In-depth Analysis of Carrot (Daucus carota L.) Samples: Carbohydrates, Enzymes, Heavy Metals, Hydrogen Peroxide Levels, and Lipid Peroxidation under Diverse Storage and Cooking Conditions

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Abstract

This study meticulously explored the phytochemical and nutritional variations in carrots (*Daucus carota L.*) under various storage and cooking methods. The investigation focused on carbohydrates, enzymes, hydrogen peroxide levels, lipid peroxidation, and specific heavy metal concentrations. The findings, which were unveiled with great significance, revealed that cooking had a profound impact on the carbohydrate content, particularly in raw carrots stored under refrigeration or freezing. Moreover, cooking significantly altered enzyme activities, notably reducing peroxidase, polyphenol oxidase, glucose oxidase, and amylase. Proline levels decreased with cooking, while hydrogen peroxide and malondialdehyde levels increased. In contrast, freezing increased proline content. Cooked carrots experienced the highest mineral loss, followed by steamed samples. Notably, heavy metals in carrot tissues were not effectively eliminated through cooking. This study's conclusions underscore the urgent need for pollution management in carrot cultivation, harvesting, and storage due to the vegetable's tendency to accumulate heavy metals. These insights are crucial for making informed dietary choices and ensuring food safety.

1. Introduction

The carrot (*Daucus carota* L.), has long been recognized as a highly nutritious and adaptable vegetable because of its abundance of vitamins, minerals, and other components. Nutritional research is increasingly focusing on carrots due to their global dietary prevalence. However, within food processing, which aims to both preserve and enhance nutritional qualities, a dual nature exists. While food processing can improve nutritional qualities, it can also lead to nutrient loss and the formation of harmful compounds that impact texture, color, or taste. Various cooking methods have unique effects on inherent phytochemical qualities and can change them for the better or worse (Zhao et al., 2019). Thermal cooking methods, like steaming and boiling, induce significant biological, physical, and chemical transformations in vegetables such as carrots. Heat catalyzes these changes, altering texture, eliminating microorganisms, increasing food digestibility, and enhancing nutrient absorption. Extensive research has explored how heat affects the phytochemical composition of vegetables, taking into account the molecular properties and the specific cooking techniques used. For instance, carrots, known for their richness in beta-carotene, the precursor of vitamin A, benefit from heat as it breaksdown cell walls, facilitating the body's access to this valuable nutrient (Palermo, Pellegrini, & Fogliano, 2014; Sharma, Karki, Thakur, & Attri, 2012).

Plant foods, highly perishable by nature, are frequently consumed in processed states. Freezing, a technological preservation method, maintains food quality and nutrients, including vitamins and antioxidants, over prolonged periods. It halts post-harvest metabolic activities, lowers enzymatic reactions, and inhibits microbial growth, thereby preserving taste, texture, and nutrients. Nonetheless, freezing may cause cell damage, releasing and degrading antioxidant compounds, leading to reduced antioxidant activity compared to fresh produce. (Neri, Faieta, Di Mattia, Sacchetti, Mastrocola, & Pittia, 2020; Purkiewicz et al., 2020).

Understanding the alterations in the nutrient content of plant-based foods during post-harvest handling and storage is essential both from the perspective of post-harvest physiology and the food industry, as well as for consumers seeking to comprehend the quality changes in nutrients after refrigerated storage. It is essential to recognize that the phytochemical content of food items can change under the influence of various post-harvest storage methods (Zhan, Zhu, & Sun, 2019).

The study extensively investigates changes in phytochemical composition and nutritional content of carrots under different treatments. It examines parameters like carbohydrates, enzymes, hydrogen peroxide, lipid peroxidation, and metal presence. Results indicate cooking and storage significantly affect carrot nutrition, particularly reducing carbohydrate content. Proline, hydrogen peroxide, and malondialdehyde are influenced by cooking, while freezing uniquely impacts proline. Enzyme activities decrease with cooking, signaling biochemical changes. Cooked carrots show pronounced reduction in mineral elements, raising concerns about heavy metal accumulation. The study underscores the need for pollution management in carrot production and storage, offering valuable insights for dietary choices and food safety practices.

2. Materials and methods

This study employed chemicals and solvents purchased from Merck and Sigma-Aldrich. Instruments utilized included a pH meter (Az 86 502, Taiwan), Shimifan model CE 148 centrifuge, Hettich centrifuge ROTOFIX 32A (Kirchlengern, Germany), vortex mixer (Labtron Ls-100, Zanjan, Iran), UV–Vis spectrophotometer (Model 6705, Jenway, UK), Optima 8300 ICP-AES (PerkinElmer, USA), GC-MS 6890N (Agilent, USA) equipped with S973N mass spectrometer, Colorimetric microtome (Reichert-Jung, Heidelberg, Germany), Eliza (DANA model, USA), COHESIVE HPLC model (Japan), Optical and fluorescent microscope (NOVEL-N800-F, Germany) equipped with a TrueChrome metric camera.

2.1. Preparation of carrot samples

Carrot samples were procured from a local market. The carrots were diced and subjected to various food processing conditions inclusive of boiling (15 min), steaming (20 min), and refrigerated storage (4°C, 20 days). Another batch of carrots (raw, steamed, and cooked samples) were frozen (-25°C, 20 days). Thereafter ethanolic extracts from each sample was prepared using the maceration method and were subsequently employed to investigate the presence of phytochemical compounds.

2.2. Preparation of carrot ethanolic extracts from samples stored under various conditions (Maceration Method)

Samples were macerated utilizing a mortar and pestle. Carrot samples (15 g) were extracted with ethanol (70%, 30 mL) for 24 hours. The resulting mixture were centrifuged (5000 rpm, 10 min). The resulting supernatant was decanted and utilized to conduct the various phytochemical tests.

2.3. Determination of electrical conductivity in carrot samples stored under different conditions

The Hampton method was employed to assess substance release from cells (Lin et al., 2016). Electrolyte leakage was measured by introducing distilled water (50 mL) to the samples (0.5 g) which were left at room temperature for 24 hours. This was performed in triplicate.

2.4. Measurement of glucose, fructose, and sucrose content in carrot samples stored under different conditions

Glucose, fructose, and sucrose levels were quantified utilizing the Fuleki method (Fuleki, Pelayo, & Palabay, 1994). Samples (2 g) were blended with distilled water (25 mL) and centrifuged. The resulting supernatant was filtered through a Millipore membrane (45 µm) and then introduced into an HPLC instrument equipped with a separating column of model SCR-101N, featuring dimensions of 300×9.7 mm. The mobile phase (water) employed was isocratic, flowing at a rate of 0.17 mL/min. Column temperature was carefully maintained at 60°C using an oven (CTO-6A). The column specifications encompassed the use of a separating column [Dikma Plastisil NH_2 model] with dimensions of 6.4×250 mm and a surface area of 1440 m^2/g.

2.5. Measurement of α-amylase enzyme activity in carrots stored under different conditions

Alpha-amylase enzyme activity was determined using the Keharom method which is based on 3,5-dinitrosalicylic acid (DNS) (Keharom, Mahachai, & Chanthai, 2016). Carrot samples (1 g) were thoroughly ground in liquid nitrogen. To the powdered samples was added phosphate buffer (10 mL, pH 7.0) and the samples centrifuged (10 min). The resulting supernatant (100 µL) was mixed with 3,5-dinitrosalicylic acid (4.0 g), sodium potassium tartrate (82.1 g), phenol (2.0 g), sodium sulfate (5.0 g), and sodium hydroxide (1.0 g). The mixture was placed in a warm water bath (10 min) and then cooled to room temperature. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. Enzyme activity was expressed as units of enzyme activity per mg of protein.

