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Chitosan enhances the production of antioxidant phenolic compounds in carrot through a synergistic effect with wounding stress

El quitosano potencializa la producción de compuestos fenólicos antioxidantes en zanahoria mediante un efecto sinérgico con el estrés de corte

L. Berumen-Guerrero[1], E. Ortega-Hernández[2], A. Gastélum-Estrada[1], A. Hurtado-Romero[1], D. Navarro-López[1], J. Benavides[2], D.A. Jacobo-Velázquez[1,2*]

[1]*Tecnologico de Monterrey, Escuela de Ingenieria y Ciencias, Av. General Ramón Corona 2514, C.P. 45201 Zapopan, Jal., Mexico.*
[2]*Tecnologico de Monterrey, Escuela de Ingenieria y Ciencias, Av. Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, N.L., Mexico*

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Abstract

In the present study, the postharvest application of wounding stress applied alone and combined with chitosan was evaluated as an innovative tool to increase the concentration of antioxidant phenolic compounds in carrots. Carrots were wounded to obtain pie-cuts and shreds. The whole and wounded tissue (300 g) was sprayed with a chitosan suspension (0.5% w/v), and the content of individual phenolics were evaluated before and after 48 h of storage at 20 °C. When the two stresses were combined (wounding+chitosan) a synergistic effect on the accumulation of phenolic compounds were obtained. For instance, after storage chlorogenic acid and *p*-coumaric acid derivative increased by 5,069.1% and 385%, respectively, in chitosan treated carrot shreds. Similarly, isocoumarin, which was not detected in carrots before storage, showed a high accumulation after storage in chitosan treated carrot shreds (1,074.8 mg/kg). Results presented herein demonstrated that the combination of wounding stress and chitosan can be used as an effective strategy to increase the content of antioxidant phenolic compounds in carrots. The stressed carrot tissue can be used as raw material to obtain value-added food products or for the extraction and purification of phenolics with application in the food and dietary supplement industries.

Keywords: chitosan, wounding stress, plants as biofactories, phenolic antioxidants, chlorogenic acid.

Resumen

En el presente estudio, se evaluó el efecto de la aplicación poscosecha de estrés de corte y quitosano sobre la acumulación de compuestos fenólicos en zanahorias. El tejido se cortó para obtener pedazos tipo pay y ralladuras y se asperjó con una suspensión de quitosano (0.5% p/v). Los compuestos fenólicos se cuantificaron antes y después del almacenamiento (48 h a 20 °C). La aplicación de estrés de corte y quitosano de forma combinada generó un efecto sinérgico sobre la acumulación de fenólicos. Por ejemplo, después del almacenamiento el ácido clorogénico y un derivado de ácido *p*-cumárico incrementaron en 5,069.1% y 385%, respectivamente, en las zanahorias ralladas tratadas con quitosano. De forma similar, la isocumarina no detectada en las muestras antes del almacenamiento, se produjo (1,074.8 mg/kg) después del almacenamiento en las ralladuras tratadas con quitosano. Los resultados obtenidos, demuestran que la combinación de ambos estreses puede ser aplicada como una estrategia efectiva para incrementar el contenido de compuestos fenólicos en zanahoria. El tejido estresado puede ser empleado como materia prima para la obtención de productos alimenticios o para la extracción de compuestos con aplicación en la industria alimentaria y de los suplementos alimenticios.

Palabras clave: quitosano, estrés de corte, plantas como biofábricas, fenólicos antioxidantes, ácido clorogénico.

* *Corresponding author. E-mail*: djacobov@tec.mx
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1 Introduction

The application of postharvest abiotic stresses (i.e. wounding stress, ultraviolet radiation, modified atmospheres) can be used as an effective tool to improve the nutraceutical content in horticultural crops (Cisneros-Zevallos, 2003). Thus, postharvest abiotic stresses have multiple applications. For instance, they can be used to improve the nutraceutical content of fresh-cut products to obtain foods with enhanced nutraceutical content. Likewise, they can be used as pre-treatment in raw materials to improve the content of nutraceuticals before processing to obtain plant foods with higher levels of antioxidants (Jacobo-Velázquez *et al*., 2017). Furthermore, postharvest abiotic stresses can be applied at extreme conditions in fruits and vegetables not meeting quality standards for human consumption in order to transform the plant tissue into biofactories of nutraceuticals that can be subjected to downstream processing to extract and purify high-value nutraceutical compounds induced by the stress (Jacobo-Velázquez and Cisneros-Zevallos, 2012; Sánchez-Rangel *et al*., 2014).

