ELISEU RODRIGUES

CAPACIDADE DESATIVADORA DE ESPÉCIES REATIVAS DE OXIGÊNIO E DE NITROGÊNIO POR CAROTENOIDES E EXTRATO DE MANÁ-CUBIU

SCAVENGING CAPACITY OF CAROTENOIDS AND EXTRACT FROM MANA-CUBIU AGAINST REACTIVE OXYGEN AND NITROGEN SPECIES

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FACULDADE DE ENGENHARIA DE ALIMENTOS

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Orientadora: Profa. Dra. Adriana Zerlotti Mercadante

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas para a obtenção do título de Doutor em Ciência de Alimentos.

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Este exemplar corresponde à versão final da tese defendida pelo aluno Eliseu Rodrigues e orientada pela Profa. Dra. Adriana Zerlotti Mercadante

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Dedico esta tese:
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RESUMO GERAL

Neste trabalho foi avaliada a capacidade antioxidante de carotenoides em sistema homogêneo e em lipossomas, de carotenoides microencapsulados e de extratos de maná-cubiu (*Solanum sessiflorum*) frente às espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS) de importância biológica. Devido à insolubilidade de carotenoides em solução tampão, utilizada como meio de reação no conhecido método *oxygen radical absorbance capacity* (ORAC), foi desenvolvido também um novo método para determinar a capacidade de carotenoides de desativarem o radical peroxila ($ROO^{\bullet}$). Em geral, as microcápsulas de goma arábica e de maltodextrina (20 DE) contendo compostos antioxidantes (trolox, α-tocoferol, β-caroteno, apo-8'-carotenal e apo-12'-carotenal) foram capazes de desativar as ROS e RNS, sendo a eficiência de desativação influenciada pelo material de parede, pela espécie reativa ($ROO^{\bullet}$, peróxido de hidrogênio (H_2O_2), radical hidroxila ($HO^{\bullet}$), ácido hipocloroso (HOCl) e ânion peroxinitrito ($ONOO^-$) e pela estrutura do composto antioxidante. Interessantemente, as microcápsulas vazias também foram capazes de desativar as ROS e RNS, sendo as de goma arábica mais eficazes que as de maltodextrina. A incorporação de apo-8'-carotenal promoveu o maior aumento na capacidade das microcápsulas de desativarem ROS e RNS, variando de 50% a 132% e de 39% a 85% para goma arábica e maltodextrina, respectivamente. Este fato sugere que o apo-8'-carotenal apresenta o melhor balanço entre a sua localização no interior das microcápsulas e a reatividade frente às espécies reativas. A localização também foi um fator importante na eficiência dos carotenoides desativarem ROS e RNS em lipossomas. Neste sistema, os carotenoides com grupos hidroxila foram geralmente mais potentes na desativação do $ROO^{\bullet}$, $HO^{\bullet}$ e HOCl que carotenos, com destaque para a astaxantina, enquanto o β-caroteno foi mais eficiente na desativação do $ONOO^-$. Para o estudo dos carotenoides em sistema homogêneo foi desenvolvido e validado com sucesso um novo método para avaliação da capacidade de carotenoides de desativarem o $ROO^{\bullet}$, o qual é baseado na perda de fluorescência da sonda ácido 4,4-difluoro-5-(4-fenil-1,3-butadienil)-4-bora-3a,4a-diazo-s-indaceno-3-undecanoico (C_{11}-$BODIPY^{581/591}$) devido à oxidação pelo $ROO^{\bullet}$, que é gerado pela termodecomposição do azobisisobutironitrila (AIBN). A aplicação deste novo método permitiu o estudo da relação entre a estrutura química de diferentes carotenoides e a capacidade de desativar o $ROO^{\bullet}$. Foi demonstrado que neste

sistema os carotenoides são potentes desativadores desta espécie reativa, sendo a eficiência influenciada principalmente pela abertura do anel β-ionona e pela extensão do cromóforo. O licopeno foi o mais potente desativador de $ROO^{\bullet}$ (8,67 ± 0,74), porém não tão eficiente quanto os carotenoides do maná-cubiu (9,80 ± 0,80). Os compostos bioativos de maná-cubiu foram determinados por cromatografia líquida de alta eficiência acoplada aos detectores de arranjo de diodos e espectrômetro de massas (HPLC-DAD-MS/MS). Esta fruta apresentou β-caroteno (7,15 μg/g) e luteína (2,41 μg/g) como carotenoides majoritários. Por fim, o extrato fenólico de maná-cubiu, contendo o ácido 5-cafeoilquínico (1298 μg/g) como composto fenólico principal, apresentou grande eficiência em desativar o H_2O_2 (IC_{50} = 305 ± 17 μg/mL) e o HOCl (IC_{50} = 13 ± 0,8 μg/mL). Os resultados do presente estudo mostram que os carotenoides foram capazes de desativar todas as ROS e RNS de relevância biológica avaliadas neste trabalho, sendo a eficiência de desativação influenciada pela concentração e estrutura química do carotenoide, pelo nível de organização do sistema (homogêneo ou multifásico) e pelo tipo de espécie reativa.

SUMMARY

In the present study, the antioxidant capacity of carotenoids in homogeneous system and in liposomes, of microencapsulated carotenoids and of extracts from mana-cubiu (*Solanum sessiflorum*) were evaluated against reactive oxygen (ROS) and reactive nitrogen (RNS) species of biological relevance. Due to the insolubility of carotenoids in aqueous buffers, which is used as reaction medium in the well known oxygen radical absorbance capacity method (ORAC), a novel method was also developed to determine the peroxyl radicals ($ROO^{\bullet}$) scavenging capacity of carotenoids. In general, the gum arabic and maltodextrin (20 DE) microcapsules containing antioxidant compounds (trolox, α-tocopherol, β-carotene, apo-8'-carotenal and apo-12'-carotenal) were capable to scavenge ROS and RNS. The scavenging efficiency of the microcapsules was influenced by the wall material, the reactive species ($ROO^{\bullet}$, hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl) and peroxynitrite anion ($ONOO^-$) and the structure of the antioxidant compound. Interestingly, the empty microcapsules were also able to scavenge the ROS and RNS, being the gum arabic microcapsules more efficient than the maltodextrin ones. The incorporation of apo-8'-carotenal resulted in the largest increase in the microcapsules scavenging capacity, varying from 50% to 132% and from 39% to 85% in the gum Arabic and maltodextrin microcapsules, respectively. These findings suggest that the apo-8'-carotenal presented the best balance between its location in the microcapsules interior and the reactivity against the reactive species. The location was also an important factor influencing the efficiency of carotenoids to scavenge ROS and RNS in liposomes. In this system, the carotenoids with hydroxyl groups were usually more potent $ROO^{\bullet}$, $HO^{\bullet}$ and HOCl scavengers than the carotenes, especially astaxanthin, whilst β-carotene was the most efficient $ONOO^-$ scavenger. To study the carotenoids in homogeneous system, a new $ROO^{\bullet}$ scavenging assay, based on the loss of fluorescence of the probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-$BODIPY^{581/591}$) due to its oxidation induced by $ROO^{\bullet}$ generated by the thermo decomposition of azobisisobutyronitrile (AIBN), was successfully developed and validated. This new method allowed the establishment of the relationship between the structure of different carotenoids and their $ROO^{\bullet}$ scavenging capacity. In this system, carotenoids showed to be potent scavengers of this reactive species, and their scavenging

capacity was influenced mainly by the opening of the β-ionone ring and by the chromophore extension. Lycopene was the most potent $ROO^{\bullet}$ scavenger (8.67 ± 0.74); however, it was not so efficient as the carotenoids from mana-cubiu (9.80 ± 0.80). The bioactive compounds of mana-cubiu were determined by high performance liquid chromatography coupled to diode array and mass spectrometry detectors (HPLC-DAD-MS/MS). The major carotenoids of this fruit were β-carotene (7.15 μg/g) and lutein (2.41 μg/g). Finally, the main phenolic compound found in the phenolic extract from mana-cubiu was 5-caffeoylquinic acid (1298 μg/g), which showed to be a good H_2O_2 (IC_{50} = 305 ± 17 μg/mL) and HOCl (IC_{50} = 13 ± 0.8 μg/mL) scavenger. The results of this thesis showed that the carotenoids are able to scavenge all the evaluated ROS and RNS of biological relevance and that their efficiency depends on the carotenoid structure and concentration, level of organization of the reaction medium (homogeneous or multiphase) and type of reactive species.

INTRODUÇÃO GERAL

Espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS) são produtos do metabolismo normal e são bem conhecidas por apresentarem um papel duplo em sistemas biológicos, onde seus efeitos podem ser benéficos ou deletérios. As ROS incluem radicais derivados do oxigênio tais como o radical ânion superóxido ($O_2^{\bullet -}$), o radical peroxila ($ROO^{\bullet}$), o radical hidroxila ($HO^{\bullet}$), e espécies não radicalares, tais como o peróxido de hidrogênio (H_2O_2), o oxigênio singlete ($^1O_2$) e o ácido hipocloroso (HOCl) (Choe & Min, 2006), enquanto as RNS incluem os derivados do radical óxido nítrico ($^{\bullet}NO$) e espécies não radicalares, como o ânion peroxinitrito ($ONOO^-$) (Halliwell & Gutteridge, 2007). Em concentrações moderadas, as ROS e RNS estão envolvidas no sistema de defesa e de sinalização celular. Porém, em concentrações elevadas, estas espécies reativas podem alterar componentes celulares e consequentemente causar danos às células e tecidos (Valko, Leibfritz, Moncol, Cronin, Mazur & Telser, 2007). Além disso, algumas ROS, tais como o $ROO^{\bullet}$, o $HO^{\bullet}$ e o $^1O_2$, podem ser geradas em alimentos e atuar como agentes oxidantes, contribuindo para a diminuição da vida útil destes produtos (Halliwell, 2001; Choe & Min, 2006).

Os antioxidantes consumidos na dieta são importantes na manutenção do equilíbrio das ROS e RNS, especialmente quando o sistema de defesa antioxidante endógeno não é capaz de desativar de forma eficapz as espécies reativas geradas no organismo (Halliwell & Gutteridge, 2007). Os carotenoides e compostos fenólicos são duas importantes classes de compostos bioativos da dieta que têm sido relacionados à redução do risco de desenvolvimento de algumas doenças crônico-degenerativas. Este efeito é atribuído, por hipótese, à capacidade destes compostos de atenuarem reações oxidativas e/ou nitrosativas que são induzidas por ROS e RNS e que estão ligadas à patogênese destas doenças (Halliwell & Gutteridge, 2007; Rock, 2009). Além disso, a presença ou adição destes compostos bioativos aos alimentos pode prevenir ou reduzir a oxidação dos seus constituintes (Halliwell, 2001; Choe & Min, 2006).

Apesar de carotenoides e compostos fenólicos terem sido largamente investigados ao longo dos anos, ainda há muitas questões que precisam ser elucidadas, sendo uma delas o potencial e a influência da estrutura dos carotenoides na desativação de ROS e RNS, tanto

em sistemas homogêneos quanto em sistemas multifásicos. Não menos importante, é a necessidade de estudos que avaliem o potencial de extratos ricos em carotenoides e fenólicos de desativarem ROS e RNS de relevância biológica, uma vez que na maioria dos estudos a capacidade antioxidante é avaliada através da desativação dos radicais estáveis 2,2-difenil-1-picrilidrazila ($DPPH^{\bullet}$) e ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfônico) ($ABTS^{\bullet+}$).

Atualmente, sabe-se que as propriedades antioxidantes dos carotenoides estão relacionadas à sua estrutura química, incluindo aspectos como número de ligações duplas conjugadas, tipos de grupos finais e substituintes contendo oxigênio (El-Agamey, Lowe, McGarvey, Mortensen, Phillip, Truscott & Young, 2004). Estas relações estão bem estabelecidas quando se considera a capacidade de suprimir o oxigênio singlete e a capacidade de desativar o radical não biológico $ABTS^{\bullet+}$ em sistema homogêneo (Di Mascio, Kaiser & Sies, 1989; Miller, Sampson, Candeias, Bramley & Rice-Evans, 1996; Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). Porém, quando se considera a capacidade dos carotenoides de desativarem o $ROO^{\bullet}$ os dados relatados na literatura são conflitantes (Naguib, 1998; Zulueta, Esteve & Frígola, 2009; Müller, Fröhlich & Böhm, 2011) devido à utilização de meios reacionais aquosos, os quais são inapropriados para o estudo de carotenoides (Zulueta et al., 2009; Müller et al., 2011).

Apesar do sistema homogêneo ser capaz de gerar informações relevantes sobre a relação entre estrutura e capacidade antioxidante dos carotenoides, ele é muito diferente do ambiente encontrado em sistemas biológicos, onde as biomoléculas estão espacialmente organizadas. Assim, é importante o uso de um sistema modelo que seja capaz de mimetizar tal ambiente. Neste contexto, estão os lipossomas, que têm sido utilizados em estudos da capacidade antioxidante de compostos bioativos devido à similaridade da sua estrutura com a estrutura em bicamada lipídica das membranas celulares. De fato, a literatura reporta trabalhos avaliando a ação de carotenoides na desativação do $ROO^{\bullet}$ em lipossomas (Woodall, Lee, Weesie, Jackson & Britton, 1997; Naguib, 2000; Zhang, Stanley & Melton, 2006), porém são escassos ou inexistentes trabalhos com outras espécies reativas tais como $HO^{\bullet}$, HOCl e $ONOO^{-}$.

Situação similar ocorre quando são consideradas microcápsulas de carotenoides. Recentemente foi relatado que microcápsulas contendo carotenoides e outros compostos

antioxidantes (trolox e α-tocoferol) foram capazes de desativar o $^1O_2$ (Faria, Mignone, Montenegro, Mercadante & Borsarelli, 2010), o que revela outra interessante propriedade das microcápsulas, além daquelas já conhecidas de aumentar a estabilidade e solubilidade dos compostos antioxidantes (Gharsallaoui, Roudaut, Chambin, Voilley & Saurel, 2007). Porém, ainda não há nenhum trabalho que tenha relatado a capacidade de desativação de outras ROS e RNS por microcápsulas contendo carotenoides.

Finalmente, considerando que os carotenoides e os compostos fenólicos são consumidos na dieta humana como misturas complexas e não como padrões isolados, é importante determinar a capacidade antioxidante de extratos naturais ricos nestes compostos, tais como aqueles provenientes de frutas. Neste contexto se encaixa o mana-cubiu (*Solanum sessiflorum*), que é uma fruta de origem Amazônica que possui várias alegações de associação do seu consumo com benefícios à saúde, o que provavelmente pode estar relacionado aos compostos bioativos presentes em sua composição. Apesar disso, não há dados na literatura sobre a composição de carotenoides e compostos fenólicos desta fruta. Além disso, diferentemente do que tem sido realizado nas últimas décadas, como a utilização de radicais estáveis que em nada lembram as espécies reativas presentes no nosso organismo e em alimentos, deve-se avaliar a capacidade antioxidante através de medidas da desativação de espécies reativas de relevância biológica.

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OBJETIVOS

Considerando o que foi exposto na introdução geral, este trabalho apresenta os seguintes objetivos:

1) Determinar a capacidade de microcápsulas contendo carotenoides de desativarem o radical peroxila ($ROO^{\bullet}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($HO^{\bullet}$), o ácido hipocloroso (HOCl) e o ânion peroxinitrito ($ONOO^-$).

2) Desenvolver e validar um novo método para determinar a capacidade de carotenoides de desativarem o radical peroxila ($ROO^{\bullet}$) e aplicar este método no estudo da relação entre a estrutura química dos carotenoides e a capacidade de desativar o $ROO^{\bullet}$.

3) Determinar a capacidade de alguns carotenoides de desativarem o radical peroxila ($ROO^{\bullet}$), o radical hidroxila ($HO^{\bullet}$), o ácido hipocloroso (HOCl) e o ânion peroxinitrito ($ONOO^-$) em lipossomas.

4) Determinar a composição de compostos bioativos majoritários (carotenoides e compostos fenólicos) do maná-cubiu, utilizando HPLC-DAD-MS/MS e avaliar a capacidade do extrato hidrossolúvel de desativar o radical peroxila ($ROO^{\bullet}$), o peróxido de hidrogênio (H_2O_2) e o ácido hipocloroso (HOCl), bem como determinar a capacidade de desativação do $ROO^{\bullet}$ pelo extrato lipossolúvel.

CAPÍTULO I

REVISÃO DE LITERATURA

1. Espécies reativas de oxigênio (ROS) e de nitrogênio (RNS)

Espécies reativas de oxigênio (ROS) é um termo que inclui radicais derivados do oxigênio, como o radical ânion superóxido ($O_2^{\bullet -}$), o radical hidroperoxila ($HOO^{\bullet}$), o radical peroxila ($ROO^{\bullet}$), o radical alcoxila ($RO^{\bullet}$) e o radical hidroxila ($HO^{\bullet}$), e derivados de oxigênio não radicalares, tais como o peróxido de hidrogênio (H_2O_2), o oxigênio singlete ($^1O_2$) e o ácido hipocloroso (HOCl) (Choe & Min, 2006). As espécies reativas de nitrogênio (RNS) incluem principalmente os radicais óxido nítrico ($^{\bullet}NO$) e a espécie não radicalar ânion peroxinitrito ($ONOO^-$) (Halliwell & Gutteridge, 2007).

As ROS e RNS são produtos do metabolismo celular normal e apresentam duplo papel, uma vez que podem ser nocivas ou benéficas para os sistemas vivos. Os efeitos benéficos destas espécies ocorrem em concentrações baixas ou moderadas e são essenciais na sinalização e regulação celular, detoxificação e função imune, sendo, portanto, continuamente produzidas pelo organismo humano (Valko, Rhodes, Moncol, Izakovic & Mazur, 2006; Winterbourn, 2008).

O efeito adverso das ROS e RNS é denominado estresse oxidativo e estresse nitrosativo, respectivamente, e ocorre em sistemas biológicos quando de um lado há uma alta produção de ROS e de RNS e de outro lado uma deficiência de antioxidantes endógenos enzimáticos e não enzimáticos. O excesso de ROS pode causar danos aos lipídeos celulares, proteínas ou DNA, alterando sua função normal (Mazzulli, Lind & Ischiropoulos, 2006; Valko, Leibfritz, Moncol, Cronin, Mazur & Telser, 2007). Já as alterações causadas pelas RNS levam à formação de macromoléculas nitradas através da adição de um grupo nitro ($-NO_2$). Os alvos biológicos modificados pela nitração incluem resíduos de tirosina e triptofano em proteínas; ácidos graxos poli-insaturados; bases do DNA e açúcares (Mazzulli et al., 2006). Devido a estes efeitos, os estresses oxidativo e nitrosativo podem estar relacionados a um número grande de doenças humanas, tais como doenças cardiovasculares e degenerativas, diabetes, câncer, quase todas as patologias hepáticas, bem como no processo de envelhecimento (Valko et al., 2007; Almeida, Fernandes, Lima, Costa & Bahia, 2009).

1.1. Radical ânion superóxido ($O_2^{\bullet-}$)

O radical ânion superóxido ($O_2^{\bullet-}$) é formado quimicamente ou enzimaticamente a partir do oxigênio triplete. A formação deste radical ocorre principalmente devido ao fluxo de elétrons gerados na cadeia de transporte de elétrons no interior da mitocôndria. Além disso, o $O_2^{\bullet-}$ é produzido pela enzima NADPH oxidase localizada nas células fagocíticas durante a resposta imune (Halliwell & Gutteridge, 2007; Winterbourn, 2008; Freitas, Lima & Fernandes, 2009). Diversas outras enzimas reduzem o O_2 até $O_2^{\bullet-}$, sendo a xantina oxidase uma das mais estudadas. Esta enzima catalisa a oxidação do substrato hipoxantina à xantina e, por sua vez, a oxidação da xantina a ácido úrico, reduzindo o O_2 em duas ROS, $O_2^{\bullet-}$ e H_2O_2 (Halliwell & Gutteridge, 2007).

O $O_2^{\bullet-}$ é uma base fraca, altamente solúvel em água e, portanto, não atravessa facilmente as membranas lipídicas. O principal destino do $O_2^{\bullet-}$ em pH fisiológico é a dismutação a H_2O_2 pela enzima superóxido dismutase (SOD), sendo que a reação de dismutação também pode ocorrer pela via não enzimática em condições ácidas (Lu, Song & Lin, 2006). O H_2O_2 pode reagir com metais como Fe^{2+} e Cu^{+} para formar $HO^{\bullet}$ através da reação de Fenton. O H_2O_2 também pode formar HOCl reagindo com Cl^{-} via mieloperoxidase em neutrófilos. Além disso, $O_2^{\bullet-}$ reage com $^{\bullet}NO$ para formar $ONOO^{-}$, um forte agente oxidante e nitrosante (Almeida et al., 2009). Portanto, apesar do $O_2^{\bullet-}$ ser relativamente pouco reativo, seus metabólitos, $HO^{\bullet}$ e $ONOO^{-}$, são altamente reativos e podem causar danos aos tecidos. Além disso, este radical pode atuar como redutor doando um elétron para metais que são catalisadores da oxidação lipídica e da reação de Fenton (Valko et al., 2007).

1.2. Peróxido de hidrogênio (H_2O_2)

O peróxido de hidrogênio (H_2O_2) também pode ser formado pela ação de diversas enzimas oxidases sobre a xantina, urato e aminoácidos (Choe & Min, 2006). O H_2O_2 é uma ROS que pode atuar como agente oxidante ou como agente redutor. Embora sua reatividade seja fraca, este pode ser citotóxico em elevadas concentrações. Esta ROS apresenta facilidade de difusão dentro e entre células e pode ser transformada em produtos altamente deletérios e reativos, como o $HO^{\bullet}$, gerado através da reação de Fenton, e o HOCl, formado pela ação da enzima mieloperoxidase (Halliwell & Gutteridge, 1985). Esta característica de

difusão possibilita que o H_2O_2, por exemplo, alcance o núcleo celular e gere o $HO^{\bullet}$ *in situ*, em razão dos metais de transição ligados ao DNA, levando a uma lesão da biomolécula (Valko et al., 2006).

1.3. Radical hidroxila ($HO^{\bullet}$)

Em sistemas biológicos, o radical hidroxila ($HO^{\bullet}$) é formado principalmente pela reação de Fenton (Reação 1) a partir do H_2O_2 na presença de ions Fe^{2+} ou Cu^{+} (Halliwell & Gutteridge, 2007). O $HO^{\bullet}$ também pode ser gerado a partir da aplicação de radiação gama sobre a água e pela fissão homolítica da ligação O-O do H_2O_2 induzida por radiação ultravioleta (UV) (Choe & Min, 2006; Halliwell & Gutteridge, 2007).

$$H_2O_2 + Fe^{2+} \xrightarrow{\text{Reação de Fenton}} Fe^{3+} + OH^{\bullet} + OH^{-} \text{ (Reação 1)}$$

O $HO^{\bullet}$ apresenta um potencial de redução muito alto (2,80 V), o que lhe confere alta reatividade e assim, um tempo de vida *in vivo* muito curto, de aproximadamente 10^{-9} s. Devido a sua alta reatividade, quando o $HO^{\bullet}$ é produzido *in vivo* ele reage próximo ao sítio de formação (Valko et al., 2007). As reações em que o $HO^{\bullet}$ participa podem ser classificadas em três tipos principais: adição de hidrogênio, abstração de hidrogênio e transferência de elétrons. As reações do $HO^{\bullet}$ com compostos aromáticos procedem-se normalmente por adição, como acontece nas reações com a guanina e timina do DNA. Assim, quando o $HO^{\bullet}$ é gerado próximo ao DNA, provoca danos nas suas bases (e na desoxirribose) e induz a quebra da cadeia (Halliwell & Gutteridge, 2007). O $HO^{\bullet}$ é o principal responsável pela iniciação da oxidação lipídica devido a sua capacidade de abstrair um hidrogênio alílico da ligação dupla de lipídeos insaturados (Choe & Min, 2006). O $HO^{\bullet}$ participa ainda de reações de transferência de elétrons com íons halogenatos, como é o caso do íon cloreto (Cl^{-}) e do íon nitrito (NO_2^{-}). A reação do $HO^{\bullet}$ com o íon carbonato (CO_3^{-2}) produz um radical ânion carbonato ($CO_3^{\bullet -}$), que é um poderoso agente oxidante (Halliwell & Gutteridge, 2007).

1.4. Radical peroxila ($ROO^{\bullet}$) e radical alcoxila ($RO^{\bullet}$)

Em geral, os radicais peroxila ($ROO^{\bullet}$) e alcoxila ($RO^{\bullet}$) são fortes agentes oxidantes com capacidade de remover hidrogênio de outras moléculas, constituindo uma reação importante na oxidação lipídica (Laguerre, Lecomte & Villeneuve, 2007).

O $ROO^{\bullet}$ é formado pela reação direta entre o oxigênio triplete com o radical alquila ($R^{\bullet}$) na oxidação de ácidos graxos insaturados. O $ROO^{\bullet}$ produz hidroperóxido pela abstração de hidrogênio de outra molécula de ácido graxo. A maioria dos hidroperóxidos são estáveis à temperatura ambiente; contudo, aquecimento, luz UV ou metais de transição aceleram a decomposição heterolítica de hidroperóxidos e produzem radicais peroxila (Reações 2 a 5). Além disso, o hidroperóxido pode sofrer decomposição homolítica gerando novas espécies radicalares, como $RO^{\bullet}$ e $OH^{\bullet}$ que irão contribuir para a iniciação de novos processos de oxidação (Choe & Min, 2006).

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \text{ (Reação 2)}$$

$$ROO^{\bullet} + R'H \longrightarrow ROOH + R'^{\bullet} \text{ (Reação 3)}$$

$$ROOH \xrightarrow{\text{Aquecimento, UV}} ROO^{\bullet} + H^{\bullet} \text{ (Reação 4)}$$

$$ROOH \xrightarrow{Fe^{+3}} ROO^{\bullet} + Fe^{+2} + H^{+} \text{ (Reação 5)}$$

1.5. Oxigênio singlete ($^1O_2$)

O oxigênio singlete ($^1O_2$) é uma molécula não radicalar, visto que não possui elétrons desemparelhados. O $^1O_2$, que possui energia de 93,6 kJ acima do estado fundamental (triplete), pode ser desativado para oxigênio triplete através da transferência de energia para o solvente ou para outra molécula orgânica. O tempo de vida do $^1O_2$ depende das propriedades químicas do solvente, com tempo de vida de 2 μs em água e de 700 μs em tetracloreto de carbono (Choe & Min, 2006).

O $^1O_2$ é formado na presença de sensibilizador e de oxigênio triplete. Se um sensibilizador em seu estado fundamental singlete (^{1}Sen) é exposto à luz ele passará para um estado singlete excitado (^{1}Sen*). Os principais sensibilizadores são azul de metileno, eosina, curcumina, clorofila, hematoporfirina e riboflavina. Os sensibilizadores no estado singlete excitado retornam ao estado fundamental via emissão de luz (fluorescência), conversão interna (aquecimento) ou cruzamento intersistema. O cruzamento intersistema do

sensibilizador no estado singlete produz sensibilizador no estado triplete excitado (Choe & Min, 2006).

O sensibilizador no estado triplete excitado pode abstrair um elétron ou um átomo de hidrogênio dos componentes alimentares e formar radicais livres (processo Tipo I). No processo Tipo II, a energia de excitação do sensibilizador no estado triplete pode ser transferida para o oxigênio triplete para produzir o oxigênio singlete e o sensibilizador retorna ao seu estado fundamental. A velocidade dos processos do Tipo I e II depende do tipo de sensibilizadores e substratos, concentração de substrato e de oxigênio no meio reacional (Choe & Min, 2006).

O principal mecanismo de formação de $^1O_2$ em alimentos é a fotossensibilização. Devido ao seu caráter eletrofílico, o $^1O_2$ pode reagir diretamente com compostos contendo uma ou mais ligações duplas, formando endoperóxidos, alil hidroperóxidos ou dioxietanos. Os tocoferóis, compostos fenólicos, ascorbato e principalmente os carotenoides podem desativar o $^1O_2$ (Di Mascio, 1989; Halliwell & Gutteridge, 2007).

1.6. Ácido hipocloroso (HOCl)

O ácido hipocloroso (HOCl) é produzido pela ação da enzima mieloperoxidase (MPO) em neutrófilos ativados, a partir do H_2O_2 e do Cl^- (Equação 6). É um poderoso agente antimicrobiano e, por isso, tem papel de destaque na defesa imunológica do organismo desencadeada pelos neutrófilos (Prütz, 1996).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \text{ (Reação 6)}$$

O HOCl apresenta alta reatividade e habilidade em causar danos às biomoléculas. Esta ROS oxida grupos sulfidrila, o ascorbato, o NADPH e promove a cloração de bases de DNA, especialmente as pirimidínicas, e dos resíduos de tirosina nas proteínas. A passagem do HOCl pelas membranas celulares provoca danos nas proteínas (especialmente nos grupos SH) e nos lipídeos durante a passagem e, se alcançar o citoplasma, também reagirá com os constituintes celulares (Prütz, 1996; Halliwell & Gutteridge, 2007).

1.7. Radical óxido nítrico ($^{\bullet}NO$) e ânion peroxinitrito ($ONOO^-$)

O radical óxido nítrico ($^{\bullet}NO$) é gerado em tecidos biológicos através da enzima óxido nítrico sintase, que metaboliza arginina à citrulina com formação de $^{\bullet}NO$ via reação oxidativa envolvendo cinco elétrons. O $^{\bullet}NO$ é um radical abundante que atua na sinalização oxidativa em uma variedade de processos fisiológicos, incluindo neurotransmissão, regulação da pressão sanguínea, mecanismos de defesa, relaxamento da musculatura lisa e regulação da resposta imunológica (Bergendi, Benes, Durackova & Ferencik, 1999). Este radical apresenta um tempo de meia-vida de poucos segundos em ambiente aquoso, porém em ambiente com baixa concentração de oxigênio, o $^{\bullet}NO$ apresenta maior estabilidade (meia-vida >15 s). Em função de sua solubilidade em meio aquoso e lipídico, este radical difunde-se rapidamente pelo citoplasma e membrana plasmática (Valko et al., 2007).

As células do sistema imunológico produzem ambos os radicais $O_2^{\bullet-}$ e $^{\bullet}NO$ através do processo oxidativo desencadeado durante o processo inflamatório. Sob estas condições, o $O_2^{\bullet-}$ e $^{\bullet}NO$ podem reagir rapidamente produzindo quantidades significativas de uma molécula reativa, o ânion peroxinitrito ($ONOO^-$), que pode causar oxidação e nitração de lipídeos, fragmentação do DNA, nitração de resíduos de aminoácidos em proteínas, bem como a oxidação de cisteína, metionina e outros resíduos. Assim, a toxicidade do $^{\bullet}NO$ está predominantemente ligada à sua capacidade de reagir com o $O_2^{\bullet-}$ (Bergendi et al., 1999; Valko et al., 2007; Halliwell & Gutteridge, 2007). A estabilidade incomum do $ONOO^-$ contribui para sua toxicidade, permitindo sua difusão para longe do seu sítio de formação, reagindo seletivamente com diferentes componentes celulares (Beckman, Chen, Ischiropoulos & Crow, 1994).

2. Antioxidantes

A exposição às espécies reativas provenientes de diversas fontes levou o organismo a desenvolver uma série de mecanismos de defesa, sendo um destes a defesa antioxidante (Valko et al., 2007). Para neutralizar o ataque das ROS e RNS, as células apresentam um sistema de defesa biológico composto por antioxidantes enzimáticos que convertem ROS e RNS em espécies menos reativas. Por exemplo, o $O_2^{\bullet-}$ é convertido a oxigênio e H_2O_2 pela superóxido dismutase, e o H_2O_2 é convertido em água e oxigênio pela catalase. Entretanto,

nenhuma ação enzimática é conhecida para desativação de algumas espécies, como do $ROO^{\bullet}$, $HO^{\bullet}$, $^{1}O_2$ e $ONOO^{-}$. Por outro lado, há um sistema de antioxidantes não enzimáticos que inclui compostos sintetizados pelo organismo humano como bilirrubina, ceruloplasmina, melatonina, ácido úrico e outros, e compostos ingeridos através da dieta ou via suplementação como o ácido ascórbico, o α-tocoferol, os compostos fenólicos e os carotenoides, que apresentam a propriedade de desativar espécies oxidantes (Huang, Ou & Prior, 2005; Halliwell & Gutteridge, 2007).

A definição mais ampla de antioxidante foi proposta por Halliwell & Gutteridge (2007), onde antioxidante é "qualquer molécula que atrasa, previne ou remove um dano oxidativo de uma molécula alvo". O termo molécula alvo engloba grande parte das moléculas encontradas em alimentos e em tecidos vivos (exceto água), como, por exemplo, proteínas, lipídeos, carboidratos e DNA.

Os antioxidantes podem atuar de diferentes formas na proteção das biomoléculas: (a) remoção das ROS e RNS por ação enzimática; (b) redução na formação de ROS; (c) supressão física de ROS e (d) desativação de ROS e RNS pelos denominados "agentes de sacrifício", que são moléculas preferencialmente oxidadas, preservando biomoléculas de maior importância biológica (Halliwell & Gutteridge, 2007). Os carotenoides e compostos fenólicos pertencem a este último grupo, visto que estas classes de compostos sofrem degradação (modificação estrutural) quando desativam algumas ROS e RNS (El-Tinay & Chichester, 1970; Handelman, Van Kuijk, Chatyerjee & Krinsky, 1991; Roche, Dufour, Mora & Dangles, 2005). Exceção é o mecanismo de desativação física do oxigênio singlete pelos carotenoides, que envolve apenas a transferência de energia e, assim, não altera a estrutura do carotenoide (Di Mascio et al., 1989).

2.1. Carotenoides

Os carotenoides são pigmentos lipossolúveis de coloração amarela a vermelha, encontrados no reino vegetal, animal e em micro-organismos. Plantas superiores e micro-organismos sintetizam esses pigmentos, enquanto que os animais são incapazes de sintetizá-los, sendo que a presença de carotenoides nestes se deve à ingestão por meio da dieta. Em geral, os carotenoides apresentam como estrutura básica um tetraterpeno com 40 átomos de carbono, formado por oito unidades isoprenoides, ligados de tal forma que a molécula é linear com simetria invertida no centro (Figura 1) (Britton, 1995).

