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Antioxidant potential of extruded snacks enriched with hyper-protein quinoa flour and vegetable extracts

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Abstract

To account for the global trend towards a healthier diet, in recent years the snack market has grown substantially, with a demand for products that are more beneficial to consumers' health. Extruded snacks from a mixture of cereals, quinoa, and corn, with and without the addition of vegetables were used. Snacks made from a mixture of rice flour and quinoa high protein flour (HHP) supplied by SEGALCO S.A.S., (Popayán, Colombia) were studied. Thus, the use of sources of phenolic compounds such as Beet, Broccoli, Avocado, and Spinach, combined with sources of protein such as quinoa, can increase the nutritional quality of snack products. A combination of cereals and vegetables can produce nutrient-rich products. In this paper, phenolic compounds (given in mg AGE/g sample d.b.) and antioxidant capacity were determined using $ABTS^{\bullet+}$, DPPH and FRAP (in mg AEAC /g sample d.b.). The highest content of phenolic compounds was found in Spinach 4% and HHP snacks (5.7 ± 0.3 and 3.5 ± 0.2 AGE/g sample d.b., respectively). Kale (2%) and Beet (4%) snacks showed a significant increase in antioxidant capacity using the $ABTS^{\bullet+}$ method. The antioxidant capacity determined using the DPPH method increased significantly in snacks made from Beet (4%), Broccoli (4%), Avocado (4%), and Spinach (4%). Using FRAP, the antioxidant capacity showed a significant increase in Kale snacks (2%) and a significant decrease in Spinach snacks (4%). In conclusion, snacks with an elevated antioxidant potential can be produced from vegetables such as kale, which can be an alternative for the food industry to develop healthier products and satisfy market trends.

Keywords: quinoa flour; snack; antioxidant; kale.

Practical Application: The development of healthy products from vegetables and quinoa hyper-protein flour.

1 Introduction

Snacks are one of the most popular foods for consumers across all ages, and especially children. In fact, most of the increase in caloric intake over the past few decades has come from snacks, defined in this paper as foods eaten between meals (Schlinkert et al., 2020). Consequently, a large number of people are overweight and suffer from risks associated with chronic diseases such as cardiovascular disease, type 2 diabetes, strokes, and cancer (Schlinkert et al., 2020; Amrein et al., 2021). Extruded snacks account for a large part of the products consumed worldwide. These snacks are derived mainly from cereals and often are not nutritionally balanced. For example, they are high in calories and low in vitamins, minor minerals, dietary fiber, essential amino acids, and other bioactive compounds (Renoldi et al., 2020). The global trend towards a healthier diet is increasingly permeating different levels of society, culture, and education, and expanding across nationalities (Popkin et al., 2021). The supply of foods that meet these types of consumer demands is changing to meet to these market needs. In this vein, snacks (particularly extruded ones) have become an important part of people's diets, and are an excellent vehicle for incorporating components with functional properties into the body that help improve consumer health (Félix-Medina et al., 2020).

Vegetables such as kale, broccoli, spinach, and beet, have bioactive components such as phenols, and specifically flavonoids. Flavonoids are not only powerful antioxidants but are also highly anti-inflammatory compounds (Osorio et al., 2017; Sánchez-Riaño et al., 2020). As such, these compounds are essential in the prevention of chronic inflammation and oxidative stress in cells, slowing or preventing certain cancers and other cardiovascular or neurodegenerative diseases (Pérez-Cabeza et al., 2018; Duque-Cifuentes et al., 2018). Avocado is a fruit with a high content of monounsaturated fatty acids (MFA), which can help reduce the risk of cardiovascular diseases, as well as having an elevated antioxidant potential (Vivero S et al., 2019). Spirulina also exhibits antioxidant properties, is a good source of protein, and does not have hard cellulose, which improves its digestibility (Guillen-Martín Del Campo et al., 2020). Quinoa, on the other hand, is a pseudo cereal of great interest, due to its attractive nutritional composition (Garcia-Parra et al., 2020). Quinoa contains lipids (2.0% and 9.5%), carbohydrates (52% and 69%), polyphenols, dietary fiber (7 to 9.7%), soluble fiber (1.3 to 6.1%) and especially proteins (13.8 to 16.5%) (Garcia-Parra et al., 2020; Filho et al., 2017) . Quinoa proteins have been reported to be rich in essential amino acids and have antioxidants and antitumor compounds (Valencia et al., 2017).

