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ORIGINAL CONTRIBUTION

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Effect of domestic cooking on human bioavailability of naringenin, chlorogenic acid, lycopene and β-carotene in cherry tomatoes

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Summary *Background* Epidemiological data showed that tomato and tomato product (sauce, paste) consumption is associated with a protective effect against the development of some chronic-degenerative diseases. Tomato antioxidant bioactive molecules such as carotenoids and polyphenols could be responsible, at least in part, for the healthy effect observed. The bioavailability of these compounds is an essential requirement to sustain their *in vivo* role. While it is well known that many factors can influence the bioaccessibility of carotenoids from the food matrix, there is little information about the factors affecting phenolic compounds' bioaccessibility. *Aim of the study* This investigation was carried out to evaluate the effect of domestic cooking on the bioavailability in humans of antioxidant molecules after the administration of a test meal containing cherry tomatoes. *Methods* A cross-over design was conducted. Subjects (3 females and 2 males) consumed experimental meals containing fresh and cooked cherry tomatoes. Blood collection was performed at different time intervals (0, 2, 4, 6, 8 and 24 h). *Results* Carotenoid and phenol plasma concentrations were measured. Plasma levels of lycopene and β-carotene were not significantly different with respect to the baseline after ingestion of both the test meals, while plasma concentrations of naringenin and chlorogenic acid increased significantly with respect to the baseline ($P < 0.05$) after administration of cooked cherry tomatoes, but not after administration of fresh cherry tomatoes. *Conclusions* The present study indicated that domestically cooked tomatoes significantly increase naringenin and chlorogenic acid plasma levels. Considering that both naringenin and chlorogenic acid are widely studied for their potential healthy properties, evidence of their bioavailability and of the factors influencing their bioaccessibility is an important tool to sustain the possibility that these polyphenols play a biological role in human physiology.

Key words tomato – domestic cooking – carotenoids – naringenin – chlorogenic acid – bioavailability

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Introduction

Cherry tomatoes represent one of the most widely consumed fresh or mildly cooked food items, especially in the Mediterranean basin [1]. They are mainly cultivated in the Sicily region, grown in warm greenhouses and produced all the year round. Thanks to the peculiar conditions in terms of climate (e. g., high UV radiation level) and irrigation water (e. g., high salinity) these tomatoes are tasty, sweet and palatable. Cherry tomatoes are widely available at the national and international level covering more than 25 % of the market of tomatoes for fresh consumption and its commercial importance is continuously increasing [2, 3].

Epidemiological studies [4] have demonstrated that the consumption of raw tomatoes and tomato products (paste, puree) is associated with a reduction in the incidence of chronic-degenerative diseases. This protective effect has been attributed to their high content of various dietary components, e. g., carotenoids and polyphenolic compounds that exert different bioactive properties.

Lycopene and β-carotene are the most prevalent carotenoid pigments in tomatoes, lycopene content being more than ten-fold higher than β-carotene. Tomatoes are the main source of lycopene in the human diet, containing between 40–180 mg/kg (fresh weight) [5].

Both lycopene and β-carotene exert antioxidant properties *in vitro*, acting as $^1O_2$ quenchers and radical trapping agents [6]. Beta-carotene serves as an important source of vitamin A, while recent epidemiological data show a significant inverse correlation between lycopene intake and the incidence of, among the others, prostate and gastrointestinal tract cancers [7].

Tomatoes are also a good source of polyphenolic compounds, such as flavonoids and hydroxycinnamic acids. Among them, naringenin (flavanone) and chlorogenic acid (hydroxycinnamic acid) are the most abundant; their amounts range between 8–42 and 13–38 mg/kg of whole red fresh tomatoes respectively [5]. Both the above mentioned compounds have been extensively studied during the last few years for their biological and antioxidant properties that could interact with human physiology, exerting beneficial effects [8–10]. While it is well known that a lot of factors can influence the bioaccessibility of carotenoids from the food matrix, among them heat and mechanical treatments of the food and the presence of fat in the meal [11–13], there is little information about the factors affecting phenolic compounds' bioaccessibility. The present study was conducted to determine whether domestic cooking of this widely consumed tomato variety affects the plasma carotenoid and polyphenol concentrations in humans, by single administration of cooked cherry tomatoes compared to fresh tomatoes.

