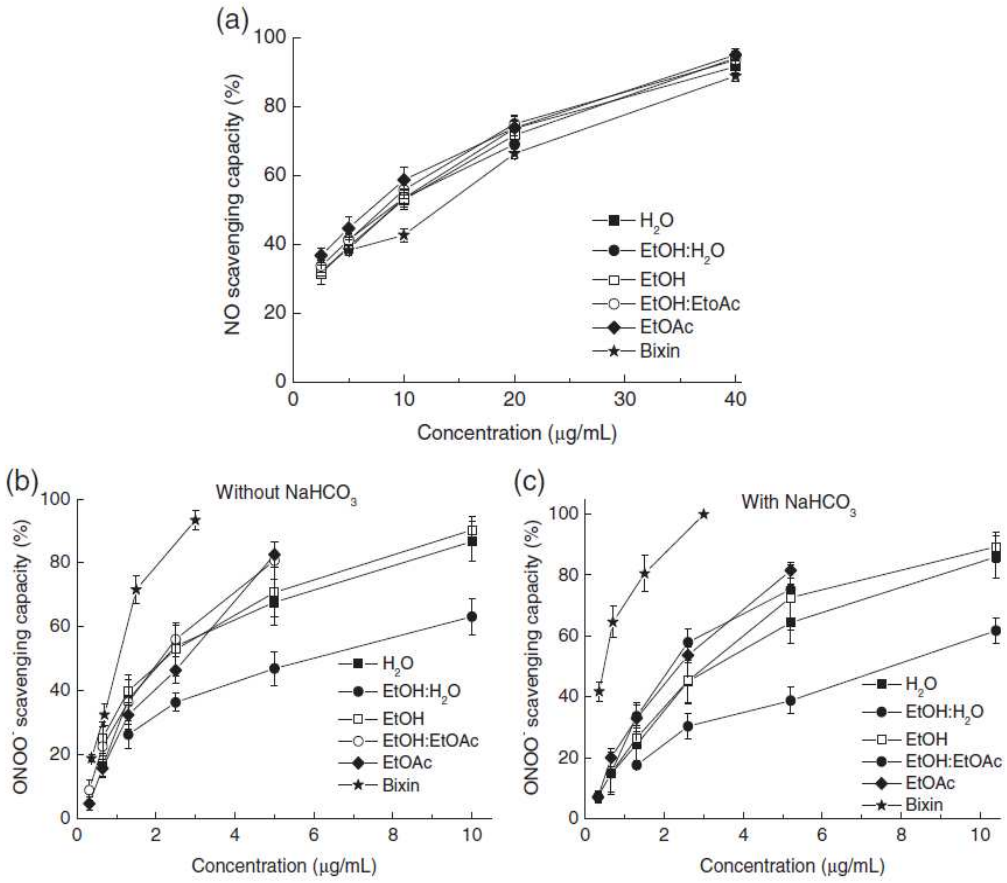

Fig. 4. (a) Nitric oxide (·NO), (b) peroxynitrite ($ONOO^-$) without $NaHCO_3$ and (c) peroxynitrite ($ONOO^-$) with $NaHCO_3$ scavenging capacities of the annatto extracts. Each point shows the standard error bars and represents the values obtained from four experiments, in five concentrations, performed in triplicate.

other hand, a putative scavenging effect on $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ may increase the efficiency of the compounds (Gomes, Costa, Lima, & Fernandes, 2006).

The results obtained in the present *in vitro* study clearly demonstrated that the annatto extracts are effective scavengers against the tested ROS and RNS. In general, the IC_{50} values for ROS and RNS showed that annatto extracts presented better scavenging capacity for non-radical species (H_2O_2, HOCl, $^1O_2$ and $ONOO^-$) than radical species ($O_2^{\cdot-}$ and ·NO). The possible mechanisms that should be considered when carotenoids are exposed to free radicals, such as peroxyl radicals and other oxidising agents, are the electron transfer due to the presence of many conjugated double bonds (11 in the bixin structure) or the hydrogen abstraction from carotenoid molecule functioning this way as chain breaking antioxidants (Burton, 1989; Squadrito & Pryor, 1998; Yeum, Aldini, Russell, & Krinsky, 2009).

Bixin standard was the best scavenger when compared to all the extracts. The great scavenging capacity of bixin, found in this *in vitro* study, can explain the *in vivo* effects of bixin reported in the literature, supporting that it may have future clinical application. The carotenoid bixin was shown to be capable of acting as an antioxidant by intercepting free radicals generated by the commonly used chemotherapeutic drug, clastogen cisplatin, such as superoxide radical and hydroxyl radical (Antunes, Pascoal, Bianchi, & Dias, 2005), and by inhibiting the ROS generation by DNA–cisplatin interaction in a concentration-dependent manner (Rios, Antunes, & Bianchi, 2009). Bixin also reduced the total number of chromosome aberrations, inhibited the increase in lipid peroxidation, and inhibited renal glutathione depletion induced by cisplatin (Silva, Antunes, & Bianchi, 2001). In addition, annatto is neither genotoxic nor carcinogenic at the highest concentration tested (1000 μg/mL) (Agner, Barbisan, Scolastici, & Salvadori, 2004).

In summary, the annatto extracts or its main bioactive constituent, bixin, can be used as an easily accessible source of natural antioxidants. Based on the results of this study, ethanol:ethyl acetate mixture and ethyl acetate are the most promising solvents to obtain annatto extracts with the highest efficiency as antioxidants. This study shows that the annatto extracts can also be effective in the prevention of lipid peroxidation and protection of food, excipient bases and medicines against oxidative damage.

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CAPÍTULO V

Identification and quantification by HPLC-DAD-MS/MS of carotenoids and phenolic compounds from an Amazonian fruit (*Caryocar villosum)*

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Identification and quantification by HPLC-DAD-MS/MS of carotenoids and phenolic compounds from an Amazonian fruit (*Caryocar villosum)*

Running head: Bioactive compounds from *Caryocar villosum*

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ABSTRACT

This study reports the profiles of carotenoids and phenolic compounds of *Caryocar villosum* fruit pulp as well as the chemical, phytochemical composition and antioxidant capacity through the Oxygen Radical Absorbance Capacity (ORAC) assay. According to the nutritional composition, water (52 %) and lipids (25 %) were the major components found in the pulp and the total energetic value was 291 Kcal/100 g. Regarding the bioactive compounds, the pulp presented (dry basis) higher content of phenolic compounds (236 mg GAE/100 g), total flavonoids (67 mg CE/100 g) and total tannins (60 mg TAE/100 g) than total carotenoids (7 mg/100 g and α-tochopherol (1 mg/100 g). The major phenolic compounds identified by HPLC-DAD-MS/MS, were gallic acid (182 μg/g pulp), followed by ellagic acid rhamnoside (107 μg/g pulp) and ellagic acid (104 μg/g pulp). The main carotenoids identified were all-*trans*-antheraxanthin (3 μg/g), all-*trans*-zeaxanthin (3 μg/g), all-*trans*-neoxanthin (2 μg/g), all-*trans*-violaxanthin (1 μg/g) and all-*trans*-β-carotene (0.7 μg/g). The antioxidant capacity (ORAC) of the pulp (3.7 mMol Trolox/100 g) indicates that it can be considered a good peroxyl radical scavenger.

KEYWORDS: piquiá, Caryocaraceae, ESI, APCI, ORAC, phenolic acids, xanthophylls.

INTRODUCTION

Caryocar villosum (Aubl.) Pers. (Brazilian name: piquiá), from the Caryocaraceae family, is a very large tree (up to 40-50 m high) native from the Amazonian forest, with a very abundant occurrence in Pará State (North of Brazil) (*1, 2*). The main commercial use for the trees of *Caryocar* genus is the wood, due to the high quality of its timber, frequently used in the construction of houses and boats. However, the pulp of its fruits represent the main popular use, providing edible oil that can be used for cooking as a substitute for

butter, regional dishes (usually with rice), for homemade soaps, and for cosmetic applications (*1, 3*). The endocarp surrounds normally one seed, whereas sometimes there can be up to four. In the Amazonian region, the flowering occurs in the dry season (from July to November) with fruiting in the rainy season (from March to May) (*1*).

The fruits of *Caryocar villosum* can be considered an interesting source of bioactive compounds, since piquiá showed the highest contents of total phenolic compounds, flavonoids and antioxidant activity as compared to other 18 tropical fruits (nine of them from the Amazonian region) in a previous screening performed by our research group (*4*). Furthermore, Marx et al. (*2*) reported the chemical composition and the main components from *Caryocar villosum* pulp, such as carbohydrates (starch, glucose, sucrose, fructose), free amino acids (asparagine, γ-aminobutyric acid, alanine, leucine, valine), minerals and trace elements (calcium, magnesium, phosphorus), unsaponifiable matter (sterols), biogenic amines (*O*-phosphoethanolamine, taurine) and volatile compounds (*trans*-nerolidol, 2-heptanone, β-bisabolene). Regarding to other phytochemicals, the triterpenoid saponins (compounds with detergent and surfactant properties) were isolated from the methanol extract of dry peel, pulp and stem bark of *Caryocar villosum* and their toxicity, antimicrobial (*5*), cytotoxic and lipolytic activities (*6*) were reported. Additionally, seven new phenolic glycosides, most of them galloyl and ellagic acid derivatives were isolated from the stem bark of *Caryocar villosum* and *Caryocar glabrum*, along with 15 known compounds, and the mushroom tyrosinase inhibitory activity was determined (*7*).

Bioactive compounds, such as carotenoids and phenolic compounds have been associated to the reduction of the risks of various chronic degenerative disorders, such as cancer, inflammations, cardiovascular diseases, cataracts, and macular degeneration (*8-10*). Although *Caryocar villosum* pulps are reported to contain phenolic compounds and carotenoids (*4*), no information about the composition of phenolics and carotenoids was found yet. However, the composition of carotenoids and phenolic compounds of the pulp of *Caryocar brasiliense* (Brazilian name: pequi), another fruit from the same family (Caryocaraceae) was reported in the literature (*11, 12*).

Considering that the essential role of biodiversity for its sustainable use in food security and nutrition is world-wide recognized (4), and that Brazil has a wide variety of native, wild and non-commercially cultivated fruits and few information about their constituents is available, this study was designed to identify and quantify the phenolic compounds and carotenoids by high performance liquid chromatography coupled to diode array and mass spectrometric detectors (HPLC-DAD-MS/MS). Additionally, the chemical

and phytochemical composition, as well as the peroxyl radical scavenging (ROO•) capacity of *Caryocar villosum* pulp was determined in this work.

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), bovine serum albumin (BSA), catechin, epicatechin, quinic acid, rutin, taxifolin, naringin, hesperidin, neohesperidin, luteolin, quercetin, naringenin, apigenin, rhamnetin, tannic acid, ascorbic acid, fluorescein, AAPH (α,α′-Azodiisobutyramidine dihydrochloride), and all-*trans*-β-carotene were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau reagent was from Dinamica (São Paulo, Brazil). Methanol, methyl tert-butyl ether (MTBE), acetonitrile, hexane and isopropyl alcohol, all of chromatographic grade, were obtained from J.T. Baker (Phillipsburg, NJ) and ultrapure water was obtained from the Millipore system (Billerica, MA). The gallic acid, ellagic acid, hydroxybenzoic acid, caffeic acid, 4-coumaric acid, ferulic acid, myricetin and kaempferol standards were purchased from Extrasynthèse (Lyon Nord, France). Standards of all-*trans*-lutein and all-*trans*-zeaxanthin were provided by DSM Nutritional Products (Basel, Switzerland). The carotenoid standards of 9-*cis*-neoxanthin, all-*trans*-violaxanthin and all-*trans*-antheraxanthin were purchased from CaroteNature (Lupsingen, Switzerland). The standards of α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol were purchased from Calbiochem (Darmstadt, Germany). All standards showed at least 95 % of purity, determined by HPLC-DAD. All other chemical salts and solvents of analytical grade were purchased from Synth (São Paulo, Brazil) or Merck (Darmstadt, Germany). For chromatographic analysis, samples and solvents were filtered using, respectively, membranes of 0.22 and 0.45 μm, both from Millipore.

Materials. The *Caryocar villosum* fruits (piquiá) were acquired at the “Ver-O-Peso” market located in the city of Belém, Pará State, Brazil (latitude 01°27’21’’ and longitude 48°30’16’’) in March 2010. All ripe fruits (9 kg) were cut in half and the shells were manually removed from the pulp and seeds. The yellow coloured pulp was separated from the seeds, weighted, grinded and divided in two parts: one was used for the proximate composition (wet basis) and the other one was immediately lyophilized (dry basis) in a freeze-drier (Liobras equipment, São Paulo, Brazil) for all other analysis. Both, fresh and lyophilized materials were thoroughly mixed, vacuum packed and stored under light-free conditions at -36 °C until analysis.

