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Consumption of commonly used artificial food dyes increases activity and oxidative stress in the animal model *Caenorhabditis elegans*

M. Alejandra Guerrero-Rubio [1], Samanta Hernández-García, Francisco García-Carmona, Fernando Gandía-Herrero [*]

Departamento de Bioquímica y Biología Molecular A, Unidad Docente de Biología, Facultad de Veterinaria, Regional Campus of International Excellence "Campus Mare Nostrum", Universidad de Murcia, Murcia, Spain

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ABSTRACT

In recent decades, the consumption of artificial colorants in foods and beverages has increased despite of concerns in the general population raised by studies that have shown possible injurious effects. In this study, tartrazine, sunset yellow, quinoline yellow, ponceau 4R, carmoisine and allura red were employed as pure compounds to explore their effects *in vivo* in the animal model *Caenorhabditis elegans*. The exposition of *C. elegans* to these artificial dyes produced damage related with aging such as oxidative stress and lipofuscin accumulation, as well as a heavy shortening of lifespan, alterations in movement patterns and alterations in the production of dopamine receptors. Besides, microarray analysis performed with worms treated with tartrazine and ponceau 4R showed how the consumption of synthetic colorants is able to alter the expression of genes involved in resistance to oxidative stress and neurodegeneration.

1. Introduction

In the current world of stimuli, appearance has become an important topic in all aspects, including food. It is essential that foods present colours that make them appetizing and visually attractive and the use of dyes has spread in response. There are different natural pigments that are used by the food industry like β-carotene, betanin or turmeric. However, the requirements of the food industry are so high that obtaining these natural pigments is not economically feasible. In addition, they have low durability in the food matrix and cannot cover the whole range of necessary colours. Therefore, in recent decades the consumption of artificial food dyes (AFDs) has increased significantly (Stevens et al., 2015). These compounds are found in foods and beverages consumed by the entire world population and they especially influence children due to their presence in candies and desserts. Some studies have shown that children consume AFDs in higher amounts than recommended by official agencies such as EFSA (European Food Safety Authority) or FDA (United States Food and Drug Administration) (Husain et al., 2006; Stevens et al., 2015). The lack of awareness by the consumer may be responsible for this high intake since several studies have shown that an excessive consumption can be harmful to health (Khayyat et al., 2017; Macioszek & Kononowicz, 2004; Sasaki et al., 2002). One of the most relevant studies in this field is known as the "Southampton studies" (McCann et al., 2007) where mixtures of six of the most used AFDs were related to an increase in the hyperactivity of children diagnosed with attention deficit hyperactivity disorder (ADHD). After these studies, EFSA re-evaluated and decreased the ADI (Acceptable Daily Intake) of three dyes: quinoline yellow, sunset yellow and ponceau 4R (Aguilar et al., 2009a–d) (Table S1). However, this measure could be considered preventive because the study did not show a direct relationship between hyperactivity and consumption of these dyes. In addition, the "Southampton studies" lack individual assays that may highlight the effect of each dye used in their mixtures. Based on these studies and in view of the increased consumption of artificial food colorants, the aim of this paper is to provide information about the *in vivo* effect of six AFDs (Fig. 1) in the animal model *Caenorhabditis elegans*. The simple nematode is widely used as genetic animal model since it provides relevant information about human biology and diseases (Markaki & Tavernarakis, 2010) due to the presence of evolutionarily conserved pathways and ortholog genes (Shaye & Greenwald, 2011).

* Corresponding author.
E-mail address: fgandia@um.es (F. Gandía-Herrero).
[1] Current address: Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom.

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2. Materials and methods

2.1. Chemicals

All chemicals and reagents employed in this work were obtained from Sigma-Aldrich (St. Louis, MO, USA) except TRIzol® and RNA extraction kit which were acquired from Fisher Scientific (Dublin, Ireland). A Milli-Q system (Millipore, Bedford, MA, USA) was employed to purify distilled water.

2.2. Colorants

Six artificial food dyes (AFDs) were used in this study: carmoisine (E112, FD&C Red 10), ponceau 4R (E124, FD&C Red 3), allura red (E129, FD&C Red 40), tartrazine (E102, FD&C Yellow No. 5), sunset yellow (E110, FD&C Yellow 6) and quinoline yellow (E104, FD&C Yellow 10). These six colorants were purchased from Sigma-Aldrich and diluted in distilled water and employed at different concentrations. The methodology used to evaluate the absorbance and fluorescence properties of the dyes is explained in the supplementary method section of the Supplementary Material. The obtained spectra are shown in the Supplementary Material.

2.3. C. elegans strains and culture conditions

The wild type strain N2 and the genetically engineered strains TJ375 (*hsp-16.2::GFP*) and BZ555 (*dat-1p::GFP*) were obtained from the *Caenorhabditis* Genetic Center (CGC). Solid nematode growth medium (NGM) was used for the maintenance of worms and the experiments were performed in a liquid S medium (see supplementary methods section). In both cases, *Escherichia coli* OP50 was employed as a food source. *E. coli* was grown overnight in liquid Luria-Bertani (LB) medium and then concentrated 10X in fresh M9 buffer. *E. coli* cells were then inactivated by incubating the cultures for 30 min at 65 °C.

This work has received approval for research ethics from "Committee on Animal Research and Ethics" (CEEA), University of Murcia (Spain).

2.4. Synchronous worm culture

Worms used in all experiments were age-synchronized. Synchronic cohorts of *C. elegans* were prepared using the bleaching method. Briefly, stock plates with many gravid adults were collected with 3.5 mL of sterile M9 buffer in a 15 mL centrifuge tube, and then 1.5 mL of freshly prepared household bleach/5N NaOH solution (2:1) was added. The mix was incubated at room temperature for ten minutes mixing by vortex every two minutes. The bleached eggs were then centrifuged at 7,500 g and washed twice with 5 mL of M9 buffer. The eggs were left to hatch overnight in 10 mL of the same buffer under orbital shaking at 20 °C.

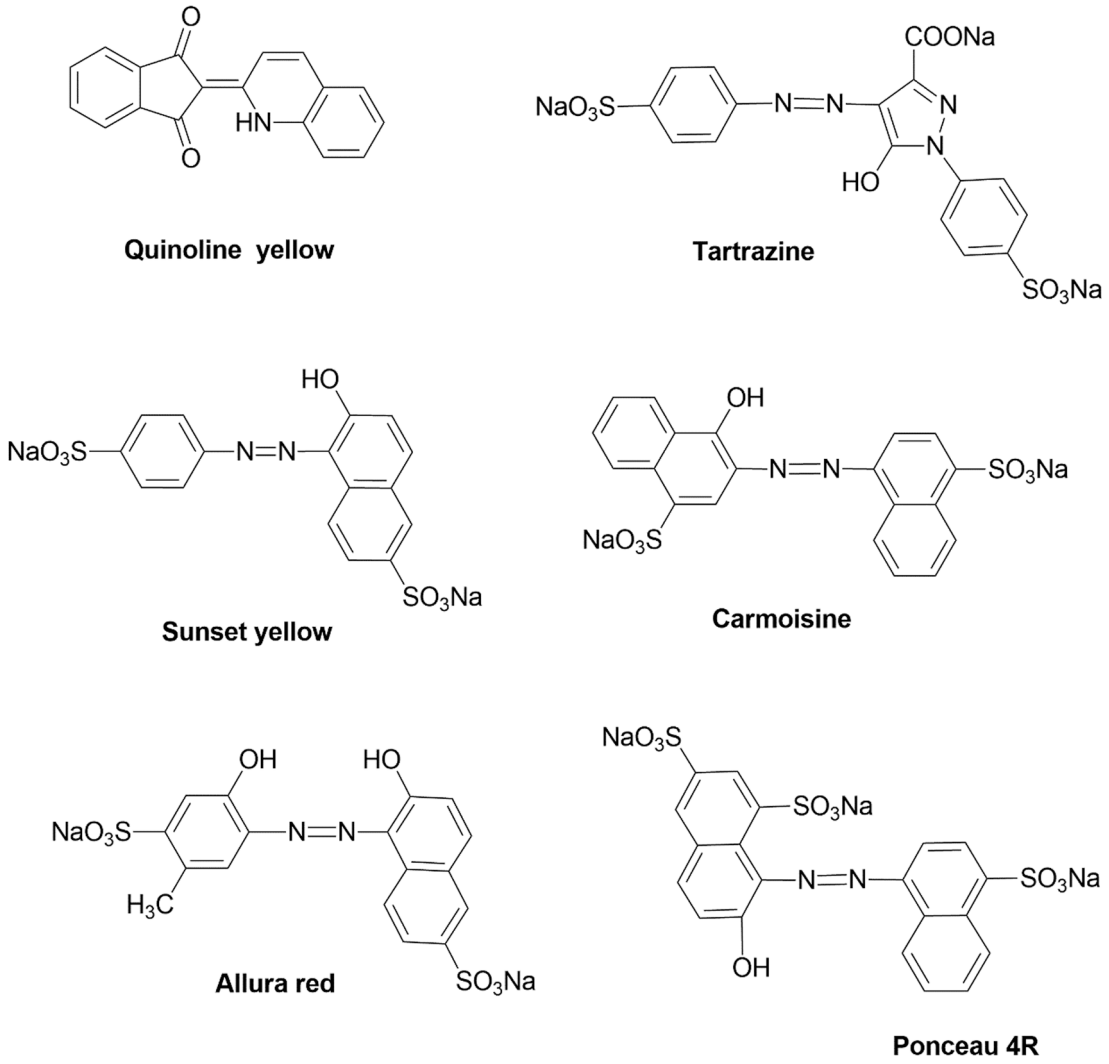

Fig. 1. Chemical structures for the artificial food dyes (AFDs) employed in this study.