Materials and methods

Chemicals and reagents

All chromatographic standards, β-glucoronidase and sulfatase were purchased from Sigma (St. Louis, MO, USA). Methanol, formic acid and ammonium acetate were obtained from Merck (Darmstadt, Germany). Orthophosphoric acid (85 %) and sodium dihydrogen orthophosphate 1-hydrate were purchased from BDH (Poole, UK). All chemicals and solvents used were of analytical or HPLC grade. Distilled water was purified using a Milli-Q (18 MΩ) water purification system (Millipore, Milan, Italy).

Subjects

Five healthy non-smoking subjects (3 females and 2 males), average age 27 ± 3 years, body weight 61 ± 10 kg and body mass index 22 ± 3 kg/m^2, were recruited on the basis of their clinical anamnesis and normal blood values. Ethical approval for the study was obtained by the Ethical Committee of the National Institute for Food and Nutrition Research and all subjects gave their signed informed consent prior to participation in the study. The number of subjects was considered sufficient for the study as several studies [12–15] have shown a significant effect of treatment with a small number of subjects. Subjects did not use any medications that could interfere with the absorption; they did not use dietary supplements (such as vitamins or minerals) and did not report gastrointestinal disturbances. During the three days before each experimental day, subjects were asked to follow a controlled diet avoiding tomatoes, orange fruit and grapefruit (rich in naringenin glycosides), coffee (rich in chlorogenic acid) and all their derived products, and limiting fruit and vegetable consumption to not more than 200 g/day. A dietician surveyed food consumption during the three days of wash out by both the recall and the record method and checked that the contribution of each macronutrient to the total energy content of the diet was balanced (15 % by protein, 25 % by lipids, 60 % by carbohydrates).

Study design

All subjects, after fasting overnight, consumed 500 g of fresh cherry tomatoes together with 70 g of pasta, 25 g of extra virgin olive oil and not more than 50 g of bread.

The experimental meal was served at 8:30 in the morning, after fasting blood samples had been drawn. One week later the same subjects received 500 g of cherry tomatoes cooked for 15 min at 100 °C, together with the same quantity of pasta, extra virgin olive oil and

bread. The total energy content and macronutrient composition of the meals were calculated for each subject (considering that bread consumption was not the same for all subjects) by using the Food Composition Tables [16]. The total energy content, fiber and macronutrient composition, lycopene, β-carotene, naringenin and chlorogenic acid contents of the meals are reported in Table 1. The tomato portion (500 g) was chosen on the basis of the available literature data and our experience [12, 17–19], in such a way as to have an ingestion of carotenoids and polyphenols suitable to observe a plasma response following a single administration.

During the experimental days, a diet composed of fried and boiled potatoes together with olive oil, low in carotenoids and phenols, was consumed 6 h after the experimental meal.

Tomato sampling and analysis

Greenhouse-grown cherry tomatoes (cv. Naomi F1) were obtained during April 2001 from southeastern Sicily, a region of Italy widely exploited for tomato greenhouse cultivation. To avoid effects of growing conditions and techniques on berries composition, fruits were harvested from the same farm chosen because it was representative of the growing area. After harvesting, tomatoes were selected on the basis of external color (i. e. L: $\sum$ a*: $\sum$ and b*: $\sum$. chromatic coordinates) in order to have samples at the same ripening stage corresponding to the marketing ripening. After harvesting fruits were kept for 2 days at ambient temperature before the experiment (in order to simulate the minimum time elapsing between harvesting and home consumption).

Fresh and cooked tomatoes, as consumed, have been analyzed for carotenoid and polyphenol contents in triplicate.

The determination of carotenoids in the tomatoes was carried out in agreement with Sharpless et al. [20], and the quantification was performed by HPLC.

