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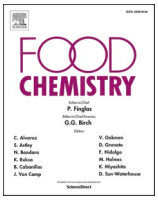

Characterization of betalain-loaded liposomes and its bioactive potential *in vivo* after ingestion

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ABSTRACT

Betalains are plant pigments characterized by showing a wide range of beneficial properties for health. Its bioactive potential has been studied for the first time after its encapsulation in liposomes and subsequent administration to the animal model *Caenorhabditis elegans*. Phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin encapsulated at concentrations of 25 and 500 μM managed to reduce lipid accumulation and oxidative stress in the nematodes. Highly antioxidant betalains dopaxanthin and betanidin were also included in the survival analyses. The results showed that phenylalanine-betaxanthin was the most effective betalain by increasing the lifespan of *C. elegans* by 21.8%. In addition, the administration of encapsulated natural betanidin increased the nematodes' survival rate by up to 13.8%. The preservation of the bioactive properties of betalains manifested in this study means that the stabilization of the plant pigments through encapsulation in liposomes can be postulated as a new way for administration in pharmacological and food applications.

1. Introduction

Betalains are nitrogenous compounds derived from the secondary metabolism of plants that provide vivid coloration to most species of the order Caryophyllales. These pigments are characterized by the presence of a molecule of betalamic acid as structural and functional unit. The condensation of betalamic acid with different compounds defines the existence of two types of betalains: the yellow betaxanthins when it is condensed with amines or their derivatives; and the violet betacyanins, if condensation occurs with indole derivates like *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) or its glycoside derivatives (Khan & Giridhar, 2015). The absorbance spectra differ between the two types of pigments, with betaxanthins and betacyanins having maximum wavelengths (λ) at 480 and 536 nm, respectively. The coloration of these compounds is due to the system of conjugated double bonds present in betalamic acid and, in the case of betacyanins, extended to the side chain leading to red-violet shades. Yellow betaxanthins additionally have a strong fluorescence which is increased by the electron acceptor character of the substituents of amines or amino acids (Gandía-Herrero, Escribano, & García-Carmona, 2010). The biosynthetic pathway of betalains begins with the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by the action of cytochrome P450-type enzymes (Sunnadeniya et al., 2016). Next, the enzyme 4,5-DOPA-extradiol-dioxygenase (4,5-DODA), considered a key enzyme in the biosynthetic route, carries out the oxidation and opening of the aromatic ring of L-DOPA giving rise to 4,5-seco-DOPA. This intermediate molecule undergoes rapid spontaneous cyclization to betalamic acid. Its further condensation with *cyclo*-DOPA or with amines and amino acids gives rise to betacyanins and betaxanthins, respectively (Fischer & Dreiding, 1972).

Interest in betalains as bioactive compounds has raised in recent years. Their antioxidant (Cai, Sun, & Corke, 2003; Gliszczyńska-Swigło, Szymusiak, & Malinowska, 2006), anti-inflammatory (Macias-Ceja et al., 2017; Martinez et al., 2015; Tan, Wang, Bai, Yang, & Han, 2015), chemopreventive (Lechner et al., 2010; Zhang, Pan, Wang, Lubet, & You, 2013) and antilipidemic (Song, Chu, Xu, Xu, & Zheng, 2016)

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activities have been studied in numerous research papers. Their use in, both *in vitro* and *in vivo* studies, has increased the knowledge of the health-promoting potential of these pigments (Martínez-Rodríguez, Guerrero-Rubio, Henarejos-Escudero, García-Carmona, & Gandía-Herrero, 2022). In addition, the use of some betalains as pigments in the food industry causes considerable interest in their purification and characterization. However, current methods for obtaining pure betalains are ineffective, which means that many investigations use extracts instead of individual compounds (Martínez-Rodríguez et al., 2022). It was necessary to develop production systems to obtain pure pigments in order to demonstrate the unequivocal involvement of betalains in the observed health-promoting effects. Here, a biotechnological method takes advantage of the key role of the enzyme 4,5-DODA in the biosynthetic route for obtaining betalains in microbial factories (Guerrero-Rubio, López-Llorca, Henarejos-Escudero, García-Carmona, & Gandía-Herrero, 2019). Thus, pure betaxanthins and betacyanins are selected as pigment models and their bioactivity is characterized after purification.

In vivo evaluations are performed in the animal *Caenorhabditis elegans*, a model for the study of the bioactive potential of molecules after ingestion recently used for the characterization of betalains (Guerrero-Rubio et al., 2020; Henarejos-Escudero, Hernández-García, Guerrero-Rubio, García-Carmona, & Gandía-Herrero, 2020). This small nematode is characterized by a fast life cycle and a very short half-life. In addition, the simplicity of its genome and, consequently, the ease of genetic manipulation, added to the existence of numerous metabolic pathways equivalent to those of the human being, makes *C. elegans* an ideal organism for use in biomedical research (Shaye & Greenwald, 2011). As for the field of food research, it is a preclinical model of help in the screening and evaluation of bioactive ingredients compliant with the 3Rs and Animal Testing Welfare Act. Testing first in this model allows the establishment of doses, toxicity, and mechanisms of action prior to testing in murine models, and finally in humans. Moreover, *C. elegans* has been used for studies of food digestion and nutrient absorption (Suzuki, Kikuchi, Numayama-Tsuruta, & Ishikawa, 2022). The comparatively simple *C. elegans* intestine has many of the complex functions of the mammalian digestive tract, liver, and fat tissues. The lumen contains several enzymes, such lysozymes, proteases, and lipases which digest and metabolize the food. Interestingly the lumen also has glucose transporters. Thus, the model offers a simple approach for the evaluation of bioactive compounds.

In this work, pure betalains are administered encapsulated in lipid vesicles or liposomes. These spherical vesicles are formed by one or several phospholipid bilayers and their use as distribution systems for molecules or drugs has become one of the most important advances in nanomedicine in recent years (Bozzuto & Molinari, 2015). Previous research has already used liposomes as a method of administering molecules with bioactive potential in *C. elegans* (Shibamura, Ikeda, & Nishikawa, 2009). However, it remains to be seen whether the health-promoting capacity of betalains is manifested by this new method of administration.

Two betaxanthins, dopaxanthin and phenylalanine-betaxanthin, and two betacyanins, indoline carboxylic acid-betacyanin and betanidin, were used in this work. Indoline carboxylic acid-betacyanin is a synthetic molecule but structurally related to the other betacyanin tested and found in nature, betanidin. The other two betalains used (dopaxanthin and phenylalanine-betaxanthin) are found naturally in roots of *Beta vulgaris* or in fruits of *Opuntia ficus-indica*, plant species widely consumed by humans and of known nutritional interest (Gandía-Herrero & García-Carmona, 2013). The mentioned betalains were encapsulated inside liposomes and their bioactive potential was tested *in vivo* in the animal model *C. elegans*. Dopaxanthin, phenylalanine-betaxanthin, and indoline carboxylic acid-betacyanin were chosen for being outstanding antioxidants and health promoters *in vivo* in *C. elegans*. Betanidin was included because it is a great antioxidant but one of the most labile betalains unable to show significant effects on nematode survival when administered in free form (Guerrero-Rubio et al., 2020).

2. Materials and methods

2.1. Chemicals and bacterial strains

Kanamycin (Km), chloramphenicol (Cm), isopropyl-β-D-1-thio-galactopyranoside (IPTG), 3,4-dihydroxy-L-phenylalanine (L-DOPA), sodium ascorbate, L-phenylalanine, L-indoline-2-carboxylic acid, juglone, sodium azide, Oil Red O, rhodamine 6G, Nile red, nystatin, ampicillin and 2′-deoxy-5-fluorouridine were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-α-phosphatidylcholine was acquired from Avanti Polar Lipids (Alabaster, Alabama, USA). Acetonitrile used for the HPLC gradient, methanol, and ethyl acetate were obtained from Fisher Scientific (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). For the establishment of microbial bioreactors, *Escherichia coli* Rossetta 2, previously transformed with the expression vector pET28a which contains the gene encoding the enzyme 4,5-DODA of *Gluconacetobacter diazotrophicus* (pET28a-GdDODA) (Contreras-Llano, Guerrero-Rubio, Lozada-Ramírez, García-Carmona, & Gandía-Herrero, 2019), was used.

