Low nanomolar concentrations of a quercetin oxidation product, which naturally occurs in onion peel, protect cells against oxidative damage

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1. Introduction

Flavonoids have attracted the attention of food chemists and biomedical researchers due to their ubiquitous presence in edible plants and their potential to induce an array of health-promoting biological actions (Del Rio et al., 2013). Recognition of the health potential of these compounds has emerged from a vast number of epidemiological studies in which inverse correlations have been established between the intake of flavonoid-rich foods and the relative risk of developing diverse cardiovascular and tumoral diseases, neurodegenerative disorders and type 2 diabetes (Alkhalidy, Wang, & Liu, 2018; Del Rio et al., 2013; Wang et al., 2016). Such studies have also found major support in a number of animal studies, in vitro cell mechanistic studies and a growing number of human intervention studies (Landete, 2012; Santhakumara, Battinob, & Alvarez-Suarez, 2018).

Considering the most commonly consumed plant foods, nearly five hundred flavonoids have been described to date. Amongst these flavonoids, due to its relative abundance, low toxicity in humans (Harwood et al., 2007), and broad range of bioactivities (Vauzour, 2010; Wang et al., 2016), quercetin (Fig. 1A) remains the single most studied flavonoid. Quercetin has been shown to exhibit, among other properties, anti-inflammatory, antiplatelet aggregation, anti-atherogenic, anti-mutagenic, anti-angiogenic, blood vessel-dilating and anti-hyperglycemic actions (Del Rio et al., 2013; Gormaz, Quintremil, & Rodrigo, 2015; Li et al., 2016; Vauzour et al., 2010). In most cases, the exact mechanisms underlying these bioactivities remain elusive. Based on the well-established capacity of quercetin to scavenge free radicals (Rice-Evans, Miller, & Paganga, 1996), inhibit the activity of certain ROS-generating enzymes and/or upregulate the expression of genes that encode various ROS-removing and/or antioxidant-synthesizing enzymes (Dinkova-Kostova, Holtzclaw, & Kensler, 2005; Dinkova-Kostova & Talalay, 2008), several studies have attempted to relate some of the bioactivities mentioned above to the antioxidant properties of quercetin (Costa, Garrick, Roqué, & Pellacani, 2016; Croft et al., 2017). However, the actual possibility that quercetin acts as an antioxidant in vivo has been increasingly questioned on the basis of the large difference that exists between the concentrations of quercetin required to exert its...
antioxidant actions in vitro (within the μM range) and those attained (low nM) after the ingestion of quercetin-rich foods in vivo (Forman, Davies, & Ursini, 2014; Galleano, Verstraeten, Oteiza, & Fraga, 2010; Manach et al., 1998). The low plasma concentrations attained in vivo reflect the limited gastrointestinal absorption and the vast bio-transformation that systemically affects this flavonoid (Manach et al., 1998; Wang et al., 2016). Furthermore, the fraction of quercetin that is not absorbed undergoes a series of catabolic reactions that are catalyzed by enzymes present in the colonic microbiota (Fraga, Croft, Kennedy, & Tomás-Barberán, 2019). Interestingly, some of these reactions lead to the formation of a set of metabolites of quercetin whose bioactivities could account for several of its beneficial health effects (Espín, González-Sarrías, & Tomás-Barberán, 2017). Regarding the antioxidant properties of these metabolic products, Amić et al. (2017) recently reported that quercetin colonic metabolites with a catecholic structure might constitute a group of scavengers that are more potent than quercetin itself. In addition to the interest that arises from such metabolites, a significant interest has also recently been placed on the antioxidant activity displayed by certain metabolites that result from the oxidation of quercetin (Atala, Fuentes, Wehrhahn, & Speisky, 2017). The oxidation of this flavonoid leads to the formation of some electrophilic intermediates (i.e. quinones and quinone methides) that are potentially capable of activating the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) pathway (Bolton & Dunlap, 2017; Lee-Hilz et al., 2006). The activation of Nrf2 by such intermediates may account for the already established ability of quercetin to upregulate the expression of genes encoding ROS-removing and/or antioxidant-synthesizing enzymes (Kang et al., 2009; Tanigawa, Fujii, & Hou, 2007). The capacity of quercetin to activate Nrf2 is shared by several flavonoids (Lee-Hilz et al., 2006) and by numerous other nonflavonoid diphenols that are easy to oxidize (Dinkova-Kostova & Wang, 2011).