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2.6. Measurement of polyphenol oxidase enzyme (PPO) activity in carrots stored under different conditions

Carrot extract (1 mL) was mixed with 0.05 M phosphate buffer (2 mL, pH 7.0) and catechol (0.5 M, 0.5 mL) at 24°C (2 min). The absorbance was measured using a spectrophotometer at a wavelength of 398 nm. PPO enzyme activity was expressed in units of enzyme activity per mg of protein (Wu, Wan, Shah, Fahad, & Huang, 2014).

2.7. Measurement of peroxidase enzyme (POD) activity in carrots stored under different conditions

POD enzyme activity was measured by mixing hydrogen peroxide (3%, 1 mL), guaiacol (2.0%, 950 µL), sodium phosphate buffer (pH 7.0, 50 mM, 1 mL,), and enzyme extract (50 µL) [10]. The absorbance of the mixture was recorded after 60 seconds at a wavelength of 470 nm (Keharom et al., 2016).

2.8. Measurement of glucose oxidase enzyme (GOX) activity in carrots stored under different conditions

The assay kit (code MAK097A) provides a simple and direct method for measuring glucose oxidase (GOX) activity in biological samples [10]. GOX activity was determined utilizing a coupled enzyme assay in which GOX oxidizes D-glucose, producing hydrogen peroxide that reacts with a probe, generating a color change measured at a wavelength of 570 nm (Wu et al., 2014).

2.9. Measurement of proline in carrot samples stored under different conditions

Thakur and colleagues utilized a modified method to determine proline content (Thakur & Sharma, 2005). In this method, free amino acid proline reacts under acidic conditions with ninhydrin reagent, at a temperature of 144°C, to produce the colored compound, di-ketohydrin di-leucine, which forms two distinct colored phases in the presence of toluene. Solution A was prepared with sulfosalicylic acid (3 g), dissolved and diluted with distilled water (100 mL). To this solution, orthophosphoric acid (6 M, 20 mL) was added. Solution B was prepared by dissolving ninhydrin (25.1 g) in glacial acetic acid (30 mL) and heating in a warm water bath (30 min).

To determine proline content, carrot samples (5.1 g) were mixed with solution A (6 mL). After centrifugation, ninhydrin (5.1 mL) was added. The mixture was vortexed (15 min) afterwhich it was placed in a hot water bath (100°C, 60 min) and immediately placed on ice (10 min). In the final step toluene (5.1 mL) was added and the mixture vortexed (10 s) to form two phases. The proline concentration was measured utilizing the upper phase (pink) which contained toluene and proline. The absorbance of this solution was read at a wavelength of 520 nm. The toluene solution was used as a control. Proline levels were determined utilizing a standard curve.

2.10. Measurement of hydrogen peroxide (H_2O_2) levels in carrot samples stored under different conditions

The Khan method was employed to determine hydrogen peroxide concentration (Khan, Yusuf, & Fariduddin, 2018). The required reagents included trichloroacetic acid (0.1 g) dissolved in distilled water (100 mL). Potassium iodide (16.6 g) was dissolved in distilled water (100 mL). Potassium phosphate buffer (10 mM, pH 7.0) was also prepared. To carrot extracts (1 mL) trichloroacetic acid solution (5 mL),

potassium iodide solution (2 mL) and potassium phosphate solution (1 mL) were added. The absorption of hydrogen peroxide was measured using a spectrophotometer at a wavelength of 390 nm.

2.11. Measurement of lipid peroxidation levels (MDA) in carrot samples stored under different conditions

Malondialdehyde (MDA) was measured using the modified Stewart method (Stewart & Bewley, 1980). The following solutions were prepared. Solution A consisted of thiobarbituric acid solution (5.0%) containing trichloroacetic acid (20%). To prepare this solution, thiobarbituric acid (1.0 g) and trichloroacetic acid (20 g) were dissolved in distilled water to a final volume of 200 mL. Phosphate buffer (1.0 M, 10 mL) was added to the carrot samples (2 g). After vortexing, the mixture was centrifuged (4000 rpm, 15 min). Solution A (1 mL) was added to the supernatant (1 mL) and the resulting solution incubated in a water bath (95°C, 30 min) followed by cooling on ice (10 min). The appearance of a brown color indicated the presence of malondialdehyde. The absorbance of the solution was measured at two wavelengths (532 nm and 600 nm) using a spectrophotometer. The quantity of malondialdehyde was reported as mmol/g based on the weight.

MDA [mol.g^{-1} FW] =] A532 - A600 [× DF /[D × Q]

Where:

DF = 10

D = 1

Q = 155

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2.12. Measurement of heavy metal levels in carrot samples stored under different conditions

Measurement of the levels of heavy metal in carrot samples stored under different conditions was conducted using the method described by Daniel and colleagues (Dániel, Kovács, Prokisch, & Győri, 1997). Nitric acid (65%, 10 mL) was added to dried carrot samples (5.0 g), and the mixture kept under a hood for 24 hours. The solution was heated until acidic fumes were expelled. After cooling, hydrogen peroxide (30%, 1 mL) was added to the solution until completely transparent. The mixture was further heated until all the acidic fumes were brown. Distilled water was added and extracts were brought to a final volume of 25 mL with distilled water. The absorption of the acidic extracts was measured using an ICP device to determine the concentration of heavy metals which was reported as mg/100 g.

2.13. Statistical analysis of the data

Statistical analysis of the data was conducted using SPSS software (version 20). Mean comparisons of the data were examined using the Duncan method. Mean comparison charts were plotted using Microsoft Excel.

3. Results and discussion

3.1. Investigating the electrical conductivity of carrot samples stored under different conditions

A significant difference was observed in the electrical conductivity of carrot samples stored under different conditions. Steamed carrots exhibited the highest electrical conductivity. Lowest electrical conductivity was observed in raw carrot samples which were frozen for 20 days. The electrical conductivity of carrot samples which were cooked, cooked and frozen (20 days), steamed and frozen (days) showed no significant difference. There was a significant difference in the electrical conductivity of fresh and raw carrot samples which were refrigerated for 20 days ($p < 0.05$) (Fig. 1).

Electrical conductivity measures the food matrix's ability to conduct and pass electrical current and is essential for food quality evaluation. Several factors, including temperature increase, sensitivity to cold, freezing tolerance, and the nature of the product itself, affects the electrical conductivity of food, fruit, and vegetables. This phenomenon occurs due to structural changes in the tissue, such as cell wall protopectin decomposition, non-conductive gas bubble discharge, and reduction of cell membrane integrity. Electrical conductivity increases due to increased ion mobility and temperature increase (Banti, 2020). The increase in electrical conductivity observed in steamed and boiled carrot samples is attributed to heat effects, causing loss of membrane integrity and cell wall breakdown. This aligns with findings from Wang et al.'s study on lettuce stems (Wang, Zhang, Mujumdar, Mothibe, & Azam, 2012). Cooling and freezing reduce ion mobility, leading to decreased conductivity, while freezing below 0°C forms ice crystals, further reducing ion mobility. In the study, raw frozen samples exhibited a sharp decrease in conductivity due to freezing effects.

3.2. Investigation of the levels of glucose, fructose, and sucrose in carrot samples stored under different conditions

A study of the levels of glucose, fructose, and sucrose in carrot samples stored under different conditions showed a significant difference. The highest levels of glucose was observed in steamed carrots, and the lowest in cooked carrots that were in frozen storage for 20 days. The glucose content of cooked and raw carrots in refrigerated storage for 20 days showed no significant difference. The glucose content of raw carrots in frozen storage for 20 days and fresh samples did not reveal any significant difference ($p < 0.05$) (Fig. 2a).