2.2. Extraction/production of betalains

Betanidin was extracted from violet flowers of *Lampranthus productus*. The extraction process was carried out in sodium acetate buffer 10 mM pH 5.0, using pulses of 5 s at medium speed using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). Subsequently, the resulting homogenate was filtered through a nylon fabric and centrifuged at 8,000 rpm for 10 min. The supernatant was stored at −20 °C until purification. The rest of betalains were obtained by a biotechnological process in microbial factories containing *E. coli* (pET28a-GdDODA) (Guerrero-Rubio et al., 2019). To do this, the bacterial culture was performed in Luria-Bertani medium (LB) supplemented with kanamycin (100 μg/mL) and chloramphenicol (34 μg/mL). The culture was incubated at 37 °C under orbital shaking until an optical density at 600 nm (OD 600 nm) of 0.8–1 was obtained. Subsequently, IPTG 1 mM was added to the culture and kept under shaking at 20 °C for 15 h. The culture was then centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and the bacterial pellet obtained was resuspended in Milli-Q water, at a volume similar to that used for bacterial growth in LB. Subsequently, and considering the optimal conditions established for the production of betalains, the reagents L-DOPA 7.6 mM, sodium ascorbate 15 mM, and phenylalanine 38 mM or indoline-carboxylic acid 3.8 mM were added for the production of phenylalanine-betaxanthin or indoline carboxylic acid-betacyanin, respectively. For the production of dopaxanthin only L-DOPA and sodium ascorbate, at the same concentrations as above, were added. The bioreactors were incubated at 20 °C under constant stirring (120 rpm) until the maximum production of phenylalanine-betaxanthin (72 h), indoline carboxylic acid-betacyanin (96 h), and dopaxanthin (96 h) was reached. After incubation, the contents were centrifuged at 7,500 rpm for 10 min and the bacterial pellets were discarded, keeping the supernatants at −20 °C until the beginning of the purification stage.

2.3. Purification, quantification, and analysis of betalains

Betalains were purified by sequential chromatographic techniques. First, the samples were subjected to solid-phase extraction (SPE) in C-18 endcapped columns (35 mL, Waters, MA, USA), with particle and pore sizes of 55–105 μm and 125 Å, respectively. The columns were conditioned with 70 mL of ethanol followed by 70 mL of Milli-Q water. Betalains were added to the column and eluted with ethanol. The resulting fractions were subjected to an anion exchange liquid chromatography in an FPLC-type system. For this, an Äkta purifier device (General Electric Healthcare, Milwaukee, USA) was used in conjunction

with a PC equipped with UNIKORN software version 3.00. A Q-Sepharose Fast Flow column with a stationary phase derivatized with quaternary ammonium and 20 mL capacity was used. The samples were eluted by forming a gradient between the solvents phosphate buffer 20 mM pH 6.0 (solvent A) and phosphate buffer 20 mM pH 6.0 supplemented with NaCl 2 M (solvent B). The elution process was as follows: 0% B up to 10 mL; after washing, a linear gradient of 120 mL was carried out from 0% to 26% B, and fractions of 3 mL were collected. The flow rate was 2 mL/min and the injection volume was 20 mL. The elution process was followed at 280, 480, and 536 nm. The resulting fractions containing the pigment were pooled and the salts were removed by SPE using the same C-18 cartridges and the same cleaning procedure as previously described (Gandía-Herrero, García-Carmona, & Escribano, 2006). Then, the samples were subjected to rotary evaporation to remove the ethanol and subsequently dissolved in Milli-Q water for analysis by UV–vis spectrophotometry. A Jasco V-650 spectrophotometer (Easton, MD, USA) was used to calculate the concentration of the pigments after purification. Spectra measurements were performed at wavelengths between 300 and 700 nm. For the quantification of pure betalains, the molar extinction coefficients (ε) used were 46,000, 49,000, 76,000, and 54,000 $M^{-1}{\cdot}cm^{-1}$ for dopaxanthin, phenylalanine-betaxanthin, indoline carboxylic acid-betacyanin and betanidin, respectively (Gandía-Herrero et al., 2010). The analysis of the fluorescence emitted by betaxanthins was performed with a Shimadzu RF-6000 spectrofluorimeter (Kyoto, Japan). Quartz cuvettes were employed, and the pigments were diluted in Milli-Q water to a final concentration of 3 μM. Excitation spectra were obtained through emission at the λ_{max} of emission. The emission spectra were obtained by exciting at the corresponding excitation λ_{max}. The study and separation of the sample components were carried out using reverse-phase high-performance liquid chromatography (HPLC) in a Shimadzu LC-10A apparatus (Kyoto, Japan) equipped with an SPD-M10A PDA detector. A Kinetex C-18 column of 250 × 4.6 mm and 5 μm particle size (Phenomenex, Torrance, CA, USA) was used. The elution gradients were formed with water (solvent A) and acetonitrile (solvent B), both supplemented with 0.05% trifluoroacetic acid (TFA). A linear gradient was performed from 0% to 35% of solvent B for 25 min. The flow rate was 1 mL/min at a fixed temperature of 30 °C, and an injection volume of 50 μL. Finally, for the structural confirmation of the pigments, electrospray ionization mass analyses (ESI-MS) were carried out. An Agilent VL 1100 with LC/MSD Trap (Agilent Technologies, Palo Alto, CA, USA) was used for this purpose. A Kinetex 5 μm C-18 column with a flow rate of 0.8 mL/min was used. Elution conditions were the same as those described for HPLC analysis. Vaporizer temperature was 350 °C, and voltage was maintained at 3.5 kV. The sheath gas was nitrogen, operated at a pressure of 45 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range m/z 50–600. The electron multiplier voltage for detection was 1350 V.

2.4. Formation of betalain-loaded liposomes

Liposomes were prepared using a mini extruder "Avanti Mini-Extruder" (Avanti Polar Lipids, Alabaster, USA), following the manufacturer's instructions. Betalains dissolved in Milli-Q water were added to 24 mg of L-α-phosphatidylcholine up to a final volume of 500 μL (final concentration of lipid: 48 mg/mL). The pigments were encapsulated at 25, 50, 100, and 500 μM, depending on the assay. Solutions containing phosphatidylcholine and betalains were loaded into one of the syringes and the two syringes were then inserted into the mini extruder. The contents were passed a minimum of 11 times across the membrane to ensure homogeneity of liposomes formed. Subsequently, the entire resulting volume was collected and liposomes already formed and containing betalains were used in the subsequent experiments.

2.5. Analysis of the content of betalain-loaded liposomes

The content of the prepared liposomes was evaluated by breakdown and subsequent HPLC analysis of the released content. For this purpose, liposomes were fabricated with phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin encapsulated at a concentration of 500 μM. Subsequently, the liposomes were washed by centrifugation (5 min at 13,000 rpm). The supernatant was collected and analyzed by HPLC for the determination of encapsulation efficiency. To simulate the conditions of *C. elegans* assays, the liposome pellet was resuspended in saline buffer (50 mM NaCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, potassium phosphate 25 mM, pH 6.0) with the same chemical composition of the NGM medium (used for maintenance of nematodes). The liposomes were incubated for 48 h at 20 °C. At 0, 24, and 48 h, 250 μL aliquots were taken and centrifuged at 13,000 rpm for 3 min. The supernatant was collected for HPLC analysis, and the pellet was resuspended in 250 μL of Milli-Q water. The liposomes were then broken with 83 μL of methanol (Miao et al., 2015), releasing the pigments to the supernatant. Then, to avoid interferences in the HPLC detection, the lipids were extracted from the supernatant by adding 375 μL of ethyl acetate at 4 °C. The organic phase was discarded and the aqueous phase containing the pigments was analyzed by HPLC.

