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# Antioxidant capacity and hydrogen peroxide formation by black and orange carrots

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Anthocyanins are powerful antioxidants with numerous beneficial health effects. However, their autoxidation may produce hydrogen peroxide. Black carrots owe their colour to the high anthocyanin content. The aim of this study was to compare the antioxidant capacity of and hydrogen peroxide generation by black and orange carrots. Black carrots were found to have a significantly higher anthocyanin content and antioxidant capacity estimated by the FRAP assay, DPPH scavenging and ABTS$^{\bullet}$ scavenging. Carrot, like other vegetables, generates hydrogen peroxide upon cooking. Black carrots generated much more hydrogen peroxide than orange carrots (55.0 ± 2.6 µM vs 6.0 ± 2.1 µM in phosphate buffer, 8.7 ± 1.2 vs 0.3 ± 0.1 µM in water, in 1:5 (w/v) homogenates. These small amounts of hydrogen peroxide are not likely to exert deleterious health effects but may have antimicrobial activity.

*Key words*: carrot, FRAP, ABTS• decolorization, DPPH, $H_2O_2$ generation

## Introduction

Anthocyanins are polyphenolic pigments belonging to the flavonoid group, formed by phenylpropopanoid pathway from phenylalanine. Anthocyanins are potent antioxidants *in vitro*, demonstrated to scavenge superoxide (Sichel et al. 1991, Tsuda et al. 1996) and peroxyl radicals (Wang et al. 1997) as well as nitric oxide (van Acker et al. 1995), and inhibit lipid peroxidation induced by various factors (Tsuda et al. 1996, Narayan et al. 1999, Liu et al. 2015, Sousa et al. 2016). Higher anthocyanin intake is associated with a decreased risk of all-cause mortality (Grosso et al. 2017) which can be mainly accounted for by a reduced cardiovascular mortality risk (Wang et al. 2014). Several meta-analyses associate greater anthocyanin intake with reduced cardiovascular disease (CVD) and with improved markers of cardiovascular health (Cassidy et al. 2011, 2013, Jennings et al. 2012). Greater anthocyanin intake is also associated with a reduced risk of type 2 diabetes (Wedick et al. 2012) and better weight maintenance (Bertoia et al. 2016). Apart from cardiovascular and metabolic functions, anthocyanin intake is also associated with a delayed decline in cognition during aging (Devore et al. 2012). Increased dietary uptake of anthocyanins seems thus highly desirable. Black carrots may be one of dietary sources of anthocyanins.

The carrot is a root vegetable widely consumed in human diet, either as fresh or processed in meals and beverages. Carrots are an important dietary source of carotenoids, mostly α-carotene and β-carotene, also known as provitamin A, as they can be converted to vitamin A once in the body. Anthocyanin-rich black carrot (*Daucus carota* subsp. *sativus* var. *atrorubens* Alef) is grown in Middle Asia, Far East and in Europe (Kammerer et al. 2004, Türkyılmaz et al. 2012). Black carrots have been reintroduced into modern production (Simon 1990) and may have potential health benefits due to their phenolic content. The colour of black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) comes from the presence of seven anthocyanins, derivatives of cyanidin, pelargonidin as well as peonidin (Charron et al. 2009, Mizgier et al. 2016). Black carrots contain also phenolic acids and their derivatives (Mizgier et al. 2016). Among beneficial effects of black carrots extracts, amelioration of cadmium toxicity in rats (Claudio et al. 2016), protection against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (Soares et al. 2018) and suppression of lipopolysaccharide-induced inflammation in the co-culture of intestinal Caco-2 and macrophage RAW264.7 cells (Olejnik et al. 2016a) can be mentioned. Black carrot extract subjected to simulated gastrointestinal digestion partly retained its antioxidant activity and reduced oxidative DNA damage in colon mucosa cells (Olejnik et al. 2016b).

In view of these benefits of anthocyanin-containing vegetables, which may be related to their antioxidant properties, it was of interest to compare the antioxidant capacities of commercially available black carrots, and of their anthocyanin-poor more often consumed orange carrots. In our opinion, such an information could be of value for a consumer intending to buy carrots in a local supermarket. Another aim of this study was to compare the

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generation of hydrogen peroxide upon cooking of anthocyanin-rich and anthocyanin-poor carrots. We recently found generation of hydrogen peroxide in cooked vegetables (Bartosz et al. 2022). Anthocyanins, like other polyphenols, generate hydrogen peroxide upon autoxidation (Grzesik et al. 2019). However, anthocyanins react also with hydrogen peroxide, which is a part of their antioxidant action (Bartosz et al. 2020) so the net effect of high anthocyanin content on the hydrogen peroxide generation in potatoes and carrots was hard to predict a priori.