Examination of the levels of fructose in carrot samples stored under different conditions showed a significant difference. The highest levels of fructose was observed in steamed carrots, and the lowest in

cooked carrots which were subjected to frozen storage for 20 days. Raw carrots which were stored in the freezer and fresh carrots showed no significant difference. Examination of the levels of fructose in raw carrots kept in refrigerated storage for 20 days, cooked and steamed carrots frozen for 20 days showed a significant difference ($p < 0.05$) (Fig. 2b).

The highest quantity of sucrose was observed in steamed carrots which were frozen for 20 days, and the lowest quantity in cooked carrots. The levels of sucrose in the raw samples that were refrigerated for 20 days and the cooked samples which were frozen for 20 days showed no significant difference. The sucrose content of fresh, steamed, and raw carrot samples stored in the freezer for 20 days showed a significant difference ($p < 0.05$) (Fig. 2c).

Carbohydrates are the primary source of energy in diets. Information regarding changes in carbohydrate composition during the storage and preparation of vegetables is therefore essential. Cultivation, weather conditions, carrot variety, and harvest time affect the carbohydrate content of different carrot cultivars (Obdiebube et al., 2023). Starch, the primary source of calories in many diets, is swollen and degraded by the cooking process. Heat results in the gelatinization of starch grains, partial hydrolysis of hemicelluloses, and dissolution of protopectins after polymerization, which impacts the overall carbohydrate content.

When water is utilized in the cooking process, sugars are dissolved and transferred to the cooking liquid, which decreases the overall carbohydrate content. Among the various cooking methods, steaming exhibits superior retention of soluble sugars compared to alternative techniques, as it involves minimal direct exposure to water, thereby enhancing shelf life. Suni *et al.*'s study on the effect of temperature on the content of sucrose, glucose, and fructose in apples revealed that sucrose hydrolyzes faster than glucose and fructose at higher temperatures (Suni, Nyman, Eriksson, Björk, & Björck, 2000).

The study attributes the decrease in total carbohydrate content to starch hydrolysis, with cooking leading to the dissolution of glucose, fructose, and sucrose. Steamed carrots showed a more intense increase in glucose and fructose compared to sucrose, possibly due to less water contact during steaming and increased starch hydrolysis. Additionally, refrigerated storage led to decreased carbohydrate content and increased sucrose levels, attributed to heightened amylase activity at lower temperatures. Research on the alfalfa plant examining the response of different metabolic pathways to freezing showed that water deficit significantly increased the plant's tolerance in freezing conditions (Xu, Li, Tong, He, & Li, 2020). This phenomenon increases the soluble sugars, amino acids, and fat content, inclusive of fat related molecules. In conditions where the plant lacks water there is an observed increase of soluble sugars, amino acids, lipids, and lipid molecules. Low temperature causes significant damage to plasma membranes. The lipid phase of the membrane which is very fluid, is converted to a hard gel, which can increase membrane permeability and eventually destroy it. This causes the release of compounds into the plant's cell membrane.

Under cold stress, plants accumulate sucrose, which shields cell membranes from damage and protein inactivation by regulating sucrose biosynthesis and minimizing enzyme degradation. Fructose,

possessing superior antioxidant properties, serves as a defense against freeze stress. The increase in sucrose relative to glucose and fructose during freezer stress is justified by these mechanisms. These dynamics elucidate alterations in carbohydrate levels in steamed and cooked frozen carrots.

3.3. Investigation of α-amylase enzyme activity in carrot samples stored under different conditions

There was a significant difference in the level of amylase activity in carrot samples stored under different conditions. The highest level of amylase enzyme activity was observed in raw carrots which were frozen for 20 days. The lowest level of amylase activity was observed in cooked carrots. Investigation of the amylase activity of steamed carrots which were frozen for 20 days and cooked carrot samples frozen for 20 days did not show any significant difference. Examination of the amylase activity of fresh, steamed, and raw carrots which were refrigerated for 20 days showed a significant difference ($p < 0.05$) (Fig. 3A).

Wei and colleagues' study found that cooking potatoes led to the breakdown of starch by amylase, converting it into soluble sugars. Higher cooking temperatures and longer durations negatively affected amylase activity, with 100°C cooking resulting in lower activity compared to 40°C and 80°C cooking. The high temperatures caused irreversible denaturation and structural changes in the enzyme. (Wei, Lu, & Cao, 2017).

In this study, the decrease in amylase activity in steamed and cooked samples was linked to enzyme denaturation during thermal processing. Conversely, the observed increase in amylase activity in refrigerated and frozen raw carrot samples is justified. The decrease in amylase activity in steamed and cooked frozen samples may be attributed to both the thermal process before freezing and the impact of cold stress on the expression of the amylase enzyme-inducing gene.

3.4. Investigation of peroxidase (POD) activity in carrot samples stored under different conditions

The level of peroxidase activity in carrot samples stored under different conditions showed a significant difference. The highest level of peroxidase activity was observed in fresh carrots, and the lowest level of peroxidase enzyme activity was observed in cooked carrots, steamed carrots that were frozen for 20 days, and cooked carrots that were frozen for 20 days. There was no significant difference between the peroxidase activity of refrigerated raw carrots (20 days) and frozen raw carrots (20 days). The level of peroxidase activity in cooked carrots, steamed samples frozen for 20 days and cooked carrots frozen for 20 days was not significantly different ($p < 0.05$) (Fig. 3B).

Enzymatic browning reactions can be problematic when preserving nutrients in fruits and vegetables. Browning reactions are caused by the activity of peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase (PAL) enzymes in the presence of oxygen. Conventional techniques such as blanching, freezing, and thermal processes minimize enzyme activity and counteract browning reactions (Sui, Meng, Dong, Fan, & Wang, 2023). Heat causes adverse changes in physicochemical properties, such as

color, texture, and nutrient content, as well as destruction of membrane integrity and partial degradation of cell wall polymers in vegetables.

Low temperatures induce the production of reactive oxygen species, prompting rapid reactions between proteins and lipids. Plants employ fundamental mechanisms to eliminate these reactive oxygen species during cold stress, involving enzymatic and non-enzymatic pathways. Research indicates that enzymes like peroxidase, superoxide dismutase, and catalase, along with antioxidants such as ascorbic acid, glutathione, phenolic compounds, carotenoids, and tocopherols, are crucial for neutralizing the toxic effects of reactive oxygen species. The peroxidase enzyme acts as a consumer of hydrogen peroxide to counteract stresses such as freezing (de Oliveira, Santos, & Buffon, 2021).

Fresh vegetables are ideally stored for a short duration at temperatures below 0°C to mitigate enzyme activity, particularly that of peroxidase. Thermal treatments like blanching before freezing are effective in minimizing browning reactions. The observed alterations in peroxidase levels in steamed and cooked frozen samples can be attributed to enzyme denaturation during heat processing and an increase in protein synthesis to counteract the adverse effects of freezing.

3.5. Investigation of polyphenol oxidase (PPO) activity in carrot samples stored under different conditions

The levels of polyphenol oxidase enzyme activity in carrot samples stored under different conditions were significantly difference. The highest levels of polyphenol oxidase activity was observed in fresh carrots and cooked samples that were frozen for 20 days. The lowest levels of polyphenol oxidase activity was observed in raw carrots that were frozen for 20 days. The level of polyphenol oxidase activity in cooked carrots and steamed samples which were frozen for 20 days showed no significant difference. Investigation of polyphenol oxidase activity in steamed carrots and raw samples that were refrigerated for 20 days showed a significant difference ($p < 0.05$) (Fig. 3C).