2.6. Visualization of liposomes using transmission electron microscopy (TEM)

The structural characterization of liposomes was performed by staining and observing them under a TEM microscopy. Briefly, 10 μL of liposomes (3 mg/mL) were placed in a formvar/carbon-supported nickel grid and allowed to settle for 1 min. The excess of sample was removed by capillarity with filter paper. Then, the liposomes were stained with 10 μL of uranyl acetate solution (2%) for 2 min. Finally, the excess of stain was removed and the grids were air-dried. The grids were mounted in the holder of the Philips Tecnai 12 TEM (Amsterdam, The Netherlands), and visualized with an acceleration voltage of 100 kV. Digital images were obtained using a megaview III digital camera. Control liposomes and liposomes loaded with phenylalanine-betaxanthin were visualized at a concentration of 25 μM. The shape and size of liposomes were analyzed using ImageJ software.

2.7. Differential scanning calorimetry (DSC)

DSC analyses were performed for the study and comparison of the thermal behavior involved in phase transitions between control liposomes and those loaded with betalains. Control liposomes (5 mg/mL) and liposomes loaded with phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin were analyzed, both at a concentration of 25 μM. Liposomal solutions were prepared using PBS (phosphate buffered saline) buffer as a solvent. The samples and the same volume of PBS buffer used as a reference were degassed for 5 min before introducing them in the calorimeter. Calorimetric analysis was performed using a Microcal VP scanning calorimeter (Microcal, Northampton, MA, USA). Three scans were carried out for each sample, starting with temperatures of −20 °C. The scan was in the range from −20 °C to 20 °C and the heating speed was 1 °C/min. The resulting thermograms were analyzed using Microcal Origin 5.0 software.

2.8. Visualization of encapsulated phenylalanine-betaxanthin using confocal microscopy

The location of the fluorescent betalain phenylalanine-betaxanthin in liposomes was performed by confocal microscopy. Liposomes (0.1 mg/mL) were prepared with phenylalanine-betaxanthin encapsulated at a concentration of 40 μM. Subsequently, liposomes were centrifuged for 3 min at 13,000 rpm. Then, the supernatant was discarded and a Nile red solution (0.01 mg/mL) was added in enough volume to cover the

liposome pellet, allowing it to incubate for 30 min in darkness. After incubation, the mixture was centrifuged for 1 min at 13,000 rpm, the supernatant was discarded and 500 μL of Milli-Q water were added. Finally, 10 μL of liposomes were placed on prepared agarose pad slides to limit the mobility of the vesicles (Kim, Sun, Gabel, & Fang-Yen, 2013). Liposomes were visualized using a Leica Stellaris 8 confocal microscopy (Leica Microsystems, Wetzlar, Germany). To determine the localization of betaxanthin in the labeled liposomes, the fluorescence of the Nile red stain with $\lambda_{ex} = 549$ nm and $\lambda_{em} = 628$ nm and the fluorescence of phenylalanine-betaxanthin with $\lambda_{ex} = 471$ nm and $\lambda_{em} = 509$ nm were observed.

2.9. *C. elegans* strains and culture conditions

The strains of *C. elegans* used for the present work include the wild-type N2, used for survival assays and lipid accumulation; and the mutant strain TJ375 (*hsp-16.2::GFP*), used for the study of oxidative stress. All strains were obtained from the *Caenorhabditis* Genetic Center (CGC, St. Paul, MN, USA), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The maintenance of the worms was carried out at 20 °C in solid growth medium NGM (Stiernagle, 2006) where *E. coli* OP50, previously grown in LB medium for 12 h at 37 °C, was employed as a food source.

2.10. Synchronization of *C. elegans* culture

All experiments were performed with nematodes of the same age, following the published protocols (Stiernagle, 2006). Briefly, worms grown on plates for three days were washed with 10 mL of M9 buffer and collected in 15 mL conical tubes. Subsequently, the nematodes were centrifuged for 1 min at 3,000 rpm, the supernatant was discarded and the volume was adjusted to 2 mL with M9. Then, 2 mL of commercial bleach and 1 mL of NaOH were added and mixed with vortex every 2 min up to a maximum of 10 min. Next, 5 mL of M9 buffer was added and centrifuged for 5 min at 8,000 rpm. Then, the supernatant was discarded and 10 mL of M9 were added and the eggs were centrifuged again, the washing step was performed twice. Finally, the resulting mixture was added to a sterile flask and left incubating under continuous orbital shaking for 12 h at 20 °C for synchronous hatching of the eggs.

2.11. Administration of encapsulated betalains to *C. elegans*

To carry out the different assays, larvae in stage L1 obtained by synchronization were used (Guerrero-Rubio, Hernández-García, García-Carmona, & Gandía-Herrero, 2019). The L1 nematodes were transferred to 55 mm NGM plates supplemented with 100 μL *E. coli* OP50 concentrated 10X (Guerrero-Rubio et al., 2020), as a food source, and 10 μL of the prepared liposomes (48 mg/mL). The plates were incubated at 20 °C for the duration of the experiment. In this work, liposomes loaded with phenylalanine-betaxanthin, indoline carboxylic acid-betacyanin, dopaxanthin, and betanidin were used at different concentrations. Additionally, liposomes loaded with water were tested as a control. The exposure time depended on the type of trial, being 48 h for oxidative stress (TJ375) and lipid accumulation (N2) tests, and continuous exposure throughout the experiment (25 days) in survival trials (N2).

2.12. Liposome-labeling and intake assays

Intake of liposomes in *C. elegans* N2 was evaluated by their administration as previously labeled particles with the lipophilic fluorescent probe rhodamine 6G. First, liposomes (48 mg/mL) were centrifuged for 5 min at 13,000 rpm. Subsequently, the supernatant was discarded and rhodamine 6G (1 mg/mL) was added in a sufficient volume to cover the pellet, for 5 min at room temperature. The mixture was then centrifuged for 1 min at 13,000 rpm. After that, the supernatant was discarded and 500 μL of Milli-Q water was added. Then, 5, 10, 50, and 100 μL of labeled liposomes were administered to nematodes. As a control, 10 μL of non-labeled liposomes were administered. At 15 min, nematodes were washed with M9 buffer and transferred to 1.5 mL tubes placed on ice to interrupt intake. The same procedure was performed 60 min after the administration of liposomes. Finally, nematodes were placed on glass slides to which 10 mM sodium azide was previously added to reduce their mobility. The fluorescence of rhodamine 6G in the body of a minimum of 10 individuals was measured for each condition. The images were taken at constant exposure times using an I3 filter of a Leica DM 2500 LED microscopy equipped with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany). The quantification of fluorescence was carried out by analyzing the images using ImageJ software (Schindelin et al., 2012). Statistical analysis was performed with ANOVA test using SigmaPlot 14 software.

2.13. Quantification of lipid accumulation in *C. elegans* N2

Staining of the body lipids of *C. elegans* was carried out using the dye Oil Red O (ORO) (O'Rourke, Soukas, Carr, & Ruvkun, 2009). Before the staining procedures, the *C. elegans* N2 strain was previously fed with different concentrations of encapsulated betalains for 48 h at 20 °C. Then, the nematodes were washed twice with M9 and fixed with a paraformaldehyde solution in PBS buffer (4%) at 4 °C for 24 h. Subsequently, the fixative was removed and tris/HCl buffer (pH 7.4) supplemented with β-mercaptoethanol (5%) and Triton X-100 (1%) was added to permeabilize the animals at 37 °C for 24 h. After that, they were washed twice with M9 buffer and stained with an ORO solution (5 mg/mL). Subsequently, they were centrifuged at 12,500 rpm, the supernatant was discarded, and worms were washed twice in the same way with M9 buffer. Subsequently, nematodes were placed on glass slides. Two independent experiments were conducted, in which lipid levels were measured for a minimum of 10 individuals for each condition. Specifically, the antilipidemic effect of phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin, encapsulated at a concentration of 25 and 500 μM, was tested. Brightfield images of the posterior region of the nematodes' bulb were taken at constant exposure times using the microscopy referred to in Section 2.12. The quantification of lipid levels was carried out by analyzing the acquired images using the ImageJ software and the statistical analysis was carried out as stated in Section 2.12.

