Emirates Journal of Food and Agriculture. 2017. 29(11): 833-845
doi: 10.9755/ejfa.2017.v29.i11.1479
http://www.ejfa.me/

REGULAR ARTICLE

Identification and antioxidant activity of carotenoids from superfine powder of *Rhodobacter Sphaeroides*

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ABSTRACT

The interest in carotenoids from the natural-antioxidant point of view has recently risen sharply because of their substantial health benefits. Here, we report the identification and antioxidant activity of carotenoids extracted by superfine grinding from *Rhodobacter sphaeroides* 3757 which is a species of photosynthetic bacteria. After separation and purification by silica gel column chromatography and reversed-phase high performance liquid chromatography (RP-HPLC), the four major carotenoids from the superfine powder of *R. sphaeroide* 3757 were identified as bixin, hydroxyspheroidenone, 3,3,4¢-tetrahydrospirilloxanthin-20-al, and spheroidenone by reversed phase - high performance liquid chromatography - atmospheric pressure chemical ionization - mass spectrometry (RP-HPLC-APCI-MS). The antioxidant activity of the carotenoids extracted after superfine grinding of dry biomass of *R. sphaeroide* 3757 was higher than that after ultrasonic treatment. When the ratio solvent-to-solid was 30, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, reducing power, and lipid peroxidation inhibitory activity of the extracts after superfine grinding were 74.0% ± 3.1%, 0.497 ± 0.022, and 77.6% ± 3.2%, respectively. By contrast, the extracts after ultrasonic treatment, these numbers were 61.0% ± 2.5%, 0.328 ± 0.014, and 55.2% ± 2.3%, respectively. These results indicate that carotenoids from *R. sphaeroide* 3757 show a significant antioxidant activity *in vitro* in a concentration-dependent manner, and that superfine grinding is the optimal extraction method. We hope to provide scientific guidance for commercial production of natural- antioxidant and functional food from carotenoids of *R. sphaeroide.*

Keywords: *Rhodobacter sphaeroides*; Carotenoids; Superfine grinding; HPLC-DAD-APCI-MS; Antioxidant activity

INTRODUCTION

Carotenoids represent a vital class of lipid-soluble functional compounds and are widely distributed in plants, animals and microbes (Liu et al., 2014). Carotenoids have been described as possessing several important functional properties, mainly an antioxidant activity as well as prevention of cardiovascular diseases, cancer, and macular degeneration and in some cases, provitamin A activity (Giuffrida et al., 2013; Kljak and Grbeša, 2015). These properties make carotenoids ideal for the ever growing functional food industry as well as for promotion of the consumption of natural products in which these compounds are present (Giuffrida et al., 2013). Currently, most of the carotenoids sold on the market are synthesized chemically and cannot meet consumers' demand for natural carotenoids. Thus, researchers shifted attention from chemical synthesis to isolation of carotenoids from biological sources (Yoo et al., 2016). Therefore, commercial production of carotenoids using carotenoid-producing microorganisms has been taken into consideration owing to the highly efficient and easy manipulation of processing schemes (Liu et al., 2015; Yoo et al., 2016). Some studies have been reported in the last few years on carotenoid synthesis by microorganisms (Chiu and Liu, 2014; Liu et al., 2015; Yoo et al., 2016).

Rhodobacter sphaeroides, being highly versatile, has attracted considerable attention in energy, environment, breeding, food and pharmaceutical industries because it can grow under either aerobic or anaerobic respiration, fermentation, or photosynthesis conditions (Chiu and Liu, 2014). *R. sphaeroides* is a species of photosynthetic bacteria that are nontoxic. A commercial carotenoid product from the extract of probiotic

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Received: 01 May 2017; **Revised:** 25 October 2017; **Accepted:** 29 October 2017; **Published Online:** 5 November 2017

R. sphaeroides mutant strain WLAPD911 has been used to enhance tilapia growth performance via immune regulation **(**Li et al., 2016**)**. In light of the impact of carotenoids on human health, it is crucial to devise an appropriate technique for quantification of various carotenoids in *R. sphaeroides* **(**Liu et al., 2014**)**. Liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) is becoming more and more popular as a tool for identification of bioactive substances because of its high specificity and sensitivity as well as excellent separation of target compounds **(**Wang et al., 2012; Sánchez et al., 2016). Atmospheric pressure chemical ionization(APCI) has become the most widely used ionization technique for carotenoids and shows high sensitivity for their analysis **(**Rivera and Canela-Garayoa, 2012**)**. Nevertheless an analysis of carotenoids in *R. sphaeroides* by high performance liquid chromatography – photo diode array detection – atmospheric pressure chemical ionization - mass spectrometry (HPLC-DAD-APCI-MS) has not been conducted so far.

After superfine grinding, the solubility of nutritive components increases, and they are easily absorbed by the human body (Lee et al., 2013). In recent years, the possible use of micro- or nanotechnology in food research attracted much attention and became the focus of research in many countries (Zhu et al., 2010; Lee et al., 2013). While research on this topic is limited (Zhao et al., 2015; Ramachandraiah and Chin, 2016). Many studies have revealed that oxidative stress plays an important role in the pathogenesis of various chronic diseases, such as diabetes, cancer, and cardiovascular diseases, and it has been suggested that antioxidants perform a protective function against various diseases by defending cells against oxidative damage (Ma et al., 2016; Venetsanou et al., 2017). More recently, growing interest has been focused on the natural antioxidant compounds present in plants, animals, and even microbes (Ramachandraiah and Chin, 2016; Sequeda-Castañeda et al., 2016; Silva and Lidon. 2016; Manzano et al., 2017). So far, the antioxidant activities of the carotenoids extracted from superfine powder of *R. sphaeroides* remain unknown.

The aim of this study was to explore the identification of major carotenoids of superfine powder from *R. sphaeroides* by HPLC-DAD- APCI-MS. We also compared the effects of superfine grinding technology and of ultrasonic technology on the antioxidative properties of carotenoids from *R. sphaeroides*.

MATERIALS AND METHODS

Materials and reagents

A strain of photosynthetic bacterium *R. sphaeroides 3757* was stored by the China General Microbiological Culture Collection Center, CGMCC No. 3757.