Recently, our laboratory reported that 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone (BZF, Fig. 1B), a major polar metabolite that results from the oxidation of quercetin (Jørgensen, Cornett, Justesen, Skibsted, & Dragsted, 1998), is at least 200-fold more potent as an antioxidant than quercetin when tested in ROS-exposed cells (Fuentes, Atala, Pastene, Carrasco-Pozo, & Speisky, 2017). Considering the notably high antioxidant potency of BZF and the fact that this metabolite can easily be formed during the exposure of quercetin to the polyphenol-oxidase (PPO) enzyme (Kubo, Nihei, & Shimizu, 2004), in the present study, we explored the occurrence of BZF in a large number of quercetin-rich plant foods. Subsequently, we investigated the potential of aqueous extracts obtained from selected BZF-containing foods in protecting cells against oxidative damage. Finally, we assessed the extent to which the antioxidant capacity of such extracts relies on the presence of BZF in the plant food studied.

2. Materials and methods

2.1. Plant food materials

Almonds (Nonpareil), apples (Fuji and Granny Smith), capers (French Nonpareil), chives (White Lisbon), clove (Zanzibar), curcuma (Curcuma longa L.), white garlic (Valenciano), ginger (Roscoe), goji (Ningxian), mushrooms (Portobello), yellow onions (Valenciana), purple onions (Ruby), oregano (Greek), potatoes (Desiree), radishes (Rover), yellow shallots (Mikor), purple shallots (Jermor), spinach (Giant Winter) and walnuts (Serr) were obtained from major local supermarkets (Santiago, Chile).

2.2. Chemicals

Quercetin (≥95%) and 3,4-dihydroxybenzoic acid (≥97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and ethanol were HPLC-grade and purchased from Merck (Darmstadt, Germany). Formic acid, sodium hydroxide, indomethacin, hydrogen peroxide, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), 2,4,6-trihydroxybenzoic acid, quercetin-4′-glucose, dimethyl sulfoxide and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. All other reagents were of analytical grade.

2.3. Plant food extracts

Ethanol-water (EW, 70:30% v:v) extracts from each of the plant foods mentioned above were prepared by adding 1.5 g of either the whole food (for capers, chives, cloves, goji, mushrooms, oregano and spinach), the peeled food (for almonds, apples, curcuma, garlic, ginger, onions, potatoes, radishes, shallots and walnuts) or the peel of the plants to 25 mL of a EW solution. After homogenization with an Ultraturrax (T18; IKA, Wilmington, NC, USA), each extract was subjected to two successive centrifugations, the first centrifugation at 3500g for 5 min and the second one at 21,000g for 10 min (both run at 5 °C). The resulting supernatant was immediately subjected to chromatographic analysis to quantify the BZF and quercetin. Additionally, aqueous extracts from the outer scales of yellow onions (OAE) and yellow shallots (SAE) were prepared as above using sodium phosphate buffer (25 mM, pH 6.0) and immediately subjected to HPLC-DAD analysis.

