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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.

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## 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·, $H_2O_2$, HO·, 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 = $3.5 \times 10^5$ 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%.

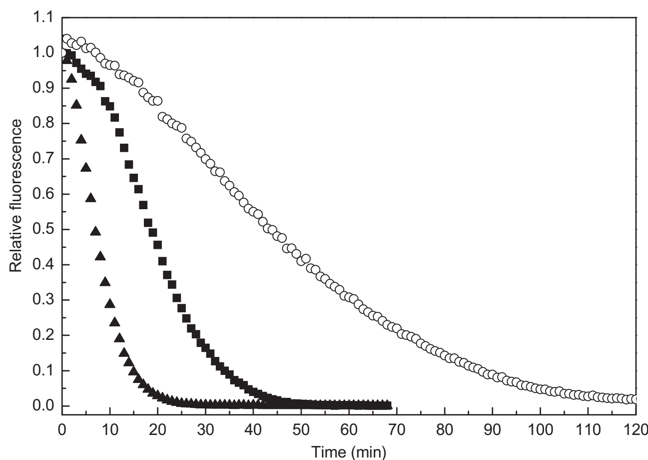


**Fig. 1.** Fluorescence decay of fluorescein induced by peroxyl radicals at 37 °C, in the presence of empty microcapsules of GA and MD, both at the same concentration (1.25 mg/ml). Blank: filled triangle, MD microcapsules: filled square, GA microcapsules: open circle.

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.