Most of the solvents and chemicals used here were of analytical grade, and were purchased from Beijing Chemical Reagents Company. Biological reagents were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd.. Acetonitrile (MeCN), methanol (MeOH), and methyl tert-butyl ether (MTBE) used during HPLC analysis were of HPLC grade, and were purchased from Dikma Technologies, Beijing, China. Silica gel GF254 for TLC was obtained from Qingdao Haiyang Chemical Company, Quingdao, China.

Preparation of dry biomass from *R. sphaeroides*

For the seed culture, *R. sphaeroides* 3757 was inoculated into the seed medium for 24 h and shaken at 180 ×g and 32 °C. The composition of the seed medium was as follows (g/L): Glucose 20, yeast extract powder 10, tryptone 10, sodium chloride 5. The fermentative medium was composed of the following substances (g/L): sodium malate 4, glucose 20, $(NH_4)_2SO_4$ 7, yeast extract powder 10, K_2HPO_4 0.9, KH_2PO_4 0.6, a growth factor solution (10 mL) consisting of vitamin B_1 1.0 (g/L), vitamin PP 1.0 (g/L), and vitamin H 0.016 (g/L). After inoculation with 10% (v/v) of the seed liquid, the cultures were incubated for 40 h at 180 ×g and 32 °C. After centrifuged at 7000 ×g for 10 min, the sediment was collected and washed twice with distilled water, then dried in a Laboratory freeze-dryer (Biocool Company , China) for 72 h at -50 °C and pressure <1 Pa.

Superfine grinding and ultrasonic treatment

The dry biomass of *R. sphaeroides* 3757 was ground into superfine powder at 4 °C for 8 h in the superfine grinder (Taijihuan company, China). 5 g of a superfine powder sample was mixed with acetone in different ratios of solvent to solid in a flask and ultrasonically processed in an ultrasonic cleaner (25 °C) for 1 h.

After the dry biomass of *R. sphaeroides* was mixed with acetone in a 10-mL container, the carotenoids were extracted by an ultrasonic crasher (Sonics & Materials Inc. USA) in various ratios of solvent to solid. And the ultrasonic amplitude, work/rest time, and duration were set to 30%, 30s/30s, and 10 min, respectively. Then the sample was incubated in an ultrasonic cleaner (25 °C, 1 h).

Finally, the samples were centrifuged at 12000 ×g for 10 min to obtain the supernatant containing carotenoids.

Extraction and separation of carotenoids

The carotenoids were extracted from 5 g superfine powder of *R. sphaeroides* 3757 as described above with the ratio of solvent to solid at 25. The extraction procedure was repeated until the supernatant became colorless. Then all the supernatants were pooled and concentrated in a rotary

evaporator (T < 30 °C), flushed with N_2, and kept at −20 °C in the dark until separation. All the extraction procedures were conducted in triplicate.

The concentrated extract was applied to a silica gel column, and carotenoids were eluted using increasing percentages of acetone in petroleum ether. Thirteen fractions were collected according to thin layer chromatography (TLC) analysis with a mixture of petroleum ether : Acetone (10:1 v/v) as the developing agent. The fractions were evaporated to dry, dissolved in acetone, passed through a 0.22-μm membrane filter for further analysis.

Further separation of carotenoids was performed using an Agilent 1260 Series system equipped with a DAD. The chromatographic analysis was conducted on a YMC carotenoid C30 column (250 mm × 4.6 mm internal diameter, i.d.)(Waters, USA). The injection volume was 20 μL. The mobile phase was MeCN : MeOH (solution A) (3 : 1, v/v) and MTBE (solution B). Samples were initially eluted with 100% of A, changing by gradient to 20% of A and 80% of B in 20 min. The flow rate was 1 mL/min. DAD detection was performed at 480 nm.

The 300–650 nm absorbance spectra of the main carotenoids were acquired by means of a Waters 1525 HPLC system and 2996 PDA detector (Waters, USA).

HPLC-APCI-MS/MS analysis

LC-MS was performed on a Thermo LTQ XL mass spectrometer (Thermo Fisher, USA) connected to an Agilent 1200 HPLC system and DAD detector (Agilent, USA). The HPLC analytical method for carotenoids has been described above. Carotenoids were detected with APCI in the positive-ionization mode. Source conditions were set as follows: Capillary temperature 300 °C, source voltage 4.5 kV, and capillary voltage 25 V. Nitrogen was utilized as the sheath gas and set to 50 arb for the positive mode. Nitrogen also served as an auxiliary gas and sweep gas, set to 5 arb, respectively. Full scan (100−1000) and ion-trap MS/MS of the most intense ion from the parent mass list were carried out using CID with a normalized collision energy of 35. Identification of major carotenoids from *R. sphaeroides* 3757 was based upon the chromatographic elution on a C30 HPLC column, spectrum characteristics, and mass spectral characteristics.

DPPH radical-scavenging activity

The (2,2-diphenyl-1-picrylhydrazyl) DPPH inhibition by carotenoids extracted from dry biomass of *R. sphaeroides* 3757 after superfine grinding or ultrasonic treatment was measured according to the method reported previously(Xia et al., 2012; Zhuang et al., 2013).

Reducing power

The reducing power of carotenoids was assayed according to the method reported elsewhere (Xia et al., 2012).

Lipid peroxidation-inhibitory activity

This activity of carotenoids was measured in a linoleic acid emulsion system according to the method reported previously (Zhuang et al., 2013).

Statistical analysis

The statistical software SPSS18.0 (IBM, USA) was used. Means and standard deviations were calculated.

RESULTS

Particle size

Small-angle X-ray scattering (SAXS) analysis is an effective method by which the particle size distribution of an ultrafine powder in a sample can be determined (Zhu et al., 2010**)**. All the particle sizes of superfine powder from *R. sphaeroides* 3757 determined by the SAXS method were below 620 nm, while 87.8% of the particle sizes were below 300 nm. These results revealed that pulverization by high-energy-nano-ball-milling can effectively reduce sizes of the particles of *R. sphaeroides* 3757 to a submicron range to manufacture ultrafine powder. Similarly, it has been reported that the particle size of nanopowdered ginseng (NPG) is in the range 600 to 1000 nm (Lee et al., 2013). Therefore, carotenoids of superfine powder from *R. sphaeroides* 3757 can be easily dispersed into a solution, and the yield considerably increased.