Polyphenol oxidase enzymes, crucial in plant physiology and food science, catalyze undesirable browning reactions in fruits and vegetables by oxidizing phenolic compounds to quinone, resulting in melanin pigment formation. These reactions degrade product quality and alter nutritional and sensory properties. Tissue damage triggers polyphenol oxidase activity, causing intracellular separation and oxygen penetration. Reducing enzyme activity is essential to minimize losses from browning reactions in plant foods. (Sui et al., 2023). One of the techniques used to lower the activity of polyphenol oxidase is the use of thermal processing. The polyphenol oxidase enzyme found in fruits and vegetables is deactivated during thermal processing by heating up to 90°C (Iqbal, Murtaza, Hu, Ahmad, Ahmed, & Xu, 2019).

Vela *et al.*'s study showed that cold stress resulted in the disintegration of mango cell structure and thylakoid's separation from the the cell's chloroplast. This causes more release of the polyphenol oxidase enzyme and increases the level of contact with phenolic substrates in the plant cell (Vela, León,

García, & La Cruz, 2003). In the current study, the increase in polyphenol oxidase activity in carrots exposed to refrigerated temperature storage can be attributed to this phenomena.

Freezing reduces the activity of polyphenol oxidase by slowing its molecular movement and disrupting enzyme-substrate interaction (Al-Jeddawi, Northcutt, & Dawson, 2023). A study conducted on the effect of frozen storage on peaches revealed that the lower the frozen storage temperature, the less cell damage due to the formation of smaller ice crystals. Higher freezing temperatures can damage cells and tissues leading to more enzymatic oxidation due to the release of phenolic compounds, oxygen, and enzymes from the tissue. In the current study, the decrease in polyphenol oxidase activity in frozen carrot samples can be attributed to the damage caused by ice crystal formation and enzymatic oxidation. Due to more contact with water, enzyme degradation occurred more when the carrots were blanched as compared to when they were steamed. In the current study, the reason for the decrease in polyphenol oxidase enzyme activity in steamed and cooked frozen samples is due to the effect of the thermal processing and freezing.

3.6. Investigation of glucose oxidase (GOX) activity in carrot samples stored under different conditions

There was a significant difference in the level of glucose oxidase activity in carrot samples stored under different conditions. Peak activity of glucose oxidase was observed in fresh carrots and raw samples that were frozen for 20 days. Lowest enzyme activity was observed in cooked samples. There was a significant difference in the level of glucose oxidase activity in cooked, steamed carrots and raw carrot samples which were refrigerated for 20 days. Glucose oxidase activity in frozen steamed carrots (20 days) and frozen cooked samples (20 days) did not show any significant difference ($p < 0.05$) (Fig. 4).

Enzyme activity and stability are influenced by pH, temperature, substrate concentration, the presence of metal ions, and enzyme concentration. Glucose oxidase is a subset of oxidoreductase enzymes that as a natural agent, effectively prevents oxidation and spoilage of fruits and vegetables and inhibits browning reactions. Glucose oxidase and glucose dehydrogenase (GDHs) are defined as key members of this group. Glucose oxidase is a specific oxidoreductase that can utilize oxygen as an electron acceptor (Khatami et al., 2022). The enzyme requires optimal temperature and pH conditions for proper activity. High temperature and disproportionate pH during food processing, such as thermal processing, causes disruption of enzyme function (İlter, Altay, Köprüalan, Ertekin, & Jafari, 2023). Reduced glucose oxidase activity in cooked and steamed carrots and the high enzyme activity in fresh and raw carrots samples confirm the above observations.

Plants that are exposed to cold stress act through specific processes. These include the expression of genes responding to cold stress which ultimately increases the stability of plant cells under cold stress. One of the challenges faced by plants exposed to low temperatures is the excessive production of reactive oxygen species such as superoxide radicals (O^{2-}), hydrogen peroxide, and hydroxyl radicals (OH), which damages cell membrane phospholipids leading to cell death. Enzymatic and non-enzymatic antioxidant systems protect the plant cell against the destructive effects of free radicals during cold

stress. Glucose oxidase catalyzes the production of hydrogen peroxide by oxidizing glucose in the presence of molecular oxygen (Manasa S, Panigrahy, Panigrahi, & Rout, 2022).

In the present study, the observed decline in glucose oxidase enzyme activity in steamed and cooked frozen samples can also be rationalized by the reasons mentioned about heat processing and freezing.

3.7. Investigation of proline content in carrot samples stored under different conditions

The study found significant variations in proline levels among carrot samples stored under different conditions. Raw carrots frozen for 20 days exhibited the highest proline levels, while cooked carrots and those frozen for 20 days had the lowest. Additionally, there was a significant difference in proline levels between fresh carrots and raw carrots refrigerated for 20 days. However, no significant difference was observed between steamed carrots and those frozen for 20 days.Cooked carrots and cooked carrots that were frozen (20 days) also did not show any significant difference ($p < 0.05$) (Fig. 5).

Many studies show a positive correlation between the accumulation of proline in plant tissue as a response to environmental stress. Determination of the level of free proline is a helpful measure to check the physiological status of the plant and to evaluate the plant's tolerance to environmental stressors. Proline stabilizes protein structure, prevents accumulation, and protects cell structures and membranes by removing reactive oxygen species and reducing oxidative stress damage caused by drought, salinity, low temperature, heavy metals, and UV radiation. Additionally, it increases plant tolerance against environmental stress by maintaining osmotic balance (Zuo et al., 2021). Very few studies have been done on the effect of thermal stress on the proline content of plants. Zuo *et al.* found that proline content increased in zucchini that were stored at 4°C. The enzymes pyrroline-5-carboxylate synthetase (P5CS), proline dehydrogenase (PDH) and ornithine-delta aminotransferase (OAT) are responsible for proline synthesis in plant cells which are exposed to cold stress conditions (Zuo et al., 2021). In the present study, the increase in proline content of carrot samples that were refrigerated can be attributed to enzyme activity responsible for proline synthesis. Freezing potentially causes stress in plant cells resulting in a change plant metabolite composition including proline. In the present study, increased proline synthesis in frozen raw samples can be attributed to the protective role of proline against the destructive effects of freezing. The decline in the proline content of steamed and cooked frozen samples can be attributed to heating before freezing which reduces proline amino acids.

3.8. Investigation of the levels of hydrogen peroxide (H_2O_2) in carrot samples stored under different conditions

Investigation of the levels of hydrogen peroxide in carrot samples stored under different conditions showed a significant difference. The highest levels of hydrogen peroxide was observed in raw carrot samples which were refrigerated for 20 days. The lowest levels of hydrogen peroxide was observed in the fresh carrot samples. There was no significant difference in hydrogen peroxide content of steamed carrots and steamed carrots that were frozen for 20 days. No significant difference was observed in

cooked carrot samples and cooked carrot samples which were frozen for 20 days. The levels of hydrogen peroxide in the fresh and raw carrots that were frozen for 20 days showed a significant difference ($p < 0.05$) (Fig. 6).

Oxidation is a natural process in living organisms, including plants, occurring during normal cellular reactions like respiration and metabolism. Under certain conditions, oxygen can generate single-electron species and free radicals, especially under stressors like heat. Free radicals, such as superoxide, hydroxyl, and oxygen derivatives like hydrogen peroxide, are highly unstable and reactive compounds. Hydrogen peroxide, a toxic substance produced in various cellular mechanisms, must be swiftly eliminated as its accumulation can harm cells and tissues. Catalase (CAT) is a key defensive enzyme that acts on hydrogen peroxide, rapidly breaking it down to mitigate its destructive effects. Fresh vegetables typically lack hydrogen peroxide due to the activity of catalase and peroxidase enzymes, which remove it from living cells. However, cooking and heat can deactivate these enzymes, exposing redox-active compounds to oxygen in the environment. As a result, hydrogen peroxide accumulates as a by-product of the self-oxidation of polyphenols in cooked vegetables and causes destructive effects on the structure and components of cells (Bartosz, Rajzer, Grzesik-Pietrasiewicz, & Sadowska-Bartosz, 2022). In the current study, the observed increase in hydrogen peroxide in steamed and cooked carrot samples can be attributed to the impact of the heating process on enzyme activity and the rise in production of free radicals and oxidative stress.

