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Investigation of the antioxidant capacity of caramels: Combination of laboratory assays and *C. elegans* model

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ABSTRACT

Caramel is one of the most current additive/ingredient obtained by controlled heat treatment of carbohydrates.

A combination of *in vitro* and *in vivo* methods showed that aromatic caramels and caramel colours are antioxidant. The highest value is obtained for Class III caramel colours for *in vitro* TEAC and KRL tests (401 μ mol Trolox equivalent/g and 5 366 mg gallic acid equivalent/100 g, respectively). In *in vivo* test, caramels also increase the resistance of *C. elegans* to oxidant attack, with until 40% live worms after 18 h of treatment in the presence of caramels against no live worms after 12 h of treatment without.

In conclusion, this study explore benefit effect of caramels and showed an antioxidant activity linked to their color intensity. The molecules involved remain to be explored.

1. Introduction

The word “caramel” refers to various products depending on recipes and applications (confectionaries, ingredients for flavouring, additives for colouring). Aromatic caramels and caramel colours are widely used in the food industry for their technological properties. Aromatic caramels are light to dark brown liquids or solids considered as food ingredients. Their main functionality is to enhance taste in diverse food applications including dairy products, ice creams, sauces or spirits. Caramel colours are used to provide a yellow to brown color in a large range of foodstuffs. These latter have been classified into 4 classes of food additives: Class I-IV (or E150a, b, c and d at the European Union level) according to the manufacturing reactants used. Caramels are produced by heating carbohydrates from vegetable sources (glucose, sucrose, invert sugar, etc.) in the presence or not of caramelization promoters (acids, alkalis, ammonium or sulfite compounds). The caramelization process includes a multitude of chemical reactions leading to the formation of a complex mixture of molecules, with low molecular weight ones responsible for the aromatic feature and high molecular

weight ones (melanoidins) responsible for the colourant feature of caramels (Golon & Kuhnert, 2012; Licht et al., 1992; Moretton, 2009; Sengar & Sharma, 2014). These reactions are specific in heat treatments and nature of the used sugar.

On the one hand, caramel colours are subject to specific monitoring due to the presence of adverse constituents at levels that may be of safety concern in a context of high-level exposure: 2-acetyl-4-tetrahydroxybutylimidazole (THI) and 4-methylimidazole (4-MEI). These molecules are regulated by national and international authorities at levels of the ppm range. But on the other hand, some molecules of caramel have been associated with nutritional and health benefits. Studies have shown potential prebiotic effects for fructose dianhydrides and some other oligosaccharides, present in the order of several hundred grams/kg in some caramels (Arribas et al., 2010; Orban, Patterson, Sutton, & Richards, 1997; Peinado et al., 2013; Rubio et al., 2014; Saito & Tomita, 2000). Caramels have also been recently highlighted as potential antioxidants (Sengar & Sharma, 2014). Antioxidants are molecules that counteract the harmful effects of free radicals and other oxidants, responsible for causing a large number of diseases by oxidizing nucleic

Abbreviations: ABTS, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AC, Aromatic caramel; BS, Burnt Sugar; CC, Caramel Colour; EBC colour, European Brewing Convention colour; GAEq, Gallic Acid Equivalent; KRL, Kit Radicaux Libres; TEAC, Trolox Equivalent Antioxidant Capacity.

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acids, proteins, lipids and DNA. Antioxidants inhibit or delay the oxidative process by blocking both the initiation and propagation of oxidizing chain reactions. There are endogenous antioxidants (super-oxide dismutase, glutathione peroxidase ...) and antioxidants from exogenous food sources (vitamins C and E, carotenoids, β -carotene, polyphenols ...) (Finley, Kong, Hintze, & Je, 2011).

Brenna et al. aimed to ascertain the contribution to the antioxidant activity of some caramel-containing soft drinks, such as cola drinks, and chinotto, an original Italian soft drink (Brenna, Ceppi, & Giovanelli, 2009). The results showed that even if soft drinks have a lower antioxidant activity than other beverages such as tea, coffee or chocolate, they may contribute to the antioxidant pool assumed with the diet. Tsai et al. heated 4 sugars (including monosaccharides and disaccharides) with different concentrations (1–40%) at pH 3, 7, and 10 at 90 °C for various durations (0–42 h). Results from 240 samples indicated that alkaline condition (pH 10 vs pH 3) as well as high sugar concentration (40% vs 20%) preferably enhance the antioxidant capacity of caramels, evaluated by ferric reducing antioxidant power (FRAP) method and DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging ability (Tsai, Yu, Chen, Liu, & Sun, 2009). A correlation between browning intensity and antioxidant was also shown.

Compared to the caramels previously studied under experimental conditions, caramels manufactured by industrial processes are heated at higher sugar concentration and to higher temperatures but for shorter durations. In this context, the objective of this study was to evaluate the global antioxidant capacity of different types of caramels marketed as aromatic ingredients or food additives. To demonstrate the global antioxidant capacity of caramels, we used *in vitro* methods well validated; the TEAC/ABTS assay (Trolox equivalent antioxidant capacity / 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and the KRL™ (Kit Radicaux Libres) tests, respectively. The nematode *Caenorhabditis elegans* was used as an *in vivo* model for studying caramel-dependent resistance to oxidative stress.

2. Material and methods

2.1. Materials

2.1.1. Samples

Aromatic caramels and caramel colours were provided by Nigay SAS (Feurs, France).

Table 1 shows composition and European Brewing Convention (EBC) colour of the caramel samples. Different raw materials were tested: sucrose for aromatic caramel and burnt sugars, sucrose or glucose for caramel colours with an addition of reactants such as alkali, sulfite or ammonia.

The colour intensity and the colouring power increase depending on the type of caramel and the raw materials used (EBC range from 570

Table 1
Raw materials and EBC colour of caramel samples.