2.4. Preparation and identification of pure BZF

BZF was prepared by oxidizing quercetin under alkaline conditions, as previously described by us (Fuentes et al., 2017). In brief, quercetin was dissolved in NaOH (pH 12) and kept in such medium at 22 °C during 13 min. Immediately after, the solution was brought to pH 5 by addition of HCl and subjected to chromatographic separation. A semi-preparative HPLC suited with a YL9111S binary pump system, a YL9120S UV–Vis detector and a reversed phase Kromasil 100–5–C18 (250 × 10 mm, i.d., 5 μm) column was used. The mobile phase consisted of a mixture of acetonitrile (A) and water/formic acid (0.1%) (B), whose composition (v:v) varied using the following HPLC gradient.
program: 10% A and 90% B (0–15 min), linear increase to 60% A over 50 min, and a return to the starting conditions within 10 min (Fuentes et al., 2017). The flow rate was 3.78 mL/min and the eluate was monitored at 294 nm (Atala et al., 2017; Jungbluth & Ternes, 2000). The fraction corresponding to the putative BZF peak was collected, brought to dryness under a stream of nitrogen at 40 °C and kept at −80 °C. The identity and purity of the isolated BZF was confirmed by HPLC-DAD-ESI-MS/MS and 1H and 13C NMR analyses. The HPLC-ESI-MS/MS analysis was performed on an Ekspert™ UltraLC 100 coupled with an AB Sciex Triple Quad™ 4500, a triple-quadrupole tandem mass spectrometer equipped with turbo spray ionization source (AB Sciex, Ontario, Canada). The mass spectrometer was operated in negative ionization mode, and the data were acquired in multiple reaction monitoring mode. The source temperature was 650 °C, and the ESI voltage and entrance potential were set at −4.5 kV and −10 V, respectively. The gas flows were as follows: ion spray gas, 50 psi; heater gas, 40 psi; curtain gas, 20 psi; and collision gas, 7 psi. The de-clustering potential, collision energy, and collision exit potential for each compound were adjusted at each tandem MS stage to determine the optimum parameters at which a maximum amount of signal information could be obtained, and a scanning rate of 150 ms was used. The ESI-MS/MS system was controlled by Analyst 1.6.2 software (AB Sciex, Darmstadt, Germany). The molecular ion [M−H]− and qualification transitions of BZF were m/z at 317 and 163/191. These results are identical to those reported earlier by us for another pure BZF preparation (Fuentes et al., 2017). To perform the 1H and 13C NMR analyses, 5 mg of the pure BZF preparation were dissolved in 600 µL of DMSO-d6 and transferred into 5 mm NMR tubes. NMR spectral data were acquired at 300 °K, chemical shifts and coupling constants were present in ppm and Hz, respectively, data were processed using a TopSpin 3.2 software. Results corresponding to the 1H and 13C NMR analyses of the pure BZF preparation were identical to those described in the Results and discussion section for a BZF isolated from OAE.

2.5. HPLC analysis of BZF and quercetin in plant food extracts

Samples of increasing concentrations of the above-obtained pure BZF (1–150 µM) or of quercetin (1–300 µM), both prepared in EW, were separately injected into an Agilent 1200 series HPLC to build calibration curves. After plotting the area of the peaks versus the concentration of BZF or quercetin, correlation coefficients equal to or greater than 0.99 were in each case systematically obtained (data not shown). The HPLC was equipped with an autosampler and a photodiode array detector (Santa Clara, CA, USA). The system was controlled by an Agilent ChemStation (Agilent Technologies 2010). BZF and quercetin (its aglycone form) were separated using a mobile phase mixture and a gradient program identical to that described above (subsection 2.4). Other chromatographic conditions were as follows: flow rate of 0.8 mL/min, column (250 × 4.6 mm i.d., 5 µm, RP-18e Purospher® Star, Merck, Darmstadt, Germany), and column oven temperature of 25 °C. The absorbance of the eluate was monitored at 294 nm for BZF and at 370 nm for quercetin (Atala et al., 2017). To estimate the concentrations of BZF and quercetin in plant food extracts, the area of the HPLC peak corresponding to each of these compounds was interpolated in the respective calibration curve. The limits of detection were 1.0 mg/100 g of dry weight for BZF and 0.25 mg/100 g of dry weight for quercetin. The water contents of the bulbs of onions and shallots, assayed at 65 °C for 6 h (or until attaining constant weigh), were 89% and 87%.
respectively. In the case of the aqueous and EW extracts of onions and shallots, the identity of BZF and quercetin was confirmed by HPLC-ESI-MS/MS, employing chromatographic conditions identical to those described in subsection 2.4.

2.6. Collection and processing of the chromatographic peaks eluted during the HPLC-DAD analysis of the onion aqueous extract

As will be explained in the Results and Discussion section, the extract obtained from the outer scale of yellow onions was selected to investigate which of its major components would account for the associated antioxidant capacity with it. During the HPLC-DAD analysis of OAE, fractions that separately comprised each of the major chromatographic peaks of the extract were collected using an Agilent 1260 Infinity automated fraction collector coupled to the HPLC system. The eluting fractions were collected as follows: peak 1 from 9.5 to 11.0 min, peak 2 from 11.1 to 12.5 min, peak 3 from 27.3 to 27.8 min, peak 4 from 33.3 to 34.5 min, and peak 5 from 38.0 to 39.6 min. Immediately after collection, the fractions were brought to dryness under a stream of nitrogen at 40 °C and reconstituted in a small volume of a mixture of a mobile phase whose composition was A/B (30:70% v:v). The chemical identity of the molecule(s) present in the reconstituted peaks was established by conducting a new ESI-MS/MS analysis. In the case of peak 3, the identity of BZF was further confirmed by 1H and 13C NMR analyses. After reconstituting each peak in a small volume of sodium phosphate-saline buffer (PBS) and before assessing them as antioxidants, the peaks were subjected to HPLC-DAD analysis, using experimental conditions identical to those described in subsection 2.4.