Chemical and phytochemical analysis. The recommended methods of the Association of Official Analytical Chemists (*13*) were adopted to determine the moisture, ash and total protein (conversion factor of 6.25 from total nitrogen to total protein) in *Caryocar villosum* fresh pulp. Lipid content was determined by the methodology of Bligh and Dyer (*14*). The content of total carbohydrates was obtained by difference of the sum of the percentage contents of moisture, ashes, total lipids and proteins (*15*). All the experiments of proximate composition were performed in triplicate and expressed in g/100 g of fresh pulp (%). The total energetic value was calculated according to the Atwater specific energy conversion factors for fruits (*15*), as follows: total energetic value (Kcal/100 g) = (protein percentage x 3.36 Kcal/g) + (lipid percentage x 8.37 Kcal/g) + (total carbohydrate percentage x 3.60 Kcal/g).

The total phenolic compounds were determined according to Singleton et al. (*16*), using the Folin-Ciocalteau colorimetric method, and the result was expressed as milligrams of gallic acid equivalent (GAE) per 100 g of freeze-dried pulp. The total flavonoids were determined by a colorimetric assay described by Zhishen et al. (*17*) and the result was expressed as milligrams of catechin equivalent (CE) per 100 g of freeze-dried pulp. The tannins were determined according to the methodology described by Brune et al. (*18*) (hydrolysable tannins) and Waterman and Mole (*19*) (condensed tannins) and the results were expressed as milligrams of tannic acid equivalent (TAE) per 100 g of freeze-dried pulp. The total carotenoids were determined according to De Rosso and Mercadante (*20*). Briefly, the carotenoids were exhaustively extracted from the freeze-dried pulp with acetone, transferred to petroleum ether:diethyl ether (1:2, v/v), and saponified overnight at room temperature with 10 % methanolic KOH. Due to the high oil content in the pulp of *Caryocar villosum*, it was necessary to physically remove the oil, as follows: prior to ether transference, the carotenoid extract was kept in the freezer (temperature < -36 °C) for 2 h, followed by filtration using cold glassware and washing with cold acetone. The total carotenoid content was calculated by using the specific extinction coefficient of zeaxanthin in ethanol ($E_{1cm}^{1\%}$ = 2540) (*21*) and expressed as milligrams of carotenoid per 100 g of freeze-dried pulp. All measurements were performed in triplicate.

In order to estimate the antioxidant capacity of *Caryocar villosum* pulp, the peroxyl radical scavenging assay (ROO$^{\bullet}$) was carried out by monitoring the effect of the bioactive compounds from the pulp, extracted with methanol:water (8:2, v/v), on the fluorescence decay resulting from ROO$^{\bullet}$-induced oxidation of fluorescein and expressed as the “Oxygen Radical Absorbance Capacity” (ORAC) (*22*). Trolox was used as the standard control and the concentration in the eight-point analytical curve varied from 8 to 96 µM. The results

were expressed as mMol Trolox equivalent per 100 g of freeze-dried pulp. All measurements were performed in triplicate in three different microplates using a microplate reader (Synergy MX, Biotek, Winooski, VT).

HPLC-DAD analysis of ascorbic acid, tocopherols and tocotrienols. The ascorbic acid was extracted from 3 g of freeze-dried pulp with an aqueous solution of 1 % oxalic acid and determined in a Shimadzu HPLC (Kyoto, Japan) with diode array detector (DAD) (SPD-M20A) at 244 nm, as described by Benassi and Antunes (*23*). The HPLC was equipped with quaternary pumps (LC-20AD), a degasser unit (DGU-20A5) and a Rheodyne injection valve with a 20 μL loop. The ascorbic acid was separated on a C_{18} Shim-pack column (5μm, 250 x 4.6 mm, Shimadzu, Kyoto, Japan) with an isocratic mobile phase containing sulphuric acid solution at pH 2.5 at 1.0 mL/min and column temperature set at 25 °C. The identification of ascorbic acid was carried out based on the retention time in the C_{18} column compared with the standard and by co-chromatography. The ascorbic acid was quantified by comparison to external standard using eight-point analytical curves based on standard solutions (measurements in duplicate), with concentrations varying from 5 to 60 μg/mL of ascorbic acid (r^2 = 0.99, limit of detection = 0.05 μg/mL and limit of quantification = 0.14 μg/mL, calculated using the parameters of the analytical curve).

The tocopherol and tocotrienol contents were analysed in accordance with AOCS (*24*) by using a normal phase HPLC system (Perkin Elmer, Waltham, MA), after diluting the oil from *Caryocar villosum* pulp, extracted according to Bligh and Dyer (*14*), in hexane. The Perkin Elmer HPLC consisted of an isocratic pump (Series 200) equipped with a spectrofluorometric detector (Series 200a) set at 290 nm for excitation and 330 nm for emission. The tocopherols and tocotrienols were separated on a Lichrosorb Si60 column (5 μm, 250 x 4 mm, Hibar Fertigsaule RT, Darmstadt, Germany) with an isocratic mobile phase consisted of hexane:isopropanol (99:1, v/v) at 1.0 mL/min. The peaks of tocopherols and tocotrienols were identified by comparison of retention times and co-elution with the available standards (α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol). The tocopherols and tocotrienols were quantified by comparison to external standard using five-point analytical curves based on standard solutions (measurements in duplicate), with concentrations varying from 1.7 to 11.9 μg/mL for all standards (r^2=0.99, limit of detection = 0.04 μg/mL and limit of quantification = 0.13 μg/mL, calculated using the parameters of the analytical curve).

The contents of ascorbic acid, tocopherols and tocotrienols were expressed as μg/g of freeze-dried pulp. All measurements were performed in triplicate.

HPLC-DAD-MS/MS analysis of carotenoids and phenolic compounds. The same extracts obtained for the spectrophotometric quantification of total phenolic compounds and total carotenoids were used to the separation, quantification and identification by HPLC-DAD-MS/MS. Both carotenoids and phenolic compounds extracts were analyzed in the same Shimadzu HPLC-DAD used for ascorbic acid determination, and connected in series to a mass spectrometer from Bruker Daltonics (Esquire 4000 model, Bremen, Germany) with APCI (Atmospheric Pressure Chemical Ionization) and ESI (Electrospray Ionization) sources, and an ion-trap analyzer.

After spectrophotometric measurement of total carotenoids, an aliquot of the extract was evaporated under N_2 flow, dissolved in MeOH:MTBE (70:30, v/v) and injected into the chromatographic system. The carotenoids were separated on a C_{30} YMC column (5 µm, 250 mm x 4.6 mm) using as mobile phase a linear gradient of MeOH:MTBE from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min (*20*). The flow rate was 0.9 mL/min and the column temperature was set at 29 ºC. The spectra were obtained between 200 and 600 nm and the chromatograms were processed at 450 nm. The MS parameters were set as follows: APCI source in the positive ion mode, current corona: 4000 nA, source temperature: 450 °C, dry gas N_2-temperature: 350 °C, flow: 5 L/min, nebulizer: 60 psi and MS/MS fragmentation energy: 1.4 V. The mass spectra were acquired with scan range of *m/z* from 100 to 800. The combined results of the following parameters were considered for carotenoid identification: elution order on the C_{30} column, UV/vis spectrum features (maximum absorption wavelength (λ_{max}), spectral fine structure (%III/II) and peak *cis* intensity (%A_B/A_{II})), and MS spectrum characteristics as compared to standards analyzed under the same conditions and data available in the literature. The carotenoids were quantified by HPLC, using external seven-point analytical curves (in duplicate) for 9-*cis*-neoxanthin (0.9-17.1 µg/mL), all-*trans*-violaxanthin (0.7-13.6 µg/mL), all-*trans*-antheraxanthin (0.8-15.9 µg/mL), all-*trans*-lutein (1.0–59.5 µg/mL), all-*trans*-zeaxanthin (1.3–59.7 µg/mL) and all-*trans*-β-carotene (1.1-30.2 µg/mL). All other carotenoids were estimated using the curve of zeaxanthin and the *cis*-isomers using the corresponding curve of the all-*trans*-carotenoid. For all analytical curves of carotenoids, the $r^2 = 0.99$ and the limit of detection was 0.1 µg/mL and the limit of quantification was 0.5 µg/mL. The NAS-IOM (*25*) conversion factor was used to calculate the vitamin A value, with 12 µg of dietary all-*trans*-β-carotene corresponding to 1 µg of retinol activity equivalent (RAE), and the activity used was 100 % for all-*trans*-β-carotene.

For the phenolic compounds, an aliquot of the hydromethanolic extract used for the Folin-Ciocalteau reaction was analyzed in the same HPLC-DAD-MS/MS described above.

In order to provide additional information, the extract was also subjected to acid hydrolysis (*26*) and analyzed by HPLC. The phenolic compounds were separated on a C_{18} Synergi Hydro column (4 µm, 250 x 4.6 mm, Phenomenex) at 0.9 mL/min, column temperature at 29 °C, with 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 linear gradient from A:B 99:1 to 50:50 in 50 min; then from 50:50 to 1:99 in 5 min. This latter ratio (1:99) was maintained for an additional 5 min. The column eluate was split to allow only 0.15 mL/min to enter the ESI interface. The spectra were obtained between 200 and 600 nm and the chromatograms were processed at 271 and 367 nm. The mass spectra were acquired with a scan range from *m/z* 100 to 800; the MS parameters were set as follows: ESI source in positive and negative ion modes; capillary voltage: 2000 V, end plate offset: -500 V, capillary exit: -110 V, skimmer 1: 10 V, skimmer 2: 5 V, dry gas (N_2) temperature: 310° C, flow rate: 5 L/min, nebulizer: 30 psi; MS/MS fragmentation energy: 1.4 V. The phenolic compounds were identified based on the following information: elution order and retention time of the peaks in relation to standards, in the reversed phase column, UV-visible and mass spectra features as compared to standards analyzed under the same conditions and data available in the literature. Phenolic compounds were quantified by comparison to external standards using seven-point analytical curves (in duplicate) for gallic acid (0.5-51.5 µg/mL), 4-coumaric acid (0.5-49.5 µg/mL), ellagic acid (0.5-52 µg/mL) and methyl quercetin (0.2-19.2 µg/mL). For all these compounds, $r^2 = 0.99$, limit of detection was 0.1 µg/mL and limit of quantification was 0.4 µg/mL, calculated using the parameters of the analytical curve.

The contents of carotenoids and phenolic compounds, as determined by HPLC-DAD, were expressed as µg/g of freeze-dried pulp. All measurements were performed in triplicate.

Statistical analysis. The mean and standard deviation (SD) results related to the chemical, phytochemical and antioxidant capacity analysis of *Caryocar villosum* pulp were calculated with the Statistica 6.0 software.

RESULTS AND DISCUSSION

Chemical and phytochemical composition of *Caryocar villosum* pulp. The fruits were irregularly oblong-globose, approximately 7-9 cm in diameter, the yellowish pulp represented approximately 12 % of total weight from the non-processed fruit (24 % of seed and 64 % of shell), in accordance with the values previously reported (*1, 2*).

The analysis of the main chemical constituents of *Caryocar villosum* pulp (piquiá), which is widely consumed in the Amazonian region of Brazil, were performed in order to obtain an overview about the chemical composition and nutritive value.

According to the nutritional composition (Figure 1a), water and lipids are the major components from *Caryocar villosum* pulp. However, the water content of piquiá (51.7 %) can be considered low as compared to other common fruits (> 80 %) (*27*). On the other hand, the nutritional composition of piquiá is similar to those from other Amazonian fruits with high lipid contents (12.8 – 47.2 %), such as *Jessenia pataua* (patauá), *Endopleura uxi* (uxi), *Bactris gasipaes* (pupunha), *Poraqueiba sericea* (mari) and *Astrocaryum vulgare* (tucumã), which presented low water contents (35.6 - 55.6 %) and with total energetic values from 252.4 to 474.0 Kcal/100 g (*28*). Due to its high lipid content, the nutritive value of *Caryocar villosum* pulp seems to be substantially determined by the composition of the lipid fraction (mainly palmitic and oleic acid) (*2*). Furthermore, piquiá pulp showed higher contents of water, proteins, ashes and carbohydrates than pequi one (*Caryocar brasiliense*) (*29*).