Caramel	Abbreviation	Raw materials	EBC colour
Aromatic caramel	AC	Sucrose	570
Burnt sugar	BS	Sucrose	11,000
Class I caramel colour	CC-I	Sucrose - alkali	17,000
Class II caramel colour	CC-II	Sucrose - sodium sulfite	19,000
Class III caramel colour	CC-III a	Glucose - ammonium hydroxide	22,000
Class III caramel colour	CC-III b	Glucose - ammonium hydroxide	35,000
Class IV caramel colour	CC-IV a	Glucose - ammonium sulfite	21,000
Class IV caramel colour	CC-IV b	Glucose - ammonium sulfite	46,000

(aromatic caramel) to 46 000 (class IV caramel colour)).

2.1.2. Chemicals

ABTS, potassium persulfate, ascorbic acid, di-potassium hydrogenophosphate and potassium dihydrogenophosphate were purchased from VWR chemicals (Fontenay sous bois, France). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Paraquat dichloride hydrate (PQ) and trivalent Arsenic chloride (AsIII) were purchased from Acros Organics (Geel, Belgium). Gallic acid and *tert*-Butyl hydroperoxide 70% aqueous solution (TBHP) were purchased from Alfa Aesar (Kandel, Germany), KRL™ test kit from Kirial International (Couternon, France).

2.1.3. *C. elegans* and bacterial strains

The Bristol N2 wild type strain and the OP50 *Escherichia coli* feeding strain were obtained from the Caenorhabditis Genetics Center (cgc.umn.edu).

2.2. Methods

2.2.1. Colour intensity EBC units

The EBC colour, initially used for beer, is a method developed by the Institute of Brewing and the European Brewing Convention to measure the colour of products. The caramel is diluted with a factor of dilution to obtain a colour visually comparable with a colour scale (colored standard discs from 6 to 27, from light yellow to dark brown). The EBC value is then calculated by the following formula:

$$EBC = \text{disc value} * 25 / (\text{solution concentration} * \text{optical pathlength})$$

2.2.2. Antioxidant capacity determinations

2.2.2.1. Trolox equivalent antioxidant capacity (TEAC/ABTS assay).

Various chemical methods are used for the evaluation of the antioxidant capacity of foods (Alam, Bristi, & Rafiquzzaman, 2013; Pisoschi & Negulescu, 2012). Most of them use colorimetric technique. The absorbance of caramels in UV and visible region decrease as wavelength increases so the choice fell on the TEAC/ABTS method with a color measurement at high wavelength (734 nm).

The determination of ABTS radical inhibition was based on the method of Re et al. (Re et al., 1999). Stock water solutions of ABTS and potassium persulfate were prepared at 5 mmol/L and 2 mmol/L, respectively. The radical cation ABTS⁺ was obtained by reacting ABTS solution with potassium persulfate solution (50/50). Before use, the mix was kept at room temperature in darkness for 12–16 h.

One hundred and fifty microliters of ABTS⁺ solution was diluted in 4.85 mL phosphate buffer 100 mM pH7 to obtain a blank absorbance around 0.8 at 734 nm. Absorbance was read using Thermo AquaMate spectrophotometer.

Antioxidants reduce the blue ABTS radical cation to its colorless neutral form. Trolox, a water-soluble analog of vitamin E, was used as a standard antioxidant to draw a linear correlation between the absorbance decrease and the concentration of Trolox in μmol . One hundred microliters of the sample solution were added to the ABTS⁺/phosphate buffer mix. The sample is diluted appropriately to produce between 20% and 80% inhibition of the blank absorbance.

Results were expressed as μmol Trolox equivalents /g of sample (TEAC).

2.2.2.2. Kit Radicaux Libres (KRL) test.

The antioxidant activity of caramel solutions was tested using the patented KRL test (Phan-Thi, Durand, Prost, Prost, & Waché, 2016; Prost, 1992). The biological KRL™ test is based on the ability of blood cells to resist against free radicals. Initially used to allow the dynamic evaluation of the overall antioxidant defense potential of an individual, it is also used for the assessment of antioxidant capacity of plant extracts and many other antioxidants products (Brahmi et al., 2018; Cases et al., 2017; Phan-Thi et al., 2016).

A control blood was subjected to free radical attack under controlled and standardized conditions with the KRL Reader (37 °C, orbital shaking) in the presence of caramel.

A caramel solution (120 µL, 2250 mg/L) was mixed with whole horse blood solution (50 µL) and KRL Reagent solution (100 µL) in 96-wells plate. Gallic acid was used as a reference compound in antioxidant activity assessment.

Measuring the decrease in absorbance (620 nm) allows the monitoring of the progressive disappearance of the cells. The resistance of blood cells against free radical attack is expressed by the time necessary to lyse 50% of the blood cells (HT50 in min).

Results of antioxidant activity (KRL AO) were expressed in mg Gallic Acid Equivalent /100 g of caramel (GAEq mg/100 g).

2.2.2.3. *Caenorhabditis elegans* model

2.2.2.3.1. *C. elegans* culture. Worms were maintained at 20 °C on nematode growth medium (NGM) agar plates seeded with OP50 bacteria. Before each experiment, young adult worms were obtained after synchronization of eggs released from gravid worms by lysis with sodium hypochlorite. Except for maintenance and larval development, feeding bacteria were 10 times concentrated and heat-killed 30 min at 65 °C.

2.2.2.3.2. Survival assays in severe oxidative stress. Three days adults were incubated 24 h at 20 °C in 24-well plates (approximately 30 worms /well) plus NGM containing none, 0.04%, 0.2% and 1% caramel solution with FUDR (50 µM) (FUDR was used to inhibit the development of progeny) and heat-killed bacteria. Three different oxidizing chemicals, Paraquat (PQ), *tert*-Butyl hydroperoxide (TBHP) or trivalent Arsenic chloride (AsIII) in M9 buffer (NaCl 86 mM, Na₂HPO₄ 42 mM, KH₂PO₄ 22 mM and MgSO₄ 1 mM), were then added to a final concentration of 200 mM, 15.4 mM or 5 mM, respectively. Worms grown without oxidant in medium with or without caramel served as additional controls. The worms were scored every hour and they were considered as dead when they did not move after repeated stimuli. Three independent experiments were performed with more than 50 animals each.

