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Original article

Changes in the bioaccessibility of citrus polyphenols during industrial freezing process

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Summary Citrus fruits can be consumed fresh or they can be subjected to various processes including freezing to extend their shelf life. The aim of the current study was to evaluate the bioaccessibility of polyphenols in samples collected at different stages of the industrial freezing process of oranges and grapefruits. The major polyphenols were identified and quantified using UPLC-ESI-MS/MS and HPLC-PDA, respectively. In addition, spectrophotometric methods were employed to determine the total phenolic and flavonoid contents along with total antioxidant capacity. The results showed that the bioaccessibility of flavonoids from frozen citrus fruits (26%–64%) was found to be lower than that of fresh fruits (27%–127%). Similarly, bioaccessibility of phenolic acids from frozen oranges (64%–76%) was significantly lower than that of fresh fruits (126%–152%). On the contrary, phenolic acids of frozen grapefruits were found to be more bioaccessible compared to fresh fruits (64%–217% and 50%–95%, respectively).

Keywords Grapefruit (*Citrus paradisi* L.), hesperidin, *in vitro* digestion, individual quick freezing, naringin, orange (*Citrus sinensis* L.).

Introduction

Citrus fruits are classified within the Rutaceae family and originate in Southeast Asia, which are later spread to different parts of the world from here. Today, citrus fruits are among the most important commercial products of the Mediterranean region (Langgut, 2017). According to the latest statistics, oranges and grapefruits rank among the citrus fruits with the highest global production rates. The world's leading orange producer is Brazil, whereas China provides more than 50% of the total grapefruit production. Türkiye, on the other hand, ranks 8th in the world in total grapefruit production with 3% contribution rate as well as being 2nd and 7th in the world with more than 400 thousand tons of grapefruit and orange exports worth approximately 290 and 105 million dollars, respectively (FAOSTAT, 2023a, 2023b).

Citrus fruits can be consumed fresh, or they can be subjected to various processes in order to increase their shelf life. Freezing process is widely recognised as a highly effective technique for preserving food, as it slows down the deterioration process by reducing enzymatic and microbial activities, oxidation, and respiration (Kamiloglu, 2019). Commercially, citrus fruits are commonly subjected to freezing process on a conveyor belt within a blast freezer that operates at low temperatures and high air velocity to produce individually quick frozen (IQF) products. In some cases, chemical peeling, also known as lye peeling or caustic peeling, may be applied as a pretreatment. In chemical peeling, in order to soften the segmental membranes of fruits, they are immersed in a concentrated hot alkali solution for a certain period of time. Subsequently, the membrane wall of the chemically treated fruit can be eliminated by rinsing it off using a water spray (Kohli *et al.*, 2021). Citric acid treatment may also be performed prior to freezing in order to extend the shelf life of peeled citrus fruits (De Corato, 2020).

Citrus fruits are rich in polyphenols, especially flavanone glycosides such as hesperidin, narirutin, and naringin (Feng *et al.*, 2020; Stabrauskiene *et al.*, 2023). These compounds are reported to exert health-promoting properties such as antioxidant, anti-inflammatory, anticancer, and cardiovascular protection activities (Yi *et al.*, 2017). In addition, hesperidin has also been proposed as a drug substance with promising activity against the coronavirus disease, which has turned into a global pandemic in recent years (Meneguzzo *et al.*, 2020). On the other hand, it

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is widely acknowledged that the health-promoting activities of polyphenols rely on their bioaccessibility within the gastrointestinal tract (Arfaoui, 2021). Various *in vitro* digestion models have been devised to investigate the bioaccessibility of polyphenols. These models have the advantages of being rapid and cheap, and they do not have ethical restrictions as human studies (Wojtunik-Kulesza *et al*., 2020). The differences in the conditions of *in vitro* digestion models led to the need for harmonisation of digestion methods. Accordingly, the standardised INFOGEST protocol, which incorporates fixed ratios and concentrations of food and enzymes, was developed based on relevant *in vivo* data (Minekus *et al*., 2014).

There are many factors affecting the bioaccessibility of polyphenols, one of which is food processing. Despite the presence of a limited number of studies in the existing literature that examined the changes in the bioaccessibility of citrus polyphenols as a result of processing such as pasteurisation and fermentation (Stinco *et al*., 2020; Andrade Barreto *et al*., 2023), to the best of our knowledge no previous study investigated the changes in bioaccessibility of orange and grapefruit polyphenols during industrial freezing process. Considering that, the aim of the current study was to evaluate the bioaccessibility of polyphenols in samples collected at different stages of the industrial freezing process of oranges and grapefruits using the standardised INFOGEST *in vitro* digestion protocol.

Materials and methods

Plant material

Oranges (*Citrus sinensis* L. cv. Washington Navel) and grapefruits (*Citrus paradisi* L. var. Star Ruby) underwent freezing process using an IQF (FloFreeze® MX; Frigoscandia, Helsingborg, Sweden) with respect to the flow chart given in Figs. S1 and S2, respectively. Over the course of 3 separate days, samples were gathered from the 11 designated points illustrated on the flow charts (Figs. S1 and S2). The samples were then transported to the laboratory under meticulously monitored conditions to ensure minimal degradation in quality. All samples were finely ground into a powder using a grinder (IKA A11 basic; IKA-Werke GmbH & Co., Staufen, Germany), and subsequently, the powdered samples were stored at −80 °C (Ultra-Low Temperature Freezer MDF-U5386S; Panasonic Co., Osaka, Japan).

Chemicals

All chemicals utilised in this study were of analytical, HPLC or LC–MS grade and acquired from Sigma-Aldrich Chemie GmbH & Co. KG (Steinheim, Germany), unless otherwise indicated.

