

Influence of Phenylalanine and Chitosan Edible Coatings on the Metabolism of Mandarin 'Chahara' – Impact on Bioactive Compounds, Organic Acids and Enzymatic Activity

Marina Jurić^a, Luna Maslov Bandić^b, Marko Vuković^c, Francesco Donsì^d, Kristina Radić^a, Slaven Jurić^{b,*}

^aUniversity of Zagreb Faculty of Pharmacy and Biochemistry, Department of Nutrition and Dietetics, Zagreb, Croatia

^bUniversity of Zagreb Faculty of Agriculture, Department of Chemistry, Zagreb, Croatia

^cUniversity of Zagreb Faculty of Agriculture, Department of Pomology, Zagreb, Croatia

^dUniversity of Salerno Department of Industrial Engineering, Fisciano, Italy

sjuric@agr.unizg.hr

Satsuma mandarins (*Citrus unshiu* Marc.) are renowned for their delicate taste, distinct flavor and high nutritional value. However, their perishable nature necessitates innovative preservation methods. Edible coatings have shown promise in improving fruit quality and extending shelf life, but their metabolic effects remain poorly understood. This study investigates the impact of coatings prepared with Chitosan, and a combination of chitosan and phenylalanine (Chitosan/Phe), compared to the use of a phenylalanine dipping solution (Phe), on mandarin metabolism during 21 days of cold storage. The analysis included total polyphenolic and total flavonoid content, antioxidant activity, total proteins, and organic acids in mandarin fruit. Enzymatic activity and malondialdehyde levels were also assessed in mandarin peels. Significant differences were observed among treatments regarding bioactive compounds, organic acids, enzymatic activity, and malondialdehyde levels. Notably, Phe-treated mandarins showed increased bioactive compounds and organic acids content, as well as antioxidant activity, compared to the control. Chitosan or Chitosan/Phe coatings resulted in the highest polyphenol oxidase and catalase activity, while Phe and Chitosan treatments resulted in the lowest malondialdehyde levels at the end of the storage period. These findings highlight the potential of Phe and chitosan-based coatings in enhancing mandarin bioactive profile, antioxidant activity and enzymatic regulation.

1. Introduction

Mandarins have gained worldwide popularity due to their delicate taste, distinctive flavor, and nutritional density (Feng et al., 2018). The health benefits associated with mandarin consumption are attributed to its rich content of phytochemicals, such as polyphenols, flavonoids, carotenoids, limonoids, amino acids and organic acids (Tietel et al., 2020). However, mandarins are highly-perishable, non-climacteric fruits that rapidly lose quality limiting their storage duration (Rokaya et al., 2016). To mitigate fruit loss and decay, combining cold storage with alternative postharvest treatments is a viable approach. Edible coatings (ECs) have garnered significant attention for protecting fruits from mechanical, physical, chemical, and microbial damage, while also influencing fruit quality by affecting important sensory attributes (Jurić et al., 2023). Chitosan is a widely used biopolymer in ECs due to its ability to inhibit fungal growth, reduce water loss, and delay browning (Obianom et al., 2019). The performance of chitosan-based ECs can be further improved by incorporating natural additives. For example, postharvest application of phenylalanine has been used to stimulate defense mechanisms in fruits, (Kumar Patel et al., 2020), thereby inhibiting fungal development and postharvest decay (Saidi et al., 2021). Phenylalanine contributes to the synthesis of antioxidants and aromatic compounds, directly influencing plant metabolism through the phenylpropanoid pathway (Aghaei et al., 2019). In this study, various postharvest treatments were investigated using ECs based on chitosan alone (Chitosan), and a combination of chitosan and phenylalanine (Chitosan/Phe), compared to a phenylalanine dipping solution (Phe), to maintain the quality of 'Chahara' Satsuma mandarins during cold storage. The primary objective was to evaluate the impact of these

treatments on mandarin metabolism, focusing on changes in bioactive compound content, antioxidant activity, organic acid composition and enzymatic activity over a 21-day cold storage period.

2. Materials and Methods

2.1 Plant material and chemicals

Satsuma mandarins 'Chahara' (*Citrus unshiu* Marc.) were obtained from a commercial orchard (Mandarinko d.o.o.) located at Opuzen, Neretva valley, Croatia (Latitude: 43.0176, Longitude: 17.5623; 43°1'3" North, 17°33'44" East). The fruits were harvested at optimal maturity and immediately delivered to the laboratory for further processing. High molecular weight chitosan (CAS Number: 9012-76-4, molecular weight: 310000–375000 Da; 800–2000 cP, 1 wt% in 1 % acetic acid (25 °C, Brookfield)) was purchased from Sigma-Aldrich (USA). L-Phenylalanine, 98.5-101.0 %, was obtained from Thermo Scientific Chemicals (USA), while all other reactants were purchased from Sigma-Aldrich.