2.2.2.3.3. Survival assays in moderate oxidative stress. Synchronized young adults were incubated at 20 °C in 96-well plates (approximately 10 worms /well) plus NGM containing none or 0.2% caramel solution with FUDR (50 µM) and heat-killed bacteria. PQ was added to a final concentration of 4 mM. Control conditions contained none or 0.2% caramel solution without PQ. The worms were scored every other day and they were considered as dead when they did not move after repeated stimuli. Animals were censored when they crawled off the well, exploded or contained internally hatched larvae. Three independent experiments were performed with more than 60 animals each.

2.2.2.3.4. Culture conditions and isolation of total RNA for gene expression analysis. Day3 adult worms were grown 24 h at 20 °C in 6-well plates (approximately 1000 worms / well) containing NGM alone or NGM plus 0,04 or 1% caramel solutions. All the media were

supplemented with FUDR (50 µM) and heat-killed bacteria. PQ, TBHP or AsIII in M9 buffer were then added to a final of 200 mM, 15.4 mM or 5 mM, respectively. Worms were incubated 2 h at 20 °C in those oxidative conditions before to be pelleted and frozen in liquid nitrogen. The worms were then homogenized in Trizol (Ambion) with acid-washed glass beads (425–600 µm; B. Sigma-Aldrich) with Precellys®24 (Bertin Technologies) at full speed, 4 times 10 s, 3 cycles. Total RNA was extracted as previously described (Fradin et al., 2003).

2.2.2.3.5. Gene expression analysis. RNAs were treated with DNaseI (Thermo Scientific) prior to be reverse transcribed to cDNA (High-capacity cDNA reverse transcription kits, Applied Biosystems). Quantitative real-time PCR was performed using PowerUp™ SYBR® Green 2X Mater Mix (Applied Biosystems). Gene expression levels were normalized to *pmp-3* and relative expression was calculated by the 2^{-ΔΔCT} method. The relative expression of genes was analyzed by comparing either the condition with oxidizing chemical to the same condition without the chemical or the caramel conditions to the condition without caramel. Primers are listed in Table S1.

2.3. Statistics

KRL test and TEAC assay: All experiments were performed in duplicate (KRL) or triplicate (TEAC) and the results were expressed as mean ± standard deviation (SD). Significant differences (P < 0.05) between the means were determined by the ANOVA test of software Graphpad Prism.

Caenorhabditis elegans model: Kaplan-Meier survival curve and statistical analysis were generated by bioinformatics software GraphPad Prism. Survival analysis and P values for gene expression are based on log-rank and two-way ANOVA tests, respectively. P < 0.05 was considered statistically significant.

3. Results and discussion

For valid assessment, the antioxidant capacity of caramel samples was determined using three complementary methods: TEAC/ABTS assay, the KRL™Test, and *Caenorhabditis elegans* model.

3.1. Results of the antioxidant capacity evaluated by in vitro assays

3.1.1. TEAC assay

3.1.1.1. Kinetic measurements. Among chemical assays, TEAC was first developed as a simple and convenient method for assessment of the total antioxidant capacity. The first step was to examine the kinetic behavior of reactions between ABTS⁺ and caramels or common antioxidants. Absorbance readings at 734 nm were taken during 20 min. Percentage of inhibition versus time graphs are shown in Fig. 1.

Trolox and ascorbic acid reacted immediately with ABTS⁺ (Fig. 1A).

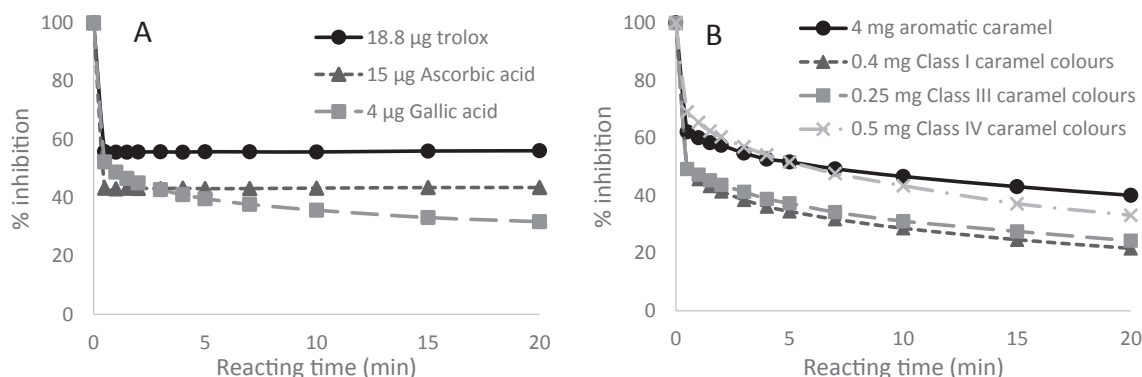


Fig. 1. Rate of reaction of antioxidants with ABTS⁺: A: common antioxidants - B: caramels.

The inhibition percentage was constant over time (56.0 and 43.5% inhibition, respectively). Gallic acid had a fast initial rate but the end point of inhibition was not reached after 20 min. Similar results of reaction rates were obtained by Walker and Everette using Trolox, ascorbic acid and plant polyphenols (Walker & Everette, 2009).

For aromatic caramel and caramel colours, the curves were comparable to the gallic acid curve (Fig. 1B). In the same way, the end point was not reached after 20 min. To compare different caramels, the reacting time / color measurement was fixed at 5 min as generally set in previous publications (Alam et al., 2013; Amigoni et al., 2017; Seeram et al., 2006).

3.1.1.2. TEAC of well-known antioxidants. As a quality control, the TEAC value of two well-known antioxidants were determined. TEAC of ascorbic acid was evaluated at 6.2 ± 0.6 mmol Trolox equivalent/g, this result was comparable to previous studies which reported mean values between 4.6 and 6.0 mmol/g (Miller & Rice-Evans, 1997; Rice-Evans, Miller, & Paganga, 1997; Walker & Everette, 2009).

TEAC of gallic acid was evaluated at 24.0 ± 2 mmol/g, this result was also comparable to published studies indicating values between 15.3 and 34.4 mmol/g (Miller & Rice-Evans, 1997; Rice-Evans et al., 1997; Walker & Everette, 2009).