In vitro digestion model

In vitro digestion model of INFOGEST was applied in triplicate using a shaking water bath (Nüve ST 30; Ankara, Türkiye). The compositions of the salivary, gastric, and intestinal fluids remained consistent with the specifications outlined in the protocol (Minekus *et al*., 2014). For simulation of oral digestion (OD), 10 g of samples were mixed with 7 mL salivary fluid, 1 mL of 1500 U mL^{-1} α-amylase (from human saliva), 0.05 mL of 0.3 M $CaCl_2$ (Tekkim, Bursa, Türkiye), and the volumes were completed to 10 mL with distilled water. The mixtures were incubated at 37 °C for 2 min, and following the completion of OD, 4 mL aliquots were collected. For simulation of gastric digestion (GD), 12 mL of gastric fluid, 2.56 mL of 25 000 U mL^{-1} pepsin (from porcine gastric mucosa), and 0.008 mL of 0.3 M $CaCl_2$ were added to the remaining mixtures. The pH was adjusted to 3 with 1 M HCl and the volumes were completed to 16 mL with distilled water. The mixtures were incubated at 37 °C for 2 h, and following the completion of GD, 4 mL aliquots were collected. For simulation of intestinal digestion (ID), 15.4 mL of intestinal fluid, 7 mL of 800 U mL^{-1} of pancreatin (from porcine pancreas), 3.5 mL of 160 mM bile (from porcine), and 0.056 mL of 0.3 M $CaCl_2$ were added to the remaining mixtures. The pH was adjusted to 7 with 1 M NaOH, and the volumes were completed to 28 mL with distilled water. The mixtures were again incubated at 37 °C for 2 h and following the completion of ID. Concurrently, a blank sample without the inclusion of citrus samples was incubated under identical conditions and used to correct interferences originating from the digestive fluids. The pH of all collected samples after OD, GD, and ID was adjusted to 2 with formic acid and centrifuged at 20 000 ***g*** for 5 min. The supernatants were stored at −20 °C.

Extraction of polyphenols

The extraction of polyphenols in undigested (UD) citrus samples was carried out as previously described (Kamiloglu, 2019). Five mL of 75% methanol with 0.1% formic acid was added to 2 g of the sample. The mixture was placed in an ultrasonic bath (Elma LC30H; Darmstadt, Germany) that is cooled with ice batteries and treated continuously at frequency of 50 Hz for 15 min, later centrifuged at 4 °C and 10 000 ***g*** for 10 min (Hitachi CF15RN; Tokyo, Japan). The supernatants were collected, and the procedure was repeated once again for the pellet. The two collected supernatants were pooled, and the final extracts were kept at −20 °C.

Identification of polyphenols with UPLC-ESI-MS/MS

Identification of citrus polyphenols was carried out using UPLC-ESI-MS/MS (Shimadzu LC–MS/MS 8060;

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Kyoto, Japan) equipped with C18 column (100 × 3 mm, 3 μm; GL Sciences, Tokyo, Japan) as previously described (Akpinar Bayizit *et al.*, 2023). Ten microliters of filtered samples were injected into the system using MQ water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) at a flow rate of 0.4 mL min^{-1}. The gradient was as the following: 0 min, 20% B; 0.0–0.5 min, 20% B, isocratic; 0.5–7.0 min, 20%–50% B, linear; 7.0–12.0 min, 50%–95% B, linear; 12.0–12.1 min, 95%–20% B, linear; 12.1–15.0 min, 20% B, isocratic. The temperature of the column was kept constant at 40 °C, while the autosampler temperature was maintained at 10 °C. Negative ESI mode was employed for the operation of the MS with the following set of operating conditions: nebulising gas (N_2) flow 3.0 L min^{-1}; drying gas (N_2) flow 10.0 L min^{-1}; interface voltage 4.0 kV; desolvation line temperature 250 °C; interface temperature 300 °C; and heat block temperature 400 °C. The spectra of the samples were recorded using multiple reaction monitoring (MRM) mode, and data acquisition was carried out with LabSolution software (Shimadzu Co.).

Quantification of polyphenols with HPLC-PDA

Citrus polyphenols were quantified using HPLC-PDA (-Shimadzu LC-2030; Kyoto, Japan) equipped with C18 column (250 × 4.6 mm, 5 μm; MZ-Analysentechnik, Mainz, Germany) as previously described (Kamiloglu, 2019). Ten microliters of filtered samples were injected into the system using MQ water with 0.1% trifluoracetic acid (TFA) (mobile phase A) and acetonitrile with 0.1% TFA (mobile phase B) at a flow rate of 1 mL min^{-1}. The linear gradient was as the following: 0 min, 5% B; 0–45 min, 35% B; 45–47 min, 75% B; 47–49 min, 35% B; 50 min, 5% B. The temperature of the column was kept constant at 40 °C, while the autosampler temperature was maintained at 10 °C. Spectral measurements were carried out at 280, 312, and 360 nm, and quantification was done using the following external standards: rutin (>97%, Acros Organics, New Jersey, USA), narirutin (99.9%, MedChemExpress, New Jersey, USA), naringin (90.5%, Dr. Ehrenstorfer, Augsburg, Germany), hesperidin (86.6%, Dr. Ehrenstorfer), neohesperidin (98.5%, MedChemExpress), gallic acid (Merck, Darmstadt, Germany), chlorogenic acid (TRC, Ontario, Canada), caffeic acid (HPC Standards, Borsdorf, Germany), and ferulic acid (TRC). Table S1 shows the analytical parameters of the standards that are used for the quantification of polyphenols. The results were expressed as mg kg^{-1} sample.

Spectrophotometric assays

All assays described below were carried out with a UV–visible spectrophotometer (Agilent Cary 60, Santa Clara, California, USA).

Total phenolic content (TPC) was assessed using Folin–Ciocalteu reagent as described previously (Velioglu *et al.*, 1998). TPC of the citrus samples was calculated using a gallic acid standard curve (linear range: 10–600 mg L^{-1}, $R^2 = 0.9999$), and results were reported as mg gallic acid equivalent (GAE) per 100 g of sample.

Total flavonoid content (TFC) was determined using $AlCl_3$ as described previously (Kim *et al.*, 2003). TFC of the citrus samples was calculated using a rutin standard curve (linear range: 1–800 mg L^{-1}, $R^2 = 0.9958$), and results were reported as mg rutin equivalent (RE) per 100 g of sample.

Total antioxidant capacity (TAC) was determined using 3 assays: Cupric ion reducing antioxidant capacity (CUPRAC) (Apak *et al.*, 2004), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Kumaran & Karunakaran, 2006) and ferric reducing antioxidant power (FRAP) (Benzie & Strain, 1996). TAC of the citrus samples was calculated using Trolox® standard curves (linear ranges: 10–800, 10–100 and 1–200 mg L^{-1}, respectively, $R^2 \geq 0.992$), and the results were reported as mg Trolox® equivalent (TE) per 100 g of sample.