2.7. Cell culture conditions

The human colonic adenocarcinoma cell line, Caco2 cells (ATCC® HTB-37™), between the 10th and 15th passages, were cultured at 37 °C (5% CO2/95% air) in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% fetal bovine serum (Biological Industries USA, Inc.). Cells were trypsinized when they reached near 90% confluence and were used for experiments.

2.8. Oxidative status experiments

Cells were seeded (1 × 10^5/well) in a 96-well microplate, and after culturing for 24 h (time at which a near 90% of cell confluence was reached), their intracellular oxidative status was assessed. As previously described in Fuentes et al. (2017), DCFH-DA was used as a ROS-reactive probe. The cells were loaded with DCFH-DA (50 μM) for 30 min, and after being washed with PBS, the cells were exposed for 40 min to indomethacin (275 μM) or hydrogen peroxide (1.75 mM) in the absence or presence of OAE or SAE, using the CytoTox-One™ homogeneous membrane integrity assay (excitation 560 nm/emission 590 nm), carried out as previously reported (Fuentes et al., 2017). The results are expressed as percentage of LDH leaked into the extracellular media. A basal value of 6% was estimated in PBS-treated cells. MTT reduction, which evaluates the mitochondrial dehydrogenase activity, was evaluated in the adherent cells. After washing with PBS, 20 μL of 2.5 mg/mL MTT and 80 μL of PBS were added to each well, and cells were further incubated for 150 min at 37 °C (5% CO2/95% air). The supernatant was discarded, and 100 μL of dimethyl sulfoxide was added to dissolve the formazan. After 10 min incubation at 37 °C, the absorbance at 540 nm was measured (Carrasco-Pozo, Gotteland, & Speisky, 2010). The results are expressed as the percentage of MTT reduction. A 100% MTT reduction was estimated in the PBS-treated cells.

2.9. Cell toxicity and cell viability assessments

Lactate dehydrogenase (LDH) leakage was used as a marker of cell toxicity, and MTT reduction was used as a marker of cell viability. LDH was evaluated in the supernatants of cells exposed for 40 min to either PBS or indomethacin, in the absence or presence of OAE or SAE, using the CytoTox-One™ homogeneous membrane integrity assay (excitation 560 nm/emission 590 nm), carried out as previously reported (Fuentes et al., 2017). The results are expressed as percentage of LDH leaked into the extracellular media. A basal value of 6% was estimated in PBS-treated cells. MTT reduction, which evaluates the mitochondrial dehydrogenase activity, was evaluated in the adherent cells. After washing with PBS, 20 μL of 2.5 mg/mL MTT and 80 μL of PBS were added to each well, and cells were further incubated for 150 min at 37 °C (5% CO2/95% air). The supernatant was discarded, and 100 μL of dimethyl sulfoxide was added to dissolve the formazan. After 10 min incubation at 37 °C, the absorbance at 540 nm was measured (Carrasco-Pozo, Gotteland, & Speisky, 2010). The results are expressed as the percentage of MTT reduction. A 100% MTT reduction was estimated in the PBS-treated cells.

2.10. Statistical analysis

The values in Table 1 and data points plotted in Figs. 3–5 represent the means of at least three independent experiments, each conducted in octuplicate. For the sake of simplicity, since the standard deviations represented less than 10% of the means, these values were omitted from the former figures. Statistical significance of the differences between the experimental conditions was assessed with unpaired t-tests or analysis of variance (ANOVA), as appropriate. Data were analyzed using the GraphPad Prism 5 statistical software (La Jolla, CA, USA).