2.5. Survival assays

Lifespan Machine, the platform for the automatic control of the organism *C. elegans* mobility, was used in the survival assays. For lifespan assays, both acute and chronic expositions to colorants were considered. In the acute assay, the obtained arrested larvae L1 were collected and transferred to a final volume of 5 mL of sterile S medium which contained 10, 25, 50 and 100 μM of each AFD. After 48 h in liquid culture worms were centrifuged at 2,000 g and then washed with M9 buffer three times. Then, 30–50 worms were transferred to 35 mm dishes, containing 8 mL of NGM agar, 30 μg/mL of nystatin and 100 μM of ampicillin to avoid contamination, 10 μg/mL of FUdR (2′-Deoxy-5-fluorouridine) to avoid progeny and 100 μL of heat inactivated *E. coli* OP50. For chronic assays, exposition to dyes was performed during the whole lifespan of the L4 worms in the plates. Dyes were added to the 8 mL of NGM agar at concentrations of 10, 50 and 100 μM and then poured directly on the 35 mm plates. The same concentrations of nystatin, ampicillin and FUdR as described above were added and 30–50 synchronized-worms were placed into each plate. All conditions, both for acute and chronic assays, used in the Lifespan Machine were performed in triplicate. Plates were closed and incubated at 20 °C. After 20 min, closed lid plates were loaded into the machine's scanners. Experiments were set at 25 °C for 25 days. Lifespan Machine was used following the constructor instructions described by Stroustrup et al., (2013) with the modifications described by Guerrero-Rubio et al., (2019).

2.6. Quantification of hsp-16.2::GFP expression in C. elegans TJ375

The mutant strain TJ375 *(hsp-16.2::GFP*) was cultivated in liquid S medium at 20 °C and the expression of *hsp-16.2* was measured by observing the fluorescence of the green fluorescent protein (GFP). The day after hatching, synchronous worms were treated with individual AFDs at a concentration of 100 μM for 72 h. Oxidative stress induced by 20 μM juglone was employed as positive control. The oxidative stress produced by juglone in combination with individual dyes was also measured. The age-synchronized worms were treated for 48 h with the individual AFDs at a concentration of 100 μM. Then, worms were transferred to a new liquid medium, and 20 μM juglone was added. The worms were kept at 20 °C and after 24 h the oxidative stress was measured. The fluorescence of TJ375 worms was observed with a Leica DM 2500 LED microscope using filtercube I3 and a 20x objective lens. Images were taken with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany) fitted to the microscope. The glass slides containing worms were supplemented with 10 mM sodium azide to reduce their mobility. Fluorescence's images of TJ375 worms included the anterior part of the worms from the back of the pharynx. To quantify GFP fluorescence, images were analysed using ImageJ software. For each raw image, worms were outlined black-to-white inverted and the mean pixel density was measured.

2.7. Neurological evaluation

Age-synchronized BZ555 (*dat-1p::GFP*) worms were kept in 5 mL of liquid S medium supplemented with 100 μM of tartrazine, sunset yellow, quinoline yellow, carmoisine, ponceau 4R or allura red, *E. coli* as food, and FUdR to avoid progeny. After 6 days at 20 °C, worms were washed with M9 buffer and mounted onto glass slides containing 10 mM sodium azide. Images were taken using filtercube I3 and the 20x lens in a Leica DM 2500 LED microscope fitted with a Leica DFC550 camera (Leica Microsystems). Images of 12–13 worms per condition were taken and analysed using ImageJ software.

2.8. Lipofuscin detection

The accumulation of lipofuscin was measured as an aging marker. Age-synchronized N2 worms were kept in 5 mL of liquid S medium supplemented with 100 μM of tartrazine, sunset yellow, quinoline yellow, carmoisine, ponceau 4R or allura red, *E. coli* as food, and FUdR to avoid progeny. After 4 days at 20 °C, worms were washed with M9 buffer and mounted onto glass slides with 10 mM sodium azide. Images were taken using filtercube A (Leica, excitation range: 340–380 nm) at constant exposure times and the 20x lens in a Leica DM 2500 LED microscope above mentioned. Images of 7–8 worms per condition were taken and analysed using ImageJ software.

2.9. Motility analysis

The effects of the six AFDs on the motility of *C. elegans* were measured by evaluating the average speed and maximum speed of the animals. Synchronized worms in the first larva stage L1 were treated with 100 μM of each colorant in S basal medium supplemented with *E. coli* as a food source. After 48 h, plates were acclimated to room temperature for 30 min and then, videos of worms' movements were recorded using an Optika SZO series stereomicroscope (OPTIKA S.r.l., Italy) fitted with an Optika H series camera (OPTIKA). Image analysis was performed by using the worMTracker -wrMTrck- plugin of imageJ software following the Pederson's protocol.

2.10. RNA extraction

The day after hatching, synchronized-worms in liquid S medium were treated with 100 μM of AFDs and kept at 20 °C with rotary shaking at 120 rpm. After 48 h, worms were washed with M9 buffer ten times for the complete elimination of *E. coli* bacteria present in the medium. Then, worms were resuspended in 250 μL of M9 buffer and 750 μL of reactive TRIzol® was employed for RNA extraction. RNA extraction and its purification were realized following the protocol of PureLinkTM RNA Mini Kit from Invitrogen (Carlsbad, California, USA). The amount and quality of RNA was checked by Bioanalyzer (Agilent Technologies) and was then used for microarray analysis.

2.11. Microarray analysis

Ss-cDNA was synthetized from 3.5 ng of each sample using the GeneChip WT Pico Reagent kit (Affymetrix, P/N 703262, CA, USA) purchased from Thermo Fisher Scientific (Thermo Fisher Scientific Inc., MA, USA). With these purposes, instructions from the manufacturer were followed. The amount and quality of ss-cDNA were checked by Nanodrop and Bioanalyzer. Ss-cDNA targets were cleaned up, and after fragmentation and terminal labelling, 3.75 μg of fragmented and biotinylated ss-cDNA were included in the hybridization mix, using the GenAtlas Hybridization, Wash and Stain kit for WT Array Strips (Affymetrix, P/N 901667) according to the recommendations of the manufacturer. The resulting preparations were hybridized to GeneChip® *C. elegans* Gene 1.1 ST Array Strip (Affymetrix, 902157) with 26 unique sequences of each transcript. After applying hybridization (spike controls) and labelling tests it was observed that the 6 chips had fulfilled the quality criteria.

After scanning, microarrays data were processed using Affymetrix Expression Command Console (Affymetrix) and all samples were within bounds for hybridization and labelling tests. Two independent RNA samples were employed. Samples from worms treated with food colorants were grouped as "treatment" and worms without colorant exposition were grouped as "control". Data analysis was then performed with RMA (Robust Multiarray Average) allowing raw intensity values to be background corrected, log2 transformed and then quantile normalized in order to obtain an individual intensity value for each probe set. Partek Genomics Suite and Partek Pathways software (Partek Incorporated, St. Louis, USA) were used for the statistical analysis and an ANOVA test was applied with a restrictive threshold at p-value ≤ 0.05. The molecular interaction, reaction and relation networks that showed differentially

expressed genes (DEGs) were then analysed using KEGG Pathways Kyoto Encyclopedia of Genes and Genomes. The microarray data have been deposited at the GEO database (NCBI) [https://www.ncbi.nlm.nih.gov/geo/] and assigned the identifier GSE143611.

2.12. Statistical analysis

Mathematical analysis of data from the Lifespan Machine's results was realized using the on-line tool OASIS 2 (Supplementary Material), where the Kaplan-Meier estimator, Boschloo's Test, Kolmogorov-Smirnov Test and Survival Time F-Test were performed. One-way ANOVA Calculator for independents measures was performed for numeric data while Chi-square Calculator Contingency Table was used for nominal data. Both analyses were performed using the online Social Sciences Calculator https://www.socscistatistics.com. The significance level for all the data was 0.05.