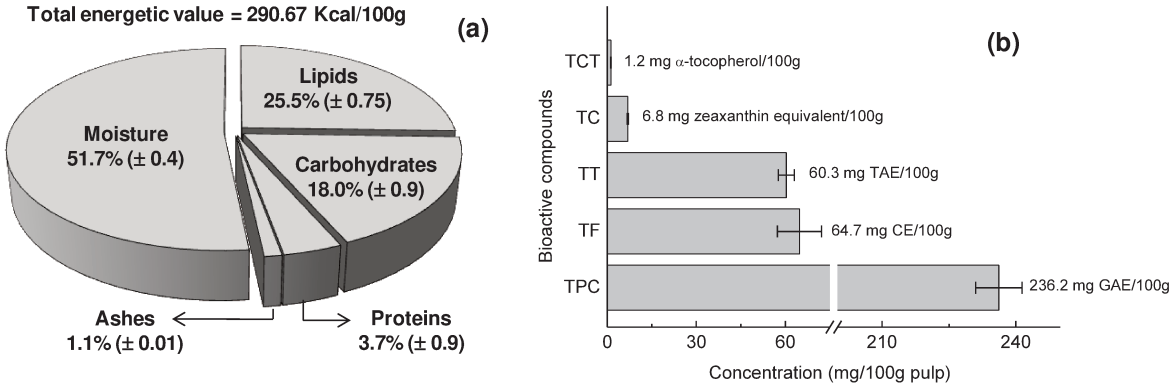

Figure 1. (a) Centesimal composition (wet basis) and (b) bioactive compounds (dry basis) from *Caryocar villosum* pulp. TPC = total phenolic compounds (GAE, gallic acid equivalent), TF = total flavonoids (CE, catechin equivalent), TT = total tannins (TAE, tannic acid equivalent), TC = total carotenoids and TCT = total tocopherols.

Regarding the bioactive compounds (Figure 1b), the contents (all in dry basis) of total phenolic compounds of piquiá pulp (236.2 mg GAE/100 g) presented intermediary values in comparison to other common fruits (47 – 795 mg GAE/100 g) (*27*). The *Caryocar villosum* pulp presented contents of total phenolic compounds and total carotenoids in the same range as those found for *Caryocar brasiliense* (209 mg GAE/100 g and 7.25 mg β-carotene equivalent/100 g, respectively) (*29*). However, the *Caryocar villosum* pulp

presented higher contents of total carotenoid and lower contents of total phenolic compounds and total flavonoids than those reported by Barreto et al. (*4*).

Among the tocopherols and tocotrienols searched, α-tocopherol was the only one found in *Caryocar villosum* pulp, and its content was similar to other 51 fruits analyzed by Chun et al. (*30*), whereas lower than other oily fruits, such as avocadoes and olives. In relation to the investigated tannins, only hydrolysable tannins were detected in piquiá pulp (60.3 mg TAE/100 g) and the content was lower than that found in fresh myrtle (*Myrtus communis* L.) (*31*). No condensed tannins were detected in piquiá pulp since no precipitate was formed after the addition of BSA. Furthermore, ascorbic acid was not detected.

The antioxidant capacity of piquiá pulp, as ORAC, was 3.74 ± 1.09 mMol Trolox/100 g, and its antioxidant capacity was higher than avocado (1.38 mMol Trolox/100 g), similar to the strawberry pulp (3.54 mMol Trolox/100 g) and, in general, presenting higher $ROO^{\bullet}$ scavenging capacity in comparison to the average reported value for 41 different fruits (2.7 mMol Trolox/100 g) (*27*). Wu et al. (*27*) used the ratio between ORAC values and total phenolic contents (TPC) (ORAC/TPC) to rank the raw food samples of the United States into four groups (0-5, 5-10, 10-15 and >15). Most ratio values of fruits and vegetables were close to 10-15, indicating a strong positive linear correlation between TPC and antioxidant capacity. The highest scavenging capacity was related to high ORAC/TPC ratio value. Considering this classification, the *Caryocar villosum* pulp was ranked into the fourth group (>15) (ORAC/TPC = 15.8) indicating high $ROO^{\bullet}$ scavenging capacity, with similar values reported for mustard seed (15.6), pepper (17.2) and turmeric (18.9) (*27*).

Identification and quantification of phenolic compounds. The phenolic compounds in *Caryocar villosum* pulp were tentatively identified by comparison of their UV-vis and mass spectra to those of available standards and data from the literature (Table 1). The retention time, UV-vis and mass spectra of 20 standards of phenolic compounds (gallic acid, catechin, hydroxybenzoic acid, caffeic acid, (-)-epicatechin, rutin, 4-coumaric acid, ellagic acid, ferulic acid, taxifolin, naringin, hesperidin, neohesperidin, myricetin, luteolin, quercetin, naringenin, apigenin, kaempferol and rhamnetin) were considered for the identification (data not shown).

Table 1. Chromatographic, spectroscopic characteristics and content of phenolic compounds from *Caryocar villosum* pulp.

Peaks	Compound	Concentration (μg/g pulp)[a]	t_R range (min)[b]	λ_{max} (nm)[c]	$[M-H]^-$ (*m/z*)	MS/MS (-) *(m/z)*[d]	Identity confirmation
1	Monogalloyl glucoside[1]	21.1 ± 1.4	8.6-8.8	274	331	**169,** 125	(*36*)
2	Gallic acid[1]	182.4 ± 17.0	9.9-10.1	271	169	**125**	Standard
3	HHDP glucoside[1]	20.4 ± 2.4	12.6-12.8	269	481	463, **319**, 301	(*37*)
4	Coumaroyl-galloyl glucoside[2]	4.5 ± 0.6	12.9-13.1	276, 303	477	**307**, 163, 145	(*37*)
5	Coumaroyl quinic acid[2]	2.7 ± 0.6	16.3-16.5	278	337	191, 173, **163**, 119	(*38*)
6	HHDP diglucoside[1]	17.4 ± 2.4	17.0-17.2	278	625	607, 581, 541, **463**, 301, 271, 257, 245	(*36*)
7	Di-galloyl glucoside[1]	18.4 ± 5.7	17.6-17.8	273	483	331, **313**, 271, 193, 169	(*36*)
8	Galloyl-HHDP glucoside[1]	20.6 ± 4.8	19.9-20.0	279	633	615, 591, 481, 463, **301**, 275, 247, 231	(*34, 36*)
9	Ellagic acid glucoside[3]	16.9 ± 2.0	21.3-21.5	360	463	**301**, 283, 257	(*34, 36*)
10	Ellagic acid arabinoside[3]	9.3 ± 1.8	23.8-23.9	361	433	415, 391, 373, 365, **301**, 271, 187	(*34, 36*)
11	Ellagic acid rhamnoside[3]	107.0 ± 9.4	24.5-24.7	253, 300(sh), 361	447	**301,** 257, 235, 217	(*34, 36*)
12	Not identified[1]	29.3 ± 3.6	25.4-25.6	285	495	467, 449, **427,** 383, 359	-
13	Ellagic acid[3]	104.0 ± 10.5	25.9-26.1	253, 300(sh), 367	301	284, 271, **257**, 229, 213, 185, 157	Standard
14	Methyl ellagic acid arabinoside[3]	8.2 ± 2.2	26.4-26.6	279, 366	447	**315**, 300	(*40*)
15	Methyl ellagic acid rhamnoside[3]	10.5 ± 2.1	28.4-28.6	300(sh), 366	461	417, **315**, 300	(*7, 40*)
16	Methyl quercetin diglucoside[4]	6.3 ± 1.1	29.4-29.6	365	639	595, 459, **315**, 300, 165	(*41*)
17	Di-methyl ellagic acid arabinoside[3]	10.3 ± 1.6	29.9-30.1	360	461	417, **329**, 315, 300	(*7, 40*)
Total phenolic compounds (μg/g pulp)		589.4 ± 41.5					

[a]n = 6 (dry basis). [b]Retention time on the C_{18} Synergi Hydro (4μm) column. [c]Solvent: gradient of 0.5 % formic acid in water and acetonitrile with 0.5 % formic acid. [d]In the MS^2, the most abundant ion is shown in boldface. HHDP = Hexahydroxydiphenoyl. The peaks were quantified as equivalent of gallic acid[1], coumaric acid[2], ellagic acid[3] and methyl quercetin[4].

The HPLC-DAD chromatograms of hydromethanolic extract recorded at 271 and 367 nm (Figure 2) illustrate the increasing selectivity in detection at higher wavelengths. All phenolic compounds of *Caryocar villosum* pulp showed absorption near the wavelength at 271 nm and/or at 367 nm. Phenolic compounds containing at least one aromatic ring (A-ring) absorb UV light with maximum at 240–285 nm range. Due to the substitution pattern and conjugation of the C-ring, the second maximum is at 300–370 nm range (with an additional maximum wavelength at 500–525 nm range if anthocyanidins are included) (*32*). The major phenolic compounds found in the pulp (dry basis) of *Caryocar villosum* were gallic acid (182.4 μg/g pulp), followed by ellagic acid rhamnoside (107.0 μg/g pulp) and ellagic acid (104.0 μg/g pulp). In relation to the identification of the peaks described in Table 1, HPLC coupled to mass spectrometry, using electrospray ionization (ESI), proved to be extremely useful for peak assignment and further characterization of individual compounds, once that only gallic and ellagic acids were available as reference compounds. The ESI in the negative ion mode provided a very sensitive, selective method and produced by far the most characteristic data for the identification of phenolic compounds in this pulp extract. In our study, no ionization in the MS was obtained in positive ion mode, in accordance with the identification of similar phenolic compounds in previous studies (*32, 34*).

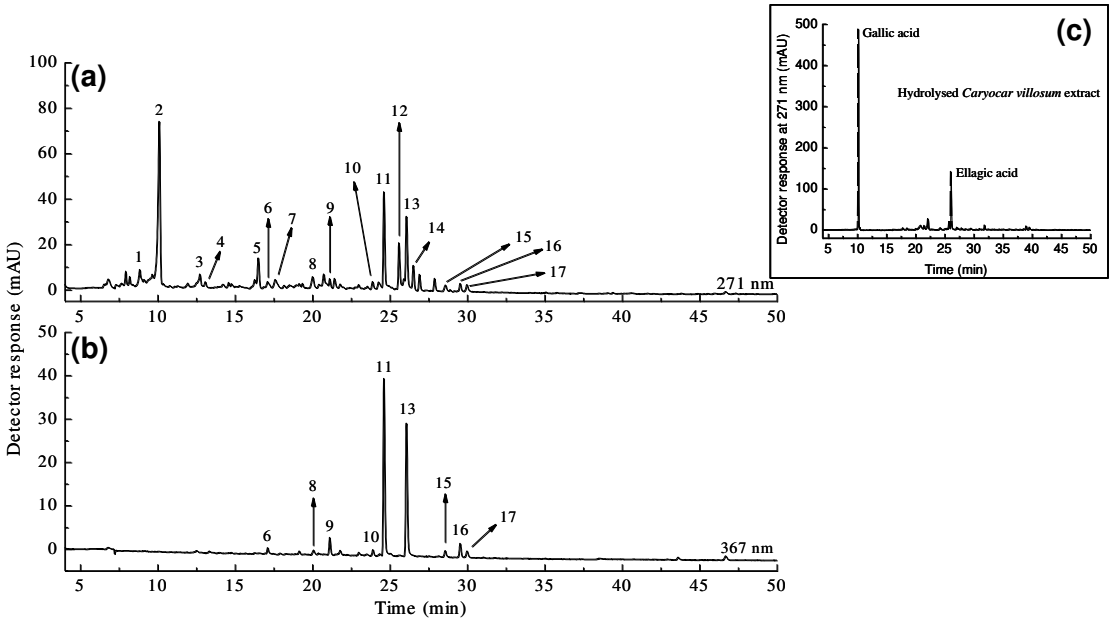

Figure 2. Chromatograms obtained by HPLC–DAD of phenolic compounds from *Caryocar villosum* pulp, (a) processed at 271 nm, (b) processed at 367 nm and (c) hydrolyzed extract processed at 271 nm. Chromatographic conditions: see text. Peak characterization is given in **Table 1**.

Phenolic compounds in nature generally occur as conjugates of sugars, usually *O*-glycosides. Glucose, fructose and sucrose were the sugar molecules already reported in the pulp of *Caryocar villosum* (*2*). Additionally, rhamnose and glucose conjugated with phenolic acids were identified in its stem bark (*7*) and arabinose was found in the peel extract of the fruit (*35*). In the MS/MS analysis of the phenolic compounds from the pulp of *Caryocar villosum*, cleavage of the glycosidic linkage with concomitant H rearrangement leads to elimination of the sugar residue, that is, 162 u (an hexose: glucose or galactose), 146 u (a deoxyhexose: rhamnose), 132 u (a pentose: xylose or arabinose). The identity of the sugar moiety could not be determined by the methodology used in the present study; thus, designation of sugar molecules was based on literature reports for *Caryocar sp*.