Separation of the major carotenoids

It has been well documented that a C30 column is superior to C18 column in terms of simultaneous separation of both positional and geometrical isomers of carotenoids because of greater hydrophobic interaction between carotenoid isomers and the C30 stationary phase (Liu et al., 2014). Due to the complexity of carotenoids in photosynthetic bacteria, a gradient solvent system consisting of acetonitrile/methanol (3:1, v/v) and MTBE (as described above) was developed on the basis of a RP-C30 stationary phase, and four main carotenoids from *R. sphaeroides 3757* were completely resolved within 20 min. The representative chromatograms of the four major carotenoids are illustrated in Supplemental Fig. 1 and Table 1, and their retention times were 17.571, 16.689, 8.432, and 15.480 min, respectively.

Structure elucidation of major carotenoids

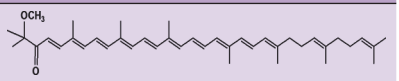
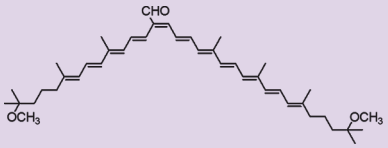
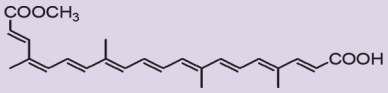
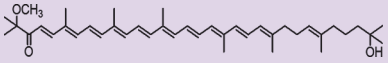
The chromatographic elution on a C30 HPLC column, spectrum characteristics, and mass spectral characteristics are shown in (Supplemental Figs. 1-6 and Table 1). The structure elucidation of unknown major carotenoids was

Table 1: Chromatographic and UV–vis spectra and mass characteristics of carotenoids from *R. sphaeroides* 3757 obtained by RP-HPLC-APCI-MS/MS

Carotenoid	RT (min)[a]	λ_{max} (nm)[b]	$[M+H]^+$(m/z)	MS(+)(m/z)[c]	MS^2(+)(m/z)[d]
Spheroidenone	17.571	376,485	583	551[M+H-32],491[M+H-92], 477[M+H-106]	551[M+H-32]
3,4,3',4'-Tetrah-ydrospirilloxant-hin-20-al	16.689	370,485	615	583[M+H-32],551[M+H-32-32], 509[M+H-106],477[M+H-106-32], 445[M+H-32-32-106], 385[M+H-32-92-106]	583[M+H-32]
Bixin	8.432	358,460,486	395	363[M+H-32]	377[M+H-18],363[M+H-32], 345[M+H-32-18], 335[M+H-32-28], 317[M+H-32-28-18], 289[M-106]
Hydroxysphero-idenone	15.480	375,484	601	583[M+H-18],569[M+H-32], 509[M+H-92,495[M+H-106], 477[M+H-18-106], 385[M+H-18-92-106]	583[M+H-18]

[a]Retention time on the C30 column, [b]Linear gradient of acetonitrile-methanol (3:1)/MTBE, [c]In MS, the most abundant ion is shown in boldface, [d]In MS/MS, the most abundant ion is shown in boldface

Table 2: Formulas and chemical structures of carotenoids from *R. sphaeroides* 3757

Carotenoid	Formula	Chemical structure
Spheroidenone	$C_{41}H_{58}O_2$	
3,4,3',4'-Tetrahydro-spirilloxanthin-20-al	$C_{42}H_62O_3$	
Bixin	$C_{25}H_{30}O_4$	
Hydroxyspheroidenone	$C_{41}H_{60}O_3$	

undertaken according to their chromatographic behaviors (such as retention time), electronic absorption spectral characteristics (e.g. the maximum absorption wavelength and spectral shapes) and mass spectral characteristics (e.g. the m/z value of parent ions and the pattern of fragmentation), in comparison with those of published data (Sánchez et al., 2016).

Spheroidenone (Rt = 17.571 min): This is a major carotenoid produced by *R. sphaeroides* during anaerobic or aerobic growth (Yeliseev and Kaplan, 1997; Chi et al., 2015). The identification of this carotenoid (Rt = 17.571 min) was based on the UV–visible spectrum characteristics (Table 1). This carotenoid has a UV–visible spectrum with λ_{max} at 485 nm Supplemental Fig. 2; the shape of the spectrum was similar to that of spheroidenone (Takaichi et al., 1991). Furthermore, this carotenoid was identified as spheroidenone by comparing the mass spectra with those given in the literature (Enzell et al., 1969). The mass spectra of this carotenoid obtained in the positive ion mode (Table 1) showed a protonated molecule at m/z 583 and fragment ions in the MS/MS at m/z 551 $[M + H-32]^+$, m/z 491 $[M + H-92]^+$, and m/z 477 $[M + H-106]^+$ Supplemental Fig. 3, corresponding to the loss of one methoxy group, toluene group, and xylene from the polyene chain, respectively.

3,4,3',4'-Tetrahydrospirilloxanthin-20-al (Rt = 16.689 min): This carotenoid was identified considering the UV–visible and mass spectral characteristics. In the positive ion mode (Table 1), the mass spectra showed a protonated molecule at *m/z* 615 and fragments at m/z 583 $[M+H-32]^+$, m/z 551 $[M+H-32-32]^+$, m/z 509 $[M+H-106]^+$, m/z 477 $[M+H-106-32]^+$, m/z 445 $[M+H-32-32-106]^+$, and m/z 385 $[M+H-32-92-106]^+$ Supplemental Fig. 4, corresponding to the loss of one methoxy group, two methoxy groups, xylene, one methoxy group + xylene, and two methoxy groups + xylene from the polyene chain, respectively. These mass spectral characteristics of 3,4,3',4'-tetrahydrospirilloxanthin-20-al have already been reported in the literature (Francis and Liaaen-Jensen, 1970).