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The food extrusion process is a high temperature, short duration (HTST) process, which is carried out continuously and homogeneously. Mechanical work, heat, and shear force is applied to food, resulting in changes in its shape, structure, and composition. This process makes it possible to develop foods for practical consumption with a variety of shapes, textures, and flavors (Lazou & Krokida, 2010). The enrichment of extruded snacks with the addition of ingredients that contain bioactive components is a promising strategy to enhance their health benefits (Yuksel et al., 2020). Supplementing extruded snacks with vegetables such as kale, broccoli, spinach, beets, with algae such as spirulina (blue-green algae), and with pseudocereals such as quinoa improve their antioxidant properties, which may have a beneficial effect on human health (Repo-Carrasco et al., 2011; Nazzaro et al., 2014; Bisharat et al., 2014a; Singh et al., 2016; Shevkani et al., 2019; Silva et al., 2021). The objective of this paper was to determine the phenolic content and antioxidant activity of extruded snacks enriched with hyper-protein quinoa flour and vegetables.

2 Materials and methods

2.1 Materials

Extruded snacks were used from a mixture of cereals, and quinoa, with and without the addition of vegetables. The paper also studied snacks made from a mixture of rice flour and hyper-protein quinoa flour. These snacks were supplied by Seguridad Alimentaria de Occidente SEGALCO S.A.S., a company based in the city of Popayán, Colombia. All snacks were prepared following the company's methodology (The proportion of vegetables was selected by the company based on studies of sensory analysis and production costs, which showed that amounts greater than 4% were not favorable). A total of eight different types of snacks were used: six with the addition of dry vegetables (Kale and Spirulina 2%; Beet, Spinach, Broccoli and Avocado 4%), a seventh without vegetables, and an eighth with a mixture of rice and hyper-protein quinoa flours (Roa et al., 2020).

2.2 Obtaining the extracts

To determine the extractable phenolic compounds, an extract was obtained by weighing 4g of a previously macerated sample, followed by adding 6 mL of NaOH 2 M. The sample was left at room temperature (± 24 °C) for 18 hours, and then 1.14 mL of HCL 2 (Garcia-Parra et al., 2020). M was added to neutralize it. The sample was then centrifuged at 10,000 rpm at 6 °C for 15 minutes and the supernatant was recovered using filtration.

To determine the antioxidant capacity, an extract was obtained by weighing 3 g of sample previously macerated for DPPH and 4 g for $ABTS^{\bullet+}$ and FRAP. After this, 6 mL of ethanol (89 °G) was added, a vortex was applied for 15 seconds, and the sample was left in refrigeration (± 4 °C) for 18 hours. The sample was then centrifuged at 5000 rpm at 6 °C for 20 minutes and the supernatant was recovered via filtration.

2.3 Determination of extractable phenolic compounds.

The extractable phenolic compounds were determined using the Folin-Ciocalteau reagent, following the methodology described by Duque-Cifuentes et al. (2018), with some modifications. The phenolic content was quantified using a calibration curve with a 0.1 g / 50 mL gallic acid standard solution. Folin-Ciocalteau (1:10) and $NaHCO_3$ 3.75 g / 50 mL solutions were then prepared. For the reaction, 40 µL of the gallic acid extract or standard solution were mixed with 1800 µL of the Folin-Ciocalteau solution, and after 5 minutes 1200 µL of $NaHCO_3$ solution was added. After 60 minutes in the dark, the absorbance was measured at a wavelength of 765 nm at room temperature. The results were reported as mg gallic acid equivalent per 1 g of sample (mg AGE/g sample d.b.). The determinations were made in triplicate. The equation used for the quantification of the extractable phenolic compounds was as follows, (Equation 1):