From the different postharvest abiotic stresses applied, wounding stress is one the most effective to activate the secondary metabolism of horticultural crops, inducing the biosynthesis of antioxidant phenolic compounds (Jacobo-Velázquez and Cisneros-Zevallos, 2012). Furthermore, it has been demonstrated that when wounding is applied in combination with other stresses such as modified atmospheres (Jacobo-Velázquez *et al*., 2011), UV radiation (Surjadinata *et al*., 2017; Ortega-Hernández *et al*., 2018, 2019), and phytohormones (Villarreal-García *et al*., 2016) the wound-induced accumulation of phenolic compounds is enhanced in crops such as carrot, prickly pear, and broccoli.

Chitosan is another compound that can be used as elicitor of the plant secondary metabolism (Ramos-Guerrero *et al*., 2020). Chitosan, is a natural polysaccharide obtained from partial or full deacetylation of chitin (Monter-Miranda *et al*., 2019). This component is an important structural element in organisms that predate plants, thus its presence triggers the activation of defense mechanisms against biotic threats in plants (Chávez-Magdaleno *et al*., 2018), leading to the accumulation of secondary metabolites. However, to the best of our knowledge there are no reports in literature evaluating the combined effect of chitosan treatment and wounding on the accumulation of antioxidant phenolic compounds in carrots, with the intention of using them as biofactories of health-promoting properties.

Therefore, the objective of the present study was to determine the effect of chitosan and wounding stress applied alone and combined on the accumulation of total and individual phenolic compounds in carrots.

2 Materials and methods

2.1 Plant material and chemicals

Carrots (*Daucus carota*) of commercial maturity with no visual fungi development or significant damage were obtained from a local market (bodega Santa Rosa, Guadalajara, Jalisco, México). All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Chlorine (Cloralex®, 6% sodium hypochlorite) was obtained from a local market (Walmart, Zapopan, Jalisco, México).

2.2 Wounding and chitosan treatments

Carrots were sorted, washed, and disinfected by submerging the tissue in an aqueous sodium hypochlorite solution (250 ppm, pH 6.5) for 5 min and dried with paper towel. Carrots were cut in two different styles and divided into three groups: whole carrots, pies, and shreds. Shreds were obtained using a food processor (Waring Commercial, WFP11, Torrington, CT, USA). For pies, slices were first obtained with a food processor and then cut manually into four equal pieces.

Chitosan suspension (0.5% w/v, 200 mL) was prepared in a 500 mL beaker. Suspension was put inside a spray bottle (ZEP brand 1L spray bottle, UK), with continuous agitation to assure a homogeneous concentration of chitosan. To apply chitosan, carrot tissue (300 g) was spread in a shallow aluminum tray (40 x 30 x 5 cm). Thereafter, chitosan suspension (200 mL) was sprayed to carrot from a distance of 15 cm while revolving the tissue to promote an even distribution of chitosan. After treatment, exceeding chitosan suspension was drained on an aluminum mesh, and the carrot tissue was centrifuged using a salad spinner (Ilko, México) to further remove excess moisture. Application of chitosan in whole carrots followed a similar procedure where three carrots (~300 g) were sprayed with 200 mL of chitosan

suspension on the tray while turning to ensure uniform coverage.

The tissue treated with chitosan was placed in hermetically sealed 4 L plastic containers and stored at 20 °C for 48 h in a Symphony incubator (VWR, Radnor, PA, USA). Containers were opened every 12 h to avoid CO_2 accumulation ($\geq 0.5\%$) in the headspace. Samples were taken at 0 and 48 h of storage and kept at -80°C until analyses. Samples used for phenolic compound analysis were taken from freeze storage, and lyophilized in a FreeZone Triad Freeze Dryers (LABCONCO, USA). Carrots sprayed with water, instead of chitosan suspension, were used as control.