Peak **1** was assigned as a monogalloyl glucoside, showing deprotonated molecule at *m/z* 331 and fragment at *m/z* 169 (loss of hexose), which corresponds to the gallic acid moiety, and the fragment at *m/z* 125, which corresponds to the consecutive losses of 162 u (hexose) and 44 u (CO_2), as reported for phenolic compounds in seed, skin and pulp of muscadine grapes (*36*). Peak **2** was positively identified as gallic acid on the basis of co-elution and comparison of UV and mass spectra with authentic standard, and the MS spectrum showed $[M-H]^-$ at *m/z* 169 with MS/MS fragment at *m/z* 125, corresponding to the neutral loss of 44 u (CO_2). Moreover, the gallic acid was the major compound identified in the *Caryocar villosum* extract after acid hydrolysis (Figure 2c). Gallic acid was also identified as one of the major phenolic compounds in ethanolic extract from the peel of *Caryocar brasiliense* fruit (*35*). Peak **3**, identified as a hexahydroxydiphenoyl glucoside (HHDP), showed $[M-H]^-$ at *m/z* 481 and MS/MS fragments at *m/z* 463 (loss of H_2O), *m/z* 319 (loss of hexose), which corresponds to the HHDP moiety, and the fragment at *m/z* 301 corresponding to the consecutive neutral losses of hexose and water molecules. Peak **4** was identified as a coumaroyl-galloyl glucoside, once that the deprotonated molecule showed $[M-H]^-$ at *m/z* 477 and MS/MS fragments at *m/z* 307 (loss of a gallic acid moiety), *m/z* 163 (loss of a galloyl glucose), which corresponds to the coumaric acid moiety, and *m/z* 145 (loss of H_2O from *m/z* 163), as previously reported for phenolic compounds of strawberry (*33*). Peak **5** was identified as a coumaroyl quinic acid, based on the similar characteristics found for the same compound in *Erigeron breviscapus* (*38*), i.e., the presence of $[M-H]^-$ at *m/z* 337 and MS/MS fragments at *m/z* 191 (loss of a coumaroyl group), which corresponds to the presence of quinic acid, *m/z* 173 (loss of H_2O from quinic acid), *m/z* 163 originated from coumaric acid moiety and *m/z* 119 (loss of CO_2 from coumaric acid). Peak **6** had a deprotonated molecule at *m/z* 625 $[M- H]^-$ that dissociated to give an intense MS/MS at *m/z* 463 $[M- H- 162]^-$, indicating the loss of a hexose unit, and

the presence of the ion at *m/z* 301 suggesting the loss of another hexose unit [M-H-162-162]$^-$. On the basis of fragmentation data, peak **6** was tentatively identified as HHDP diglucoside. Peak **7** was assigned as a di-galloyl glucoside due to the presence of the deprotonated molecule at *m/z* 483 [M-H]$^-$ and MS/MS fragments at *m/z* 331 [M-H-152]$^-$ and at *m/z* 169 [M- H-152-162]$^-$, after sequential removal of the galloyl group (152 u) and the hexose moiety (162 u), respectively. Peak **8**, identified as a galloyl-HHDP glucoside, had an ion at *m/z* 633 [M-H]$^-$ and MS/MS fragments at *m/z* 481, indicating the presence of HHDP-glucose via the loss of a galloyl unit [M-H-152]$^-$, and at *m/z* 301, suggesting the loss of a glucose unit. The loss of a galloyl unit suggested that this unit was attached directly to the glucose, because a galloyl bond via a *m*-depside bond is more cleavable and would give loss of a galloyl unit (152 u) (*34; 36*). Peaks **9**, **10** and **11** were identified as ellagic acid glucoside ([M-H]$^-$ at *m/z* 463), ellagic acid arabinoside ([M-H]$^-$ at *m/z* 433) and ellagic acid rhamnoside ([M-H]$^-$ at *m/z* 447), respectively. These compounds showed MS/MS fragment at *m/z* 301 as the most abundant ion, suggesting neutral losses of glucose (loss of 162 u from [M-H]$^-$ of peak 9), arabinose (loss of 132 u from [M-H]$^-$ of peak 10) and rhamnose (loss of 146 u from [M-H]$^-$ of peak 11), all in accordance with that previously reported in the literature (*34, 36*). Peak **12** was not identified, whereas presented absorption maximum at 285 nm and showed [M-H]$^-$ at *m/z* 495 and the most abundant ion in MS/MS at *m/z* 427 (loss of 68 u). Peak **13** was identified as ellagic acid on the basis of co-elution and comparison of UV and mass spectra with authentic standard. This peak presented [M-H]$^-$ at *m/z* 301 and typical MS/MS fragmentation ions of ellagic acid (*34, 36, 39*) at *m/z* 257 (loss of CO_2), 229 (loss of CO_2 and CO), and 185 (loss of two CO_2 and one CO). Furthermore, the ellagic acid was the second major compound identified in the *Caryocar villosum* extract after acid hydrolysis (Figure 2c). Peak **14**, identified as a methyl ellagic acid arabinoside, showed [M-H]$^-$ at *m/z* 447 and presented MS/MS fragments at *m/z* 315 (loss of an arabinose moiety), and at *m/z* 300, corresponding to the ellagic acid after neutral loss of one methyl group from the fragment at *m/z* 315. Peaks **15** and **17** were identified as methyl ellagic acid rhamnoside and di-methyl ellagic acid arabinoside, respectively, both with deprotonated molecule at *m/z* 461 ([M-H]$^-$) and the first MS/MS fragment at *m/z* 417 ([M-H-CO_2]$^-$). However, in peak **15**, the neutral loss of rhamnose can be seen by the MS/MS fragment at *m/z* 315 ([M-H-146]$^-$), and in peak **17** the fragment at *m/z* 329 ([M-H-132]$^-$) can be attributed to the neutral loss of arabinose. Additionally, peaks **15** and **17** showed the same MS/MS fragment at *m/z* 300, probably due to the neutral loss of one methyl group (peak **15**) or two methyl groups (peak **17**) after losing their sugar moieties. The structure of 3-O-methyl-4'-(3''-O-acetyl)-α-L-rhamnopyranosyl ellagic acid

was previously reported as the phenolic glycoside from the stem bark of *Caryocar villosum* and *Caryocar glabrum* (*7*), confirming the assignment of peaks **11** (ellagic acid rhamnoside) and **15** (methyl ellagic acid rhamnoside). Finally, peak **16**, with $[M-H]^-$ at *m/z* 639, was identified as methyl quercetin diglucoside after considering the MS/MS fragments at *m/z* 595 (loss of CO_2), at *m/z* 315 (loss of diglucoside moiety, 324 u) and at *m/z* 300 (loss of methyl group after losing the diglucoside moiety), also observed in Sicilian wines (*41*). The fragment at *m/z* 300 found in peak **16** was considered as quercetin and not as ellagic acid, once that a methyl ellagic acid diglucoside should eluted before peak **15**. The basic chemical structures of the plenolic compounds proposed for *Caryocar villosum* pulp are shown in Figure 3.

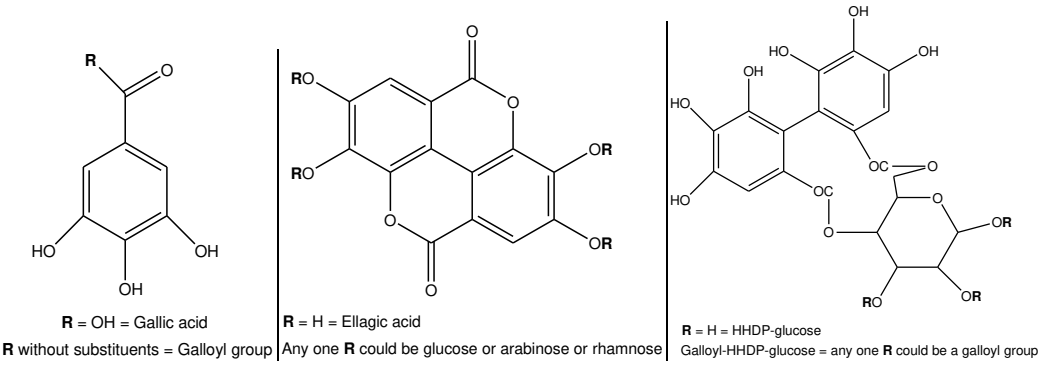

Figure 3. Chemical structures of phenolic compounds proposed for *Caryocar villosum* pulp.

Identification and quantification of carotenoids. The carotenoids separated from *Caryocar villosum* pulp (Figure 4) were tentatively identified and the MS/MS experiments confirmed the assignment of the protonated molecule ($[M+H]^+$) of all identified peaks through the fragments expected for the carotenoid polyene chain and functional groups (Table 2). Although in-source fragmentation occurred in the APCI interface, the MS/MS unambiguously allowed the assignments of some functional groups due to the specific losses from the protonated molecule. Considering that a detailed description of carotenoid identification using the above information was already reported (*20, 42, 43*), only some of the most important aspects were discussed below.

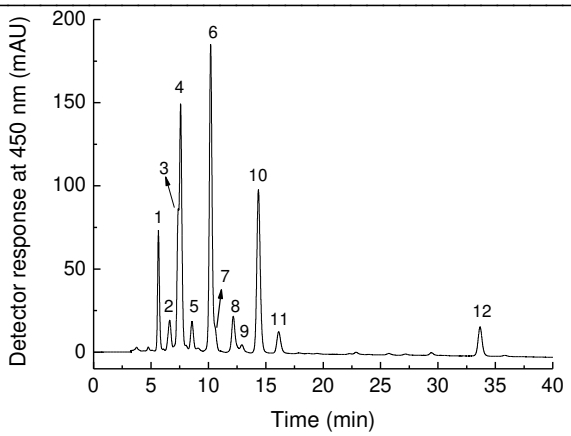

Figure 4. Chromatogram obtained by HPLC–DAD of carotenoids from *Caryocar villossum*. Chromatographic conditions: see text. Peak characterization is given in **Table 2**.

All identified carotenoids presented two hydroxyl groups, with exception of β-carotene (peak **12**), and as expected for reversed-phase columns, the xanthophylls eluted before this carotene. The carotenoids with 5,6-epoxide and/or 5,8-furanoid groups located at the 3- or 3′-hydroxy β-rings, all-*trans*-neoxanthin (peak **1**), 9-*cis*-isomers of neoxanthin (peaks **2** and **5**), all-*trans*-violaxanthin (peak **3**), 9-*cis*-violaxanthin (peaks **7**), all-*trans*-antheraxanthin (peak **6**), 9-*cis*-mutatoxanthin (peak **9**) and 9-*cis*-antheraxanthin (peak **11**), showed UV-Vis and mass spectra features similar to those reported in the literature (*20, 42, 43, 44*). The peaks 9-*cis* and *9'-cis* isomers of neoxanthin was also tentatively identified by comparison with 9-*cis* isomers of neoxanthin reisolated from spinach, characterized by HPLC, UV-Vis and NMR and submitted to photoinduced stereomutation in the presence of iodine or diphenyl diselenide at conditions not involving isomerization of the allenic bond (*44*). The identification of 9-*cis*-neoxanthin, all-*trans*-violaxanthin, all-*trans*-antheraxanthin and all-*trans*-β-carotene (peak **12**) were also confirmed through co-elution with authentic standards, and based on UV-Vis and mass spectra features in comparison to standards and to the literature (*20, 42, 43*). Since the major carotenoids identified in the pulp of *Caryocar villosum* presented epoxide groups, all analysis were performed again in both fresh and freeze-dried pulps, with and without addition of $NaHCO_3$ during extraction step, and the carotenoid profiles did not show any differences.

Table 2. Chromatographic, UV-Vis, mass spectroscopy characteristics and content (dry basis) of carotenoids from *Caryocar villossum*, obtained by HPLC-DAD-APCI-MS/MS.