$$Y = \left(\frac{X - 0,0105}{1,5424} \right) \times \frac{V}{M} \quad (1)$$

Where, extractable phenolic compounds in mg AGE/g sample (d.b.) (Y), absorbance at 765 nm (X), volume (mL) of solvent used (V), and Mass (g) of sample (d.b.) (M).

2.4 Antioxidant capacity

The Antioxidant capacity was determined using the $ABTS^{\bullet+}$, DPPH, and FRAP spectrophotometric methods. The $ABTS^{\bullet+}$ was determined according to the methodology described by Kuskoski et al. (2005), with some modifications. The quantification of the antioxidant capacity was carried out using a calibration curve with a standard solution of 2 mM of ascorbic acid in ethanol (8.8 mg of ascorbic acid / 25 mL of ethanol). The reagent preparation was carried out by obtained 20 mM of acetate buffer (pH 4.5). The $ABTS^{\bullet+}$ radical cation was obtained by reacting 11.84 mg of ABTS with 1.29 mg potassium persulfate, which was individually dissolved in 2.86 mL of acetate buffer (in the absence of light), for subsequent mixing. Then, 5.1 mL of this mixture was added to a 250 mL flask with the acetate buffer and was allowed to incubate in refrigeration (± 4 °C) in the absence of light for 18 h. For the reaction, 4 mL of the reagent and 135 µL of the extract of each sample or ascorbic acid standard solution were taken, placed in a thermostatic bath at 37 °C for 30 minutes in the absence of light and the absorbance was measured at 730 nm. The results were expressed in terms of mg of L-ascorbic acid equivalents (AEAC) per 1 g of sample (mg AEAC /g sample d.b.). The determinations were made in triplicate. The equation used for the antioxidant capacity by $ABTS^{\bullet+}$ was as follows, (Equation 2):

$$Y = \left(\frac{X - 0,7897}{-0,0046} \right) \times \frac{V}{M \times 1000} \quad (2)$$

Where, extractable compounds in mg AEAC /g sample (d.b.) (Y), absorbance at 730 nm (X), volume (mL) of solvent used (V), and Mass (g) of sample on a dry weight basis (d.b.) (M).

The antioxidant capacity DPPH was measured according to the methodology described by Guldiken et al. (2016), with some modifications. The reduction of the absorbance of a solution of the DPPH 6×10^{-5} M radical in ethanol was verified

at a wavelength of 517 nm. The quantification of the antioxidant capacity was carried out by means of a calibration curve with a standard solution of 2 mM of ascorbic acid in ethanol. 0.1 mL of the ascorbic acid extract or standard solution was mixed with 3.9 mL of the DPPH solution, which was then homogenized and placed in a thermostat bath at 37 °C for 30 minutes in the absence of light. The results were expressed in terms of mg of L-ascorbic acid equivalents (AEAC) per 1 g of sample (mg AEAC /g sample d.b.). The determinations were made in triplicate. The equation used for the antioxidant capacity by DPPH was as follows, (Equation 3):

$$Y = \left(\frac{X - 0,5567}{-0,0031} \right) \times \frac{V}{M \times 1000} \quad (3)$$

Where, extractable compounds in mg AEAC/g sample (d.b.) (*Y*), absorbance at 517 nm (*X*), volume (mL) of solvent used (*V*), and Mass (g) of sample on a dry weight basis (d.b.) (*M*).