Bixin (Rt = 8.432 min): This carotenoid was identified by comparing the UV–visible spectrum (λ_{max} at 358, 460, 486 nm) (Supplemental Fig. 2 and Table 1) with that given in the database. In the positive ion mode (Table 1), the mass spectra showed a protonated molecule at *m/z* 395 and the most abundant fragment ion in the MS/MS spectrum at m/z 363 ($[M + H-32]^+$), corresponding to cleavage of the methoxy group. The mass spectra showed the fragment ions m/z 377 $[M+H-18]^+$, m/z 363 $[M+H-32]^+$, m/z 345 $[M+H-32-18]^+$, m/z 335 $[M+H-32-28]^+$, m/z 317 $[M+H-32-28-18]^+$, and m/z 289 $[M-106]^+$, corresponding to the loss of H_2O, CH_3OH, $CH_3OH + H_2O$, CH_3OH + CO, $CH_3OH + CO + H_2O$, and xylene from the polyene chain, respectively Supplemental Fig. 5. This fragmentation pattern for bixin has already been reported in the literature (Takaichi et al., 1993; Chiste et al., 2011).

Hydroxyspheroidenone (Rt = 15.480 min): This carotenoid was tentatively identified as hydroxyspheroidenone by comparing the UV–visible spectrum with that given in the database and literature (Manwarning et al., 1980). Moreover, the mass spectra of this carotenoid obtained in the positive ion mode (Table 1) showed the protonated molecule at m/z 601 and fragment ions in the MS/MS spectrum at m/z 583 $[M + H-18]^+$, m/z 569 $[M + H-32]^+$, m/z 509 $[M + H-92]^+$, m/z 495 $[M + H-106]^+$, m/z 477 $[M+H-18-106]^+$, and 385 $[M+H-18-92-106]^+$, corresponding to the loss of one hydroxyl group, methoxy group, toluene group, xylene, H_2O + xylene, and H_2O + toluene + xylene from the polyene chain, respectively.

The formulas and molecular structures of the four major carotenoids from superfine powder of *R. sphaeroides* 3757 are shown in Table 2.

Antioxidant properties

The antioxidant properties of carotenoids extracts obtained by either superfine grinding (ESG) or ultrasonic treatment (EUT) from superfine powder of *R. sphaeroides* 3757 were evaluated based on their *in vitro* indexes including DPPH radical-scavenging activity, reducing power and lipid peroxidation-inhibitory activity, and the results are shown in Fig. 1.

The assay for activity of scavenging of DPPH radicals is a widely used, easy-to-perform, and highly reproducible method for testing the antiradical activity of a large variety of compounds and natural extracts (Stajc˘ic´ et al., 2015; Zarza-García et al., 2017). As shown in Fig. 1, all the extracts showed a concentration-dependent DPPH radical-scavenging activity. The scavenging activity increased with the increase in the concentration of dry biomass of *R. sphaeroides* 3757. The ESG samples showed higher DPPH radical-scavenging activity than EUT samples did. When the ratio solvent-to-solid was 30, the scavenging activities of ESG and EUT samples were 74.0% ± 3.1% and 61.0% ± 2.5%, respectively.

The reducing-power assay is often used to evaluate the ability of natural antioxidants to donate an electron or hydrogen (Xia et al., 2012). As shown in Fig. 1, the ESG samples possessed higher reducing power than EUT samples did. When the ratio solvent-to-solid was 30, the reducing power of the ESG and EUT samples was 0.497 ± 0.022 and 0.328 ± 0.014, respectively. The reason may be the higher carotenoid concentration extracted from the same amount of dry biomass of *R. sphaeroides* 3757. Reducing power of both ESG samples and EUT samples increased obviously (0.242 ± 0.007 to 0.743 ± 0.036 and 0.128 ± 0.005 to 0.437 ± 0.021, respectively) with a decrease in the ratio solvent-to-solid from 70 to 20. The reducing capacity of the ESG samples and EUT samples was enhanced sharply depending on the concentration of dry biomass from *R. sphaeroides* 3757. The results indicated that the reducing power of the extracts from different concentrations of dry biomass of *R. sphaeroides* 3757 showed a dose-dependent effect.

The inhibition of lipid peroxidation is important for prevention of the deterioration of food quality and for prevention of certain human disease processes involving free radicals (Liu et al., 2005). As shown in Fig. 1, the ESG samples showed a more substantial inhibitory effect upon linoleic-acid peroxidation than did the EUT samples. When the ratio solvent-to-solid was 30, the inhibitory effects of ESG and EUT samples upon linoleic acid peroxidation were 77.6% ± 3.2% and 55.2% ± 2.3%, respectively. This result may be due to the higher carotenoid concentration extracted from the same amount of dry biomass of *R. sphaeroides* 3757. As shown in Fig. 1, there was a dose-dependent relation between extract concentration and its lipid peroxidation-inhibitory activity; and the lipid peroxidation-inhibitory activities of the extracts increased with the increasing concentrations of dry biomass of *R. sphaeroides* 3757. As the ratio solvent-to-solid decreased from 60 to 20, the lipid peroxidation-inhibitory activities of both ESG and EUT samples increased noticeably (35.9% ± 1.4% to 82.1% ± 3.7% and 13.1% ± 0.5% to 65.2% ± 2.8%), respectively. This finding indicated that superfine grinding is a much more efficient extraction method in terms of inhibition of lipid peroxidation by the resulting product.