Peak[a]	Carotenoid	Concentration (μg/g pulp)	t_R range (min)[b]	Area (%)	λ_{max} (nm)[c]	%III/II	$\%A_B/A_{II}$	$[M+H]^+$ (*m/z*)	MS/MS (+) (*m/z*)[d]
1	All-*trans*-neoxanthin[1]	2.3 ± 0.6	5.6-5.7	9.0	416, 440, 469	90	0	601	**583 [M+H-18]+,** 565 $[M+H-18-18]^+$, 547 $[M+H-18-18-18]^+$, 509 $[M+H-92]^+$, 221
2	9-*cis*-neoxanthin[1]	0.8 ± 0.2	6.5-6.6	2.6	327, 416, 440, 469	83	13	601	**583 [M+H-18]+,** 565 $[M+H-18-18]^+$, 547 $[M+H-18-18-18]^+$, 509 $[M+H-92]^+$, 221
3	All-*trans*-violaxanthin[2]	1.1 ± 0.2	7.2-7.4	6.1	415, 439, 468	92	0	601	**583 [M+H-18]+,** 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491 $[M+H-92-18]^+$, 221
4	Not identified[4]	2.8 ± 0.4	7.5-7.6	22.1	420, 445, 473	55	0	nd	**583 $[M+H-18]^{+e}$,** 565 [M+H-18-18]+, 547 [M+H-18-18]+, 491 [M+H-92-18]+
5	9'-*cis*-neoxanthin[1]	0.8 ± 0.2	8.5-8.6	2.8	325, 416, 439, 467	64	6	601	**583 [M+H-18]+,** 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491 $[M+H-92-18]^+$, 221
6	All-*trans*-antheraxanthin[3]	3.4 ± 0.8	10.1-10.2	27.3	420, 444, 472	61	0	585	**567 $[M+H-18]^+$**, 549 $[M+H-18-18]^+$, 529 $[M+H-56]^+$, 221
7	9-*cis*-violaxanthin[2]	0.4 ± 0.1	10.6-10.7	1.4	325, 413, 435, 464	73	8	601	**583 [M+H-18]+,** 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 221
8	All-*trans*-lutein[5]	0.9 ± 0.2	12.1-12.2	3.4	420, 444, 471	55	0	nd	**551 $[M+H-18]^{+e}$**, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$
9	9-*cis*-mutatoxanthin[4]	0.6 ± 0.5	12.8-12.9	1.5	310, 405, 427, 452	78	11	585	**567 $[M+H-18]^+$**, 549 $[M+H-18-18]^+$, 493 [M+H-92], 221
10	All-*trans*-zeaxanthin[4]	2.9 ± 0.3	14.3-14.4	18.9	425, 450, 476	33	0	569	**551 $[M+H-18]^+$**, 533 $[M+H-18-18]^+$, 477 $[M+H-92]^+$
11	9-*cis*-antheraxanthin[3]	0.6 ± 0.2	16.1-16.2	1.3	325, 417, 440, 468	63	7	585	**567 $[M+H-18]^+$**, 549 $[M+H-18-18]^+$, 221
12	All-*trans*-β-carotene[6]	0.7 ± 0.04	33.6-33.7	3.7	425, 451, 477	40	0	537	**444 $[M+H-92]^+$**
Total carotenoids (μg/g pulp)		17.3 ± 2.4							
Vit. A value (μg RAE/g pulp)		0.06 ± 0.003							

[a]Numbered according to the chromatogram shown in **Figure 4**. [b]Retention time on the C_{30} column. [c]Linear gradient of methanol/MTBE. [d]In the MS^2, the most abundant ion is shown in boldface. [e]In-source detected fragment. nd = not detected. RAE = retinol activity equivalent. The peaks were quantified as equivalent of 9-*cis*-Neoxanthin[1], violaxanthin[2], antheraxanthin[3], zeaxanthin[4], lutein[5] and β-carotene[6].

Despite all-*trans*-lutein (peak **8**) and all-*trans*-zeaxanthin (peak **10**) have the same chemical formula ($C_{40}H_{56}O_2$) and, therefore, identical protonated molecule (*m/z* 569), zeaxanthin possess two β-rings, while lutein has one β-ring and one ε-ring. Thus, one of the hydroxyl groups of lutein is allylic to the double bound in the ε-ring and not conjugated with the polyene chain, resulting in ten conjugated double bonds (c.d.b.). On the other hand, zeaxanthin has both double bonds in β-ring conjugated to the polyene chain, and consequently its cromophore shows eleven c.d.b. Therefore, it is possible to distinguish them by UV-visible and also mass spectra fragmentations. As expected, zeaxanthin showed λ_{max} values (425, 450, 476 nm) higher than those of lutein (420, 444, 471 nm). The mass spectrum of zeaxanthin showed a more intense protonated molecule peak (*m/z* 569) in comparison to the fragment at *m/z* 551 $[M+H-18]^+$, whereas the contrary was observed for lutein, as previously reported by De Rosso and Mercadante (20, 43). Moreover, the identities of the peaks of lutein and zeaxanthin were confirmed by co-elution with authentic standards

A non-identified carotenoid (peak **4**) was also found in piquiá (2.84 μg/g), presenting λ_{max} at 445 nm in methanol/MTBE (%III/II = 55 and no *cis* peak) and an in-source fragment at *m/z* 583 ($[M+H-18]^+$). This peak did not co-eluted neither with the available standards (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein and β-carotene) nor with the carotenoids from saponified extracts of kale, mango and flowers of chrysanthemum, with and without HCl addition for 5,8 epoxide formation, or heat treatment for *cis* isomerization. Thus, the isolation of this compound by chromatographic techniques as well as the identification by nuclear magnetic resonance must be performed in order to establish the correct structure of this possible new carotenoid.

This is the first report about the carotenoid composition from *Caryocar villosum* (piquiá) pulp. All-*trans*-antheraxanthin was the major carotenoid, followed by peak **4** (not identified) and all-*trans*-zeaxanthin. Azevedo-Meleiro and Rodriguez-Amaya (*11*) reported the carotenoid composition of *Caryocar brasiliense* (pequi), and the main carotenoids were violaxanthin, lutein and zeaxanthin, with smaller amounts of β-cryptoxanthin, β-carotene and neoxanthin. Although having similar popular names, these fruits are different and, therefore, the compositional differences are not surprising.

For vitamin A activity, a carotenoid must have at least one unsubstituted β-ionone ring with an attached polyene side chain of at least eleven carbons. According to these chemical structural requirements, among the identified carotenoids of *Caryocar villosum* pulp, only β-carotene possess vitamin A activity (0.06 μg RAE/g pulp), which was higher than that found for jackfruit (0.03 μg RAE/g pulp) (*45*) and lower than those reported for

other Amazonian fruits, such as buriti (72.8 µg RAE/g pulp), mamey (6.9 µg RAE/g pulp), marimari (6.0 µg RAE/g pulp), palm oil (15.3 µg RAE/g pulp), peach palm (14.9 µg RAE/g pulp), physalis (11.1 µg RAE/g pulp) and tucuma (8.5 µg RAE/g pulp) (*20*). Thus, the pulp of *Caryocar villosum* should not be considered as a good pro-vitamin A source.

Based on these results, the pulp of piquiá presents a promising chemical composition for the research of bioactive compounds with antioxidant properties. Additionally, due to its nutritional composition and energetic value, the consumption of *Caryocar villosum* pulp can be considered important to the population who lives around the occurrence area of this species.

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CAPÍTULO VI

Classification of different extracts from an Amazonian fruit (*Caryocar villosum)* using multivariate statistics based on antioxidant and colour properties

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Classification of different extracts from an Amazonian fruit (*Caryocar villosum)* using multivariate statistics based on antioxidant and colour properties

Short title: Bioactive compounds from *Caryocar villosum* extracts.

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ABSTRACT

Caryocar villosum was reported to be a potential source of bioactive compounds, such as phenolic compounds and carotenoids. In this study, the main purpose was to obtain natural extracts from the freeze-dried pulp of *Caryocar villosum* fruits using five solvents with different polarities (water, ethanol:water, ethanol, ethanol:ethyl acetate and ethyl acetate). All natural extracts were characterised in relation to the contents of bioactive compounds (total phenolic compounds, flavonoids, tannins, carotenoids and tocopherols). In addition, the scavenging capacity against peroxyl radical ($ROO^{\bullet}$) through the Oxygen Radical Absorbance Capacity (ORAC) assay, as well as the quenching activity against singlet oxygen ($^1O_2$) was determined for all the extracts. Colour parameters were measured in the CIELAB system. All data were used for extract classification applying multivariate statistical analysis. The water and ethanol:water extracts presented the highest levels of total phenolic compounds (9.2 and 6.3 mg gallic acid equivalent/g, respectively), total flavonoids (3.8 and 2.5 mg catechin equivalent/g, respectively) and total tannins (7.6 and 2.4 mg tannic acid/g, respectively). The ethanol:water extract also showed the highest scavenging capacity against $ROO^{\bullet}$ (ORAC) (0.3 mmol Trolox equivalent/g extract). On the other hand, the ethanol extract, which was classified as the most vivid and yellow one (C^*_{ab} = 13.7 and b^* = 13.3), presented the highest level of total carotenoids (0.1 mg/g) and the highest percentage of protection against $^1O_2$ (10.6 %). Based on the results of this study, ethanol:water mixture, water and ethanol are the most promising solvents to obtain *Caryocar villosum* extracts with high content of bioactive compounds, protection against singlet oxygen and peroxyl radical scavenging capacity.

Keywords: Caryocaraceae, phenolic compounds, carotenoids, antioxidant capacity, PCA, HCA.

INTRODUCTION

Piquiá (*Caryocar villosum* (Aubl.) Pers.), a native fruit from Amazonia region, is used in regional dishes (usually with rice) and homemade soap, and can be used as a substitute of butter due to the characteristic of providing edible oil and in cosmetic applications (Clement, 1993; Pianovski et al., 2008). The pulp of *Caryocar villosum* was reported to contain 50 % of water, and in dry matter possess 65 % of oil, 3 % of available carbohydrate, 83 mg calcium/100 g, 52 mg magnesium/100 g and 41 mg phosphorus/100 g (Marx et al., 1997). Additionally, piquiá showed the highest values of total phenolic compounds, flavonoids and antioxidant activity (ABTS assay) in comparison to other 18 tropical fruits (nine of them from the Amazonian region) in a previous screening performed by our research group (Barreto et al., 2009). Chisté and Mercadante (***Chapter V***) reported that the major phenolic compounds identified in *Caryocar villosum* pulp were gallic acid, followed by ellagic acid rhamnoside and ellagic acid (Figure 1). These same authors also reported the carotenoid composition and the main compounds identified were all-*trans*-antheraxanthin, all-*trans*-zeaxanthin and all-*trans*-neoxanthin (Figure 1).

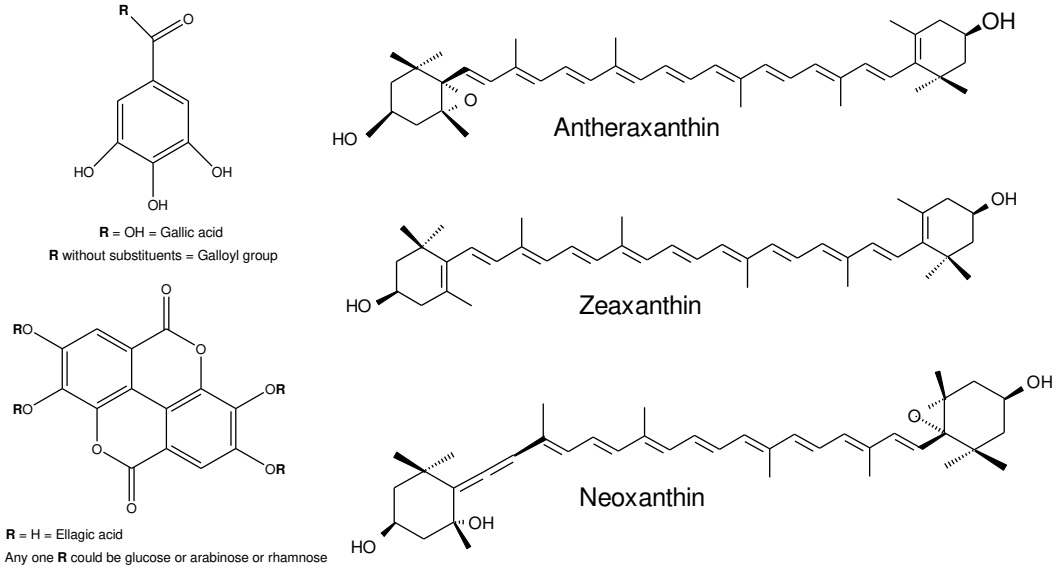

Figure 1. Chemical structures of gallic acid ($C_7H_6O_5$), ellagic acid ($C_{14}H_6O_8$), antheraxanthin ($C_{40}H_{56}O_3$), zeaxanthin ($C_{40}H_{56}O_2$) and neoxanthin ($C_{40}H_{56}O_4$) found in *Caryocar villosum* pulp.

High ingestion of fruits has been associated with the low incidence of degenerative diseases (Serdula et al., 1996). This effect is associated not only to the presence of antioxidants such as vitamins C and E, but also to other natural bioactive compounds, such as carotenoids, flavonoids and other phenolic compounds, which show scavenging capacity against both reactive oxygen and nitrogen species as well as quenching property against singlet oxygen and the triplet state of sensitizers (Krinsky, 1994; Huang et al., 2005; Chisté et al., 2011a; Finley et al., 2011). Thus, the production of natural extracts with high levels of bioactive compounds, from accessible natural sources, can be considered a very interesting approach to the food, pharmaceutical and cosmetic industries.

In addition, natural extracts may show, depending on the type of extraction and solvent employed, other compounds with beneficial actions to human health, which may be an advantage when compared to the colouring agents obtained by chemical synthesis with high purity degree (Chisté et al., 2011b). According to the Department of Health and Human Services, Food and Drug Administration (FDA, USA) in a Federal Register published on December 1997 (Vol. 62, No. 247) (FDA, 1997), the solvents are divided into three classes: class 1: solvents to be avoided (known human carcinogens, strongly suspected human carcinogens, and environmental hazards), class 2: solvents to be limited (nongenotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity) and class 3: solvents with low toxic potential (solvents with low toxic potential to man; no health-based exposure limit is needed). Class 3 solvents, which comprise ethanol and ethyl acetate, have allowed daily exposure of 50 milligrams or more per day and may be regarded as less toxic and with lower risk to human health.