The antioxidant capacity FRAP is based on the reduction of the ferric iron (Fe^{+3}) present in the FRAP reagent until it reaches a ferrous form of Fe^{+2} due to the presence of antioxidants (Rioja-Antezana et al., 2018). The reduction of ferric iron (Fe^{+3}) was determined according to the methodology described by Rioja-Antezana et al. (2018), with some modifications. The quantification of the antioxidant capacity was carried out using a calibration curve with a standard solution of 0.5 mM of ascorbic acid in ethanol (2.2 mg of ascorbic acid / 25 mL of ethanol). The reagent preparation was carried out by making a 0.3M acetate buffer (pH 3.6). Then 90 mL of 0.3 M acetate buffer was mixed with 9 mL of 10 mM TPTZ (31.2 g of TPTZ / 10 mL of 40 mM HCl) and 9 mL of 20 mM $FeCl_3.6H_2O$ (54.1 mg of $FeCl_3.6H_2O$ / 10 mL of distilled water). For the reaction, 105 µL of the sample or standard solution of ascorbic acid and 315 µL of distilled water were taken and a vortex was applied for 10 seconds. After this, 3.15 mL of FRAP reagent was added and was brought to a thermostatic bath at 37 °C for 30 minutes and absorbance was measured at 595 nm. The results were expressed in terms of mg of L-ascorbic acid equivalents (AEAC) per 1 g of sample (mg AEAC /g sample d.b.). The determinations were made in triplicate. The equation used for the antioxidant capacity by FRAP was as follows, (Equation 4):

$$Y = \left(\frac{X - 0,3387}{0,0095} \right) \times \frac{V}{M \times 1000} \quad (4)$$

Where, extractable phenolic compounds in mg AGE/g sample (d.b.) (*Y*), absorbance at 595 nm (*X*), volume (mL) of solvent used (*V*), and Mass (g) of sample on a dry weight basis (d.b.) (*M*). All absorbance measurements were registered at room temperature on a UV-Vis spectrophotometer (GENESYS 10S, Thermoscientific, Germany), using ethanol as blank.

2.5 Experimental design and statistical analysis

Completely randomized design with repeated measurements was used. All data was expressed as the mean ± standard deviation (SD). The Shapiro-Wilks test was used to determine the normality of the distribution with a significance level of 0.05. The samples with normal distribution were subjected to an ANOVA analysis of variance, measuring significantly acceptable differences at a 95% confidence level ($p < 0.05$). While that, samples with no-normal distribution were subjected to an non-parametric test. Tukey's multiple comparison test ($\alpha = 0.05$) was applied to determine the differences between means. The results were processed by GraphPad Prism version 6 software.

3 Results and discussion

In addition to its developmental and energy functions, food also can protect structures against the formation of free radicals. This process, which entails the cellular oxidation derived from the appearance of these radicals, is linked both to physiological aging in general and to a series of diseases (cardiovascular, degenerative, Alzheimer's, Parkinson's, as well as different types of cancer). The antioxidants present in food can help to prevent some of these processes and alleviate or slow down some of the diseases associated with them (Fuentes-Berrio et al., 2015).

The results of the extractable phenolic compounds were expressed as mg of Gallic acid equivalent (mg GAE) per g of the extruded snacks (d.b). While, the antioxidant capacity were determined by $ABTS^{\bullet+}$, DPPH, and FRAP methods. The results can be shown in Figure 1, which shows statistically significant differences ($p < 0.05$) between the samples.

3.1. Extractable phenolic compounds.

Phenolic compounds constitute a large and diversified group of secondary metabolites that are mainly found in plants, although they can also be synthesized by other organisms, such as algae. Knowledge of the mechanisms of action of these compounds and their interaction with the human body are relevant by their potential health-promoting effects on human health (Shahidi & Ambigaipalan, 2015; Jimenez-Lopez et al., 2021). Polyphenols are primary antioxidants known as free radical terminators. These compounds can neutralize free radicals by acting as a rapid donor from hydrogen to radical atom, delaying action or preventing the oxidation of lipids, proteins and DNA by reactive oxygen species produced in cells during oxidation (Singh et al., 2017).