DISCUSSION

Despite the availability of a variety of natural and synthetic carotenoids, there is currently a renewed

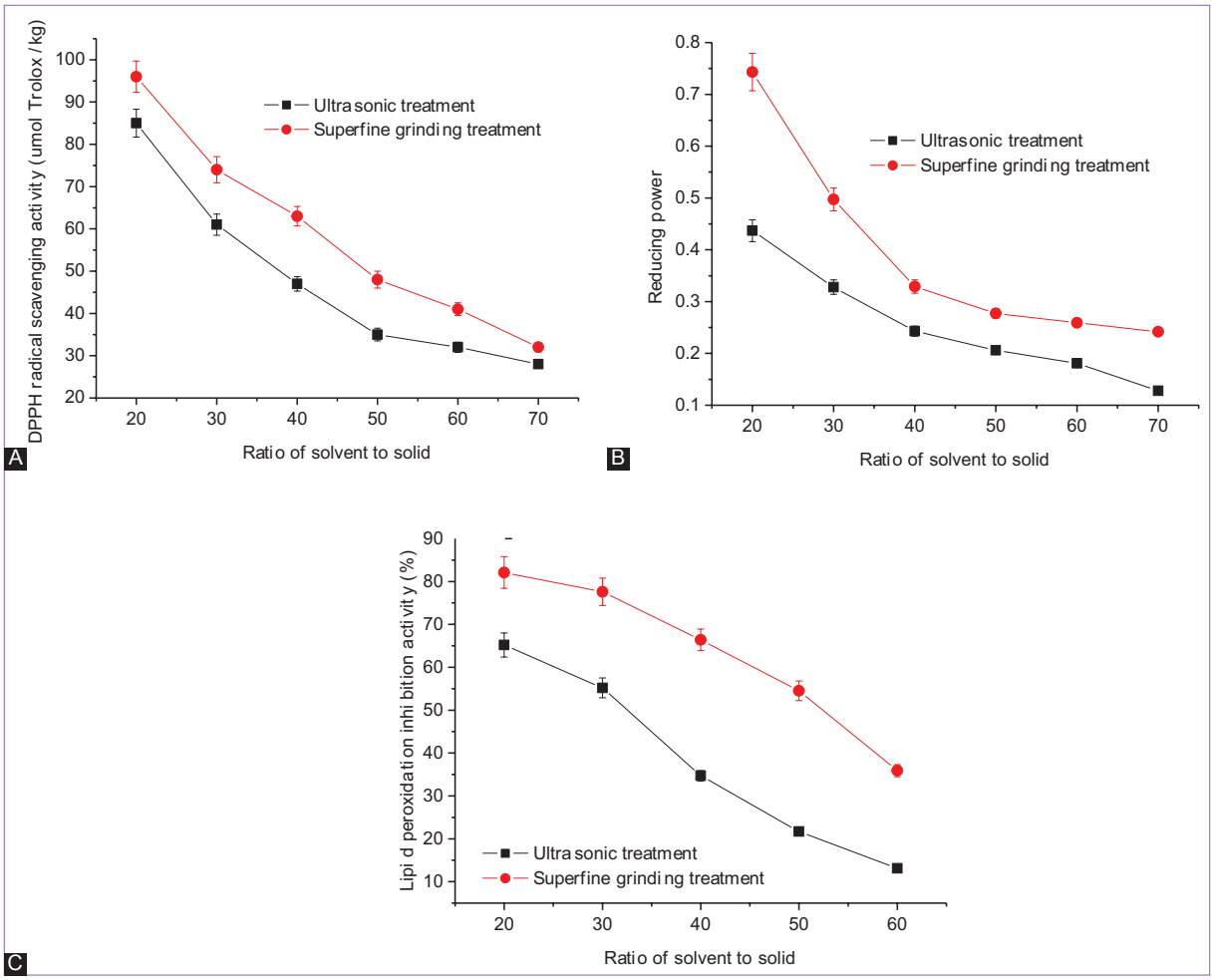


Fig 1. The *in vitro* antioxidant activity of carotenoids from *R. sphaeroides* 3757 (A: DPPH radical-scavenging activity; B: reducing power; C: lipid peroxidation inhibitory activity)

interest in microbial sources of pigments because of the problems of seasonal and geographical variability in plant origin, and great demands have been placed on their identification and determination (Sivathanu and Palaniswamy, 2012). The present results reveal the presence of carotenoids including bixin, hydroxyspheroidenone, 3,3′,4′-tetrahydrospirilloxanthin-20-al, and spheroidenone in *R. sphaeroides* 3757. This is the first chromatographic analysis of the carotenoid composition from the superfine powder of *R. sphaeroides* by HPLC-DAD-APCI-MS. Similarly, bixin was identified to be the main carotenoid in annatto seeds by HPLC–DAD-MS/MS, and the antioxidant capacity of bixin results from its ability to quench singlet oxygen, deactivate the excited triplet state of sensitizers and scavenge free radicals (Chiste et al., 2011). HPLC-DAD-APCI-MS was a powerful tool for the detection and characterization of carotenoids in rose hip fruit, and 23 carotenoid esters and 21 carotenoids were detected in the unsaponified and saponified extract, respectively (Zhong et al., 2016). The various carotenoids in *Scutellaria barbata* were identified by HPLC-DAD-APIC-MS using a YMC C30 column, and all-*trans*-lutein and its *cis* isomers constituted the largest portion, followed by all-*trans*-β-carotene and its *cis* isomers (Liu et al., 2014). However, none of the above mentioned compositions of carotenoids included hydroxyspheroidenone, 3,3,4,-tetrahydrospirilloxanthin-20-al and spheroidenone.

Superfine grinding technology is applied in functional food processing as a novel technology. This is the first report of the antioxidant properties of carotenoid extracts obtained by superfine grinding (ESG) from *R. sphaeroides* 3757 according to DPPH radical-scavenging activity, reducing power and lipid peroxidation-inhibitory activity. The present results reveal that the antioxidant activity of the carotenoids extracted by superfine grinding from *R. sphaeroides* 3757 was higher than that by ultrasonic treatment, and that the carotenoids from *R. sphaeroides* 3757 show a significant antioxidant activity *in vitro* in a concentration-dependent manner, probably because of the higher carotenoid concentration extracted from the same amount of dry biomass of *R. sphaeroides* 3757. This finding indicated that superfine grinding was a much more efficient extraction method in terms of scavenging of

DPPH radicals, reducing power and lipid peroxidation-inhibitory activity by the resulting product. The superfine grinder we used belongs to ball-milling which is an efficient cost-effective grinding method that is not detrimental to the environment (Zhao et al., 2015). The contents of polyphenols and flavanol in red grape pomace powders (GPP) increased with particle size decreasing during superfine grinding, which could enhance GPP antioxidant activities (Ramachandraiah and Chin, 2016). This is similar to our results. It suggests that superfine grinding technology has a great potential application in food industry, such as functional, manufacture and convenient foods.