2.14. Quantification of the expression of *hsp-16.2::GFP* in *C. elegans* TJ375

The quantification of the fluorescence emitted by the green fluorescent protein (GFP) allowed to use of the expression of *hsp-16.2::GFP* as a marker of oxidative stress. L1 larvae of the strain TJ375 were treated with different concentrations of encapsulated betalains for 48 h at 20 °C on NGM plates. Subsequently, the nematodes were transferred to S basal medium where oxidative stress was induced by the addition of 20 μM juglone. After 24 h, the individuals were washed with M9 buffer, placed on microscope glass slides containing 10 mM sodium azide, and visualized under fluorescence microscopy. Two independent experiments were conducted, in which the fluorescence of a minimum of 10 individuals was measured for each concentration of betalains. Specifically, the antioxidant effect of phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin encapsulated at a concentration of 25 and 500 μM was tested. The acquisition and analysis of images were carried out following the procedure described in Section 2.12.

2.15. Measurement of *C. elegans* N2 lifespan

After 48 h of incubation following administration of the first dose of liposomes, the nematodes were centrifuged at 3,500 rpm and washed with M9 buffer. Next, about 50 worms were transferred to 35 mm analysis plates with 8 mL of NGM supplemented with nystatin (30 μg/

mL), ampicillin (100 μg/mL) and 2′-deoxy-5-fluorouridine (FUdR) (10 μg/mL) to avoid progeny. As a food source, 100 μL of an overnight *E. coli* OP50 culture, concentrated 10X in M9 buffer, and inactivated at 65 °C for 30 min was added. A second dose of liposomes at the same concentration as the previous one was also added. The plates were then closed and incubated for 20 min at 20 °C. Finally, the closed plates were introduced into the Lifespan Machine device for the study of the lifespan of the nematodes. Lifespan Machine is a tool capable of automatically tracking each individual nematode during the life cycle (16–20 days) and providing the lifetime (Stroustrup et al., 2013). The duration of the experiments was 25 days, and the plates were kept at 25 °C. The survival and lifespan curves were obtained by Kaplan-Meier adjustment and statistical analysis with the Log-Rank test of the data generated by Lifespan Machine, using SigmaPlot 14 software.

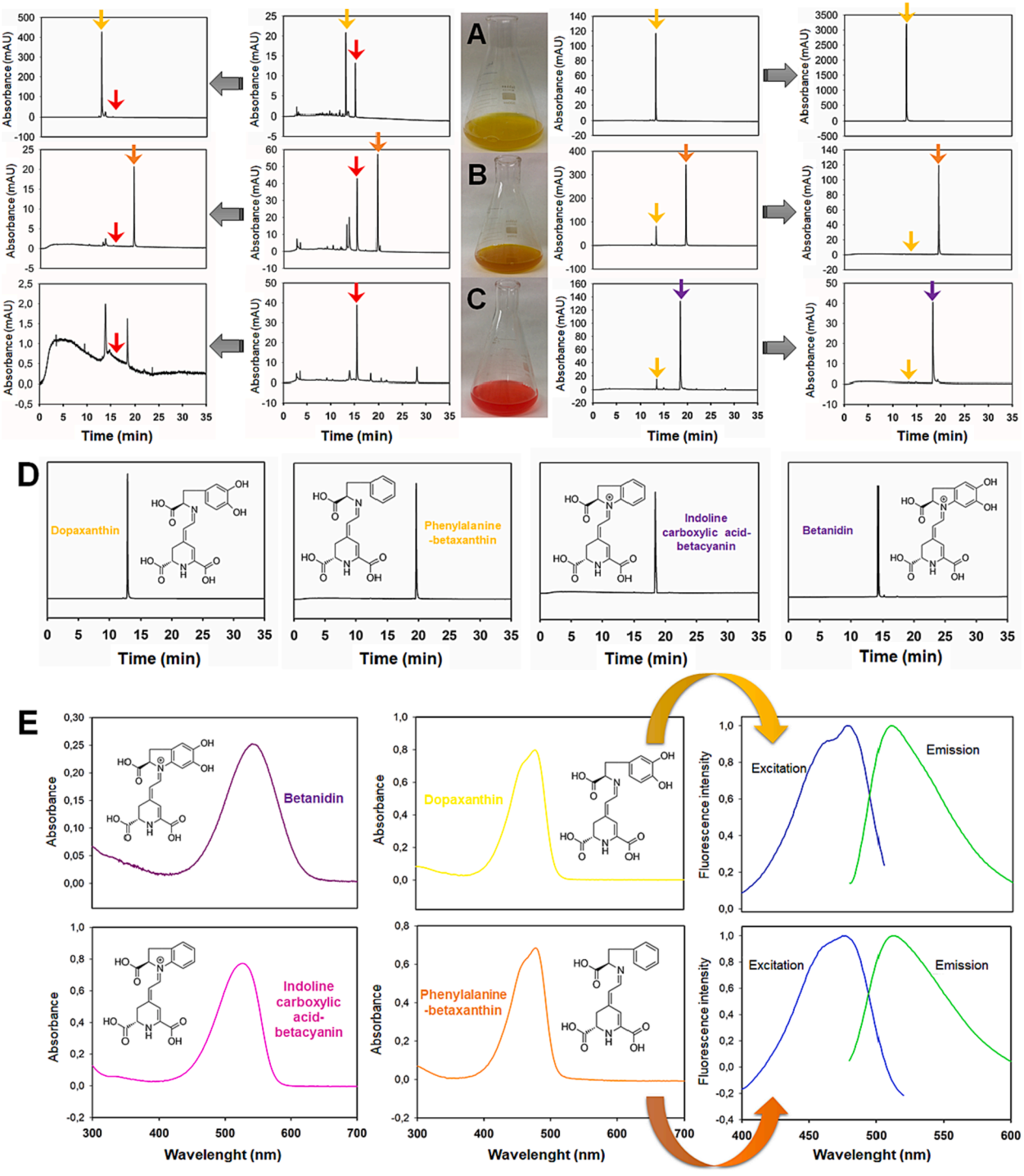

Fig. 1. Betalains production, purification, and characterization. Purification of dopaxanthin (A), phenylalanine-betaxanthin (B), and indoline carboxylic acid-betacyanin (C) after production in microbial factories. HPLC chromatograms are shown at λ = 405 nm (left) and λ = 480 nm (right), both before and after the purification process (indicated by the grey arrow). Betalamic acid (red arrow), dopaxanthin (yellow arrow), phenylalanine-betaxanthin (orange arrow), and indoline carboxylic acid-betacyanin (violet arrow) are shown. (D) Chemical structures and chromatograms obtained from the four individual betalains used in this work. Chromatograms are shown at λ = 480 nm for betaxanthins and λ = 536 nm for betacyanins. (E) Absorbance and fluorescence spectra (in the case of betaxanthins) of the betalains used in this work

3. Results and discussion

3.1. Extraction, production, and purification of betalains

The extraction and purification of betanidin from violet flowers of *L. productus* yielded 27 μg of pigment for each gram of petals processed. After the purification process, HPLC analysis showed a single peak with a retention time of 14.3 min, corresponding to betanidin. In the biotechnological production of the rest of the betalains used, the supplementation of the LB medium with IPTG induced the heterologous expression of the betalamic acid-forming enzyme 4,5-DODA in *E. coli* cells. After incubation with L-DOPA and sodium ascorbate, the reaction medium acquired a yellow coloration, corresponding to the formation of dopaxanthin. Moreover, media containing L-DOPA and sodium ascorbate supplemented with phenylalanine or indoline carboxylic acid showed orange and red-violet coloration, respectively. The addition of L-DOPA as a substrate of the reaction catalyzed by 4,5-DODA allowed the formation of betalamic acid, which was condensed with L-phenylalanine to produce phenylalanine-betaxanthin; with indoline carboxylic acid to obtain indoline carboxylic acid-betacyanin, and with L-DOPA itself to produce dopaxanthin. The replacement of the LB medium by water as a reaction medium was carried out with the aim of reducing the number of unwanted reactions between the betalamic acid and other components present in LB (Guerrero-Rubio et al., 2019). Sodium ascorbate was added to prevent spontaneous oxidation of L-DOPA, and incubation was performed in agitation in order to provide the oxygen necessary for the enzymatic activity of 4,5-DODA and the formation of betalains (Guerrero-Rubio et al., 2019). HPLC analysis confirmed the presence of the betalains of interest, in addition to other secondary compounds. Betalamic acid, betaxanthins, and betacyanins were detected at $\lambda = 405$, 480, and 536 nm, respectively. The retention times obtained were 15.5, 13.4, 19.8, and 18.5 min for betalamic acid, dopaxanthin, phenylalanine-betaxanthin, and indoline carboxylic acid-betacyanin, respectively. After sequential purification steps, HPLC analyses showed chromatograms containing only the betalain of interest (Fig. 1). Betalamic acid and dopaxanthin were eliminated after the purification process of phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin. For the purification of dopaxanthin, only betalamic acid was removed. The high concentrations of dopaxanthin and phenylalanine-betaxanthin allowed their detection at $\lambda = 405$ nm. The chromatographic purity obtained by HPLC was 96.31, 95.08, 96.38, and 93.40% for dopaxanthin, phenylalanine-betaxanthin, indoline carboxylic acid-betacyanin and betanidin, respectively (Fig. S1). All analytical data for purified betalains used in this work are given in Table S1. The mass values obtained by ESI-MS were 391, 359, 357, and 389 *m/z* at 13.4, 19.8, 18.5 and 14.3 min, expected values for the protonated molecular ions $[M + H]^+$ of dopaxanthin, phenylalanine-betaxanthin, indoline carboxylic acid-betacyanin and betanidin, respectively, thus confirming the nature of the structures proposed.