Statistical analysis

All analyses were performed in triplicates, and the results were expressed as mean ± standard deviation. The data were analysed using SPSS software (version 24; Chicago, IL, USA). One-way ANOVA followed by a Tukey *post hoc* test was used to compare differences between treatments. ($P < 0.05$).

Results and discussion

Flavonoids

UPLC–ESI–MS/MS analysis of oranges and grapefruits led to the tentative identification of five major flavonoids (Table 1). Four of the identified flavonoids were belonging to flavanones, namely narirutin, naringin, hesperidin, and neohesperidin. Hesperidin was only identified in oranges, whereas naringin and neohesperidin were present in grapefruits. On the other hand, narirutin was found both in oranges and grapefruits. Besides flavanones, rutin, a flavonol, was also identified in oranges. Flavonoids were identified in negative mode by analysing the MS data, fragmentation pattern, and through comparison with existing literature data (Deng *et al.*, 2022; Xu *et al.*, 2022).

The changes in the bioaccessibility of flavonoids of oranges and grapefruits at different stages of industrial freezing process are presented in Tables 2 and 3, respectively. For UD oranges, hesperidin was determined as the predominant flavonoid accounting for 59%–84% of the flavonoids, whereas for UD grapefruits, naringin

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Table 1 Identification of the major polyphenols detected in the oranges (*Citrus sinensis* L.) and grapefruits (*Citrus paradisi* L.) using UPLC–ESI–MS/MS

Retention time (min)	Mass (m/z)	Other fragmental ions (m/z)	Tentative identification	Oranges (*Citrus sinensis* L.)	Grapefruits (*Citrus paradisi* L.)	Reference(s)
Flavonoids						
3.102	(−) 609.10	**300.00**, 271.10	Rutin	X		Xu *et al.* (2022)
3.585	(−) 579.30	**271.10**, 151.10	Narirutin	X	X	Deng *et al.* (2022); Xu *et al.* (2022)
3.808	(−) 578.90	459.20, **271.10**	Naringin		X	Deng *et al.* (2022)
3.913	(−) 609.0	303.10, **301.10**	Hesperidin	X		Xu *et al.* (2022)
4.106	(−) 609.30	**301.10**, 164.00	Neohesperidin		X	Deng *et al.* (2022)
Phenolic acids						
1.741	(−) 168.90	**125.10**, 79.0	Gallic acid	X		Hou *et al.* (2021)
2.010	(−) 353.10	**191.10**, 110.90	Chlorogenic acid	X	X	Hou *et al.* (2021); Xi *et al.* (2015)
2.670	(−) 178.80	**135.10**, 89.20	Caffeic acid	X	X	Hou *et al.* (2021); Xi *et al.* (2015)
3.794	(−) 192.90	178.10, **134.10**	Ferulic acid	X	X	Hou *et al.* (2021); Xi *et al.* (2015)

Main fragments are shown in bold.

was the dominant flavonoid representing 67%–91% of the flavonoids. Fruit peels contained significantly higher levels of flavonoids compared to fruit pulps ($P < 0.05$). This finding was in agreement with those reported previously (Chen *et al.*, 2020). After OD, significantly lower levels of flavonoids were obtained compared to UD samples, indicating that the short incubation time applied for OD (2 min) may be insufficient for the release of all flavonoids from the food matrix. Following GD, release of flavonoids generally increased compared to OD, while ID further enhanced the flavonoid levels in grapefruits. On the other hand, with a few exceptions, ID generally reduced the flavonoid levels in oranges, which may be partially explained by their conversion into chalcones in alkaline conditions (Fernández-Fernández *et al.*, 2021).

Pretreatments applied prior to freezing had different effects on citrus flavonoids. In particular, blanching significantly reduced the flavonoid content in oranges both before and after the completion of gastrointestinal digestion (13%–61%; $P < 0.05$). The elevated temperature of the blanching water likely resulted in the disruption of cell walls and the degradation of flavonoids, causing them to be released into the blanching water. The variations observed in the extent of flavonoid loss following the blanching treatment may be attributed to differences in the solubility and thermal stability of individual flavonoids in hot water (Irondi *et al.*, 2017). The presence of NaOH in blanching water could also contribute to loss of flavonoids through increment of pH. Citric acid treatment following blanching further reduced the level of flavonoids for undigested oranges, whereas for digested samples presence of citric acid had a promoting effect on flavonoid levels (up to 87% increase). Enhancement of digestive stability of flavonoids in the presence of citric acid has been reported previously (Shim *et al.*, 2012), which can be explained by the antioxidant effect of citric acid on flavonoids that can undergo degradation when exposed to the alkaline environment of the upper intestinal tract (Kamiloglu *et al.*, 2021). For grapefruits, removal of the segment membrane significantly reduced the levels of flavonoids at all stages of digestion (14%–58%; $P < 0.05$). This observation was in agreement with the literature reporting higher levels of flavonoids in segment membranes of grapefruits compared to juice vesicles (Xi *et al.*, 2015).

Freezing process had varying effects on orange flavonoids. Before digestion, higher levels of hesperidin were determined in frozen oranges compared to fresh fruit (27%; $P < 0.05$), whereas narirutin and rutin levels reduced significantly after freezing (53%–65%; $P < 0.05$). Following digestion, there was statistically no significant difference between hesperidin and rutin contents of fresh and frozen oranges ($P > 0.05$), while narirutin levels decreased significantly (66%; $P < 0.05$). The trend observed for grapefruit flavonoids was more consistent. Freezing enhanced the flavonoid levels for undigested grapefruits (53%–104%), while for digested samples frozen grapefruits contained less flavonoids compared to fresh fruit (21%–54%). Overall, the bioaccessibility of flavonoids from frozen citrus fruits (26%–64%) was found to be lower than that of fresh fruits (27%–127%). It is possible that freezing might have favoured the cleavage of glycosylated flavonoids during digestion. Moreover, formation of ice crystals during freezing damages the food matrix. This damage can potentially lead to cell wall breakdown, facilitating the release of flavonoids during digestion. However, it also increases their sensitivity to oxidation and degradation (Oliveira *et al.*, 2016; Kamiloglu, 2020).