The body requires a supplement of dietary antioxidants which can be obtained only by the consumption of an antioxidant-rich diet. Many carotenoids are found to be strong antioxidants because of their unsaturated groups, and much research has been done in the area of antioxidative assays of carotenoids (Fu et al., 2011). Tomato waste extracts which contained considerable amounts of carotenoids (lycopene and β-carotene) exhibited good antioxidant, and the carotenoid contents exhibited a strong correlation with antioxidant (Stajc˘ic´ et al., 2015). This is in accordance with our results. Both composition and antioxidant activity of carotenoids extracted from various samples had been studied recently. Lutein and zeaxanthin were the predominant carotenoids determined using HPLC, and antioxidant activity in both assays increased linearly with total carotenoid content (Giuffrida et al., 2013). These are similar to our results, but the predominant carotenoids in *R. sphaeroides* 3757 are different from what had been reported above.

CONCLUSIONS

After separation and purification by silica gel column chromatography and RP-HPLC on a C30 chromatographic column, the four major carotenoids from superfine powders of *R. sphaeroides* 3757 were identified as bixin, hydroxyspheroidenone, 3,3,4,-tetrahydrospirilloxanthin-20-al, and spheroidenone by RP-HPLC-APCI-MS. The antioxidant activity of the carotenoids extracted by superfine grinding from dry biomass of *R. sphaeroides* 3757 is higher than that of extracts prepared by ultrasonic treatment according to their *in vitro* indexes of DPPH radical-scavenging activity, reducing power and lipid peroxidation-inhibitory activity. These results revealed that carotenoids from *R. sphaeroides* 3757 show a significant antioxidant activity *in vitro* in a concentration-dependent manner, and that superfine grinding is the optimal extraction method. Thus, based on the present results, carotenoids from superfine powder of *R. sphaeroides* might be applied in natural antioxidant and functional food with good antioxidant activities.

ACKNOWLEDGMENTS

This paper was supported by the National Natural Science Foundation of China [grant number 3157020423 and 3157020721], China Scholarship Council Foundation [grant number 201708110129], Beijing Natural Science Foundation [grant number 6173033] and Beijing Municipal Commission of Education [grant number KM20131141 7007 and PXM2013_ 014209_07_ 000082]. We also gratefully acknowledge financial support of Scientific Research Project from Facing Characteristic Discipline of Beijing Union University (grant number KYDE40201703).

Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

Zuming Li and Zhihui Bai conceived and designed the experiments; Lina Kong performed the experiments; Lina Kong, Bodi Hui and Zuming Li analyzed the data; Dong Wang contributed materials; Zuming Li and Lina Kong wrote the paper; Xiaoya Shang, Liping Gao, Na Luan and Xuliang Zhuang gave important suggestions and support, Zuming Li, Zhihui Bai and Bodi Hui revised the manuscript.

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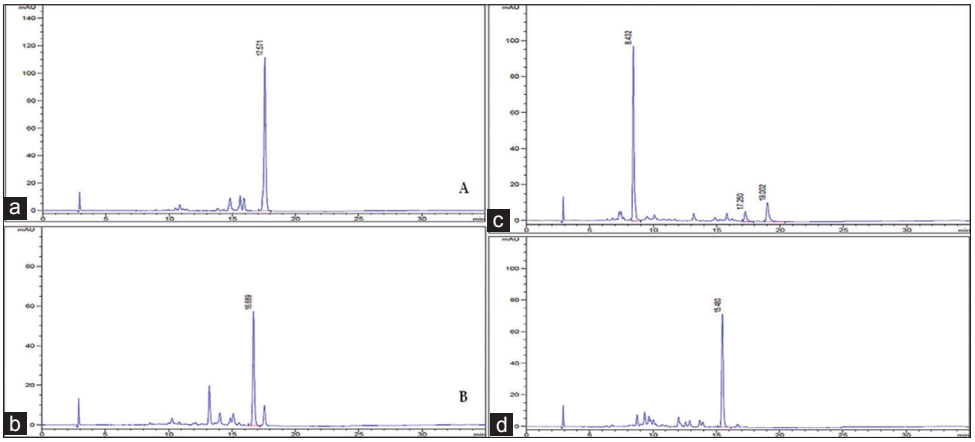
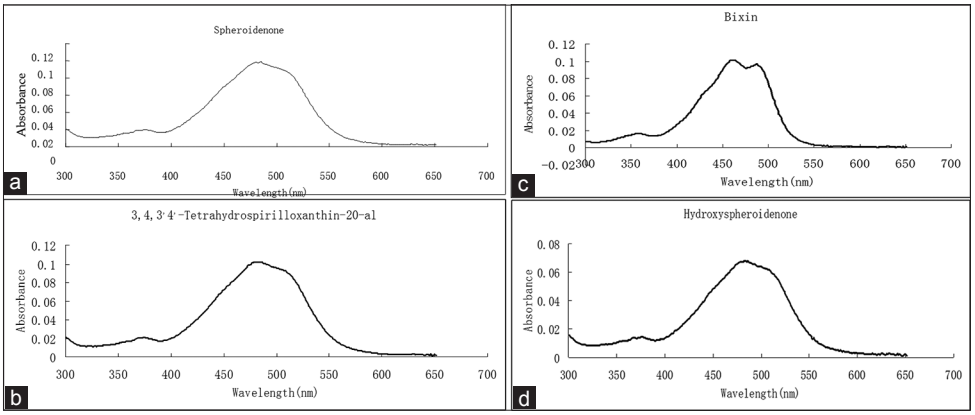
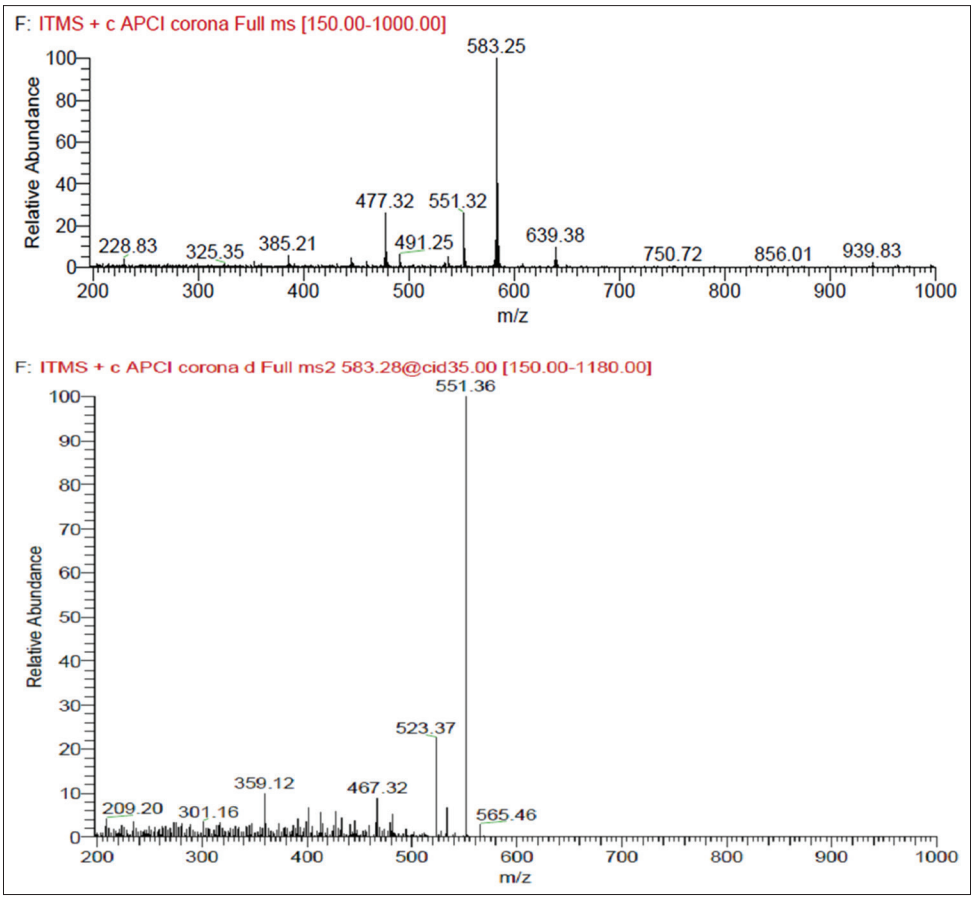
SUPPLEMENTAL FIGURES