Polyphenols were both hydrolyzed and not, in order to obtain free and conjugated forms, and extracted as described by Hertog et al. [21]. We verified the suitability of the method also for hydroxycinnamic acid carrying out the same validation procedure as described by Hertog et al. [21]. Quantitative analysis was performed using an ESA HPLC system. The HPLC system used consisted of an ESA MODEL 540 refrigerated autoinjector (4 °C), an ESA MODEL 580 solvent delivery module with two pumps, an ESA 5600 eight-channels coulometric electrode array detector and the ESA coularray operating software which controlled all the equipment and carried out data processing (ESA, Chelmsford, MA, USA). A Supelcosil LC-18 column (25 × 4.6, 5 μm) with a Perisorb Supelguard LC-18 (Supelco, Milan, Italy) was used. Chromatography was performed at 30 °C, at a flow rate of 1 ml min^{-1} using the following solvent system: A 0.03 mol/l sodium phosphate adjusted to pH 2.8 with 85 % orthophosphoric acid; B methanol. The linear gradient used consisted of 87 % of solvent A, decreasing to 60 % over 13.5 min and to 10 % over 25.5 min reaching the final condition of 0 % 3 min later, after that returning to 87 % of solvent A over 3 min and maintaining this condition for 4 min.

The flow rate of the eluent was constant at 1 ml/min and the setting potentials were 60, 120, 200, 340, 480, 620, 760 and 900 mV. Sample peaks were analyzed with the method of external standard.

Table 1 Macronutrients and fibre composition, total energy content, and lycopene, β-carotene, naringenin and chlorogenic acid contents of the experimental meals. Total energy content and macronutrients were calculated by using Food Composition Tables (National Institute for Food and Nutrition Research, 2000) and are reported as mean values ± SD (n = 5 subjects) . Lycopene, β-carotene, naringenin and chlorogenic acid are reported as mean values ± SD (n = 3 replicate analysis)

	Meal containing fresh tomatoes	Meal containing cooked tomatoes
Protein (g)	18.0 ± 2.2	18.7 ± 2.4
Lipid (g)	26.4 ± 0.2	26.5 ± 0.2
Carbohydrate (g)	114.9 ± 15.2	120.3 ± 16.3
Fiber (g)	7.8 ± 0.7	8.0 ± 0.8
Calories (g)	741 ± 67	764 ± 72
Lycopene (mg)	36.9 ± 4.5	21.5 ± 3.0
β-carotene (mg)	6.6 ± 0.6	7.1 ± 1.5
Chlorogenic acid (mg)	45.9 ± 8.1	31.3 ± 3.5
Naringenin (mg)	19.5 ± 7.3	17.0 ± 3.8

Plasma sample preparation and analysis

Blood samples were drawn into vacutainers containing anticoagulant (heparin and EDTA) before (0 h) and 2, 4, 6, 8 and 24 h after ingestion of the tomato meal. Blood samples were centrifuged, the plasma obtained was separated in aliquots of 2 ml and stored at –80 °C until analysis.

In all plasma samples, lycopene, β-carotene, naringenin, chlorogenic acid were analyzed in duplicate. Carotenoid contents (lycopene and β-carotene) were evaluated by HPLC as described by Maiani et al. [22].

Single phenol levels (naringenin and chlorogenic acid) were evaluated by HPLC with electrochemical detector ED (HPLC-ED) after enzymatic hydrolysis as described by Bugianesi et al. [17]. An amount of 0.5 ml of plasma was incubated at 37 °C for 45 min with 0.5 ml of an enzymatic solution containing 5.5×10^5 U/l sulfatase and 1.0×10^7 U/l β-glucoronidase (sulfase S 3009 type H-5 from Helix Pomatia, Sigma, St. Louis, MO, USA), in 0.2 mol/l acetate buffer (pH 5). Immediately after, 1 ml of 3

mol/l HCl – MeOH 1:1 (*v/v*) was added to obtain protein precipitation. Polyphenols were extracted by adding 2 ml of ethylacetate, followed by vortex mixing for 3 min and sonication for 1 min before centrifugation at 3000 rpm for 5 min. The extraction procedure was repeated three times and the three organic layers were combined and evaporated under a flow of nitrogen. The residue was dissolved into 250 µl of mobile phase (phosphate buffer pH 2.8 and methanol 1:1 *v/v*). Analyses were performed by using the same equipment and chromatography described for polyphenols in food. In order to confirm the presence of chlorogenic acid in plasma an HPLC-ESI-MS analysis was performed. Chromatography was performed by two LC Micro Pump Series 200, autosampler Series 200 and 785/A UV/VIS detector (Perkin Elmer, Norwalk, CT, USA) using a Luna 10 µ Phenyl-Hexyl column 250 × 4.6 mm (Phenomenex, USA), a flow rate of 0.8 ml min^{-1} and detection at 280 nm. The residue was dissolved in 100 µl of MeOH and 20 µl of sample were separated using a two phase system (A: H_2O/formic acid pH 2.5, 5 mmol/l ammonium acetate; B: MeOH/phase A 80:20 *v/v*), and chlorogenic acid elution was achieved as follows: linear gradient from 5 to 50 % of B in 10 min, from 50 % to 60 % of B in 10 min, from 60 % to 100 % of B in 4 min. After 5 min at 100 % B, the mobile phase was taken back to the starting conditions in six minutes.