The results showed that the maximum content of extractable phenolic compounds was found in Spinach (4%) (5.6883 ± 0.29 mg AGE/g sample d.b.), while Avocado (4%) showed the lowest content (1.8739 ± 0.26 mg AGE/g sample d.b.). The samples were compared with the Control (2.0017 ± 0.1684 mg AGE/g sample d.b.) and a significant increase in phenolic compounds was seen in the snacks with Spinach (4%) and HHP (3.5313 ± 0.23 mg AGE/g shows d.b.) (Figure 1A).

The high value of phenolic compounds in the Spinach (4%) snack may be related to the high content of flavonoids and phenolic acids (mainly ferulic acid and p-coumaric acid) present in spinach (Pérez-Cabeza et al., 2018; Roberts & Moreau, 2016). Furthermore, this value can be attributed to the reactivity of the Folin-Ciolcateu reagent with Fe^{+2} ions (Everette et al., 2010), since spinach is a good source of iron, of which a considerable part is in the Fe^{+2} ferrous form (El-Sherif et al., 1984; Crispin and Varey, 2002; Arango-Ruiz et al., 2012; Rodriguez-Ramiro et al., 2019).

Hyper-protein quinoa flour is one of the raw materials used in the HHP snack (Roa et al., 2020). Quinoa, like spinach, has phenolic compounds such as phenolic acids (ferulic, caffeic, p-coumaric, vanillic, and benzoic acids) and flavonoids (kaempferol, myricetin, and quercetin) (Pellegrini et al., 2017; Song et al., 2022).

These compounds may be responsible for the significant increase in the values observed in these compounds. However, this increase can also be attributed to the reactivity of the Folin-Ciolcateu reagent with other non-phenolic compounds such as amino acids and proteins (Everette et al., 2010), that are found in Quinoa (Galindo-Luján et al., 2021). Shevkani et al. (2019) reported that the total phenolic content increased significantly when adding 4% spinach to extruded corn snacks. Jiapong & Ruttarattanamongkol (2021) reported that snacks containing 40% Sacha inchi seed flour (rich in protein) and 60% rice flour, showed a higher amount of total phenolic compounds than that of rice flour snacks reported by Gat & Ananthanarayan (2015).

The data shows the mean values ± the standard deviation (SD) of three independent extractions from each sample (d.b). The different letters at the top of the bars represent statistically significant differences ($p < 0.05$).

Lisiecka & Wójtowicz (2021) found that the supplementation of rice snacks with fresh kale pulp at 2.5% (w/w), did not significantly affect the content of total phenolic compounds. However, the addition of 5% beet significantly increased the content of these compounds.

3.2. Antioxidant capacity.

Antioxidant properties are an important characteristic to consider when seeking to improve the nutritional quality of foods (Singh et al., 2017). The antioxidant capacity of a food product is determined by interactions between compounds with different mechanisms of action. For this reason, the determination of the antioxidant capacity of complex extracts is usually carried out by different complementary methods, which evaluate various mechanisms of action (Mercado-Mercado et al., 2013).

The antioxidant capacity through the capture of free radicals $ABTS^{\bullet+}$ showed a higher content in Kale (2%) (0.31 ± 0.04 mg AEAC/g sample d.b.) and a lower content in Avocado 4% (0.22 ± 0.01 mg AEAC/g shows d.b.). When comparing the samples with the Control (0.24 ± 0.04 mg AEAC/g sample d.b.), a significant increase was observed in the snacks with 2% Kale and 4% Beet (0.3116 ± 0.0003 mg AEAC/g sample d.b.) (Figure 1B). The 4% Beet snack showed the highest antioxidant capacity to reduce the DPPH radical with a value of 0.17 ± 0.02 mg AEAC /g sample d.b., as opposed to the Control, which showed the lowest antioxidant capacity among the samples with a value 0.12 ± 0.01 mg AEAC /g sample d.b.. The snacks using 4% Beet, 4% Broccoli, 4% Avocado, and 4% Spinach showed a significant increase compared to the Control (Figure 1C). The ferric reducing antioxidant power (FRAP) test showed that the 2% Kale snack had the maximum antioxidant capacity (0.29 ± 0.02 mg AEAC /g sample d.b.) among the samples. Spinach (4%) exhibited the lowest content (0.10 ± 0.01 mg AEAC /g sample d.b.). Likewise, these snacks were the only ones that showed a significant difference when compared with the Control (0.22 ± 0.01 mg AEAC /g sample d.b.) (Figure 1D).