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Table 2 Changes in the bioaccessibility of individual polyphenols of oranges (*Citrus sinensis* L.) at different stages of individual quick freezing (IQF) process

	Processing stage					
Digestion stage	**Fruit with peel**	**Fruit without peel**	**Peel**	**Blanching**	**Citric acid treatment**	**Freezing**
Flavonoids						
Rutin (mg kg^{-1})						
UD	16.7 ± 0.1A,b	9.2 ± 0.2A,c	63.8 ± 3.7A,a	5.0 ± 0.02A,d	3.6 ± 0.03A,d	4.3 ± 0.03A,d
OD	9.6 ± 0.2B,b	2.8 ± 0.2C,d	12.5 ± 0.2C,a	4.1 ± 0.02B,c	2.2 ± 0.3B,e	4.4 ± 0.02A,c
GD	17.5 ± 3.5A,b	5.9 ± 0.8B,c	45.5 ± 1.7B,a	3.9 ± 0.1C,c	2.2 ± 0.4B,c	3.0 ± 0.4B,c
ID	3.4 ± 0.1C,b	2.5 ± 0.1C,c	5.7 ± 0.2D,a	2.1 ± 0.1D,d	2.4 ± 0.1B,cd	2.8 ± 0.03B,c
Narirutin (mg kg^{-1})						
UD	194.2 ± 17.5A,c	253.3 ± 0.5A,b	543.7 ± 10.9A,a	121.5 ± 1.8A,d	78.6 ± 0.04A,e	89.6 ± 0.1A,e
OD	84.9 ± 0.7B,b	61.9 ± 7.9C,c	118.9 ± 1.0D,a	82.2 ± 2.9B,b	43.6 ± 8.2B,d	74.5 ± 0.3B,bc
GD	200.4 ± 25.8A,b	162.8 ± 5.2B,b	338.8 ± 24.0B,a	86.9 ± 1.4B,c	46.6 ± 7.6B,c	58.7 ± 4.6C,c
ID	99.3 ± 5.5B,b	148.0 ± 10.5B,a	159.6 ± 10.5C,a	58.0 ± 2.7C,cd	70.0 ± 2.9A,c	50.5 ± 0.5D,d
Hesperidin (mg kg^{-1})						
UD	514.6 ± 29.5A,b	382.6 ± 8.7A,c	988.9 ± 24.7A,a	331.0 ± 5.4A,d	300.6 ± 0.2A,d	487.0 ± 0.5A,b
OD	84.8 ± 12.5D,b	43.3 ± 4.8C,b	84.8 ± 18.8C,b	85.1 ± 0.7C,b	89.4 ± 12.8C,b	160.3 ± 35.6B,a
GD	175.8 ± 10.5B,a	125.3 ± 5.2B,c	165.9 ± 3.3B,ab	105.8 ± 1.6B,c	80.8 ± 14.6C,d	148.2 ± 5.2B,b
ID	129.9 ± 1.4C,bc	124.1 ± 1.0B,c	135.7 ± 6.5B,b	86.4 ± 4.3C,d	161.9 ± 6.3B,a	124.4 ± 1.4B,bc
Phenolic acids						
Gallic acid (mg kg^{-1})						
UD	12.0 ± 0.3A,b	6.7 ± 0.2A,c	31.7 ± 0.7A,a	2.8 ± 0.1B,d	2.4 ± 0.01B,d	3.0 ± 0.01B,d
OD	5.2 ± 1.1B,b	2.5 ± 0.3B,cd	8.8 ± 1.0C,a	2.5 ± 0.2B,cd	1.8 ± 0.2B,d	3.7 ± 0.1B,bc
GD	11.3 ± 2.6A,b	7.6 ± 0.7A,c	22.4 ± 1.3B,a	3.8 ± 0.1A,d	2.3 ± 0.4B,d	2.9 ± 0.5B,d
ID	5.3 ± 0.5B,cd	3.4 ± 0.1B,e	8.4 ± 0.5C,a	4.5 ± 0.6A,de	6.6 ± 0.1A,b	6.5 ± 0.5A,bc
Chlorogenic acid (mg kg^{-1})						
UD	42.2 ± 0.2A,b	12.3 ± 0.3A,c	112.3 ± 2.3A,a	7.7 ± 0.2A,d	7.0 ± 0.04A,d	8.8. ± 0.03A,d
OD	18.9 ± 1.3C,b	3.8 ± 0.5D,d	23.1 ± 1.3D,a	5.7 ± 0.03C,d	4.3 ± 0.6B,d	8.9 ± 0.1A,c
GD	35.4 ± 4.6B,b	8.7 ± 0.5B,c	73.8 ± 5.2B,a	6.5 ± 0.1B,c	4.5 ± 0.8B,c	6.6 ± 1.1A,c
ID	21.2 ± 1.6C,b	7.6 ± 0.2C,cd	39.8 ± 1.6C,a	3.9 ± 0.2D,d	5.2 ± 0.3B,d	9.9 ± 2.8A,c
Caffeic acid (mg kg^{-1})						
UD	8.3 ± 0.04A,b	5.7 ± 0.2A,c	20.3 ± 0.4A,a	2.5 ± 0.1B,e	3.3 ± 0.02B,d	3.8 ± 0.02B,d
OD	3.1 ± 0.1C,b	1.3 ± 0.1C,d	3.7 ± 0.1C,a	1.7 ± 0.01D,c	2.0 ± 0.3C,c	3.4 ± 0.02C,ab
GD	6.6 ± 1.5AB,b	3.6 ± 0.2B,c	10.1 ± 0.6B,a	2.1 ± 0.01C,c	2.2 ± 0.4C,c	2.8 ± 0.2D,c
ID	6.2 ± 0.2B,bcd	5.3 ± 0.1A,cd	9.8 ± 1.7B,a	4.3 ± 0.2A,d	6.4 ± 0.4A,bc	8.0 ± 0.1A,ab
Phenolic acids						
Ferulic acid (mg kg^{-1})						
UD	21.6 ± 0.3A,b	12.8 ± 0.3A,c	61.8 ± 2.5A,a	9.4 ± 0.2A,d	8.5 ± 0.01A,d	11.0 ± 0.01A,cd
OD	5.8 ± 0.5B,b	3.3 ± 0.2D,c	6.4 ± 1.0D,b	6.0 ± 0.01C,b	4.9 ± 0.8B,bc	8.2 ± 0.03B,a
GD	19.8 ± 4.3A,b	7.3 ± 0.3B,c	30.9 ± 2.4B,a	6.6 ± 0.1B,c	5.0 ± 1.0B,c	4.6 ± 0.7D,c
ID	9.9 ± 0.1B,b	6.4 ± 0.2C,c	23.6 ± 1.0C,a	4.2 ± 0.2D,d	6.5 ± 0.3B,c	7.0 ± 0.1C,c

The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in columns and small letters in the rows represent statistically significant differences ($P < 0.05$). GD, gastric digestion; ID, intestinal digestion; OD, oral digestion; UD, undigested.