2.3 Analysis of phenolic compounds

2.3.1 Extraction

Freeze-dried carrot (0.5 g) was homogenized with methanol (20 mL) utilizing an Ultra-Turrax homogenizer (IKA Works, Inc. USA) then centrifuged at 11,000 xg for 1h at 4°C. The supernatant was recovered, passed through nylon membranes of 0.2 μm (Sigma-Aldrich, USA) and used for the chromatographic analyses of phenolic compounds.

2.3.2 Identification and quantification of phenolic compounds by high-performance liquid chromatograhy - diode array detection (HPLC-DAD)

The HPLC system used for the identification and quantification of phenolic compounds was composed of a quaternary pump, an autosampler, and a diode array detector (DAD, 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA). The column used was a 4.6 × 250 mm, 5 μm C18 reverse phase column (Luna, Phenomenex, Torrance, CA, USA). Two mobile phases were used: phase A was water while phase B was methanol-water (60:40, v/v, phase B) adjusted to pH 2.4 with orthophosphoric acid. The gradient solvent system was 0/100, 3/70, 8/50, 35/30, 40/20, 45/0, 50/0, and 60/100 (min/% phase A) with a flow rate of 0.8 mL/min. Injection volume was 10 μL. The identification of individual phenolic compounds was based on their DAD spectra as compared with authentic standards and previous reports (Becerra-Moreno *et al*., 2015; Viacava *et al*., 2020). For the quantification of individual phenolics, standard curves of chlorogenic acid, protocateuric acid, galic acid, and *p*-coumaric acid were prepared at a range of 5-250 mg/L (ppm). Total phenolics were expressed as the sum of the concentration of all individual phenolic compounds. Data was processed using OpenLAB CDS ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

2.4 Statistical analyses

Statistical analyses were performed using three replicates. Treatments were run concurrently. Results represent mean values ± standard error of the mean. One-way analysis of variance (ANOVA) was conducted to determine significant difference between mean values followed by Tukey's HSD test ($p < 0.05$). Statistical analyses were performed using JMP software version 14 (SAS Institute Inc.,Cary, NC, USA)

3 Results and discussion

3.1 Effect of chitosan application on the total phenolic content of carrot wholes, pies and shreds

The combined effect of chitosan treatment and different wounding intensities (whole, pies, and shreds) on the accumulation of total phenolics is shown in Figure 1. Carrots sprayed with water were used as control for samples treated with chitosan suspension, since it has been reported that adenosine triphosphate (ATP), released from wounded cells [further referred as extracellular ATP (eATP)], acts as the primary wound signal that binds to adjacent cells of unwounded cells, eliciting the wound response (Song 2006; Jacobo-Velázquez *et al*. 2011, 2015, 2017; Gastélum-Estrada *et al*. 2020). Thus, carrots sprayed with water were used as a control to determine a possible removal of the primary wound-signal due to water spraying.

Results indicated that the accumulation of total phenolics after storage increased with wounding intensity, showing increases of 278.9% and 1,516.9% for pie-cuts and shreds, respectively, as compared with the control before storage (further referred as CBS), whereas whole carrots did not show a significant increase after storage (Figure 1). Spraying carrot tissue with water decreased the wound-induced accumulation of phenolics in shredded carrots, where increases of 1,267.7% in phenolic accumulation were quantified as compared with CBS. When chitosan was applied in the tissue the wound-induced accumulation of phenolics was further enhanced, showing increases

of 917.4% and 2,332.7% for pie-cuts and shreds, respectively, as compared with CBS. Interestingly, after storage whole carrots treated with chitosan showed a significant increase (119.5%) in the total phenolic content as compared with CBS.