3. Results and discussion

3.1. Identification and quantification of BZF and quercetin in several plant foods

The presence of BZF was investigated in EW extracts prepared from the peel and/or flesh of the following quercetin-rich plant foods: almonds, apples, capers, chives, cloves, curcuma, garlic, ginger, goji, mushrooms, onions, oregano, potatoes, radishes, shallots, spinach, and walnuts. As shown in Table 1, among all these foods, the presence of BZF could be established (HPLC-ESI-MS/MS) and quantified (HPLC-DAD) exclusively in onions (Allium cepa L. cepa group) and shallots (Allium cepa L. aggregatum group). Interestingly, BZF was detected only in the dry outer scales (not in the bulbs) of these foods. In the case of onions, differences in BZF content were detected among its varieties. While BZF contents were 110% higher in purple onions compared to yellow onions, no difference was seen between purple and yellow shallots. Table 1 also describes the quercetin content, assayed in its

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Benzofuranone and quercetin contents in the outer scales and bulbs of the yellow and purple varieties of onions and shallots.</th>
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<tbody>
<tr>
<td>Plant Food</td>
<td>BZF</td>
</tr>
<tr>
<td>Yellow Shallot outer scales</td>
<td>438^1</td>
</tr>
<tr>
<td>Yellow Shallot bulb</td>
<td>nd</td>
</tr>
<tr>
<td>Purple Shallot outer scales</td>
<td>418^1</td>
</tr>
<tr>
<td>Purple Shallot bulb</td>
<td>nd</td>
</tr>
<tr>
<td>Yellow onion outer scales</td>
<td>394^1</td>
</tr>
<tr>
<td>Yellow onion bulb</td>
<td>nd</td>
</tr>
<tr>
<td>Purple onion outer scales</td>
<td>826</td>
</tr>
<tr>
<td>Purple onion bulb</td>
<td>nd</td>
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1 mg/100 g of dry weight.
2 mg/100 g of fresh weight.
aglycone form, in the dry outer scales and the fresh bulb of the two varieties of onions and shallots. Compared to the bulbs, quercetin content was substantially higher in the dry outer scales of yellow and purple onions, 1750-fold and 7564-fold, respectively. In the outer scales of yellow and purple shallots, quercetin contents were 476-fold and 104-fold higher, respectively. When such comparisons are made on a 100 g of dry weight basis, the values given for the four differences are reduced to approximately one-tenth. The above-described results not only confirm the early report by Ly et al. (2005) on the presence of BZF in the dry outer onion scales but also reveal that, in addition to these vegetables, shallots also exhibit comparably high BZF contents. While the outer scales of onions and shallots may serve to protect the bulb of these foods against pathogens by providing both a physical and biochemical barrier, the actual reason why BZF is contained only in these two plant foods and its presence limited to their outer scales remains to be established. However, in the case of onions and shallots, the phenolic composition of their outer layer could undergo dynamic changes during the post-storage period. At least in onion bulbs, once the dormancy phase is broken, the glycosides start to be hydrolyzed faster and the concentration of quercetin increases (Sharma, Assefa, Kim, Ko, & Park, 2014). Considering the latter, and the fact that the peroxidase activity of the onion peel is approximately 20-fold greater in the first compared to the fifth scales of the abaxial epidermis (Hirota, Shimoda, & Takahama, 1998), the existence of a localized oxidative environment capable of oxidizing quercetin into BZF seems reasonable to be assumed. On the other hand, the observation that chives and capers, despite containing 10- and 100-fold higher quercetin content than onions (Phenol-Explorer 3.6.), contain no BZF, suggests that the richness in quercetin (aglycone) of any given plant food cannot be assumed to be a marker of the natural presence of BZF in its tissues.
3.2. Chromatographic and NMR identification of BZF in onion peel