2.2 Preparation of dipping solutions

A 2 % (w/v) chitosan dipping solution was prepared by dissolving chitosan in sterile citric acid (2 %, w/v), followed by the addition of glycerol (2 % v/v) as a plasticizer (Chitosan). The Chitosan/Phe dipping solution was prepared by dissolving phenylalanine (6 mM) directly into the chitosan solution. The Phe dipping solution, used as a separate treatment, was prepared by dissolving phenylalanine (6 mM) in distilled water. The concentration of phenylalanine was chosen based on previous studies (Kumar Patel et al., 2020).

Before the post-harvest treatments, mandarins were washed in tap water and 45 mandarin fruits (15 per biological replicate) were randomly selected for each treatment and the control. The mandarins were submerged in the dipping solutions for three minutes, then drained and allowed to dry in a well-ventilated room. Treated mandarins were stored in a chamber at 5°C with a low relative humidity of 75-85% for up to 21 days, since up to this time and under these conditions most significant changes in bioactive compounds are expected to occur.

2.3 Mandarin preparation for chemical analysis

Nine mandarins (three from each biological replicate) were used for juice preparation, yielding a total of three juices per treatment per sampling day. Mandarin fruits were weighted, peeled, and homogenized using a FOSS homogenizer 2094 (Hillerød, Denmark). The weight of each fruit was recorded before and after peeling. The resulting puree was transferred to Falcon tubes and centrifuged at 11,180 × g and 4°C, for 10 min. The supernatant was filtered under vacuum using Whatman No.4 filter paper and used for further analysis. Meanwhile, mandarin peels were freeze-dried and micronized using a laboratory mixer for subsequent enzymatic activity and malondialdehyde determination.

2.4 Determination of bioactive compounds, antioxidant activity and organic acids in mandarins

Fruit weight loss and decay rate were assessed by measuring the mandarins before treatment and after each sampling day for the remaining fruits. Total soluble solids (TSS) of the mandarins were determined using a digital hand refractometer (PAL-1; Atago, Tokyo, Japan) and expressed as a percentage (%). Total acids (TA) content was determined through titration, following the method reported by Jurić et al. (2023). The share of edible fruit part (%) was calculated as the ratio of the edible part to the total fruit mass.

Total proteins (TP) in mandarins were determined using the Lowry method (Lowry et al., 1951). The modified Folin Ciocalteu's method (Singleton et al., 1999) was used to assess total polyphenolic content (TPC). Total flavonoid content (TFC) was determined using a modified spectrophotometric method at 360 nm (Ivanova et al., 2010). Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reagents (Re et al., 1999). All data were expressed as mg of standard equivalents per 100 g of mandarins (edible part).

For the determination of oxalic acid (OA), malic acid (MA), ascorbic acid (AA), and citric acid (CA), a modified HPLC method was used (Nour et al., 2010). Diluted supernatants were filtered through 0.45 µm syringe filters before analysis. The Agilent 1260 Infinity II System (Agilent, Waldbronn, Germany), equipped with an autosampler, column thermostat, and DAD (diode-array detector) was used. Organic acids were separated using a COSMOSIL C18-PAQ column (250 mm × 4.6 mm i.d., 5 µm) at 40°C. Detection wavelengths were set at 254 nm for ascorbic acid and 210 nm for the other acids, with an injected volume of 20 µL. The mobile phase consisted of a 50 mM phosphate buffer with isocratic elution at a flow rate of 0.7 mL/min.

2.5 Determination of enzymatic activity and malondialdehyde levels in mandarin peels

Polyphenol oxidase (PPO) and peroxidase (POD) activity in the mandarin peel powder were determined using a modified method of Chen et al. (2019). Catalase activity (CAT) and malondialdehyde (MDA) content in the

mandarin peel powder were determined according to the modified methods by Petriccione et al. (2015) and Kim et al. (2022).