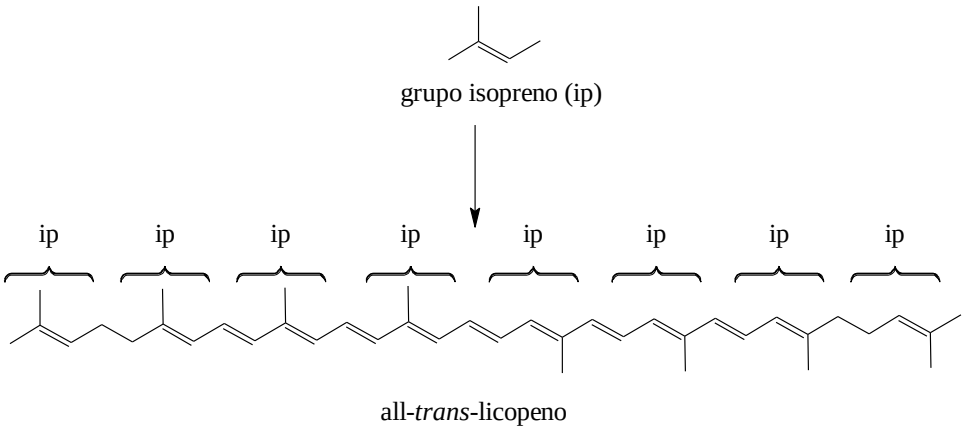

Figura 1. Estrutura básica dos carotenoides. Fonte: Delgado-Vargas, Jiménez & Paredes-López (2000).

Os carotenoides são divididos em dois grupos, os carotenos que são hidrocarbonetos e as xantofilas que são derivados oxigenados. Neste último grupo estão incluídos pigmentos que possuem em sua estrutura grupos hidroxílicos, carbonílicos, carboxílicos e/ou epóxidos. Os grupos terminais podem ser acíclicos, monocíclicos ou bicíclicos. Muitas outras modificações estruturais ainda são possíveis, resultando na grande diversidade de estruturas químicas, com mais de 700 carotenoides já relatados (Figura 2) (Britton, 1995; Britton, Liaaen-Jensen & Pfander, 2004).

Os carotenoides apresentam na sua cadeia uma série de ligações duplas conjugadas (l.d.c), gerando por ressonância um sistema de elétrons π que se desloca sobre toda a cadeia poliênica, proporcionando a estas substâncias alta reatividade química e absorção de luz na região do visível. Quanto maior o número de l.d.c na estrutura do carotenoide, mais extenso é o cromóforo e, por conseguinte, maior o comprimento de onda de máxima absorção ($\lambda_{máx}$) (Britton, 1995).

A alta reatividade química proporcionada pelo sistema de l.d.c torna esses pigmentos susceptíveis à degradação quando submetidos a condições de alta temperatura, meio ácido, presença de luz, entre outros fatores, podendo sofrer isomerização geométrica e oxidação. Essas alterações na molécula do carotenoide levam à perda ou diminuição do poder corante e da sua atividade biológica como, por exemplo, da atividade provitamina A, ou ainda podem levar à formação de compostos de baixo peso molecular que influenciam no aroma dos alimentos (Mercadante, 2008). Por outro lado, a elevada reatividade química dos carotenoides também lhes confere a capacidade de desativar diferentes ROS e RNS, o

que lhes dá propriedades antioxidantes tanto em sistemas biológicos quanto em alimentos (El-Agamey, Lowe, McGarvey, Mortensen, Phillip, Truscott & Young, 2004).

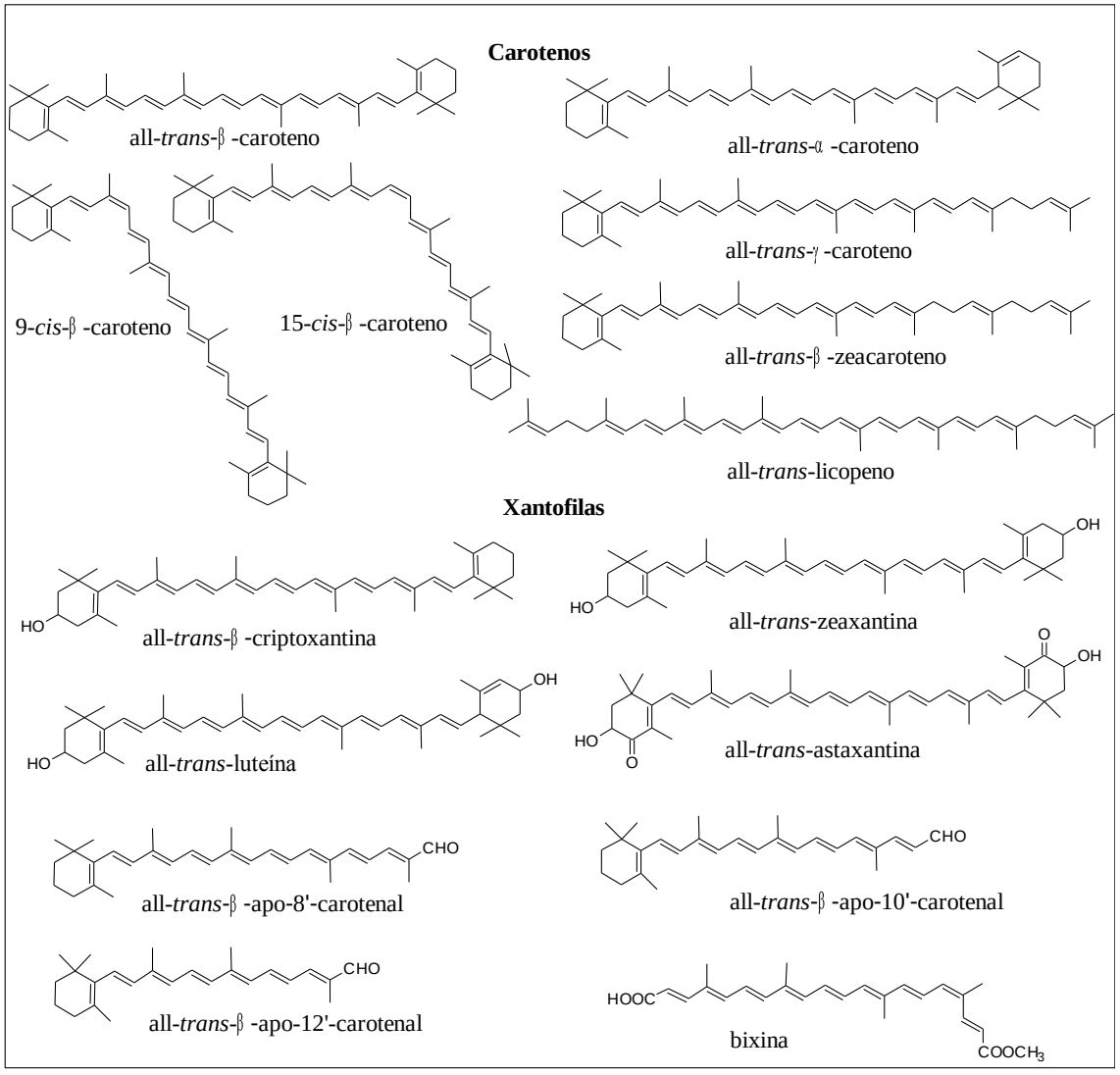

Figura 2. Estrutura químicas de alguns carotenoides. Fonte: Britton et al. (2004).

Atualmente, sabe-se que as propriedades antioxidantes dos carotenoides em sistema homogêneo estão relacionadas à sua estrutura química, incluindo aspectos como o número de l.d.c., tipos de grupos terminais e substituintes contendo oxigênio (Di Mascio et al., 1989; Miller, Sampson, Candeias, Bramley & Rice-Evans, 1996; El-Agamey et al., 2004). Neste sistema simples a estrutura dos carotenoides afeta a reatividade frente a uma determinada espécie reativa e também a estabilidade dos radicais formados após a reação com uma determinada ROS e RNS (El-Agamey et al., 2004). Em lipossomas, além destes fatores, a estrutura do carotenoide também determina a distribuição da molécula do carotenoide no interior da membrana, o que afeta diretamente a capacidade antioxidante dos carotenoides (Gruszecki, 2009).

Três mecanismos são sugeridos para a desativação de radicais como o $ROO^{\bullet}$ e $HO^{\bullet}$ pelos carotenoides: transferência de elétrons (Equação 7), abstração de hidrogênio alílico (Equação 8) e adição do radical às l.d.c (Equação 9) (El-Agamey et al., 2004; Jomová et al., 2009). A ocorrência de um ou outro mecanismo depende das características do sistema reacional (homogêneo ou multifásico), polaridade do solvente e estrutura do carotenoide (El-Agamey et al., 2004, Guo & Hu, 2010).

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet +} + R^{-} \text{ (transferência de elétrons)} \qquad \text{(Reação 7)}$$

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet} + RH \text{ (abstração de hidrogênio)} \qquad \text{(Reação 8)}$$

$$CAR + R^{\bullet} \rightarrow R - CAR^{\bullet} \text{ (adição)} \qquad \text{(Reação 9)}$$

A reação dos carotenoides com o $ONOO^-$ é relativamente lenta e apresenta uma cinética de primeira ordem. Foram propostos dois mecanismos para a desativação do $ONOO^-$ pelos carotenoides. No primeiro, o carotenoide recebe energia do $ONOO^-$ e chega a um estado excitado (bi-radical), e quando retorna ao estado fundamental produz isômeros *cis*. No segundo, o carotenoide reage diretamente com o $ONOO^-$, produzindo um dioxietano que se cliva formando apocaroteno ou sofre metanólise, gerando um metóxi-carotenoide (Yokota et al., 2004). A formação de nitro-carotenoides também foi reportada como resultado da reação entre carotenoides e $ONOO^-$ (Yoshioka et al., 2006; Tsuboi et al., 2010).

Recentemente foi proposto um mecanismo para a desativação do HOCl pelo licopeno, no qual o átomo de Cl atua como um eletrófilo e as ligações duplas conjugadas como nucleófilos. Quando o átomo de Cl é adicionado à dupla ligação, através de um carbocátion, um íon clorônio é gerado. Então, a adição de um íon hidróxido ocorre, formando uma cloroidrina, que sofre uma reação de substituição do tipo SN2 com substituição do Cl, originando um epóxido de licopeno. Este epóxido pode reagir com outra molécula de HOCl, causando clivagem da ligação C-C, o que gera um aldeído (Pennathur et al., 2010).

2.2. Compostos fenólicos

Os compostos fenólicos são metabólitos secundários sintetizados por plantas durante o desenvolvimento normal e em resposta a condições de estresse, tais como infecções, ferimentos e radiação UV. Estes compostos estão presentes em todas as plantas, constituindo um grupo diversificado de fitoquímicos (Beckman, 2000; Naczk & Shahidi, 2004).

As plantas contêm em sua composição fenóis simples, ácidos fenólicos (derivados do ácido benzóico e cinâmico), cumarinas, flavonoides, estilbenos, taninos, lignanas e ligninas. Estes compostos fenólicos podem atuar atraindo polinizadores, contribuindo na pigmentação, como antioxidantes, e como agentes protetores contra luz UV, patógenos e predadores. Em alimentos, os fenólicos podem contribuir para o amargor, adstringência, cor, *flavor*, odor e estabilidade oxidativa de produtos (Bravo, 1998; Naczk & Shahidi, 2004).

Os compostos fenólicos também exibem uma variedade de propriedades fisiológicas, tais como antialergênica, antiaterogênica, antiinflamatória, antimicrobiana, antitrombótica, efeito cardioprotetor, vasodilatador e antioxidante (Benavente-Garcia, Castillo, Marin, Ortuno & Del Rio, 1997; Puupponen-Pimiä, Nohynek, Meier, Kähkönen, Heinonen & Hopia, 2001). Todas estas propriedades justificam o grande número de trabalhos determinando a composição de fenólicos de diferentes frutas e vegetais.

Os principais compostos fenólicos da dieta humana são os ácidos fenólicos, flavonoides e taninos, os quais ocorrem nos alimentos em quantidades aproximadas de 1-3 mg/Kg de alimento. A avaliação da composição desses compostos é dificultada pelo grande número de compostos fenólicos e por diversos fatores que afetam seu conteúdo, como:

espécie e cultivar, sistema de cultivo, localização geográfica, estado de maturação, partes avaliadas e condições de armazenamento (King & Young, 1999).

Os ácidos fenólicos (Figura 3) caracterizam-se por possuírem um anel benzênico, um grupamento carboxílico, e um ou mais grupamentos hidroxila e/ou metoxila na molécula (Natella, Nardini, Di Felice & Scaccini, 1999). São divididos em três grupos. O primeiro é composto pelos ácidos benzoicos, que possuem sete átomos de carbono (C_6-C_1) e são os ácidos fenólicos mais simples encontrados na natureza. O segundo é formado pelos ácidos cinâmicos, que possuem nove átomos de carbono (C_6-C_3). O terceiro são as cumarinas, derivadas do ácido cinâmico por ciclização da cadeia lateral do ácido o-cumárico (Soares, 2002).

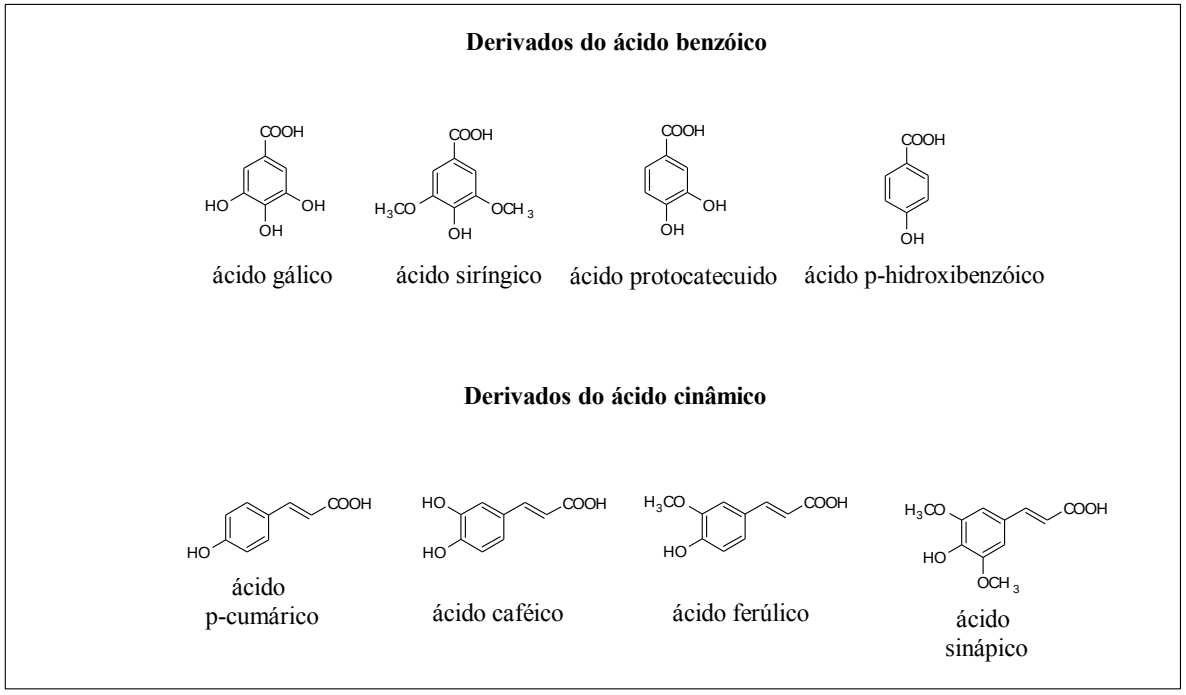

Figura 3. Estruturas químicas de alguns ácidos fenólicos. Fonte: Balasundram, Sundram & Samman (2006).

Os flavonoides constituem um grupo enorme de fenólicos de plantas, representando mais da metade dos compostos fenólicos que ocorrem naturalmente (Benavente-Garcia et al., 1997; Bravo, 1998; Balasundram et al., 2006). Caracterizam-se estruturalmente por um esqueleto carbônico C_6-C_3-C_6 (Figura 4), no qual os três carbonos entre os grupos fenil são ciclizados com oxigênio. As várias classes de flavonoides diferem

no nível de oxidação e no padrão de substituição no anel C, enquanto compostos individuais dentro de uma classe diferem no padrão de substituição nos anéis A e B (Pietta, 2000). Assim, dividem-se em seis classes: flavanonas, flavonas, flavonois, isoflavonas, flavanois e antocianinas (Hollman & Katan, 1999).

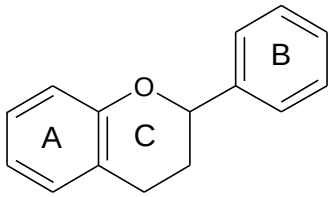

Figura 4. Estrutura básica dos flavonóides. Fonte: Cook & Samman (1996).

Os compostos fenólicos são conhecidos como ótimos desativadores de espécies radicalares, sendo relatados dois mecanismos principais: transferência de um átomo de hidrogênio (HAT) e transferência de um elétron (SET) (Huang et al., 2005; Anouar, Kosinová, Kozlowski, Mokrini, Duroux & Trouillas, 2009). Recentemente foi relatado que os compostos fenólicos também são capazes de desativar radicais através da formação de aduto (Anouar et al., 2009).

3. Maná-cubiu (*Solanum sessiflorum*)

Maná-cubiu (*Solanum sessiflorum*) é uma fruta (Figura 5) que pertence à família *Solanaceae*. Esta fruta é nativa da região Amazônica e largamente distribuída nas regiões úmidas equatoriais do Brasil, Peru e Colômbia (Schuelter et al., 2009). As frutas são conhecidas como topiro/tupiro no Peru, cocona na Venezuela, tomate da índia no nordeste do Brasil, cubiu na região Amazônica brasileira e oricono ou *apple*/*peach tomato* em países de língua inglesa. As frutas podem ser arredondas ou achatadas, apresentando diâmetros variando de 5-6 cm e massa entre 30 e 400 g. A fração comestível representa aproximadamente 91% (m/m) da massa fresca total (9% m/m de casca), sendo que 90% (m/m) da fração comestível correspondem à água (Marx, Andrade & Maia, 1998).

A composição química e os principais componentes da polpa de maná-cubiu já foi relatada, como sendo, carboidratos (amido, glucose, frutose, sacarose e traços de inositol), ácidos orgânicos (ácido cítrico), aminoácidos livres (asparagina, serina e glutamina),

minerais (ferro, zinco e manganês), aminas biogênicas (etanolamina e ornitina) e compostos voláteis (safrol) (Marx et al., 1998). Devido à alta concentração de ácido cítrico, a polpa possui sabor ácido, lembrando frutas cítricas e usualmente é consumida como salada, suco ou geleia (Marx et al., 1998).

Figura 5. Frutos de maná-cubiu (*Solanum sessiflorum*).

Os carotenoides do maná-cubiu ainda não foram relatados na literatura. Porém, foi reportada a composição de carotenoides da fruta naranjilla (*Solanum quitoense* Lam. Var. Puyo Hybrid), que é do mesmo gênero do maná-cubiu. Foi identificado, utilizando HPLC-DAD-MS/MS, como carotenoide majoritário o all-*trans*-β-caroteno, seguido de all-*trans*-luteína, 13-*cis*-β-caroteno e 9-*cis*-β-caroteno (Gancel, Alter, Dhuique-Mayer, Ruales & Vaillant, 2008). Porém, os carotenoides minoritários presentes na naranjilla não foram identificados.

Os compostos fenólicos do maná-cubiu também não foram relatados na literatura. No entanto, a composição fenólica da naranjilla determinada por HPLC-DAD-MS/MS foi relatada previamente. Foram identificados como compostos fenólicos majoritários os ácidos clorogênicos e seus hexosídeos. Interessantemente, também foram identificados muitos conjugados de ácido cafeico com espermidinas (Gancel et al., 2008).

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CAPÍTULO II

Microcapsules containing antioxidant molecules as scavengers of reactive oxygen and nitrogen species

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Microcapsules containing antioxidant molecules as scavengers of reactive oxygen and nitrogen species

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ABSTRACT

The antioxidant capacities of gum arabic and maltodextrin microcapsules containing antioxidant molecules (trolox, α-tocopherol, β-carotene, apo-8′-carotenal and apo-12′-carotenal) against reactive oxygen and nitrogen species were evaluated. The scavenging capacities were influenced by the wall material, the reactive species, namely ROO·, H_2O_2, HO·, HOCl and $ONOO^-$, and the antioxidant molecule. In general, a more pronounced enhancement of the antioxidant capacity due to incorporation of antioxidant molecules was observed in gum arabic microcapsules. The empty microcapsules showed capacity to scavenge all the studied ROS and RNS, being gum arabic a more potent antioxidant than maltodextrin. Apo-8′-carotenal incorporation promoted the highest increase in the scavenging capacities among the evaluated antioxidants, varying from 50% to 132% and from 39% to 85% for gum arabic and maltodextrin microcapsules, respectively, suggesting that this carotenoid presented the best balance between the molecule localization inside the microcapsules and the reactivity against the specific reactive species.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism and they are well recognized for playing a dual role in living systems once their effects can be either harmful or beneficial. The term ROS includes oxygen-derived radicals such as superoxide radical ($O_2^{\cdot -}$), peroxyl radical (ROO·), hydroxyl radical (HO·), and non-radical species, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) (Choe & Min, 2006), whilst RNS includes mainly the nitric oxide radical (·NO) and non-radical species, such as peroxynitrite anion ($ONOO^-$) (Halliwell & Gutteridge, 2007, chap. 9). At moderate concentrations, ROS and RNS can be involved in cellular responses to injury, e.g. in the defense against infectious agents, and also in cellular signalling systems. A balance between the generation of ROS and RNS and the endogenous antioxidant defense system (enzymatic and non-enzymatic) occurs at normal physiological state; however, some events, such as infections, can induce an overproduction of ROS and RNS that can either play their role in combating the invading organism or cause damage in the organism cell components and tissue injuries (Valko et al., 2007). Moreover, some ROS, such as ROO·, HO· and $^1O_2$, can also be generated in food and cosmetics and act as oxidant agents contributing to the degradation of these products (Choe & Min, 2006).

The antioxidants consumed in the diet are important in maintaining the balance between ROS and RNS, especially when the endogenous antioxidant defense system is not able to scavenge the proper amounts of generated reactive species. Carotenoids and tocopherols (Supplementary Fig. S1) are two important classes of bioactive compounds present in the diet that are associated with a reduced risk of chronic degenerative diseases. This effect is mainly attributed to the attenuation of oxidative and/or nitrosative events linked to these diseases pathogenesis (Rock, 2009). Moreover, food and cosmetic products can also benefit from the addition of these bioactive compounds due to their antioxidant capacity in the prevention of the oxidation of lipids, proteins, vitamins, among other constituents. The application of lipophilic antioxidant compounds in such products is not easy due to their low solubility in aqueous systems and high susceptibility to degradation by high temperature, low pH and presence of light and oxygen, especially the carotenoids (Mercadante, 2008).

Microencapsulation by spray-drying is a technique widely used in the industry to provide stability and to allow the incorporation of ingredients with low solubility in water, such as flavours, lipids, vitamins and carotenoids, into food products (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). Besides, as antioxidant compounds are able to maintain, at least partially, their antioxidant capacity when microencapsulated, it becomes possible to add lipophilic compounds into aqueous systems to scavenge ROS

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and RNS (Faria, Mignone, Montenegro, Mercadante, & Borsarelli, 2010; Montenegro, Nunes, Mercadante, & Borsarelli, 2007). Recently our research group produced and characterized microcapsules with gum arabic (GA) and maltodextrin DE 20 (MD), as wall materials, containing β-carotene, apo-8′-carotenal, apo-12′-carotenal, α-tocopherol and trolox, and verified a significant ability to quench $^1O_2$ (Faria et al., 2010). To continue this previous study, the antioxidant capacity of these microcapsules against other ROS and RNS of biological relevance, namely ROO$^\cdot$, H_2O_2, HO$^\cdot$, HOCl and $ONOO^-$, was evaluated in the present study. Furthermore, this is the first time that the capacity of microcapsules containing antioxidants molecules to scavenge these ROS and RNS is reported.

2. Materials and methods

2.1. Materials

The carotenoid standards used to prepare the microcapsules were β-carotene (98% purity), α-tocopherol (97% purity), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, 99.5% purity), purchased from Sigma–Aldrich (Missouri, USA), and apo-8′-carotenal (96% purity) and apo-12′-carotenal (91% purity), kindly donated by DSM Nutritional Products (Basel, Switzerland). These compounds were used as received, with the exception of β-carotene, which was recrystallized up to 98% purity, and the purity was determined by high-performance liquid chromatography with diode array detector (HPLC–DAD) (Supplementary Figs. S2, S3 and Table S1). The following chemicals: ascorbic acid (99% purity), cysteine (97% purity), α,α′-azodiisobutyramidine dihydrochloride (AAPH), sodium phosphate tribasic dodecahydrate ($Na_3PO_4.12H_2O$), dihydrorhodamine 123 (DHR), lucigenin, luminol, sodium hypochlorite with 13% available chlorine, 30% (w/w) hydrogen peroxide solution, fluorescein sodium salt, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) were supplied by Sigma–Aldrich and gallic acid by Extrasynthèse (Genay, France). Ultrapure water was obtained from the Millipore system (Massachusetts, USA). Powdered GA (MW = 3.5×10^5 g/mol) was supplied by Colloids Naturels Brazil (São Paulo, Brazil) and maltodextrin 20 DE (MW = 1000 g/mol) by Corn Products Brazil (São Paulo, Brazil).

2.2. Preparation of microcapsules

The microcapsules used in this study were the same prepared and characterized in a previously study (Faria et al., 2010). Five compounds, β-carotene, apo-8′-carotenal, apo-12′-carotenal, α-tocopherol and trolox, were microencapsulated using MD and GA as wall material, totalling 10 microcapsules. In addition, two empty microcapsules (without antioxidant), one using MD and the other using GA, were prepared. Solutions of each biopolymer (200 ml, 30% w/v) were prepared in water at 45 °C and were kept under continuous stirring until temperature reached 30 °C. In order to obtain antioxidant solutions with similar molar concentrations, 15–63 mg of each carotenoid, trolox and α-tocopherol was dissolved in a solvent in which each compound is highly soluble (dichloromethane for carotenoids and ethanol for α-tocopherol and trolox), and added to the polymer solution. The mixture was homogenized at 7000 rpm for 30 min and the resulting emulsion was diluted with water to obtain a 20% (w/v) biopolymer solution. The emulsion was submitted to a spray-dryer (Lab Plant SD-04, Huddersfield, United Kingdom) under slow agitation. The microcapsules were immediately stored under N_2 atmosphere and kept at −36 °C until analysis. The final core concentration (μmol/g of biopolymer) of antioxidants in the microcapsules were: trolox 2.60 and 1.88, α-tocopherol 1.55 and 2.13, β-carotene 1.39 and 1.04, apo-8′-carotenal 0.37 and 0.35, and apo-12′-carotenal 1.67 and 1.06, in GA and MD microcapsules, respectively.

The residual water of the microcapsules was determined in an oven at 80 °C for 16 h (Polavarapu, Oliver, Ajlouni, & Augustin, 2011). The average and standard deviation of triplicate analysis of residual water contents (g/100 g of microcapsule) were 2.10 ± 0.07 in GA and 2.40 ± 0.06 in MD empty microcapsules. The GA microcapsules with antioxidants had very similar residual water contents (g/100 g of microcapsule): 2.30 ± 0.06 for trolox, 2.30 ± 0.09 for α-tocopherol, 2.40 ± 0.08 for β-carotene, 2.40 ± 0.03 for apo-8′-carotenal and 2.10 ± 0.06 for apo-12′-carotenal. The residual water contents in g/100 g of MD microcapsules were also similar: 2.30 ± 0.13 for trolox, 2.20 ± 0.07 for α-tocopherol, 2.00 ± 0.07 for β-carotene, 2.30 ± 0.11 for apo-8′-carotenal and 2.40 ± 0.04 for apo-12′-carotenal.

The composition of the antioxidant compounds in the microcapsules was determined in order to verify composition changes after microencapsulation. In order to release the carotenoids, around 0.20 g of the MD microcapsules were dispersed in 5 ml of water, whilst 0.10 g of the GA ones were dispersed in 5 ml of water:methanol (2:3, v/v). The carotenoids were extracted exhaustively with dichloromethane from the microcapsule solution; the organic phases were recovered in a separation funnel and the residual water was removed with anhydrous Na_2SO_4. α-Tocopherol and trolox were extracted straight from 0.20 g of the microcapsule powder with 5 ml of ethanol by sonication (1 min), vortexing (5 min) and centrifugation (Beckman Coulter, California, USA) at 20000 *g* during 5 min. Afterward, the residual water of the supernatant was removed with anhydrous Na_2SO_4 and filtered. The solvent was removed under vacuum in a rotary evaporator ($T < 35$ °C). The dry extracts were redissolved, carotenoids in methanol:methyl *tert*-butyl-ether (1:1, v/v), α-tocopherol in methanol and trolox in methanol:water:formic acid (70:29.5:0.5, v/v/v), and analyzed by HPLC–DAD–MS/MS. These results are presented at Supplementary Figs. S2, S3 and Table S1.

2.3. ROS and RNS scavenging assays

2.3.1. General

The experiments were conducted immediately after the preparation of fresh microcapsules aqueous solutions to avoid their slow collapse in solution since in our previous study, these microcapsules presented a half-life of 17 ± 3 h and around 60 h for the complete release of pyrene molecules (Faria et al., 2010). The assays were carried out in a microplate reader (Synergy Mx, BioTek, Vermont, USA) for fluorescence, UV/vis and luminescence measurements, equipped with a thermostat set at 37 °C and dual reagent dispenser. Two control assays were conducted in all microplates, one of them to verify the interaction among the probe and the microcapsules, without radical generator or reactive species addition and the other one as quality analytical control (positive control), adding a compound with known capacity to scavenge the specific reactive species. No interaction between the probes and the microcapsules was observed and the maximum variation in the response of the positive controls during the assays was ⩽10%. Each ROS and RNS scavenging assay corresponds to two independent experiments, performed in duplicate. Except for peroxyl radical scavenging capacity, the results are presented as percent of inhibition, IC_{50} or IC_{20} values, calculated by non-linear regression analysis using the GraphPad Prism 5 software. The increase in scavenging capacity due to addition of antioxidant molecules was calculated by Eq. (1).

$$\text{Increase in scavenging capacity } (\%/\mu\text{mol antioxidant g biopolymer}) = \left(\left(\frac{\text{net scavenging capacity}}{\text{empty microcapsules scavenging capacity}}\right) \times 100\right) \times [\text{Aox}]^{-1} \quad (1)$$

where, net scavenging capacity = antioxidant microcapsules slope – empty microcapsules slope for peroxyl radical and IC_{50} or IC_{20} of empty microcapsules – IC_{50} or IC_{20} of antioxidant microcapsules

for the other ROS and RNS; [Aox] = concentration of antioxidant compound in microcapsules (μmol antioxidant/g biopolymer).

2.3.2. Peroxyl radical scavenging assay

The ROO· scavenging capacity was measured by monitoring the effect of the microcapsules on the fluorescence decay resulting from ROO·-induced oxidation of fluorescein (Ou, Hampsch-Woodill, & Prior, 2001). ROO· was generated by thermodecomposition of AAPH at 37 °C. Reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 200 μl): fluorescein (61 nM), AAPH solution in phosphate buffer (19 mM) and microcapsules aqueous solutions (four concentrations). The mixture was preincubated in the microplate reader during 10 min before AAPH addition. The fluorescence signal was monitored every minute for the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm, until 180 min. Trolox was used as positive control (Net area (64 μM) = 23).

2.3.3. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity was measured by monitoring the H_2O_2-induced oxidation of lucigenin (Gomes et al., 2007). Reaction mixtures contained the following reagents at final concentrations (final volume of 300 μl): 50 mM Tris–HCl buffer (pH 7.4), lucigenin solution in Tris–HCl buffer (0.8 mM), 1% (w/w) H_2O_2 and aqueous solutions of antioxidant microcapsules or trolox (five concentrations). The chemiluminescence signal was detected in the microplate reader after 5 min of incubation. Ascorbic acid was used as positive control (IC_{50} = 171 μg/ml).

2.3.4. Hydroxyl radical scavenging assay

The HO· scavenging capacity was measured by monitoring the HO·-induced oxidation of luminol (Costa, Marques, Reis, Lima, & Fernandes, 2006). The HO· was generated by a Fenton system ($FeCl_2$–EDTA–H_2O_2). Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 250 μl): luminol (20 mM), $FeCl_2$–EDTA (25, 100 μM), H_2O_2 (3.5 mM) and aqueous solutions of antioxidant microcapsules or trolox (five concentrations). The chemiluminescence signal was detected in the microplate reader after 5 min of incubation. Gallic acid was used as positive control (IC_{50} = 0.11 μg/ml).

2.3.5. Hypochlorous acid scavenging assay

The HOCl scavenging capacity was measured by monitoring the HOCl-induced oxidation of DHR to rhodamine 123 (Gomes et al., 2007). HOCl was prepared by adjusting the pH of a 1% (w/v) solution of NaOCl to 6.2, with 10% H_2SO_4 (v/v). The concentration of HOCl was determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} and further dilutions were made in 100 mM phosphate buffer (pH 7.4). Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 μl): DHR (5 μM), HOCl (5 μM) and aqueous solutions of antioxidant microcapsules or trolox (five concentrations). The fluorescence signals were measured in the microplate reader at 528 ± 20 nm for emission and 485 ± 20 nm for excitation. The fluorescence signal was measured immediately after HOCl addition. Cysteine was used as positive control (IC_{50} = 0.07 μg/ml).

2.3.6. Peroxynitrite scavenging assay

The $ONOO^-$ scavenging capacity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine (Gomes et al., 2007). $ONOO^-$ was synthesized as previously described by Gomes, Costa, Lima, and Fernandes (2006). Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 μl): DHR (5 μM), $ONOO^-$ (600 nM) and aqueous solutions of antioxidant microcapsules or trolox (five concentrations). The fluorescence signal was measured in the microplate reader after 5 min incubation, with wavelengths of emission 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 concentration. This evaluation is important because, under physiological conditions, the reaction between $ONOO^-$ and bicarbonate is predominant ($k = 3–5.8 \times 10^4$ M^{-1} s^{-1}), generating nitrogen dioxide ($^{\cdot}NO_2$) and carbonate radical anion ($CO_3^{\cdot-}$). Ascorbic acid was used as positive control (IC_{50} = 0.22 μg/ml and IC_{50} = 0.31 μg/ml in the absence and presence of $NaHCO_3$, respectively).