An EW extract obtained from the dry outer scales of yellow onions (Allium cepa L. cepa group) was subjected to HPLC-DAD analysis. As shown in Fig. 2A, five major peaks were detected when the eluate was monitored at 294 nm, a wavelength at which the absorption of the quercetin-derived benzofurane is maximum (Jungbluth & Ternes, 2000). Inserted in this figure is the HPLC chromatogram that results from injecting a pure BZF preparation. The tR of the latter compound and the tR of peak 3 were almost identical (27.6 and 27.5 min, respectively). A total overlap was also found between the tR of peaks 1, 2 and 5, and those obtained after injecting 2,4,6-trihydroxybenzoic acid (9.9 min), 3,4-dihydroxybenzoic acid (11.4 min) and quercetin (38.9 min), respectively. As described below, peak 4, whose tR was 34.1 min, was identified as quercetin-4′-glucoside. To confirm that peak 3 corresponded to BZF, a sample of the above-referred extract was further analyzed by HPLC-ESI-MS/MS, using the same chromatographic conditions as those used in the HPLC-DAD analysis. The chromatogram thus generated, shown in Fig. 2B, was, in terms of its tR, almost identical to that obtained in Fig. 2A for peaks 1, 2, 3, 4 and 5. The molecular ion [M−H]− and qualification transitions of these peaks were as follows: m/z at 169 and 151/83 for peak 1 (2,4,6-trihydroxybenzoic acid), m/z at 153 and 109/79 for peak 2 (3,4-dihydroxybenzoic acid), m/z at 317 and 163/191 for peak 3 (2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofurane), m/z at 463 and 301/151 for peak 4 (quercetin-4′-glucoside), and m/z at 301 and 151/273 for peak 5 (quercetin). These data are consistent with those previously reported by Ly et al. (2005) and by Khiari and Makris (2012) in 100% methanolic extracts from the dry outer scale of onions. The identity of BZF in onion peel was also assessed by 1H and 13C NMR analyses. Results obtained were: 1H NMR (400 MHz, DMSO-d6) δ (ppm): 5.90 (d, J = 2 Hz, 1H, H-6); 5.95 (d, J = 2 Hz, 1H, H-8); 6.79 (d, J = 8 Hz, 1H, H-5); 7.52-7.55 (m, J = 1.90 Hz, 2H, H-2′ and H-6′). 13C NMR (100 MHz, DMSO-d6) δ (ppm): 90.34 (C-8); 96.38 (C-6); 100.48 (C-4a); 104.58 (C-3); 114.89 (C-5′); 117.34 (C-2′); 123.81 (C-6′); 125.02 (C-1′); 144.73 (C-3′); 151.36 (C-4′); 158.55 (C-8a); 168.47 (C-5); 171.87 (C-7); 189.86 (C-4′); 190.25 (C-2′). These data are coincident with those reported earlier by Jørgensen et al. (1998) for a pure BZF. Spectra corresponding to the above 1H and 13C NMR results are shown in Supplementary Data.

3.3. Evaluation of the antioxidant and cytoprotective properties of onion and shallot extracts

Considering the recently reported particularly high antioxidant potency of the BZF molecule (Fuentes et al., 2017), extracts prepared from the dry outer scales of the yellow variety of onions and shallots were assessed for their potential to act as antioxidants in ROS-exposed cells. However, to prevent the exposure of the cells to a residual concentration of ethanol, aqueous extracts were first prepared and chromatographically characterized. Fig. 2C and D depict the HPLC-DAD chromatograms of aqueous extracts obtained from the dry outer scales of onions and shallots, respectively. The chromatographic profiles and tR of the five major peaks depicted by these figures are virtually identical and do not seem to differ from the profiles already obtained in the analysis of a yellow onion EW extract (Fig. 2A). In aqueous extracts prepared at a 60 mg/L concentration, the BZF and quercetin concentrations were estimated to be 560 nM and 125 nM in OAE, and 527 nM and 104 nM in SAE, respectively. These values reveal that extracting the yellow outer scales of onions and shallots with an aqueous solvent shifts the BZF to quercetin ratio from near 0.5, estimated from data on the EW extracts shown in Table 1, to 4.5 and 5.0, respectively. Fig. 3A shows the results from assessing the capacity of OAE and SAE to protect cells against oxidative distress (i.e.; increase in DCHF oxidation) induced by the ROS generator indomethacin (Carrasco-Pozo et al., 2010). A 100% value corresponds to the increase in oxidative tone induced by indomethacin. In the absence of this agent, the sole exposure of cells to 3 µg/L of OAE or SAE alone caused no significant increase of the oxidative tone. However, when these extracts were added together with indomethacin, OAE afforded a 59% and a 78% protection against the increase in oxidative tone at 3 ng/L and 3 µg/L, respectively. In the case of SAE, a 51% and 71% protection was observed at 3 ng/L and 3 µg/L, respectively. Fig. 3A also reveals that when pure quercetin is assayed at 0.09 µM, for the concentration at which this flavonoid is present in the 3 µg/L OAE, no protection is afforded.