3. Results

3.1. Effect of individual AFD on C. elegans lifespan

The wild-type N2 strain was used to study the effect of each individual dye in the lifespan of *C. elegans*. Wild-type worms were exposed for 48 h to concentrations of 10, 25, 50 and 100 μM of colorants in acute exposure assay (Table S1, Fig. S5-S10). The worms were then transferred to fresh NGM supplemented with *E. coli* as a food source and incubated at 25 °C in the Lifespan Machine. Acute exposure to colorants negatively affected the mean lifespan of worms (Fig. 2). Fig. 2 shows relative

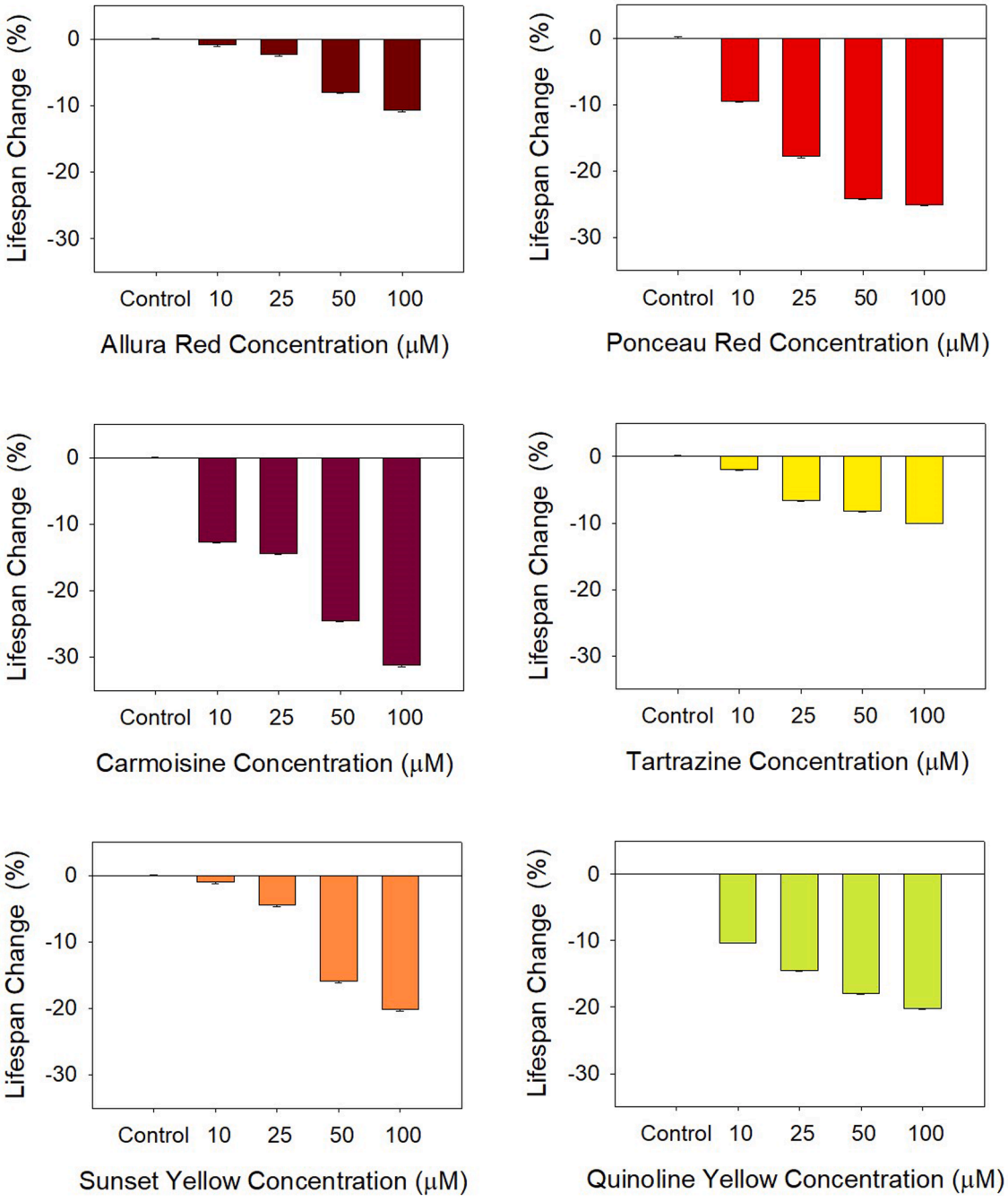

Fig. 2. Mean lifespan changes of *C. elegans* wild-type strain obtained after an acute exposure to individual AFDs. Histograms show the negative effect produced by an exposure of 48 h to individual artificial food dyes at 10, 25, 50 and 100 μM concentrations.

decrease of lifespan in the presence of AFDs. Absolute values are represented in Fig. S1. Survival curves are included in the Supplementary Material (Fig. S2). The negative effect shown was dose-dependent and proportional to colorant concentration. At the highest concentration, mean lifespan decreased 10.6% for allura red exposure, 25.0% for ponceau, 31.2% for carmoisine, 10.0% for tartrazine, 20.2% for sunset yellow and 20.2% for quinoline yellow with respect to non-treated worms. This reduction in the lifespan was higher when chronic exposure of colorants was assayed (Fig. 3, Fig. S4 and Table S2). Concentrations of 10, 50 and 100 μM were used directly on the plates in order to evaluate the effects of a chronic exposure to individual colorants, producing a decrease of up to 28.6% with respect to the control worms with the exposure at 100 μM of allura red and ponceau (Fig. 3). This corresponds to 2.4 days less of the mean lifespan in the model animal. Supplementary Fig. 3 shows the absolute values obtained for these experiments. For carmoisine exposure, a decrease in lifespan of 26.8% was experienced by *C. elegans*. The yellow colorants provoked a decrease of 25.6% for tartrazine, 23.0% for sunset yellow and 24.4% for quinoline yellow.

3.2. Lipofuscin accumulation

Worms treated with AFDs presented a higher accumulation of lipofuscin than untreated worms. For comparative purposes, the blue

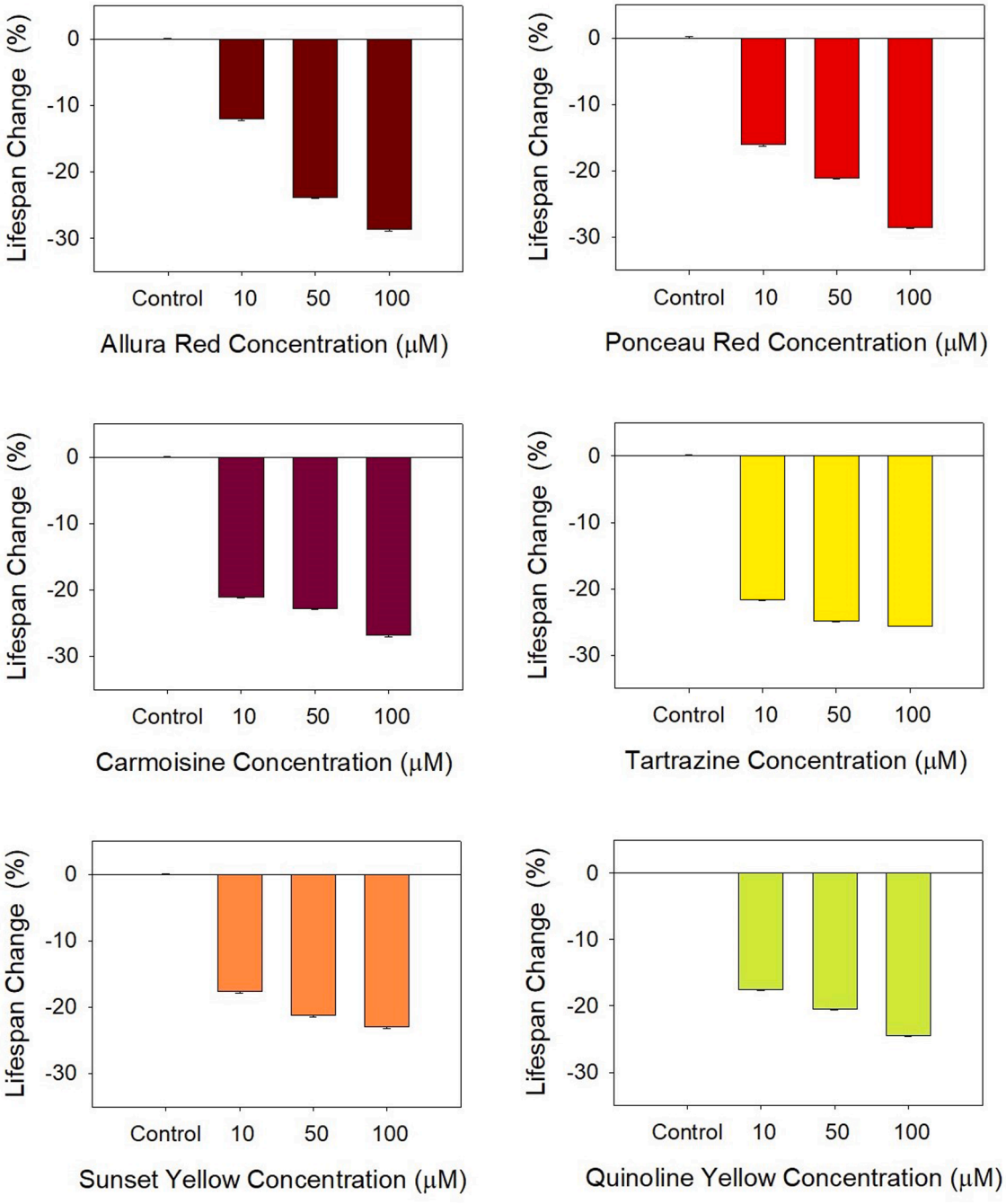

Fig. 3. Mean lifespan changes of *C. elegans* wild-type strain obtained after chronic exposure to different concentrations of individual AFDs. Histograms show the negative effect produced by continuous exposition to individual artificial food dyes at 10, 50 and 100 μM concentrations. Data represented as average ± S.D.