3.1.1.3. TEAC of caramels. As shown in Fig. 2, all the caramels tested exhibited antioxidant activity according to the TEAC test, between 20.5 ± 1.7 for standard aromatic caramel to 401 ± 30 μ mol Trolox equivalent /g for Class III caramel colour.

For caramels without ammonium or sulphite compounds (AC, BS, CC-I, CC-II), the antioxidant capacity increases significantly with EBC colour. In the same way, inside Class III and IV caramel colours (CC-III, CC-IV), the antioxidant capacity increases with colour intensity. Among caramel colours, Class IV type showed lower antioxidant capacity and Class III had the highest antioxidant activity. These results confirm those obtained by Brenna et al. on commercial caramel colours using the FRAP and DPPH* methods (Brenna et al., 2009).

To compare, chocolates had a TEAC value between 10 and 100 μ mol Trolox equivalent /g, dark chocolates showed a greater antioxidant capacity (Laličić-Petronijević, 2016; Todorovic et al., 2015). Honey had a TEAC value between 0.3 and 8 μ mol/g (Marylenlid et al., n.d.; Sancho et al., 2016; Vit, 2009). Coffee was a well-known antioxidant. The TEAC value of ground coffee had been reported between 100 and 300 μ mol/g (Brezová, Šlebođová, & Staško, 2009; da Cruz et al., 2018).

3.1.2. Protection of red blood cells evaluated by the KRL™ test

TEAC test is a validated *in vitro* chemical method to evaluate the overall antioxidant capacity of foods. But among limitations, the TEAC assay has been challenged for its lack of biological relevance and lower

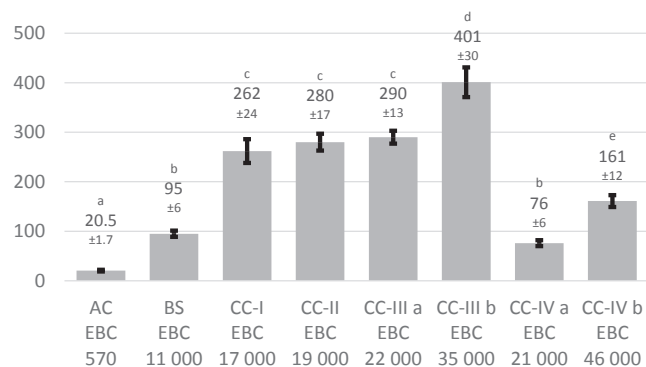


Fig. 2. Antioxidant activity of caramel samples by TEAC test data are expressed in Trolox equivalent (μ mol/g of sample) and presented as the means \pm standard deviation. Bars with no letters in common are significantly different ($p < 0.05$, ANOVA test).

sensitivity compared to the ORAC test (Shahidi & Zhong, 2015). We decided then to evaluate the antioxidant activity of caramels with a biological test. For this method, whole blood is submitted to an oxidant stress induced by free radical attack. Haemolysis is recorded by optical density decay with the KRL microplate reader. The overall antioxidant effectiveness of caramels in biological conditions was determined.

Main results of the KRL™ test are presented in Fig. 3. We found that all the caramel samples (aromatic, burnt sugar, class I, II, III, IV) exhibited an antioxidant capacity from 308 ± 1 to 5366 ± 147 mg GAEq/100 g.

As for the TEAC test, CC-III b had the highest antioxidant activity and the antioxidant activity increases with the colour for each category of caramels.

Other food matrices were previously analyzed with the same KRL method. The results in wine were between 120 and 350 mg GAEq/100 g. In coffee beverage and orange juice, the antioxidant activity has been evaluated at 65–700 and 75–80 mg GAEq/100 g respectively (Unpublished data from LARA-SPiRAL and Prost et al., 2017).

3.1.3. Comparison of results between methods and with other foods

To check the reliability of our data, the results obtained with the 8 marketed caramel samples using TEAC and KRL-test were compared. Fig. 4 shows a strong linear correlation between the two sets of results ($r = 0.966$). Both methods, one rather chemical and one rather biological, confirm the antioxidant potential of caramels.

The antioxidant effects of caramels are equivalent or superior to those obtained with the same measurement techniques in other food products recognized as antioxidant. In Europe, the combined mean exposures to the four caramel colours (E 150a-150d) was evaluated from 15 to 57 mg/kg bw/day for adults, which could be equivalent to 1–4 g for a weight of 70 kg (EFSA, 2012). Based on our results, the antioxidant capacity from the daily ingestion of caramel colours is almost equivalent to a glass of orange juice (140 vs. 160 mg gallic acid equivalent for fruit juice) (Unpublished data from LARA-SPiRAL, Debabbi et al., 2017). The exposure to caramel colours would reach 41–165 mg/kg bw/day among the highest consumers (95th percentile), the equivalent of a 3–11 g daily intake. This consumption could be compared to a cup of coffee in term of antioxidant activity. The consumption of aromatic caramels is more difficult to assess but they also contribute to the exposure. Even when related to its level of consumption, caramels could consequently be a significant contributor to the daily intake of antioxidant compounds. As an interesting point about the risk/benefit balance, it should be noted that the level of consumption recorded in Europe remains below the group acceptable daily intake applicable to combined four colours (300 mg/kg bw/day (EFSA, 2012)).

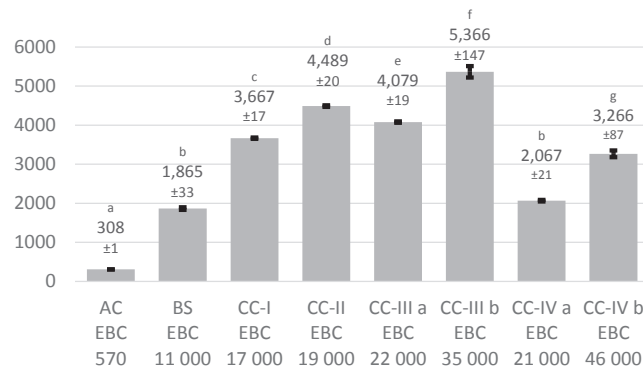


Fig. 3. Antioxidant activity of caramel samples by KRL™ test data are expressed in gallic acid equivalent (mg/100 g caramel) and presented as the means \pm standard deviation (SD). Bars with no letters in common are significantly different ($p < 0.05$, ANOVA test).