# Materials and methods

## Reagents and equipment

All reagents were purchased from Merck (Poznań, Poland) except for xylenol orange (POCh, Gliwice, Poland) and 2,4,6 tri-2-pirydrylo-s-triazine (Fluka, Buchs, Switzerland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA). Transparent and black flat-bottom 96-well plates (Greiner, Kremsmünster, Austria) were used for the assays. Fluorometric and absorptiometric measurements were done in a Spark multimode microplate reader (Tecan Group Ltd, Mannedorf, Switzerland). In all cases, controls contained all the reactants, except for the carrot extracts.

## Material

Vegetables: orange carrot (*Daucus carota* L. ssp. *sativus*) and black carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) were purchased in a local supermarket in Rzeszów.

### Sample preparation

Deionized water or 50 mM sodium phosphate buffer, pH 7.4, (to stabilize pH) was added to the vegetables in a ratio of 5 ml water/buffer per gram of a vegetable and homogenized. Part of the homogenate was subjected to heating in a water bath at 95 °C for 10 minutes simulating cooking. Samples of the homogenates were then centrifuged (12 100 x g, 5 min) in an Eppendorf MiniSpin centrifuge (Eppendorf Poland, Warsaw).

### Estimation of the anthocyanin content

Anthocyanin content was estimated according to Shehata et al. (2020) with small modifications. Briefly, 500 µl aliquots of the supernatant were pipetted to two Eppendorf tubes and mixed with 500 µl of 2 M HCl or 500 µl of 0.1 M sodium acetate buffer, pH 4.5. After 3-min incubation, the samples were centrifuged (12 000 × *g*, 5 min), and absorbance of the supernatants was measured at 510 nm and 700 nm (to correct for turbidity).

Anthocyanin content C (mg $dm^{-3}$) was calculated as

$$C = (A_{520} - A_{700})_{pH\ 1} - (A_{520} - A_{700})_{pH\ 4.5} \times R$$

where $R = 10^3 \times MW \times (\text{dilution factor})/\varepsilon \times d$, MW – molecular weight of cyanidin-3-glucoside (449.2 g $mol^{-1}$), $\varepsilon$ = 26.900 $M^{-1}$ $cm^{-1}$ (absorption coefficient for cyanidin-3-glucoside), d – length of the optical path.

### Ferric ion reducing antioxidant power (FRAP) assay

The Ferric Ion Reducing Antioxidant Potential (FRAP) was determined by a slightly modified method of Benzie and Strain (1996). Briefly, appropriate volumes of the supernatants or Trolox solution were added to 200 µl a reaction mixture obtained by combining 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM $FeCl_3$ in a 10:1:1 vol ratio. Absorbance was measured at the wavelength of 593 nm after 30-min incubation at room temperature.

### DPPH scavenging assay

Aliquots (200 µl) of 0.3 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol were added to various volumes of a supernatant or Trolox solution and incubated for 30 minutes in the dark at ambient temperature. The decrease in absorbance at 517 nm was measured.

### ABTS• scavenging assay

ABTS• was prepared by oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulfate (Re at al. 1999). The stock ABTS• solution was diluted with phosphate-buffered saline (PBS) so that the absorbance of 200 µl of the solution in a well of a 96-well microplate was 1.0 at 734 nm. Several volumes of the supernatants or Trolox (0.5 mM) as a standard antioxidant were added to 200-µl aliquots of ABTS• solution, incubated for 30 minutes in the dark and decrease in absorbance was measured.

In all assays, using ratio of the dependence of the absorbance decrease or increase on the volume of homogenates and the respective dependence for the standard Trolox solutions, the amount of Trolox equivalents per volume of the supernatants was calculated and related to the original material considering the dilution.

### $H_2O_2$ generation

The homogenates were incubated at room temperature in the dark for 3 h. Thereafter, 180 µl aliquots of the solution were pipetted into wells of a 96-well plate. Then, 2 µl of PBS were added to one well, and 2 µl of catalase (1 mg $cm^{-3}$ PBS) to another. The plate was incubated for 15 min to decompose hydrogen peroxide in catalase-containing samples. Then the hydrogen peroxide was determined in the samples according to Gay and Gebicki (2002). Briefly, 20.2 µl of the Xylenol Orange Reagent (2.5 mM xylenol orange/2.5 mM Mohr salt (ferrous ammonium sulfate) in 1.1 M perchloric acid) were added to the wells, the plate was incubated for 30 min and absorbance at 560 nm was measured. The concentration of hydrogen peroxide was calculated using a standard curve.

## Results and discussion

The anthocyanin content of orange carrots was negligible for the orange carrots and significant for the black carrots. In both cases much higher values were found for boiled than fresh homogenate, apparently due to a better release of cellular contents after boiling. Homogenization of buffer resulted in a more effective release of anthocyanins than homogenization in water (Table 1).