fluorescence emitted by the intestinal region of untreated worms was taken as 100% of fluorescence and then compared with the emission from treated worms (Fig. 4). The amount of lipofuscin increased in worms exposed to quinoline yellow (154%), ponceau 4R (182%) and carmoisine (169%) with respect to untreated worms while sunset yellow did not produce a higher accumulation of lipofuscin (88%). Allura red and tartrazine exposition produced the highest values (277% and 344%, respectively) in the accumulation of lipofuscin. Spectrophotometric and fluorescent measures of the AFDs have been made to rule out a possible interference of their intrinsic fluorescence in the experiments. The colorants had an absorbance maximum in the UV zone (200–250 nm) (Fig. S11.). However, when excited at 340 nm (the microscope excitation light for the A filter cube) they did not exhibit fluorescence as shown in the fluorescence spectra of Fig. S12.

3.3. *Effects of AFDs in the antioxidant system of C. elegans.*

The effects of the exposition to colorants in the antioxidant system of *C. elegans* were studied measuring the fluorescence intensity emitted by *C. elegans* strain TJ375. This strain contains the *hsp-16.2::GFP* construction, responsible for the fluorescence emitted in the pharynx cells under oxidative stress. In this assay, exposition to juglone

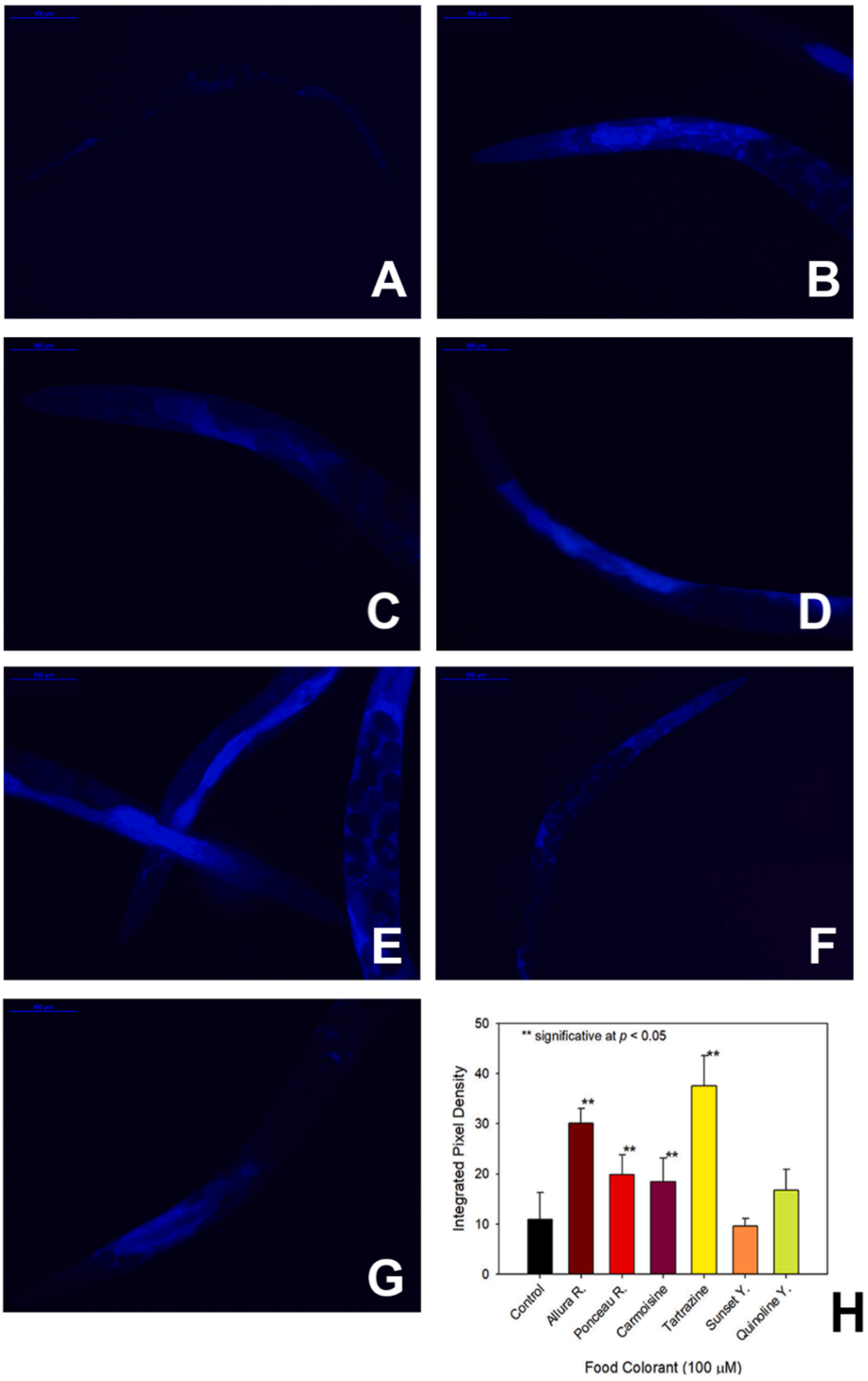

Fig. 4. Accumulation of lipofuscin in 4-day old worms. A-G show representative images of untreated worms (**A**) and worms treated with 100 μM of allura red (**B**), ponceau 4R (**C**), carmoisine (**D**), tartrazine (**E**), sunset yellow (**F**) and quinoline yellow (**G**). Scale bar: 100 μm, objective magnification 20X. **H**: Quantification of fluorescence emitted by lipofuscin accumulation. Data represented as average ± S.D., ** significant at $p \leq 0.05$. 10–12 worms per condition and experiment were assayed, two independent experiments were performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(naphthoquinone) 20 μM was employed as a positive control of oxidative stress. The addition of the six colorants produced worms with green fluorescence in the pharynx, an indication of oxidative stress. Tartrazine, carmoisine and quinoline yellow were less harmful dyes, producing a fluorescence of 19.5%, 19.6% and 18.7%, respectively, with respect to the juglone effect (100%). The fluorescence values due to ponceau 4R, sunset yellow, and allura red exposure were 31.9%, 36.7%, and 44.8%, respectively, while in the absence of juglone or dyes there was no response (Fig. 5A). The added oxidative effect produced by the presence of juglone in combination with each AFD was also assayed. In this case, the fluorescence of TJ375 worms increased as a result of a higher oxidative stress. The combination of juglone and colorants yielded the following values (respect to juglone only control): 184.5% for tartrazine, 316.2% for sunset yellow, 282.5% for quinoline yellow, 232.9% for ponceau 4R, 190.5% for carmoisine and 321.6% for allura red (Fig. 5B).

3.4. Effects of AFC on C. elegans ROS levels

The possible direct effect of AFC on the presence of reactive oxygen species in the model animal was evaluated *in vivo* through the use of specific fluorescent probes (Supplementary Material). Total ROS were measured using the fluorescent probe 2,7-dichloro-dihydrofluorescein diacetate (DHF-DA), which is non-polar, crosses the cell membrane and presents low fluorescence. Then inside cells, the esterases deacetylate it to 2′,7′-dichlorodihydrofluorescein (DCFH) which is a polar species that does not cross the cell membrane and also has low fluorescence. In the presence of ROS this species is oxidized to give the highly fluorescent species 2′,7′-dichlorofluorescein (DCF) and thus allows its detection. Overall, all the colorants increased the ROS levels inside the animals around 3-fold, except tartrazine and sunset yellow that increased the ROS production in the nematodes by a 5 and 14-fold respectively (Fig S15).