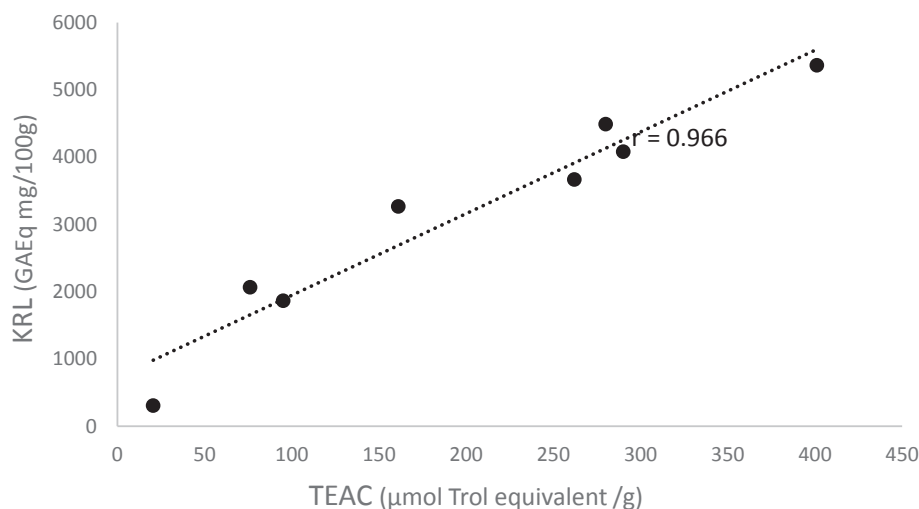


Fig. 4. Comparison of results of the antioxidant activity obtained with the TEAC method ($\mu\text{mol Trolox equivalent/g}$ of sample) and the KRL method ($\text{mg GAEq}/100\text{ g}$ of sample).

3.2. Effect of caramels on survival and gene expression in *C. Elegans* model

Four samples of the eight tested for KRL and TEAC studies were selected for our investigation on *C. elegans*. Thus, we performed analyses with aromatic caramel, burnt sugar and caramels colours class III and class IV, the latter showing the highest colour intensity (EBC 35 000 and 46 000, respectively).

3.2.1. Caramel solutions increase survival of *C. Elegans* in response to severe oxidative conditions

In order to highlight any direct or indirect antioxidant property of caramels, we tested the resistance of worms to different oxidative stresses in the presence of caramel solutions. We scored the worms' survival during treatment with different chemicals at a concentration that generate severe oxidative stress.

Under physiological condition, the different concentrations of caramels didn't decrease the worms' survival, suggesting that no caramel has a toxic effect on *C. elegans* (Fig. 5). Consistent with this finding, non-toxicity of class IV caramels had been highlighted using *in vitro* assays

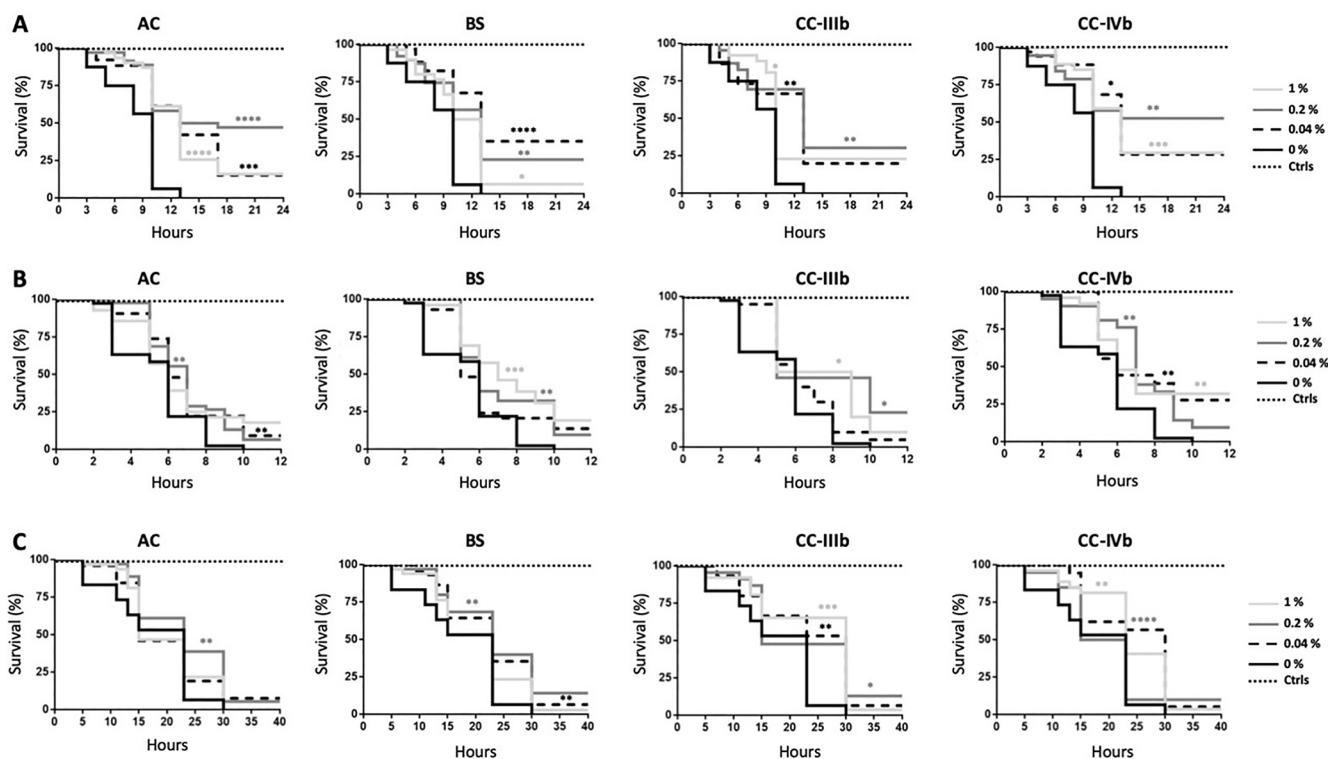


Fig. 5. Survival of worms to severe oxidative stresses in presence of caramels. Worms were pretreated with none (0%), 0.04%, 0.2% or 1% of caramel AC, caramel BS, caramel CC-IIIb or caramel CC-IVb before addition of oxidizing chemicals: paraquat/PQ (A), *tert*-Butyl hydroperoxide/TBHP (B) or trivalent Arsenic/AsIII (C). Worms incubated without oxidant in medium with or without caramel served as control (Ctrls): both survival curves are superimposed at 100% over the experimentation time. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to control survival.