In addition to assessing the antioxidant capacity of OAE and SAE, the potential of these extracts to protect Caco-2 cells against the cell lytic (i.e. LDH leakage) and mitochondrial disturbing (i.e. MTT reduction-inhibition) effects of indomethacin were evaluated (Fig. 3B and C). As shown in Fig. 3B, the basal leakage of LDH (corresponding to PBS-treated cells) was increased by indomethacin by nearly 3-fold. This effect was completely prevented when OAE or SAE was added at a 3 µg/L concentration (no such effect was seen at 0.3 ng/L). A similar protection pattern was seen when OAE and SAE were assayed for their capacity to protect against the inhibition of MTT reduction induced by indomethacin (Fig. 3C). In additional experiments, the antioxidant capacities of OAE and SAE were also assessed using hydrogen peroxide (1.75 mM) instead of indomethacin (275 µM). Under these conditions, a 6% and a 74% of protection was seen when OAE was added at 0.3 ng/L and 3 µg/L, respectively. At these concentrations, the antioxidant protection afforded by SAE was 2% and 67%, respectively (data not shown).

The protection of cells afforded by the aqueous onion scale extract is in line with the data reported earlier by us (Carrasco-Pozo et al., 2010) using a quercetin-rich apple peel extract. However, when these two extracts are compared on a phenolic concentration basis, the concentration of OAE (0.044 µg of gallic acid equivalents/L) needed to afford a degree of protection similar to that reported (Carrasco-Pozo et al., 2010) with the apple peel extract is 1360-fold lower. In the present study, the effectiveness of OAE to afford antioxidant protection cannot be attributed to the presence of quercetin since its concentration in such an extract (0.09 µM) is at least two orders of magnitude lower than the concentration reported by us (10 µM) to be required to protect cells against indomethacin-induced damage (Fuentes et al., 2017). Thus, we decided to explore which compounds, in addition to or other than quercetin, that are present in OAE could individually account for its antioxidant effects.

3.4. Assessment of the antioxidant capacity of different chromatographic fractions eluted during the HPLC-DAD analysis of OAE

In our search to elucidate which molecule(s) could account for the protection afforded by OAE, we subjected this extract to HPLC-DAD chromatography placing specific attention on those peaks that emerged as its major components (Fig. 2C). In this analysis, we collected and subjected to dryness those chromatographic fractions that comprised peaks 1, 2, 3, 4 and 5 (as described in Material and Methods). The dried fractions were immediately reconstituted in PBS and assayed for their antioxidant capacity (Fig. 4). The fractions were reconstituted in a volume that brought the compound present in each peak to a concentration identical to the concentration the concentration initially present in the 3 µg/L aqueous extract. Comparatively, peaks 1, 2, 4 and 5 afforded no significant protection against the increase in the oxidative tone induced by indomethacin. In turn, peak 3 protected the cells against the increase in DCHF oxidation induced by indomethacin by nearly 80%. An identical protection was also observed when a pool of peaks 1, 2, 3, 4 and 5 was dried, duly reconstituted in PBS and assayed for its antioxidant capacity, suggesting that the drying and subsequent reconstitution of the collected peaks did not affect the original antioxidant capacity of the extract. The latter result reveals that, presumably, those components of the OAE that were not collected play a negligible role in the protective effect of the extract. Interestingly, when
a peak 3-free pool of all peaks (obtained by chemical subtraction) was evaluated, absolutely no protection was observed, indirectly revealing that the antioxidant capacity of OAE would reside in the presence of its BZF. A more direct support for the latter assertion was given by the demonstration that after isolating the BZF from the OAE, and testing it at a 0.03 nM concentration, namely, at a concentration identical to that present in the 3 µg/L extract, its antioxidant capacity did not differ from the latter. We reached the same conclusion when chemical subtraction experiments were conducted with the aqueous extract obtained from the outer scale of shallots (not shown).