3.2 Effect of chitosan application on the accumulation of individual phenolic compounds in carrot wholes, pies and shreds

The combined effect of chitosan treatment and different wounding intensities (whole, pies, and shreds) on the accumulation of individual phenolic compounds in carrots is shown in Figure 2. The major phenolic compounds identified in non-stressed carrot tissue (CBS) was chlorogenic acid (220.6 mg/kg, Figure 2A), followed by p-coumaric acid derivative (112.9 mg/kg, Figure 2B), *p*-coumaric acid (99.5 mg/kg, Figure 2D), gallic acid (71.8 mg/kg, Figure 2F) and protocatechuic acid (39.3 mg/kg, Figure 2E).

After storage, the application of chitosan induced the accumulation of isocoumarin in whole carrots (27.9 mg/kg), which was not detected in CBS (Figure 2C). An additional phenolic compound that showed accumulation due to chitosan application in whole carrots were chlorogenic acid, and protocatechuic acid, showing increases of 284.6% and 9.95%, respectively, as compared with CBS. As observed for total phenolics (Figure 1), the accumulation of certain individual phenolics increased with the wounding intensity applied. For carrot pies not treated with chitosan, phenolics that showed increases in concentration after storage were the chlorogenic acid (670.0%), *p*-coumaric acid derivative (29.43%). Furthermore, biosynthesis of isocoumarin was also detected in carrot pies (45.9 mg/kg) due to wounding stress. Likewise, other compounds such as *p*-coumaric acid and protocatechuic acid did not show significant increase after storage of carrot pies, whereas the concentration of gallic acid decreased by 50.1%.

On the other hand, shredded carrots not treated with chitosan, showed higher accumulation of most individual phenolics as compared with carrot pies (Figure 2). In this context, shreds showed increases of chlorogenic acid (3,470.0%), *p*-coumaric acid derivative (200.8%), and *p*-coumaric acid (17.2%) as compared with CBS. Likewise, shredded carrots showed a high accumulation of isocoumarin after storage (377.5 mg/kg), which was 723.1% higher than isocumarin content in stored carrot pies. As observed for carrot pies, shreds did not show significant difference in the protocatechuic acid content after storage, whereas gallic acid concentration decreased by 32.9% as compared with CBS.

When the two stresses were combined (wounding+chitosan) a synergistic effect on the accumulation of certain phenolic compounds was obtained. For instance, after storage chlorogenic acid increased by 1,991.3% and 5,069.1% in chitosan treated carrot pies and shreds, respectively; whereas *p*-coumaric acid derivative increased by 173.7% and 385.6% in chitosan treated carrot pies and shreds, respectively. Similarly, isocoumarin, which was not detected in CBS, showed a high accumulation after storage of chitosan treated carrot pies (413 mg/kg) and shreds (1074.8 mg/kg). For *p*-coumaric acid, also a slight increase was detected in chitosan treated pies (10.3%) and shreds (23.7%) as compared with CBS. Protocatechuic acid showed a significant increase (22.0%) after storage of pie-cuts treated with chitosan as compared with CBS, whereas chitosan treated shreds did not show a significant increase after storage. Finally, gallic acid was the only phenolic compound that showed decreased concentration after storage of wounded tissue. However, chitosan application in carrot pies slightly impeded this decrease in concentration, where carrot pies treated with chitosan showed 18.7% higher gallic acid content as compared with non-chitosan treated carrot pies stored for 48 h.

4 Discussion

4.1 Effect of wounding and chitosan on total phenolics accumulation in carrots

Wounding stress has been reported as one of the postharvest abiotic stresses with the highest capacity to activate the primary and secondary metabolism of plants, leading to the accumulation of secondary metabolites with health promoting-properties, such as the phenolics (Jacobo-Velázquez and Cisneros-Zevallos 2012; Sánchez-Rangel *et al.*, 2014). In the present study, the application of wounding stress in carrots to produce pies and shreds increased the accumulation of total phenolics (Figure 1). As the wounding intensity increased, the accumulation of phenolics was higher (shreds > pies > wholes) after 48 h of storage at 20°C. These results agree with previous studies reporting that the biosynthesis of phenolic antioxidants in carrot tissue increases