2.3.7. Protein content and amino acid profile of gum arabic

Protein content was determined according to the Kjeldahl method (AOAC, 1997), using the conversion factor of 6.25. The

Table 1
ROS and RNS scavenging capacity of gum arabic and maltodextrin microcapsules containing antioxidant molecules.

Biopolymer	Antioxidant molecule (c.d.b.)[a]	ROO· (slope)[b]	IC_{20}[c] (mg/ml) H_2O_2	IC_{50}[d] (mg/ml)			
				HO·	HOCl	$ONOO^-$	
						Absence of $NaHCO_3$	Presence of $NaHCO_3$
Gum arabic	Trolox (3)	0.046	3.70 ± 0.13	0.77 ± 0.01	0.68 ± 0.06	0.16 ± 0.01	0.22 ± 0.03
	α-Tocopherol (3)	0.044	3.98 ± 0.23	0.73 ± 0.03	0.81 ± 0.06	1.40 ± 0.09	0.82 ± 0.04
	Apo-12′-carotenal (8)	0.042	3.31 ± 0.09	0.66 ± 0.05	0.84 ± 0.07	1.08 ± 0.05	0.93 ± 0.10
	Apo-8′-carotenal (10)	0.042	2.66 ± 0.05	0.64 ± 0.05	0.65 ± 0.01	0.82 ± 0.01	0.93 ± 0.09
	β-Carotene (11)	0.033	0.35 ± 0.00	0.39 ± 0.02	0.51 ± 0.01	0.66 ± 0.03	0.64 ± 0.05
	None	0.031	5.11 ± 0.32	1.05 ± 0.06	0.80 ± 0.05	1.61 ± 0.08	1.01 ± 0.10
Maltodextrin	Trolox (3)	0.010	INT[e]	5.04 ± 0.16	0.83 ± 0.03	0.37 ± 0.02	0.38 ± 0.01
	α-Tocopherol (3)	0.010	INT	6.07 ± 0.55	0.91 ± 0.08	4.44 ± 0.31	3.96 ± 0.04
	Apo-12′-carotenal (8)	0.010	INT	3.97 ± 0.32	0.96 ± 0.04	3.76 ± 0.11	3.00 ± 0.23
	Apo-8′-carotenal (10)	0.010	INT	4.18 ± 0.07	0.85 ± 0.00	3.11 ± 0.20	2.82 ± 0.15
	β-Carotene (11)	0.010	INT	4.70 ± 0.08	0.86 ± 0.08	3.21 ± 0.05	3.38 ± 0.13
	None	0.010	INT	5.95 ± 0.28	0.87 ± 0.01	3.60 ± 0.24	3.61 ± 0.35

[a] c.d.b. = Number of conjugated double bonds.
[b] slope = Slope of the curve of the concentrations of microcapsule (mg microcapsule/ml water) against the net area under the curve, meaning that the higher the slope the higher the capacity to scavenge ROO·.
[c] IC_{20} = concentration (mg microcapsule/ml water) necessary to inhibit oxidation by 20%.
[d] IC_{50} = concentration (mg microcapsule/ml water) necessary to inhibit oxidation by 50%.
[e] INT = interference with the probe of the methodology.

Table 2
Effect of the incorporation of different antioxidant molecules on ROS and RNS scavenging capacity of gum arabic and maltodextrin microcapsules.

Biopolymer	Antioxidant molecule	Increase in scavenging capacity (%/μmol g)					
		$ROO^{\cdot}$	H_2O_2	$HO^{\cdot}$	HOCl	$ONOO^-$	
						Absence of $NaHCO_3$	Presence of $NaHCO_3$
Gum arabic	Trolox	19	11	10	6	35	30
	α-Tocopherol	27	14	20	NI	8	12
	Apo-12′-carotenal	21	21	22	NI	20	NI
	Apo-8′-carotenal	97	130	105	50	132	NI
	β-Carotene	NI[a]	67	45	26	43	26
Maltodextrin	Trolox	NI	INT[b]	8	NI	48	47
	α-Tocopherol	NI	INT	NI	NI	NI	NI
	Apo-12′-carotenal	NI	INT	31	NI	NI	16
	Apo-8′-carotenal	NI	INT	85	NI	39	62
	β-Carotene	NI	INT	20	NI	10	6

[a] NI = no increase, meaning that the antioxidant capacity of the microcapsules containing antioxidant is the same as that of the empty microcapsules.
[b] INT = interference with the methodology.

amino acid analysis was carried out according to White, Hart, and Kry (1986). Both analyses were performed in duplicate.

3. Results

3.1. General

Two approaches were used to present and discuss the capacity of GA and MD microcapsules to scavenge ROS and RNS. The first one aimed to compare the antioxidant capacity of the microcapsules as a whole, regardless the fact that they do not have the same antioxidant concentration (Table 1). The second approach discusses the effects of the addition of 1 μmol of antioxidant molecule per gramme of biopolymer (GA or MD) in comparison to the biopolymer alone (empty microcapsule) (Table 2).

Except for trolox, it is not possible to compare the microencapsulated antioxidants with the correspondent not microencapsulated ones since carotenoids and tocopherol are lipophilic, thus they are not soluble in the solvents used in the methods. Microencapsulation, both using GA and MD as wall material, resulted in suppression of trolox scavenging capacities against $HO^{\cdot}$ and $ONOO^-$ (Table 3). However, microencapsulation of trolox with GA improved the $ROO^{\cdot}$, H_2O_2 and HOCl scavenging capacity as compared to trolox alone, being about 2-, 57- and 96-fold more potent, respectively (Table 3).

3.2. Peroxyl radical scavenging capacity

Both empty microcapsules presented capacity to scavenge $ROO^{\cdot}$, although GA was more potent than MD (Fig. 1). GA microcapsules containing α-tocopherol and trolox, apo-8′-carotenal and apo-12′-carotenal presented similar capacity to scavenge $ROO^{\cdot}$ and were better $ROO^{\cdot}$ scavengers than β-carotene microcapsules. However, all MD microcapsules, with or without antioxidants, presented no differences among each other as $ROO^{\cdot}$ scavengers, i.e. carotenoids, α-tocopherol and trolox did not improve the capacity of MD microcapsules themselves to scavenge $ROO^{\cdot}$ (Table 1). Incorporation of apo-8′-carotenal promoted the major increase, 97%/μmol g, in the GA microcapsules scavenging capacity (Table 2).

3.3. Hydrogen peroxide scavenging capacity

With the exception of the microcapsules containing β-carotene, all the other GA microcapsules did not reach a 50% decay effect at the maximum tested concentration (Fig. 2a) due to the limited solubility of the microcapsules in water. For this reason, the H_2O_2 scavenging capacity was calculated as IC_{20}. The use of other solvents was avoided in order to prevent microcapsules collapse. The β-carotene microcapsules showed the highest capacity to scavenge H_2O_2, whilst the other microcapsules with antioxidants were ten times less efficient than those containing β-carotene (Table 1). As can be seen in Table 2, all antioxidants improved the capacity of GA microcapsules to scavenge H_2O_2. In fact, incorporation of apo-8′-carotenal promoted the major increase (Table 2).

It was not possible to evaluate the MD microcapsules using this assay because they interfered with the methodology, provoking an increase in the chemiluminescence signal in a concentration-dependent manner. This effect occurred only in the presence of H_2O_2, indicating that this increase in the analytical signal did not result from direct oxidation of lucigenin by MD microcapsules, but probably these microcapsules directly react with H_2O_2, generating products that are able to oxidize lucigenin, as previously reported for the β-adrenergic antagonists, atenolol, carvedilol and pindolol (Gomes et al., 2006).

3.4. Hydroxyl radical scavenging capacity

Fig. 2a and b shows the $HO^{\cdot}$ scavenging capacities of GA and MD microcapsules, respectively. Empty GA microcapsules showed about six times higher capacity to scavenge $HO^{\cdot}$ than MD

Table 3
Effect of microencapsulation on the scavenging capacity of ROS and RNS by trolox.

Reactive species	Trolox in solution	GA microcapsules	MD microcapsules
$ROO^{\cdot}$[a]	0.36	0.78	–
H_2O_2[b]	440	7.69	–
$HO^{\cdot}$[c]	0.35	2.00	9.60
HOCl[c]	530	5.53	–
$ONOO^-$ (absence of $NaHCO_3$)[c]	0.13	0.42	0.70
$ONOO^-$ (presence of $NaHCO_3$)[c]	0.14	0.58	0.80

[a] slope = Slope of the curve of concentrations of microcapsule (mg microcapsule/ml water) against the net area under the curve, meaning that the higher the slope the higher the capacity to scavenge $ROO^{\cdot}$.
[b] IC_{20} = concentration (mg microcapsule/ml water) necessary to inhibit oxidation by 20%.
[c] IC_{50} = concentration (mg microcapsule/ml water) necessary to inhibit oxidation by 50%.

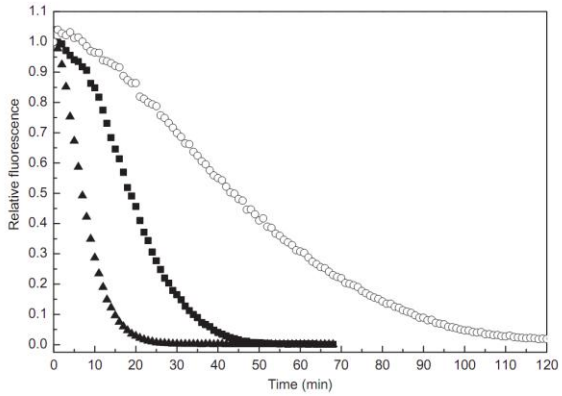

Fig. 1. Fluorescence decay of fluorescein induced by peroxyl radicals at 37 °C, in the presence of empty microcapsules of GA and MD, both at the same concentration (1.25 mg/ml). Blank: filled triangle, MD microcapsules: filled square, GA microcapsules: open circle.

microcapsules (Table 1). GA microcapsules with β-carotene were the most effective, whilst MD microcapsules with α-tocopherol presented the lowest scavenging capacity (Table 1). In fact, the scavenging capacity of MD microcapsules with α-tocopherol was similar to that of the empty MD microcapsules. Incorporation of apo-8′-carotenal promoted the major increase in the scavenging capacity of both GA and MD microcapsules, 105 and 85%/μmol g, respectively, whilst α-tocopherol incorporation resulted in an increase of 20%/μmol g when added to GA microcapsules but had no effect on MD microcapsules. The incorporation of β-carotene to GA microcapsules resulted in an increase of 45%/μmol g, but only half of this, 20%/μmol g, when incorporated to MD microcapsules (Table 2).

3.5. Hypochlorous acid scavenging capacity

The HOCl scavenging capacities of GA and MD microcapsules are shown in Figs. 2c and 3b, respectively. GA microcapsules containing β-carotene presented the highest capacity to scavenge HOCl, whilst α-tocopherol and apo-12′-carotenal GA microcapsules did not differ from empty GA microcapsules. MD microcapsules containing all tested antioxidant compounds presented the same scavenging capacity than empty MD microcapsules (Table 1). Incorporation of apo-8′-carotenal to GA microcapsules promoted a 50%/μmol g increase in the scavenging capacity, and β-carotene contributed with less than 30%/μmol g (Table 2).

3.6. Peroxynitrite scavenging capacity

The empty GA microcapsules presented a marked difference, 60%, between the $ONOO^-$ scavenging capacity in the presence and absence of $NaHCO_3$, but the empty MD microcapsules did not (Table 1). The GA microcapsules containing trolox were the most effective $ONOO^-$ scavengers, both in the presence and absence of $NaHCO_3$ (Table 1).

The incorporation of apo-8′-carotenal and β-carotene to GA microcapsules increased the capacity to scavenge $ONOO^-$, without $NaHCO_3$, in 132 and 43%/μmol g, respectively; meanwhile, when these carotenoids were incorporated to MD microcapsules, the increase was only 39%/μmol g for apo-8′-carotenal and 10%/μmol g for β-carotene. Interestingly, when $NaHCO_3$ was added to the reaction system, the incorporation of apo-8′-carotenal did not affect the scavenging capacity of GA microcapsules; however, in MD microcapsules, the scavenging capacity raised 62%/μmol g. A similar effect was observed when apo-12′-carotenal was incorporated to MD microcapsules.

4. Discussion

The polymers used as wall materials for microencapsulation were in the past considered inert and their main functions were to protect and to control the liberation of the encapsulated compounds. However, recent studies have shown that some polymers used as wall materials, such as gum arabic, agar-agar, alginic acid, guar and xanthan gums, possess antioxidant capacity (Faria et al., 2010; Montenegro et al., 2007; Trommer & Nerbert, 2005). For example, microencapsulated GA was able to delay photo-oxidation in skimmed milk by efficiently quenching the riboflavin triplet state (Montenegro et al., 2007). The previous study carried out by Faria et al. (2010) also showed that the empty microcapsules of GA and MD were able to quench singlet oxygen. In the present study, the empty microcapsules also showed capacity to scavenge all the studied ROS and RNS in a concentration dependent manner. In general, when the capacity to scavenge ROS and RNS was compared, considering the microcapsule concentration in mg of biopolymer per ml of water, GA showed to be a more potent ROS and RNS scavenger than MD. GA is a complex and variable mixture of arabinogalactan oligosaccharides, polysaccharides and glycoproteins, resulting in a high molecular weight biopolymer (MW ≈ 350 kDa) (Renard, Lavenant-Gourgeon, Ralet, & Sanchez, 2006), whilst MD (MW ≈ 1 kDa) is a mixture of short polymers of D-glucose (3–20 units), in which the α-D-glucopyranosyl monomers are joined by (1 → 4) linkages to give linear chains with a certain degree of chain branching due to (1 → 6) bonding (Kennedy, Noy, Stead, & White, 1985). Thus, when a comparison is made considering the molar concentration of the wall material, the capacity of GA empty microcapsules to scavenge ROS and RNS is at least 350 times higher than MD empty microcapsules. These results are in agreement with those reported by Faria et al. (2010), since the capacity of the empty GA microcapsules to quench $^1O_2$ was about 300 times higher than empty MD microcapsules. The difference between the antioxidant capacities of the biopolymers can be attributed to the protein fraction of GA that corresponds to 0.76% (w/w) of this biopolymer (Supplementary Table S3). The amino acids tyrosine, histidine and methionine seem to be the main responsibles for the antioxidant capacity of GA against ROS and RNS (Atmaca, 2004; Meucci & Mele, 1997; Yilmaz & Toledo, 2005). In addition, the low antioxidant capacity of MD is probably related to the lack of functional groups that are able to donate electrons or hydrogen to ROS and RNS (Phillips, Carlsen, & Blomhoff, 2009).

Our *in vitro* findings reinforce the results of some *in vivo* studies that showed a positive relation between GA ingestion and the reduction of oxidative stress induced by gentamicin in rats, which was related to the capacity of GA to scavenge the ROS and RNS generated by this drug (Al-Majed, Mostafa, Al-Rikabi, & Al-Shabanah, 2002; Gamal el-din, Mostafa, Al-Shabanah, Al-Bekairi, & Nagi, 2003).

The incorporation of carotenoids, α-tocopherol and trolox into the microcapsules resulted in different effects on the ROS and RNS scavenging capacity, depending on the wall material, the reactive species tested and the antioxidant compound. In general, a more pronounced enhance of the antioxidant capacity due to incorporation of antioxidant compounds was observed in GA microcapsules. This biopolymer probably allows better interaction between the microencapsulated compounds and the ROS and RNS as compared to MD. The GA wall acts as membranes semipermeable to oxygen (Bertolini, Siani, & Grosso, 2001) and, possibly, the reactive species with similar molecular volumes to oxygen can diffuse into the interior of the microcapsules where they are scavenged by the antioxidants.

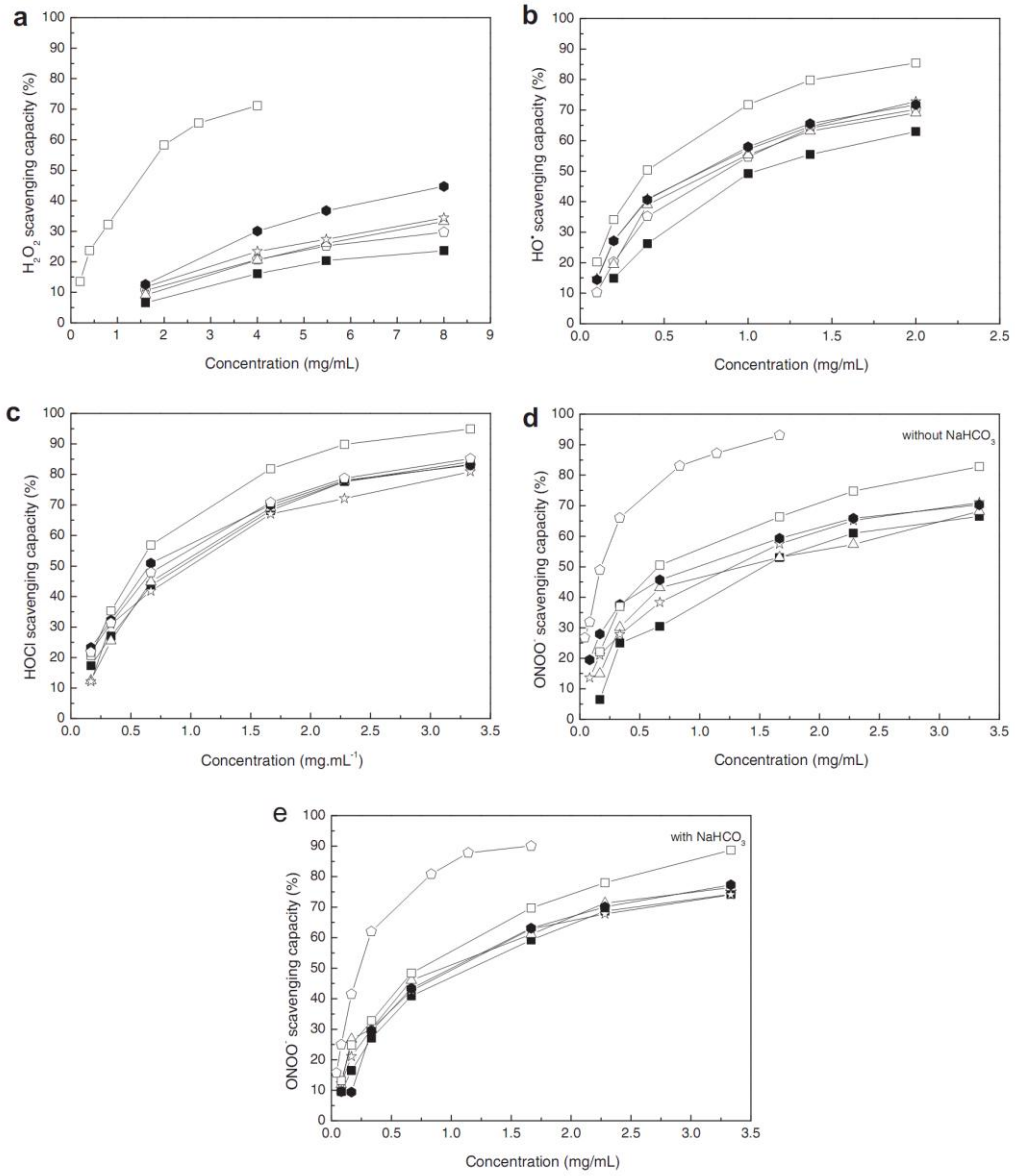

Fig. 2. Gum arabic microcapsules scavenging capacity of (a) hydrogen peroxide (H_2O_2), (b) hydroxyl radical ($HO^•$), (c) hypochlorous acid (HOCl), (d) anion peroxynitrite ($ONOO^-$) without $NaHCO_3$ and (e) $ONOO^-$ with $NaHCO_3$. Legend: filled square: empty microcapsule (no antioxidant molecule), open triangle: tocopherol microcapsule, open star: apo-12′-carotenal microcapsule, filled hexagon: apo-8′-carotenal microcapsule, open square: β-carotene microcapsule, open pentagon: trolox microcapsule.

The antioxidant capacity of carotenoids against ROS and RNS includes mainly one of the following mechanisms: electron transfer, hydrogen abstraction and addition of reactive species to form carotenoid-radical adducts (Burton & Ingold, 1984; El-Agamey et al., 2004; Jomová et al., 2009). Several factors, including the nature of the ROS and RNS, system polarity, carotenoid structure, the location and orientation of the carotenoids into the microcapsules, have probably an influence on the preferential antioxidant mechanism; however, these interactions are not totally elucidated. Among the three reaction pathways for carotenoids to scavenge radical species, electron transfer leading to the formation of the carotenoid radical cations appears to be the best accepted mechanism for polar systems as used in the present study.

Considering all the studied reactive species, apo-8′-carotenal promoted the highest increases in the scavenging capacity when incorporated into both microcapsules. This fact suggests that this carotenoid presents the best balance between the localization of the molecule inside the microcapsules and the reactivity against the specific ROS and RNS. The carbonyl group (CHO) in apo-8′-carotenal structure probably allows this carotenoid to hold strategic positions in the microcapsules facilitating the interaction with the ROS and RNS, and, in addition to the number of conjugated

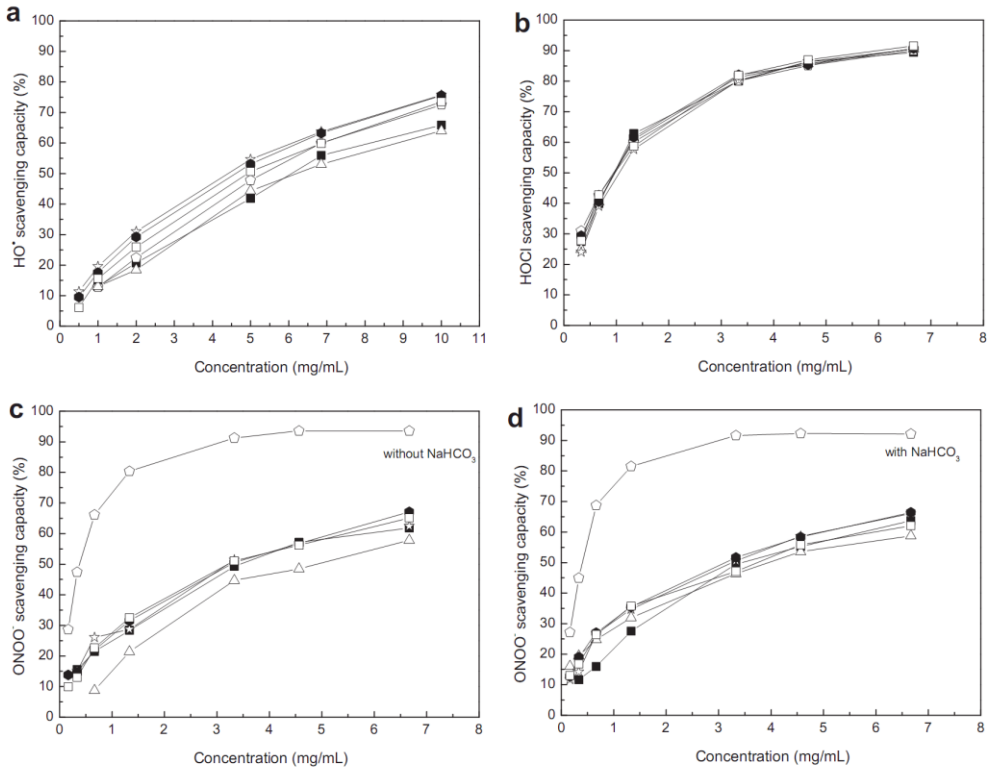

Fig. 3. Maltodextrin microcapsules scavenging capacity of (a) hydroxyl radical (HO•), (b) hypochlorous acid (HOCl), (c) anion peroxynitrite ($ONOO^-$) without $NaHCO_3$ and (d) $ONOO^-$ with $NaHCO_3$. Legend: filled square: empty microcapsule (no antioxidant molecule), open triangle: tocopherol microcapsule, open star: apo-12′-carotenal microcapsule, filled hexagon: apo-8′-carotenal microcapsule, open square: β-carotene microcapsule, open pentagon: trolox microcapsule.

double bonds, simultaneously facilitates electron donation. The increase of the capacity to scavenge ROO• radicals by insertion of carbonyl functions into the polyene molecule was recently described by Müller, Fröhlich and Böhm (2011).

The structures of trolox and α-tocopherol are very similar (Supplementary Fig. S1), the alkyl side chain of α-tocopherol is replaced by a carboxyl group in trolox, increasing the polarity, but not modifying the phenolic hydroxyl group involved in the antioxidant mechanism of both α-tocopherol and trolox. In this case, the mechanisms to scavenge ROS and RNS comprise donation of the phenolic hydrogen, generating a hydroperoxide and an antioxidant radical stabilized by resonance, or electron transfer (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). Despite the structural similarity, trolox and α-tocopherol presented distinct behaviours against the studied reactive species. In general, α-tocopherol was more potent than the empty microcapsule only as radical ROS scavenger, whilst trolox showed a better antioxidant capacity than the empty microcapsules for both radical and non-radical species. These evidences suggest that the polarity of these molecules directly affects their antioxidant capacity, probably due to its influence on the positioning of the antioxidant molecules into the microcapsule interior.

The decrease of trolox scavenging capacity against HO• and $ONOO^-$ due to microencapsulation is in agreement to the findings of Faria et al. (2010) for $^1O_2$ quenching. On the other hand, the raise in the capacity of trolox after microencapsulation to scavenge ROO•, H_2O_2 and HOCl suggests the occurrence of a synergistic effect between the biopolymers and the antioxidant molecules, probably involving the formation of high stable antioxidant radicals facilitating the scavenging of these reactive species. A similar effect was observed for the inclusion complexes of β-cyclodextrin and catechins, in which stable semiquinone radical species were characterized by electron spin resonance (Folch-Cano et al., 2011).

In summary, GA and MD microcapsules containing carotenoids, α-tocopherol and trolox are able to scavenge ROO•, HO•, HOCl, $ONOO^-$ and possibly, $^{\cdot}NO_2$ and $CO_3^{\cdot-}$. Moreover, the biopolymers GA and MD are also ROS and RNS scavengers themselves, which is an important characteristic for food and drug ingredients. The results of the present work, along with the high singlet oxygen quenching capacity (Faria et al., 2010), contribute to the development of multi-functional microcapsules that are able to scavenge a broad range of reactive species of biological relevance, serving as a dietary supplement or as antioxidants for food products, and can also be used as colourants in hydrophilic matrices, such as foods and drugs, without raising the fat content.

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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.foodchem.2012.02.163.

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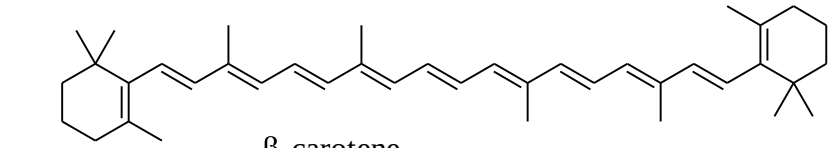
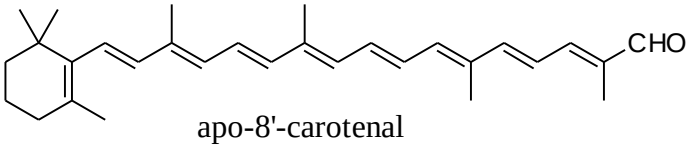
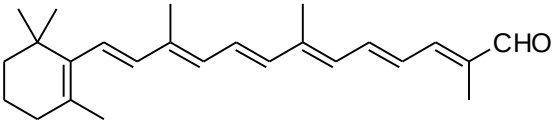


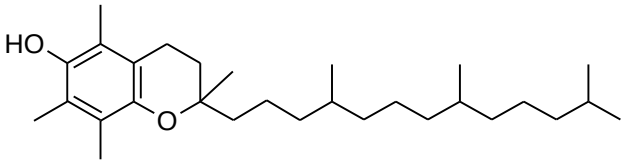
β-carotene



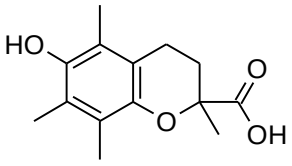
apo-8'-carotenal



apo-12'-carotenal

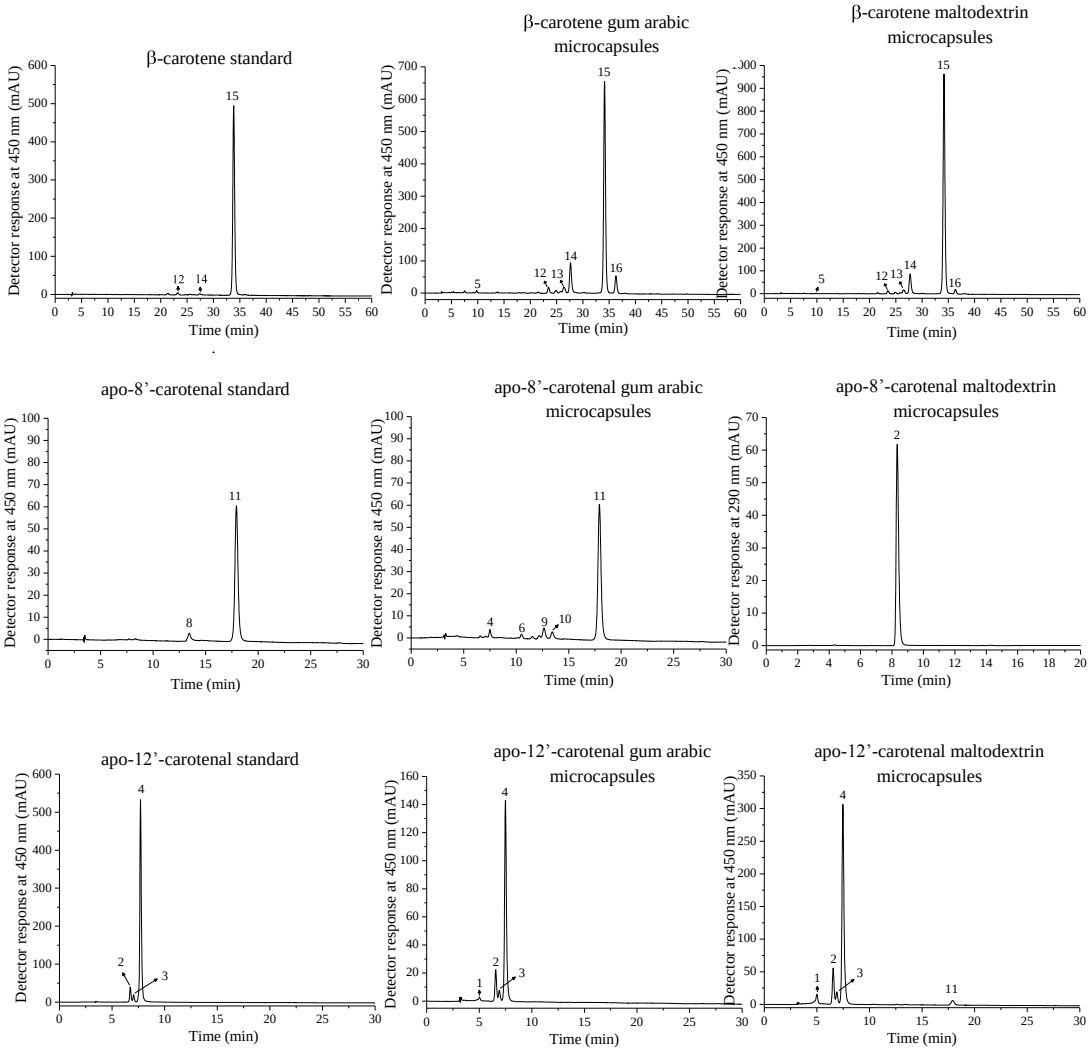


α-tocopherol

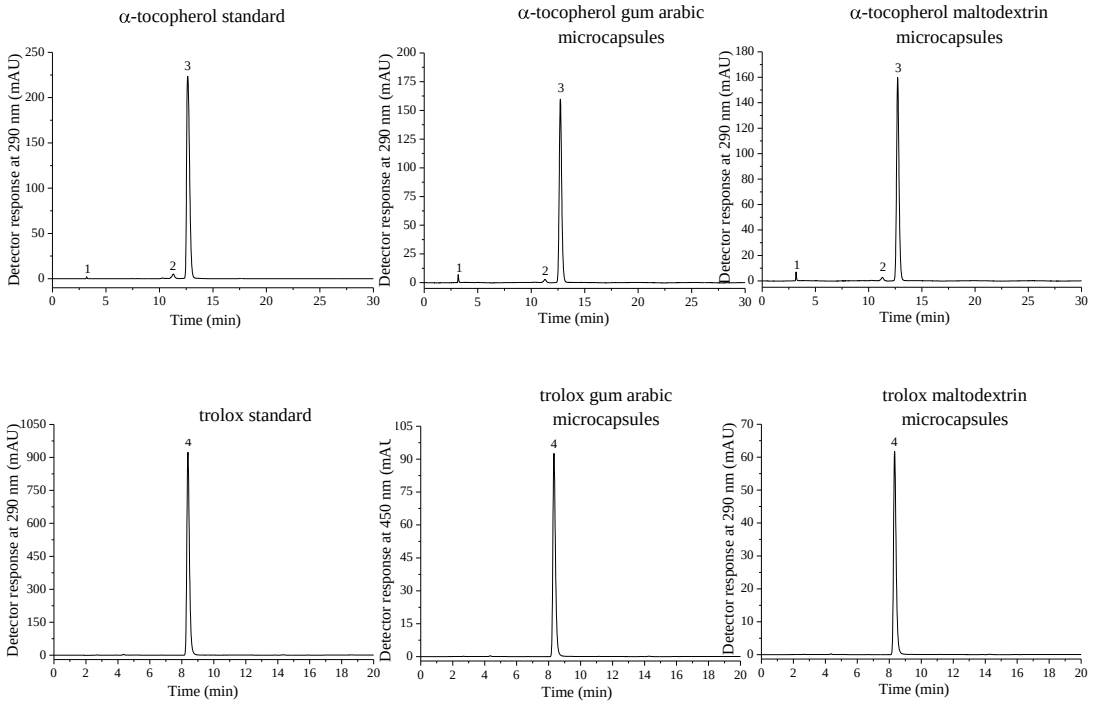


trolox

Supplementary Figure S1. Structures of carotenoids, α-tocopherol and trolox.