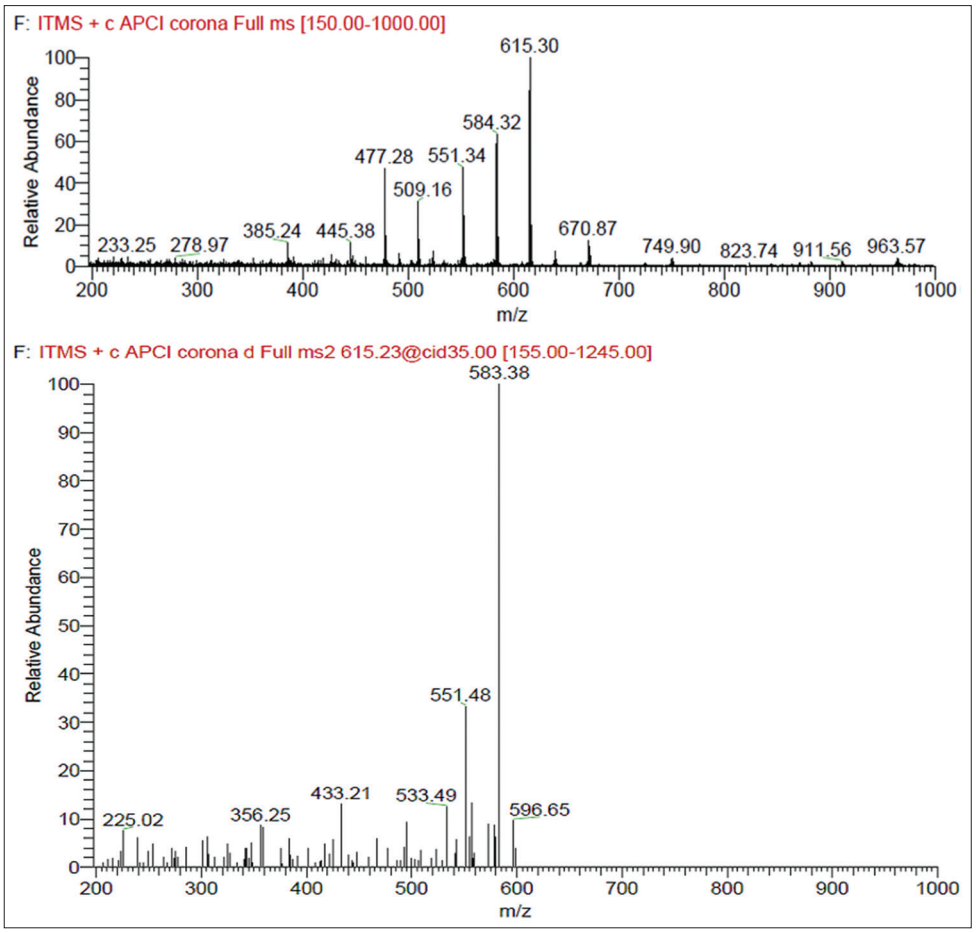
Supplemental Fig 1. HPLC chromatograms of the four main carotenoids from *R. sphaeroides* 3757 (A: peak 1; B: peak 2; C: peak 3; D: peak 4). Peak identification is shown in Table 1.