3. Results and Discussion

3.1 Fruit quality, bioactive compounds, antioxidant activity and protein content changes in mandarins over cold storage period

Fruit quality includes several parameters, including freshness, which is often associated with a glossy appearance (Althaus and Blanke, 2021). Glossiness is an important external fruit quality attribute (Zhai et al., 2022). An initial sensory evaluation of fruit glossiness was conducted by twelve sensory-trained panelists on control fruits and after the ECs application, before cold storage. Remarkably, this initial sensory evaluation of fruit glossiness, evaluated by twelve sensory-trained panelists (data not shown) revealed that Chitosan and Chitosan/Phe collected higher consumer preferences compared to control or Phe-treated mandarins. This aligns with previous findings for chitosan-based ECs applied to mandarins (Jurić et al., 2023). Regarding primary fruit quality traits, such as TSS, TA, the TSS to TA ratio, the share of edible fruit part, and weight loss, no significant differences were observed among treatments (data not shown). However, the treatments significantly impacted the content of bioactive compounds in mandarins, as shown in Table 1.

Table 1: Total polyphenolic and total flavonoid content, antioxidant activity (DPPH and ABTS) and total proteins in mandarins initially (day 0) and after cold storage time up to 21 days.

Treatment	Day 0	Day 7	Day 14	Day 21
Total Polyphenolic Content (TPC) (mg GAE/100 g)				
Control		22.50±1.64 ^c	20.68±2.97 ^c	19.07±4.74 ^c
Phe	25.05±0.85	30.10±2.98 ^a	28.74±1.18 ^a	26.76±1.94 ^a
Chitosan		23.65±3.97 ^{bc}	24.98±1.53 ^b	20.65±1.68 ^{bc}
Chitosan/Phe		25.49±1.63 ^b	23.44±2.14 ^{bc}	23.12±1.80 ^b
Total Flavonoid Content (TFC) (mg QE/100 g)				
Control		13.89±0.87 ^c	11.83±1.47 ^c	12.62±3.09 ^c
Phe	13.60±0.06	19.48±1.53 ^a	19.25±0.62 ^a	17.97±1.16 ^a
Chitosan		14.53±2.58 ^{bc}	16.23±0.93 ^b	14.28±0.79 ^{bc}
Chitosan/Phe		15.72±1.62 ^b	12.91±1.07 ^c	15.98±0.62 ^b
Antioxidant activity (DPPH) (µmol TE/100 g)				
Control		80.71±7.60 ^d	83.14±18.89 ^b	74.26±10.05 ^d
Phe	64.75±2.75	129.77±10.63 ^a	110.39±11.59 ^a	105.54±7.58 ^a
Chitosan		92.75±10.30 ^c	86.88±9.36 ^b	95.79±5.61 ^b
Chitosan/Phe		102.28±4.77 ^b	81.38±7.19 ^b	85.20±6.71 ^c
Antioxidant activity (ABTS) (µmol TE/100 g)				
Control		117.58±9.86 ^c	106.03±13.76 ^a	83.89±10.94 ^c
Phe	126.08±10.48	169.80±12.89 ^a	121.51±14.43 ^b	145.71±13.03 ^a
Chitosan		123.84±14.61 ^c	113.54±11.46 ^{ab}	118.81±5.90 ^b
Chitosan/Phe		138.17±4.25 ^b	114.49±4.14 ^{ab}	116.89±4.21 ^b
Total Proteins (TP) (g chymosin/100 g)				
Control		1.10±0.12 ^b	1.04±0.14 ^c	0.97±0.23 ^b
Phe	1.02±0.04	1.48±0.09 ^a	1.47±0.03 ^a	1.31±0.09 ^a
Chitosan		1.16±0.13 ^b	1.25±0.08 ^b	1.11±0.07 ^b
Chitosan/Phe		1.18±0.15 ^b	1.09±0.03 ^c	1.05±1.13 ^b

Values superscripted with the same letter within a column (respectively to the method) are not significantly different according to the post hoc t-test ($p < 0.05$).

Throughout the cold storage period, control samples exhibited a gradual decline in bioactive compounds (TPC and TFC) and antioxidant activity (DPPH and ABTS). In contrast, Phe-treated mandarins showed a significant initial increase (day 7), followed by a slower decrease after 14 and 21 days of storage. Compared to the control, Phe-treated mandarins displayed a notable increase in bioactive compounds, up to 40.3% for TPC, up to 62.7% for TFC, and enhanced antioxidant activity, up to 60.8% for DPPH and up to 74.3% for ABTS. Additionally, total proteins increased by up to 35.1%. Chitosan and Chitosan/Phe mandarins also showed significant increases in TPC and TFC content on some of the sampling days which is in line with previous studies that have indicated chitosan coatings influence on fruit defense responses (Saidi et al., 2021). By the end of the storage period,

only Chitosan/Phe and Phe treatments maintained significantly higher values compared to the control samples for bioactive compounds. For antioxidant activity, all treatments showed significantly higher values than the control on the last day. Notably, only the Phe treatment consistently maintained elevated total protein levels in mandarins, which were significantly higher than those in control samples and other treatments.