Electrospray mass spectrometry detection of HPLC eluent was obtained using an API-100 single quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada) equipped with TurboIonSpray (TIS) ion sources set at 400 °C, introducing 200 µl min^{-1} of eluent into the ion source.

The eluate was analyzed using a probe voltage of 5.0 kV and a declustering potential of 40 V. Data acquisition was performed in negative mode selected ion monitoring (SIM), selecting the mass to charge ratio of 353 corresponding to the deprotonated molecule of chlorogenic acid $(M\text{-}H)^-$, using a dwell time of 300 ms. The mass-to-charge ratio scale of the instrument was calibrated with the ions of polypropylene glycol ammonium adducts. Data were processed through the Bio Multi View software (Sciex).

Method precision and absolute recovery were calculated by analyzing spiked samples at four concentrations ranging in the interval in which experimental values fall, in replicates of four on two separate occasions. Spiked samples were processed and analyzed exactly as described above. Repeatability was estimated as the coefficient of variation (CV%) of the replicate measurements. Absolute recovery > 80 %, CV% within days < 5 % and between days < 9 % were found at each concentration tested for both naringenin and chlorogenic acid. Limits of detection were 0.02 µmol/l and 0.03 µmol/l for naringenin and chlorogenic acid respectively. We considered these parameters suitable to accept the information derived from our experiment. The method of the external standard was used for the calibration.

Statistics

Data are given as the mean ± SD. Statistical analysis was performed using the non-parametric Friedman ANOVA test and the Wilcoxon matched pairs test. Differences were considered significant at $P \leq 0.05$. The computer program used was STATISTICA for Windows, release 4.5.

Results

The composition of both fresh and cooked tomato meals administered to subjects is reported in Table 1. In order to evaluate the effect of domestic cooking on the content of lycopene, β-carotene, naringenin and chlorogenic acid, tomatoes were analyzed both before and after cooking without observing significant variations. There were no differences in naringenin content between hydrolyzed and unhydrolyzed extractions of fresh and cooked tomatoes, demonstrating the absence of naringenin glycosides. Fig. 1 shows the plasma concentration of lycopene after consumption of the fresh and cooked tomato meals. No changes in plasma lycopene concentrations were observed. Similarly, no difference in plasma β-carotene levels before and after consumption of both fresh and cooked tomato was observed (Fig. 2). Fig. 3 shows naringenin plasma levels after consumption of fresh and cooked tomato meals. A significant ($P < 0.05$) increase with respect to baseline was observed 2 h after administration of cooked tomato, when there was the plasma naringenin peak (0.06 ± 0.02 µmol/l), while no significant change was found after fresh tomato consumption. Fig. 4 shows chlorogenic acid plasma levels after administration of

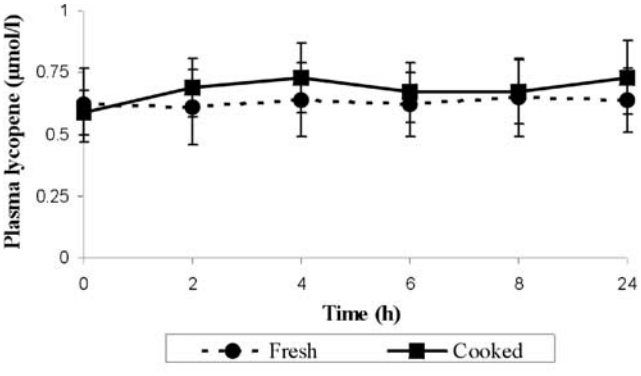

Fig. 1 Plasma lycopene concentration over time in subjects consuming a single portion of fresh and cooked tomato meals. Values are means of five subjects and bars indicate standard deviation