3.2. Characterization of betalain-loaded liposomes

The encapsulation of betalains in liposomes took place at 65 °C in order to increase the solubility of phosphatidylcholine. The effect of this temperature on betalains was analyzed by HPLC (Fig. S2A). The results obtained showed a decrease in the integrated peak area of 5.1 and 26.4% for phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin, respectively, when they were heated at 65 °C for 10 min (equivalent to the time taken for liposome fabrication). The supernatants obtained after formation and centrifugation of the liposomes allowed determining the encapsulation efficiency, being 84.48 and 80.71% for phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin, respectively. On the other hand, the rupture of liposomes loaded with both betalains allowed analysis of their contents by HPLC (Fig. S2B). The pigments were detected and no degradation products were observed. In addition, the decrease of betalains content inside liposomes at 24 and 48 h reveals a possible release of betalains under the conditions of *in vivo* assays (Fig. S2C). To confirm this, the supernatants obtained after centrifugation of liposomes were analyzed. Specifically, 34.9 and 5.2% of phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin were released from the liposomes after one day of incubation. The results obtained are comparable with previous studies on the release of other liposome-encapsulated betalains (Amjadi, Mesgari Abbasi, Shokouhi, Ghorbani, & Hamishehkar, 2019). The observed lower release of indoline carboxylic acid-betacyanin suggests that there is an influence of the structure on the retention of the encapsulated molecules inside the vesicles. Previous studies on the release of bioactive compounds encapsulated in liposomes highlight this fact (Li, Ling, Peng, Feng, Chen, Zhu, Qing, Zou, Qiang, Liu, & Liu, Mei., 2018; Liu et al., 2018).

Once the liposomes were formed, their presence was confirmed by TEM (Fig. 2A). The vesicles had heterogeneous sizes (high polydispersity) and a predominant spherical morphology. Image analysis by ImageJ resulted in an average diameter of 0.63 ± 0.1 μm, not exceeding the pore diameter of the membrane/filter used for its formation (1 μm). Therefore, liposomes were of the large unilamellar (LUV) type (Rideau, Dimova, Schwille, Wurm, & Landfester, 2018). Polydispersity may be due to the passing of small liposomes no larger than the size of the pore, through the membrane. No morphological differences were found between control liposomes and liposomes loaded with phenylalanine-betaxanthin. The images also showed aggregates of liposomes (predominantly with the smaller liposomes around the larger ones) promoted by the hydrophobicity of the lipid bilayers.

The analysis by DSC allowed to know the temperature at which the lipids of the liposomal membrane changed from gel phase to liquid-crystalline or fluid phase (transition temperature) by measuring the heat capacity of the liposomal solution as a function of temperature (thermogram). The arrangement of the hydrocarbon chains of phosphatidylcholine defines both transition states, being rigid in the gel phase and mobile in the fluid phase (Mabrey & Sturtevant, 1978). The transition temperature is sensitive to the presence of other biomolecules immersed in the lipid bilayer so these analyses allowed to know what position is occupied by the betalains encapsulated in the liposome (i.e. aqueous compartment or within the bilayer) when comparing the thermograms of the different samples. The liposomes were prepared using PBS buffer instead of water to ensure the stability of the membranes and to prevent the formation of ice crystals during scans. The results showed similar endothermic transitions between the control liposomes and the betalain-loaded liposomes samples (Fig. 2B). The heat capacity (Cp) was constant in the same phase and increased when the transition occurred, indicating that more energy was needed to increase the same amount of temperature with respect to the reference (PBS buffer). This is because some of the energy was used to change the phase (endothermic transition). The transition temperatures (Tm) obtained were −10.3, −10.5, and −10.5 °C for unfilled liposomes, liposomes loaded with phenylalanine-betaxanthin, and those loaded with indoline carboxylic acid-betacyanin, respectively. The Tm values obtained are in accordance with the results of previous studies on phase transitions of phosphatidylcholine (Koynova & Caffrey, 1998). The similarity of the thermograms obtained and the minimal differences in the Tm confirm that the encapsulated betalains are located into the aqueous compartment of the liposomes without affecting to the lipid bilayer.

The labeling of liposomes was successful due the Nile red is a fluorescent lipophilic dye and its fluorescence was combined with the natural fluorescence emitted by the more hydrophilic molecule phenylalanine-betaxanthin (Gandía-Herrero et al., 2010). These characteristics allowed visual studies of localization of the pigment within the vesicles. The images showed the location of the betalain fluorescence inside the lipid vesicles, that were surface-labeled with Nile red (Fig. 2C1). In addition, the recording of several images in different planes of the same particles allowed the 3D reconstruction of liposomes with encapsulated phenylalanine-betaxanthin (Fig. 2C2 and Video S1). The internal location of the encapsulated betalain was clearly confirmed