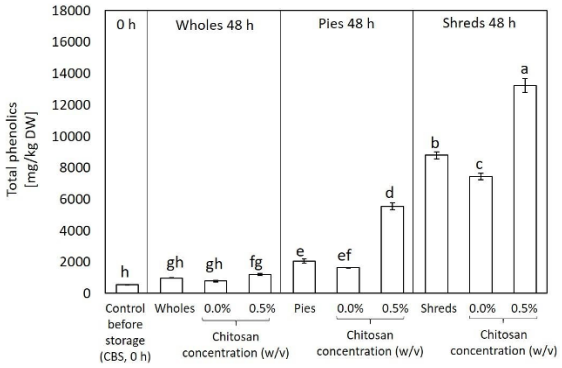

Figure 1. Total phenolic content before and after storage (48 h at 20°C) of carrot wholes, pies and shreds sprayed with chitosan suspension (0.5% w/v). Bars are means of three replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p<0.05$).

with wounding intensity (Surjadinata and Cisneros-Zevallos 2012; Jacobo-Velázquez *et al*., 2011). The physiological mechanisms inducing the accumulation of phenolic compounds in plants as a response to wounding stress has been previously elucidated (Song, 2006; Jacobo-Velázquez *et al*., 2011). Upon the application of wounding, eATP is released from the cytosol of wounded-cells, and then bind to plasma membrane ATP receptors of unwounded cells to elicit the wound-response. ATP binding to receptors induce a release of cytosolic Ca^{2+} that activates NADPH oxidase, which induce the production of superoxide radical (O_2^-) (Song *et al*., 2006, Jacobo-Velázquez *et al*., 2011; Gastélum-Estrada *et al*., 2020). O_2^- is then transformed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Hydrogen peroxide produced serves as the secondary wound-signal and induces the activation of genes related with the biosynthesis of phenolic compounds (Jacobo-Velázquez *et al*., 2015). Spraying shredded carrots with water decreased the wound-induced accumulation of phenolics, suggesting the partial removal of the primary wound-signal (eATP). Interestingly, the reduction in phenolics accumulation due to water-spraying was not observed in carrot pies, indicating that the lower area of exposure to water impeded the removal of the primary wound-signal.

Chitosan is a natural polysaccharide obtained from partial or full deacetylation of chitin, which is an important structural element in the exoskeleton of arthropods and fungi cell wall. When chitosan is applied to fruit and vegetable tissue stimulates the activation of the plant secondary metabolism (Hidangmayum *et al*., 2019). As shown in Figure 1, spraying whole carrots with chitosan suspension induced a significant increase (119.5%) in total phenolic content of whole carrots. These results agree with previous reports where the application of chitosan increased the accumulation of phenolics in *Eriobotrya japonica* (Ghasemnezhad *et al*., 2011), *Lycopersicon esculentum* Mill (Liu *et al*. 2007), and *Prunus salicina* L. (Chang *et al*., 2019). Chitosan induces the activation of the plant secondary metabolism by binding to its own plasma membrane receptor, known as the chitin/chitosan elicitor binding proteins (CEBiP) found in several plants. In this context, chitosan acts similarly to eATP produced by wounding. Chitosan binding to CEBiPs create a variety of responses including the release of Ca^{2+} into the cytosol, and thus the production of hydrogen peroxide via NADPH oxidase and SOD as described for wounding (Lin *et al*., 2005).

When the two stresses were applied in carrots, a synergistic effect on the accumulation of total phenolics was detected (Figure 1). This synergistic effect could be attributed to the increased levels H_2O_2 accumulated in the tissue when the two stresses were applied at the same time. To the best of our knowledge, this is the first report in literature evaluating the combined effect of wounding and chitosan on the accumulation of phenolics in a horticultural crop. Previous reports have demonstrated that combining wounding with additional stresses such as UV radiation (Formica-Oliveira *et al*., 2016; Surjadinata *et al*., 2017; Ortega-Hernández *et al*., 2019) and hyperoxia (Jacobo-Velázquez *et al*., 2011) induce higher accumulation of reactive oxygen species (ROS, i.e. hydrogen peroxide) in fruits and vegetables, increasing oxidative stress, resulting on an enhancement of the wound-induced accumulation of phenolic antioxidants. The results obtained herein, suggest that a similar phenomenon is induced when chitosan is applied in wounded carrots.