and *Drosophila melanogaster* model (Mateo-Fernández et al., 2019). On the contrary, the different concentrations of caramels, whatever their nature, increased worm survival under the 3 oxidizing environments (PQ, TBHP and AsIII), the level of protection depends on the type of oxidative stress induced (Fig. 5).

PQ generates superoxide radicals after reduction in the mitochondria. No worm survived a 12-hour exposure to PQ without caramel. Caramels AC and CC-IVb showed higher resistance to PQ than caramels BS and CC-IIIb with more than 40% live worms after 18 h of treatment (Fig. 5A). The 0.2% concentration of caramel appeared to be the optimal concentration for PQ resistance.

TBHP is a strong oxidant, often more effective than hydrogen peroxide. All worms were killed after 10-hours treatment with TBHP without caramel (Fig. 5B). Although survival curves are significantly increased with the different caramels, the median survival of worms to TBHP is not changed with any of the different caramel solutions. By contrast, the maximum survival of worms at treatment is increased in the presence of the caramels. Whatever their concentrations, caramels BS, CC-IIIb and CC-IVb had the most positive effect.

Results similar to TBHP were obtained after treatment of worms with AsIII (Fig. 5C).

The antioxidant effects of each caramel *in vivo* depend on the oxidizing conditions tested, preventing the classification of the different caramels according to an antioxidant activity. It is therefore difficult to compare these results with those obtained with *in vitro* tests. In addition, the possible metabolism of caramels by the worm can certainly modify their antioxidant properties. The results show that caramels of class III and IV have an antioxidant effect *in vivo*.

3.2.2. Caramels induce resistance to moderate paraquat exposure

Knowing the positive effect of caramels on severe PQ exposure, we investigated the resistance of worms to moderate PQ exposure (4 mM) in

presence of the caramel solutions. The caramels were used at the optimal concentration of survival in severe oxidative conditions: 0.2%. Under physiological condition, caramels did not decrease the worms' lifespan, suggesting that no caramel has a toxic effect on *C. elegans* (Ctrl vs AC/BS/CC on Fig. 6). On the contrary, caramel AC significantly increased the longevity of the worms.

In moderate oxidative conditions and without caramels, the longevity of worms logically decreased (Ctrl vs Ctrl + PQ, Fig. 6). With caramels, the longevity of worms has not changed for burnt sugar, whereas the 3 other caramels significantly increased the lifespan of worms under this oxidizing condition (Ctrl + PQ vs AC/BS/CC + PQ, Fig. 6).

These results confirm the beneficial effect of those caramels to prevent damage caused by oxidants. The result with aromatic caramel suggests that this caramel has a positive effect on the longevity of worms which is independent of oxidative stress and/or which is partly dependent on physiological oxidative stress. Worms incubated with caramels CC-IIIb and CC-IVb have the same longevity whether they are incubated with or without PQ, showing that these caramels protect the worms from induced moderate oxidative stress.

Although surprising, the results obtained with burnt sugar show that the antioxidant properties of any molecule is conditioned by various factors including oxidative stress itself. Caramel BS either has oxidative properties which deteriorate over time or is metabolized by the worm, inducing an antioxidant effect in the short term (against severe stress) and not in the long term (against moderate stress). Whatever the cause, this result reinforces the antioxidant effect of the 3 other caramels tested against moderate oxidative stress which is induced by PQ.

3.2.3. Expression of genes coding response to severe oxidative stress is partially modulated by caramels

We further explored the antioxidant effects of the caramel solutions.