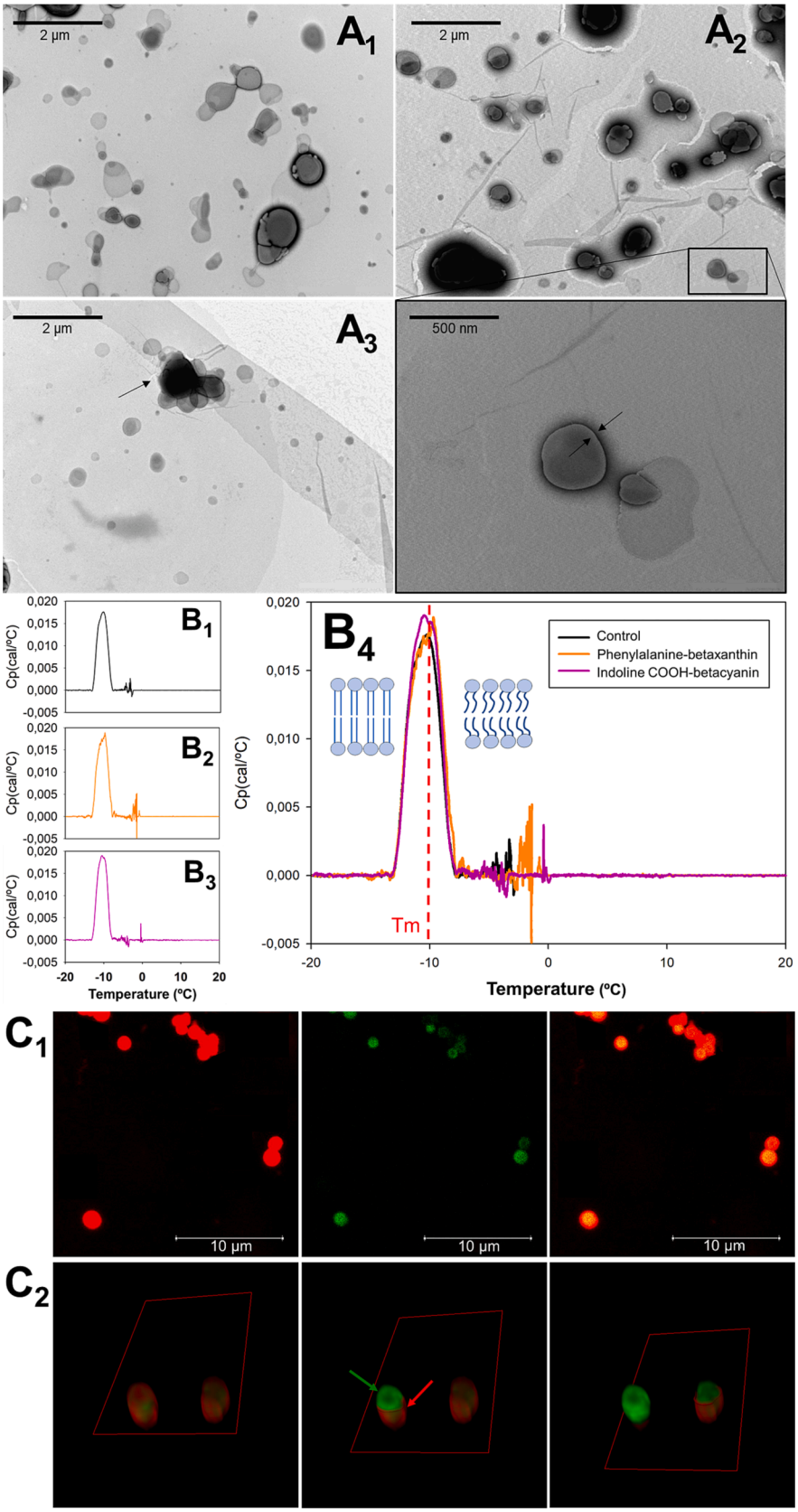

Fig. 2. Characterization of betalain-loaded liposomes. (A) Liposomes saw under transmission electron microscopy (TEM). (A_1) Control liposomes; (A_2) Liposomes with phenylalanine-betaxanthin encapsulated. The arrows point out to the lipid bilayer of a liposome seen at greater magnification; (A_3) Aggregation of liposomes promoted by hydrophobic forces. (B) Thermograms obtained after DSC analysis of control liposomes (B_1), loaded with phenylalanine-betaxanthin (B_2), and loaded with indoline carboxylic acid-betacyanin (B_3). The overlapping thermograms are also shown (B_4). (C) Images of liposomes loaded with phenylalanine-betaxanthin obtained by confocal microscopy. (C_1) Same liposomes excited with the laser at 549 nm (red) and 471 nm (green) and colocalization (yellow) after merging of the images, scale bar: 10 µm; (C_2) 3D reconstruction of liposomes labeled with Nile red (red arrow) and with phenylalanine-betaxanthin (green arrow) encapsulated inside. Sections of the lipid bilayer made at different planes (indicated with the red rectangle) are shown.

visually, like a green fluorescent vesicle with a red cover of lipids, in agreement with the results previously obtained by DSC. This is the first direct evidence of the formation of betalain-loaded vesicles.

3.3. Time and dose effects on liposomes intake by C. elegans

The use of rhodamine 6G as a fluorescent probe to label liposomes and the transparency of *C. elegans* allowed to study of the intake of vesicles by wild-type N2 nematodes. Animals that consumed labeled liposomes showed fluorescence along the digestive tract (Fig. 3A–E), not being detected in control individuals after administration of 10 μL of unlabeled liposomes (Fig. 3F). The results obtained after quantification of fluorescence showed higher levels of incorporated fluorescence as the dose of liposomes increased (Fig. 3G). Likewise, nematodes that were in contact with the vesicles for 60 min fed for longer and showed higher fluorescence levels compared to those which interrupted feeding after 15 min. It is concluded that increased fluorescence results in a greater number of ingested liposomes. These results confirm the intake of liposomes by the feeding nematodes, and its positive dependence on the time of contact with the vesicles and on the dose administered. The bacteriophage behavior of *C. elegans* leads worms to absorb the solutions, retain the bacteria and spit out the rest of the contents (Avery & Thomas, 1997). During the feeding of the nematodes, liposomes seem to simulate bacteria, being ingested and assimilated in the same way. In this sense, the use of liposomes as vehicles is postulated as an efficient alternative for the administration of chemical compounds with respect to solutions.

3.4. Effect of encapsulated betalains on lipid accumulation in vivo

The use of the ORO staining procedure allowed to dye the lipids present within the nematodes. Wild-type N2 individuals fed with control liposomes appeared to show greater fat accumulation than untreated ones, due to the lipidic nature of the vesicles. However, when the administered liposomes were loaded with betalains, lipid abundance in the animals was reduced (Fig. 4A–C). The quantification of lipid levels showed that betalains managed to reduce lipid accumulation significantly, presenting a greater effect when the encapsulated betalain concentration was 500 μM (Fig. 4D). At this concentration, phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin reduced nematode fat by 48.1 and 52.5%, respectively, compared to control individuals treated with control liposomes. This is a significant antilipidemic effect. Interestingly, the compound's mechanism of action (MOA) involves different longevity pathways in *C. elegans*, Guerrero-Rubio and coauthors (2020) reported that indoline carboxylic acid-betacyanin treatment produced the overexpression (1.64-fold) and translocation the transcription factor SKN-1 (Guerrero-Rubio et al., 2020), that is involved in oxidative stress resistance and detoxification. Moreover, the microarray assays performed by the authors and deposited in GEO database (NCBI) [https://www.ncbi.nlm.nih.gov/geo/] with the identifier GSE134614, showed that indoline carboxylic acid-betacyanin treatment was able to modulate several genes involved in fat metabolism as *fat-7* (-4.4-fold *vs* control), *lipl-4* (12.06-fold *vs* control), or *pha-4* (3.09-fold *vs* control). The gene *fat-7* encodes a Δ9 fatty acid desaturase which is indispensable for the synthesis of monounsaturated fatty acids, required for animals' viability and normal growth. It has been reported that *fat-6/fat-7* double mutants showed a decrease in lipid storage compared with wild-type animals (Brock, Browse, & Watts, 2007). Furthermore, *lipl-4* encodes a triglyceride lipase, which is predicted to function in lipid hydrolysis, lifespan and positive regulation of autophagy dependent of the FoxA transcription factor *pha-4* (Lapierre, Meléndez, & Hansen, 2012). Therefore the decrease in fat levels observed in nematodes fed with indoline carboxylic acid-betacyanin encapsulated in liposomes could be explained by the compound modulation at genetic level. The results obtained support the antilipidemic nature of the betalains tested (Martínez-Rodríguez et al., 2022).

3.5. Effect of encapsulated betalains on oxidative stress in vivo

TJ375 strain presents the GFP transgene of *Aequorea victoria* coupled to the promoter of the *hsp-16.2* gene, expressed in the pharynx of animals under conditions of oxidative stress. Such stress can be caused by exposure to quinones such as juglone (Link, Cypser, Johnson, & Johnson, 1999). The antioxidant properties of the betalain pigments were studied by quantifying the fluorescence emitted by the animals after treatment with encapsulated betalains and subsequent administration of juglone 20 μM. The results showed reductions in fluorescence intensity when the animals were fed liposomes loaded with phenylalanine-betaxanthin and indoline carboxylic acid-betacyanin (Fig. 5). This fact translates into a lower expression of *hsp-16.2::GFP* and, therefore, into a decrease in oxidative stress mediated by the ingestion of betalain-loaded liposomes. Pigments encapsulated at a concentration of 500 μM had a greater antioxidant effect and reduced fluorescence by 37.2 (phenylalanine-betaxanthin) and 48.5% (indoline carboxylic acid-betacyanin), compared to control worms treated with juglone and control liposomes. In addition, the results obtained showed GFP expression when the animals were treated only with control liposomes, indicating that the intake of the lipid vesicles alone produces additional oxidative stress in