4.2 Effect of wounding and chitosan on the qualitative and quantitative phenolic profile of carrots

The major phenolic compounds identified in carrot tissue was chlorogenic acid, followed by *p*-coumaric acid derivative, *p*-coumaric acid, gallic acid and protocatechuic acid (Figure 2). Phenolic compounds identified agree with the phenolic profile of carrots previously reported (Becerra-Moreno *et al*., 2015;

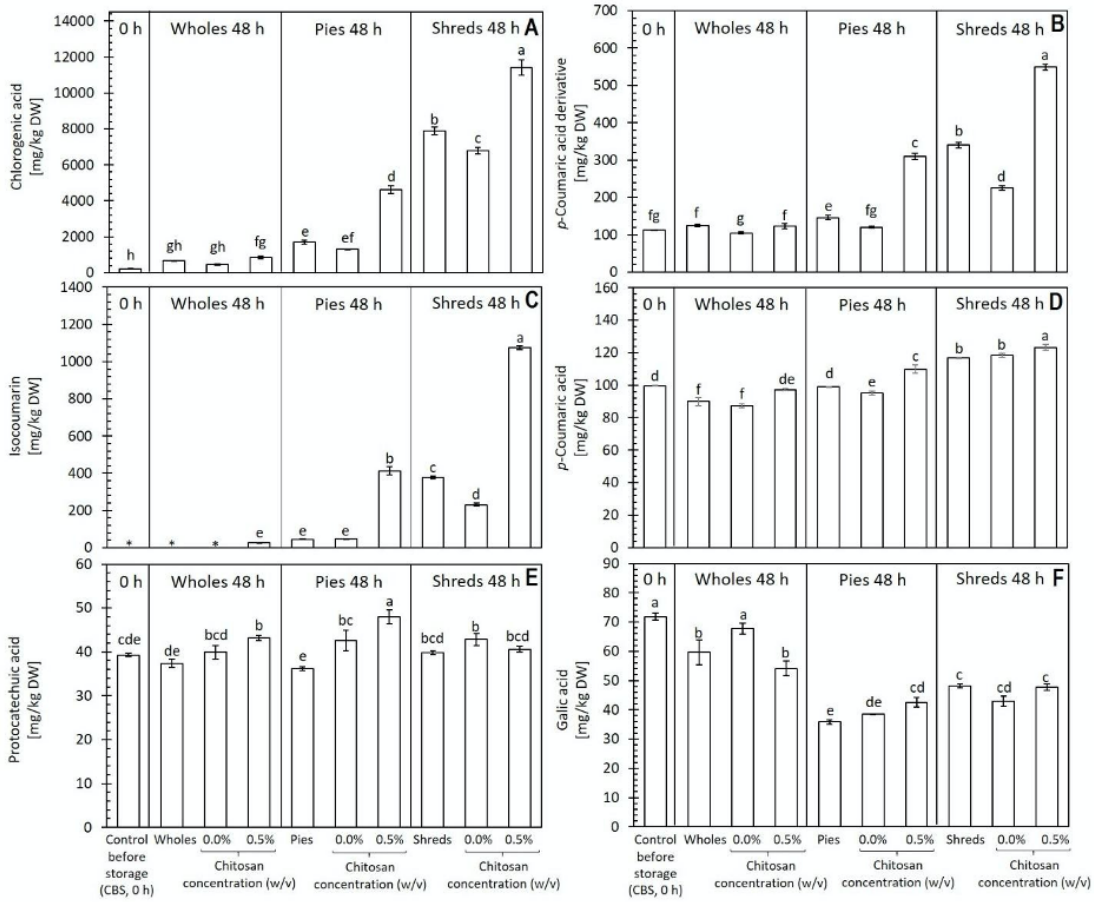

Figure 2. Content of individual phenolics before and after storage (48 h at 20°C) of carrot wholes, pies and shreds sprayed with chitosan suspension (0.5% w/v). Chlorogenic acid (A), *p*-coumaric acid derivative (B), isocoumarin (C), *p*-coumaric acid (D), protocatechuic acid (E), gallic acid (F). Bars represent the means of 3 replicates ± standard error. Samples with an asterisk (*) mean that the compound was not detected. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p<0.05$).