Phenolic acids

Four major phenolic acids were tentatively identified in oranges and grapefruits using UPLC–ESI–MS/MS in negative mode (Table 1). Three of the identified phenolic acids including chlorogenic, caffeic, and ferulic acids belonging to hydroxycinnamic acids were present both in oranges and grapefruits. On the other hand, gallic acid, a hydroxybenzoic acid, was only identified in oranges. The phenolic acid profile of oranges and grapefruits was compatible with those reported previously (Xi *et al.*, 2015; Hou *et al.*, 2021).

The changes in the bioaccessibility of phenolic acids of oranges and grapefruits at different stages of industrial freezing process are presented in Tables 2 and 3, respectively. For UD oranges and grapefruits, chlorogenic and ferulic acid were determined as the predominant compounds accounting for 33%–63% and 16%–42% of the total phenolic acids, respectively. Fruit peels contained significantly higher levels of phenolic acids

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Table 3 Changes in the bioaccessibility of individual polyphenols of grapefruits (*Citrus paradisi* L.) at different stages of individual quick freezing (IQF) process

	Processing stage				
Digestion stage	**Fruit with peel**	**Fruit without peel**	**Peel**	**Segment membrane removal**	**Freezing**
Flavonoids					
Narirutin (mg kg^{-1})					
UD	590.9 ± 1.3A,a	133.9 ± 0.1B,d	491.2 ± 0.1B,b	85.8 ± 0.1B,e	211.3 ± 0.1A,c
OD	275.4 ± 26.6C,a	77.3 ± 3.4C,c	128.1 ± 5.4D,b	58.8 ± 0.5C,c	60.3 ± 8.7C,c
GD	416.8 ± 15.7B,a	58.6 ± 4.1D,d	276.3 ± 4.7C,b	69.1 ± 1.2C,d	113.3 ± 27.8B,c
ID	631.8 ± 13.5A,b	162.6 ± 3.5A,c	824.2 ± 14.6A,a	113.9 ± 7.9A,d	128.1 ± 2.1B,d
Naringin (mg kg^{-1})					
UD	4292.2 ± 0.1B,b	461.4 ± 0.3B,d	5295.9 ± 1.9B,a	193.7 ± 0.1B,e	811.6 ± 5.4A,c
OD	1490.4 ± 131.2C,a	212.9 ± 15.3C,c	1242.8 ± 116.8D,b	137.9 ± 1.2C,c	222.3 ± 48.7C,c
GD	1494.8 ± 51.1C,b	219.9 ± 3.6C,A,d	3268.1 ± 64.3C,a	150.8 ± 5.1C,d	444.5 ± 119.7B,c
ID	4685.7 ± 107.0A,b	548.8 ± 11.5A,c	7912.0 ± 696.9A,a	243.6 ± 17.3A,c	249.9 ± 34.2C,c
Neohesperidin (mg kg^{-1})					
UD	41.2 ± 0.8B,a	15.2 ± 0.01B,d	34.2 ± 0.01B,b	8.3 ± 0.02B,e	31.0 ± 0.1A,c
OD	17.1 ± 3.7C,a	7.9 ± 0.3C,b	7.6 ± 0.7D,b	5.4 ± 0.1D,b	8.7 ± 1.7C,b
GD	21.8 ± 0.4C,a	8.3 ± 0.1C,b	21.4 ± 0.2C,a	7.1 ± 0.1C,b	17.2 ± 4.0B,a
ID	48.9 ± 1.3A,a	19.3 ± 0.5A,b	51.2 ± 1.2A,a	10.8 ± 0.7A,c	10.9 ± 0.2C,c
Phenolic acids					
Chlorogenic acid (mg kg^{-1})					
UD	37.6 ± 0.2B,b	13.2 ± 0.1B,e	61.3 ± 0.1B,a	14.6 ± 0.1B,d	22.0 ± 0.03A,c
OD	22.1 ± 3.2C,a	9.4 ± 0.8C,b	18.9 ± 1.6D,a	8.4 ± 0.02C,b	7.6 ± 0.5C,b
GD	32.6 ± 2.0B,a	6.7 ± 0.4D,c	36.8 ± 0.2C,a	9.2 ± 0.1C,bc	13.5 ± 3.1B,b
ID	50.1 ± 0.8A,b	19.9 ± 0.3A,c	96.0 ± 1.3A,a	16.7 ± 1.1A,d	16.7 ± 0.4B,d
Caffeic acid (mg kg^{-1})					
UD	19.1 ± 0.1B,a	11.0 ± 0.02B,e	13.8 ± 0.4B,c	11.5 ± 0.02A,d	17.0 ± 0.01A,b
OD	10.0 ± 1.2D,a	7.0 ± 0.4C,b	4.0 ± 0.4D,c	6.8 ± 0.01B,b	5.9 ± 0.9C,b
GD	13.3 ± 0.1C,a	6.9 ± 0.2C,b	8.0 ± 0.02C,b	7.8 ± 0.1B,b	11.4 ± 2.6B,a
ID	20.7 ± 0.3A,a	16.7 ± 0.2A,b	16.0 ± 0.2A,b	11.9 ± 0.8A,c	10.9 ± 0.6B,c
Ferulic acid (mg kg^{-1})					
UD	11.0 ± 0.1B,b	6.1 ± 0.02B,d	22.8 ± 0.1B,a	5.8 ± 0.01A,e	8.3 ± 0.01A,c
OD	6.3 ± 1.1D,a	3.9 ± 0.2C,b	6.9 ± 0.6D,a	3.4 ± 0.03C,b	2.8 ± 0.3C,b
GD	9.2 ± 0.2C,b	2.8 ± 0.02D,d	12.5 ± 0.1C,a	4.2 ± 0.05B,cd	5.6 ± 1.4B,c
ID	14.5 ± 0.3A,b	7.7 ± 0.2A,c	40.6 ± 0.4A,a	6.7 ± 0.5A,d	6.1 ± 0.4AB,d