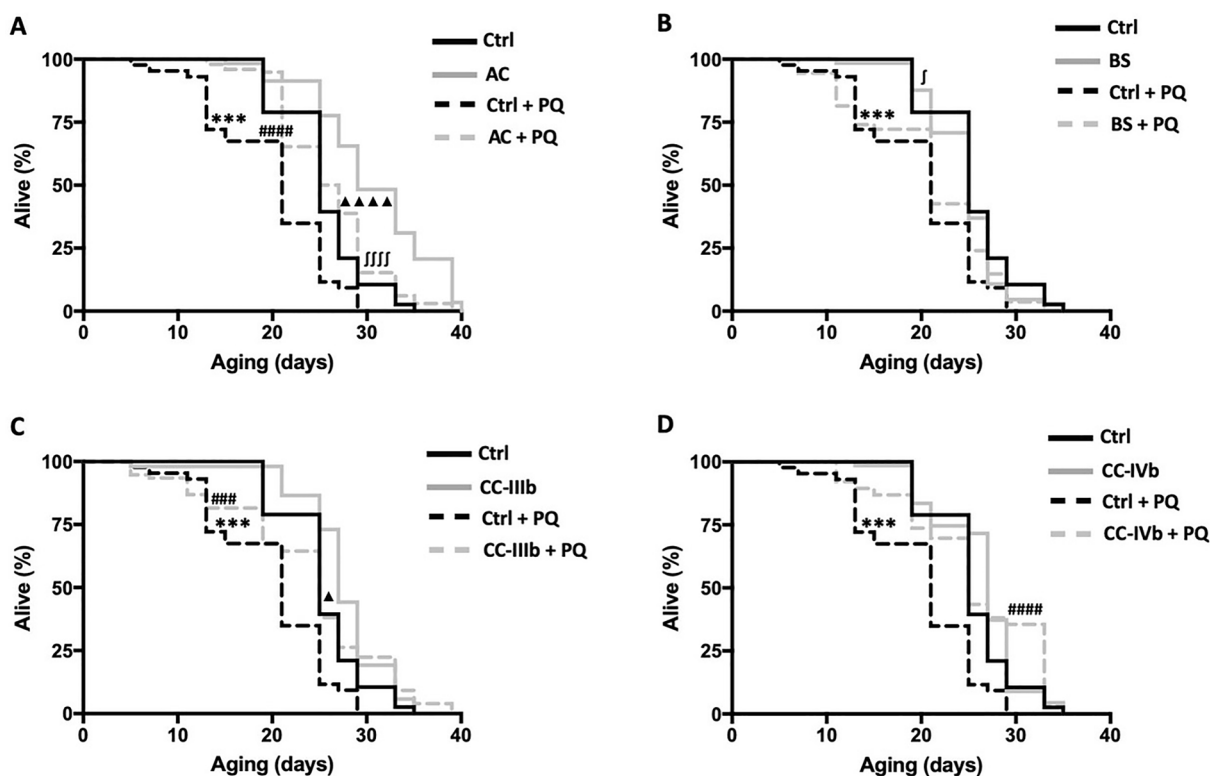


Fig. 6. Effects of caramels on lifespan of *C. elegans* in moderate oxidative stress. Young adults of N2 strain were grown on NGM plates containing none or 4 mM paraquat/PQ with no caramel (Ctrl) or 0.2% caramel AC (A), caramel BS (B), caramel CC-IIIb (C) or caramel CC-IVb (D) solutions. *** $p < 0.001$: Ctrl vs Ctrl + PQ. f $p < 0.05$: caramel vs Ctrl. #### $p < 0.0001$ and ##### $p < 0.001$: caramel + PQ vs Ctrl + PQ.

We verified if caramels could directly or indirectly modulate gene expression of enzymes involved in antioxidant response of *C. elegans*. Severe oxidative stress was then administered to the worms with the 3 oxidizing substances for 2 h with or without pretreatment with the caramel solutions.

Targeted antioxidant response was analyzed with the measurement of the relative expression of genes encoding some worm's key antioxidant enzymes: superoxide dismutase SOD-3 and glutathione-S-transferases GST-4 and GST-14 (40). GSTs have an important role in antioxidant recycling but also deactivate electrophilic xenobiotics or metabolites. Expression of the gene encoding glutamate-cysteine ligase GCS-1, an important enzyme for glutathione synthesis, was also measured (Ferguson & Bridge, 2019).

3.2.3.1. Modulation of oxidative stress response by severe oxidative stress or caramels. In the absence of caramel, exposure of worms with each oxidizing chemical induced the expression of genes coding for the antioxidant enzymes apart from *sod-3* whose expression is not enhanced by TBHP and AsIII (Control bars on Figs. 7 and S1), which is in line with the oxidizing species generated and their subcellular localization.

In a non-oxidative condition, the caramels did not influence the expression of the genes encoding the antioxidant enzymes (Fig. S2). These results suggest that caramels do not directly regulate the expression of these genes. Although non-significant, the highest concentration of caramel CC-IVb down-regulated expression of the genes *sod-3*, *gst-4* and *gst-14*, implying a possible effect of this caramel on the level of physiological stress of the worms.