Viacava *et al*., 2020). As previously described, the main phenolic compound accumulated in carrots due to wounding stress was the chlorogenic acid, followed by *p*-coumaric acid derivative, isocoumarin and *p*-coumaric acid. Except for isocoumarin, the phenolic compounds accumulated as a response to wounding are hydroxycinnamic acids that serve as precursors for the lignin biosynthesis. Lignin is a complex organic polymer produced during the wound-healing process to prevent water loss (Becerra-Moreno *et al*., 2015). Thus, the accumulation of phenolic compounds observed can be attributed to a higher biosynthesis rate that its utilization rate for lignin production. In the specific case of isocumarin, it is a phytoalexin with antifungal properties that is produced in carrots as a response to wounding and other abiotic stresses (i.e. UV radiation and exogenous ethylene) (Mercier *et al*., 1994). This compound was not detected in the whole carrot tissue, however, a large increase in concentration was observed due to wounding.

Chitosan applied in whole tissue, induced the accumulation of isocoumarin in the whole tissue, indicating that the carrot peel can perceive chitosan through the CEBiP. Isocumarin production could be the result of a defense mechanism against pathogen attack. When the two stresses were applied at the same time, a large increase in the accumulation of chlorogenic acid, *p*-coumaric acid derivate, and isocoumarin was detected (Figure 2) indicating, as previously described for total phenolics, that both stressors (wounding+chitosan) are acting synergistically to induce the accumulation of phenolic antioxidants that protect the plant cell against oxidative stress, and pathogen attack.