3.5. Comparative antioxidant effects of OAE and isolated BZF

Prompted by the extremely low concentration of BZF (0.03 nM) needed to protect cells against the oxidative damage induced by indomethacin, we decided to investigate whether this effect follows a concentration dependent pattern and whether the same applies to the BZF-containing OAE. Fig. 5 depicts the percentage of protection against the increase in oxidative tone induced by indomethacin afforded by increasing concentrations of BZF (from 10⁻⁸ nM to 10⁻⁵ nM), added in its pure form or as part of the OAE. While no protection was seen at a 3 × 10⁻⁶ nM BZF concentration, which in the case of OAE corresponds to 0.3 ng of outer scale onion/L, a significant and sustained protection (from 70% to 80%) is shown within the 3 × 10⁻⁴ nM to 10⁻³ nM BZF concentration range. In the case of OAE, such protection was abruptly lost when the concentration of BZF in the extract reached 2 × 10⁻³ nM, remaining at zero until 5 × 10⁻² nM. However, at concentrations of BZF in OAE greater than 5 × 10⁻³ nM, the protection was increasingly recovered to attain a full protection at 10⁻¹ nM. In the case of cells treated with pure BZF, a slow but complete and permanent loss was seen at concentrations equal to or greater than 5 × 10⁻² nM. From these results, several points can be noted. First, the remarkably close overlap that is seen between the pure BZF and the OAE curves until the 10⁻² nM concentration mentioned above at which both the pure BZF and the OAE start to lose their protecting capacities. Such a value represents a threshold concentration from which the cells respond, shifting from a previous direct- into an inverse-concentration-dependent antioxidant response. Such biphasic behavior suggests that BZF triggers a “para-hormetic” response (Forman et al., 2014), where this molecule is able to induce opposite biological effects at different concentrations (Calabrese et al., 2007). BZF efficiently increases the antioxidant cell capacity at low concentrations and promotes such an effect less efficiently, to reach zero at higher concentrations. On the other hand, the differences in the slopes and the no-protection BZF concentration levels associated with the two decay curves suggest that beyond the 10² nM BZF threshold concentration, cells respond in a different manner, depending on whether they also face components of the extract other than the BZF. In the absence of such components, an increase of five-fold in the concentration of BZF (from 10² nM to 5 × 10² nM) added to the cells was associated with a total loss of the antioxidant protection. In turn, in their presence, just doubling the concentration of BZF (from 10² nM to 2 × 10² nM) and the concentration of other components of the extract (some of which are phenolics) seen by the cells was enough to see a total loss. One may speculate that under the latter conditions the cells “sense” the components of the extract as a sign that there is no need to upregulate the endogenous antioxidant capacity. However, once the antioxidant protection has been lost, the cells are able to respond to further increases in BZF only when the concentration of this compound is higher than 5 × 10² nM. The data from Fig. 5 reveals that a protection of near 100% can be attained when the cells face a BZF concentration of 10⁴ nM in the extract. Interestingly, under such conditions, the concentration of quercetin seen by the cells raises to 2 µM. Presumably, as cells approach to such a concentration of quercetin, this flavonoid and the other phenolics present in OAE accumulate to act as antioxidants via an ROS-scavenging mechanism.

In vitro studies can be an important tool to investigate the bioactivity, and possible underlying mechanisms of action, of pure molecules or food extracts which contain them. However, it is important to note that in vitro studies do not consider a number of physiological parameters which take place in vivo and that can significantly affect the metabolism and/or the bioavailability of the bioactive compounds (Martins, Barros, & Ferreira, 2016). In order to project the eventual applicability of the here-shown extremely potent antioxidant properties of BZF and OAE, in vivo studies addressing the latter should be conducted.

4. Conclusions

Out of twenty quercetin-rich plant foods analyzed (by HPLC-DAD-ESI-MS/MS), only onions and shallots were found to contain BZF, revealing that the high presence of quercetin in a given plant food alone cannot be interpreted as an indication of the presence of BZF is the food. Interestingly, the presence of BZF was limited, in both plants to their dry outer scales. In line with the recently recognized high antioxidant potency of BZF (Fuentes et al., 2017), aqueous extracts prepared from the dry outer scales of yellow onions showed potent antioxidant and cytoprotective effects at a 3 µg/L concentration. At this concentration, the BZF content in the extract was 0.03 nM. By means of chemical subtraction of the BZF peak from the extract and its subsequent isolation, this compound was found to account fully and exclusively for the antioxidant protection seen with the whole extract. Finally, the antioxidant effect of the onion extract and that of a pure BZF preparation were tightly described by two perfectly overlapping curves whose concentration-dependence was within the 3 × 10⁻⁴ to 10⁰ nM BZF concentration range. At higher concentrations, the antioxidant capacity of the extract and that of a pure BZF preparation decayed swiftly and completely. The biphasic concentration-dependence behavior of the extract and the pure BZF preparation suggests that the latter compound exerts its antioxidant effects through a para-hormetic
mechanism of action. The data reveal that BZF can exert, in its pure form or as part of a plant food extract, antioxidant effects at extremely low nM concentrations. The unprecedented low range of concentrations at which BZF affords its antioxidant effects places the BZF-containing plant foods shown here on the frontier of the search for novel sources of natural antioxidants.

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

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References