Low temperatures can damage plant tissue and cell membranes, disrupting their activity and metabolism. Cold stress increases the production of active oxygen molecules, leading to oxidation and damage to cellular macromolecules. This imposes secondary stress, known as plant oxidative stress, on the plant. Hydrogen peroxide, a free radical, affects membrane permeability by peroxidizing unsaturated fatty acids in membrane lipids, leading to damage to cell membranes.The cell produces antioxidants and activates antioxidant enzymes such as dysmotase, catalase, ascorbate, and peroxidase, which increases the plant's resistance to cold and freezing stress (Kamran, Toorchi, Moghadam, & Mohammadi, 2015). In the present study, the increase in hydrogen peroxide in raw specimens stored at refrigerated temperatures can be attributed to the increased production of active oxygen species due to cold stress and freezing, as was also observed in steamed and cooked samples that were frozen. Thermal processing and low temperatures led to more production of hydrogen peroxide.

3.9. Investigating the levels of lipid peroxidation in carrot samples stored under different conditions

Investigation of the levels of lipid peroxidation in carrot samples stored under different conditions showed a significant difference. The highest level of lipid peroxidation was observed in carrot samples that were cooked and frozen (20 days), whereas the lowest levels of lipid peroxidation was observed in the fresh carrot samples. There was no significant difference in lipid peroxidation in cooked and steamed carrots that were frozen for 20 days. A significant difference was observed in the level of lipid

peroxidation in raw carrots which were refrigerated for 20 days, steamed carrots and raw carrots that were frozen for 20 days ($p < 0.05$) (Fig. 7).

During the processing and storage of fruits and vegetables, the production of reactive oxygen species leads to a reduction in antioxidants due to numerous oxidation reactions. Mitochondria, chloroplasts, and peroxisomes are the primary organelles in plant cells responsible for producing reactive oxygen species. Free radicals initiate lipid peroxidation, a consequence of oxidative stress, which releases additional free radicals from cell membrane lipids' electrons. Consequently, fats become free radicals themselves. Various factors such as aging, pathogen attacks, high temperature, low temperature, and freezing intensify lipid peroxidation, resulting in the destruction of membrane lipids. This leads to a loss of membrane integrity and changes in the physical structure and fluidity of the membrane (Kamran et al., 2015). In the current study, the observed increase in lipid peroxidation in carrot samples exposed to heat stress, refrigeration temperatures, and freezing is one of the consequences of oxidative stress.

3.10. Investigating the heavy metals content of carrot samples stored under different conditions

The heavy metal analysis of carrot samples under different storage conditions revealed significant variations in mercury, lead, zinc, cadmium, and copper levels. Mercury, lead, zinc, cadmium, and copper concentrations were highest in fresh and raw samples stored in the refrigerator or freezer for 20 days. Cooked samples and those stored in the freezer for 20 days after cooking showed the lowest levels of mercury, lead, zinc, cadmium, and copper. No significant difference in copper content was observed in steamed samples stored in the freezer for 20 days ($p < 0.05$, Fig. 8).

Plants absorb heavy metals from contaminated soils, introducing these elements into the food chain. Additionally, the overuse of nitrogen, mineral, and organic fertilizers in agriculture can result in elevated levels of nitrates, anions, and heavy metals in plants, surpassing their physiological requirements and posing health hazards to humans and animals. Heavy metals resist decomposition and accumulate in various parts of the plant, such as stems, roots, and leaves.

Given the potential health risks associated with heavy metal consumption, it is imperative to analyze their presence and quantities in vegetables. Research by Shariatifar *et al.* indicates that various cooking methods can reduce rice's cadmium, mercury, and lead levels. Cooking processes break chemical bonds and dissolve heavy metals, transferring some to cooking water (Shariatifar, Rezaei, Alizadeh Sani, Alimohammadi, & Arabameri, 2020).

In the current study, the decrease in mercury, lead, zinc, cadmium, and copper concentrations during cooking and steaming, was notably lower in the steaming method than cooking, and can be attributed to the dissolution of these elements during thermal processes. Zhang *et al.* observed that heavy metal concentrations in wheat remain primarily unaffected by refrigeration and freezing, which is consistent with the findings of this study (Zhang, Xu, Yang, & Zhang, 2021). Changes in heavy metal levels in steamed and cooked frozen samples may result from pre-freezing heat exposure.

4. Conclusion

The study probed into the impact of storage and cooking methods on carrot samples, revealing significant changes in their nutritional profile. Glucose and fructose levels declined during storage, with steaming increasing them while freezing intensified the decrease in cooked carrots. Cooking reduced carbohydrate levels, but sucrose levels increased during cold storage and freezing. Oxidative stress indicators indicated metabolic pathway alterations related to free radicals during storage. Cooking reduced proline content, while freezing increased it, and hydrogen peroxide and malondialdehyde content rose due to cooking and storage. Enzyme activity changes reflected biochemical pathway shifts, with cooking decreasing peroxidase activity and affecting other enzymes differently. The presence of heavy metals in carrot tissue was underscored, highlighting the need to manage heavy metal contamination during planting, harvesting, and storage due to carrots' ability to accumulate these elements as storage roots. These findings call for further research to fully understand the implications and to develop effective strategies for managing these changes.

Declarations

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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Figures

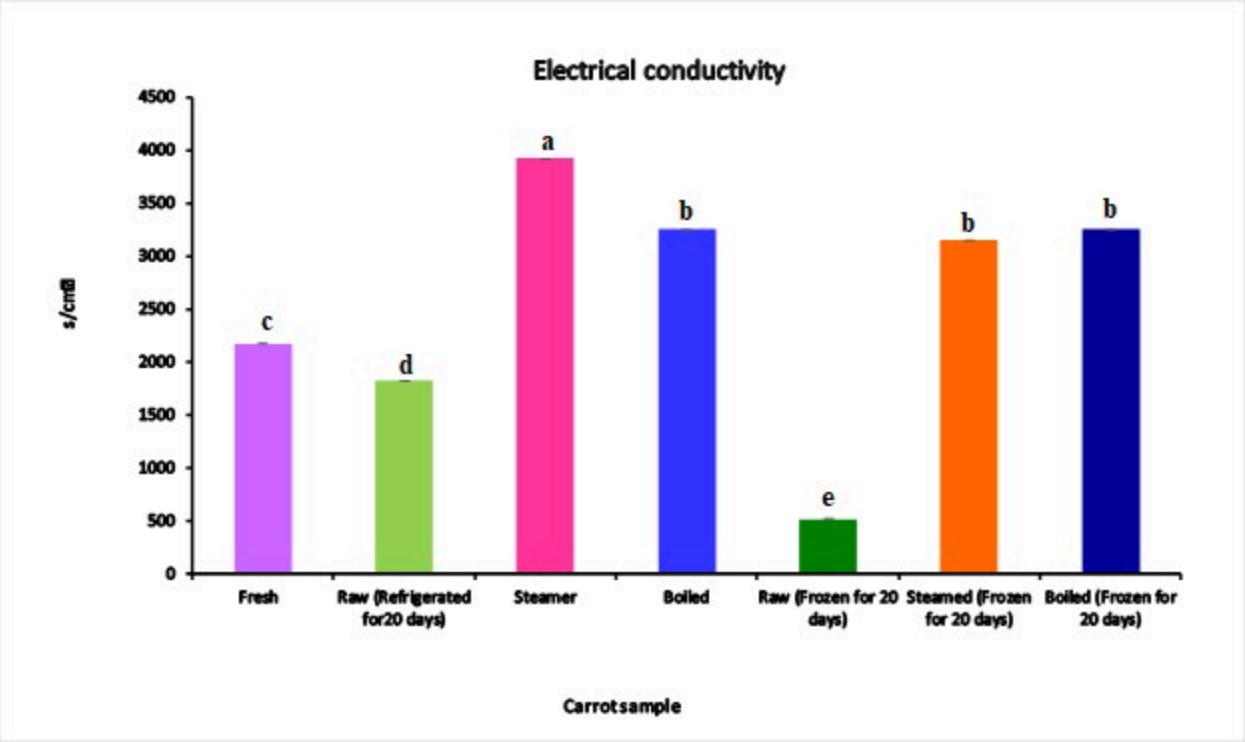

Figure 1

Electrical conductivity of carrot samples stored under different conditions. The values are the average of 3 replicates ± standard error. Similar letters at the top of the columns indicate no significant difference between the means.