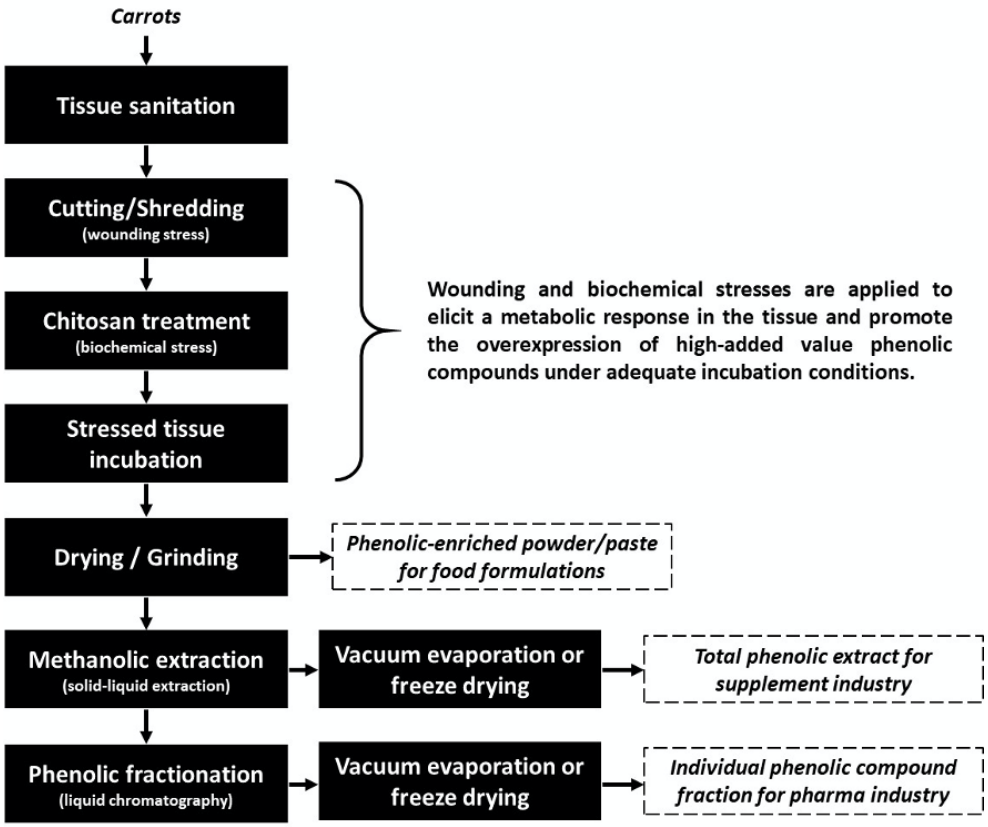

Figure 3. Unit operation sequence for the obtention of different phenolic-enriched products from a stressed carrot tissue using wounding and chitosan treatment.

The results in the present research work can be easily extrapolated into a production process in which carrot tissue is used as a biofactory for the synthesis of high-added value phenolic compounds with application in the supplement and pharmaceutical industry. Due to the nature of the stresses applied to the tissue (wounding and biochemical elicitation) conventional, well-characterized, technology may be readily incorporated into a transformation process in which carrots not meeting the quality requirement to be commercialized as fresh produce (about 10% of the total carrot production is typically lost due to non-optimal postharvest handling). This would prevent a direct competition with the food supply chain, making use of a resource consider a waste in the agricultural industry and rendering a much more sustainable process. Another important aspect to remark is the versatility of the process designed, based on the intended application. The final application of the product dictates how extensive the downstream process needs to be, going from a phenolic enriched paste/powder (for food formulations), passing through a total phenolic extract (for supplement formulations) until reaching a highly pure fraction of a individual phenolic compound (for pharmaceutical formulations). Figure 3 depicts how the downstream processing sequence can be adapted based on the intended final product. Even for the application that requires the highest purity the process would not require a large number of unit operations, particularly using novel chromatographic matrixes that may exploit more than one biochemical characteristic in order to efficiently separate complex mixtures in a single step.

Conclusions

The results presented herein show that wounding stress and chitosan treatment act synergistically to induce the biosynthesis of antioxidant phenolic compounds in carrot. The most significant result of the study was the accumulation of total phenolics

(2332.7% higher than CBS) when chitosan was applied in shredded carrots. From the individual phenolics accumulated chlorogenic acid showed the highest significant increase (5069.1%) as compared with CBS. Another interesting result was the production of isocoumarin, which was not detected in CBS and high accumulation was obtained in shredded carrots treated with chitosan (1074.8 mg/kg). It is important to point out the nutraceutical properties of chlorogenic acid, since it is the main phenolic compound induced by wounding combined with chitosan treatment. According with previous reports, chlorogenic acid can be used for the prevention of the metabolic syndrome (Santana-Gálvez *et al*., 2017), for the prevention of colon cancer when metabolized to dihydrocaffeic acid by the gut microbiota (Santana-Gálvez *et al*., 2020a, 2020b), and also as an enhancer of cognitive ability and neural development according with results from *in vivo* studies (López-Martínez *et al*., 2020).

Future research could be focused on finding alternative method of chitosan delivery (or even chitin) on the wounded tissue to induce higher stress response. Variations in molecular weight could allow obtaining solutions to produce full coatings, which would result in more surface interaction enhancing the interaction between chitosan and its cell receptor. It would also be valuable to stablish the effectiveness of this effect when the source of chitin/chitosan is not pure and comes from a more unrefined source like another processed food product. Chitin/chitosan can be found in several popular foodstuffs like seafood but novel health foods like cricket flour should produce a similar effect as the one observed herein. This would allow the obtention of novel nutraceutical product of great added value, especially when using cricket flour which contains high protein value.

Acknowledgements

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Nomenclature

ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CBS	Control before storage
CEBiP	Chitin/chitosan elicitor binding proteins
CO_2	Carbon dioxide
DAD	Diode array detector
eATP	Extracellular adenosine triphosphate
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
O_2^-	Superoxide radical
SOD	Superoxide dismutase
UV	Ultraviolet

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