Supplementary Figure S2. Chromatograms, obtained by HPLC-DAD-MS/MS, of standard and microencapsulated carotenoids. Peak identification is shown in **Supplementary Table 1.** Chromatographic conditions: carotenoids were separated in a C_{30} YMC column (5 μm, 250 x 4.6 mm i.d.) (Waters, Wilmington, NC) using as mobile phase a linear gradient of methanol/methyl *tert*-butyl-ether from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min. The flow rate was 0.9 mL/min and column temperature was set at 29 ºC. The UV-vis spectra were obtained between 250 and 650 nm and the chromatograms were processed at 450 nm.



Supplementary Figure S3. Chromatograms, obtained by HPLC–DAD-MS/MS, of standard and microencapsulated α-tocopherol and trolox. Peak identification is shown in **Supplementary Table 2.** Chromatographic conditions: α-tocopherol and trolox were separated in a C_{18}(2) Luna column (5 μm, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA). Isocratic elution for α-tocopherol analysis was carried out using methanol at 0.9 mL/min flow rate and for trolox analysis, using methanol/water/formic acid (70:29.5:0.5) at 0.7 mL/min. The UV-vis spectra were obtained between 250 and 650 nm and the chromatograms were processed at 290 nm.

Supplementary Table S1. Chromatographic, UV-Vis, and mass spectroscopy characteristics of microencapsulated carotenoids and standards.

peak[a]	carotenoid	t_r (min)[b]	% area			λ_{max} (nm)[c]	% III/II	% A_B/A_{II}	$[M+H]^+$ (m/z)[i]	MS/MS (+) (m/z)[j]
			STD[d]	GA[e]	MD[f]					
1	not identified 1	5.0	n.d.[g]	0.8	4.4	414	0	0	367	349 $[M+H-18]^+$, 205
2	*cis*-apo-12'-carotenal 1	6.6	6.1	11.8	11.9	296, 417	0	47	351	333 $[M+H-18]^+$
3	*cis*-apo-12'-carotenal 2	6.9	3.0	2.4	4.3	295, 419	0	39	351	333 $[M+H-18]^+$
4	all-*trans*-apo-12'-carotenal	7.5	90.8	85.0	77.5	426	0	0	351	333 $[M+H-18]^+$
5	all-*trans*-apo-10'-carotenal	9.8	n.d.	0.5	0.1	430-436	0	0	377	359 $[M+H-18]^+$
6	epoxy-apo-8'-carotenal	10.5	n.d.	1.5	3.3	450-454	n.c.[h]	n.c.	433	415 $[M+H-18]^+$, 205
7	not identified 2	11.5	n.d.	n.d.	1.6	443	0	0	417	399 $[M+H-18]^+$
8	*cis*-apo-8'-carotenal 1	12.2	n.d.	n.d.	2.4	334, 453	0	63	417	399 $[M+H-18]^+$
9	*cis*-apo-8'-carotenal 2	12.6	n.d.	4.6	9.9	335, 453	0	48	417	399 $[M+H-18]^+$
10	*cis*-apo-8'-carotenal 3	13.4	2.7	3.1	6.9	334, 449	0	44	417	399 $[M+H-18]^+$
11	all-*trans*-apo-8'-carotenal	17.9	96.1	87.3	75.6	462	0	0	417	399 $[M+H-18]^+$
12	5,6-epoxy-β-carotene	23.5	0.6	1.9	1.1	419, 445, 473	56	0	553	535 $[M+H-18]^+$, 205
13	15-*cis*-β-carotene	26.4	n.d.	2.6	1.8	337, 420, 447, 474	n.c.	45	537	444 $[M-92]^+$, 399 $[M-137]^+$
14	13-*cis*-β-carotene	27.6	0.2	11.8	8.6	338, 420, 444, 470	17	47	537	444 $[M-92]^+$, 399 $[M-137]^+$
15	all-*trans*-β-carotene	34.1	98.7	75.2	86.2	423, 451, 478	30	0	537	444 $[M-92]^+$, 399 $[M-137]^+$
16	9-*cis*-β-carotene	36.3	n.d.	6.0	1.5	341, 420, 446, 473	30	7	537	444 $[M-92]^+$, 399 $[M-137]^+$

[a]Numbered according to Supplemental Figure S2. [b]Elution time on the C_{30} column. [c]Linear gradient methanol/methyl *tert*-butyl-ether. [d]STD: standard. [e]GA: carotenoid microencapsulated with gum arabic. [f]MD: carotenoid microencapsulated with maltodextrin. [g]n.d.: not detected. [h]n.c.: not calculated . [i]The carotenoids were ionized by APCI and parameters were set as follows: positive mode; current corona: 4 μA; source temperature: 450 ºC; dry gas (N_2) temperature: 350 ºC, flow: 5 L/min; nebulizer: 60 psi. The mass spectra were acquired with scan range of *m/z* from 100 to 600. [j]The MS/MS was set in automatic mode applying 1.4 V fragmentation energy.

Supplementary Table S2. Chromatographic, UV-Vis, and mass spectroscopy characteristics of microencapsulated α-tocopherol and trolox and standards.

peak[a]	compound	tr (min)[b]	% area			λ_{max} (nm)[c]	$[M-H]^-$ (m/z)[h]	MS/MS (-) (m/z)[i]
			STD[d]	GA[e]	MD[f]			
1	not identified 1	3.2	0.2	1.2	0.7	270	377	359 $[M-H-18]^-$
2	not identified 2	11.3	2.2	1.6	1.5	294	n.d.[g]	n.d.
3	α-tocopherol	12.6	97.6	97.9	97.2	291	429	414, 163
4	trolox	8.3	99.5	99.5	99.5	289	249	205, 176, 163

[a] Numbered according to Supplemental Figure 2. [b]Elution time on the C_{18} column. [c]α-tocopherol: methanol, trolox: methanol/water/formic acid (70:29.5:0.5, v/v/v). [d]STD: standard. [e]GA: α-tocopherol and trolox microencapsulated with gum arabic. [f] MD: α-tocopherol and trolox microencapsulated with maltodextrin. [g]n.d.: not detected. [h]The α-tocopherol and trolox were ionized in ESI and parameters set as follows: negative mode; end plate offset: -500 V; dry gas (N_2) temperature: 325 °C, flow: 8 L/min; nebulizer: 30 psi. The mass spectra were acquired with scan range of *m/z* from 100 to 600. [i]The MS/MS was set in automatic mode applying 1.2 V fragmentation energy.

Supplementary Table S3. Protein content and amino acid composition of gum arabic.

Determination	g/100 g
asparagine	0.023
glutamine	0.019
serine	0.143
glycine	0.008
histidine	0.019
arginine	0.010
threonine	0.025
alanine	0.025
proline	0.062
tyrosine	0.008
valine	0.025
methionine	0.004
isoleucine	0.016
leucine	0.048
phenylalanine	0.018
lysine	0.007
total amino acids	**0.455**
total protein	**0.760**

CAPÍTULO III

Development of a novel micro-assay for evaluation of peroxyl radical scavenger capacity: application to carotenoids and structure-activity relationship

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Abstract

A micro-assay was developed and validated, using a microplate reader in 96-well format, C_{11}-BODIPY$^{581/591}$ as fluorescent probe and AIBN as $ROO^{\bullet}$ generator. The structure-activity relationship was established for 15 carotenoid standards, indicating that the opening of the β-ionone ring and the increase of chromophore extension in the carotenoid structure were the major factors leading to the increase of $ROO^{\bullet}$ scavenging capacity. The values for $ROO^{\bullet}$ scavenging capacity were calculated using α-tocopherol as reference compound. Among the studied carotenoids, all-*trans*-lycopene was the most efficient $ROO^{\bullet}$ scavenger (8.67 ± 0.74) followed by all-*trans*-astaxanthin (6.50 ± 0.62). All the carotenoids showed to be more effective $ROO^{\bullet}$ scavengers than α-tocopherol and some hydrophilic compounds. Finally, the method was successfully applied to assay the $ROO^{\bullet}$ scavenging capacity of carotenoid extracts from two Amazonian fruits, peach palm (7.83 ± 0.21) and mamey (6.90 ± 0.44).

Keywords: carotenes; xanthophylls; antioxidant capacity; ROS; SAR; Amazonian fruit

1 Introduction

Epidemiological evidence has associated the fruit intake with the decrease of the incidence of cardiovascular diseases and certain types of cancer (Voutilainen, Nurmi, Mursu & Rissanen, 2006; Zhang et al., 2007; Karppi, Kurl, Laukkanen, Rissanen & Kauhanen, 2011). This effect is hypothetically attributed to the antioxidant properties of the carotenoids and other bioactive compounds, which minimise the *in vivo* oxidative damages induced by reactive oxygen and nitrogen species (Rock, 2009). Moreover, in food matrices, carotenoids can also minimise or delay lipid oxidation, consequently, increasing the shelf-life of food products (Choe & Min, 2006).

The carotenoids are yellow to red liposoluble pigments found in plants, animals and microorganisms (Britton, 1995). The basic structure of such compounds consists in a tetraterpene with a series of conjugated double bonds (c.d.b.), which generates a resonance system of π electrons moving along the entire polyene chain. Due to these structural characteristics, these compounds are very reactive and absorb light in the visible region of the spectrum (390-750 nm) (Mercadante, 2008). Basically, there are two types of carotenoids **(Fig. 1)**: carotenes, which contain only carbon and hydrogen atoms, and xanthophylls, which also contain one or more oxygen atoms (Mercadante, 2008).

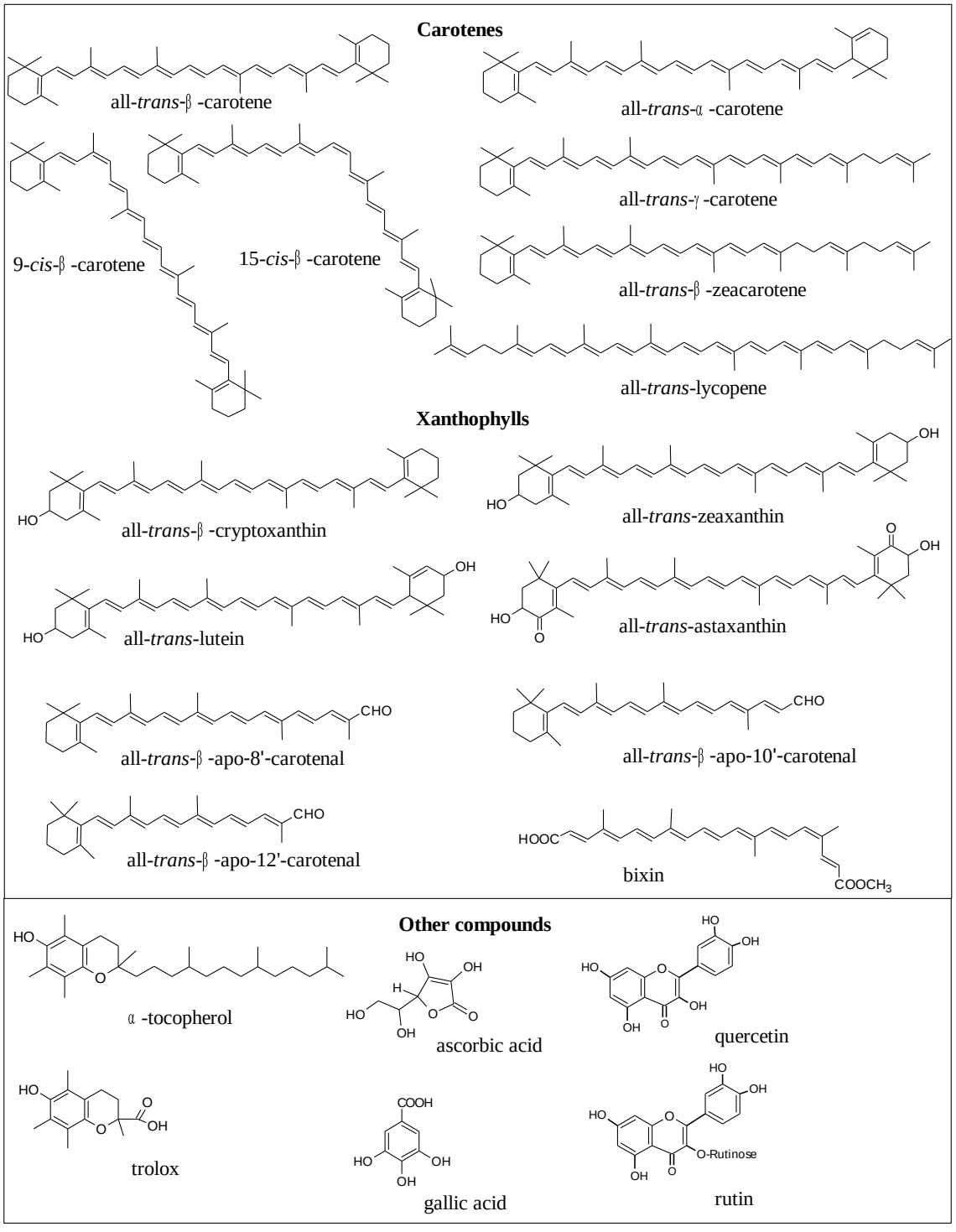

Fig. 1 Structure of the carotenoids and other analysed compounds.

Although the carotenoids have been studied for many years, there are still some unanswered questions concerning their antioxidant properties that need to be elucidated. Currently, it is known that the antioxidant properties of the carotenoids are closely related to their chemical structure, including aspects such as the number of c.d.b., type of structural end-groups, and oxygen-containing substituents (El-Agamey et al., 2004). These relations are well-established when the capacity to quench singlet oxygen and to scavenge the non-physiological radical 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) are considered (Di Mascio, Kaiser & Sies, 1989; Miller, Sampson, Candeias, Bramley & Rice-Evans, 1996; Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). However, literature data regarding the capacity of carotenoids to scavenge peroxyl radicals ($ROO^{\bullet}$) are not consistent, once conflicting results are presented. For example, comparing the $ROO^{\bullet}$ scavenging capacity of all-*trans*-β-carotene and all-*trans*-lutein assayed by three different methods (Naguib, 1998; Zulueta, Esteve & Frígola, 2009; Müller, Fröhlich & Böhm, 2011), the results showed that all-*trans*-β-carotene was 114 % (Zulueta et al., 2009) or 33 % (Naguib, 1998) more efficient as $ROO^{\bullet}$ scavenger than all-*trans*-lutein or that both carotenoids presented the same capacity (Müller et al., 2011). These conflicting results are probably generated by the use of different reaction means, i.e. aqueous buffers (Zulueta et al., 2009; Müller et al., 2011), which are not appropriated for carotenoids analysis, or organic solvents (Naguib, 1998). Moreover, different probes, such as fluorescein (hydrophilic) (Zulueta et al., 2009; Müller et al., 2011) and C_{11}-$BODIPY^{581/591}$ (Naguib, 1998) (lipophilic), and different radical generators, such as 2,2'-azobis-2,4-dimethyl valeronitrile (AMVN) (lipophilic) (Woodall, Lee, Weesie, Jackson & Britton, 1997; Naguib, 1998;) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH)

(Zulueta et al., 2009; Müller et al., 2011) (hydrophilic) were used. In fact, organic solvents, such as hexane, hexane/isopropanol/tetrahydrofuran (6:5:2, *v/v/v*) and chlorobenzene, were successfully used to study the oxidative degradation of carotenoids induced by $ROO^{\bullet}$ (Woodall et al., 1997).

The most appropriated method to evaluate the capacity of carotenoids to scavenge $ROO^{\bullet}$ using a homogeneous system should: (1) be able to maintain all the reactants and the carotenoids properly dissolved during analysis and do not allow carotenoid aggregation, (2) use a probe with behaviour similar to that of lipids, (3) use a lipophilic radical generator, (4) properly assay carotenoid extracts from food matrices and (5) minimise analysis residue generation.

The semi-automated micro-assay proposed in the present work was developed to simulate, in a quite simple manner, a biological system containing an oxidable substrate (the probe - a fatty acid analogue, C_{11}-$BODIPY^{581/591}$), a reactive species ($ROO^{\bullet}$ generated by the thermodecomposition of AIBN) and the antioxidant. In this system, in the absence of the antioxidant, the $ROO^{\bullet}$ reacts with the probe generating non-fluorescent products with excitation at 540 ± 20 nm and emission at 600 ± 20 nm (Drummen, Van Liebergen, Op Den Kamp & Post, 2002), and a fast decay in the fluorescence signal is observed. When an antioxidant capable to scavenge $ROO^{\bullet}$ is added to the reaction system, the rate of fluorescence decay slows down due to the inhibition of the probe oxidation by $ROO^{\bullet}$. In addition, the relation between the structure of carotenoid standards and their potential as $ROO^{\bullet}$ scavengers was also discussed. Furthermore, the method was successfully applied to carotenoid extracts from Amazonian fruits.

2 Material and methods

2.1 Standards and reagents

Standards of all-*trans*-β-carotene (99.9 %), all-*trans*-astaxanthin (97.4 %), α-tocopherol (97.6 %), ascorbic acid (99.0 %), rutin (98.1 %), quercetin (98.5 %) and trolox (99.5 %) were purchased from Sigma-Aldrich (Missouri, USA). Gallic acid (98.0 %) was purchased from Extrasynthèse (Genay, France). All-*trans*-β-cryptoxanthin (99.5 %), all-*trans*-lutein (98.8 %), all-*trans*-zeaxanthin (97.4 %), all-*trans*-β-apo-8'-carotenal (96.1 %), all-*trans*-β-apo-10'-carotenal (99.9 %), all-*trans*-β-apo-12'-carotenal (90.8 %), all-*trans*-β-zeacarotene (98.9 %), all-*trans*-lycopene (99.9 %), all-*trans*-γ-carotene (99.9 %), 15-*cis*-β-carotene (95.3 %), 9-*cis*-β-carotene (99.9 %) and all-*trans*-α-carotene (99.7 %) were kindly donated by DSM Nutritional Products (Basel, Switzerland). All-*trans*-violaxanthin (95.0 %) was acquired from CaroteNature (Lupsingen, Switzerland). The bixin standard was isolated in our laboratory (Rios & Mercadante, 2004) and re-crystallized to achieve 98 % purity. All these compounds were used as received. The purity was determined by HPLC-DAD-MS/MS **(Supplementary Tables S1 and S2)**. The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$, MW = 504.43 g/mol) was purchased from Invitrogen (Oregon, USA). Dimethyl sulfoxide (DMSO) was acquired from Sigma-Aldrich (Missouri, USA); methanol and ethanol from Synth (São Paulo, Brazil) and methyl *tert*-butyl ether (MTBE) from J.T. Baker (New Jersey, USA), azobisisobutyronitrile (AIBN) was donated by Mig Quimica (São Paulo, SP, Brazil).

2.2 Development of the method

The micro-assay was developed based upon the method previously used by Naguib (1998) for carotenoids. In this method, the reaction mixtures in the 1-cm quartz cuvettes (final volume of 3 mL) contained the C_{11}-BODIPY$^{581/591}$ (probe), AMVN (radical generator), both in octane/butyronitrile (9:1, *v/v*), and the carotenoids in chloroform. In this new method, modifications were made in the solvent (reaction medium) in order to adapt the method to be carried out in a microplate reader in 96-well format using polystyrene microplates and to assure that the carotenoids were completely dissolved, and also in the radical generator, since AMVN is not commercially available in Latin America. Moreover, no differences were verified in the carotenoid reactivity towards $ROO^{\bullet}$ generated by thermodecomposition of either AMVN or AIBN (Woodall et al., 1997).

Five solvents were tested: octane/butyronitrile (9:1, *v/v*), methanol, ethanol, methanol/ethanol (1:1, *v/v*) and DMSO/MTBE (10:1, *v/v*). The original $ROO^{\bullet}$ generator, AMVN, was replaced by AIBN. Besides, four AIBN final concentrations in the microplate well (78, 116, 175 and 262 mM) were tested.

The assays were carried out in a microplate reader equipped with a thermostat set at 41 ºC and dual reagent dispenser (Synergy Mx, BioTek, Vermont, USA) using black polystyrene microplates (Costar, Corning, New York, USA). The fluorescence measurements were expressed as relative fluorescence, using the fluorescence signal measured after 1 min of incubation as the initial reference, and measuring each 2 min until reach 0.5 % of the initial fluorescence signal. The α-tocopherol (35 μM) was used as standard for analytical quality control and was assayed in triplicate in each microplate.

The relative $ROO^{\bullet}$ scavenging capacity was calculated as the ratio between the slope of the curve representing the sample concentration (carotenoid standard or extract) against the net area under the curve (net AUC) (S_{sample}) and the slope of the curve representing α-tocopherol or trolox concentration against the net AUC ($S_{\alpha\text{-tocopherol or trolox}}$) (Eq. 1). The area under the curve (AUC) was calculated by Eq. 2 and the net AUC was calculated by the difference between the AUC of the sample and the AUC of the blank. The blank assays were carried out replacing the sample by the same volume of DMSO/MTBE (10:1, *v/v*) for carotenoid standards and extracts or DMSO for the other compounds (gallic acid, rutin, quercetin and ascorbic acid).

$$\text{Peroxyl radical scavenging capacity} = \frac{S_{sample}}{S_{\alpha\text{-tocopherol or trolox}}} \quad (1)$$

Where:

S_{sample}= slope of the carotenoid standard or extract curve;

$S_{\alpha\text{-tocopherol or trolox}}$ = slope of α-tocopherol or trolox curve.

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + f_n/f_0 \quad (2)$$

Where:

f_0 = initial fluorescence (fluorescence signal after 1 min of incubation);

f_n = fluorescence signal at time n (time when the fluorescence signal reaches 0.5% of the initial signal).

2.3 Method Validation

Both α-tocopherol and trolox were used as standards to validate the method and the following parameters were evaluated: linearity, limits of detection (LOD) and quantification (LOQ), repeatability and recovery. Linearity was observed through the determination coefficients (R^2, $p < 0.05$) of the analytical curves with six concentrations of standard solutions (six replicates), with final concentrations in the microplate well ranging from 12 to 143 μM. The recovery analyses were carried out in three levels, with 4 replicates for each level, at the final concentrations in the microplate well of 35, 65 and 97 μM for α-tocopherol and 35, 70 and 105 μM for trolox. Repeatability was evaluated using the relative standard deviations (RSD). LOD and LOQ were calculated using the analytical curves according to Ribani, Bottoli, Collins, Jardim & Melo (2004).

2.4 Preparation of samples

The solutions of α-tocopherol, trolox, gallic acid, rutin, quercetin and ascorbic acid were prepared in DMSO, with concentrations varying from 63 to 1260 μM. Stock solutions of carotenoid standards were prepared in dichloromethane, an aliquot was evaporated under N_2 flow and redissolved in petroleum ether (all-*trans*-β-carotene, all-*trans*-β-cryptoxanthin, all-*trans*-zeaxanthin, all-*trans*-β-apo-8'-carotenal, all-*trans*-β-apo-10'-carotenal, all-*trans*-β-apo-12'-carotenal, all-*trans*-β-zeacarotene, all-*trans*-lycopene, all-*trans*-γ-carotene, 15-*cis*-β-carotene, 9-*cis*-β-carotene and all-*trans*-α-carotene), ethanol (all-*trans*-lutein), dichloromethane (bixin) or hexane (all-*trans*-astaxanthin) and their concentrations were spectrophotometrically (Agilent model 8453, Missouri, USA) determined using their

specific absorption coefficients ($A_{1cm}^{1\%}$) (Davies, 1976). Appropriate aliquots were taken from the stock solutions to prepare the working solutions in five concentrations (40, 77, 153, 248 and 310 μM), evaporated under N_2 flow, redissolved in DMSO/MTBE (10:1, *v/v*) and sonicated for 30 s. To verify if all the carotenoid crystals were completely dissolved, besides visual inspection, the UV-Vis spectra were recorded using a cuvette with optical path of 1 mm **(Supplementary Fig. S2)**.

To prepare the fruit extracts, 1 kg of mamey (*Mammea americana*) and 1 kg of palm peach (*Bactrys gasipaes*) were acquired at the "Ver-O-Peso" market located in the city of Belém, Pará State, Brazil in September 2011. The fruits were washed, peeled, cut into small pieces and immediately frozen in liquid nitrogen before lyophilisation (Liobras model K105, São Paulo, Brazil). Lyophilisation was conducted during 96 h at a temperature of -60 °C with pressure < 40 mmHg. The freeze-dried fruits were grinded into powder in a domestic food mixer (Black & Decker, Minas Gerais, Brazil), divided into portions, vacuum packed in polyethylene plastic bags (Jumbo Plus, Selovac, São Paulo, Brazil) and stored at -37 °C until analysis. The carotenoid extract was prepared from the freeze-dried pulps according to De Rosso and Mercadante (2007). Briefly, the extraction was performed with acetone, transferred with partition to petroleum ether/diethyl ether (1:1, *v/v*) and saponified overnight (~16 hours) at room temperature with 10% (w/v) methanolic KOH. After saponification, the extracts were again partitioned and the alkali was removed by washing with water, and then the solvent was evaporated in a rotary evaporator (T < 38 °C). The dried extracts were stored under nitrogen atmosphere (99.9 % purity), protected against the luminosity at -37 °C until analysis. The dried extracts were dissolved in petroleum ether (stock solutions) and the total carotenoid concentrations were

spectrophotometrically determined using the specific absorption coefficients ($A_{1cm}^{1\%}$=2396) of all-*trans*-β-carotene (Davies, 1976). To determine the carotenoid composition, an aliquot of the stock solution was evaporated under N_2 flow, redissolved in MeOH/MTBE (70:30, *v/v*) and analyzed by HPLC-DAD-MS/MS (De Rosso & Mercadante, 2007). To determine the $ROO^{\bullet}$ scavenging capacity, appropriate aliquots were taken from the stock solutions to prepare the working solutions in five concentrations (40, 77, 153, 248 and 310 μM), evaporated under N_2 flow, redissolved in DMSO/MTBE (10:1, *v/v*) and sonicated for 30 s. To verify if all the carotenoid extract was completely dissolved, besides visual inspection, the UV-vis spectra were recorded using a cuvette with optical path of 1 mm **(Supplementary Fig. S2)**.

2.5 *HPLC-DAD-MS/MS* analysis of carotenoids

The carotenoid composition of both extracts of Amazonian fruits (mamey and peach palm) were determined in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), on-line degasser and a Rheodyne (Rheodyne LCC, Robert Park, CA, USA) injection valve with a 20 μl loop, connected in series to a DAD detector (model SPD-M20A) and a mass spectrometer with an ion trap analyser and atmospheric pressure chemical ionisation (APCI) source (Bruker Daltonics, model Esquire 4000, Bremen, Germany). The carotenoids were separated on a C_{30} YMC column (5 μm, 250 mm x 4.6 mm) using a linear gradient of MeOH/MTBE as the mobile phase from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min, according to the procedure previously described by De Rosso and Mercadante (2007) for carotenoids from Amazonian fruits.

The carotenoids were quantified by HPLC-DAD, using external seven-point analytical curves (in duplicate) for all-*trans*-violaxanthin (0.7-13.6 μg/mL), all-*trans*-lutein (1.0-59.5 μg/mL) and all-*trans*-β-carotene (1.1-30.2 μg/mL). For all the 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.

2.6 Statistical analysis

The results were submitted to analyses of variance using one-way ANOVA and the means were classified by Tukey's test at the level of 95 % of significance. The analytical curves were plotted by linear regression ($p < 0.05$). The Software Origin® 8 was used for all calculations.

3 Results

3.1 Method development

The solvent choice to determine the antioxidant capacity of lipophilic compounds, especially carotenoids, is a critical factor since three basic characteristics are required: allow the complete dissolution of the reagents and carotenoids, do not react with the microplate material (polystyrene) and do not evaporate under the temperature used for radical generation. The last characteristic is extremely relevant, especially to microplate reader assays, which constitute open systems.

The microplates used in fluorescent assays are made of polystyrene, thus the following solvents were not considered due to chemical incompatibility: acetone, acetonitrile, benzene, chlorobenzene, chloroform, cyclohexane, diethyl ether, hexane, ethyl

acetate and petroleum ether (http://www.perkinelmer.com/CMSResources/Images/44-73405GDE_MicroplateChemicalCompatibility.pdf). Among the five tested solvents (octane/butyronitrile (9:1, *v/v*) (Naguib, 2000), methanol (Brand-Williams, Cuvelier & Berset, 1995), ethanol (Re et al., 1999), methanol/ethanol (1:1, *v/v*) and DMSO/MTBE (10:1, *v/v*), the only appropriate solvent was DMSO/MTBE (10:1, *v/v*) which presented all the desired characteristics. The use of other ratios of DMSO/MTBE was disregarded due to solvent evaporation observed during analysis. Since octane/butyronitrile (9:1, *v/v*) reacted with the microplate and the alcohols evaporated during analysis, they were considered inappropriate (data not shown). The solvent interaction with the microplate material was verified by the fast decrease of the fluorescence signal in the control assay (probe without AIBN addition), the solvent evaporation was both visually observed (dry wells) and an increase of the fluorescence signal over time in the control assay also occurred.

In general, the carotenoids were completely dissolved in DMSO/MTBE (10:1, *v/v*) until the maximum concentration of 310 μM. Exceptions were noticed for all-*trans*-β-carotene, all-*trans*-α-carotene and all-*trans*-lycopene, which only dissolved completely at this concentration first with the addition of 100, 200 and 400 μL of MTBE, respectively, followed by 900 μL of DMSO/MTBE (10:1, *v/v*) and sonication for 2 min. The UV-visible absorption spectra **(Supplementary Fig. S2)** of the all carotenoid solutions showed no evidence of carotenoid aggregation, since no dramatic changes in their absorption spectra were observed, e.g., shift to shorter wavelengths (blue shift) or shift to longer wavelengths (red shift) of the absorption spectrum (Köhn et al., 2008).

The $ROO^{\bullet}$ scavenging capacity was measured by monitoring the effect of the samples on the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of the C_{11}-

BODIPY$^{581/591}$. The thermodecomposition of AIBN follows a homolytic scission mechanism that has been extensively investigated, generating N_2 and carbon-centered radicals (Hammond, Sen & Boozer, 1955; Bevington, Fridd & Tabner, 1982). In the presence of oxygen, these radicals are rapidly converted to ROO$^{\bullet}$. In the range of the four AIBN concentrations tested, an inverse linear relationship was observed between AIBN concentrations and AUC when the experiment was conducted at 41 ºC in the absence of antioxidants (blank assay) (Fig. 2). The AIBN concentration of 175 mM was chosen in order to achieve 0.5 % of the initial fluorescence signal in about 60 min in the blank assay. The C_{11}-BODIPY$^{581/591}$ remained stable during the whole analysis time (~120 min) when no AIBN was added (Fig. 2); showing that the fluorescence decay is exclusively due to the probe oxidation by ROO$^{\bullet}$ generated during the thermodecomposition of AIBN and not provided by its thermal or photodegradation.

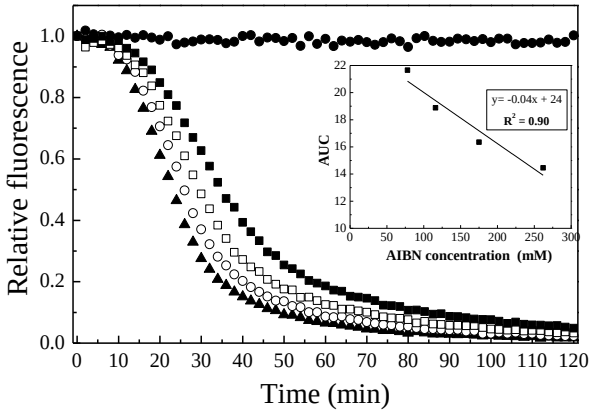

Fig. 2 Fluorescence decay of C_{11}-BODIPY$^{581/591}$ induced by peroxyl radical generated from thermodecomposition of different concentrations of AIBN at 41 °C: no AIBN (*filled circle*), 78 mM (*filled square*), 116 mM (*open square*), 175 mM (*open circle*), 262 mM (*filled triangle*). **Inset:** relationship between AIBN concentrations and AUC values from the fluorescence decay curves of C_{11}-BODIPY$^{581/591}$ oxidation.

The methodology was established so the reaction mixtures in the wells contained the following reagents: 100 μL of 0.4 μM C_{11}-BODIPY$^{581/591}$ in DMSO, 100 μL of 394 mM AIBN in DMSO/MTBE (10:1, *v/v*) and 25 μL of the sample (five different concentrations) either in DMSO or DMSO/MTBE (10:1, *v/v*). The assays were carried out at 41 ºC and fluorescence was measured until reached 0.5 % of the initial signal.

3.2 Method validation

The method validation was carried out using two standard compounds, α-tocopherol and trolox, which are known to possess good antioxidant capacity and possess the same basic structure, but different polarities. The α-tocopherol is a lipophilic compound, whilst trolox is its hydrophilic analogue, in which the long alkyl chain of α-tocopherol was replaced by a carboxyl group (Fig. 1). The linearity between the antioxidant concentration and the net AUC presented a determination coefficient (R^2) higher than 0.99 ($p < 0.05$), within the range of the tested concentrations, for both α-tocopherol and trolox (Fig. 3). The LOD and LOQ of α-tocopherol were 9 and 26 μM, respectively, which were lower than those of trolox, 11 and 35 μM, respectively (Table 1). In the three concentration levels, for both standards, recovery was close to 100 % and the repeatability, measure as RSD, was within ± 15 % (Table 1). The method performance was compared with the Oxygen Radical Absorbance Capacity (ORAC) (Ou, Hampsch-Woodill & Prior, 2001) method validated in our laboratory (**Supplementary Table S4**).