Kale (2%) was the only snack that showed the highest common significant increase in the $ABTS^{\bullet+}$ and FRAP methods. These methods are similar since they are based on the transfer of electrons donated by the different antioxidant compounds

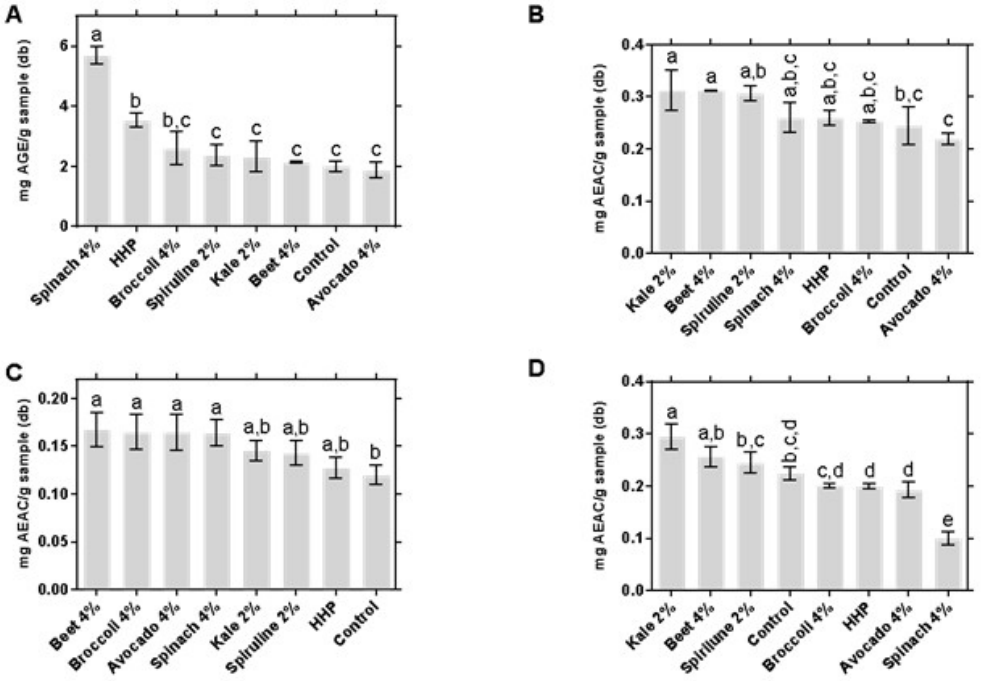

Figure 1. Extractable phenolic compounds (A) and antioxidant capacity by $ABTS^{\bullet+}$ (B), DPPH (C), and FRAP (D) of extruded snacks.

(Chen et al., 2014). FRAP is the only test that measures antioxidants directly compared to other tests, which measure the inhibition of free radicals, and is directly proportional to the concentration of the electron-donating antioxidants (Hsieh & Rajashekaraiah, 2021). Kale has a high content of bioactive compounds such as vitamin C, phenolic compounds, glucosinolates, carotenoids (lutein and zeaxanthin), and antioxidant minerals such as selenium with beneficial health properties. Furthermore, it has received much attention from scientists for being a vegetable with very high antioxidant activity (De Ancos et al., 2016;

Boerzhijin et al. (2020). The above may explain the high antioxidant content found in this snack using the $ABTS^{\bullet+}$ and FRAP methods.