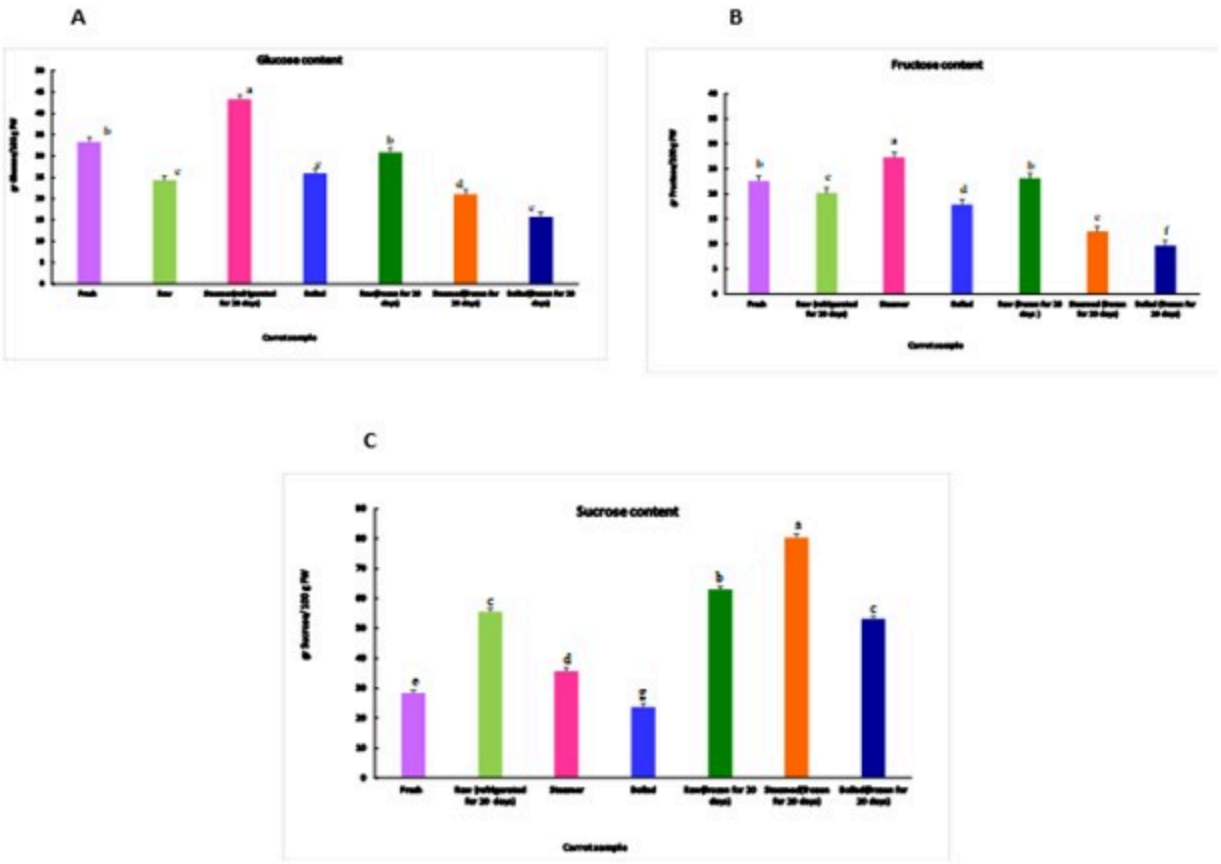

Figure 2

The levels of glucose (A), fructose (B), and sucrose (C) in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard deviation. Similar letters at the top of the columns indicate no significant difference between the means.

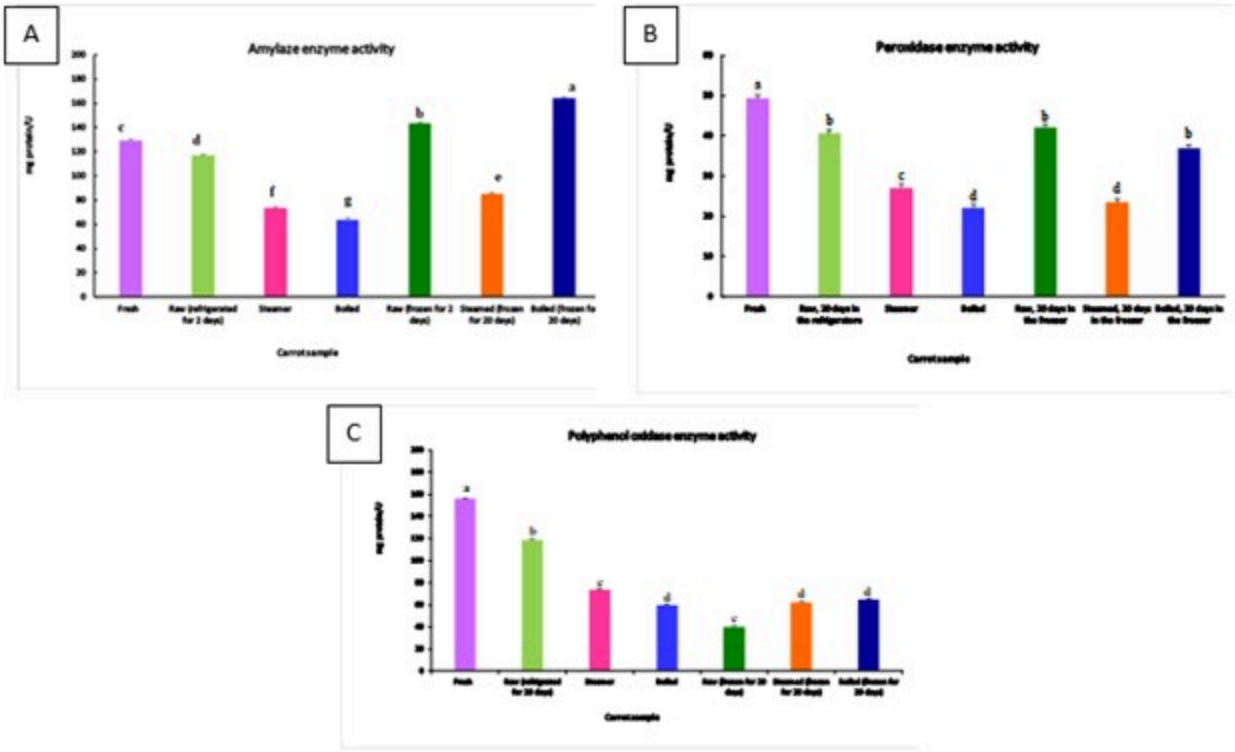

Figure 3

The levels of α-amylase enzyme (A), Peroxidase enzyme (POD, B), and Polyphenol oxidase enzyme (PPO, C) in carrot samples stored under different conditions. activity in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard deviation. Similar letters at the top of the columns indicate no significant difference between the means.