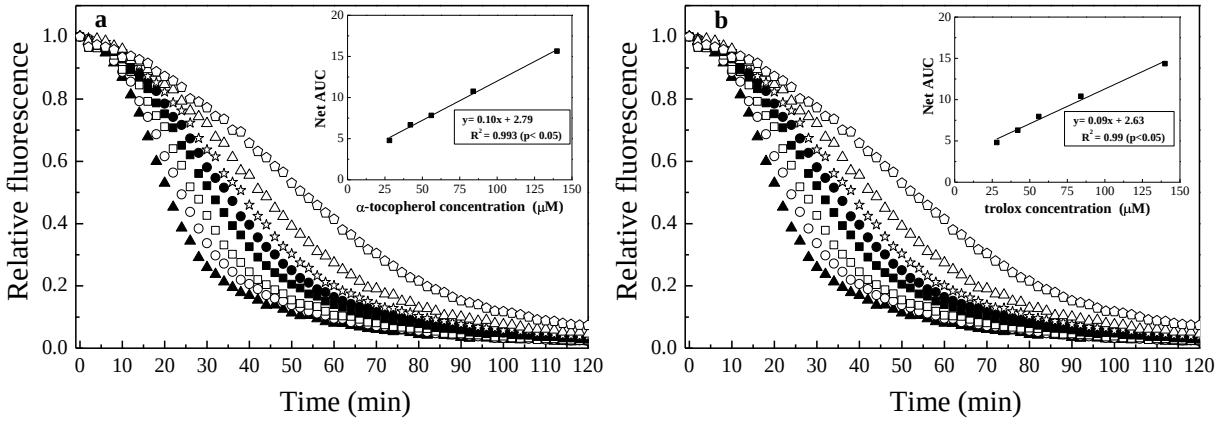

Fig. 3 Fluorescence decay of C_{11}-BODIPY$^{581/591}$ induced by peroxyl radicals in the presence of different concentrations of **(a)** α-tocopherol and **(b)** trolox. Legend: blank (*filled triangle*), 7 μM (*open circle*), 14 μM (*open square*), 28 μM (*filled square*), 42 μM (*filled circle*), 56 μM (*open star*), 84 μM (*open triangle*) and 140 μM (*open hexagon*). **Inset:** linear relationship between (a) α-tocopherol or (b) trolox concentrations and net AUC values from the fluorescence decay curves of C_{11}-BODIPY$^{581/591}$ oxidation.

Table 1. Parameters of validation of the micro-assay for carotenoid ROO• scavenging capacity

Parameter		α-tocopherol	trolox
linearity range (μM)		12 - 119	14 - 143
slope [a]		0.10 (4) [g]	0.09 (3)
intercept [a]		2.79 (42)	2.63 (8)
R^2 [a]		0.993	0.992
LOD (μM) [b]		9	11
LOQ (μM) [c]		26	35
recovery (%)	level 1 [d]	110 (4)	105 (10)
	level 2 [e]	99 (10)	106 (8)
	level 3 [f]	99 (9)	98 (3)
repeatability (%)	level 1	4	11
	level 2	12	8
	level 3	6	4

[a] For each analytical curve, the equation y = ax + b was applied, where y = net area under curve (AUC), x = final concentration of compounds, a = slope, b = intercept and R^2 = determination coefficient ($p<0.05$). [b]LOD = limit of detection. [c]LOQ = limit of quantification. [d]35 μM. [e]65 μM of α-tocopherol and 70 μM of trolox. [f]97 μM of α-tocopherol and 105 μM of trolox. [g] Relative standard deviation (RSD, %) are given in parentheses (slope and intercept: n = 6; recovery: n=4).

3.3 Carotenoid $ROO^{\bullet}$ scavenging capacity and the structure-activity relationship

Table 2 shows the capacity to scavenge $ROO^{\bullet}$ of 15 carotenoids, as well as of other compounds known to possess antioxidant capacity, such as α-tocopherol, trolox, ascorbic acid, gallic acid, rutin and quercetin. All the carotenoids presented higher capacity to scavenge $ROO^{\bullet}$ (4-20 times superior) than the other tested compounds, and all-*trans*-lycopene was the most efficient $ROO^{\bullet}$ scavenger. The efficiency of the carotenoids as $ROO^{\bullet}$ scavengers varied considerably according to their chemical structures and was influenced by the type of terminal group, *cis-trans* isomer configuration, presence of oxygen substituents and chromophore extension.

Table 2. ROO• scavenging capacity of carotenoids and other analysed compounds

Compound	end ring	Number			Value[A]	
		c.d.b[B]	OH groups	CO groups	α-tocopherol relative	trolox relative
all-*trans*-lycopene	0	11	0	0	8.67 ± 0.74 [a]	9.63 ± 0.82 [a]
all-*trans*-astaxanthin	2β	13	2	2	6.50 ± 0.62 [b]	7.22 ± 0.69 [b]
all-*trans*-γ-carotene	2β	11	0	0	6.07 ± 0.35 [b]	6.74 ± 0.35 [b]
bixin	0	11	1	2	5.20 ± 0.15 [c]	5.80 ± 0.15 [c]
ll-*trans*-apo-10'-carotenal	1β	9	0	1	5.03 ± 0.21 [c]	5.59 ± 0.23 [c]
ıll-*trans*-apo-8'-carotenal	1β	10	0	1	4.90 ± 0.14 [c]	5.44 ± 0.16 [c]
all-*trans*-zeaxanthin	2β	11	2	0	3.52 ± 0.39 [d]	3.91 ± 0.43 [d]
all-*trans*-β-carotene	2β	11	0	0	3.24 ± 0.22 [e]	3.60 ± 0.24 [e]
ıll-*trans*-β-cryptoxanthin	2β	11	1	0	2.85 ± 0.29 [ef]	3.17 ± 0.33 [ef]
9-*cis*-β-carotene	2β	11	0	0	2.65 ± 0.05 [f]	2.94 ± 0.06 [f]
15-*cis*-β-carotene	2β	11	0	0	2.67 ± 0.21 [f]	2.96 ± 0.23 [f]
all-*trans*-α-carotene	1β, 1ε	10	0	0	2.62 ± 0.24 [fg]	2.91 ± 0.27 [fg]
all-*trans*-lutein	1β, 1ε	10	1	0	1.90 ± 0.17 [g]	2.11 ± 0.19 [g]
ll-*trans*-apo-12'-carotenal	1β	8	0	1	1.56 ± 0.19 [g]	1.73 ± 0.22 [g]
all-*trans*-β-zeacarotene	1β	9	0	0	1.52 ± 0.10 [g]	1.69 ± 0.11 [g]
α-tocopherol	-	3	1	0	1 [h]	1.11 ± 0.00 [h]
trolox	-	3	1	1	0.90 ± 0.00 [h]	1 [h]
ascorbic acid	-	2	2	0	0.87 ± 0.06 [h]	0.96 ± 0.06 [h]
quercetin	-	6	7	1	0.63 ± 0.01 [i]	0.69 ± 0.01 [i]
gallic acid	-	3	3	1	0.54 ± 0.05 [i]	0.60 ± 0.05 [i]
rutin	-	6	10	1	0.39 ± 0.01 [j]	0.43 ± 0.01 [j]

[A]Calculated using equation 1. Mean ± standard deviation (n= 3). Means with the different superscript letters at the same column are significantly different ($p<0.05$).

[B]c.d.b = conjugated double bonds.

The opening of the β-ionone ring in the carotenoid structure resulted in an increase of $ROO^{\bullet}$ scavenging capacity. All-*trans*-lycopene has a linear chain and was 1.4 and 2.7-fold more potent than all-*trans*-γ-carotene (1 β-ionone ring) and all-*trans*-β-carotene (2 β-ionone rings), respectively.

Cis isomers were less efficient as $ROO^{\bullet}$ scavengers than the corresponding *trans* isomers. The effect of *cis*-*trans* isomerization can be observed by the comparison among the antioxidant capacity of three β-carotene isomers, all-*trans*, 9-*cis* and 15-*cis*-β-carotene. The *cis* isomers presented values about 20 % lower than that found for the all-*trans*-β-carotene.

The influence of the addition of oxygenated functional groups, such as hydroxyl (OH) and keto (CO) groups, in the terminal ring on the capacity to scavenge $ROO^{\bullet}$ depended on the number, type of functional groups and if it is part or not of the chromophore. Comparing three carotenoids with the same chromophore structure, i.e. all-*trans*-β-carotene (no oxygen substituent), all-*trans*-β-cryptoxanthin (1 OH) and all-*trans*-zeaxanthin (2 OH), the addition of one OH did not affect ($p>0.05$) the scavenging capacity; however, when 2 OH were added, there was an increase of less than 10 % in relation to that of all-*trans*-β-carotene. On the other hand, the addition of two OH to all-*trans*-α-carotene structure, originating lutein (same chromophore), did not result in any change ($p>0.05$) in the $ROO^{\bullet}$ scavenging capacity. In all the above examples, the addition of OH did not change the chromophore. All-*trans*-astaxanthin (2OH, 2CO), originated by the addition of two OH and two CO groups to the all-*trans*-β-carotene structure, was 100 % more effective than all-*trans*-β-carotene. If only the addition of the CO groups is considered, comparing

all-*trans*-astaxanthin and all-*trans*-zeaxanthin, the increase was about 85 %, since these groups are part of the molecule chromophore.

The reduction in the chromophore extension is related to the decrease of the capacity to scavenge $ROO^{\bullet}$. The decrease in the scavenging capacity became more evident, between 70 and 75 %, when four hydrogen atoms were introduced in the carotenoid structure, with the consequent decrease of two c.d.b., for instance, comparing the pairs all-*trans*-γ-carotene (11 c.d.b.) with all-*trans*-β-zeacarotene (9 c.d.b.), and all-*trans*-apo-8'-carotenal (10 c.d.b.) with all-*trans*-apo-12'-carotenal (8 c.d.b.). However, the loss of one c.d.b. resulted in a low (20%) or none ($p > 0.05$) decrease in the scavenging capacity, as can be observed when comparing all-*trans*-β-carotene (11 c.d.b.) with all-*trans*-α-carotene (10 c.d.b.), and all-*trans*-β-apo-8'-carotenal with all-*trans*-β-apo-10'-carotenal (9 c.d.b.). The most pronounced reduction in the scavenging capacity (46 %) was noticed when comparing all-*trans*-zeaxanthin (11 c.d.b.) and all-*trans*-lutein (10 c.d.b.).

Interestingly, the $ROO^{\bullet}$ scavenging capacity values of the apocarotenoids, all-*trans*-β-apo-8'-carotenal, all-*trans*-β-apo-10'-carotenal and bixin, were all high, but did not differ among themselves ($p > 0.05$) despite the different number of c.d.b.. Their scavenging capacities were was just lower than those of all-*trans*-lycopene, all-*trans*-astaxanthin and all-*trans*-γ-carotene.

3.4 Scavenging capacity of carotenoid extracts from Amazonian fruits

The extracts from mamey presented higher carotenoid concentration (318.03 ± 25.90 μg/g pulp) than peach palm extract (52.50 ± 2.22 μg/g pulp). The carotenoid composition of these extracts was determined by HPLC-DAD-MS/MS (**Supplementary**

Table S3 and Fig. S1) and the carotenoid profiles were very similar to those found by our research group in a previous study (De Rosso & Mercadante, 2007). Considering that a detailed description of carotenoid identification was already reported (De Rosso & Mercadante, 2007), the HPLC-DAD-MS/MS will not be discussed in the present study. The major carotenoids found in mamey extract were all-*trans*-violaxanthin (24 %), 9-*cis*-violaxanthin (17 %) and *cis*-β-apo-8'-carotenal (20 %); whilst all-*trans*-β-carotene (54 %) was the major one in peach palm.

The net AUC obtained for the carotenoid extracts from both peach palm and mamey was linearly dependent on the carotenoid concentration ($R^2 > 0.99$, $p < 0.05$) (Fig. 4). The value of the $ROO^{\bullet}$ scavenging capacity found for the carotenoid extract from peach palm was 7.83 ± 0.21 (α-tocopherol relative), which was slightly superior to that found for the mamey extract (6.90 ± 0.44, α-tocopherol relative). These values are lower than the values found for the $ROO^{\bullet}$ scavenging capacity of all-*trans*-lycopene and higher than the other carotenoid standards assayed and also higher than α-tocopherol, trolox, ascorbic acid, gallic acid, rutin and quercetin (Table 2).

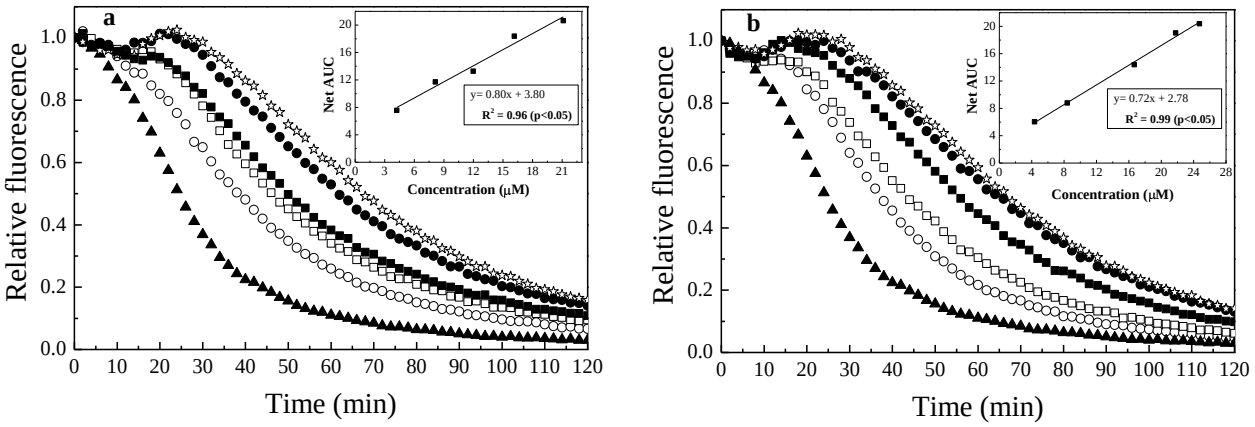

Fig. 4 Fluorescence decay of C_{11}-BODIPY$^{581/591}$ induced by peroxyl radicals in the presence of different concentrations of carotenoid extracts from **(a)** palm peach (*Bactrys gasipaes*): blank (*filled triangle*), 4 μM (*open circle*), 8 μM (open square), 12 μM (*filled square*), 16 μM (*filled circle*) and 21 μM (*open star*) and **(b)** mamey (*Mammea americana*): blank (*filled triangle*), 4 μM (*open circle*), 8 μM (*open square*), 17 μM (*filled square*), 22 μM (*filled circle*) and 25 μM (*open star*). **Inset:** linear relationship between the carotenoid concentrations of (a) peach palm or (b) mamey extract and net AUC values from the fluorescence decay curves of C_{11}-BODIPY$^{581/591}$ oxidation.

4 Discussion

The method proposed in the present study was developed considering the most recent, comprehensive and well accepted definition of antioxidant proposed by Halliwell and Gutteridge (2007): "antioxidant is any substance that delays, prevents or removes oxidative damage to a target molecule". Morover, the developed method presented all the desired characteristics of an appropriated method to determine the antioxidant capacity of carotenoids in homogeneous systems: (i) it is able to maintain all the reactants and the carotenoids completely dissolved during the whole analysis, not allowing carotenoid aggregation; (ii) it uses C_{11}-BODIPY$^{581/591}$, which is a fatty acid analogue, as fluorescent probe; (iii) it uses AIBN as ROO$^{\bullet}$ lipophilic generator; (iv) it can be successfully applied to

carotenoid extracts from food matrices; and (v) it uses a reduced volume of reagents and solvents (approximately 13 times less than in traditional methods using 3 mL cuvettes), consequently reducing the residue generation and analysis cost, since it is a micro-assay using 96-well format microplates. The validation parameters of the new method, using both trolox and α-tocopherol as standard, were similar to the results obtained in the in-house validation of the classical ORAC method using trolox as standard **(Supplementary Table S4)**. The validation data demonstrated that this new micro-assay provides good linearity, accuracy and repeatability within the acceptable criteria as compared to the classical method to determine the antioxidant capacity of hydrophilic compounds, the original ORAC method (Ou et al., 2001).

The scavenging of peroxyl radicals are of particular interest because of the prominent role of such radicals in lipid peroxidation. The lipid peroxidation occurs by a chain reaction mechanism during which $ROO^{\bullet}$ is generated. Chain-breaking antioxidants can interrupt these reactions by scavenging the lipid peroxyl radicals. The most known chain-breaking antioxidants are α-tocopherol and phenolic compounds, which scavenge lipid peroxyl radicals by donating a phenolic hydrogen atom, forming a lipid hydroperoxide and a resonance-stabilised antioxidant radical (Ou et al., 2001; Müller, Theile & Böhm, 2010). The main compounds formed by the reaction of phenolic compounds and $ROO^{\bullet}$ are dimers, trimers and even oligomers (Roche, Dufour, Mora & Dangles, 2005).

Carotenoids, which are known to be efficient singlet oxygen quenchers (Di Mascio et al., 1989), can also scavenge $ROO^{\bullet}$ by three main mechanisms: electron transfer (equation 3), allylic hydrogen abstraction (equation 4) and radical addition to the c.d.b. system (equation 5) (El-Agamey et al., 2004; Jomová et al., 2009). The occurrence of one

or another mechanism depends on the characteristics of the reactional system (homogeneous or multi-phase), solvent polarity and carotenoid structure (El-Agamey et al., 2004; Guo & Hu, 2010). The main reaction products formed by oxidation of all-*trans*-β-carotene induced by $ROO^{\bullet}$ (generated by thermodecomposition of AIBN) were apocarotenoids (β-apo-13-carotenone, β-apo-15-carotenal, β-apo-14'-carotenal, β-apo-12'-carotenal, β-apo-10'-carotenal), epoxycarotenoids (β-carotene-5,6-epoxide, β-carotene-5,8-epoxide, β-carotene-5,6,5',6'-diepoxide, β-carotene-15,15'-epoxide) and *cis*-β-carotene (Stratton, Schaefer & Liebler, 1993; Handelman, Van Kuijk, Chatyerjee & Krinsky, 1991; El-Tinay & Chichester, 1970).

$$CAR + ROO^{\bullet} \rightarrow CAR^{\bullet+} + ROO^{-} \text{ (electron transfer)} \quad (3)$$

$$CAR + ROO^{\bullet} \rightarrow CAR^{\bullet} + ROOH \text{ (hydrogen abstraction)} \quad (4)$$

$$CAR + ROO^{\bullet} \rightarrow ROO\text{-}CAR^{\bullet} \text{ (addition)} \quad (5)$$

All-*trans*-lycopene was the most efficient $ROO^{\bullet}$ scavenger among the 15 studied carotenoids, followed by all-*trans*-astaxanthin, all-*trans*-γ-carotene and bixin. All-*trans*-lycopene was also previously found to be the most effective $ROO^{\bullet}$ scavenger among 7 carotenoids in a kinetic study (Woodall et al., 1997). However, all-*trans*-lycopene presented low capacity to scavenge $ROO^{\bullet}$, being less effective than all-*trans*-β-carotene, all-*trans*-α-carotene and all-*trans*-lutein in other studies (Naguib, 2000; Müller et al., 2011). Bixin and all-*trans*-astaxanthin have been previously reported to be potent $ROO^{\bullet}$ scavengers (Naguib, 2000; Müller et al., 2011) and no data on $ROO^{\bullet}$ scavenging capacity of all-*trans*-γ-carotene was found in the literature. In our study, all-*trans*-β-carotene was

around 1.2-fold better $ROO^{\bullet}$ scavenger than all-*trans*-α-carotene; however, Naguib (1998) found that all-*trans*-α-carotene presented a scavenging capacity around twice as higher than all-*trans*-β-carotene.

The conflicting results are possibly related to the different solvents employed, such as DMSO/MTBE (10:1, *v/v*) in the present study, octane/butyronitrile (9:1, *v/v*) (Naguib, 1998; Naguib, 2000) and borate buffer/DMSO (9:1, *v/v*) (Müller et al., 2011), which influenced the carotenoid solubility and also the mechanism of $ROO^{\bullet}$ scavenging. Although DMSO/MTBE (10:1, *v/v*) is an organic solvent system more polar than octane/butyronitrile (9:1, *v/v*), both of them are capable to completely dissolve the carotenoids; however, using the first solvent, the carotenoids can act by the three antioxidant mechanisms and using the second, electron transfer does not occur, since it is thermodynamically infeasible because of the non-polar medium, which will not support charge separation (El-Agamey et al., 2004). The borate buffer/DMSO (9:1, *v/v*) constitute a hydrated polar solvent mixture and it is well known that carotenoids present low solubility in this kind of system. Moreover, even the dissolved carotenoids will present a high tendency to form aggregates in this system, which will dramatically change their properties (Köhn et al., 2008).

Interestingly, the carotenoids showed to be more effective $ROO^{\bullet}$ scavengers than α-tocopherol and the hydrophilic compounds, such as trolox, ascorbic acid, gallic acid, rutin and quercetin. This result could be attributed probably to the organic reaction medium used in the developed method in comparison to the hydrophilic buffered systems usually used in the other methods described in the literature (Ou et al., 2001), which favours the action of hydrophilic compounds due to the formation of the phenolate anion at pH values around 7 (Amorati, Pedulli, Cabrini, Zambonin & Landi, 2006). These methods probably have

underestimated the capacity of the carotenoids to scavenge ROO•, since the carotenoids are not totally dissolved in the system and could be in suspension or as aggregates.

Considering the relationship of the carotenoid structure and the capacity to scavenge ROO•, the positive effect observed due to the opening of the β-ionone ring can be attributed to the increase of the carotenoid reactivity to ROO•, since the reaction rate constant (*k*) for the reaction between the carotenoid and ROO• was reported to be 0.41 μM/min for all-*trans*-β-carotene and 0.58 μM/min for all-*trans*-lycopene (Woodall et al., 1997). The high reactivity of all-*trans*-lycopene is related to the easy addition of ROO• to its long system of c.d.b. and mainly to the facility to donate the allylic hydrogen at C-4 since the hydrogen abstraction at other positions is energetically unfavored (Simic, 1992). Although all-*trans*-β-carotene, all-*trans*-γ-carotene and all-*trans*-lycopene possess 11 c.d.b, in the first two carotenoids, the c.d.b. at the β-ionone ring are not coplanar to the polyenic chain, decreasing the orbital overlay and, consequently, decreasing the ability of delocalization of unpaired electrons formed at C-4 position. On the other hand, in lycopene, all the C=C bonds are coplanar, allowing that the unpaired electrons formed at C-4 to be readily delocalized (Woodall et al., 1997). Similarly, a negative effect was observed in relation to the isomerization of all-*trans*-β-carotene to 9-*cis* and 13-*cis*-β-carotene, possibly due to the decreasing of the orbital overlap, resulting in sterical hindrance.

The capacity of three carotenoids with the same chromophore, i.e. all-*trans*-β-carotene (no oxygen substituent), all-*trans*-β-cryptoxanthin (1 OH) and all-*trans*-zeaxanthin (2 OH), to scavenge ROO• is expected to be very close if electron donation and radical addition to the c.d.b. system are considered as the main antioxidant mechanisms. In

the present study, all-*trans*-zeaxanthin showed to be 10% more potent than all-*trans*-β-carotene and all-*trans*-β-cryptoxanthin, suggesting that another mechanism could be involved in the $ROO^{\bullet}$ scavenging (Woodall et al., 1997), possibly by the hydrogen donation from the hydroxyl group.

The extension of the chromophore seems to be one of the most important factors affecting the carotenoid $ROO^{\bullet}$ scavenging capacity. The increase in the c.d.b. system results in an increase of the scavenging capacity, generally around 20% for the addition of 1 c.d.b. and around 70 % for the addition of 2 c.d.b. The three antioxidant mechanisms (Eqs. 3-5) are thermodynamically favored by the increase of the c.d.b. system, since the resulting reaction products are more stable (El-Agamey et al., 2004). The increase in the scavenging capacity observed after the addition of two CO groups to all-*trans*-zeaxanthin, generating all-*trans*-astaxanthin, is related to the extension of the chromophore due to the two free electron pairs, which might contribute to the electron resonance system of c.d.b. (Di Mascio et al., 1989). In addition, the activation of the hydroxyl groups, due to a balance between the keto and enol forms of all-*trans*-astaxanthin, possible occurs. Such balance would result in the formation of an ortho-dihydroxy-conjugate polyene system acting as a chain breaking antioxidant in a similar way to α-tocopherol (Naguib, 2000).

The influence of the carotenoid structure on the antioxidant capacity, considering $ABTS^{\bullet+}$ scavenging and $^1O_2$ quenching, presents some trends similar to that of $ROO^{\bullet}$ scavenging. In general, the opening of the β-ionone ring from all-*trans*-β-carotene and the increase of the chromophore extension have a positive effect on the capacity to scavenge the three reactive species, $ROO^{\bullet}$, $ABTS^{\bullet+}$ and $^1O_2$ (Di Mascio et al., 1989; Miller et al., 1996). The addition of hydroxyl groups resulted in a positive effect on the $ROO^{\bullet}$

scavenging capacity; however, the effect was almost negligible for $ABTS^{\bullet+}$ and $^1O_2$. A positive effect was observed by the addition of 2 OH and of 2 CO for $ROO^{\bullet}$ scavenging and $^1O_2$ quenching capacities; whilst a negative effect occurred for $ABTS^{\bullet+}$. No differences were observed between the capacity of c*is* and *trans* carotenoid isomers to scavenge $ABTS^{\bullet+}$ (Bohm, Puspitasari-Nienaber, Ferruzzi & Schwartz, 2002). However, the *cis* isomers showed a slightly lower capacity to quench $^1O_2$ (Conn, Schalch, & Truscott, 1991) and to scavenge $ROO^{\bullet}$ than the *trans* isomers.

Finally, the method was successfully applied to assay the $ROO^{\bullet}$ scavenging capacity of carotenoid extracts from food matrices, such as fruits. There is a lack of studies evaluating the actual isolated contribution of the carotenoids on the antioxidant activity of foods, since generally the results presented in the literature deal with extracts also containing phenolic compounds and apply methods appropriate to hydrophilic compounds. In this sense, no comparisons to previous data reported in the literature were made.

5 Conclusion

A semi-automated micro-assay, using a microplate reader in 96-well format, to determine the capacity of carotenoids to scavenge $ROO^{\bullet}$ was successfully developed and validated. A marked relationship between carotenoid structure and the capacity to scavenge $ROO^{\bullet}$ was observed. The opening of the β-ionone ring and the chromophore extension seemed to be the factors with major impact on the $ROO^{\bullet}$ scavenging capacity. The addition of oxygenated functional groups exerted a less pronounced effect when such groups were not conjugated to the chromophore, such as the addition of hydroxyl groups; however, when the groups were part of the chromophore, increasing the number of c.d.b. such as the

keto groups in all-*trans*-astaxanthin, the increase in the scavenging capacity was quite significant. Moreover, the method was successfully applied to carotenoid extracts from Amazonian fruits.

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Appendix A Supplementary data

Supplementary Table S1. Chromatographic, UV-Vis, and mass spectroscopy characteristics of carotenoid standards.

Supplementary Table S2. Chromatographic, UV-Vis, and mass spectroscopy characteristics of the α-tocopherol, trolox and phenolic standards.

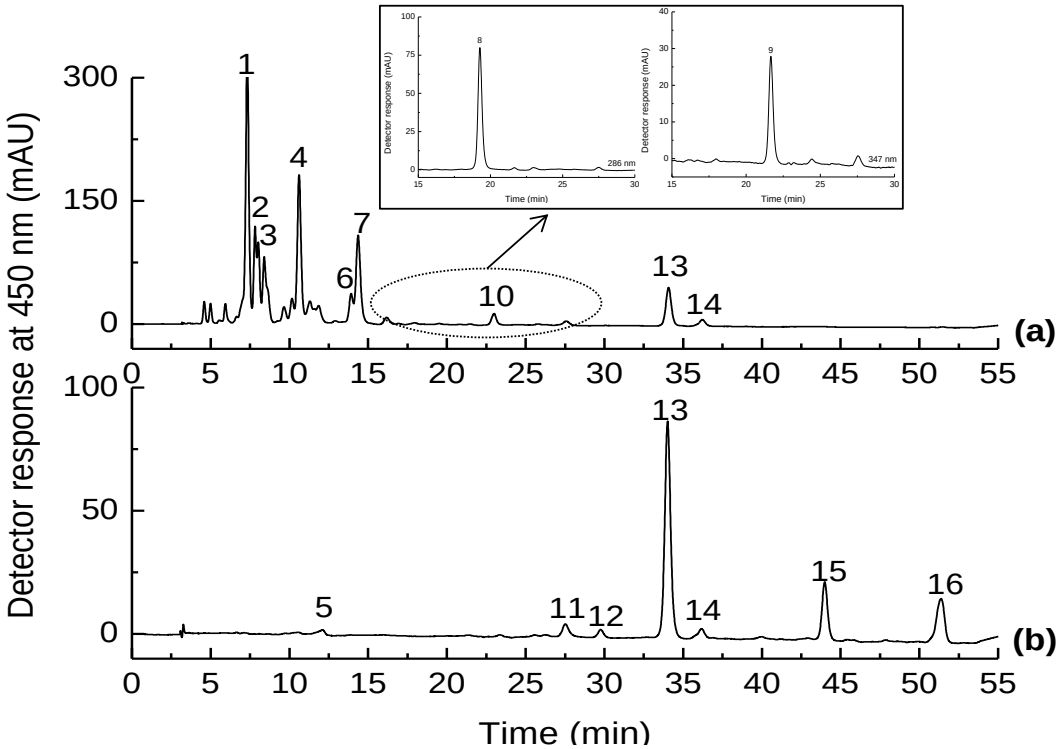
Supplementary Table S3. Chromatographic, UV-Vis, mass spectroscopy characteristics and content (dry basis) of carotenoids from mamey and palm peach extracts, obtained by HPLC-DAD-APCI-MS/MS.

Supplementary Table S4. In-house **v**alidation parameters of the Oxygen Radical Absorbance Capacity (ORAC) method.

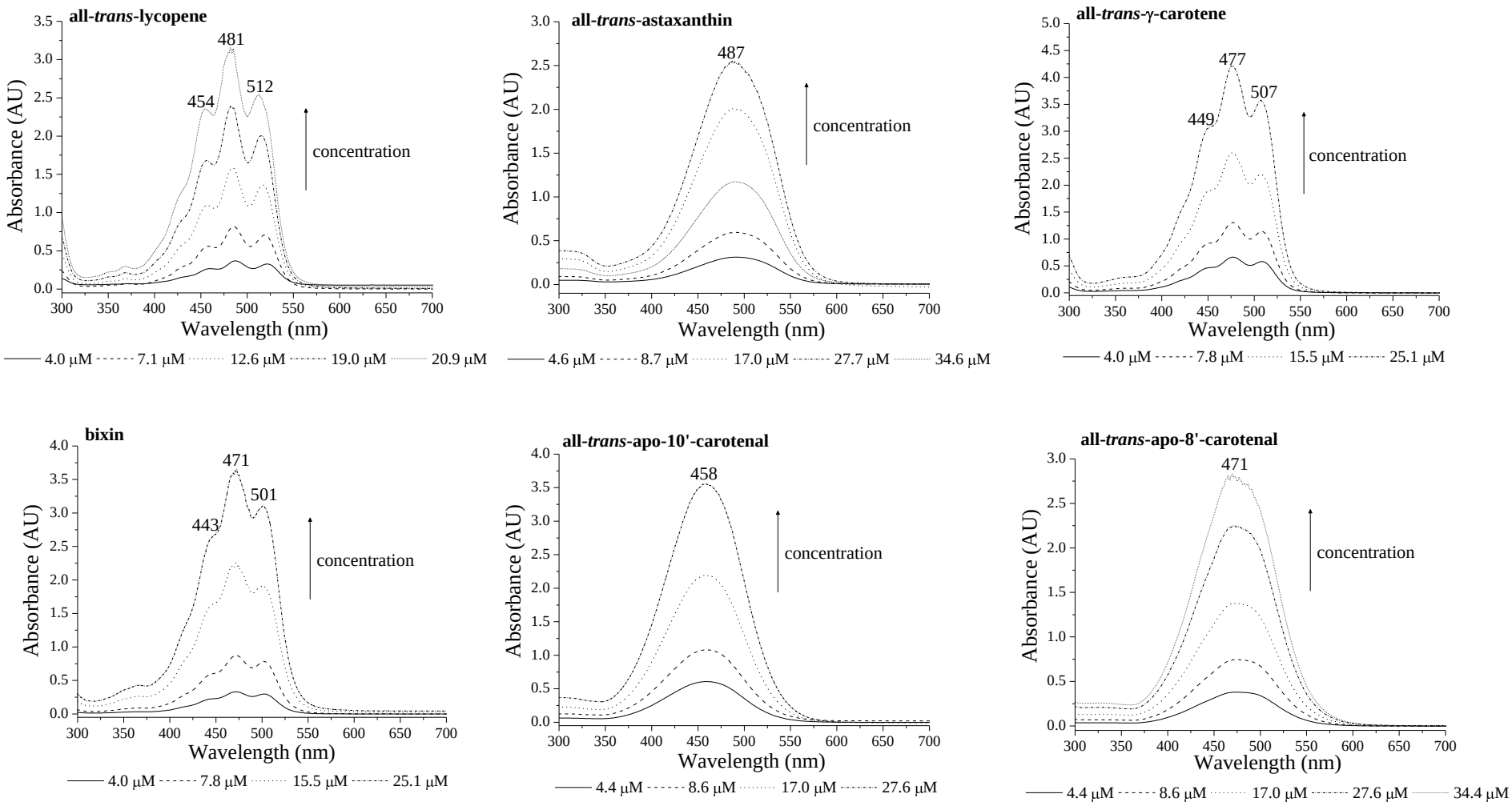
Supplementary Fig. S1. Chromatogram obtained by HPLC–DAD of carotenoids from (a) mamey and (b) peach palm. Chromatographic conditions: see text. Peak characterization is given in **Table S3**.

Supplementary Fig. S2. UV-vis spectra of the carotenoid standards and extracts in DMSO/MTBE (10:1, *v/v*).