Using chitosan/glycerol as carrier coatings may enhance the effectiveness of phenylalanine, by prolonging its diffusion and absorption rate over time. Kumar Patel et al. (2020) found that phenylalanine concentrations in treated and untreated fruits became similar after five hours, indicating rapid penetration in the fruit. Based on the results obtained, embedding phenylalanine in a chitosan coating shows promise, but longer storage times may reveal its full potential. Given that phenylalanine-treated mandarins have been shown to enhance defense responses and inhibit postharvest decay caused by fungal pathogens (Kumar Patel et al., 2020), this work focused on how phenylalanine affects mandarin metabolism in terms of bioactive compounds, organic acids, antioxidants and enzymatic activity. Previous studies have demonstrated that combining polysaccharide coatings with phenylalanine yields optimal results for avocado fruits stored at room temperature and in cold storage, improving resistance to fungal pathogens and chilling (Saidi et al., 2021). Phenylalanine seems to induce fruit responses to chilling, including the expression of phenylpropanoid-related transcripts. The metabolism of Phe results in a variety of metabolites, unique to each plant and plant organ, likely contributing to increased accumulation of phenylalanine-derived phenylpropanoids (Oliva et al., 2019).

3.2 Organic acid composition in mandarins throughout the cold storage period

The dynamic changes in organic acids in citrus fruits are complex and are influenced by various storage conditions over time (Wei et al., 2020). A decline in organic acid content during postharvest storage is a major factor negatively affecting the quality and storage performance of citrus fruits (Ma et al., 2020). Organic acids serve as a respiratory substrate, which can lead to a decrease in their content during storage (Eroğul et al., 2023). Overall, untreated mandarins exhibited a decline in organic acids after 21 days of storage (Figure 1), with the most pronounced effects observed for OA, MA and AA. However, all treatments were able to either maintain or increase the content of organic acids.

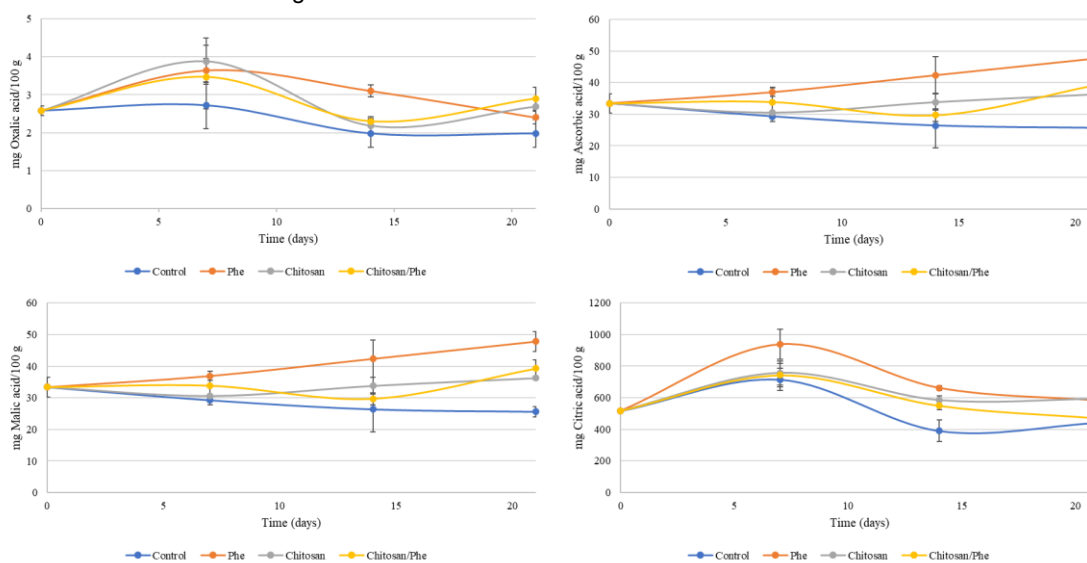


Figure 1: Organic acid (Oxalic, Malic, Ascorbic and Citric) composition in mandarins throughout the cold storage.