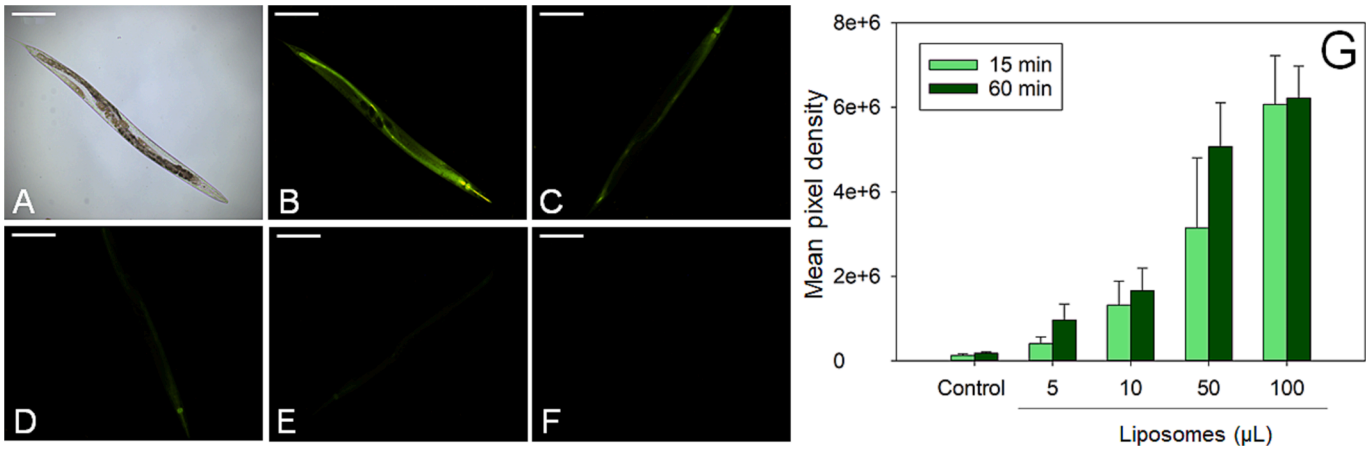

Fig. 3. Intake of liposomes by *C. elegans* N2. (A) Representative image of a nematode acquired by brightfield microscopy 15 min after administration of 100 μL of labeled liposomes. (B) The same nematode visualized under fluorescence microscopy. Fluorescence representative images of nematodes fed with 50 (C), 10 (D), 5 μL (E) of labeled liposomes, and control liposomes (F). (G) Quantification of liposome intake by integrating fluorescence for each condition (data represented as mean ± S.D.). Scale bar: 200 μm.

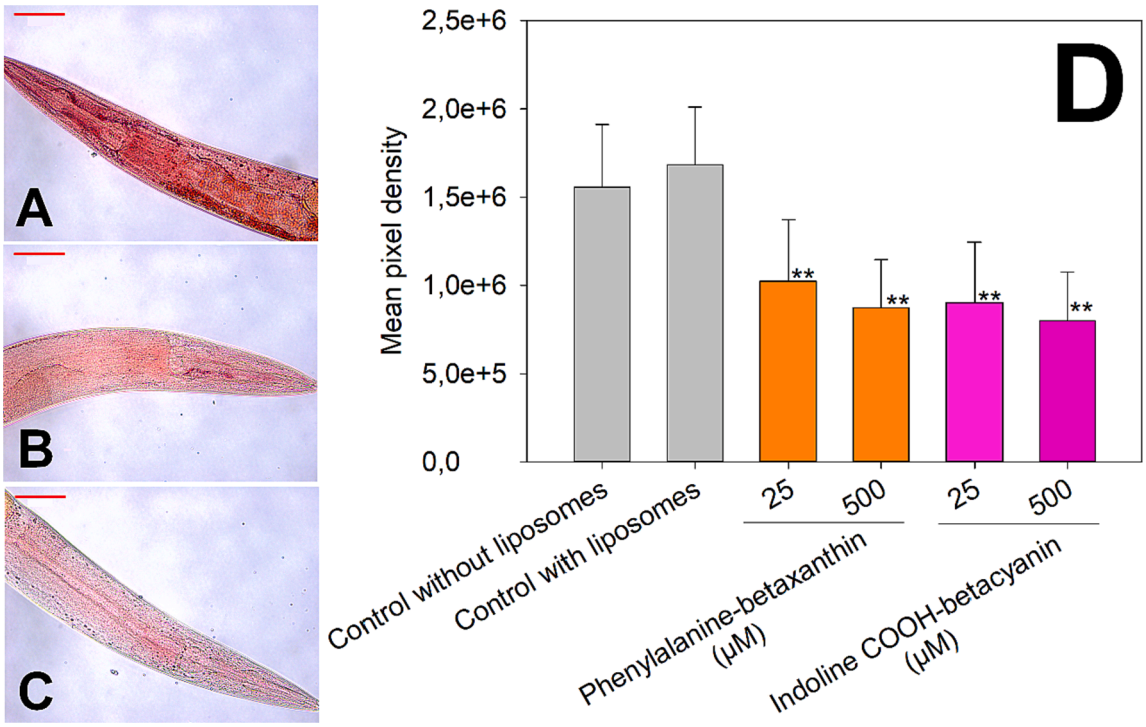

Fig. 4. Reduction of lipid accumulation in *C. elegans* N2. Representative images of ORO-stained nematodes fed with control liposomes (A), loaded with phenylalanine-betaxanthin (500 μM) (B), and loaded with indoline carboxylic acid-betacyanin (500 μM) (C). (D) Quantification of the average pixel density for each condition. Data represented as mean ± S.D. ** Significant at $p < 0.05$. Scale bar: 50 μm.

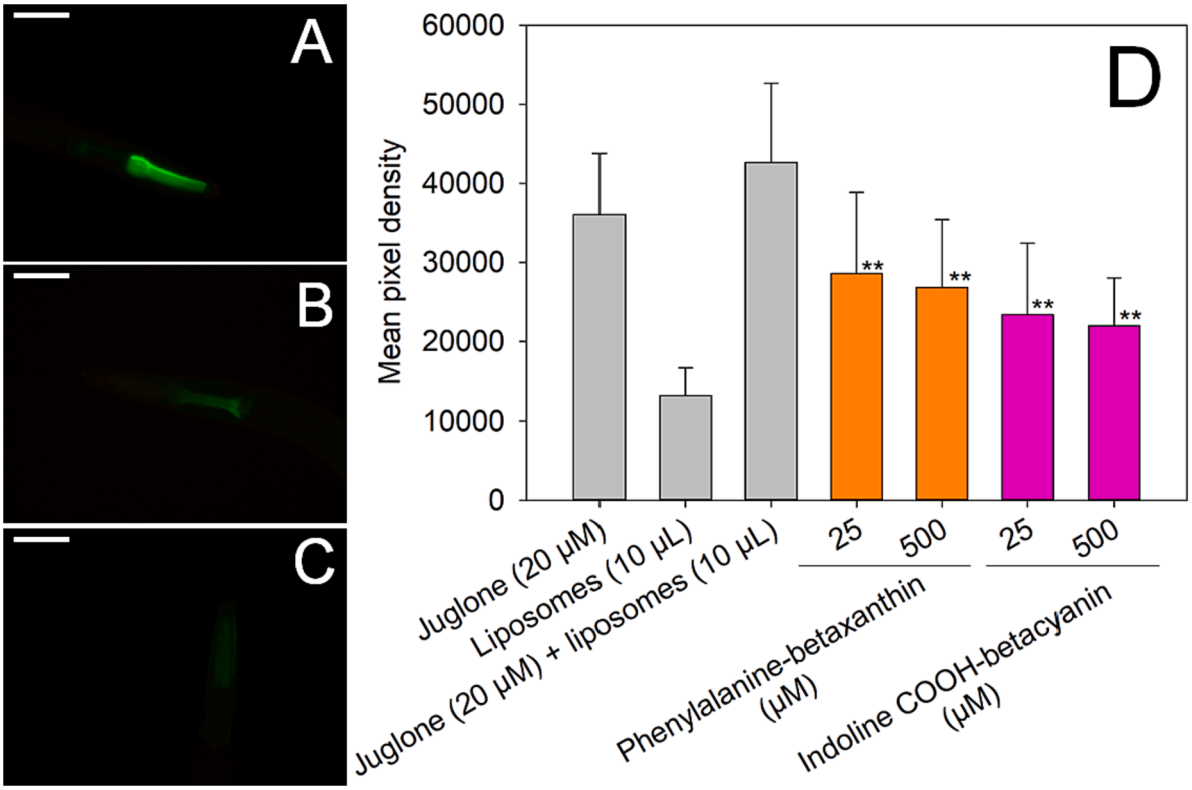

Fig. 5. Quantification of the antioxidant effect of betalains encapsulated in liposomes. Representative images of TJ375 animals treated with control liposomes (A), loaded with phenylalanine-betaxanthin (500 μM) (B), and loaded with indoline carboxylic acid-betacyanin (500 μM) (C). (D) Quantification of the fluorescence intensity for each condition (data represented as a mean ± S.D.). ** Significant at $p < 0.05$. Scale bar: 50 μm.

worms.