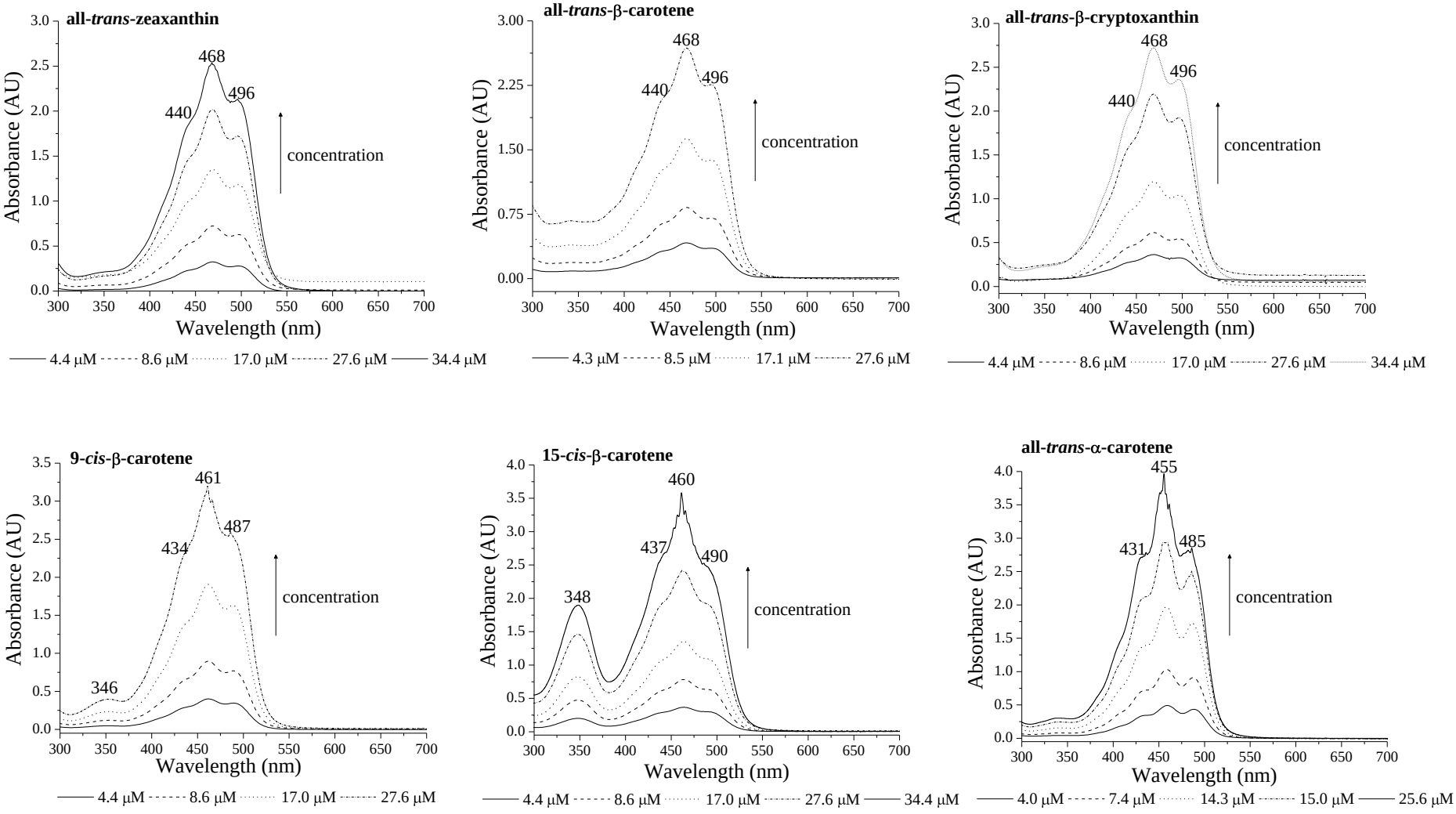
Supplementary Figure S1 Chromatogram obtained by HPLC–DAD of carotenoids from (a) mamey and (b) peach palm. Chromatographic conditions: see text. Peak characterization is given in **Supplementary Table S3**.



Supplementary Figure S2 UV-vis spectra of the carotenoid standards and extracts in DMSO/MTBE (10:1, *v/v*).



Supplementary Figure S2 Continued



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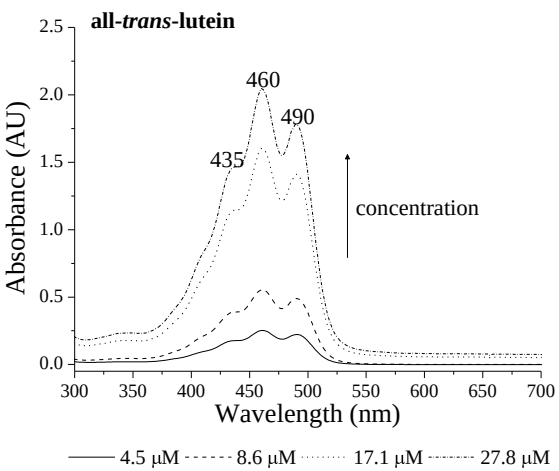

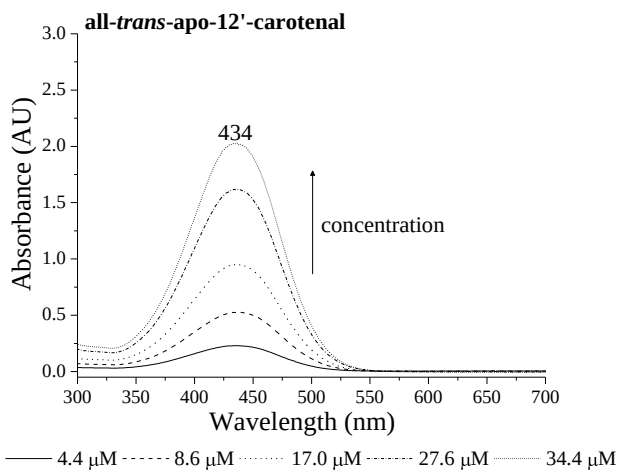

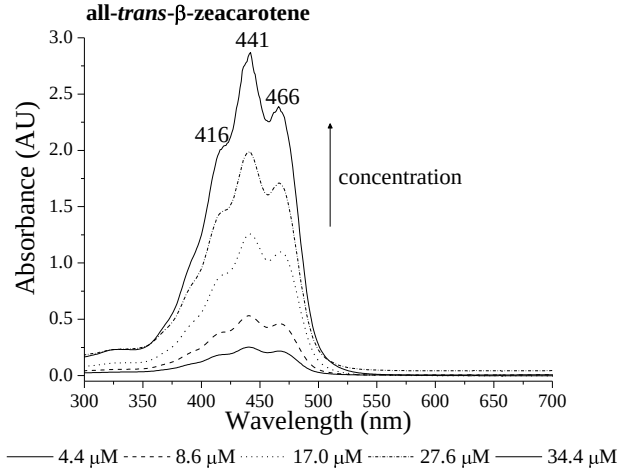

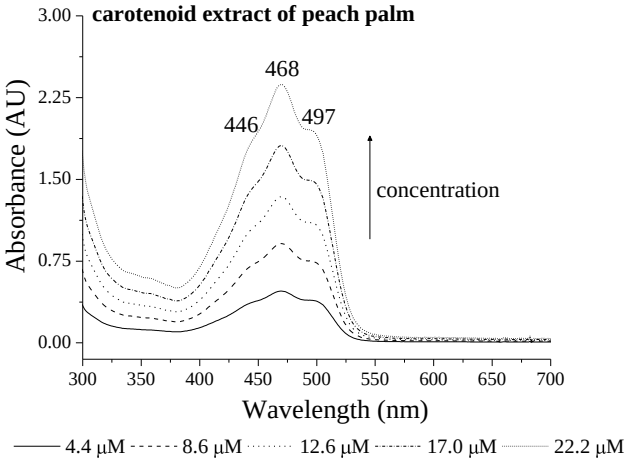

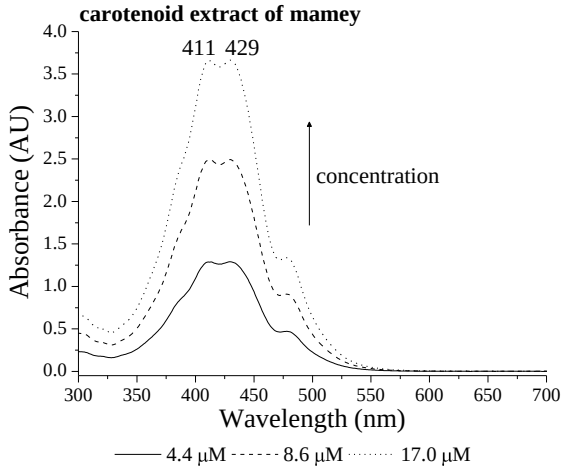

Supplementary Table S1 Chromatographic, UV-Vis, and mass spectroscopy characteristics of carotenoid standards.

carotenoid	t_R (min)[a]	Purity (%)	λ_{max} (nm)[b]	% III/II	% A_B/A_{II}	$[M+H]^+$ (m/z)[c]	MS/MS (+) (m/z)[d]
all-*trans*-β-apo-12'-carotenal	7.5	90.8	426	0	0	351	333 $[M+H-18]^+$
all-*trans*-β-apo-10'-carotenal	9.6	99.9	449	0	0	377	359 $[M+H-18]^+$
all-*trans*-astaxanthin	11.6	97.4	476	0	0	597	579 $[M+H-18]^+$, 505 $[M+H-92]^+$
all-*trans*-lutein	12.5	98.8	420, 444, 472	64	0	569	551 $[M+H-18]^+$, 533 $[M+H-18]^+$, 459 $[M+H-18-92]^+$
all-*trans*-zeaxanthin	14.7	97.4	422, 450, 477	28	0	569	551 $[M+H-18]^+$, 533 $[M+H-18]^+$, 477 $[M+H-92]^+$
all-*trans*-β-apo-8'-carotenal	17.9	96.1	462	0	0	417	399 $[M+H-18]^+$
all-*trans*-β-cryptoxanthin	22.8	99.5	422, 451, 477	28	0	553	535 $[M+H-18]^+$, 461 $[M+H-92]^+$
15-*cis*-β-carotene	24.9	95.3	337, 422, 448, 473	16	58	537	444 $[M-92]^+$
all-*trans*-α-carotene	28.8	99.7	420, 445, 473	64	0	537	481 $[M+H-56]^+$, 444 $[M-92]^+$
all-*trans*-β-carotene	32.9	99.9	422, 451, 478	20	0	537	399 $[M-137]^+$, 444 $[M-92]^+$
all-*trans*-β-zeacarotene	34.7	98.9	400, 427, 453	60	0	539	402 $[M+H-137]^+$, 310 $[M+H-137-92]^+$
9-*cis*-β-carotene	35.0	99.9	420, 446, 472	33	6	537	399 $[M-137]^+$, 444 $[M-92]^+$
all-*trans*-γ-carotene	49.9	99.9	435, 461, 492	46	0	537	399 $[M-137]^+$, 468 $[M+H-69]^+$
all-*trans*-licopene	72.6	99.9	446, 472, 503	76	0	537	444 $[M-92]^+$, 467 $[M-69]^+$
bixin	38.6[e]	98.0	430, 459, 487[f]	33	10	395[g]	377 $[M+H-18]^+$, 363 $[M+H-32]^+$, 335 $[M+H-32-28]^+$ [h]

[a]Elution time on the C_{30} column. [b]Linear gradient methanol/MTBE. [c]The carotenoids were ionized by APCI and parameters were set as follows: positive ion mode; current corona: 4 μA; source temperature: 450 ºC; dry gas (N_2) temperature: 350 ºC, flow: 5 L/min; nebulizer: 60 psi. The mass spectra were acquired with scan range of *m/z* from 100 to 600. [d]The MS/MS was set in automatic mode applying 1.4 V fragmentation energy. [e] Elution time on the C_{18} column. [f]Linear gradient of 2% formic acid in water and methanol with 2% formic acid. [g] The carotenoid bixin were ionized by ESI and parameters were set as follows: positive ion mode; capillary voltage: 1500 V; end plate offset: -500 V; dry gas (N_2) temperature: 325 °C, flow: 8 L/min; nebulizer: 30 psi. The mass spectra were acquired with scan range of *m/z* from 100 to 800. [h]The MS/MS was set in automatic mode applying 1.2 V fragmentation energy.

Supplementary Table S2 Chromatographic, UV-Vis, and mass spectroscopy characteristics of the α-tocopherol, trolox and phenolic standards.

Compound	t_R (min)[a]	Purity (%)	λ_{max} (nm)	$[M-H]^-$ (m/z)[c]	MS/MS (-) (m/z)[d]
trolox	8.3	99.5	289 [b]	249	205, 176, 163
gallic acid	10.1	98.0	271 [c]	169	125
α-tocopherol	12.6	97.6	291 [b]	429	414, 163
rutin	25.1	98.1	312, 351 [c]	609	393, 301, 271
quercetin	37.3	98.5	370 [c]	301	273, 179

[a]Elution time on the C_{18} column. [b]Isocratic: α-tocopherol in methanol, trolox in methanol/water/formic acid (70:29.5:0.5). [c]Gradient: 0.5% formic acid in water and acetonitrile with 0.5% formic acid [d]The tocopherol derivatives were ionized in ESI and parameters set as follows: negative ion mode; end plate offset: -500 V; dry gas (N_2) temperature: 325 °C, flow: 8 L/min; nebulizer: 30 psi. The mass spectra were acquired with scan range of *m/z* from 100 to 600. [e]The MS/MS was set in automatic mode applying 1.2 V fragmentation energy.

Supplementary Table S3 Chromatographic, UV-Vis, mass spectroscopy characteristics and content (dry basis) of carotenoids from mamey and palm peach extracts, obtained by HPLC-DAD-APCI-MS/MS.

Peak[a]	Carotenoid	Concentration (μg/g pulp)[b]		t_R range	λ_{max}	%III/	$\%A_B/$	$[M+H]^+$	MS/MS
		Mamey	Peach palm	(min)[c]	(nm)[d]	II	A_{II}	(*m/z*)	(*m/z*)
1	all-*trans*-violaxanthin[1]	76.61 ± 18.62	nd	7.3-7.4	414, 438, 468	87	0	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491, 221
2	10'-apo-β-caroten-10'-ol[3]	24.14 ± 2.86	nd	7.8.79	380, 400, 421	40	0	379	361 $[M+H-18]^+$, 305 $[M+H-56]^+$, 255, 223
3	*cis*-10'-apo-β-caroten-10'-ol[3]	15.56 ± 1.75	nd	8.0-8.1	375, 395, 416	21	0	379	361 $[M+H-18]^+$, 305 $[M+H-56]^+$, 255, 223
4	9-*cis*-violaxanthin[1]	53.25 ± 7.58	nd	10.6-10.7	325, 411, 435, 463	87	8	601	583 $[M+H-18]^+$, 565 $[M+H-18-18]^+$, 509 $[M+H-92]^+$, 491, 221
5	all-*trans*-lutein[2]	nd	1.11 ± 0.03	12.1-12.2	423, 444, 472	60	0	569	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495, 477 $[M+H-92]^+$
6	*cis*-8'-apo-caroten-8'-al 1[3]	11.85 ± 0.85	nd	13.9-14.0	461	0	0	417	399 $[M+H-18]^+$, 359, 333, 317
7	*cis*-8'-apo-caroten-8'-al 2[3]	52.43 ± 1.18	nd	14.4-14.5	451	0	0	417	399 $[M+H-18]^+$, 359, 333, 317
8	phytoene[3]	34.44 ± 3.47	nd	19.3-19.4	276, 286, 300	n.c.	0	545	489, 435, 395, 339 [M-205]
9	all-*trans*-phytofluene[3]	13.20 ± 1.74	nd	21.7-21.8	331, 348, 367	62	0	543	461, 406 $[M+H-137]^+$, 338 $[M+H-205]^+$
10	all-*trans*-β-cryptoxanthin[3]	6.05 ± 0.61	nd	23.0-23.1	420, 451, 478	20	0	553	535 $[M+H-18]^+$, 495, 461 $[M+H-92]^+$
11	15-*cis*-β-carotene[3]	nd	2.22 ± 0.01	27.5-27.6	338, 418, 444, 471	21	35	537	444 [M-92]
12	13-*cis*-β-carotene[3]	nd	1.65 ± 0.04	29.8-29.9	420, 446, 473	72	0	537	444 [M-92]
13	all-*trans*-β-carotene[3]	25.45 ± 0.23	28.25 ± 1.07	34.0-34.1	420, 451, 478	33	0	537	444 [M-92]
14	9-*cis*-β-carotene[3]	5.06 ± 0.22	2.25 ± 0.10	36.2-36.3	348, 420, 450, 472	33	8	537	444 [M-92]
15	all-*trans*-δ-carotene[3]	nd	7.87 ± 0.62	44.0-44.1	430, 455, 482	50	0	537	481 $[M+H-56]^+$, 444 [M-92]
16	all-*trans*-γ-carotene[3]	nd	9.16 ± 0.44	51.4-51.5	437, 461, 492	64	0	537	467 [M-69], 444 [M-92]
Total carotenoids (μg/g pulp)		318.03 ± 25.90	52.50 ± 2.22						

[a]Numbered according to the chromatogram shown in **Figure S1**. [b]The results correspond to the average (n = 2) ± standard deviation. [c]Retention time on the C_{30} column. [d]Linear gradient of methanol/MTBE. nd = not detected. The peaks were quantified as equivalent of violaxanthin[1], lutein[2] and β-carotene[3].

Supplementary Table 4. In-house **v**alidation parameters of the Oxygen Radical Absorbance Capacity (ORAC) method

Parameter		trolox
linearity range (μM)		8 - 96
slope [a]		0.29 (4) [g]
intercept [a]		2.82 (12)
R^2 [a]		0.994
LOD (μM) [b]		2
LOQ (μM) [c]		7
recovery (%)	level 1 [d]	100 (7)
	level 2 [e]	103 (5)
	level 3 [f]	97 (2)
repeatability (%)	level 1	7
	level 2	4
	level 3	2

[a] For each analytical curve, the equation y = ax + b was applied, where y = net area under curve (AUC), x = final concentration of compounds, a = slope, b = intercept and R^2 = determination coefficient ($p<0.05$). [b]LOD = limit of detection. [c]LOQ = limit of quantification. [d]20 μM of trolox. [e]40 μM of trolox. [f]80 μM of trolox. [g] Relative standard deviation (RSD, %) are given in parentheses (n=6).

CAPÍTULO IV

Scavenging capacity of marine carotenoids against reactive oxygen and nitrogen species in membrane-mimicking system

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Article

Scavenging capacity of marine carotenoids against reactive oxygen and nitrogen species in membrane-mimicking system

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Abstract: Carotenoid intake has been associated with the decrease of the incidence of some chronic diseases by minimizing the *in vivo* oxidative damages induced by reactive oxygen (ROS) and nitrogen species (RNS). The carotenoids are well-known singlet oxygen quenchers; however, their capacity to scavenge other reactive species, such as peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl) and anion peroxynitrite ($ONOO^{-}$), still needs to be more extensively studied, especially using membrane-mimicking systems, such as liposomes. Moreover, the identification of carotenoids possessing high antioxidant capacity can lead to new alternatives of drugs or nutritional supplements for prophylaxis or therapy of pathological conditions related to oxidative damages, such as cardiovascular diseases. The capacity to scavenge $ROO^{\bullet}$, $HO^{\bullet}$, HOCl and $ONOO^{-}$ of seven carotenoids found in marine organisms was determined in liposomes based on the fluorescence loss of a fluorescent lipid (C_{11}-$BODIPY^{581/591}$) due to its oxidation by these reactive species. The carotenoids bearing hydroxyl groups were generally more potent ROS scavengers than the carotenes, whilst β-carotene was the most efficient $ONOO^{-}$ scavenger. The role of astaxanthin as antioxidant should be highlighted, since it was a more potent scavenger of $ROO^{\bullet}$, HOCl and $ONOO^{-}$ than α-tocopherol.

Keywords: liposomes; astaxanthin; carotenoids; ROS; RNS

1. Introduction

Carotenoids are yellow to red fat-soluble pigments found in plants, microorganisms and animals [1-4]. Animals do not synthesize carotenoids *de novo*, and thus, those found in animals are either directly accumulated from food or partly modified through metabolic reactions. Among the 750 carotenoids found in nature identified so far, more than 250 are of marine origin and show a great structural diversity. In particular, except for neoxanthin and its derivatives, allenic carotenoids and all acetylenic carotenoids, such as fucoxanthin, are originated from marine animals and seaweeds [4].

Evidences from epidemiological studies and some supplementation human trials have associated the carotenoid intake with the decrease of the incidence of some chronic diseases [5,6], such as cardiovascular diseases and some types of cancer. This effect is hypothetically attributed to the antioxidant properties of the carotenoids, which minimize the *in vivo* oxidative damages induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [7,8].

ROS and RNS are products of the normal cellular metabolism and they are well recognized for playing a dual role in living systems once their effects can be either harmful or beneficial [9]. At moderate concentrations, ROS and RNS can be involved in cellular responses to pathogens; however, some events, such as infections, can induce an overproduction of ROS and RNS that can either play their role in combating the invading organism or cause damage in the organism cell components and tissue injuries, situation denominated oxidative stress [10].

The antioxidant properties of the carotenoids have been largely studied *in vitro* due to the complexity of *in vivo* systems. The methods to determine the antioxidant capacity of carotenoids generally use a homogenous system and measure the capacity of the carotenoids to quench singlet oxygen ($^1O_2$) [11] and to scavenge either peroxyl radicals ($ROO^{\bullet}$) [12-14] or non-biological radicals, such as ABTS [14,15]. Despite the fact that the homogeneous systems used to assay the antioxidant capacity do not resemble the cellular environment, the results obtained using these systems are often misguidedly extrapolated to the possible effects on living organisms. Living organisms are extremely complex functional systems that are made up of, at a minimum, many thousands of biomolecules in an environment that is spatially organized by membranes. In this sense, the use of liposomes as mimic systems of membranes to evaluate the antioxidant capacity of bioactive compounds has been stimulated due to the similarities between the bilayer structure of the liposomes and the lipid fraction of cell membranes [16]. In fact, the literature reports the capacity of some carotenoids to scavenge $ROO^{\bullet}$ in liposomes [12,17,18]; however, data on the capacity of carotenoids in liposomes to scavenge other reactive species, such as

hydroxyl radicals ($HO^{\bullet}$), hypochlorous acid (HOCl) and peroxynitrite anions ($ONOO^-$), are scarce or inexistent.

The identification of potent marine carotenoids as ROS and RNS scavengers can lead to new alternatives of drugs or nutritional supplements for prophylaxis or therapy for pathological conditions related to oxidative damages, such as cardiovascular diseases and some types of cancer, improving human healthcare. In the present study, the antioxidant capacities of seven carotenoids commonly present in marine sources against ROS and RNS of biological relevance, namely $ROO^{\bullet}$, $HO^{\bullet}$, HOCl and $ONOO^-$, were determined in membrane-mimic systems (liposomes). Furthermore, this is the first time that the capacity of carotenoids to scavenge HOCl and $ONOO^-$ in liposomes is reported.

2. Results

The capacity to scavenge $ROO^{\bullet}$, $HO^{\bullet}$, HOCl and $ONOO^-$ of seven marine carotenoids in liposomes, i.e. fucoxanthin, β-carotene, lycopene, astaxanthin, canthaxanthin, zeaxanthin and lutein (**Figure 1**), is shown in **Table 1**. To establish a comparison with other compounds widely known to possess antioxidant capacity, α-tocopherol, trolox, quercetin, ascorbic acid and cysteine were also analyzed (**Table 1**). Among the tested carotenoids, astaxanthin was the most efficient ROS scavenger, whilst β-carotene was the most potent RNS scavenger. The scavenging capacities were calculated (as described in the Experimental section, Equation 4) considering as reference: trolox (1.00) for $ROO^{\bullet}$ and $HO^{\bullet}$, cysteine (1.00) for HOCl and ascorbic acid (1.00) for $ONOO^-$.

Figure 1. Structure of the marine carotenoids and other antioxidant compounds.

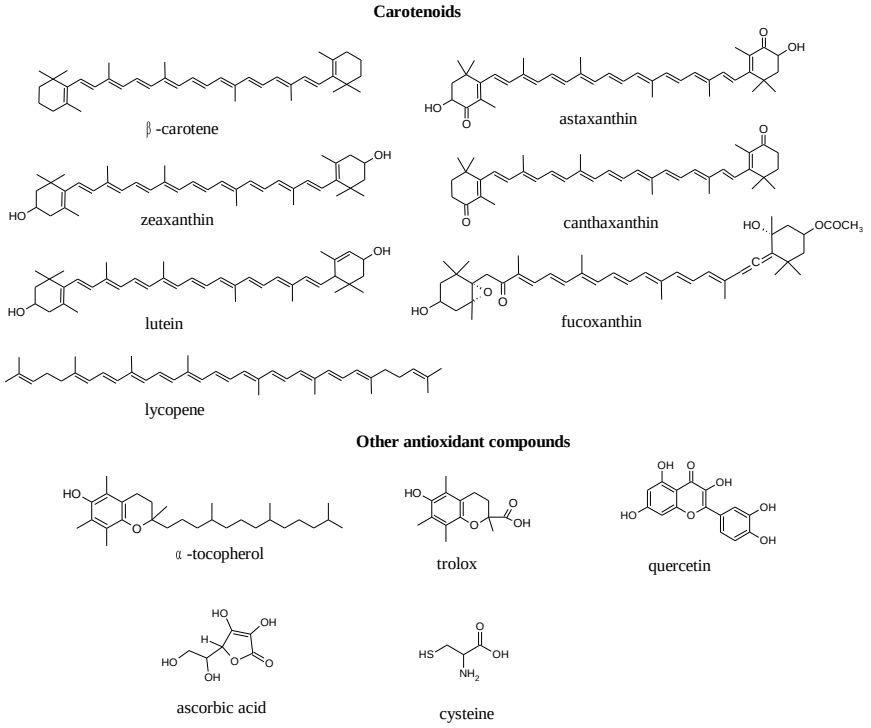

Table 1. Peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl) and peroxynitrite anion ($ONOO^{-}$) scavenging capacity of carotenoids and other compounds incorporated into liposomes, as well as partition ratio values.

Compound	Scavenging Capacity[a]				log P[b]
	$ROO^{\bullet}$	$HO^{\bullet}$	HOCl	$ONOO^{-}$	
β-carotene	0.14	0.71	NA	1.02	14.76 ± 0.43
zeaxanthin	0.56	1.41	3.87	0.77	10.92 ± 0.45
lutein	0.60	0.97	4.81	0.78	11.52 ± 0.46
lycopene	0.08	0.35	0.40	0.31	14.53 ± 0.45
fucoxanthin	0.43	1.18	6.26	NA	7.30 ± 0.65
cantaxanthin	0.04	0.28	0.10	NA	9.53 ± 0.45
astaxanthin	0.64	1.66	9.40	0.73	8.24 ± 0.59
α-tocopherol	0.48	1.77	NA	0.37	10.96 ± 0.35
trolox	**1.00**	**1.00**	NA	NA	2.46 ± 0.36
quercetin	0.84	1.42	5.63	0.97	1.99 ± 1.08
ascorbic acid	NA[c]	NA	0.41	**1.00**	-2.78 ± 0.42
cysteine	0.04	NA	**1.00**	0.02	0.08 ± 0.32

The values are mean of two independent experiments. [a]The values of the $ROO^{\bullet}$ and $HO^{\bullet}$ scavenging capacity were calculated as equivalent to trolox, HOCl was calculated as equivalent to cysteine and $ONOO^{-}$ was calculated as equivalent to ascorbic acid (equation 4). [b]Partition ratio value of the antioxidant compound between the water and octanol, calculated using the ACD/ChemSketch Freeware. [c]NA: no activity was found within the tested concentrations.

The xanthophylls astaxanthin, lutein and zeaxanthin presented similar capacities to scavenge $ROO^{\bullet}$, which were higher than those of the known antioxidants α-tocopherol, ascorbic acid and cysteine, but were less effective than quercetin and trolox (**Table 1**). On the other hand, canthaxanthin presented the lowest $ROO^{\bullet}$ scavenging capacity among all the evaluated carotenoids.

The best scavenger of $HO^{\bullet}$ was astaxanthin, followed by zeaxanthin and fucoxanthin (**Table 1**). Astaxanthin was 66% and 17% more potent than trolox and quercetin, respectively, but only 6% less potent than α-tocopherol. Once more, canthaxanthin presented the lowest $HO^{\bullet}$ scavenging capacity among the evaluated carotenoids.

With the exception of β-carotene, all the other carotenoids were able to scavenge HOCl. Moreover, α-tocopherol and trolox were also not able to scavenge HOCl. Astaxanthin, followed by fucoxanthin, lutein and zeaxanthin presented the highest HOCl scavenging capacities among the carotenoids. Canthaxanthin presented the lowest HOCl scavenging

capacity among the carotenoids, being 94-fold less efficient than astaxanthin. Lycopene also presented low capacity to scavenge HOCl, which was similar to that of ascorbic acid.

Apart from canthaxanthin, fucoxanthin, trolox and cysteine, all the other compounds were able to scavenge $ONOO^-$. The β-carotene was the best $ONOO^-$ scavenger, showing scavenging capacity similar to those of quercetin and ascorbic acid.

3. Discussion

Carotenoids, which are well-known singlet oxygen quenchers [11], can also scavenge other ROS and RNS. Currently, it is known that the antioxidant properties of the carotenoids in homogenous systems are closely related to their chemical structure, including aspects such as the number of conjugated double bonds (c.d.b), type of structural end-groups, and oxygen-containing substituents [13,17,19]. In these systems, the structure activity relationship shows that the structural characteristics of the carotenoids influence both the reactivity towards the ROS and RNS and the stability of the radicals formed after the reaction with ROS and RNS [19]. In liposomes, besides these factors, the carotenoid structure also determines the distribution and orientation of the carotenoid molecules inside the membrane, which directly affects the antioxidant capacity of the carotenoids [20]. The exact location of the carotenoids in the liposomes is still object of study. In general, the polyene chain of the carotenoids is located in the hydrophobic core of the membrane and the carotenes display a certain orientational freedom with respect to the membrane, whilst the xanthophylls polar end-groups tend to form hydrogen bonds with the lipid membrane head-groups and water, since they are located at the membrane interface close to the axis normal to the plane of the bilayer [20]. The effect of the location and orientation of the carotenoid inside the membrane on the antioxidant capacity can be clearly observed when comparing the order of the antioxidant capacity of two carotenoids in homogeneous system and in liposomes. For instance, among 15 carotenoids analyzed in homogeneous system, lycopene (carotene) and lutein (xanthophyll having two hydroxyl groups) presented the highest and the lowest capacities to scavenge $ROO^{\bullet}$, respectively [13]. However, in the present study, when these carotenoids were incorporated into liposomes, an opposite behavior occurred and lutein showed to be a much better $ROO^{\bullet}$ scavenger than lycopene. The influence of the carotenoid localization was also observed in spray-dried microcapsules of carotenoids [21,22] and in oil-in-water emulsions containing carotenoids [23]. Moreover, the hydrophilic or hydrophobic nature of the reactive species analyzed also plays an important role in the antioxidant capacity of carotenoids [17].

In the present study, the carotenoids bearing hydroxyl groups were generally more potent ROS ($ROO^{\bullet}$, $HO^{\bullet}$ and HOCl) scavengers than the more hydrophobic carotenes. This fact indicates that carotenes are most probably in a position to intercept hydrophilic ROS

entering the membrane from the aqueous phase since they are located in the hydrophobic inner core of the bilayer, whilst the carotenoids with hydroxyl end-groups span the bilayer with their end-groups located near to the hydrophobic-hydrophillic interface where the ROS attack first occurs. On the other hand, a carotene, specifically β-carotene, was the most efficient scavenger of $ONOO^-$, most probably because the reactive form of $ONOO^-$ in neutral pH is the peroxynitrous acid (ONOOH), which is able to diffuse until the hydrophobic inner core of the bilayer, where it can interact with the carotenoids present in this site [24].

The structures of trolox and α-tocopherol are very similar (Figure 1), the alkyl side chain of α-tocopherol is replaced by a carboxyl group in trolox, increasing the polarity, but not modifying the phenolic hydroxyl group involved in the antioxidant mechanism of both α-tocopherol and trolox. In fact, in homogeneous systems trolox and α-tocopherol usually present similar antioxidant capacity, e.g against ABTS radical [26], DPPH radical [27] and $ROO^•$ [13], suggesting that the scavenging capacity of trolox and α-tocopherol comprises donation of the phenolic hydrogen or electron transfer [25]. However, trolox and α-tocopherol presented distinct behaviors against the studied reactive species in the liposomes. α-Tocopherol was more potent than trolox as $ONOO^-$ and $HO^•$ scavenger, whilst trolox showed a better antioxidant capacity than the α-tocopherol against $ROO^•$. These evidences suggest that the polarity of these molecules directly affects their antioxidant capacity, probably due to its influence on the distribution and orientation positioning of the antioxidant molecules inside the membrane, which affects the antioxidant capacity of the carotenoids. Recently, our research group observed similar behavior in gum Arabic and maltodextrin microcapsules containing trolox and α-tocopherol [21,22].

The interaction of a compound with biomembranes is strongly related to its lipophilicity, which can be expressed as the partition ratio (log P), which is the ratio between the compound concentration in each of the two phases of an immiscible mixture. Considering water as hydrophilic and octanol as lipophilic solvents, in the present study, the log P values were calculated (**Table 1**) and used to inflict the position of the carotenoid in the lipid membrane and its influence on the capacity to scavenge the ROS and RNS. Interestingly, a negative correlation was found between the log P values and the capacity of the studied carotenoids to scavenge ROS, $ROO^•$ ($R = -0.50$, $p = 0.25$), $HO^•$ ($R = -0.54$, $p = 0.21$) and HOCl ($R = -0.64$, $p = 0.18$); however, a positive correlation was found for RNS scavenging capacity, $ONOO^-$ ($R = 0.47$, $p = 0.29$). This fact means that the more affinity a carotenoid has for the hydrophilic phase (lower log P values), the more potent scavenger of ROS it is, whilst the contrary occurs for RNS.

The reactive species $HO^•$ and $ROO^•$ are of particular interest because of their prominent role in lipid peroxidation, which has been related to the development of atherosclerosis and

other cardiovascular diseases [10]. The $HO^{\bullet}$ shows a very short *in vivo* half-life of approximately 10^{-9} s, thus it is a very dangerous radical due to its high reactivity [28], and when produced *in vivo*, $HO^{\bullet}$ reacts closely to its site of formation, where it can initiate the lipid peroxidation by abstracting an allylic hydrogen from an unsaturated fatty acid generating $ROO^{\bullet}$. Chain-breaking antioxidants can interrupt these reactions by scavenging the lipid $ROO^{\bullet}$. The most known chain-breaking antioxidants are α-tocopherol, ascorbic acid and phenolic compounds, which scavenge $HO^{\bullet}$ and $ROO^{\bullet}$ by donating a hydrogen atom, forming a lipid hydroperoxide and a resonance-stabilized antioxidant radical [25]. Three mechanisms are proposed for scavenging of $ROO^{\bullet}$ and $HO^{\bullet}$ by carotenoids, i.e. electron transfer (equation 1), abstraction of the allylic hydrogen (equation 2) and radical addition to the c.d.b. system (equation 3) [19]. The occurrence of one or another mechanism depends on the organization level of the reaction system and its polarity and on the carotenoid structure [19].