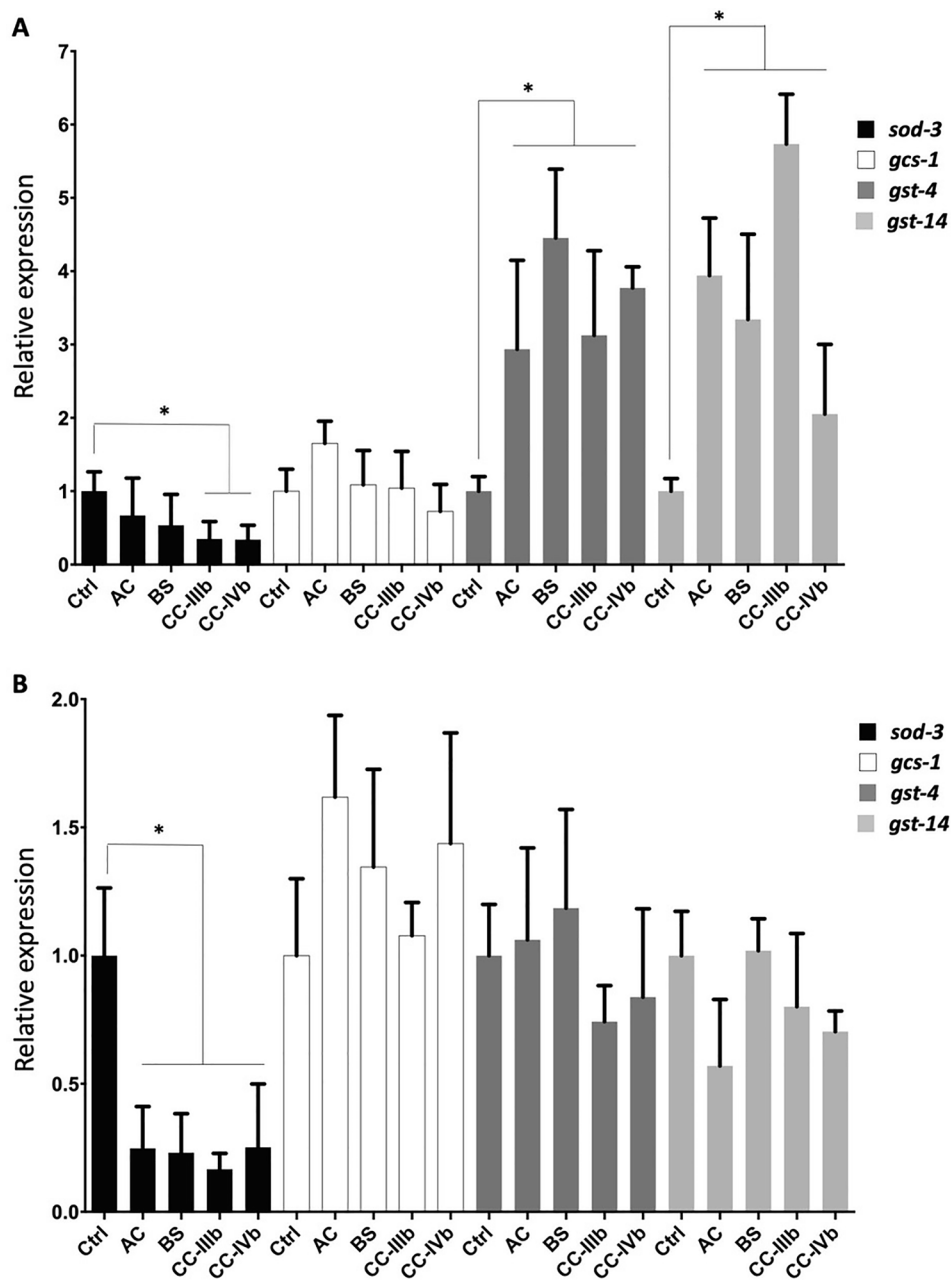


Fig. 7. Relative expression of antioxidant genes induced by severe oxidative stress in presence of caramels. Worms were pretreated with none (Ctrl) or 0.04% (A) or 1% (B) of caramel AC, caramel BS, caramel CC-IIIb or caramel CC-IVb before addition of 200 mM paraquat/PQ. After 2 h of PQ exposure, relative expression of genes was analyzed by comparing the condition with caramel to the Ctrl condition. The *pmp-3* gene was used to normalize levels of gene expression. * $p < 0.05$ (caramel vs Ctrl).

3.2.3.2. Modulation of oxidative stress response by caramels in severe oxidative stress. The caramels studied had very little effect on the transcriptional response induced after TBHP and AsIII treatments (Figs. S3 and S4) while they modulated the antioxidant response induced by PQ exposure (Fig. 7). PQ-induced *sod-3* expression was inhibited by at least one concentration of aromatic caramel, burnt sugar and class III and class IV caramels colours, suggesting an inhibition or a blocking of superoxide anion by the caramels. On the other hand, the genes coding for GST-4 and GST-14 were more expressed in the presence of the 4 caramels used at the lowest concentration (0.04%, Fig. 7A) while the highest concentration of caramels (1%, Fig. 7B) had no effect or decreased non-significantly the expression of these genes (caramel AC and CC-IIIb for the expression of *gst-14*). These results strongly suggest a direct effect of caramels on superoxide anions but also on the production of antioxidant recycling dependent on GST activities.

Induction of the *gst* genes shows that 0.04% caramels have a partial antioxidant effect. It is known that the expression of GSTs can also be regulated independently of oxidative stress (e.g. hormones, xenobiotics...). We cannot exclude such a regulation, but it would be partly dependent on oxidative stress since caramels do not induce the expression of these genes under physiological condition (Fig. S2). Caramels seem to have no effect on the upstream processes of antioxidant response because they had no significant effect on the expression of *gcs-1* whatever the induced oxidative stress (Figs. 7, S3 and S4).

Different studies have described the beneficial effects of different food compounds on different biological functions of *C. elegans* including the maintenance of protein homeostasis, mobility and longevity. These compounds such as coffee and orange extracts and thioallyls enhance the oxidative stress resistance of the worms (Amigoni et al., 2017; Dostal, Roberts, & Link, 2010; Ogawa, Kodera, Hirata, Blackwell, & Mizunuma, 2016; Wang et al., 2020). In the present study, we show that, caramels are also able to improve the resistance of worms against oxidative stress, certainly thanks to the antioxidant properties demonstrated by *in vitro* tests. A future study will demonstrate whether these additives have other properties on the biological functions of *C. elegans*.

3.3. Conclusion

Three different methods used to evaluate the antioxidant activity of marketed aromatic caramels and caramel colours showed promising results that strongly support the hypothesis that the process of sugars heating leads to compounds with beneficial antioxidant properties.

In vitro TEAC and KRL assays have demonstrated that the antioxidant activity depends on the colour of the product and the caramelization promoters used. As caramels are metabolized *in vivo*, it therefore was essential to validate that this antioxidant effect is preserved *in vivo*. *C. elegans* is considered as a relevant animal model to evaluate the *in vivo* antioxidant effects of bioactive compounds (Ayuda-Durán, González-Manzano, González-Paramás, & Santos-Buelga, 2020). Results in this model confirmed that caramels are able to prevent damage caused by oxidative stress and this activity strongly depends on the dose and type of caramel tested.

The structure of most molecules in caramels remains actually unknown but the characterization of antioxidant molecules present in products obtained by the heat treatment of sugars would probably be a next topic of interest (Rodríguez et al., 2019). They probably differ depending on the reactants used and level of heating process. A better knowledge of their content and chemical composition in caramels would partly explain the different antioxidant properties observed in our study.

Our results open up a promising field of research on the antioxidant potential of heated-formed compounds present in caramels and bring new elements that highlight the potential beneficial health effects of caramels. From a more prospective point of view and with the support of additional studies, caramels could represent a significative source of antioxidants that may prevent the effects of free radical-induced physiological stages or diseases. Future applications would particularly be of

great interest in aging prevention, clinical or sports nutrition.

Ethics statement

We have read and adhered to the Publishing Ethics regarding animal experiments.

CRediT authorship contribution statement

Cédric Moretton: Investigation, Visualization, Writing - original draft. **Cécile Gouttefangeas:** Investigation, Supervision. **Constance Dubois:** Investigation. **Frédéric Jacques Tessier:** Investigation, Writing - review & editing. **Chantal Fradin:** Investigation, Writing - review & editing. **Emmanuelle Prost-Camus:** Investigation, Writing - review & editing. **Michel Prost:** Investigation, Writing - review & editing. **Marc Haumont:** Investigation, Writing - review & editing. **Henri Nigay:** Supervision, Resources.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2020.104308>.

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