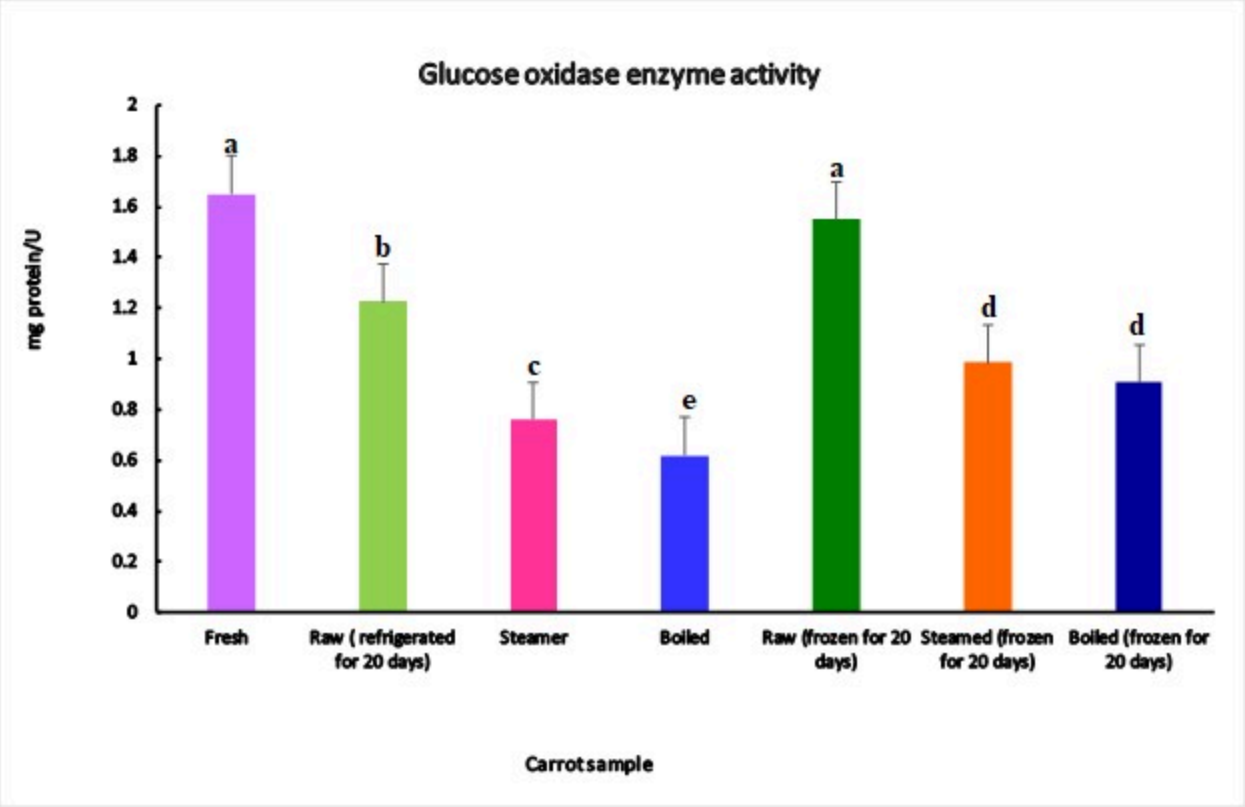

Figure 4

Glucose oxidase (GOX) activity in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard deviation. Similar letters at the top of the columns indicate no significant difference between the means.

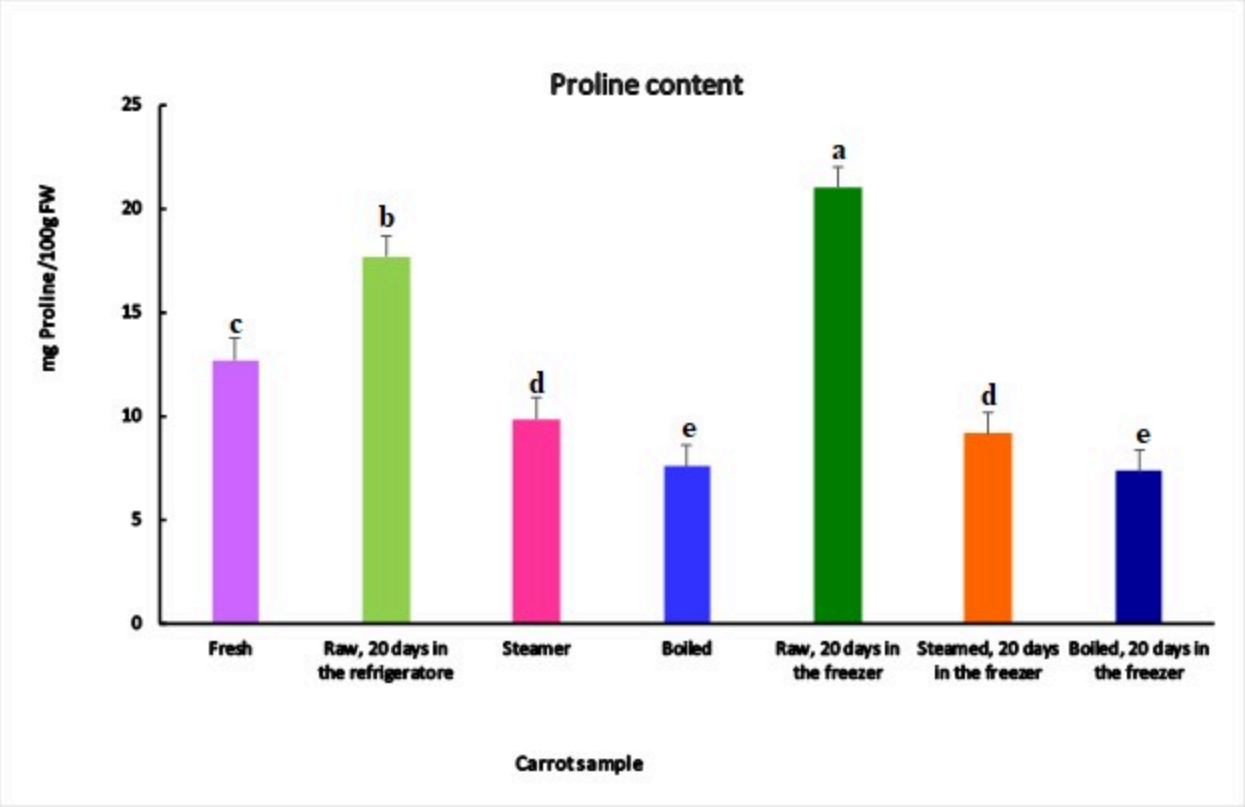

Figure 5

Proline content in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard error. Similar letters at the top of the columns indicate no significant difference between the means.