The data presented in this table consist of average values ± standard deviation of three independent batches. Different capital letters in columns and small letters in the rows represent statistically significant differences ($P < 0.05$). GD, gastric digestion; ID, intestinal digestion; OD, oral digestion; UD, undigested.

compared to fruit pulps ($P < 0.05$). With a few exceptions, a trend similar to that of flavonoids was seen during the digestion of phenolic acids. Low levels of phenolic acids observed after OD were increased after GD and ID further enhanced phenolic acid levels in grapefruits. Again, with a few exceptions, ID generally reduced the phenolic acid levels in oranges.

Blanching reduced phenolic acid levels in UD oranges (26%–58%), whereas this treatment generally caused no significant difference after digestion ($P < 0.05$). The industrial blanching process is known to remove most of the water-soluble polyphenols (Mandalari *et al.*, 2016), which could explain the reduced phenolic acid content of UD oranges. On the other hand, the remaining phenolic acids that are linked to blanched fruit matrix, hence initially inaccessible, seem to release at a higher rate compared to unblanched fruit following digestion. As in flavonoid results, citric acid treatment following blanching had a promoting effect on phenolic acid levels for digested oranges (33%–54%). For grapefruits, although effect of segment membrane removal on phenolic acid levels varied before digestion, digested fruits contained significantly less phenolic acids after removal of segment membranes (14%–29%; $P < 0.05$). Freezing reduced the phenolic acids for UD oranges (14%–55%), while increased levels of phenolic acids were observed after digestion (up to 91%). Freezing induces the formation of a crystal structure within the plant cell, leading to the disruption of the cell wall and hence higher susceptibility to oxidation and degradation

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of phenolic compounds prior to digestion. At the same time, these alterations in the matrix are likely to result in enhanced extractability of phenolic compounds during digestion (Ribas-Agustí *et al*., 2018). Confirming the findings in our study, bioaccessibility of ferulic acid in strawberries (Balasooriya *et al*., 2020) and *p*-coumaric acid derivatives in spinach (Kamiloglu, 2020) were also enhanced as a result of freezing process. For grapefruits, an opposite observation was recorded. UD-frozen grapefruits contained significantly higher levels of phenolic acids compared to fresh fruits (37%–67%; $P < 0.05$). However, freezing resulted in reduced phenolic acid content of grapefruits after digestion (16%–34%; $P < 0.05$). Overall, the bioaccessibility of phenolic acids from frozen oranges (64%–76%) was significantly lower than that of fresh fruits (126%–152%). On the contrary, phenolic acids frozen grapefruits were found to be more bioaccessible compared to fresh fruits (64%–217% and 50%–95%, respectively). These results suggest that pretreatments, particularly blanching, rather than freezing alone may be responsible from the reduced bioaccessibility of phenolic acids from oranges.

Total phenolics, flavonoids, and antioxidant capacity

The changes in the bioaccessibility of TPC, TFC, and TAC of oranges and grapefruits at different stages of industrial freezing process are shown in Tables 4 and 5, respectively. Among UD samples, fruit peels contained higher levels of TPC, TFC, and TAC compared to fruit pulps. After OD, lower levels of TPC, TFC, and TAC were observed compared to UD samples, while GD generally increased these parameters. ID reduced TPC, TFC, and TAC in oranges and majority of the grapefruit samples. Therefore, the trend observed during digestion was mostly in line with the results obtained with chromatographic analysis.

Blanching pretreatment significantly reduced TPC, TFC, and TAC of oranges both before and after gastrointestinal digestion (33%–72%; $P < 0.05$) except for

Table 4 Changes in the bioaccessibility of total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) of oranges (*Citrus sinensis* L.) at different stages of individual quick freezing (IQF) process