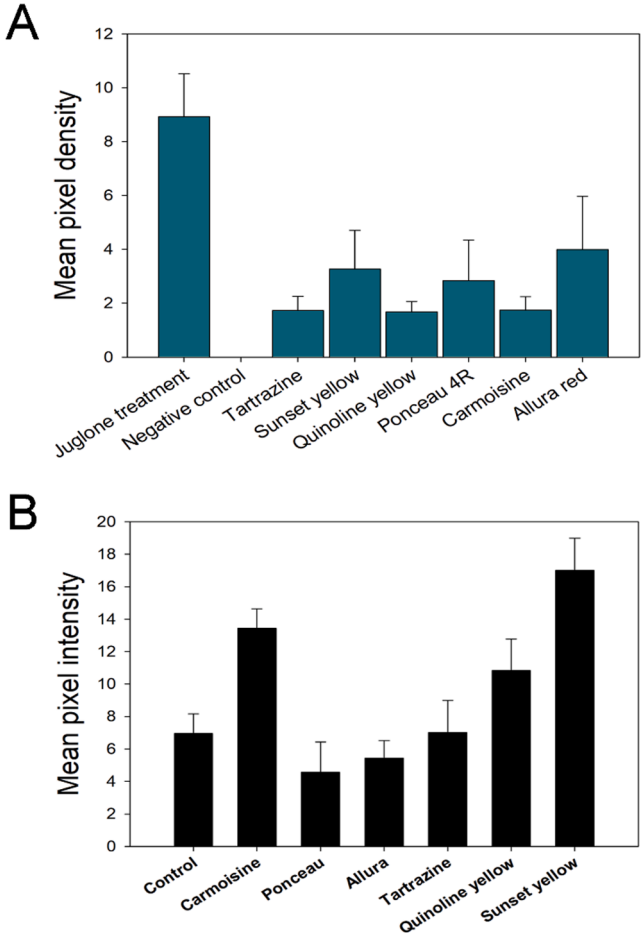

Fig. 5. Expression of HSPs measured through the fluorescence emitted in the pharynx of *C. elegans* TJ375 (*hsp-16.2::GFP*) strain. A. Effect of individual AFDs in the fluorescence located in the pharynx. **B.** Effect of exposure to individual AFDs in worms treated with juglone. In all cases, AFDs were administered at 100 μM concentration. Data represented as average ± S.D., all changes were significative at $p < 0.05$. 10–12 worms per condition and experiment were assayed, two independent experiments were performed.

Superoxide species were evaluated with the specific fluorescent probe dihydroethidium, that shows red fluorescence only in the presence of superoxide anions (Fig. S14 A-G). Quinoline yellow produced the highest increase in superoxide production, 4.3-fold. Whereas ponceau 4R did not have any effect in the superoxide production *in vivo*. The rest of the colorants increased the superoxide production between 3.5 and 1.7-fold (Fig. S14).

3.5. Neurological effects

AFDs have been related to attention deficit hyperactivity disorder (ADHD), one of the most common behavioural disorders in children (Schab & Trinh, 2004). Some reports also associate ADHD with alterations in the expression of genes involved in dopaminergic, serotonergic and noradrenergic neurotransmission systems (Banaschewski et al., 2010). In order to explore the relationship between AFDs and neuronal alterations in the animal model, BZ555 (*dat-1p::GFP*) strain of *C. elegans* was employed. This strain expresses GFP under *dat-1* promoter, which encodes a plasma membrane dopamine transporter. The administration of tartrazine or quinoline yellow produced an increase in the expression of *dat-1* promoter and, consequently, a higher accumulation of dopamine into the dopaminergic neurons (Fig. 6E and G). On the other hand, allura red, ponceau red, carmoisine and sunset yellow produced a slight decrease in the fluorescence of dopaminergic neurons.

3.6. Movement analysis

The motility of untreated worms showed an average speed of 0.21 mm/s but this value was higher in *C. elegans* treated with some of the AFDs. Worms exposed to 100 μM of tartrazine or sunset yellow had an increase of 25% (p value of 0.025) and 66% (p value $\leq$ 0.00001) in average speed, respectively (Fig. 7A). Additionally, the treatment of worms with tartrazine or sunset yellow produced an increase in their maximum speed and their number of body lengths per second (BLPS), as Fig. 7B-C and supplementary video show (see Supplementary Material). The rest of the colorants had no significant effect on the motility of the animals.

3.7. Effect of tartrazine and ponceau 4R in gene expression by RNA microarray analysis

The six colorants used in this work showed potential toxic effects in the *C. elegans* lifespan assays. Thus, RNA microarrays were used to estimate whether these toxic effects could involve changes in the gene expression of worms treated with the colorants. Tartrazine and ponceau 4R were chosen for being well-known colorants used worldwide in a wide variety of alimentary products and in cosmetic formulations and have presented high toxicity in chronic assays performed with *C. elegans*. In addition, tartrazine was selected because some reports suggest that tartrazine consumption is linked to ADHD (attention deficit/hyperactivity disorder) in children (Stevens et al., 2011).

RNA microarray of *C. elegans* treated with tartrazine showed genes of the insulin signalling pathway up or down regulated like *ins-7* (9.5-fold *vs* control), *ins-10* (3.6-fold *vs* control), *ins-19* (1.3-fold *vs* control) and *ins-11* (-2.5-fold *vs* control). Tartrazine treatment also downregulated other immune related genes, such as *sek-1* (-1.9-fold *vs* control), *pmk-1* (-2.1-fold *vs* control) and their target gene *gcs-1* (-1.25-fold *vs* control).

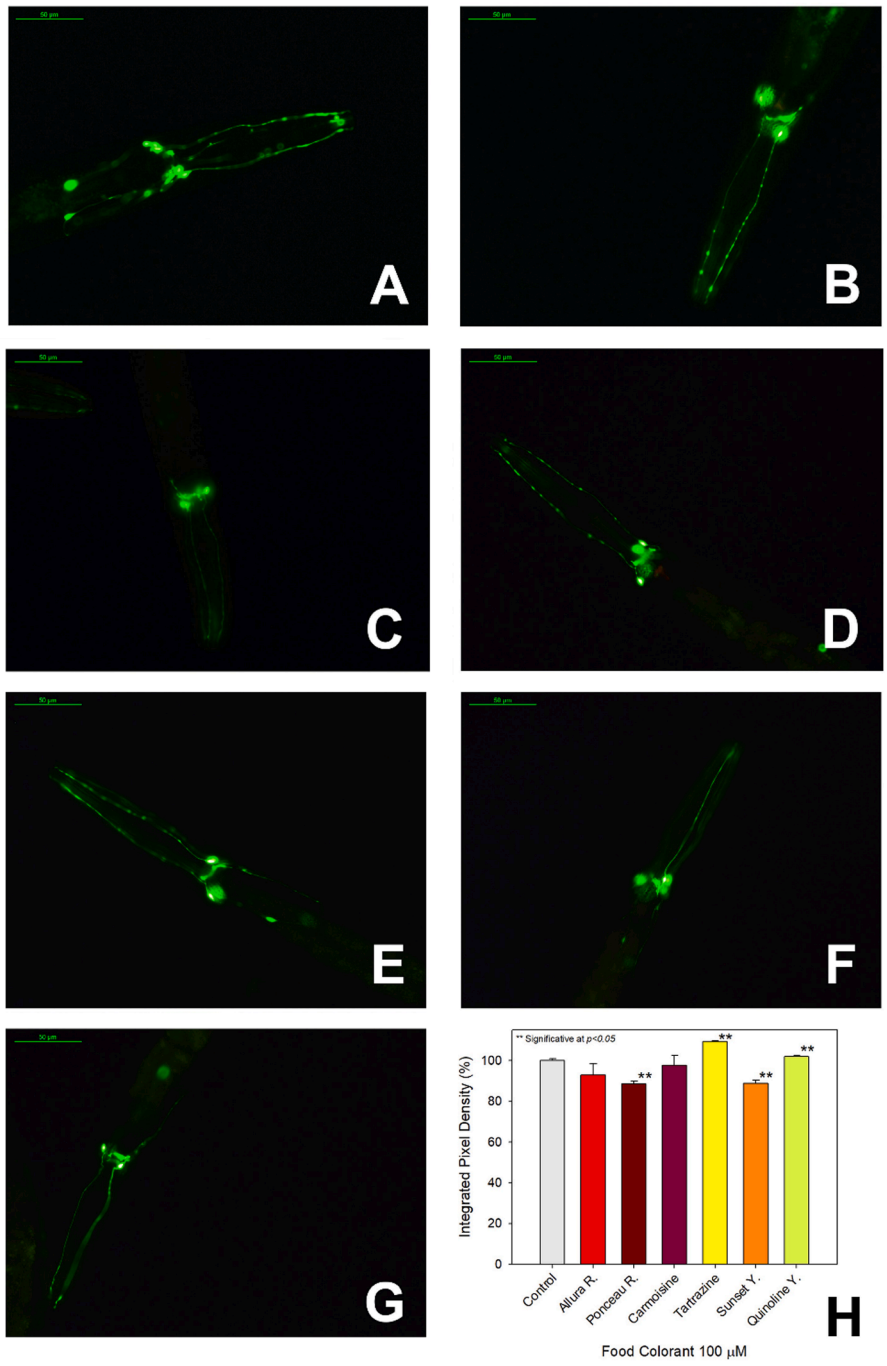

Fig. 6. Fluorescence emitted by the dopamine receptor *dat-1* expressed in dopaminergic neurons of *C. elegans* BZ555 (*dat-1p::GFP*) strain. A-G show representative images of dopaminergic neurons visualized through the fluorescence (I3 filtercube, Leica microscope) of *dat-1* receptor expressed in untreated worms (**A**) and worms treated with 100 μM of allura red (**B**), ponceau 4R (**C**), carmoisine (**D**), tartrazine (**E**), sunset yellow (**F**) and quinoline yellow (**G**). Scale bar: 50 μm, objective magnification 40X.**H**: Quantification of fluorescence emitted by expression of dat-1p::GFP. Data represented as average ± S.D., ** significant at $p \leq 0.05$. 10–12 worms per condition and experiment were assayed, two independent experiments were performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Heat shock factor (HSF) was also modulated by tartrazine, upregulating the upstream genes *hsb-1* (2.7-fold *vs* control) and *ddl-1* (2.4-fold *vs* control). Worms treated with ponceau 4R showed an alteration in the metabolic pathway (5.2-fold *vs* control). The microarrays showed that the main genes affected were *lips-17* (-1.79-fold *vs* control), the steroid dehydrogenase gen *stdh-3* (-1.4-fold *vs* control), infection response genes like *lys-7* (3.1-fold *vs* control) and *spp-1* (1.4-fold *vs* control) as well as the heat shock protein *hsp-16.2* (4.55-fold *vs* control).