Table 1. Anthocyanin content of orange and black carrots [mg/kg] calculated based on analysis and aqueous and buffer homogenates

| | Aqueous homogenates | | Buffer homogenates | |
|---|---|---|---|---|
| Carrots | Raw | Boiled | Raw | Boiled |
| Orange | 0.38 ± 0.66 | -0.64 ± 1.73 | 0.71 ± 0.62 | 1.32 ± 0.92 |
| Black | 14.03 ± 3.06** | 255.69 ± 10.99***♥ | 71.45 ± 4.43***$^c$ | 318.07 ± 5.16***$^c$♥ |

**$p$ < 0.01, ***$p$ < 0.001 (black vs orange); $^c p$ < 0.001 (buffer vs aqueous homogenates); ♥$p$ < 0.001 (boiled vs raw)

Antioxidant capacity was determined using three independent methods (see Materials and methods). The ABTS• scavenging was determined after 1 min ("fast" scavenging, dependent mainly on rapidly reacting antioxidants such as ascorbate) and after 30 min ("total" scavenging reflecting the reactions of both rapidly and slowly reacting antioxidants). Results obtained for the FRAP and DPPH assays were comparable while the values of total ABTS• scavenging were significantly higher. The antioxidant capacity of black carrots was significantly higher with respect to the orange carrots in all tests. The boiled homogenates showed higher antioxidant capacity than homogenates of raw carrots. Homogenization in buffer produced extracts of higher antioxidant capacity than homogenization in water, again apparently due to a better release of antioxidants from the cells (Fig. 1).

Incubation of homogenates lead to generation of hydrogen peroxide, which was higher in buffer than in water and higher in homogenates of black carrots than in homogenates of orange carrots (Table 2). The pH dependence of autoxidation of flavonoid, resulting in higher generation of hydrogen peroxide in neutral or alkaline than at acidic pH, has been reported (Akagawa et al. 2003, Arakawa et al. 2004). Homogenates of vegetables prepared in water have lower pH than those prepared in buffer, pH 7.4 (Bartosz et al. 2022).

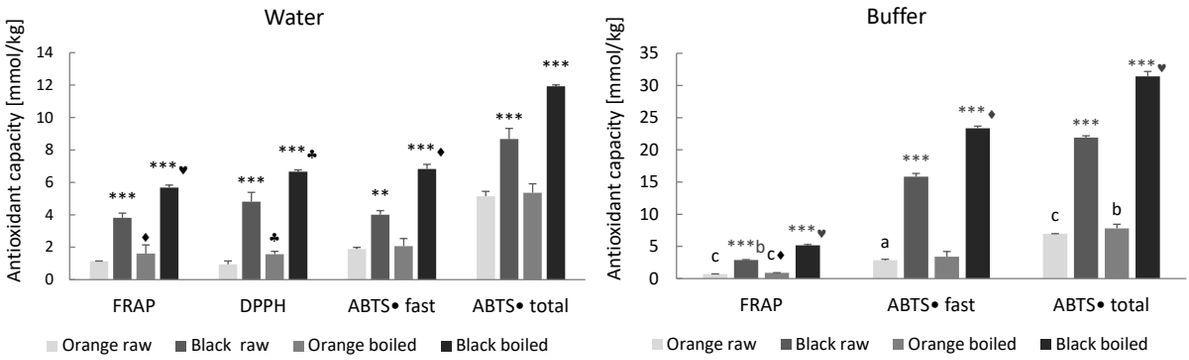


Fig. 1. Comparison of antioxidant capacity of raw and boiled extracts of orange and black carrots obtained in water and in 50 mM phosphate buffer, pH 7.4.**$p$ < 0.01, ***$p$ < 0.001 (black carrots vs orange carrots); [a]$p$ < 0.05, [b]$p$ < 0.01, [c]$p$ <0.001 (buffer vs aqueous homogenates); ♣$p$ < 0.05, ♦$p$ < 0.01, ♥$p$ < 0.001 (boiled vs raw).