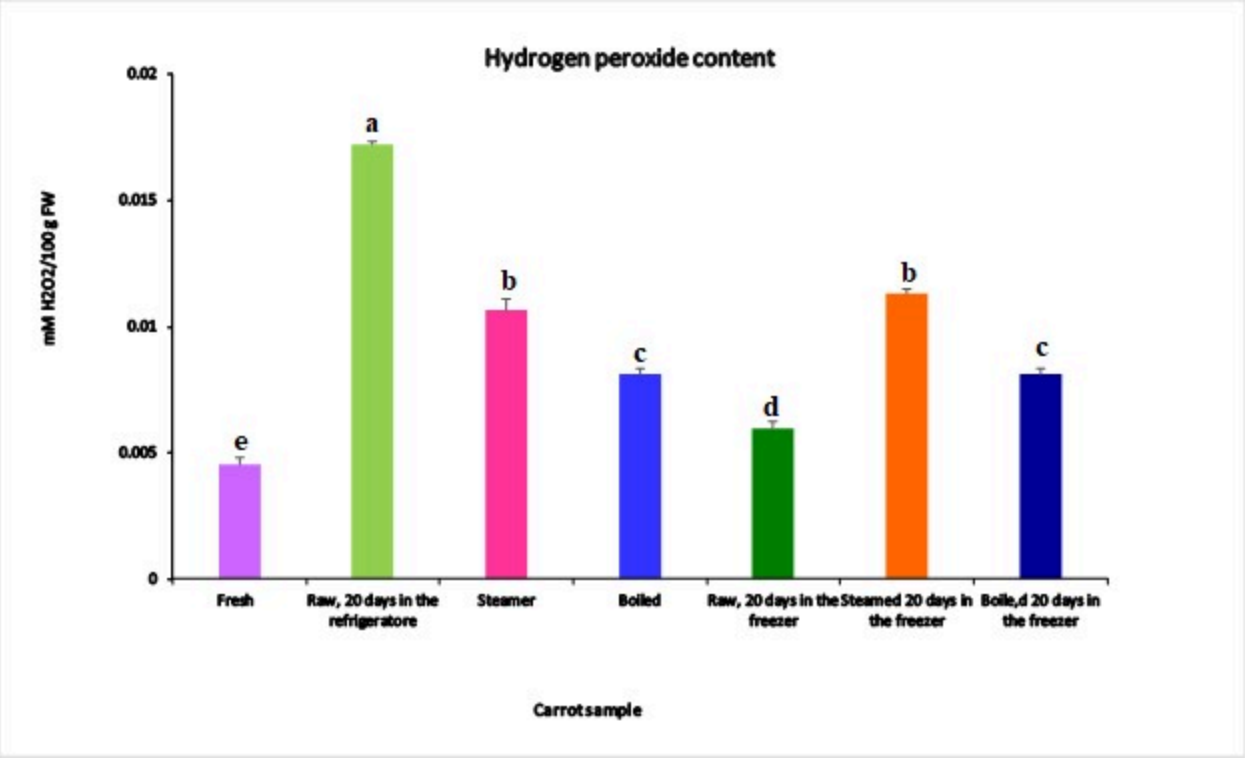

Figure 6

The levels of hydrogen peroxide in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard error. Similar letters at the top of the columns indicate no significant difference between the means.

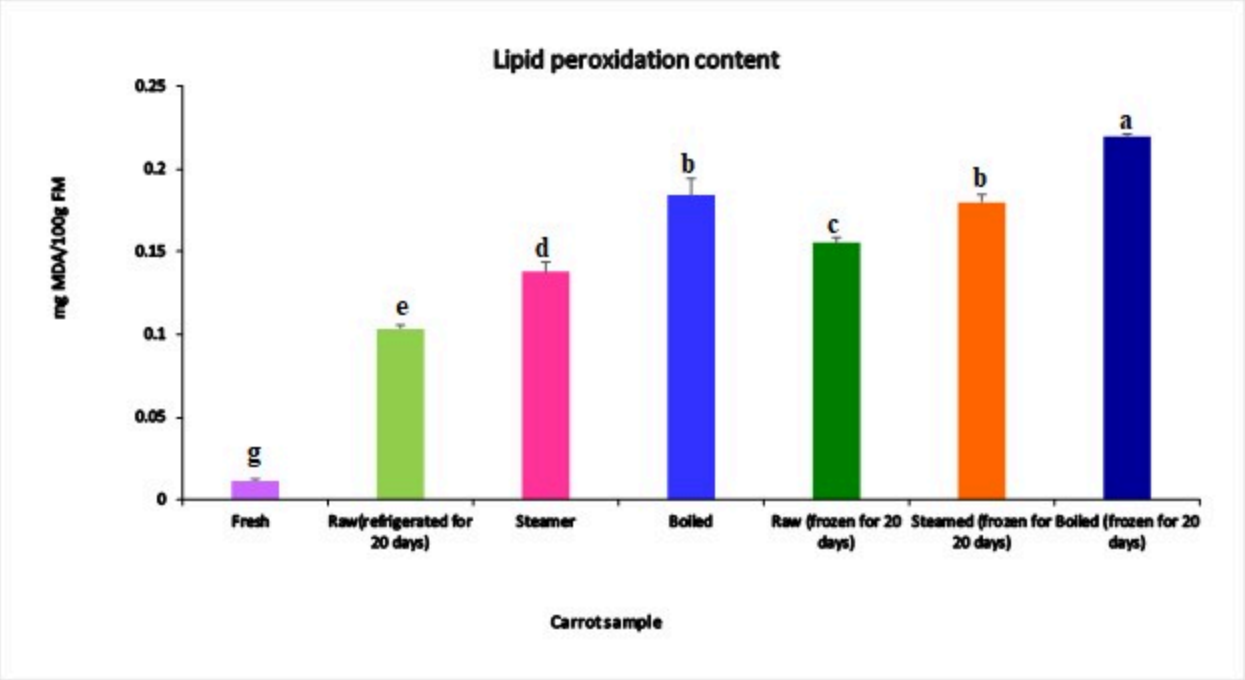

Figure 7

Lipid peroxidation in carrot samples stored under different conditions. The values are the average of 3 replicates ± standard error. Similar letters at the top of the columns indicate no significant difference between the means.

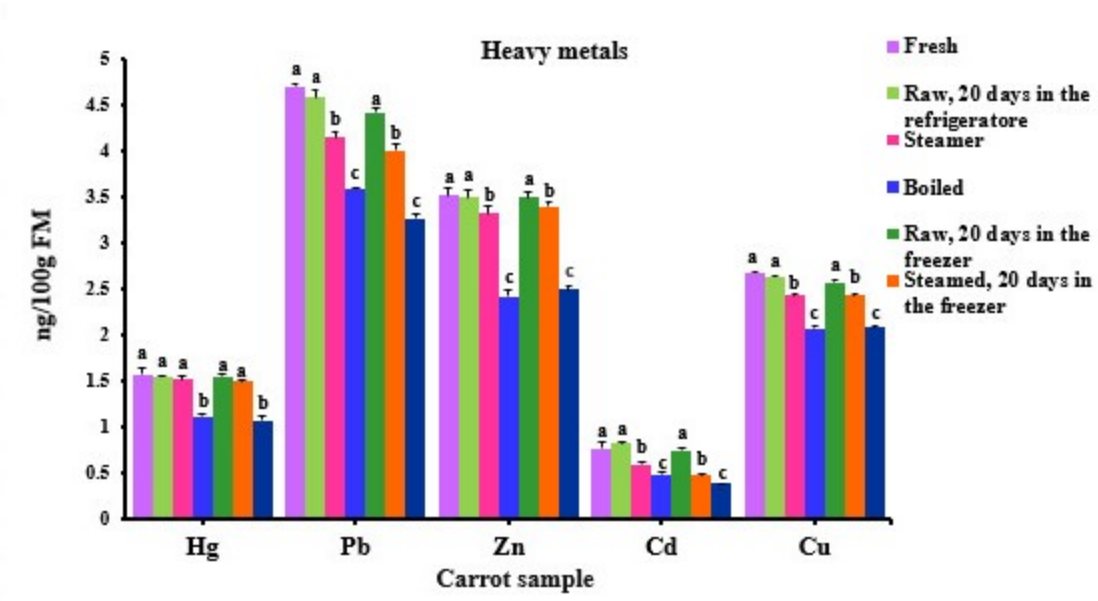

Figure 8

Heavy metal content in carrot samples stored under different conditions. The values are the average of 3 repetitions ± standard error. Similar letters at the top of the columns indicate no significant difference between the means.