Considering the potential of *Caryocar villosum* pulp as a natural potential source of phenolic compounds and carotenoids, this work was designed to obtain natural extracts with high levels of these bioactive compounds by the extraction using solvents with different polarities. Moreover, all extracts were analysed by two multivariate exploratory techniques in order to define the most promising solvent to obtain *Caryocar villosum* extracts with both antioxidant and colour properties.

EXPERIMENTAL

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), fluorescein, methylene blue, tannic acid, BSA (bovine serum albumin), AAPH (α,α′-Azodiisobutyramidine dihydrochloride) and all-*trans*-β-carotene were purchased from Sigma-Aldrich (Steinheim, Germany) and Folin-Ciocalteau reagent from Dinamica (São

Paulo, Brazil). The gallic acid, ellagic acid and catechin standards were purchased from Extrasynthèse (Lyon Nord, France). The standard of all-*trans*-zeaxanthin was provided by DSM Nutritional Products (Basel, Switzerland). The standard of all-*trans*-violaxanthin was isolated from kale by open column chromatography (Kimura & Rodriguez-Amaya, 2002). The standards of α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol were purchased from Calbiochem (Darmstadt, Germany). All standards showed at least 95 % of purity, determined by HPLC-DAD. Hexane and isopropyl alcohol of chromatographic grade were obtained from J.T. Baker (Phillipsburg, NJ). Methanol, ethanol, ethyl acetate, petroleum ether, ethyl ether all were analytical grade and purchased from Synth (São Paulo, Brazil). All other chemical salts and solvents of analytical grade were purchased from Synth (São Paulo, Brazil) or Merck (Darmstadt, Germany).

Material. The *Caryocar villosum* fruits (piquiá) were acquired at the "Ver-O-Peso" market in Belém, Pará State, Brazil (latitude 01°27'21'' and longitude 48°30'16'') in March 2010. All ripe fruits (9 kg) were cut into halves and the shells were manually removed from the pulp and seeds. The yellow coloured pulp was separated from the seeds, weighted, grinded and immediately freeze-dried (Liobras equipment, São Paulo, Brazil). The lyophilized material was thoroughly mixed, vacuum packed and stored under light-free conditions at -36 ºC until analysis.

Preparation of *Caryocar villosum* extracts. Triplicate extractions were performed in a completely randomized design, using the following solvents: water, ethanol:water (1:1, v/v), ethanol, ethanol:ethyl acetate (1:1, v/v) and ethyl acetate. These solvents were chosen considering the permissibility of residues in the extracts after evaporation, according to the Commission Directive 95/45/EC from European Communities (1995). The freeze-dried pulp of *Caryocar villosum* was weighted (5 g) and the solvents were added at the mass:solvent ratio of 1:8 (w/v), stirred on an orbital shaker MA 140/CFT (Marconi, São Paulo, Brazil) at 168 rpm for 15 hours at room temperature (25 °C). All operations were protected against luminosity. After the extraction time, the extracts were vacuum filtered and the residues in the filter were washed with the respective solvent (5 mL). The filtered extracts were transferred to volumetric flasks (50 mL) and filled with the respective solvent. Each liquid extract was subjected to colour analysis, frozen with liquid nitrogen and lyophilised in a freeze-drier (Liobras, São Paulo, Brazil). The freeze-dried extracts were

transferred to amber flasks, weighted to calculate the yield of solid extract, sealed under N_2 flow and stored at -36 ºC until analysis.

Colour measurements. Colour measurements in the liquid extracts of *Caryocar villosum* were performed using a spectrocolorimeter Color Quest XE (HunterLab, Reston, USA) using reflectance with excluded specular, equipped with the light source D65 and observation angle of 10°. Using the values obtained in the CIELAB system, *L** (lightness), and the chromatic coordinates *a** (red-green component) and *b** (yellow-blue component), the values of C^*_{ab} (chroma) and h_{ab} (hue angle) were calculated according to Equations 1 and 2. All measurements were performed in triplicate.

$$C^*_{ab} = [(a^*)^2 + (b^*)^2]^{1/2} \quad (1)$$

$$h_{ab} = \arctan\left(\frac{b^*}{a^*}\right) \quad (2)$$

Phytochemical analysis of *Caryocar villosum* extracts. The content of total phenolic compounds of each extract was determined using the Folin-Ciocalteau colorimetric method (Singleton *et al*., 1999). The *Caryocar villosum* extracts, obtained with different solvents, were weighted (0.1 g) in tubes of 15 mL and extracted with methanol:water (8:2, v/v) (1 mL) in an ultrasound equipment (Unique model, São Paulo, Brazil) for 5 min at 25 °C. The tubes were centrifuged at 2683 x g for 5 min at 20 °C. After centrifugation, the supernatant was transferred to volumetric flasks. The extraction was repeated five times and all supernatants were combined in order to obtain 5 mL as the final volume. These liquid extracts were kept in the freezer (-18 °C) for 20 min before centrifugation at 290 x g for 20 min at 20 °C. The quantification was performed with an UV-Visible spectrophotometer (Agilent, Santa Clara, USA) at 750 nm by using a seven-point analytical curve (measurements in duplicate), with concentrations varying from 1 to 8 μg/mL of gallic acid standard (r^2=0.99, limit of detection = 1.19 μg/mL and limit of quantification = 3.61 μg/mL, calculated using the parameters of the standard curve). The results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of freeze-dried extract.

The quantification of total flavonoids was carried out using the colorimetric assay described by Zhishen et al. (1999). For this purpose, an aliquot from the same liquid extract obtained for quantification of phenolic compounds was used. The quantification was performed with the same UV-Vis spectrophotometer described above at 510 nm by

using a seven-point analytical curves (measurements in duplicate), with concentrations varying from 2.5 to 20 μg/mL of catechin standard (r^2=0.99, limit of detection = 0.48 μg/mL and limit of quantification = 1.46 μg/mL, calculated using the parameters of the standard curve). The results were expressed as milligrams of catechin equivalent (CE) per 100 g of freeze-dried extract.

The total tannins (hydrolysable + condensed) were determined according to the methodology described by Brune et al. (1991) (hydrolysable tannins) and Waterman and Mole (1994) (condensed tannins). An aliquot from the same liquid extract obtained for quantification of phenolic compounds was used for the quantification of total tannins. The absorbance was measured with an UV-Visible spectrophotometer at 578 nm (hydrolysable tannins) and at 510 nm (condensed tannins). The total tannins were quantified by using six-point analytical curves (measurements in duplicate), with concentrations varying from 1.02 to 41 μg/mL of tannic acid standard (r^2=0.99, limit of detection = 3.05 μg/mL and limit of quantification = 9.25 μg/mL, calculated using the parameters of the standard curve). The results were expressed as milligrams of tannic acid (TA) per 100 g of freeze-dried extract.

In order to determine the content of total carotenoids from *Caryocar villosum* extracts, 0.1 g were weighted in a tube of 15 mL and extracted with acetone (2 mL) by agitation in a vortex for one min before centrifugation at 3864 x g for five min at 20 °C. After centrifugation, the supernatant was transferred to another flask. The extraction was exhaustively repeated until the extract was colourless. All supernatants were combined and directed to the liquid-liquid partition in a separation funnel with petroleum ether:ethyl ether (1:2, v/v) and washed with distilled water. After partition, the carotenoid extract was evaporated under vacuum (T < 38 °C) and re-suspended in ethanol for spectrophotometric quantification at 450 nm. The total carotenoid contents were calculated by using the specific extinction coefficient of zeaxanthin in ethanol ($E_{1cm}^{1\%}$ = 2540) (Davies, 1976) and expressed as milligrams of carotenoid per 100 g.

All analysis were performed in triplicate.

HPLC quantification of tocopherols and tocotrienols. The oil from *Caryocar villosum* extract was extracted and quantified, as total lipids (%), according to the Bligh and Dyer (1959) methodology. After diluting the oil samples, in hexane, the tocopherol and tocotrienol contents were quantified according to AOCS (2004) by using a normal-phase HPLC system (Perkin Elmer, Waltham, MA). The Perkin Elmer HPLC consisted of an isocratic pump (Series 200) equipped with a spectrofluorometric detector (Series 200a) set

at 290 nm for excitation and 330 nm for emission. The tocopherols and tocotrienols were separated on a Lichrosorb Si60 column (5 μm 250 x 4 mm, Hibar Fertigsaule RT, Darmstadt, Germany) with an isocratic mobile phase consisting of hexane:isopropyl alcohol (99:1, v/v) at 1.0 mL/min.The peaks of tocopherols and tocotrienols were identified by comparison of retention times and co-elution with the available standards of α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol (Calbiochem, Darmstadt, Germany). The tocopherols and tocotrienols were quantified by comparison of the external standard using five-point calibration curves based on standard solution (measurements in duplicate), with concentrations varying from 1.7 to 11.9 μg/mL for all standards (r^2=0.99, limit of detection = 0.04 μg/mL and limit of quantification = 0.13 μg/mL, calculated using the parameters of the standard curves). The contents of tocopherols and tocotrienols were expressed as μg/g of freeze-dried extract. All measurements were performed in triplicate.

***In vitro* antioxidant capacity determination**

Peroxyl radical (ROO•) scavenging capacity assay. In order to determine the antioxidant capacity of each *Caryocar villosum* extract, the peroxyl radical scavenging assay (ROO•) was carried out by monitoring the effect of the freeze-dried extracts on the fluorescence decay resulting from ROO•-induced oxidation of fluorescein and expressed as the "Oxygen Radical Absorbance Capacity" (ORAC) (Ou, Hampsch-Woodill & Prior, 2001). This assay was carried out in a microplate reader (Synergy MX, Biotek, Vermont, USA). Trolox was used as standard control and the concentration in the eight-point analytical curve varied from 8 to 96 μM (r^2=0.99, limit of detection = 2.21 μM and limit of quantification = 6.71 μM, calculated using the parameters of the standard curve). The results were expressed as mMol Trolox equivalent per 100 g of freeze-dried extracts. All measurements were performed in triplicate in three different plates.

Singlet oxygen ($^1O_2$) quenching activity. The Singlet oxygen quenching activity was determined as percentage of protection against $^1O_2$, according to Montenegro *et al*. (2004) with adaptations. The *Caryocar villosum* extract was weighted (50 mg), dissolved in 10 mL of DMSO:acetone (1:1, v/v) and then diluted in five concentrations. The standards of gallic acid, ellagic acid, violaxanthin, zeaxanthin and β-carotene were dissolved in ethanol or ethanol:methanol (1:1, v/v) or ethanol:ethyl acetate (1:1, v/v) or acetone and diluted in five concentrations. The reactions were performed in quartz cuvettes using 950 μL of 9,10-

dimethylantracene (DMA) (0.2 mM) as the actinometer, and 950 μL of methylene blue (MB) (20 μM) as sensitizer, both solutions prepared in ethanol. In addition, an aliquot (100 μL) of the different solutions of *Caryocar villosum* extracts (five concentrations) was added to the solution under moderate agitation, air atmosphere and temperature set at 25 ± 1 ºC. The blank was carried out under the same conditions, replacing 100 μL of *Caryocar villosum* extract by 100 μL of each solvent used to prepare the extracts. The excitation source used was a 150 W filament lamp coupled with red and orange cut-off filters to avoid direct excitation of light-sensitive compounds, such as carotenoids. The excitation light (> 620 nm) was focused into the sample cell, providing the excitation of the sensitizer (MB), generating singlet oxygen, which reacts with the actinometer (DMA). The intensity decay of absorbance of DMA (measured at 377 nm) was monitored with intervals of one min during 10 min with a UV-Visible spectrophotometer (Agilent, Santa Clara, USA). The kinetics data obtained from the intensity decay of the absorbance of DMA were fitted to a first order reaction (eq. 3) and the rate constants were calculated (eq. 4). The protection percentage that *Caryocar villosum* extracts (EXT) offered to the actinometer (DMA) was calculated through Equation 5.

$$Y = Y_{\infty} + A.\exp^{(-k.x)} \quad (3)$$

$$k = \frac{\ln 2}{t_{1/2}} \quad (4)$$

$$\text{protection}(\%) = \frac{k_{obs}^{DMA} - k_{obs}^{DMA+EXT}}{k_{obs}^{DMA}} x100 \quad (5)$$

where, Y is the intensity of DMA absorbance; Y_{∞} is the intensity of DMA absorbance at infinite time; A is pre-exponential factor; *k* is the pseudo-first order rate constant; x is reaction time; $t_{1/2}$ is half-life time (min), k_{obs}^{DMA} is the observed pseudo-first order rate constant fitted to DMA decay curve (obtained in the blank experiment) and $k_{obs}^{DMA+EXT}$ is the observed pseudo-first order rate constant fitted to DMA decay curve in presence of *Caryocar villosum* extract (EXT).