The antioxidant capacity by DPPH showed a significant increase in Avocado (4%), contrasting the results obtained in $ABTS^{\bullet+}$ and FRAP which were lower. The $ABTS^{\bullet+}$ method can measure the activity of hydrophilic and lipophilic compounds. On the other hand, DPPH can only be dissolved in an organic medium, so it preferentially measures the antioxidant capacity of low-polar or non-polar compounds (Mercado-Mercado et al., 2013). This last method is considered less chemically robust as a valid test to measure anti-radical activity (Huang et al., 2005). Avocado is the fruit with the highest lipophilic antioxidant capacity among fruits and vegetables (Wu et al., 2004), may be due to components such as vitamin E and carotenoids (Wang et al., 2010). The significant increase in the antioxidant capacity for the 4% avocado snack by DPPH can be attributed to the above. The DPPH method is usually used only to hydrophilic extracts. Although there was no significant decrease in the values obtained by $ABTS^{\bullet+}$ and FRAP (determine hydrophilic and hydrophobic antioxidant capacities), they were low, including the results of phenolic compounds. This phenomenon is possibly related to lipid oxidation (Shahidi & Ambigaipalan, 2015), since avocado is one of the few fruits whose main components are lipid (Wang et al., 2010; Vivero S et al., 2019), thus affecting the snack.

On the other hand, the significant decrease in the antioxidant capacity of Spinach (4%) by FRAP, as with phenolic compounds, can be attributed to the iron present in spinach. The concentration of this element in the snack extract and its ability to change valence easily ($^{+}2/^{+}3$) could also intervene in the reaction of the FRAP reagent and the antioxidant agent, giving rise to the formation of free radicals (Bartosz, 2013).

Bisharat et al. (2014b) reported that the antioxidant activity by DPPH increased with the addition of broccoli flour in corn snacks. Singh et al. (2016) reported that the inhibition of DPPH and $ABTS^{\bullet+}$ increased with the incorporation of beet powder into corn extrudates. Kolniak-Ostek et al. (2017) reported that there was a significant increase in antioxidant activity measured by the $ABTS^{\bullet+}$, DPPH, and FRAP methods in cornmeal snacks enriched with pumpkin tissue flour. Kasprzak et al. (2018) found a high potential for DPPH radical scavenging in snacks enriched with 8, 6, and 4% kale. They also observed that the unenriched snacks containing 2% kale had a lower capacity to eliminate free radicals.

Shevkani et al. (2019) reported that the addition of 4% spinach leaf powder to corn snacks did not affect their antioxidant activity (inhibition activity of DPPH and $ABTS^{\bullet+}$). Silva et al. (2021) reported that when adding spirulina hydrolysates there was a significant increase in the inhibition capacity of the $ABTS^{\bullet+}$ radical in corn snacks. However, they did not observe a significant difference compared to the control sample when they added 2% spirulina.

4 Conclusions

The phenolic content of extruded snacks enriched with spinach and hyper-protein quinoa flour increased. However, the phenolic content of snacks enriched with kale, spirulina, beets, spinach, broccoli, and avocado was not affected. The supplementation of snacks with kale increased the antioxidant capacity obtained by $ABTS^{\bullet+}$ and FRAP. The addition of beet increased the antioxidant capacity quantified by the $ABTS^{\bullet+}$ and DPPH methods. The antioxidant capacity of snacks enriched with spinach, broccoli, and avocado also increased in DPPH. On the other hand, spinach decreased the antioxidant capacity of snacks obtained using the FRAP method. Extruded snacks with an elevated antioxidant potential and higher quality characteristics such as color and texture can be produced with vegetables like Kale, which increases consumer acceptability. This can be an alternative for the food industry to develop healthier products and satisfy market trends.

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