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet +} + R^{-} \text{ (electron transfer)} \quad (1)$$

$$CAR + R^{\bullet} \rightarrow CAR^{\bullet} + RH \text{ (hydrogen abstraction)} \quad (2)$$

$$CAR + R^{\bullet} \rightarrow R - CAR^{\bullet} \text{ (addition)} \quad (3)$$

Astaxanthin was the carotenoid that presented the best scavenging capacity against $ROO^{\bullet}$ and $HO^{\bullet}$. This behavior could be explained by the activation of the hydroxyl groups, due to a possible balance between the keto and enol forms of astaxanthin, which would result in the formation of an ortho-dihydroxy-conjugate polyene system acting as a chain breaking antioxidant in a similar way that does α-tocopherol [12].

Although not a radical as $HO^{\bullet}$ and $ROO^{\bullet}$, the reactive species HOCl is a potent oxidant agent that can either act as an antimicrobial agent or cause tissue damages [29]. Moreover, the HOCl has been associated to the development of diverse pathological conditions, such as inflammatory diseases, atherosclerosis, respiratory discomfort, acute vasculitis, rheumatoid arthritis, glomerulonephritis and cancer [30,31]. The HOCl is enzymatically generated *in vivo* by myeloperoxidase (MPO), which uses the H_2O_2 produced during the respiratory burst to catalyze the chloride ions (Cl^-) [29]. Pennathur et al. [32] proposed a reaction mechanism between lycopene and HOCl in homogeneous system, in which the Cl atom acts as an electrophile and the double bonds as nucleophiles. When the Cl atom is added to the double bond, through a pseudo-secondary carbocation, a stable cloronium ion is generated. Then, the addition of a hydroxide ion occurs, forming a chlorohydrin, which undergoes a SN2-type reaction with the substitution of the Cl, originating a lycopene epoxide. This epoxide can react with another HOCl molecule (deprotonated by Cl^-),

causing the cleavage of a C-C bond, generating an aldehyde. Astaxanthin and fucoxanthin presented high capacities to scavenge HOCl, suggesting that these carotenoids are potential compounds to be used in the prevention of the development of pathological states related to inflammations.

The $ONOO^-$ is a reactive nitrogen species formed *in vivo* from superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) and it is a highly reactive oxidant that causes nitration of the aromatic ring of free tyrosine and protein tyrosine residues. Furthermore, the $ONOO^-$ was found to induce various forms of oxidative damage such as low-density lipoprotein (LDL) oxidation, lipid peroxidation, and DNA strand breakage [10]. The reaction between the carotenoid and $ONOO^-$ is relatively slow and presents a first order kinetics. There are two proposed ways of $ONOO^-$ scavenging by carotenoids. In the first one, lycopene accepts energy from $ONOO^-$ and goes to an excited state (biradical), and while returning to the ground state produces (*Z*)-isomers. In the second one, lycopene directly reacts with $ONOO^-$ to produce a dioxetane that cleaves to apo-lycopenals or undergoes methanolysis to yield methoxy-lycopene [33]. Moreover, recently the formation of nitrocarotenoids as a result of the reaction between carotenoids and $ONOO^-$ was described [34,35].

Especial attention should be paid to the allenic xanthophyll fucoxanthin, a carotenoid mainly produced by brown seaweeds and microalgae, being the most abundant carotenoid in nature, contributing to more than 10% of the estimated total carotenoid production [36]. Fucoxanthin has a peculiar structure, quite different from the carotenoids commonly found in nature, due to the presence of an allenic bond (C=C=C) and some oxygenated functional groups, such as hydroxyl, epoxy, carbonyl and carboxyl (**Figure 1**). Several biological properties have been attributed to fucoxanthin, such as anti-inflammatory, anticancer, anti-obese, anti-diabetic, anti-angiogenic, anti-malarial and antioxidant [36,37]. Recently, fucoxanthin was identified as the main carotenoid being responsible for the high antioxidant capacity of extracts from 27 species of brown seaweeds [3], and this activity was mainly attributed to the allenic bond [36]. In the present study, fucoxanthin showed to be an efficient $ROO^{\bullet}$, $HO^{\bullet}$ and HOCl scavenger.

Our *in vitro* findings reinforce the results of some animal studies [38,39] that showed that the protective effects of astaxanthin on the cardiovascular system are related to its capacity to scavenge ROS and RNS. Moreover, in our study β-carotene showed to be a less potent ROS scavenger than most of the other carotenoids, corroborating with fact that, despite presenting some anticarcinogenic activity, β-carotene seems to be less effective than other carotenoids, such as astaxanthin, zeaxanthin, lutein and fucoxanthin [40,41].

4. Experimental Section

4.1. Chemicals and standards

Standards of (all-*E*)-β-carotene (99.9%), (all-*E*)-astaxanthin (97.4%), (all-*E*)-fucoxanthin (95.0%), (all-*E*)-canthaxanthin (90.0%), α-tocopherol (97.6%), ascorbic acid (99.0%) and cysteine (97.0%) were supplied by Sigma-Aldrich (St. Louis, MO, USA), (all-*E*)-lutein (98.8%), (all-*E*)-zeaxanthin (97.4%) and (all-*E*)-lycopene (99.9%) were kindly donated by DSM Nutritional Products (Basel, Switzerland). All these compounds were used as received and the purity of the standards was determined by HPLC-DAD. All carotenoids are in (all-*E*) configuration unless stated otherwise. The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$, MW = 504.43 g/mol) was acquired from Invitrogen (Eugene, OR, USA). The chemicals 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, 99.5%), soybean L-α-phosphatidylcholine (MW ≅ 900 g/mol), α,α'-azodiisobutyramidine dihydrochloride (AAPH), sodium phosphate dibasic, potassium phosphate monobasic, solution sodium hypochlorite with 13% (w/w) available chlorine, 30% (w/w) hydrogen peroxide solution were purchased from Sigma-Aldrich. Chloroform (PA ACS grade) was purchased from Merck (Darmstadt, Germany); dichloromethane (PA ACS grade), from Labsynth (Diadema, SP, Brazil); ferrous chloride ($FeCl_2$), from JT Baker (Phillipsburg, NJ, USA) and ethylenediaminetetraacetic acid, from Quemis (Joinville, SC, Brazil). Ultrapure water was obtained from the Millipore system (Billerica, MA, USA).

4.2. Preparation and characterization of the liposomes

Fresh solutions of phosphatidylcholine in chloroform (5 mM) and of the probe C_{11}-BODIPY$^{581/591}$ in methanol (2 mM) were prepared. To prepare the liposomes, aliquots of these solutions were transferred to a round-bottomed flask in order to achieve final concentrations of 5 mM of phosphatidylcholine and 5 μM of probe in the liposomes. After that, an aliquot of the antioxidant compound solution, to give a final concentration of about 6 mol%, was added. In the case of the method validation, final concentrations from 1 to 8 mol% of trolox, 6 to 51 mol% of cysteine and 1.6- 12.8 mol% of ascorbic acid were used (**Table 2**). The antioxidant concentration in mol% was calculated dividing the number of moles of the antioxidant compound by the number of moles of phosphatidylcholine and multiplying by 100. The carotenoid solution was prepared in dichloromethane, α-tocopherol in ethanol, trolox and quercetin in methanol, and ascorbic acid and cysteine in phosphate buffered saline (PBS) (12 mM, pH 7.4). The mixture was vortexed for 2 min, sonicated for 2 min and finally vortexed for 30 s. The solvent was evaporated (T < 30 °C)

while rolling the flask in order to deposit a thin lipid film on the flask wall and let overnight in a freeze-dryer to remove any remaining solvent. For hydration, saccharose (final concentration: 26 mM) and PBS were added to the dry film and the multilamellar lipid vesicles (MLV) were produced by vortexing for 5 min and by sonication for 2 min. The MLV were freeze-thawed 3 times and extruded in a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA) by passing 21 times through a 100 nm polycarbonate membrane to obtain the large unilamelar vesicles (LUV), which were called liposomes within the text. Blank liposomes were also prepared without the addition of antioxidants.

Table 2. Validation parameters of micro-assays for the ROS and RNS scavenging capacity in liposomes.

Reactive species	Linearity range (mol%)	R^2 [a]	Slope[b]	Intercept [b]
$ROO^{\bullet}$ [c]	1.0-8.0	0.96	20.8	4.7
$HO^{\bullet}$ [c]	1.0-6.0	0.97	19.3	3.9
HOCl [d]	6.0-51.0	0.98	0.17	2.3
$ONOO^-$ [e]	1.6-12.8	0.98	3.5	9.2

[a] R^2 is the determination coefficient ($p<0.05$). [b] For each analytical curve, a equation $y = ax + b$ was obtained by linear regression, where y is the net area under curve (net AUC), a is the slope and b is the intercept. [c] Trolox was used as reference. [d]Cysteine was used as reference. [e]Ascorbic acid was used as reference.

The liposome size and zeta potential were measured in a ZetaSizer Nano (Malvern, United Kingdom). Liposomes average diameters were measured by laser light scattering and ranged from 110 to 140 nm. Zeta potential was measured by electrophoretic mobility and the surface charge was approximately zero.

The carotenoid concentration was determined in the liposomes by mixing 500 μL of liposomes with 3 mL of dichloromethane and vortexing during 1 min. This mixture was transferred to a separation funnel containing dichloromethane/water (1:1, v/v) and after 3 min, the lower phase containing the carotenoid was collected. The dichloromethane was dried under N_2 stream, the carotenoid was redissolved in petroleum ether (β-carotene, zeaxanthin, canthaxanthin, fucoxanthin and lycopene), hexane (astaxanthin) or ethanol (lutein) and the concentration was spectrophotometrically (Agilent model 8453, Missouri, USA) determined using the specific absorption coefficients for each compound [42]. In the lipossomes, the final concentrations were 6 mol% of β-carotene, zeaxanthin, canthaxanthin, fucoxanthin and astaxanthin and 5 mol% of lutein and lycopene.

4.3. ROS and RNS scavenging capacity assays

4.3.1. Adaptation and validation of the methods

The method to determine the $ROO^{\bullet}$ scavenging capacity of hydrophilic and lipophilic compounds in liposomes developed by Zhang et al. [18] was adapted to micro-assays to determine the $ROO^{\bullet}$, $HO^{\bullet}$, HOCl and $ONOO^-$ scavenging capacities. These methods are based on the loss of the fluorescence at 600 nm of the probe C_{11}-$BODIPY^{581/591}$, a fluorescent lipid, due to its oxidation by these reactive species. This probe was chosen since its sensitivity to oxidation was comparable to that of endogenous fatty acids, and the probe was found previously to be sensitive to $HO^{\bullet}$ and $ONOO^-$ but not to $O_2^{\bullet-}$, $NO^{\bullet}$, H_2O_2, transition metal ions and hydroperoxides *per se* [43]. The modifications consisted in adapting the reagent volumes to carry out the analysis of $ROO^{\bullet}$ scavenging capacity in microplates and in the use of the same probe to assay the capacity to scavenge $HO^{\bullet}$, HOCl and $ONOO^-$.

The assays were carried out in a microplate reader equipped with a thermostat set at 37 ºC and dual reagent dispenser (Synergy Mx, BioTek, Winooski, VT, USA) using 96-well black polystyrene microplates (Corning, New York, NY, USA).

For method validation, an analytical curve was constructed with five concentrations of the compound used as reference (**Table 2**) for each micro-assay. The linearity between the concentration of the reference compound and the net AUC presented a determination coefficient (R^2) higher than 0.95 ($p<0.05$), within the range of the tested concentrations, for all the reactive species (**Table 2**). No interactions between the probe and the compounds used as reference were observed and the loss of fluorescence due to probe photo-bleaching was less than 10% (Supplementary Figure S1). Repeatability was evaluated using the relative standard deviations (RSD) between two independent experiments and was within ± 10%, indicating that the developed methods are precise.

4.3.2. Peroxyl radical scavenging assay

$ROO^{\bullet}$ was generated by thermodecomposition of AAPH at 37 °C. Reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 200 μL): 100 μL of liposomes, 84 μL of PBS (12 mM, pH 7.4) and 16 μL of AAPH (40 mM) solution in PBS. The mixture was preincubated in the microplate reader during 10 min before AAPH addition. The fluorescence signal was monitored every minute for the emission wavelength at 540 ± 20 nm with excitation at 600 ± 20 nm, until 180 min. Trolox was used as reference to calculate the $ROO^{\bullet}$ scavenging capacity.

4.3.3. Hydroxyl radical scavenging assay

The $HO^{\bullet}$ was generated by the Fenton reaction ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + HO^{\bullet} + OH^-$). Five concentrations of H_2O_2 (61, 121, 183, 244 and 286 mM) were tested and the concentration of Fe^{+2}/EDTA was set at 313 μM:1250 μM. The H_2O_2 concentration of 61 mM was chosen in order to achieve about 5% of the initial fluorescence signal in 120 min in the blank assay (no antioxidant in the liposome). The probe oxidation was also evaluated using only H_2O_2 (61 mM) and only Fe^{+2}/EDTA (313 μM:1250 μM). These compounds (H_2O_2 and Fe^{+2}/EDTA) were not able to oxidize the probe by themselves, proving that the $HO^{\bullet}$ generated by the Fenton reaction was the only reagent that reacted with the probe (Supplementary Figure S1).

Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 250 μL): 100 μL of liposomes, 59 μL of PBS (12 mM, pH 7.4), 25 μL of $FeCl_2$/EDTA (313 μM:1250 μM) and 16 μL of H_2O_2 (61 mM). The mixture was preincubated in the microplate reader during 10 min before $FeCl_2$/EDTA and H_2O_2 addition. The fluorescence signal was monitored every minute for the emission wavelength at 540 ± 20 nm with excitation at 600 ± 20 nm, until 240 min. Trolox was used as reference to calculate the $HO^{\bullet}$ scavenging capacity.

4.3.4. Hypochlorous acid scavenging assay

HOCl was prepared by adjusting the pH of a 1% (v/v) solution of NaOCl to 6.2, with 10% H_2SO_4 (v/v). The concentration of HOCl was determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1}. Five concentrations of HOCl (6.9, 1.4, 2.1, 2.8 and 4.3 mM) were tested. The HOCl concentration of 2.8 mM was chosen in order to achieve about 10% of the initial fluorescence signal in about 20 min in the blank assay (no antioxidant in the liposome) (Supplementary Figure S1).

Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 200 μL): 100 μL of liposomes, 30 μL of PBS (12 mM, pH 7.4) and 70 μL HOCl (2.8 mM). The mixture was preincubated in the microplate reader during 10 min before HOCl addition. The fluorescence signal was monitored every minute for the emission wavelength at 540 ± 20 nm with excitation at 600 ± 20 nm, until 30 min. Cysteine was used as reference to calculate the HOCl scavenging capacity.

4.3.5. Peroxynitrite scavenging assay

$ONOO^-$ was synthesized as previously described by Rodrigues et al. [22]. Five concentrations of $ONOO^-$ (0.5, 50, 100, 250 and 500 μM) were tested. The $ONOO^-$ concentration of 100 μM was chosen in order to achieve about 1% of the initial

fluorescence signal in 50 min in the blank assay (no antioxidant in the liposome) (Supplementary Figure S1).

Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 200 μL): 100 μL of liposomes, 84 μL of PBS (12 mM, pH 7.4) and 16 μL of $ONOO^-$ (100 μM). The mixture was preincubated in the microplate reader during 10 min before $ONOO^-$ addition. The fluorescence signal was monitored every minute for the emission wavelength at 540 ± 20 nm with excitation at 600 ± 20 nm, until 180 min. Ascorbic acid was used as reference to calculate the HOCl scavenging capacity.

4.3.6. Calculation of the scavenging capacity

The scavenging capacity of all reactive species was calculated according to Equation 4.

$$\text{Scavenging capacity} = \left(\frac{\text{Net AUC}_{\text{antioxidant}} - b}{a} \right) x \left(\frac{1}{[\text{antioxidant}]} \right) \quad (4)$$

Where:

$$\text{Net AUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$$

$$\text{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + f_n/f_0$$

f_0 = initial fluorescence

f_n = fluorescence signal at time n

a = slope of the analytical curve of [compound used as reference] against Net AUC (**Table 2**)

b = intercept of the analytical curve of [compound used as reference] against Net AUC (**Table 2**)

[antioxidant] = concentration of the antioxidant compound in the liposomes (mol%).

[compound used as reference] = trolox for $ROO^\bullet$ and $HO^\bullet$ scavenging capacity, cysteine for HOCl scavenging capacity and ascorbic acid for $ONOO^-$ scavenging capacity.

4.4. Partition ratio (log P)

The log P values of the carotenoids and other antioxidant compounds (**Table 1**) were calculated, using water and octanol as solvents, by the ACD/ChemSketch Freeware (version 12.01).

4.5. Statistical analysis

The Software Origin® 8 was used for the calculations. The results were expressed as mean of two independent experiments (n = 2). The analytical curves were plotted by linear regression ($p<0.05$) and the correlations between the partition ratio (log P) and the antioxidant capacity were established using the Pearson's correlation coefficient.

5. Conclusions

Marine carotenoids are compounds with a great potential to scavenge several ROS and RNS in different degrees of efficiency. It is important to highlight that astaxanthin, fucoxanthin, lutein and zeaxanthin showed to be potent ROS scavengers, whilst β-carotene was the most efficient RNS scavenger. In fact, astaxanthin showed to be a more potent scavenger of $ROO^{\bullet}$, HOCl and $ONOO^{-}$ than α-tocopherol. The results of the present study reinforce the hypothesis that the antioxidant capacity of the carotenoids is one of the mechanisms responsible for the decrease of the risk of development of cardiovascular diseases and some types of cancer. Moreover, the knowledge of the behavior of the carotenoids as antioxidants in lipid bilayers can help the interpretation of the results of *in vivo* studies aiming to correlate the consumption of seafoods and seaweeds rich in carotenoids with health benefits.

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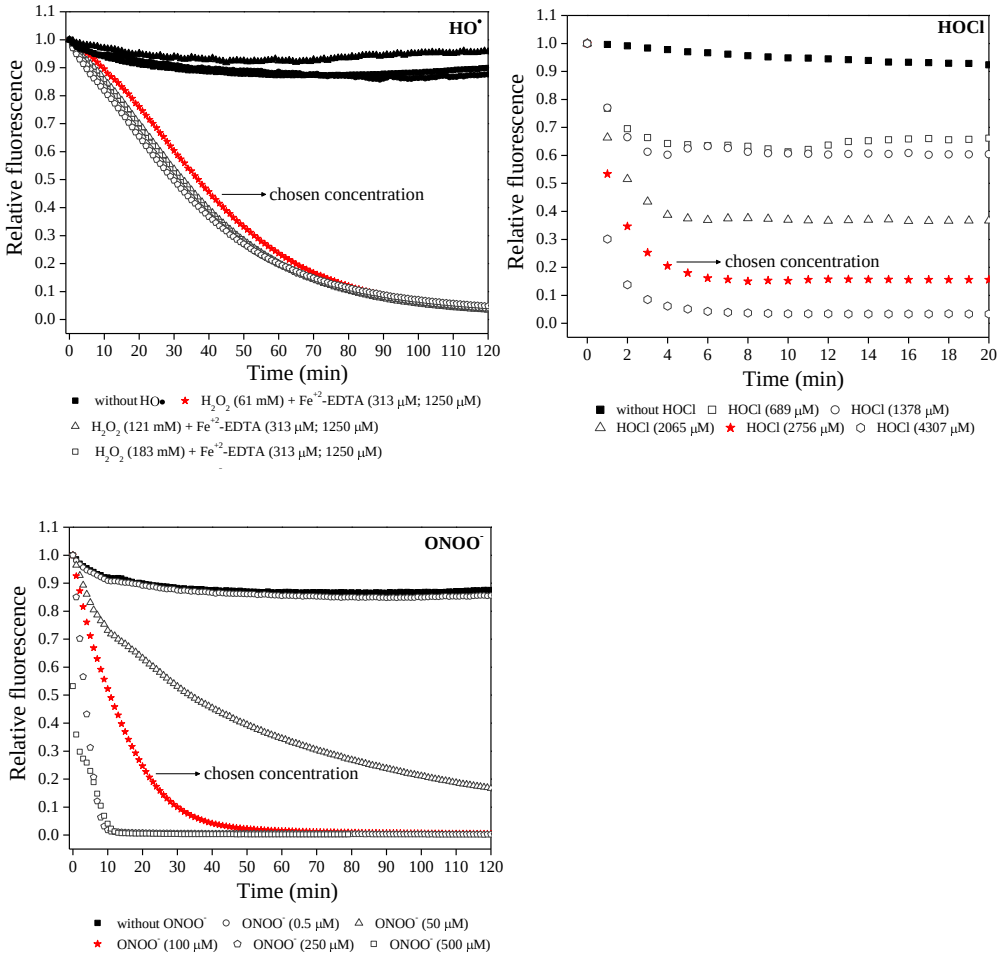
Supplementary Figure S1 Decay curves of the probe C_{11}-BODIPY$^{581/591}$ fluorescence using different concentrations of hydroxyl radical generators ($HO^{\bullet}$) and different concentrations of hypochlorous acid (HOCl) and anion peroxynitrite ($ONOO^-$). The red line corresponds to the chosen concentrations.



CAPÍTULO V

Bioactive compounds and *in vitro* scavenging capacity against reactive oxygen species from an unexploited Amazonian fruit (S*olanum sessiflorum*)

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Abstract

In this study, high-performance liquid chromatography coupled to diode array and mass spectrometry detectors (HPLC-DAD-MS/MS) was used to determine the composition of carotenoids and phenolic compounds from mana-cubiu (*Solanum sessiflorum*) fruit. The antioxidant capacities of the phenolic and carotenoid extracts against some reactive oxygen species, namely $ROO^{\bullet}$, H_2O_2 and HOCl were also determined. Seventeen carotenoids and five phenolic compounds were found in mana-cubiu. The major carotenoids found were all-*trans*-β-carotene (7.15 μg/g), all-*trans*-lutein (2.41 μg/g), 9-*cis*-luteoxanthin (1.33 μg/g), all-*trans*-luteoxanthin (1.30 μg/g) and all-*trans*-violaxanthin (0.98 μg/g). The 5-caffeoylquinic acid (1298 μg/g) was the major phenolic compound, representing more than 70 % w/w of the total phenolic compounds. Interestingly, two dihydrocaffeoyl spermidines were found in the phenolic extract. The mana-cubiu's carotenoid extract showed to be a potent scavenger of peroxyl radicals, whilst the phenolic extract was a potent hydrogen peroxide and hypochlorous acid scavenger.

Keywords: HPLC-MS; chlorogenic acid; carotenoid; Amazonian fruit; bioactive compounds; Solanaceae.

1. Introduction

Mana-cubiu (*Solanum sessiflorum*) **(Fig. 1)** is a fruit that belongs to the Solanaceae family. This fruit is native from the Amazonian region and widely distributed across the humid equatorial regions of Brazil, Peru and Colombia (Schuelter et al., 2009). The fruits, also known as topiro/tupiro in Peru, cocona in Venezuela, Indian tomato in the northeast of Brazil, cubiu in the Brazilian Amazonian region and oricono or apple/peach tomato in English speaking countries, are 5 to 6 cm in diameter, weight between 30 and 400 g and their edible fraction represents approximately 91% (w/w) of total fresh weight (9% w/w of peel). The fruit contains around 90% (w/w) of water in its composition. The remaining components are basically citric acid (14 g/100 g dry weight) and carbohydrates (32 g/100 g dry weight), where glucose and fructose are predominant (Marx, Andrade & Maia, 1998). The pulp has an acidic pleasant taste and the flavor is similar to that of citric fruits and peaches and it is usually consumed as salad, juice, jelly or it can be added to cakes. The color of the peel goes from green to orange during ripening and the pulp is light yellow due to the presence of carotenoids **(Fig. 1)** (Marx et al., 1998).

Fig. 1 Mana-cubiu fruit (*Solanum sessiflorum*).

Epidemiological evidence has associated the fruit intake with the decrease of the incidence of cardiovascular diseases and certain types of cancer (Voutilainen, Nurmi,

Mursu & Rissanen, 2006; Zhang et al., 2007; Karppi, Kurl, Laukkanen, Rissanen & Kauhanen, 2011). Moreover, plants with therapeutic actions are commonly used particularly in phytogeographic regions, such as Amazonia, and are usually known in folklore for their therapeutic potential. For instance, mana-cubiu has been used against snake bites, scorpion stings and skin infections, presented anti-hypertensive activity and reduced cholesterol, glucose and uric acid levels in the blood (Silva Filho, Noda, Yuyama, Yuyama, Aguiar & Machado, 2003; Pardo, 2004; Vandebroeka, Van Damme, Van Puyvelde, Arrazola & De Kimped, 2004).

The bioactive compounds present in mana-cubiu, are probably responsible for the benefic properties attributed to its consumption. However, no data on the carotenoid and phenolic compositions of mana-cubiu has been previously reported. Thus, the aim of this work was to identify and quantify the carotenoids and phenolic compounds from mana-cubiu by high performance liquid chromatography coupled to photodiode array and mass spectrometry detectors (HPLC-DAD-MS/MS). Moreover, the scavenging capacities of mana-cubiu extracts were evaluated against peroxyl radicals ($ROO^{\bullet}$), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2).

2. Material and Methods

2.1. Chemicals

Standards of all-*trans*-β-carotene, caffeic acid, gallic acid and 5-caffeoylquinic acid were purchased from Sigma-Aldrich (Missouri, USA). All-*trans*-lutein, all-*trans*-α-carotene and all-*trans*-β-cryptoxanthin were donated by DSM Nutritional Products (Basel, Switzerland) and standards of 9'-*cis*-neoxanthin and all-*trans*-violaxanthin were acquired from CaroteNature (Lupsingen, Switzerland). The purity of the carotenoid and phenolic

compounds standards varied from 93 to 99%, measured by HPLC-DAD. Methyl *tert*-butyl ether (MTBE) was acquired from J. T. Baker (New Jersey, USA) and the other HPLC grade solvents were obtained from Merck (Darmstadt, Germany) or Mallinckrodt Baker (Philipsburg, USA). Dihydrorhodamine 123 (DHR), 30% hydrogen peroxide, sodium hypochlorite solution with 4% available chlorine, α,α'-azodiisobutyramidine dihydrochloride (AAPH), dimethyl sulfoxide (DMSO), lucigenin and fluorescein sodium salt were obtained from Sigma-Aldrich. Azobisisobutyronitrile (AIBN) was donated by Mig Quimica (São Paulo, SP, Brazil). The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$, MW = 504.43 g/mol) was purchased from Invitrogen (Oregon, USA). The other reagents were all of analytical grade from Labsynth (Diadema, São Paulo, Brazil). Water was purified by a Milli-Q system (Billerica, USA). The samples and solvents were filtered through Millipore membranes of 0.22 and 0.45 μm, respectively.

2.2. Samples

Three batches of around 7 kg of mana-cubiu were acquired in CEAGESP (Companhia de Entrepostos e Armazéns Gerais de São Paulo, São Paulo, Brazil). The fruits were washed, peeled, cut into small pieces and immediately frozen in liquid nitrogen before lyophilization. Lyophilization was conducted in a freeze-drier (Liobras, São Paulo, Brazil) during 96 h at -60 °C and below 40 μHg. The lyophilized fruits were grinded into powder in a domestic food mixer (Black & Decker, São Paulo, Brazil). The mana-cubiu powder from the three batches was homogenized to compose a single sample. The sample was vacuum packed (Jumbo Plus, Selovac, São Paulo, Brazil) in polyethylene bags containing 50 g portions and was stored at -37 °C in the dark until analysis.

2.3. Carotenoid extraction

The lyophilized fruit was weighted (5.00 g) and the carotenoids were exhaustively extracted with acetone, transferred to petroleum ether/diethyl ether (1:1, v/v) and saponified with 10 % (w/v) methanolic KOH overnight (~16 h) at room temperature (De Rosso & Mercadante, 2007). The alkali was removed by washing the extract with distilled water, and the solvent evaporated in a rotary evaporator (T < 30 °C). The dry extract was stored at -80 °C under nitrogen atmosphere (99.9% purity) in the dark until HPLC analysis.

2.4. Extraction of phenolic compounds

The lyophilized fruit was weighted (0.10 g) in 10 mL tubes and extracted with 5 mL of methanol/water (8:2, v/v) by vortexing (Phoenix Luferco, Araraquara, São Paulo, Brazil) for 5 min at 25 °C. After centrifugation (Beckman Coulter Allegra 64R, California, USA) at 3,864 x *g* for 5 min at 20 °C, the supernatant was transferred to a 25 mL volumetric flask. The extraction procedure was performed five times and the supernatants were combined in order to obtain a final volume of 25 mL. After that, the extract was lyophilized and stored at -37 ºC until analysis. Immediately before the analysis, the lyophilized extract was suspended in water (10 mg of dry extract/mL of water), centrifuged at 37,000 x *g* for 10 min at 10 °C and the supernatant was used for ROS scavenging capacity analysis and HPLC-DAD-MS/MS analysis of the phenolic compounds.

2.5. HPLC-DAD-MS/MS analysis

A Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), on-line degasser and a Rheodyne (Rheodyne LCC, Robert Park, USA) injection

valve with a 20 μL loop, connected in series to a DAD detector (Shimadzu) and a mass spectrometer with an ion trap analyser with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) sources (Bruker Daltonics, model Esquire 4000, Bremen, Germany) was used to determine the carotenoids and the phenolic compounds.

The carotenoids were separated on a C_{30} YMC column (5 μm, 250 x 4.6 mm i.d.) (Waters, Wilmington, USA) using as mobile phase a linear gradient of methanol/MTBE from 95:5 (v/v) to 70:30 (v/v) in 30 min, followed by 50:50 (v/v) in 20 min (De Rosso & Mercadante, 2007). The flow rate was 0.9 mL/min and the column temperature was set at 29 ºC. The UV-vis spectra were obtained between 200 and 600 nm and the chromatograms were processed at 450 nm. The MS parameters were set as follows: positive mode; corona current: 4000 nA; source temperature: 450 ºC; dry gas (N_2) temperature: 350 ºC, flow: 5 L/min; nebulizer: 60 psi. The MS/MS was set in automatic mode applying 1.4 V fragmentation energy. The mass spectra were acquired with scan range from *m/z* 100 to *m/z* 800 (De Rosso & Mercadante, 2007). The identification of the carotenoids was performed considering the combination of the following parameters: 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 analysed under the same conditions and data available in the literature. The carotenoids were quantified by HPLC-DAD, using seven-point analytical curves of 9-*cis*-neoxanthin (0.9 to 17.1 μg/mL), all-*trans*-violaxanthin (0.7 to 13.6 μg/mL), all-*trans*-lutein (1.0 to 59.5 μg/mL) and all-*trans*-β-carotene (1.1 to 30.2 μg/mL). All other carotenoid contents were estimated using the curve of all-*trans*-β-carotene and the *cis*-isomers were estimated using the curve of the corresponding all-*trans*-carotenoid. All analytical curves were linear ($r^2 = 0.99$), the limit of detection was 0.1 μg/mL and the limit of quantification

was 0.5 μg/mL. The NAS-IOM (2001) 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.

The phenolic compounds were separated in a C_{18} Synergi Hydro-RP column (4 μm, 250 x 4.6 mm, Phenomenex, Torrance, CA) at a flow rate of 0.9 mL/min, column temperature at 29 °C, using a mobile phase consisting of water/formic acid (99.5:0.5, v/v) (solvent A) and acetonitrile/formic acid (99.5:0.5, v/v) (solvent B) in a linear gradient from A/B 99:1 to 50:50 in 50 min; then from 50:50 to 1:99 in 5 min. The former ratio (1:99) was maintained for additional 5 min. The column eluate was split to allow only 0.15 mL/min to enter the ESI interface. The UV-vis spectra were obtained between 200 and 600 nm and the chromatograms were processed at 280 and 320 nm. The mass spectra were acquired with a scan range from *m/z* 100 to *m/z* 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: 1.6 V (Chisté & Mercadante, 2012a). The phenolic compounds were identified based on the following information: elution order and retention time in the reversed phase column, UV-vis and mass spectra features as compared to standards analyzed under the same conditions and data available in the literature. The phenolic compounds were quantified by using a six-point analytical curve of 5-caffeoylquinic acid (5 to 200 μg/mL). The analytical curve was linear ($r^2 = 0.999$), the limit of detection was 2 μg/mL and the limit of quantification was 7 μg/mL.

2.6. ROS scavenging capacity

The assays were carried out in a microplate reader (Synergy Mx, BioTek, Vermont, USA) for fluorescence, UV-Vis and luminescence measurements, equipped with a thermostat and dual reagent dispenser. To avoid the degradation of the bioactive compounds, the experiments were conducted immediately after weighing the lyophilized phenolic extract or maximum after 24 h of carotenoid extraction. Two control assays were conducted in all microplates, one of them to verify the interaction among the probe and the extract solution, without radical generator or reactive species addition, and the other one as quality analytical control (positive control), adding a compound with known capacity to scavenge the specific reactive species. No interaction between the probes and the extract compounds was verified and the maximum variation in the response of the positive controls during the assays was less than 10%. Each ROS scavenging assay corresponds to two independent experiments, performed in triplicate. Except for peroxyl radical scavenging capacity, the results are presented as percent of inhibition (IC_{50} values) calculated by non-linear regression analysis using the GraphPad Prism 5.03 (Graph Pad Software Inc., San Diego, USA) software.

2.6.1. Peroxyl radical scavenging assay (for hydrophilic compounds)

The $ROO^{\bullet}$ scavenging capacity was measured by monitoring the effect of the phenolic extract or standard (5-caffeoylquinic acid and trolox) on the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of fluorescein (Ou, Hampsch-Woodill & Prior, 2001). $ROO^{\bullet}$ was generated by thermodecomposition of AAPH at 37 °C. Reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 200 μL): fluorescein (61 nM), AAPH solution (19 mM) and phenolic extract

(1.25, 6.25, 12.5, 25 and 50 μg/mL) or 5-caffeoylquinic acid (0.35, 0.53, 0.7 and 1.4 μg/mL) in 75 mM phosphate buffer (pH 7.4). The mixture was preincubated in the microplate reader during 10 min before AAPH addition. The fluorescence signal was monitored every min during 60 min (emission: 528 ± 20 nm, excitation: 485 ± 20 nm). The $ROO^{\bullet}$ scavenging capacity was calculated according to Ou et al. (2001) and expressed as μmol trolox equivalent (TE)/mg of sample. Trolox was used as positive control (Net area (2 μM) = 7.4 ± 0.9; Net area (4 μM) = 13.1 ± 1.1 and Net area (8 μM) = 22.6 ± 1.4).