4. Discussion

The experiments performed in this work provide a new insight into the effects of AFDs *in vivo* by using the animal model *C. elegans*. The safety of such colorants widely used by the food industry has been questioned by studies that have reported negative health effects after their consumption (Amin et al., 2010; Gao et al., 2011; Macioszek & Kononowicz, 2004; Sasaki et al., 2002; Stevens et al., 2011). *C. elegans* is a well-accepted animal model for use in experiments related to the bioactivity of low molecular weight molecules that are incorporated into their standard diet constituted by bacteria (Chen et al., 2013; García-Casas et al., 2019; Guerrero-Rubio et al., 2020). For the first time AFDs have been introduced in the nematode with this purpose. The effects of artificial food colorants after their consumption by *C. elegans* show that acute or chronic exposure to individual AFDs produced a decrease in the lifespan of worms. Lifespan of *C. elegans* is considered a standard parameter in the evaluation of the biological activity of molecules in preclinical studies. In the case of AFDs, an acute exposure produced a shortening in the worms' lifespan in a dose-dependent trend (Fig. 2,

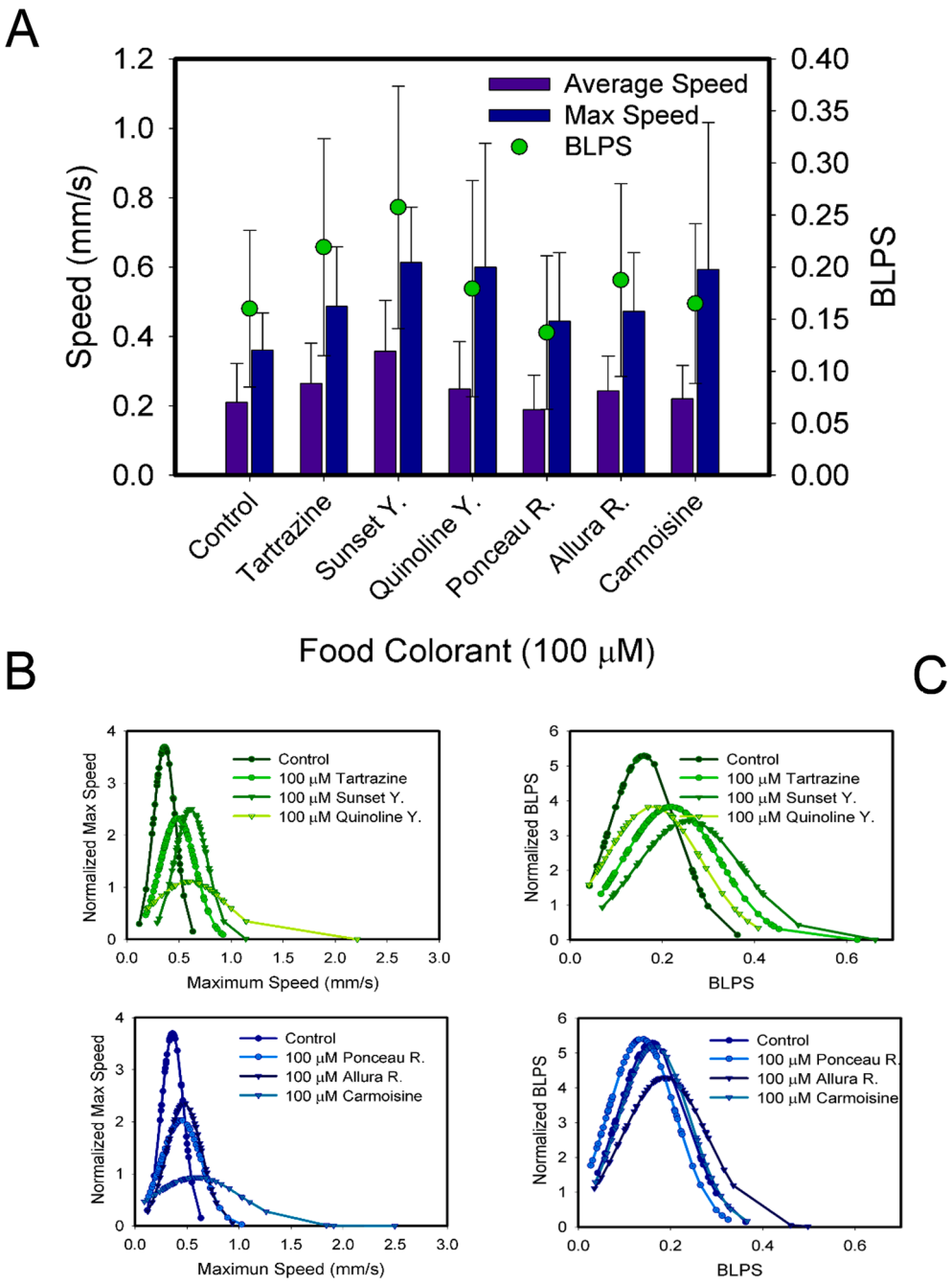

Fig. 7. Changes in motility parameters of *C. elegans* wild-type after exposure to individual AFDs. A. Effect of AFDs 100 μM in mean speed, maximum speed and blps (body length per second) of worms. Data represented as average ± S.D., 10–12 worms per condition and experiment were assayed, two independent experiments were performed. Normal distributions are shown for the maximum speed (**B**) and blps (**C**) of the control and treated worms (100 μM AFDs). Values are clustered around the mean average speed obtained in each case.

Fig. S1 and Fig. S2). This toxicity of AFDs in the worms' lifespan was higher under chronic exposure, where it produced a shorter lifespan even at low concentrations. The highest difference between acute and chronic exposure was obtained with tartrazine. In the acute assay, tartrazine was one of the least harmful dyes at 100 μM (Table S1) but its effect in the chronical experiments was close to that of the other colorants (Table S2). The maximum damage in the lifespan of *C. elegans* occurred with chronic exposure to 100 μM for all dyes tested, except carmoisine, which produced higher damage when administered in an acute manner. Mean lifespan of *C. elegans* indicates an accelerated cellular aging, where many factors intervene. Thus, it is possible that different dyes may follow different modes of action in the negative bioactivity shown in the animal model.

In this sense, the intestinal accumulation of lipofuscin is also an evidence of cellular damage during aging (Gerstbrein et al., 2005). Lipofuscin is a degradation metabolite derived from the peroxidation of polyunsaturated lipids that is progressively accumulated during aging. By following the autofluorescence of this compound in worms exposed to artificial dyes, a higher accumulation is seen in the treated animals with respect to the control. This strongly suggests an accelerated aging, which agrees with the results of lifespan. As Fig. 4 shows, after 4 days of adulthood, worms exposed to dyes were 'physiologically older' than untreated worms since the accumulation of lipofuscin is gradual until early adulthood, and rapid in midlife (Gerstbrein et al., 2005). Lipofuscin is named the age pigment as its increase is proportional to the organism age. However, the generation of lipofuscin is also related to other factors as ROS, cell damage or decreases in the activity of lysosomal enzymes (Brunk & Terman, 2002). Recent studies in eukaryotic cells and in mice (Ameur et al., 2018; dos Santos et al., 2022) model showed that tartrazine exposure caused cell damage *in vitro* and impaired the activity of fundamental enzymes such as proteases. Thus, the higher lipofuscin showed in nematodes exposed to tartrazine may be

partially explained by an increase in cell damage and reduced protease activity added to an excessive production of ROS.