	Processing stage					
Digestion stage	**Fruit with peel**	**Fruit without peel**	**Peel**	**Blanching**	**Citric acid treatment**	**Freezing**
TPC (mg GAE 100 g^{-1})						
UD	230.9 ± 18.7A,b	165.5 ± 15.6B,c	350.3 ± 16.3A,a	86.3 ± 7.6AB,d	103.1 ± 3.5A,d	101.7 ± 4.4B,d
OD	92.4 ± 1.4C,a	59.4 ± 1.8D,b	92.0 ± 2.7C,a	55.6 ± 4.3C,b	45.3 ± 0.5D,c	52.8 ± 3.1D,b
GD	185.0 ± 5.4B,b	210.0 ± 15.4A,b	278.1 ± 28.2B,a	90.9 ± 3.9 A,c	95.9 ± 1.3 B,c	111.5 ± 3.2 A,c
ID	113.5 ± 4.2 C,b	115.7 ± 6.1 C,ab	128.2 ± 3.6 C,a	71.2 ± 8.2 BC,c	60.6 ± 1.2 C,c	73.3 ± 1.9 C,c
TFC (mg RE 100 g^{-1})						
UD	34.2 ± 6.1 A,b	34.6 ± 2.5 B,b	126.6 ± 11.5 A,a	23.2 ± 5.8 A,bc	28.4 ± 0.3 A,bc	16.8 ± 4.3 A,c
OD	11.1 ± 0.4 B,b	10.1 ± 1.5 C,b	25.7 ± 1.5 C,a	7.2 ± 1.2 B,c	11.1 ± 0.4 B,b	6.9 ± 0.1 B,c
GD	32.6 ± 4.1 A,c	104.3 ± 2.8 A,a	50.6 ± 5.0 B,b	21.8 ± 3.5 A,d	10.4 ± 1.5 B,e	5.6 ± 1.1 B,e
ID	7.5 ± 0.7 B,ab	8.8 ± 1.0 C,a	4.8 ± 0.9 D,c	5.4 ± 0.5 B,bc	5.9 ± 1.0 C,bc	4.8 ± 1.1 B,c
TAC (mg TE 100 g^{-1})						
CUPRAC assay						
UD	219.6 ± 9.4A,b	181.7 ± 12.3A,c	299.1 ± 17.6A,a	87.3 ± 12.4A,d	78.2 ± 8.2A,d	104.7 ± 8.3A,d
OD	75.5 ± 8.4D,a	47.5 ± 8.2C,b	65.2 ± 3.1D,a	28.4 ± 2.8C,c	10.0 ± 0.2B,d	19.4 ± 2.1C,cd
GD	153.6 ± 3.8B,c	199.3 ± 17.2A,b	237.6 ± 11.6B,a	69.2 ± 1.7B,d	74.0 ± 12.3A,d	85.9 ± 11.5AB,d
ID	99.0 ± 9.3C,b	107.0 ± 13.2B,ab	127.0 ± 0.3C,a	41.2 ± 0.4C,d	62.0 ± 7.1A,cd	74.0 ± 9.7B,c
DPPH assay						
UD	51.2 ± 3.6B,a	56.6 ± 3.6B,a	60.2 ± 7.0B,a	57.3 ± 4.7A,a	56.1 ± 2.6B,a	61.5 ± 3.7A,a
OD	38.1 ± 0.4C,a	32.0 ± 3.1C,c	19.8 ± 1.2D,d	37.1 ± 1.9B,ab	33.0 ± 0.6C,bc	39.6 ± 0.3B,a
GD	91.7 ± 0.7A,b	107.8 ± 5.3A,a	87.2 ± 1.9A,bc	53.3 ± 6.7A,e	74.9 ± 7.8A,cd	61.4 ± 6.5A,de
ID	31.5 ± 5.9C,bc	27.9 ± 3.9C,c	43.1 ± 3.3C,a	39.7 ± 4.5B,ab	41.0 ± 1.4C,ab	44.2 ± 4.7B,a
FRAP assay						
UD	45.7 ± 1.4B,b	31.0 ± 3.1A,c	69.2 ± 1.0A,a	14.3 ± 2.9C,d	16.0 ± 2.5C,d	18.8 ± 2.5C,d
OD	40.0 ± 0.8C,a	29.9 ± 0.6A,b	23.9 ± 0.5D,c	29.4 ± 0.5B,b	24.0 ± 1.3B,c	31.3 ± 2.0B,b
GD	63.4 ± 1.9A,ab	28.4 ± 4.8AB,e	58.9 ± 1.8B,b	41.5 ± 1.7A,d	52.1 ± 0.3A,c	66.9 ± 0.8A,a
ID	27.1 ± 0.7D,a	21.4 ± 1.2B,b	29.6 ± 0.6C,a	6.0 ± 1.3D,cd	4.9 ± 1.0D,d	9.1 ± 1.9D,c

The data presented in this table consist of average values ± standard deviation of three independent batches. TPC, TFC, and TAC are expressed as mg of gallic acid equivalent (GAE), rutin equivalent (RE), and Trolox® equivalent (TE) per 100 g of sample, respectively. Different capital letters in columns and small letters in the rows represent statistically significant differences ($P < 0.05$). CUPRAC, cupric ion reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GD, gastric digestion; ID, intestinal digestion; OD, oral digestion; UD, undigested.

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Table 5 Changes in the bioaccessibility of total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) of grapefruits (*Citrus paradisi* L.) at different stages of individual quick freezing (IQF) process

	Processing stage				
Digestion stage	**Fruit with peel**	**Fruit without peel**	**Peel**	**Segment membrane removal**	**Freezing**
TPC (mg GAE 100 g^{-1})					
UD	302.7 ± 7.4C,b	92.7 ± 2.0B,d	357.3 ± 12.2B,a	89.0 ± 1.0C,d	132.6 ± 7.3A,c
OD	125.5 ± 2.6D,a	54.9 ± 2.2C,b	125.5 ± 8.8D,a	45.5 ± 1.5D,b	52.1 ± 2.9B,b
GD	320.6 ± 8.9B,a	125.5 ± 2.0A,c	272.8 ± 23.0C,b	119.6 ± 4.3B,c	125.3 ± 2.6A,c
ID	356.1 ± 2.3A,b	125.3 ± 3.4A,c	477.1 ± 20.0A,a	134.6 ± 3.6A,c	130.7 ± 4.0A,c
TFC (mg RE 100 g^{-1})					
UD	50.9 ± 3.3A,b	31.8 ± 6.5A,c	75.9 ± 10.9A,a	26.8 ± 5.3A,c	36.4 ± 4.3A,bc
OD	20.7 ± 0.1B,a	12.3 ± 1.4B,b	20.4 ± 2.3B,a	8.8 ± 1.3B,b	9.7 ± 0.3B,b
GD	48.3 ± 3.4A,b	26.9 ± 4.5A,c	69.9 ± 0.1A,a	22.4 ± 2.1A,c	28.6 ± 3.6A,c
ID	7.7 ± 0.6C,b	4.5 ± 0.3B,c	6.3 ± 0.8B,bc	10.1 ± 0.7B,a	4.3 ± 0.5B,c
TAC (mg TE 100 g^{-1})					
CUPRAC assay					
UD	235.1 ± 22.7A,a	105.3 ± 4.9B,c	261.8 ± 15.0A,a	112.2 ± 14.3A,c	177.1 ± 18.1A,b
OD	81.6 ± 7.6B,a	47.5 ± 1.2D,b	81.0 ± 3.3B,a	44.8 ± 4.1C,b	46.1 ± 5.9C,b
GD	203.6 ± 13.1A,b	128.3 ± 14.0A,c	263.7 ± 9.3A,a	126.1 ± 14.1A,c	108.8 ± 10.3B,c
ID	213.0 ± 9.9A,b	79.5 ± 6.1C,c	255.7 ± 12.2A,a	79.4 ± 6.6B,c	78.2 ± 9.2B,c
DPPH assay					
UD	71.6 ± 2.6B,a	44.7 ± 5.4C,b	76.0 ± 2.4B,a	42.2 ± 6.4B,b	41.7 ± 3.2BC,b
OD	24.8 ± 1.0D,cd	35.5 ± 1.1D,a	20.5 ± 3.3D,d	32.2 ± 0.4BC,ab	27.2 ± 4.0C,bc
GD	117.3 ± 1.8A,a	120.2 ± 2.0A,a	93.6 ± 1.7A,b	116.8 ± 4.0A,a	113.9 ± 9.2A,a
ID	63.0 ± 1.9C,a	64.7 ± 2.6B,a	65.2 ± 4.3C,a	26.8 ± 1.8C,c	45.9 ± 4.5B,b
FRAP assay					
UD	52.4 ± 6.9B,bc	39.1 ± 3.5BC,d	65.8 ± 3.4AB,a	45.9 ± 2.6AB,cd	60.7 ± 4.6A,ab
OD	25.9 ± 2.0C,bc	33.3 ± 1.0C,a	21.1 ± 3.0C,c	27.2 ± 1.7C,b	29.8 ± 1.5C,ab
GD	65.8 ± 5.0A,a	59.9 ± 3.4A,a	56.8 ± 6.9B,a	54.9 ± 8.9A,a	52.4 ± 1.4B,a
ID	60.5 ± 1.4AB,b	41.1 ± 1.8B,c	74.5 ± 4.1A,a	36.6 ± 2.5BC,cd	30.5 ± 0.5C,d