The percentage data related to the protection against $^1O_2$ provided by all extracts or standards were used to plot graphics of extract concentration (μg) *versus* percentage of protection against $^1O_2$ (%), and a polynomial fit was performed in all cases ($R^2 > 0.96$). The results were expressed as the percentage of protection (%) that each *Caryocar villosum* extract can provide to the DMA molecules against $^1O_2$ per milligrams of freeze-dried extracts. All measurements were performed in duplicate.

Statistical analysis. The results obtained from the characterisation of *Caryocar villosum* extracts (mean ± standard deviation) were analysed with the Statistica 6.0 software (Statsoft Inc., 2001) using analysis of variance (ANOVA) and Tukey test ($p<0.05$). The kinetics data obtained from the singlet oxygen assay were fitted to a pseudo-first order reaction using the Origin 8.0 software (OriginLab). In addition, two multivariate exploratory techniques: principal components analysis (PCA) and hierarchical cluster analysis (HCA) were applied for characterization of the extracts, using the Statistica 6.0 software package. For PCA, colour parameters (L^* and C^*), total phenolic compounds, flavonoids, tannins and carotenoids were used as active variables in the derivation of the principal components, and the supplementary variables (antioxidant capacity and the characteristics of the solvents) were projected onto the factor space. The hierarchical tree was obtained considering the active variables of PCA, the extracts were joined by unweighted pair-group average as the linkage rule, considering the Euclidian distances as the coefficient of similarity.

RESULTS AND DISCUSSION

Characteristics of *Caryocar villosum* extracts obtained by different solvents.

The pulp of *Caryocar villosum* used to obtain all extracts in this study was previously characterised by our research group in relation to the main chemical and phytochemical constituents (Chisté & Mercadante, **Chapter V**). The most important characteristics of the piquiá pulp can be highlighted as the high contents of total lipids (25.5 % in wet basis), total phenolic compounds (236.2 mg gallic acid equivalent/100 g), total flavonoids (64.7 mg catechin equivalent/100 g), total tannins (60.3 mg tannic acid equivalent/100 g) and total carotenoids (6.8 mg zeaxanthin equivalent/100 g).

Regarding the tocopherol derivatives, α-tocopherol was the only tocopherol detected and quantified in piquiá pulp (Chisté & Mercadante, **Chapter V**). However, in *Caryocar villosum* extracts the contents of α-tocopherol were below of the limit of quantification of the methodology applied (0.13 µg/mL) and none of the tocotrienols were detected (Table 1). In addition, condensed tannins were not detected in the *Caryocar villosum* extracts since no precipitate was formed after the addition of BSA in the condensed tannins assay. Thus, only hidrolysed tannins were considered to calculate the total tannins content.

Table 1. Bioactive compounds, CIELAB parameters and antioxidant capacity of *Caryocar villosum* pulp extracts obtained with different solvents.

Characteristics	Freeze-dried extracts				
	H_2O	**EtOH:H_2O**	**EtOH**	**EtOH:EtOAc**	**EtOAc**
Yield of solid extract (%) (from 5 g of freeze-dried pulp)	14.7 ± 0.6^{b}	10.8 ± 0.4^{c}	11.1 ± 0.6^{c}	46.4 ± 2.4^{a}	41.9 ± 2.1^{a}
Total lipids (%)*	30.3 ± 0.9^{d}	2.5 ± 0.1^{e}	78.5 ± 0.2^{c}	82.3 ± 0.3^{b}	91.1 ± 0.5^{a}
Bioactive compounds					
Total phenolic compounds (mg GAE/g)*	6.3 ± 0.2^{b}	9.2 ± 0.1^{a}	1.3 ± 0.03^{c}	0.2 ± 0.01^{d}	0.05 ± <0.01^{e}
Total flavonoids (mg CE/g)*	2.5 ± 0.1^{b}	3.8 ± 0.1^{a}	0.3 ± 0.01^{c}	0.04 ± <0.01^{d}	0.03 ± <0.01^{d}
Total tannins (mg TAE/g)*	2.4 ± 0.1^{b}	7.6 ± 0.4^{a}	0.3 ± 0.01^{c}	< LOD	< LOD
Total carotenoids (mg/g)[1,*]	0.03 ± <0.01^{d}	0.01 ± <0.01^{e}	0.1 ± <0.01^{a}	0.05 ± <0.01^{c}	0.06 ± <0.01^{b}
Total tocopherols (mg α-tocopherol/g)*	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Colour parameters (CIELAB)					
*L**	37.9 ± 0.4^{a}	11.9 ± 0.6^{d}	14.1 ± 0.4^{c}	19.1 ± 0.5^{b}	18.9 ± 0.8^{b}
*a**	-1.3 ± 0.1^{a}	-2.2 ± 0.1^{b}	-3.3 ± 0.2^{d}	-3.3 ± 0.2^{cd}	-2.9 ± 0.1^{c}
*b**	12.3 ± 0.6^{ab}	6.2 ± 0.2^{c}	13.3 ± 0.8^{a}	12.5 ± 0.3^{ab}	11.8 ± 0.7^{b}
h_{ab}	95.9 ± 0.5^{c}	109.7 ± 1.5^{a}	103.9 ± 1.2^{b}	104.6 ± 1.0^{b}	103.7 ± 1.2^{b}
C^*_{ab}	12.3 ± 0.6^{ab}	6.6 ± 0.1^{c}	13.8 ± 0.8^{a}	12.9 ± 0.3^{ab}	12.1 ± 0.6^{b}
Antioxidant Capacity					
ORAC (mMol Trolox equivalent/g extract)*	0.15 ± <0.01^{b}	0.28 ± 0.01^{a}	0.01 ± <0.01^{c}	0.004 ± <0.001^{d}	0.002 ± <0.001^{d}
Protection against $^{1}O_2$ (%)[2,*]	2.9 ± 0.8^{d}	2.5 ± 0.8^{d}	10.6 ± 0.5^{a}	8.7 ± 0.5^{c}	9.0 ± 0.4^{b}

*The results are expressed in dry basis. [1]Total carotenoids were quantified as zeaxanthin. [2]Quenching ability at the highest tested concentration (500 μg/mL). GAE = galic acid equivalent; CE = catechin equivalent; TAE = tannic acid equivalent; ORAC = Oxygen radical absorbance capacity; $^{1}O_2$ = singlet oxygen; H_2O = distilled water; EtOH:H_2O = ethanol:water; EtOH = ethanol; EtOH:EtOAc = ethanol:ethyl acetate; EtOAc = ethyl acetate. Mean ± standard deviation (triplicate). < LOD = lower than the limit of detection (3.0 μg/mL). < LOQ = lower than the limit of quantification (0.1 μg/mL). Means with the same superscript letters at the line do not present statistical difference ($p<0.05$).

The extraction of bioactive compounds from plant materials is strongly influenced by the solubility of their specific structures in the solvent used. In addition, the polarity of the solvent and other characteristics play a key role in increasing the solubility of these bioactive compounds. As can be seen in Table 1, the *Caryocar villosum* extracts yielded solid mass ranging from 10.8 to 46.4 %, depending on the solvent. The ethyl acetate, ethanol:ethyl acetate extracts presented the highest contents of total lipids as well as the highest yield in solid mass. Since piquiá pulp was reported to contain high content of lipids (Marx et al., 1997, Chisté & Mercadante, ***Chapter V***), these results are in agreement due to the high solubility of lipids in ethanol and ethyl acetate solvents.

According to Table 1, the ethanol:water extract showed the highest values of total phenolics compounds (9.2 mg GAE/g), total flavonoids (3.8 mg CE/g) and total tannins (7.6 mg TAE/g), followed by the extracts obtained with water, ethanol, ethanol:ethyl acetate, whilst the ethyl acetate extract presented the lowest contents of these compounds. This behaviour can be explained considering the profile of phenolic compounds found in *Caryocar villosum* pulp (Chisté & Mercadante, **Chapter V**), mainly phenolic acids (gallic and ellagic acid) which are compounds that possess high affinity to solvents of high polarity. It is widely known that solvents containing water or ethanol and its mixtures show high efficiency in the extraction of phenolic compounds from different plant sources (Yu et al., 2005; Vatai et al., 2009).

In relation to the contents of total carotenoids, the ethanol extract presented the highest value (0.1 mg/g), while the ethanol:water mixture showed the lowest content (0.01 mg/g). Since the composition of carotenoids from *Caryocar villosum* pulp (Chisté & Mercadante, ***Chapter V***) was reported to be given basically by carotenoids with hydroxyl groups (xanthophylls), i.e., more polar than the carotenes usually found in high amounts in fruits from Amazonia (De Rosso & Mercadante, 2007), they showed more affinity for medium polar solvents, such as ethanol. However, as expected, the use of solvents with high polarity (such as water and ethanol:water) showed low efficiency in the extraction of carotenoids.

The characteristics of the solvents used in this study, such as solvent polarity index (SPI) and the solvent selectivity triangle, described by Snyder *et al*. (1993) (Table 2) were considered to explain the extraction efficiency of bioactive compounds from *Caryocar villosum* extracts. Considering the results of Tables 1 and 2, in general, the extraction of bioactive compounds in *Caryocar villosum* pulp seems to be polarity-dependent of the solvent applied, i.e., the higher the polarity of solvent, the higher the extraction of phenolic compounds, such as flavonoids and tannins (more polar compounds) and, on the other

hand, the solvent with medium polarity (ethanol) showed the highest efficiency in the extraction of carotenoids (less polar compounds). The selectivity triangle, in which organic solvents are classified according to their ability to interact with the solute either as a dipole (dipolarity, π^*), as a proton acceptor (acidity, α), or as a proton donor (basicity, β) (Table 2) also explained the yield obtained for the different compounds with the different solvents. The solvents that present predominant property of acidity (water, ethanol:water and ethanol) showed to be more efficient for the extraction of phenolic compounds, flavonoids and tannis. The same behaviour was already reported by Chisté et al. (2011b) for the extraction of phenolic compounds and carotenoids (bixin) from annatto seeds.

Table 2. Characteristics of solvents used for the preparation of piquiá extracts.

Solvents	**Characteristics**			
	SPI[a]	**Normalized selectivity factors[b]**		
		π^*	α	β
H_2O	9.0	0.45	0.43	0.48
EtOH:H_2O	7.1	0.35	0.41	0.42
EtOH	5.2	0.25	0.39	0.36
EtOH:EtOAc	4.7	0.40	0.19	0.41
EtOAc	4.3	0.55	0.00	0.45

H_2O = distilled water; EtOH:H_2O = ethanol:water (1:1, v/v); EtOH = ethanol; EtOH:EtOAc – ethanol:ethyl acetate (1:1, v/v); EtOAc = ethyl acetate. [a]SPI = solvent polarity index (Snyder, 1974). [b]π^* = dipolarity, α = acidity and β = basicity (Snyder *et al.*, 1993). The normalized selectivity factors for the mixtures EtOH:H_2O and EtOH:EtOAc were calculated considering the solvent proportion and the respective values for pure solvents.

According to the colour parameters (Table 1), the similar values of h_{ab} (95.9 to 109.7) located all the *Caryocar villosum* extracts in the CIELAB space corresponding to the yellowish colour. This location can be explained due to the visible presence of yellowish carotenoids in all extracts. Meléndez-Martínez et al. (2007) reported similar values of h_{ab} (95.2 a 107.4) for carotenoid solutions of antheraxanthin, lutein, lutein epoxides, mutatoxanthin, neoxanthin, violaxanthin and zeaxanthin, i.e., the same compounds already found in the *Caryocar villosum* pulp (Chisté & Mercadante, ***Chapter V***). The ethanol:water extract, which showed the highest value of total phenolic compounds, total flavonoids, total tannins and the lowest value of total carotenoids was the darkest (L^* = 11.9) and the least vivid one (C^*_{ab} = 6.6). On the other hand, the ethanol

extract, which presented the highest content of total carotenoids, was the most vivid extract (C^*_{ab} = 13.7), with the highest intensity of yellow (b^* = 13.3) and the least intensity of red colour (a^* = -3.3).

Concerning the antioxidant capacity of the *Caryocar villosum* extracts, in general the extracts that presented the highest contents of total phenolic compounds, total flavonoids and total tannins (ethanol:water and water extracts) were the most efficient ones as scavengers of peroxyl radicals. Ellagic and gallic acids were reported as the major phenolic compounds identified in *Caryocar villosum* pulp (Chisté & Mercadante, ***Chapter V***), and they possess several hydroxyl groups and, in the case of gallic acid, one carboxyl group (Figure 1). The chemical properties of phenolic compounds in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers predict their high antioxidant activity (Rice-Evans et al., 1995). Since the ORAC assay is based on hydrogen atom transfer reaction between the tested antioxidant and the formed radical (Huang et al., 2005), the ethanol:water and water extracts, wtih the highest phenolic compound contents, are able to scavenge the peroxyl radical ($ROO^{\bullet}$) most probably due to the presence of available hydrogen atoms in its hydroxyl or carboxyl groups.