Jurić et al. (2023) reported that mandarins coated with chitosan-based ECs had higher organic acid content compared to untreated mandarins during both room temperature (10 days) and cold storage (28 days). Similarly, exogenous phenylalanine has been shown to inhibit the tricarboxylic acid cycle, thereby suppressing respiration and maintaining fruit quality in 'Jinfeng' pears (Wang et al., 2023). Phenylalanine suppressed the enzymatic activities and gene expression levels of key enzymes in the tricarboxylic acid cycle, such as pyruvate dehydrogenase, citrate synthase, aconitase, α -keto glutaric dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase, and malate dehydrogenase. This suppression promoted the accumulation of succinic acid, citric acid, and malic acid contents in pear fruits, ultimately delaying fruit senescence by inhibiting the tricarboxylic acid cycle (Wang et al., 2023). Based on the present findings, the combination of chitosan and phenylalanine (Chitosan/Phe) theoretically offers the best outcome, but a prolonged storage time is necessary to fully evaluate its effectiveness in retaining organic acids.

3.3. Influence of treatments on enzymatic activity and malondialdehyde content in mandarin peels

Compared to control samples, Chitosan and Chitosan/Phe-treated mandarins showed a significant increase in the activity of antioxidant enzymes, including PPO, POD and CAT, as detailed in Table 2. Chitosan and Chitosan/Phe treatments resulted in the highest PPO activity, with increases of 101.1 and 104.9%, respectively, and the highest CAT activity, with increases of 113.5 and 114.1%. Meanwhile, Phe and Chitosan treatments resulted in the lowest MDA levels, with reductions of 22.0 and 18.1%, respectively, by the end of the storage period. Similar findings were reported by Gao et al. (2018) for Ponkan mandarins treated with chitosan and chitosan/cinnamaldehyde ECs, where POD and CAT activity significantly increased after 20 days of storage at room temperature compared to control samples.

Table 2: Enzymatic activity and malondialdehyde levels in freeze-dried mandarin peels after 21 days of cold storage.

	Polyphenol oxidase (U/h/g d.w.)	Peroxidase (U/min/g d.w.)	Catalase (μ mol H ₂ O ₂ /min/g d.w.)	Malondialdehyde (mg MDA/g d.w.)
Control	9.49±1.00 ^c	30.37±1.50 ^{bc}	4.56±0.50 ^c	1.75±0.14 ^a
Phe	13.37±0.61 ^b	24.00±1.57 ^c	6.08±0.91 ^b	1.36±0.06 ^c
Chitosan	19.09±0.79 ^a	38.10±3.89 ^a	9.75±1.42 ^a	1.43±0.18 ^c
Chitosan/Phe	19.45±0.68 ^a	34.28±3.77 ^b	9.77±0.93 ^a	1.62±0.06 ^b

Values superscripted with the same letter within a column are not significantly different according to the post hoc t-test ($p < 0.05$).

MDA levels were significantly lower in treated mandarins, where Phe and Chitosan treatments had the most pronounced effect, similar to findings by Gao et al. (2018). Additionally, Phe treatment has been shown to decrease MDA content in 'Jinfeng' pear fruit during storage at room temperature for 0-10 days (Wang et al., 2023). As fruits age during storage, membrane lipid oxidation increases. Overall, ECs effectively delayed fruit senescence by reducing membrane lipid peroxidation.

3. Conclusions

The findings of this study highlight the potential of phenylalanine and chitosan-based coatings in maintaining fruit quality by enhancing the bioactive profile, antioxidant activity and enzymatic regulation of mandarins. Previous research has shown that phenylalanine can stimulate mandarin fruit defense against pathogens, and the results of this work reveal the complexity of these interactions, indicating the need for further research. Nevertheless, phenylalanine, chitosan, or their combination significantly impacted the biochemical composition of mandarins, improving overall fruit quality. Chitosan also enhanced important sensory qualities, such as glossiness, bringing the produce closer to meeting consumer expectations for vibrant, healthier and longer-lasting products. However, a limitation of this study was the relatively short storage period. Chitosan and Chitosan/Phe coatings might have a more pronounced effect on organic acids during extended storage. Developing blends of ECs and active ingredients that allow a slowed release of components, such as phenylalanine, over time is crucial. This would enable a continuous supply of stimulants while maintaining the effectiveness of ECs in retaining moisture, regulating gas exchange, and ultimately preserving fruit quality.

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