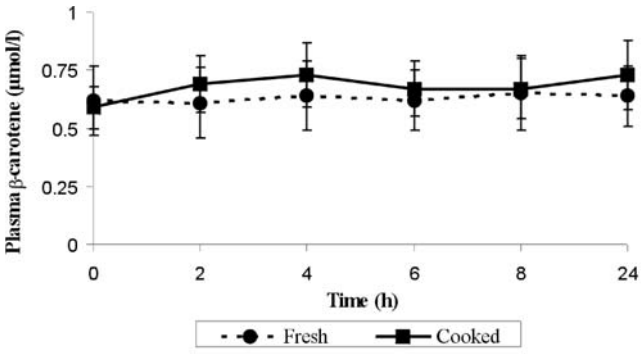

Fig. 2 Plasma β-carotene concentration over time in subjects consuming a single portion of fresh and cooked tomato meals. Values are means of five subjects and bars indicate standard deviation

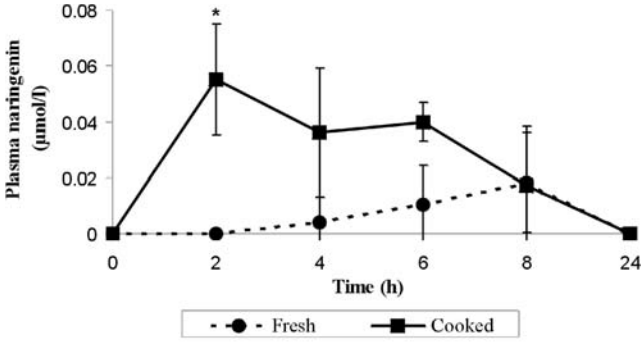

Fig. 3 Plasma naringenin concentration over time in subjects consuming a single dose of fresh and cooked tomato meals. Values are means of five subjects and bars indicate standard deviation. * $P < 0.05$ (Wilcoxon test)

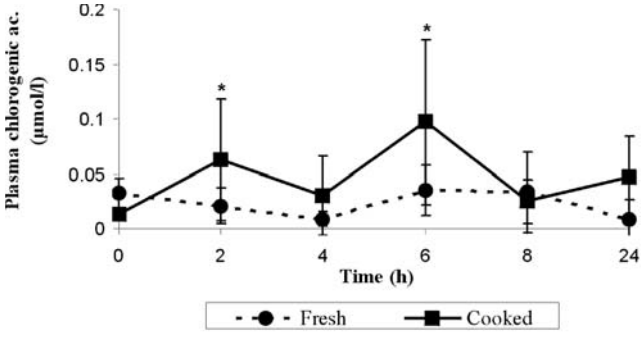

Fig. 4 Plasma cholorgenic aciol concentration over time in subjects consuming a single dose of fresh and cooked tomato meals. Values are means of five subjects and bars indicate standard deviation. * $P < 0.05$ (Wilcoxon test)

the two meals. In this case a significant ($P < 0.05$) increase at 2 h (0.06 ± 0.05 µmol/l) and a maximum peak (0.10 ± 0.07 µmol/l, $P < 0.05$) at 6 h were observed after cooked tomato consumption, while no significant change was detected when fresh tomatoes were administered. Electrospray mass spectrometry analysis confirmed the presence of chlorogenic acid in plasma samples and gave the same quantitative response obtained with the electrochemical detection.

Discussion

The main aim of this study was to evaluate the bioavailability of the most abundant tomato carotenoids and polyphenols after consumption of fresh and cooked cherry tomatoes. For both the test meals administered, no statistical difference in plasma carotenoid concentrations was observed, even maintaining constant levels of fat and other meal ingredients.

The lack of significant changes in carotenoid plasma concentrations can depend on different factors. In the first place, since the subjects participating in the study had fixed habits of consuming a high daily quantity of foods of plant origin, three days of dietary wash-out could not have been enough to lower lycopene and β-carotene basal values below the average normal values [23, 24], also if the diet composition analysis revealed that fruit and vegetable income was very low (mean value: ~ 25 g/day for each subject) and that, among foods containing carotenoids, at least lycopene sources were completely absent. It is likely that the baseline carotenoid values found were too high to observe a significant variation of plasma levels after a treatment by single ingestion.