The metabolic transformation of AFDs after ingestion could also be related to their harmful effect. Tartrazine and sunset yellow are metabolized by the intestinal microflora to sulfanilic acid, which generates reactive oxygen species (ROS) (Ameur et al., 2018). Therefore, the decrease measured in the lifespan may be due to the accumulation of sulfanilic acid and the production of ROS, causing a consequent decrease in the antioxidant capacity of cells (Himri et al., 2011). The ROS measurements *in vivo* showed that exposure to AFDs promoted the redox imbalance in the animals. Tartrazine and sunset yellow both increased its production. However, tartrazine produced more hydroxyls and peroxyls radicals while sunset yellow exposure generated more superoxide anions. In *C. elegans*, the presence of ROS directly affects to the expression of the heat shock protein *hsp-16.2*. In order to evaluate the potential effect of AFDs consumed by *C. elegans* in the expression of *hsp-16.2*, the transgenic strain TJ375 was used. The co-expression of *hsp-16.2* fused to GFP into the pharynx cells of these animals is an indication of oxidative stress *in vivo*. The *C. elegans* TJ375 strain showed harmful effect promoted by exposure to sunset yellow and tartrazine. These two artificial colorants increased the fluorescence induced by juglone exposure and produced the signal by themselves. Thus, it can be concluded that the colorants were able to induce oxidative stress by themselves and to exacerbate the damage provoked by the juglone. Previously there have been no reports of oxidative stress induced by tartrazine due to a low expression of catalase and superoxide dismutase (SOD) (Gao et al., 2011). In *C. elegans* both routes, catalase/superoxide dismutase and HSP, are regulated via the DAF-2 insulin/IGF-1 signalling pathway and DAF-16 (Murphy et al., 2003). Therefore, our results suggest that tartrazine may affect the expression of *hsp-16.2*, as will later be demonstrated by microarray experiments. The oxidative stress produced by sunset yellow exposure could be due to its metabolization to 1-amino-2-naphtol-6-sulphonic acid (ANSA) ("Scientific Opinion on the re-evaluation of Sunset Yellow FCF (E 110) as a food additive," 2009), but there is not enough evidence to relate ANSA and ROS.

Despite the lack of evidence of its metabolization or its relationship with ROS accumulation, quinoline yellow administration also produced an increase in the fluorescence of *C. elegans* TJ375. This is an indication of increased oxidative damage after feeding, to be added to its genotoxic effects (Macioszek & Kononowicz, 2004), which may explain the shortening of lifespan in *C. elegans*. Carmoisine and ponceau 4R present the same main metabolite, naphthionic acid, but there is no evidence of a possible negative effect of naphthionic acid ("Scientific Opinion on the re-evaluation of Azorubine/Carmoisine (E 122) as a food additive," 2009; "Scientific Opinion on the re-evaluation of Ponceau 4R (E 124) as a food additive," 2009). However, the consumption of carmoisine *per se* is related to renal and hepatic damage in young male rats and also produced a decrease in SOD and catalase (Amin et al., 2010). Carmoisine and ponceau 4R are present in several foods consumed by children and some studies have demonstrated that their consumption is above their ADI (Husain et al., 2006), so a deeper study on carmoisine and ponceau 4R effects and their derivatives is necessary. Regarding allura red consumption, there is not enough evidence in the bibliography about whether its metabolization could imply a harmful oxidative effect. In this sense, our results with the *C. elegans* model of oxidative stress show that allura red is the dye with the highest oxidative effect.

The accumulation of ROS is closely related to different neurogenerative diseases (Mancuso et al., 2006) like Parkinson's or Alzheimer's, also characterized by alterations in dopamine levels. Our results with the mutant strain BZ555 (*dat-1p::GFP*) of *C. elegans* show how AFDs are able to contribute to this alteration by producing a disarrangement in the dopamine receptors *dat-1*. These dopamine receptors have been related to neurodegenerative disorders and they are targets of current pharmacological treatments (Joyce & Millan, 2007). The alterations of dopamine receptors are also related to behavioural alterations such as ADHD. There is evidence suggesting that infants susceptible to ADHD show a notable increase of dopamine receptor D2 (Lou et al., 2004). Besides, it is known that ADHD symptoms improve with dietary restrictions, such as a low consumption of AFDs (Stevens et al., 2011). Our results show the relationship between the intake of artificial colorants and the disarrangement of dopamine receptors, with tartrazine administration contributing to the increase of dopamine receptors. Additionally, the analysis of the motility of worms shows how tartrazine, the main colorant related to ADHD (Stevens et al., 2011), increased the average speed of the animals. In addition, the treatment with sunset yellow, which has also been related to ADHD, produced a pronounced effect in the worms' behaviour, by increasing the worms' speed (Fig. 7). The normal distribution on Fig. 7 describes how the values of a variable are distributed. Values around the centre of the curve are more likely to occur than further values. In statistics, the normal distribution is one of the most important probability distributions since it fits many natural phenomena. The normal distribution of these curves showed how the speed of the worms is clustered around the mean of the average speeds obtained and the differences in locomotion between the animals treated with tartrazine and sunset yellow and the non-treated (control) animals is easily observed. These facts in the model animal agree with the wide bibliography which describes a correlation between hyperactivity and AFDs' intake (McCann et al., 2007; Schab & Trinh, 2004; Stevens et al., 2011, 2015).

Our results indicate that colorants may have different ways of altering processes involved in the lifespan of *C. elegans*, as ROS accumulation and antioxidant capacity, motility, or neurological changes. In order to get a deeper understanding of the effects of AFDs in the animal model *C. elegans*, gene expression was analysed after the treatment with the colorants tartrazine and ponceau 4R. The RNA microarray of *C. elegans* treated with tartrazine showed several genes of the insulin signalling pathway with altered expression (Fig. 8). INS is a family of forty insulin/IGF-1 (insulin growth factor) like peptides, which serve as hormones, neurotransmitters and growth factors during post-embryonic stages in invertebrates but also in higher animals (Antonova et al., 2012). Tartrazine treatment up-regulated the expression of *ins-7*, which seems to function as antagonist of DAF-2 insulin/IGF-1 receptor. *ins-7* is expressed in amphid sensory neurons, labial neurons, nerve ring, ventral cord and tail neurons. RNAi silencing of *ins-7* increased the lifespan of wild type worms but this effect was lost in the long-living *daf-2* mutants, suggesting that *ins-7* lifespan modulation is dependent on *daf*-2. Kawli & Tan (2008) concluded that INS-7 neuropeptide secretion from the dense core vesicles (DCVs) suppresses the immune function by activating DAF-2 signalling, suppressing DAF-16 nuclear translocation, and consequently reducing the expression of the immunity related gene.

Tartrazine treatment directly down-regulated other immune related genes such as *sek-1* (-1.9-fold *vs* control), *pmk-1* (-2.1-fold *vs* control) and their target gene *gcs-1* (-1.25-fold *vs* control). These genes appear in the longevity pathway, specifically in the phase 2 detoxification response to oxidative stress pathway that confers oxidative stress resistance in *C. elegans*. *sek-1* is an ortholog of human MAP2K3 (mitogen activated protein kinase kinase 3) expressed in several tissues of the worm including the gonads and the nervous system. This gene is related to the activation of MAPK (mitogen activated protein kinase) signalling pathway, defense response against other organisms, determination of asymmetry in nervous system (Cochella et al., 2014) and it has been shown to promote synaptic transmission in GABA (gamma-aminobutyric acid) neurons (Vashlishan et al., 2008). *pmk-1* encodes a mitogen activated kinase, which is a downstream gene of *sek-1* and also related to p38 MAPK activation. It has transcription factor binding and kinase activities and it is involved in several processes, such as defense response to other organisms or positive regulation of response to biotic stimuli. Like *sek-1*, *pmk-1* is expressed in head neurons and the intestine. *sek-1* and *pmk-1* target gene *gcs-1* was also down-regulated when worms were exposed to tartrazine. *gcs-1* encodes a gamma glutamine cysteine synthetase and it is involved in the phase 2 detoxification in the oxidative stress response pathway (Papp et al., 2012). RNAi silencing of *gcs-1*