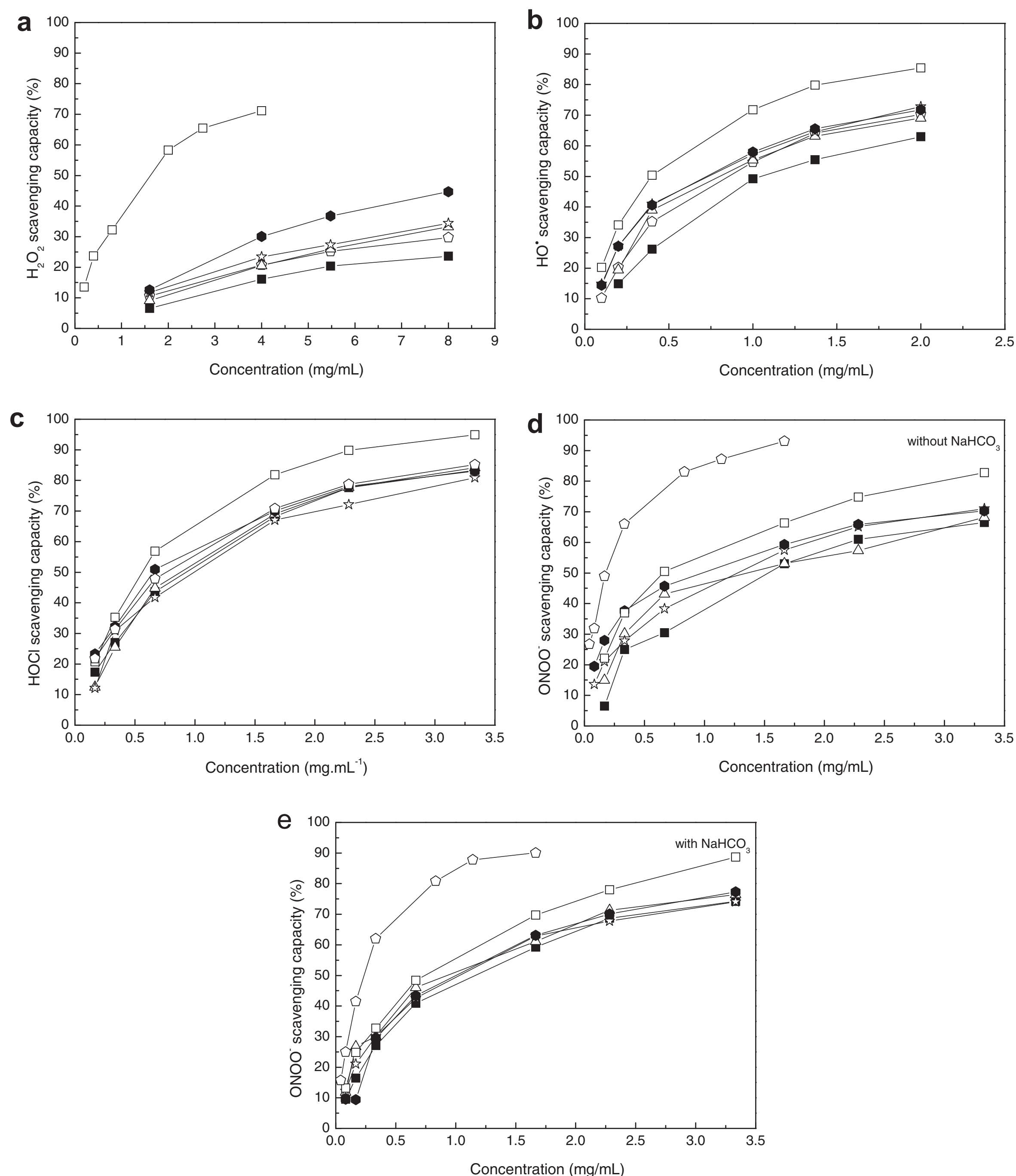


**Fig. 2.** Gum arabic microcapsules scavenging capacity of (a) hydrogen peroxide ($H_2O_2$), (b) hydroxyl radical ($HO^•$), (c) hypochlorous acid (HOCl), (d) anion peroxynitrite ($ONOO^-$) without $NaHCO_3$ and (e) $ONOO^-$ with $NaHCO_3$. Legend: filled square: empty microcapsule (no antioxidant molecule), 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

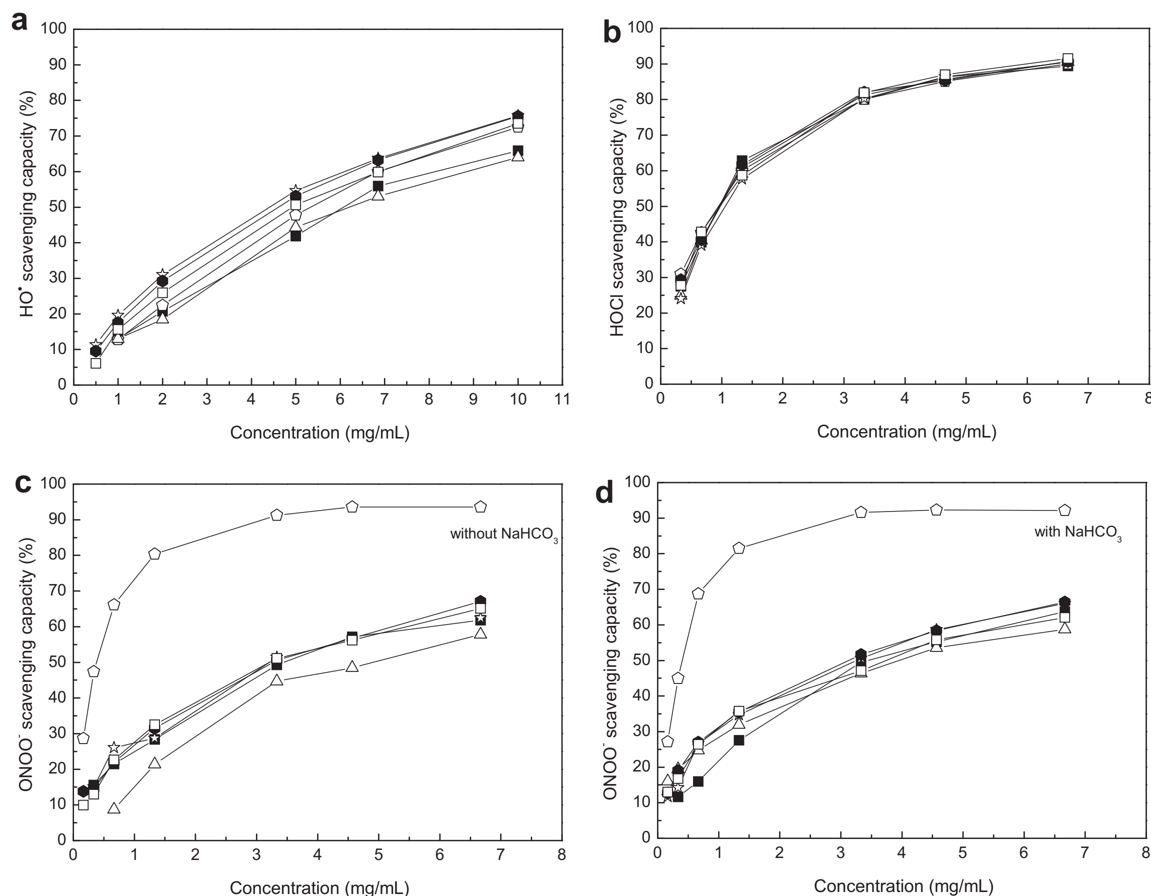


**Fig. 3.** Maltodextrin microcapsules scavenging capacity of (a) hydroxyl radical ($HO^{\cdot}$), (b) hypochlorous acid (HOCl), (c) anion peroxynitrite ($ONOO^-$) without $NaHCO_3$ and (d) $ONOO^-$ with $NaHCO_3$. Legend: filled square: empty microcapsule (no antioxidant molecule), open triangle: tocopherol 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 $ROO^{\cdot}$ 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 $HO^{\cdot}$ 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 $ROO^{\cdot}$, $H_2O_2$ 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 $ROO^{\cdot}$, $HO^{\cdot}$, 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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