However, the lack of carotenoid increase in plasma after administration of a single dose has been reported in other studies [12, 25]. Large interindividual variation in the plasma response to single carotenoids doses, or even nonresponse, have also been observed [26, 27].

Furthermore, this lack of response may also depend on the short period of cooking (only 15 min) and absence of homogenization. In fact, Stahl and Sies [13] found that heating tomato juice for 1 h before consumption led to an increase in lycopene plasma concentrations. In the same way, van Het Hof et al. [18] observed that both heating tomatoes for 1h at 100 °C and homogenizating under high pressure enhanced the lycopene plasma response significantly. These authors found similar effects to those for lycopene also for β-carotene.

The present study is the first to demonstrate the absorption of naringenin and chlorogenic acid after consumption of cooked cherry tomatoes. It appears that domestic cooking enhances the polyphenol bioavailability from tomatoes. In this study, maximum plasma concentrations (C_{max}) of naringenin (0.06 ± 0.02 µmol/l) was reached 2 h (T_{max}) after consumption of cooked cherry tomatoes containing 17 mg of naringenin, while no significant peaks were detected after ingestion of 19 mg of naringenin from fresh tomato consumption. In a previous study we observed naringenin bioavailability from cooked tomato paste [17] detecting, as in the present study, a significant peak at 2 h. However, the bioaccessi-

bility of naringenin from cooked tomato paste seems to be greater than that from cooked cherry tomatoes, since after ingestion of 3.81 mg of naringenin from tomato paste a C_{max} 0.12 ± 0.03 μmol/l was observed. Considering that no significant peak was detected after consumption of fresh cherry tomatoes, we can speculate that, as for carotenoids, naringenin bioaccessibility is limited by interactions with food matrix and improved by mechanical and heat treatments. Data on tomato fruit physiology are consistent with the last consideration. In fact, naringenin is trapped in the cutin matrix of the membrane of the ripe fruit where it strongly interacts with insoluble polyesters that are constituent of tomato fiber [28]. Mechanical and heat treatments may provide the energy necessary to break the interactions, improving naringenin bioaccessibility *in vivo*.

In the case of chlorogenic acid, plasma maximum concentration (0.1 ± 0.07 μmol/l) was reached 6 h after ingestion of 31 mg of cooked tomatoes, although a first significant peak (0.06 ± 0.05 μmol/l) appeared at 2 h. Literature data regarding the absorption of chlorogenic acid in its intact form are still fragmentary and not exhaustive. In fact, *in vitro* studies have established that in humans neither plasma, liver and intestinal mucosal nor gastric juice and duodenal fluid have esterase activity [29, 30], while colonic microflora of both animals and humans is able to hydrolyze chlorogenic acid into caffeic acid and quinic acid [29, 31]. "In vivo" studies have shown on the one hand that caffeic acid and not intact chlorogenic acid is detected in rat plasma and in human urine after chlorogenic acid consumption [32, 33], on the other hand that 33 % of the ingested dose was absorbed intact in human small intestine by using an ileostomy model [30]. Since this is the first study showing chlorogenic acid bioavailability in human plasma, the results were confirmed analyzing plasma extracts also by mass spectroscopy. Caffeic acid was not detected in plasma extracts at any time.

The two-phase absorption observed, with a first peak at 2 h and a second one at 6 h, could be due to re-absorption events by enterohepatic circulation, but this hypothesis needs to be substantiated by specific investigation of chlorogenic acid bio-transformation by the liver and the effect of colonic microflora on chlorogenic acid metabolites. As for naringenin, chlorogenic acid plasma level was significantly different from the baseline only after cooked tomato consumption, while after the ingestion of 46 mg of chlorogenic acid from fresh tomatoes no significant peaks were detected, demonstrating that a mild cooking is able to improve chlorogenic acid bioaccessibility. The concluding remarks should focus on our demonstration, for the first time, that chlorogenic acid is bioavailable in its intact form. Furthermore, we have demonstrated that mild domestic cooking improves the bioaccessibility of two bioactive compounds present in cherry tomatoes. Considering that both naringenin and chlorogenic acid are widely studied for their potential healthy properties [34–36], evidence of their bioavailability and of the factors influencing their bioaccessibility is an important tool to sustain the possibility that polyphenols play a biological role in human physiology.

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