Table 2. Generation of hydrogen peroxide in aqueous and buffer homogenates (1:5, w/v) of orange and black carrots

| | Aqueous homogenates | | Buffer homogenates | |
|---|---|---|---|---|
| Carrots | Raw | Boiled | Raw | Boiled |
| Orange | -1.81 ± 4.04 | 0.26 ± 0.09 | -0.08 ± 0.15 | 5.99 ± 2.14[b]♦ |
| Black | 0.82 ± 0.26 | 8.68 ± 1.24***♥ | 4.00 ± 0.93**[b] | 55.01 ± 2.63***[b]♥ |

[b]$p$ < 0.01 (buffer vs aqueous homogenates); **$p$ < 0.01, ***$p$ < 0.001 (black vs orange); ♦$p$ < 0.01, ♥$p$ < 0.001 (boiled vs raw)

Various results have been published on the antioxidant activity of carrots, especially black carrots which are usually difficult to compare due to differences in methodology and sample origin. Frond et al. (2019) reported ABTS• scavenging and FRAP values of < 10 mM Trolox equivalents/kg for the purple carrots. Mizgier et al. (2016) found FRAP, DPPH reduction and ABTS• reduction capacities of 3.09 ± 0.21, 1.95 ± 0.42 and 3.83 ± 0.07 mol Trolox equivalents/kg of purple carrot acetone/acetic acid extracts. The yield of extract with respect to the fresh mass was not reported so these results cannot allow for expression of antioxidant capacity per fresh mass. Blando et al. (2018) compared purple and black carrots by the ABTS• reduction assay finding values of 63.82 ± 8.24 and 1026.43 ± 142 µmol Trolox equivalents/100 g. Pereira-Caro et al. (2021) found values of about 9 and about 13 mmol Trolox equivalents/kg DW for ABTS• reduction and DPPH reduction assays for black carrots (read from Figure 1). Kamiloglu et al. (2015) found about 380 mg and about 720 mg Trolox equivalents/100 g DW for the DPPH reduction and FRAP assays (values read form Figure 1), i. e. ca 12 and 29 mmol Trolox equivalents/kg. Algarra et al. (2014) reported values of 14 ± 4 and 13 ± 5 µmol Trolox equivalents/kg for orange carrots (DPPH reduction assay and FRAP assay, respectively), and 176 ± 90 and 864 ± 80, and 2400 ± 540 and 1820 ± 270 for two varieties of black carrots. Our data point also to the effect of the method of extraction on the anthocyanin content and antioxidant capacity: extraction with buffer always resulted in higher values of these parameters than extraction with water.

Despite differences of values and between different methods, within-a-study comparisons always point to a significantly higher antioxidant activity of black carrots with respect to the orange carrots. This increase antioxidant capacity can be partly ascribed to the increased anthocyanin content; however, increased content of other polyphenols, especially phenolic acids (Algarra et al. 2014, Pandey et al. 2020) may also contribute. Irrespective of the contribution of anthocyanins, the total antioxidant capacity of black carrots is significantly higher compared to orange carrots. Moreover, due to the presence of anthocyanins, black carrots have health benefits, which may be independent of the antioxidant properties of these compounds (Bendokas et al. 2020).

Recently, we found that hydrogen peroxide is generated upon cooking of vegetables (submitted), like in the case of brewing tea (Akagawa et al. 2003, Arakawa et al. 2004) or coffee (Tsuji et al. 1991). The reason for generation of hydrogen peroxide in the beverages and vegetables is the autoxidation of polyphenols present in tea, coffee and vegetables. Black carrots containing increased content of anthocyanins and other polyphenols generated significantly more hydrogen peroxide than orange carrots (Table 2), indicating that the rate autoxidation of polyphenols is higher than the rate of their reaction with hydrogen peroxide leading to the decomposition of $H_2O_2$. As the rate of generation of hydrogen peroxide decreases with decreasing pH, the amount of hydrogen peroxide generated is lower during real cooking than that generated in the buffer of neutral pH (Akagawa et al. 2003, Arakawa et al. 2004, Grzesik et al. 2019, Bartosz et al. 2022) and can be diminished by preparing and cooking carrots at acidic pH.

The generation of low concentrations od hydrogen peroxide in cooked carrots does not appear to raise health problems and may even have beneficial effects. Hydrogen peroxide can exert bactericidal and virucidal action contributing to the mouth hygiene and health (Hernandez et al. 2019, Caruso et al. 2020) and acting on *Helicobacter pylori* in the stomach (Di et al. 2020). This compound promotes gastric motility (Fajardo et al. 2018). High $H_2O_2$ concentrations may damage colon cells but low concentrations were suggested to stimulate cell divisions in the damaged intestine, thus contributing to epithelial repair (Craven et al. 1986). In the digestive tract, $H_2O_2$ can react with available iron, form the hydroxyl radical and other free radicals, and facilitate digestion, since proteins subjected to free radical action may show enhanced susceptibility to proteolytic enzymes (Wolf and Dean 1986).

# Conclusions

Black carrots, having high anthocyanin content, have higher antioxidant capacity than orange carrots and can be recommended for preferential consumption. Black carrots produce more hydrogen peroxide upon cooking but these micromolar concentrations of hydrogen peroxide formed do not seem to have adverse health effects and may have microbicidal activity. Formation of hydrogen peroxide may be diminished by acidic environment of preparation and cooking.

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