In relation to the percentage of protection against singlet oxygen, all *Caryocar villosum* extracts and chemical standards tested presented an excellent fit to the pseudo-first order reaction (R^2 = 0.99) for the absorbance intensity decay of DMA, in the presence of the sensitizer and extracts. In general, the ethyl acetate extract delayed more efficiently the degradation of DMA by quenching the singlet oxygen generated by the photosensitization of methylene blue in the tested concentration range (Figura 2a). However, the extract obtained with ethanol showed the highest percentage of protection (10.6 %) at the highest tested concentration (500 μg/mL). As expected, the highest value of protection was associated with the highest level of total carotenoids in *Caryocar villosum* extract. The possible mechanisms that should be considered when carotenoids are exposed to free radicals, such as peroxyl radicals and other oxidising agents, are the electron transfer reaction due to the presence of several conjugated double bonds or the hydrogen abstraction from the xanthophyll molecule functioning, in this way, as a chain breaking antioxidants (Burton, 1989; Squadrito & Pryor, 1998; Yeum, Aldini, Russell, & Krinsky, 2009). Furthermore, as can be seen in Figure 2b, the chemical standards of carotenoids (zeaxanthin, β-carotene and violaxanthin) were more efficient in the *in vitro* quenching of singlet oxygen than the phenolic compounds tested (gallic acid and ellagic acid). Among the carotenoids, violaxanthin presented the lowest concentration (3.4 μg/mL) that provides 50 % of protection of DMA against $^1O_2$, followed by zeaxanthin (6.0 μg/mL)

and β-carotene (9.1 μg/mL). Both tested phenolic compounds did not provide 50 % of protection at the studied concentration range, whereas at the highest concentration (254 μg/mL) ellagic acid showed 44 % of protection against singlet oxygen and gallic acid presented less efficiency (30 %).

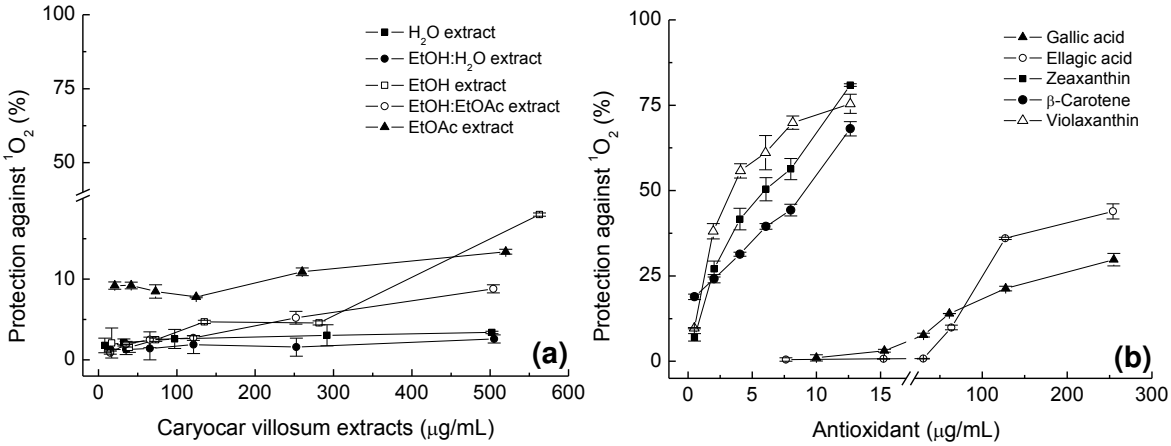

Figure 2. *In vitro* percentage of protection against singlet oxygen ($^1O_2$) provided by (a) the extracts of *Caryocar villosum* pulp and (b) chemical standards of carotenoids (zeaxanthin, β-carotene and violaxanthin) and phenolic compounds (gallic acid and ellagic acid).

Classification of *Caryocar villosum* extracts by multivariate statistical analysis

In the PCA analysis, the first two components accounted for 98 % of the explained variance. As can be seen in Figure 3a, a positive correlation was observed among total phenolic compounds (TPC), total flavonoids (TF), total tannins (TT) and the ORAC values ($r > 0.98$). The solvent polarity index (SPI), as well as the acidity property of solvent (α) also showed positive correlation with TPC, TF and TT ($r > 0.80$), i.e., the higher the acidity and the SPI values, the higher the yield of phenolic compounds (including flavonoids and tannins) in the extract. On the other hand, the total carotenoids showed positive correlation with the colour parameter chrome (*C**) ($r = 0.77$) and the values of protection against singlet oxygen ($^1O_2$) ($r = 0.90$). Considering the correlations obtained by the PCA analysis, the phenolic compounds content presented the largest contribution to the antioxidant capacity in *Caryocar villosum* extracts as determined by ORAC assay. On the other side, the carotenoids clearly demonstrated its high contribution to the quenching of singlet oxygen. Additionally, negative correlations among TPC, TF, TT and the values of protection against $^1O_2$ ($r < -0.82$) were observed, as well as, negative correlation between total carotenoids and ORAC values ($r = -0.81$).

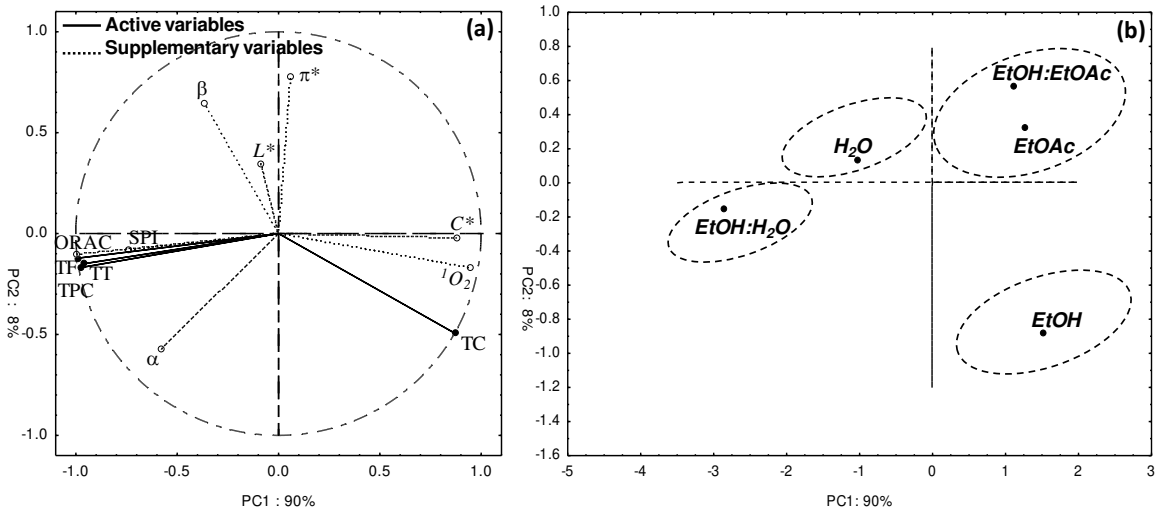

Figura 3. PCA plot of composition, colour and antioxidant properties of the different *Caryocar villosum* extracts. (a) Variable projection and (b) scatterplot for the cases with suggested drawn grouping ellipses suggested by HCA.

The *Caryocar villosum* extracts were divided into four groups (Figura 3b): one group formed by the extract obtained with ethanol:water mixture (EtOH:H_2O), one group formed by water (H_2O), one group with solvents that contain ethyl acetate in the composition (EtOH:EtOAc and EtOAc), and the last one group formed by the extracts obtained only with ethanol (EtOH). Based on the PCA analysis, Figure 3b clearly shows the localization of the extracts according to the solvent efficiency in the yield of bioactive compounds as PC1. The ethanol:water and water extracts, which presented the highest contents of total phenolic compounds, total flavonoids and total tannins, were characterised as the most efficient scavengers of peroxyl radicals, whilst ethanol extract, that presented the highest carotenoid content, showed the best colour intensity, as well as the most efficient quenching activity against singlet oxygen.

Figure 4 shows the dendrogram obtained when HCA was applied taking into consideration the composition data (bioactive compound contents) and colour characteristics (L^* and C^*_{ab}). The tree diagram provides evidence for four groups, which can be observed in PCA plot (Fig. 3b).

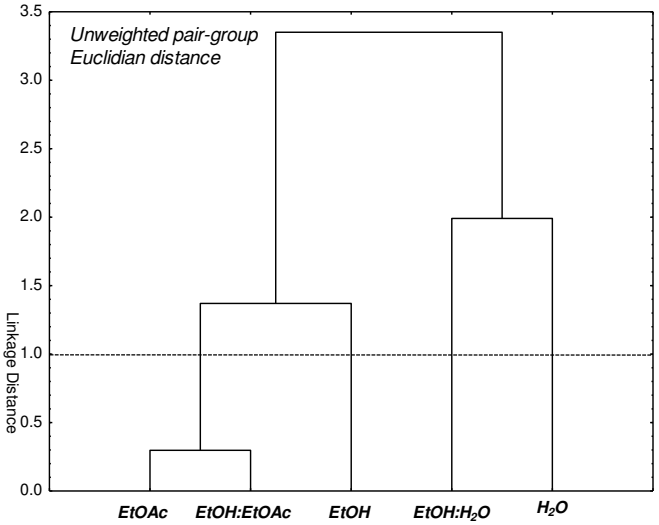

Figura 4. Dendrogram obtained by HCA analysis, from the composition, colour and antioxidant capacities (ORAC and percentage of protection against singlet oxygen) of the different *Caryocar villosum* extracts.

Based on the results of this study, the mixture ethanol:water, water and ethanol solvents produced extracts which were characterized by the greatest values of bioactive compounds, protection against singlet oxygen and free radical scavenger capacity. This information is important to the food, cosmetic and pharmaceutical industries since *Caryocar villosum* is an unexploited source of natural bioactive compounds that can be used as a potential product against oxidative damages in foods or biological systems.

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CONCLUSÃO GERAL

Os métodos propostos tanto para a extração simultânea, quanto para separação, identificação e quantificação de compostos fenólicos e de bixina em sementes e extratos de urucum foram validados com sucesso. A otimização da extração simultânea destes compostos através da metodologia de superfície de resposta forneceu as melhores condições para extrair compostos fenólicos e bixina de sementes de urucum. Além disso, a hipolaetina e um derivado do ácido caféico foram identificados pela primeira vez nas sementes de urucum.

Com relação aos extratos líquidos de urucum, o extrato obtido com acetato de etila foi caracterizado pelos maiores valores de proteção contra o $^1O_2$, capacidade antioxidante (TEAC) e intensidade de cor. Nos extratos de urucum, estas propriedades foram altamente correlacionadas com os teores de bixina. Dessa forma, a mistura etanol:acetato de etila e o acetato de etila foram os solventes mais promissores para obtenção de extratos líquidos de urucum com ambas as propriedades antioxidantes e de cor.

Os extratos liofilizados de urucum foram eficientes na desativação *in vitro* de ROS e RNS. Em geral, os valores de IC_{50} para as ROS e RNS mostraram que os extratos de urucum apresentaram melhor eficiência na desativação de espécies não-radicais (H_2O_2, HOCl, $^1O_2$ e $ONOO^-$) do que para as espécies radicais ($O_2^{\bullet-}$ e $^{\bullet}NO$). Assim, os extratos obtidos com etanol:acetato de etila e acetato de etila foram os solventes mais promissores para obtenção de extratos liofilizados de urucum com maior eficiência antioxidante. Tais informações são de grande importância para a indústria de produtos naturais, uma vez que o urucum é uma fonte acessível de compostos bioativos antioxidantes a ser mais bem explorada.

Com relação aos estudos sobre o piquiá, a polpa apresentou uma composição química promissora para a pesquisa de compostos bioativos. Além disso, foi reportada pela primeira vez a composição de compostos fenólicos (majoritários: ácido gálico, ácido elágico ramnosídeo e ácido elágico) e carotenóides (majoritários: all-*trans*-anteraxantina, all-*trans*-zeaxantina e all-*trans*-neoxanthin) presentes na polpa.

Para os extratos liofilizados de polpa de piquiá, os extratos obtidos com os solventes etanol:água, água e etanol foram caracterizados pelos maiores valores de compostos bioativos, proteção contra o $^1O_2$ e capacidade antioxidante. Esta informação é importante para as indústrias alimentícia, cosmética e farmacêutica, sabendo que o piquiá é uma fonte natural inexplorada de compostos bioativos para serem usados como um produto em potencial contra os danos oxidativos em alimentos ou sistemas biológicos.