The data presented in this table consist of average values ± standard deviation of three independent batches. TPC, TFC, and TAC are expressed as mg of gallic acid equivalent (GAE), rutin equivalent (RE), and Trolox® equivalent (TE) per 100 g of sample, respectively. Different capital letters in columns and small letters in the rows represent statistically significant differences ($P < 0.05$). CUPRAC, cupric ion reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GD, gastric digestion; ID, intestinal digestion; OD, oral digestion; UD, undigested.

the results obtained with DPPH assay. This observation might be related to the leaching of hydrophilic antioxidants into the blanching water. DPPH assay is recognised to be suitable for lipophilic antioxidants, whereas FRAP assay quantifies hydrophilic antioxidants and CUPRAC assay can determine both. Furthermore, interpretation of the results may be hindered due to the presence of overlapping spectra from certain antioxidants, such as carotenoids, that are absorbed in similar wavelength ranges as DPPH (Capanoglu *et al.*, 2022). Citric acid treatment of oranges and segment membrane removal of grapefruits mostly did not induce significant differences in TPC, TFC, and TAC ($P > 0.05$).

Freezing reduced TPC, TFC, and TAC of oranges (except for DPPH) both before and after digestion (31%–66%; $P < 0.05$). On the other hand, while TFC and DPPH assay results of frozen and fresh grapefruits were statistically not significant ($P > 0.05$), TPC as well as CUPRAC and FRAP results revealed significant enhancement for UD grapefruits as a result of freezing (36%–68%; $P < 0.05$). At this point, differences seen between the results of spectrophotometric and chromatographic assays may be due to the presence of other hydrophilic antioxidants in citrus fruits such as ascorbic acid. TPC assay based on Folin–Ciocalteu reagent lacks specificity for phenolic compounds. The presence of reducing agents like ascorbic acid, citric acid, simple sugars, or specific amino acids can potentially interfere with the analysis and lead to an overestimation of TPC (Capanoglu *et al.*, 2022). Similarly, although the spectrophotometric assay involving $AlCl_3$ is one of the most commonly applied method for the measurement of TFC, a study by Huang *et al.* (2018) reported that this method could only detect flavonols and flavones with a C-5 hydroxyl group and is insufficient for the determination of TFC in citrus fruits. Due to the lack of specificity in the

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assays employed for determining TPC and TFC, it is recommended to complement the analysis with chromatographic methods to identify and quantify polyphenols, as demonstrated in this study.

Overall, the differences observed between oranges and grapefruits seem to be largely due to the blanching pretreatment applied to oranges. Inner membrane of the citrus fruits can either be removed in blanching water containing NaOH (as in case of oranges) or this procedure can also be done by hand (as in case of grapefruit). The cost of the production increases significantly when the peeling is carried out by hand. In the industry, the production method depends on the need of the customer. However, from the results obtained in this study, it appears that removal of inner membranes by hand instead of blanching would probably eliminate or reduce the observed adverse effects on orange antioxidants.

Conclusions

The findings obtained in the current study demonstrated that the bioaccessibility of flavonoids from frozen citrus fruits was found to be lower than that of fresh fruits. For phenolic acids of oranges, a similar trend was recorded, whereas for grapefruits bioaccessibility phenolic acids were enhanced as a result of industrial freezing process. The differences observed between oranges and grapefruits appear to be largely due to the blanching pretreatment applied to oranges. Given this observation, removal of inner membranes by hand instead of blanching would likely to improve the bioaccessibility of polyphenols from oranges. Moreover, this study solely focuses on the impact of processing, while it is also crucial to investigate the alterations in citrus polyphenols during frozen storage. In addition, the outcomes obtained here only apply to oranges and grapefruits; therefore, further investigation is necessary for other citrus fruits. In conclusion, additional *in vivo* research is necessary to comprehensively comprehend the bioaccessibility of polyphenols from citrus fruits that have undergone an industrial freezing process.

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Author contributions

Nurdan Ozdemirli: Formal analysis (lead); investigation (lead); validation (lead); visualization (lead); writing – original draft (lead). **Senem Kamiloglu:** Conceptualization (lead); funding acquisition (lead); methodology (lead); project administration (lead); resources (lead); supervision (lead); writing – review and editing (lead).

Conflict of interest

The authors declare no conflicts of interest.

Ethical approval

Ethics approval was not required for this research.

Peer review

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Data availability statement

Data available on request from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flow chart outlining the steps involved in the industrial freezing process of orange (*Citrus sinensis* L.) slices. Samples examined in the current study: (1) Fruit with peel, (2) Fruit without peel, (3) Peel, (4) Blanching, (5) Citric acid treatment, (6) Freezing.

Figure S2. Flow chart outlining the steps involved in the industrial freezing process of grapefruit (*Citrus paradisi* L.) slices. Samples examined in the current study: (7) Fruit with peel, (8) Fruit without peel, (9) Peel, (10) Segment membrane removal, (11) Freezing.

Table S1. Standards used for the quantification of polyphenols.

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