3.6. Effect of encapsulated betalains on the lifespan of C. elegans

The wild-type strain N2 was used for the study of the general health-promoting effect of encapsulated betalains measured as increases in the lifespan of *C. elegans*. The Lifespan Machine was employed to measure the mean lifespan (Fig. S3). Age-synchronized nematodes were exposed from the larval stage L1 to concentrations of 25, 50, 100, and 500 μM of encapsulated betalains using a fixed dose of 10 μL of liposomes (48 mg/mL). From the statistical adjust of the survival curves, mean lifespan (average death age) and maximum lifespan (time of death for the last living) were obtained. In addition, the possible effect of lipid vesicles intake on nematode survival was studied. The results are shown in Table S2 and Fig. 6. It is important to note that the administration of increasing doses of liposomes (5, 10, 25, and 50 μL) significantly reduced the lifespan of the nematodes with respect to the control without treatment, decreasing survival by up to 14.0% when the dose of vesicles administered was maximum (50 μL). Likewise, exposure to 10 μL of liposomes was the condition that least reduced the lifespan of the nematodes, and thus it was selected for the analysis of the health-promoting potential of encapsulated betalains. In this sense, indoline carboxylic acid-betacyanin had no positive effect on the lifespan of *C. elegans*, with respect to control with control liposomes. However, this betacyanin itself did have an oxidative stress-reducing effect on nematodes, as indicated in the previous section. In contrast, betanidin encapsulated at a concentration of 25 μM managed to increase the lifespan of nematodes by up to 13.8%, increasing the maximum lifespan by 1.2 days. This fact suggests that liposomes are able to efficiently stabilize this natural and unstable betacyanin, causing its health-promoting effect to be manifested here for the first time, so far absent in *in vivo* assays after its administration in free form (Guerrero-Rubio et al., 2020). In the case of betaxanthins, encapsulated dopaxanthin increased the lifespan of nematodes by 8.1% and the maximum lifespan by 0.4 days when the highest concentration of encapsulated pigment (500 μM) was used. On the other hand, encapsulated phenylalanine-betaxanthin was the most effective betalain, increasing mean survival by up to 21.8% and maximum lifespan by 1.2 days when administered at a concentration of 100 μM. The use of the highest concentration tested (500 μM) resulted in a smaller increase in the lifespan, thus indicating that the optimal concentration for phenylalanine-betaxanthin is 100 μM when the betalain-loaded liposomes are used as health-promoting agents *in vivo*.

4. Conclusions

The bioactive properties of four pure betalains have been studied after their encapsulation in lipid vesicles. Although liposome intake slightly decreases lifespan in the animal model *C. elegans*, the first bioactivity studies of liposome-encapsulated betalains show how anti-lipidemic, antioxidant, and lifespan extension effects are not affected by this promising oral route of administration. Within the betalains tested, phenylalanine-betaxanthin showed the best performance in the experiments carried out on the different strains of *C. elegans* employed in this work. In addition, encapsulation in liposomes increased the stability of the labile pigment betanidin, causing its biological activity to be manifested for the first time in terms of increasing the average lifespan of *C. elegans* specimens. The results obtained in the present work open new paths to evaluate the health-promoting properties of betalains by showing a new strategy for the administration and characterization of other betalains and molecules with bioactive potential.

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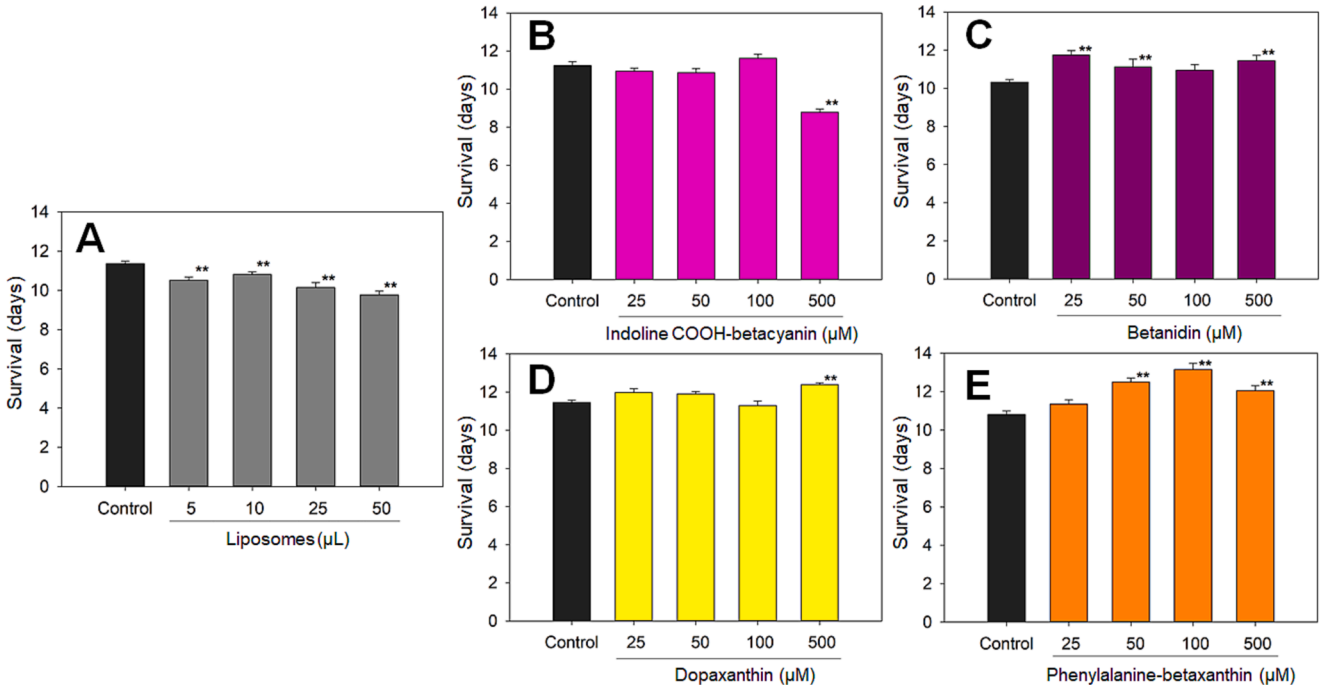

Fig. 6. Liposomes' effect on *C. elegans* lifespan. Mean lifespan of *C. elegans* N2 after exposure to increasing doses of control liposomes (A), liposomes loaded with indoline carboxylic acid-betacyanin (B), betanidin (C), dopaxanthin (D), and phenylalanine-betaxanthin (E). Data represented as a mean ± S.E. ** Significant at $p < 0.05$. The survival data, *p*-values, standard errors, and n are summarized in Supplementary Table S2.

CRediT authorship contribution statement

Pedro Martínez-Rodríguez: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **M. Alejandra Guerrero-Rubio:** Conceptualization, Formal analysis, Supervision, Validation, Methodology, Writing – original draft. **Samanta Hernández-García:** Methodology, Investigation, Resources, Writing – original draft. **Paula Henarejos-Escudero:** Methodology, Investigation, Resources, Writing – original draft. **Francisco García-Carmona:** Conceptualization, Funding acquisition, Project administration, Validation, Writing – review & editing. **Fernando Gandía-Herrero:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Some of the authors launched in 2022 the company Betaelegans Biotech, a spin-off from the public academic institution Universidad de Murcia (Spain), primarily devoted to the synthesis of betalain standards and to the screening of bioactivities in *C. elegans*. This has not biased or influenced the conception, analysis, or writing of the present research. The authors declare no other competing financial interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.135180.

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