2.6.2. Peroxyl radical scavenging assay (for lipophilic compounds)

The $ROO^{\bullet}$ scavenging capacity was measured by monitoring the effect of the carotenoid extract or α-tocopherol standard on the fluorescence decay resulting from $ROO^{\bullet}$-induced oxidation of C_{11}-$BODIPY^{581/591}$ (Rodrigues, Mariutti & Mercadante, 2012). $ROO^{\bullet}$ was generated by thermodecomposition of AIBN at 41°C. Reaction mixtures in the wells contained the following reagents at the indicated final concentrations (final volume of 225μL): 0.18μM C_{11}-$BODIPY^{581/591}$ in DMSO, 195 mM AIBN in DMSO/MTBE (10:1, *v/v*) and the carotenoid extract dissolved in DMSO (2.4, 3.2, 6.1, 12.1 and 15.8 μM). The fluorescence was measured until 120 min. The $ROO^{\bullet}$ scavenging capacity was calculated according to Rodrigues et al. (2012). α-Tocopherol was used as positive control (Net area (56μM) = 8.02 ± 0.13).

2.6.3. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging capacity was measured by monitoring the effect of the phenolic extract or standard (5-caffeoylquinic acid and ascorbic acid) on the luminescence

increase resulting from H_2O_2-induced oxidation of lucigenin (Gomes et al., 2007). Reaction mixtures contained the following reagents at final concentrations (final volume of 300 μL): 50 mMTris-HCl buffer (pH 7.4), 0.8 mM lucigenin in Tris-HCl buffer, 1% (w/w) H_2O_2 and phenolic extract (10, 40, 80, 120, 160, 200, 400 and 800 μg/mL) or 5-caffeoylquinic acid (43, 86, 173, 346, 691 and 1000 μg/mL) dissolved in Tris-HCl buffer. The chemiluminescence signal was measured in the microplate reader after 5 min of incubation at 37 °C. Ascorbic acid was used as positive control (IC_{50} = 171 μg/mL).

2.6.4. Hypochlorous acid scavenging assay

The HOCl scavenging capacity was measured by monitoring the effect of the extract or standard (5-caffeoylquinic acid and trolox) on the fluorescence increase resulting from HOCl-induced oxidation of DHR to rhodamine 123 (Gomes et al., 2007). HOCl was prepared by adjusting the pH of a 1% (w/v) solution of NaOCl to 6.2, with 10% H_2SO_4 (v/v). The concentration of HOCl was determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} and further dilutions were made in 100 mM phosphate buffer (pH 7.4). Reaction mixtures contained the following reactants at the indicated final concentrations (final volume of 300 μL): DHR (5 μM), HOCl (5 μM) and phenolic extract (1.7, 4.2, 8.3, 16.7, 33.3, 66.7 and 100 μg/mL) or 5-caffeoylquinic acid (12, 24, 48, 96, 192 and 384 μg/mL) dissolved in phosphate buffer (pH 7.4). The fluorometric measurements (emission at 528 ± 20 nm, excitation at 485 ± 20 nm) were performed at 37 °C. Trolox was used as positive control (IC_{50} = 134 ± 8 μg/mL).

3. Results and Discussion

3.1. Carotenoids and ROO• scavenging capacity

Seventeen carotenoids from mana-cubiu were separated by HPLC (**Fig. 2**) and 14 carotenoids were identified based on the combined information obtained from chromatographic elution on C_{30} column, UV-vis and mass spectra characteristics **(Table 1)**. The MS/MS fragments, characteristic of the polyenic chain and functional groups, allowed the confirmation of the assigned protonated molecules. Considering that a detailed description of carotenoid identification using the above information was already reported (De Rosso & Mercadante, 2007; Van Breemen, Dong & Pajkovic, 2012), only some of the most important aspects are discussed below.

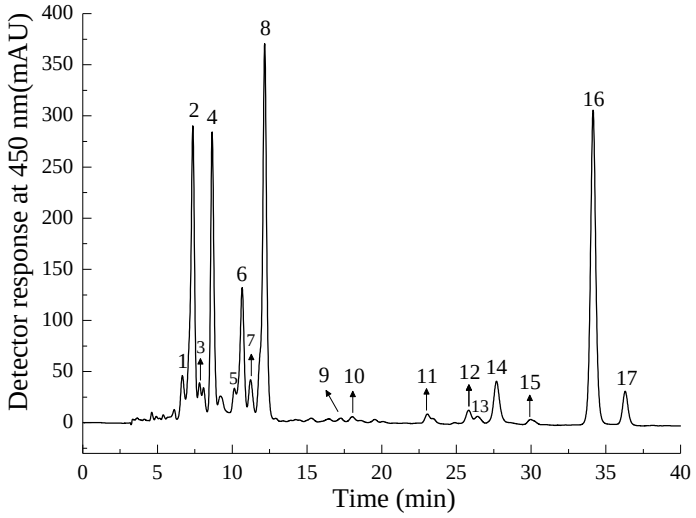


Fig. 2 Chromatogram, obtained by HPLC-DAD, of carotenoids from mana-cubiu. Chromatographic conditions: see text. Peak characterization is given in **Table 1**.

Table 1. Chromatographic, UV-Vis, mass spectroscopy characteristics and content (dry basis) of carotenoids from *Solanum sessiflorum* pulp, obtained by HPLC-DAD-APCI-MS/MS.

peak[a]	Carotenoid	concentration (μg/g pulp)	tr[b] range (min)	λmax (nm)[c]	% III/II	% A_B /A_{II}	$[M+H]^+$ (*m/z*)	fragment ions (*m/z*)
1	9'-*cis*-neoxanthin[1]	0.05 ± 0.01	6.6-6.7	330, 415, 440, 468	64	31	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 547[M+H-18-18-18]$^+$, 509[M+H-92]$^+$, 393, 221
2	all-*trans*-violaxanthin[2]	0.98 ± 0.10	7.3-7.4	415, 438, 468	92	0	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 509[M+H-92]$^+$, 221, **181**
3	mixture 1[4]	0.03 ± 0.00	7.9-8.0	328, 422, 440, 462	n.c.[d]	16	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 509[M+H-92]$^+$, 221
4	all-*trans*-luteoxanthin[2]	1.30 ± 0.06	8.6-8.7	398, 421, 448	96	0	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 509[M+H-92]$^+$, 221, **181**
5	mixture 2[4]	0.35 ± 0.01	10.0-10.1	381, 401, 425	175	0	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 509[M+H-92]$^+$, 221
6	9-*cis*-violaxanthin[2]	0.55 ± 0.03	10.5-10.6	324, 412, 434, 463	85	10	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 545[M+H-56]$^+$, 509[M+H-92]$^+$, 221, 181
7	*9-cis*-luteoxanthin[2]	1.33 ± 0.06	11.1-11.2	302, 396, 417, 443	90	0	601	583[M+H-18]$^+$, 565[M+H-18-18]$^+$, 545[M+H-56]$^+$, 509[M+H-92]$^+$, 221
8	all-*trans*-lutein[3]	2.41 ± 0.09	12.0-12.1	422, 444, 472	57	0	569	551[M+H-18]$^+$, 533 [M+H-18-18]$^+$, 477 [M+H-92]$^+$
9	5,8-epoxy-β-cryptoxanthin[2]	0.04 ± 0.00	17.2-17.3	401, 428, 452	50	0	569	551[M+H-18]$^+$, 459[M+H-18-92]$^+$, 221
10	zeinoxanthin[4]	0.09 ± 0.00	18.0-18.1	420, 445, 472	75	0	553	535[M+H-18]$^+$, 496
11	all-*trans*-β-criptoxanthin[4]	0.08 ± 0.00	22.9-23.0	418, 450, 476	33	0	553	535[M+H-18]$^+$, 495, 461 [M+H-92]$^+$,
12	mixture 3[4]	0.26 ± 0.09	25.7-25.8	405, 427, 452	100	0	553	535[M+H-18]$^+$, 205
13	15-*cis*-β-carotene[4]	0.08 ± 0.00	26.3-26.4	337, 422, 449, 473	n.c.	n.c.	537	444 [M-92]
14	13-*cis*-β-carotene[4]	0.78 ± 0.02	27.6-27.7	337, 418, 444, 470	20	47	537	444 [M-92]
15	all-*trans*-α-carotene[3]	0.05 ± 0.00	29.8-29.9	420, 445, 473	64	0	537	481 [M+H-56]$^+$
16	all-*trans*-β-carotene[4]	7.15 ± 0.21	34.0-34.1	421, 451, 478	25	0	537	444 [M-92]
17	9-*cis*-β-carotene[4]	0.57 ± 0.02	36.2-36.3	331, 420, 446, 472	33	6	537	457 [M+H-80]$^+$, 445 [M+H-92]$^+$, 399[M -137]$^+$, 400[M+H-137]$^+$
	Total carotenoids (μg/g freeze-dried pulp)	16.1± 0.50						
	Total carotenoids (μg/g fresh pulp)	1.45± 0.05						
	Vitamin A value (μg RAE/g freeze-dried pulp)[e]	**0.88 ± 0.03**						

[a] Numbered according to the chromatogram shown in **Figure 2**. [b] Retention time on the C_{30} column. [c] Linear gradient of methanol/MTBE. [d] n.c. = not calculated. [e] RAE = retinol activity equivalent.

The peaks were quantified as equivalent of 9'-*cis*-neoxanthin[1], violaxanthin[2], lutein[3] and β-carotene[4].

Peak 4 was tentatively identified as all-*trans*-luteoxanthin, based on the UV-vis spectra features, protonated molecule $[M+H]^+$ at *m/z* 601 and mass fragments in MS/MS at *m/z* 583 $[M+H-18]^+$, 565[M+H-18-18] and 509[M+H-92], corresponding to the loss of one hydroxyl group, two hydroxyl groups and toluene from the polyene chain, respectively. Moreover, the detection of a mass fragment at *m/z* 221 in the MS/MS spectrum indicated the presence of an epoxy group in the β-ionone ring with a hydroxyl substituent.

Peak 10 was tentatively identified as all-*trans*-zeinoxanthin. To differentiate between this carotenoid and its isomer all-*trans*-α-cryptoxanthin, the relation between the signal intensity of the protonated molecule at *m/z* 553 and the fragment at *m/z* 535 $[M+H-18]^+$ was compared. Zeinoxanthin mass spectrum showed higher intensity of the protonated molecule (*m/z* 553) as compared to the mass fragment at *m/z* 535$[M+H-18]^+$, whilst the contrary was observed for α-cryptoxanthin, as previously reported in the literature (De Rosso & Mercadante, 2007; Faria, De Rosso & Mercadante et al., 2009).

As far as we are concerned, this is the first report on the carotenoid composition of mana-cubiu. The major carotenoids found were all-*trans*-β-carotene, all-*trans*-lutein, all-*trans*-violaxanthin, 9-*cis*-violaxanthin and all-*trans*-luteoxanthin. All-*trans*-β-carotene and all-*trans*-lutein represented 60% (w/w) of the total carotenoid content and 25% (w/w) were epoxy carotenoids. Due to the considerable amount of carotenoids possessing epoxy groups, the carotenoid extraction was performed with and without $NaHCO_3$ addition in both fresh and freeze-dried mana-cubiu pulps and no difference was verified among the obtained carotenoid profiles.

The carotenoid composition of mana-cubiu showed to be similar to the one of other fruits belonging to the genus *Solanum*, such as naranjilla *(Solanum quitoense Lam.*), which major carotenoids are all-*trans*-lutein (45 to 55.0%) and all-*trans*-β-carotene (13.0 to 21%),

and potato (*Solanum tuberosum*), which presented a considerable amount of epoxy carotenoids (Breithaupt & Bamedi, 2002). However, a marked difference was noticed between mana-cubiu and tomato (*Solanum lycopersicum*), which major carotenoid is lycopene (Vogel, Tieman, Sims, Odabasi, Clark & Klee, 2008).

A carotenoid possesses vitamin A activity when it has at least one unsubstituted β-ionone ring bonded to the polyene chain with at least eleven carbons. Thus, to calculate the vitamin A activity of mana-cubiu (0.88 μg RAE/g pulp), the following carotenoids were considered: all-*trans*, 9-*cis*, 13-*cis* and 15-*cis*-β-carotene, α-carotene and β-cryptoxanthin. Mana-cubiu showed higher vitamin A activity than that of piquiá (0.06 μg RAE/g pulp) (Chisté et al., 2012a) but lower than those of other Amazonian fruits (3.44 to 36.4 μg RAE/g pulp) (De Rosso & Mercadante, 2007).

The carotenoid extract from mana-cubiu was able to scavenge $ROO^{\bullet}$ and the net AUC values were linearly dependent on the carotenoid concentration ($R^2 > 0.99$, $p<0.05$) **(Fig. 3)**. Mana-cubiu has a low total carotenoid content (16.1 ± 0.50 μg/g dry pulp) as compared to other Amazonian fruits, such as mamey (318.03 ± 25.90 μg/g dry pulp) and peach palm (52.50 ± 2.22 μg/g dry pulp) (Rodrigues et al., 2012). However, the carotenoid extract from mana-cubiu showed a $ROO^{\bullet}$ scavenging capacity of 9.83 ± 0.21 (Table 3), which was almost 1.5 times higher than that found for the carotenoid extracts from mamey (6.90 ± 0.44) and peach palm (7.83 ± 0.21) (Rodrigues et al., 2012). The carotenoid extract from mana-cubiu was also more potent as $ROO^{\bullet}$ scavenger than authentic standards of all-*trans*-lycopene (8.67 ± 0.74), all-*trans*-β-carotene (3.24 ± 0.22) and all-*trans*-lutein (1.90 ± 0.17) (Rodrigues et al., 2012), suggesting the occurrence of synergy among the carotenoids present in the fruit extract. The synergistic effect among carotenoids was previously

reported between astaxanthin and β-carotene, astaxanthin and lycopene, and also lycopene and lutein (Liang, Tian, Yang, Zhang & Skibsted, 2009; Stahl, Junghans, De Boer, Driomina, Briviba & Sies, 1998).

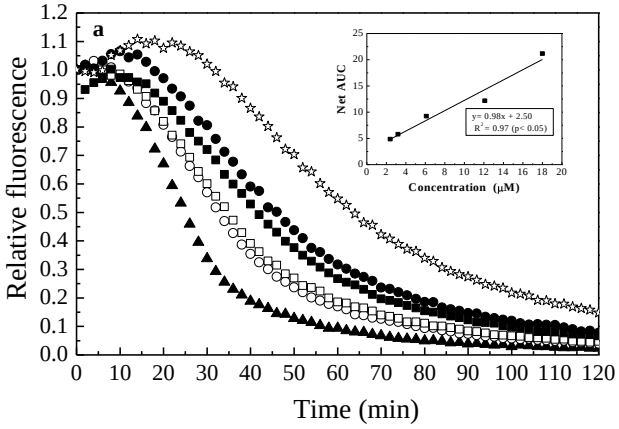


Fig. 3 Fluorescence decay of C_{11}-BODIPY$^{581/591}$ induced by peroxyl radicals in the presence of different concentrations of carotenoid extract from mana-cubiu. Legend: blank (*filled triangle*), 2.4 μM (*open circle*), 3.2 μM (*open square*), 6 μM (*filled square*), 12 μM (*filled circle*) and 16 μM (*open star*). **Inset:** linear relationship between carotenoid concentration and net AUC values from the fluorescence decay curves of C_{11}-BODIPY$^{581/591}$ oxidation.

The three main mechanisms by which carotenoids can scavenge ROO$^{\bullet}$ are electron transfer, allylic hydrogen abstraction and radical addition to the conjugated double bond system (El-Agamey et al., 2004; Jomová et al., 2009). Recently, it has been shown that the opening of the β-ionone ring and the increase of the chromophore extension in the carotenoid structure are the major factors leading to the increase of ROO$^{\bullet}$ scavenging capacity in homogeneous system (Rodrigues et al., 2012).

3.2 Phenolic compounds and ROS scavenging capacity

The phenolic compounds of mana-cubiu were determined in the freeze-dried fruit. The HPLC-DAD chromatograms were processed at 280 and 320 nm and are shown in **Fig. 4**. **Table 2** shows the tentative identification of the major compounds. The combined results of the following parameters were considered for phenolic compound identification: elution order on the C_{18} column, UV-vis spectrum features (maximum absorption wavelength (λ_{max})), spike with standard, MS spectrum characteristics as compared to standard analyzed under the same conditions and data available in the literature (Gancel, Alter, Dhuique-Mayer, Ruales & Vaillant, 2008; Clifford, Johnston, Knight & Kuhnert, 2003).

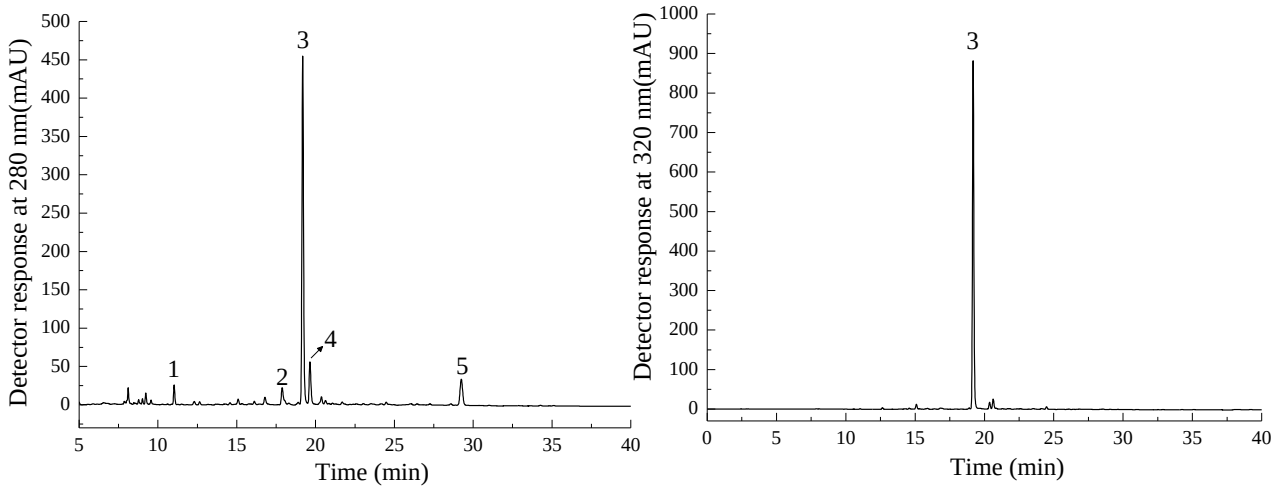


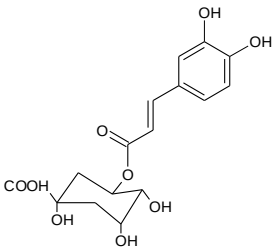
Fig. 4 Chromatograms obtained by HPLC-DAD of the phenolic compounds from mana-cubiu pulp. Chromatographic conditions: see text. Peak characterization is given in **Table 2**.

Table 2. Chromatographic, spectroscopic characteristics and content (dry basis) of phenolic compounds from *Solanum sessiflorum* pulp, obtained by HPLC-DAD-ESI-MS/MS.

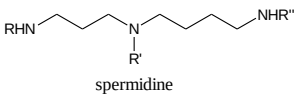
Peak[a]	Compound	concentration (μg/g dry pulp)[b]	tr range (min)[c]	λ_{max} (nm)[d]	$[M+H]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	MS/MS (-) (*m/z*)
1	not identified 1	63 ± 3	11.0-11.1	275, 298sh[e]	n.d.[f]	n.d.	n.d.
2	not identified 2	98 ± 4	17.9-18.0	277	n.d.	n.d.	n.d.
3	5-caffeoylquinic acid	1298 ± 96	18.9-20.0	300sh, 326	355	353	191
4	N^1,N^4or N^4,N^8orN^1,N^8-bis(dihydrocaffeoyl) speridine	199 ± 6	19.6-19.7	280	474	472	351, 309
5	N^1,N^4,N^8-tris(dihydrocaffeoyl) spermidine	158 ± 17	29.1-29.2	281	638	636	473
	Total phenolic compounds (μg/g freeze-dried pulp)	1816 ± 20					
	Total phenolic compounds (μg/g fresh pulp)	249 ± 5					

[a] numbered according to the chromatogram shown in F**igure 3**.[b]The phenolic compounds were quantified as 5-caffeoylquinic acid equivalent (n = 3). [c]Retention time on the C_{18}Synergi Hydro (4μm) column. [d]Solvent: gradient of 0.5 % formic acid in water and acetonitrile with 0.5 % formic acid. [e]sh: shoulder. [f]n.d.: not detected.

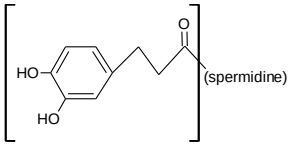
Peak 3 was identified as 5-caffeoylquinic acid (MW = 354) **(Figure 5)**, since it mass spectra obtained in the positive ionization mode showed the protonated molecule $[M+H]^+$ at *m/z* 355, the deprotonated molecule $[M-H]^-$ in the negative ionization mode at *m/z* 353 and a base peak at *m/z* 191 was observed in the MS/MS of the deprotonated molecule, corresponding to the deprotonated quinic acid (Clifford et al., 2003). Moreover, peak 3 presented the same retention time, UV-visible and MS spectra and MS/MS fragmentation pattern than that of the authentic standard of 5-caffeoylquinic acid. The identity of this compound was confirmed by co-elution with the 5-caffeoylquinic acid standard.



5-caffeoylquinic acid



spermidine



dihydrocaffeoyl-(DHC)

Compound	R	R'	R''
spermidine	-H	-H	-H
N^1,N^4-bis(dihydrocaffeoyl) spermidine	-DHC	-DHC	-H
N^4,N^8-bis(dihydrocaffeoyl) spermidine	-H	-DHC	-DHC
N^1,N^8-bis(dihydrocaffeoyl) spermidine	-DHC	-H	-DHC
N^1,N^4,N^8-tris(dihydrocaffeoyl) spermidine	-DHC	-DHC	-DHC

Fig. 5 Structures of the 5-caffeoylquinic acid and dihydrocaffeoylspermidines.

Peak 4 mass spectrum presented the protonated molecule at *m/z* 474 and the deprotonated molecule at *m/z* 472. The MS/MS spectra of the deprotonated molecule showed fragment ions at *m/z* 351 and *m/z* 308, which correspond to the loss of $C_7H_7O_2$ and dihydrocaffeoyl, respectively (Roshani & Duroy, 2006). Thus, it was tentatively identified as bis-(dihydrocaffeoyl) spermidine isomer (Gancel et al., 2008) **(Figure 5)**. Peak 5 was tentatively assigned as tris-(dihydrocaffeoyl) spermidine. This identification was based on the protonated molecule at *m/z* 638 and the deprotonated molecule at *m/z* 636 and comparison with literature data (Parr, Mellon, Colquhoun & Davies, 2005; Gancel et al., 2008).

The polyamines found in mana-cubiu are usually present in foods, especially in breast milk and meat, and are also found in plants in their free form or as hydroxycinnamic acid conjugates (Edreva, 1996). Several works report that these compounds present biological activities, such as immunologic system cell differentiation and regulation of inflammatory reactions (Larque, Sabater-Molina & Zamora, 2007).

The phenolic extract of mana-cubiu was able to scavenge $ROO^{\bullet}$, HOCl and H_2O_2 in a dose-dependent manner **(Fig. 6 and 7)** and the IC_{50} are presented in **Table 3**.

Table 3. ROS scavenging capacity of the phenolic and carotenoid extracts from mana-cubiu and other compounds.

Extracts/standard	IC_{50} (μg/mL)		$ROO^{\bullet}$	
	H_2O_2	HOCl	hydrophilic[a]	lipophilic[b]
phenolic extract	305 ± 17	13 ± 0.8	0.32 ± 0.01	nd
carotenoid extract	nd[c]	nd	nd	9.80 ± 0.80
5-caffeoylquinic acid	544 ± 10	56 ± 2.5	11.95 ± 0.31	nd
ascorbic acid	155 ± 18	0.24 ± 0.02	5.42 ± 0.30	0.87 ± 0.06
trolox	nd	134 ± 18	4.03 ± 0.30	0.90 ± 0.00
α-tocopherol	nd	nd	nd	1.00

[a]μmol trolox equivalent/mg extract or compound. [b]α-tocopherol relative. [c] nd= not determined.

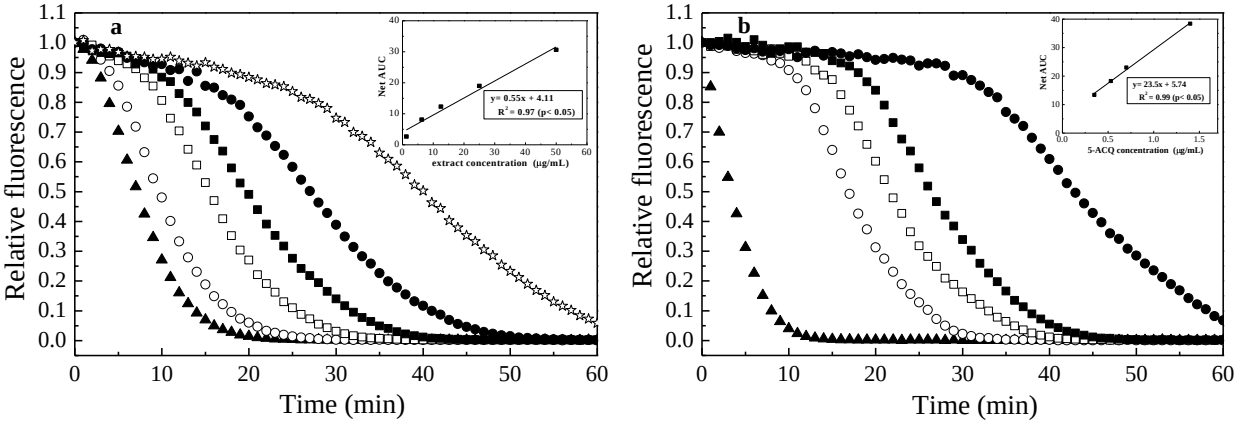


Fig. 6 Fluorescence decay of fluorescein induced by peroxyl radicals in the presence of different concentrations of **(a)** phenolic extract and **(b)** 5-caffeoylquinic acid standard. Legend **(a)**: blank (*filled triangle*), 1.25 μg/mL (*open circle*), 6.25 μg/mL (*open square*), 12.5 μg/mL (*filled square*), 25.0 μg/mL (*filled circle*) and 50.0 μg/mL (*open star*). Legend **(b)** blank (*filled triangle*), 0.35 μg/mL (*open circle*), 0.53 μg/mL (*open square*), 0.70 μg/mL (*filled square*) and 1.40 μg/mL (*filled circle*). **Inset:** linear relationship between **(a)** phenolic extract and **(b)** 5-caffeoylquinic acid concentrations and net AUC values from the fluorescence decay curves of fluorescein oxidation.

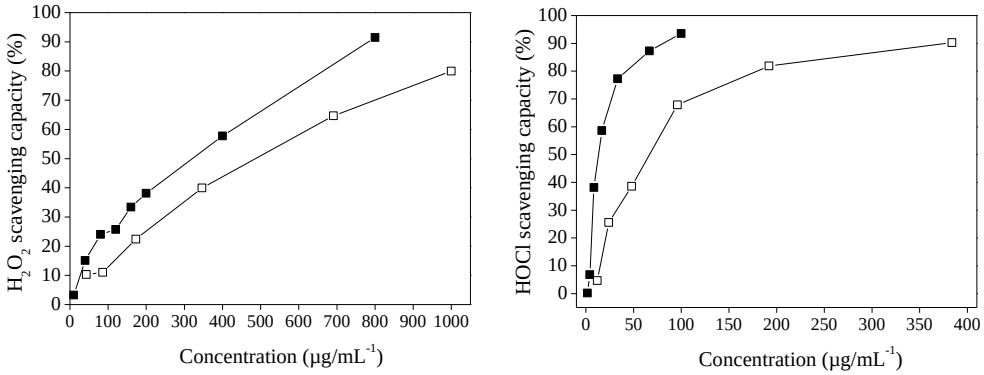


Fig. 7 Phenolic extract or 5-caffeoylquinic acid scavenging capacity of hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Legend: phenolic extract (*filled square*) and 5-caffeoylquinic acid (*open square*).

The ROO$^{\bullet}$ scavenging capacity of the phenolic extract of mana-cubiu was only 2.7% of the value presented by the 5-caffeoylquinic acid standard. Mana-cubiu was also a less potent ROO$^{\bullet}$ scavenging than other fruit extracts, such bilberry (2.64 ± 0.20 μmolTE/mg extract) and elderberry (2.22 ± 0.16 μmolTE/mg extract) (Ou et al., 2001). On the contrary, the phenolic extract of mana-cubiu showed to be a very potent scavenger of H_2O_2 and HOCl. The capacity of the mana-cubiu extract to scavenge H_2O_2 was almost twice higher than that of 5-caffeoylquinic acid. Mana-cubiu extract was also a better H_2O_2 scavenger than piquia ethanolic extract, which reached only 10% of inhibition at the concentration of 1000 μg/mL (Chisté and Mercadante, 2012b). The phenolic extract of mana-cubiu was 4- and 15-fold more potent as HOCl scavenger than 5-caffeoylquinic acid and an ethanolic extract of piquiá (199 ± 29 μg/mL) (Chisté and Mercadante, 2012b), but less efficient than ascorbic acid.

The high capacity of the mana-cubiu extract to scavenge H_2O_2 can be verified by comparing its IC_{50} value with the IC_{50} values obtained for plant extracts of well recognized

antioxidant capacity, such as tutsan (*Hypericum androsaemum*) (IC_{50}= 944 μg/mL) (Almeida, Fernandes, Lima, Costa & Bahia, 2009a), walnut (*Juglans regia*) (IC_{50}= 383 μg/mL) (Almeida, Fernandes, Lima, Costa & Bahia, 2008a), *Eucalyptus globulus* (IC_{50}= 389 μg/mL) (Almeida et al., 2009b), chestnut (*Castanea sativa*) (IC_{50}= 410 μg/mL) (Almeida et al., 2008b) and oak (*Quercus robur*) (IC_{50}= 251 μg/mL) (Almeida, Fernandes, Lima, Costa & Bahia, 2008b) extracts.

Phenolic compounds main mechanism to scavenge $ROO^{\bullet}$ involves the transfer of one H atom (HAT); however, the transfer of one electron (SET) has already been reported and more recently, the formation of adducts between the reactive species and the phenolic compound molecule was also reported (Anouar, Kosinová, Kozlowski, Mokrini, Duroux & Trouillas, 2009). The 5-caffeoylquinic acid represented more than 70% (w/w) of the phenolic compounds of the mana-cubiu hydrophilic extract and it is highly possible that it is the main responsible for its capacity to scavenge $ROO^{\bullet}$. Despite this fact, the extract presented a lower capacity to scavenge $ROO^{\bullet}$ than that of the 5-caffeoylquinic acid standard (**Table 3**), possibly because 5-caffeoylquinic acid represents only about 0.4% (w/w) of the extract total weight.

The mechanism of H_2O_2 and HOCl involves the transfer of two electrons (Winterbourn, 2008). The higher efficiency of the hydrophilic extract to scavenge H_2O_2 and HOCl as compared with that of the 5-caffeoylquinic acid standard indicates that the other compounds present in the extract, including the spermidines conjugated to caffeic acid, presents a high capacity to scavenge these two ROS. Carbohydrates, proteins and amino acids are also components of the hydrophilic extract and therefore can have a great role in the extract's scavenging capacity against H_2O_2 and HOCl, since these compounds have

been previously reported as efficient scavengers of these two ROS (Pattison & Davies, 2006).

The carotenoid and phenolic compound compositions of mana-cubiu were successfully determined by HPLC-DAD-MS/MS for the first time. Despite the low carotenoid content and vitamin A activity, the carotenoid extract from mana-cubiu was a potent $ROO^{\bullet}$ scavenger. The major phenolic compound was the 5-caffeoylquinic acid and two different poliamines (dihydrocaffeoylspermidines) were also identified. Although the phenolic extract of mana-cubiu was a less efficient $ROO^{\bullet}$ scavenger, it presented a high capacity to scavenge H_2O_2 and HOCl, possibly due to the presence of polyamines.

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CONCLUSÃO GERAL

Os resultados deste trabalho permitem afirmar que os carotenoides são capazes de desativar várias espécies reativas radicalares e não radicalares, como o $ROO^{\bullet}$, o H_2O_2, o HOCl, o $ONOO^-$ e o $HO^{\bullet}$, sendo a eficiência de desativação influenciada pela concentração e estrutura química do carotenoide, pelo nível de organização do sistema (homogêneo ou multifásico) e pelo tipo de espécie reativa.

Em sistemas homogêneos, a extensão do cromóforo e a abertura do anel β-ionona são os fatores que mais influenciam a eficiência dos carotenoides em desativarem o $ROO^{\bullet}$. A análise do comportamento do licopeno em sistemas homogêneos e em lipossomas indica que em sistemas multifásicos a distribuição e orientação dos carotenoides influencia significativamente a capacidade de desativar uma espécie reativa, o que é reforçado pelos resultados obtidos nas microcápsulas de carotenoides. A influência do tipo de espécie reativa pode ser observada através do comportamento do β-caroteno em lipossomas, onde este carotenoide apresentou eficiência pouco expressiva em desativar o $ROO^{\bullet}$, o H_2O_2, o HOCl e o $HO^{\bullet}$, porém foi o mais eficiente na desativação do $ONOO^-$.

O maná-cubiu possui o β-caroteno (7,15 μg/g) e a luteína (2,41 μg/g) como carotenóides majoritarios, e ácido o 5-cafeoilquínico (1298 μg/g) como composto fenólico principal. O estudo com extratos de maná-cubiu sugere que os carotenoides presentes nesta fruta atuam de maneira sinergística na desativação do $ROO^{\bullet}$ e também mostra que o extrato hidrossolúvel de maná-cubiu é um potente desativador de H_2O_2 e HOCl.

Finalmente, a análise dos resultados deste estudo permite afirmar que os carotenoides são capazes de desativar *in vitro* todas as ROS e RNS de relevância biológica avaliadas neste estudo. Estes resultados associados à elucidação, em um futuro próximo, do papel de cada espécie reativa na patogênese de algumas doenças ou na sinalização celular irá permitir uma melhor compreensão da ação dos carotenoides no organismo humano.