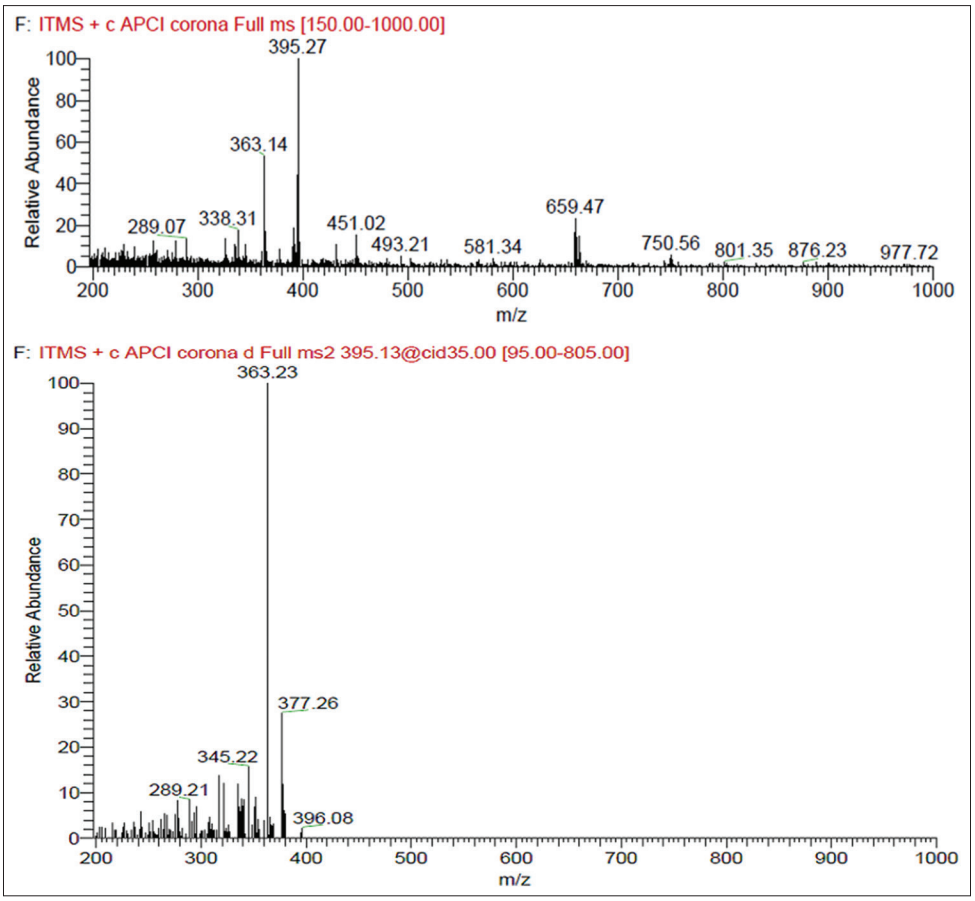
Supplemental Fig 2. UV–vis spectra of the four main carotenoids from *R. sphaeroides* 3757 obtained by HPLC-PDA (A: peak 1; B: peak 2; C: peak 3; D: peak 4). See Table 1 for peak identification.



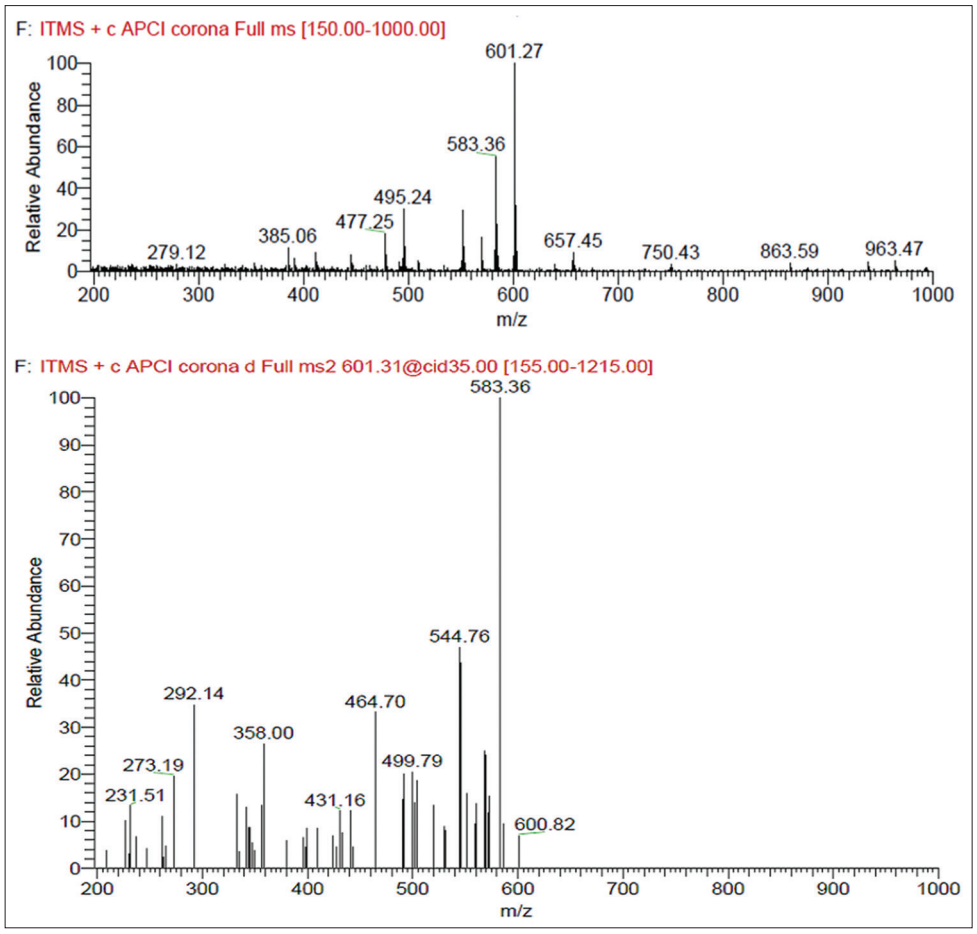
Supplemental Fig 3. APCI mass spectra of spheroidenone (Peak A).



Supplemental Fig 4. APCI mass spectra of 3,4,3′,4′-tetrahydrospirilloxanthin-20-al (peak B).



Supplemental Fig 5. APCI mass spectra of bixin (peak C).



Supplemental Fig 6. APCI mass spectra of hydroxyspheroidenone (peak D).