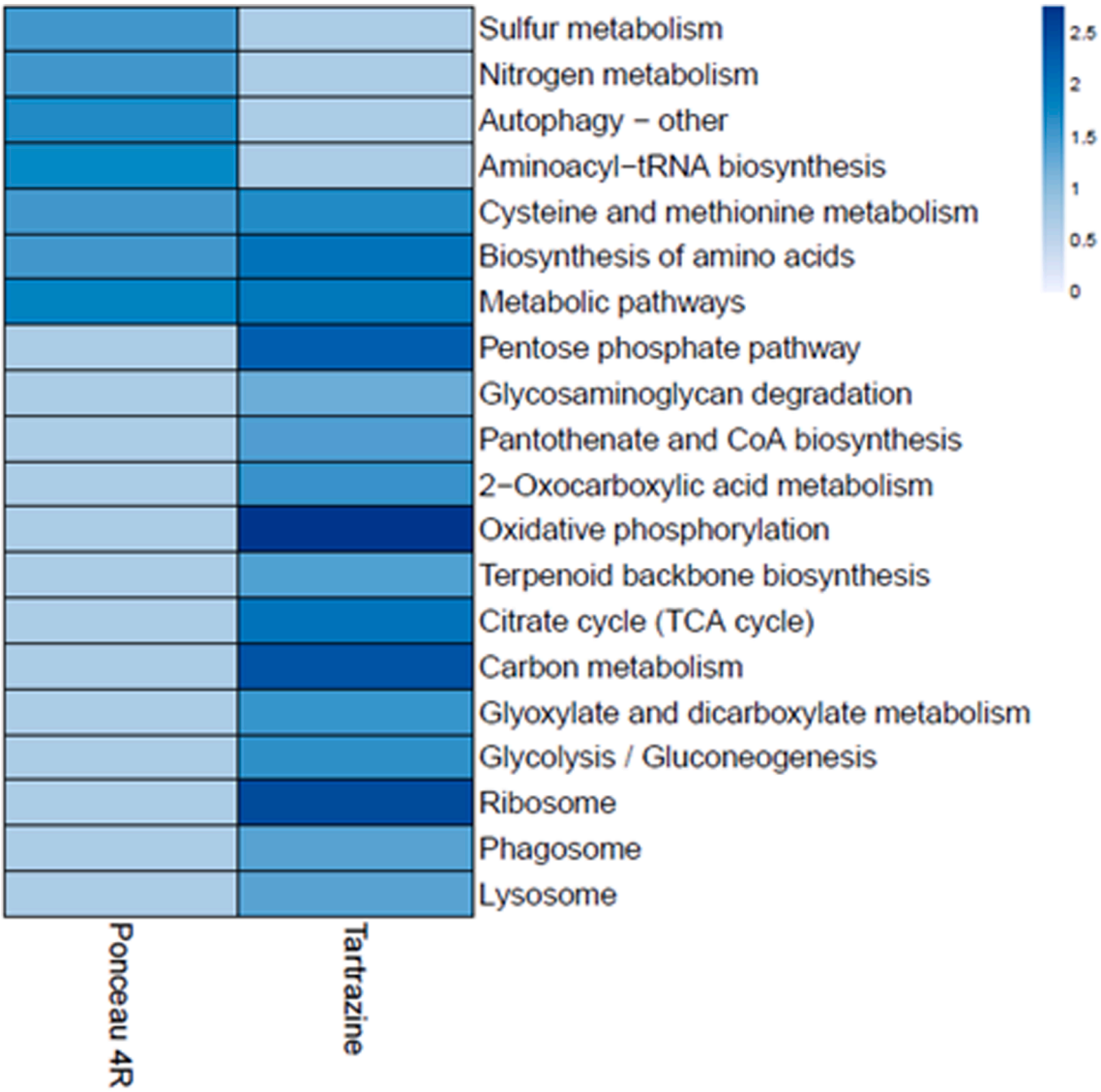

Fig. 8. Heatmap of *C. elegans* pathways altered by ponceau 4R or tartrazine treatment (100 μM) *vs* untreated control worms.

produced worms with intestinal abnormalities, an indication that *gcs-1* is also involved in post-embryonic development. Fluorescent mutants for this protein labelled with green fluorescent protein, GCS-1::GFP, showed that GCS-1 is accumulated in the intestine and ASI chemosensory neurons in response to harmful stimuli (An & Blackwell, 2003). Tartrazine also modulated heat shock factor (HSF) by up-regulating the upstream genes *hsb-1* (2.7-fold *vs* control) and *ddl-1* (2.4-fold *vs* control). Heat-shock proteins appear at high levels in inflammatory diseases as a consequence of the temperature increase. These proteins have many purposes in *C. elegans* including thermotolerance, apoptosis or enhancement of the innate immune system. HSF acts downstream DAF-2/DAF-16 and contributes to the worm immunity against bacterial pathogens, by the activation of HSPs (Singh & Aballay, 2006). *hsb-1* encodes a heat shock factor binding protein, ortholog to the human HSBP1. This gene is involved in the negative regulation of the heat-shock response and may have a role in the suppression of the activation of the stress response in aging (Satyal et al., 1998). In *C. elegans,* over-expression of this gene generates animals that are more sensitive to heat stress, and the survival rates of the mutants were reported to be 48% for the wild type worms (Satyal et al., 1998). Furthermore, *ddl-1* encodes a protein homolog to human CCDC53 (human coiled-coil domain-containing protein 53). CCDC53 modulates HSF-1 activity by forming a complex with it, but it has also been reported to form complex with the HSF-1 negative regulator of CCDC53 (Rual et al., 2005). Inhibition on *ddl-1* produced worms with longer lifespan and enhanced thermotolerance dependent on *hsf-1* and the IIS (insulin/IGF-1-like signalling) pathway (Chiang et al., 2012). Therefore, exposure to tartrazine increases its expression and decreases the lifespan and the thermotolerance in *C. elegans*. The transcription factor PHA-4, which is involved in the TOR (target of Rapamycin) pathway, was also down-regulated in worms fed with tartrazine (-4.47-fold *vs* control). PHA-4 is an ortholog of human FOXA3 (forkhead box A3), a transcription factor located in hepatocytes that regulates the expression of several genes such as AFP, albumin, tyrosine aminotransferase, or PEPCK, and it plays an essential role in early stages of development and later in the regulation of glucagon production and glucose homeostasis (Kaestner, 2000). In *C. elegans*, the suppression of *pha-4* in adult worms shortened the lifespan (Panowski et al., 2007). Overall, the microarray results for tartrazine indicate that the reduction in lifespan may be explained as a result of an alteration in key genes expression. The pathways most affected by tartrazine are those associated with resistance to oxidative stress, the innate immune system and thermotolerance, all linked by DAF-2 and INS. Interestingly, some of the altered genes are expressed in neurons involved in neuronal formation and activity.

On the other hand, worms treated with ponceau 4R showed an alteration in the metabolic pathways (5.2-fold *vs* control) (Fig. 8). The microarray showed that the gene *lips-17* is down-regulated (-1.79-fold *vs* control) along with the steroid dehydrogenase gen *stdh-3* (-1.4-fold *vs* control). The first gene is predicted to encode a triacylglycerol lipase involved in lipolysis and longevity (McCormick et al., 2012). Instead, *stdh-3* encodes a hydroxysteroid 17-beta dehydrogenase enzyme, predicted to have oxidoreductase activity (Maglioni & Ventura, 2016). The human ortholog HSD17B12 encodes a mitochondrial multifunctional enzyme (HSD10), which plays an important role in the metabolism of neuroactive steroids and the degradation of isoleucine (Yang et al., 2007), the loss of this protein is known as HSD10 disease, a mitochondrial metabolic disorder. HSD10 deficiency may cause acidosis, neurodegeneration, loss of motor skills, epilepsy or blindness (Zschocke, 2012). Indeed, some reports have shown that in *Drosophila melanogaster* (Torroja et al., 1998) and mice (Rauschenberger et al., 2010) the gene knock-down causes embryonic lethality and it is likely that the same may occur in humans, where RNAi silencing of HSD17B12 gene expression caused mitochondrial tRNA precursors accumulation (Holzmann et al., 2008). This is in agreement with our microarray results that showed an up-regulation of 4.7-fold *vs* control in the aminoacyl-tRNA biosynthesis pathway in worms treated with ponceau 4R. Although other genes are altered by ponceau 4R exposition, such as infection response genes *lys-7* (3.1-fold *vs* control) and *spp-1* (1.4-fold *vs* control) or the heat shock protein *hsp-16.2* (4.55-fold *vs* control), overall, these alterations may not have a direct impact in the *C. elegans* lifespan. The down-regulation of *stdh-3* and the accumulation of tRNA could be the main cause of premature death in worms treated with ponceau 4R.

Microarray assays have shown that the reduced lifespan of the worms treated with these AFDs could be partially explained by an effect in gene regulation. Genes and pathways affected are different and there is not a common trend (Fig. 8). Although all are artificial colorants, the chemical structures and properties are different. Ponceau was the most lethal of both in the lifespan experiments, and its effect on gene regulation may explain this result. Nevertheless, tartrazine shortened the lifespan and down-regulated longevity genes.

Altogether, these results indicate the need for new and exhaustive studies on the effects of AFDs on health, development, and genetic regulation. Our data suggest that caution should be taken to avoid excessive consumption of artificial food dyes, and the search for alternatives should be promoted. There is enough evidence for the use of dyes from fruits (Albuquerque et al., 2021) or other natural sources that are not only innocuous but also show health-promoting effects. For instance, one alternative to red colorants is betanin, the betacyanin extracted from beetroot, well-known due to its health-promoting properties (Martínez-Rodríguez et al., 2022), that is employed as the food colorant E-162 in the European Union and 73.40 in the 21 CFR section of the FDA in the USA. Our previous studies with *C. elegans* showed its extraordinary antioxidant effect in *C. elegans* (Guerrero-Rubio et al., 2019, 2020). Other natural yellow pigments such as turmeric or carotenoids are also used in the food and cosmetic industries as E-100 and E-160a, respectively. These compounds also have antioxidant and anti-inflammatory properties (Ammon et al., 1993; Stahl & Sies, 2003) and they are potential alternatives to the employment of artificial colorants.

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CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request. The microarray data from this publication have been deposited at the GEO database (NCBI) [https://www.ncbi.nlm.nih.gov/geo/] and assigned the identifier GSE143611.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112925.

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