



# The use of alternative sweeteners (sucralose and stevia) in healthy soft-drink beverages, enhances the bioavailability of polyphenols relative to the classical caloric sucrose

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## ABSTRACT

The comparison of non-caloric sweeteners (stevia and sucralose) and sucrose, on the plasma concentration and cumulative effects of phenolic compounds, was achieved. A long-term intervention, consisting of the daily intake of 330 mL of healthy citrus-maqui soft drinks, for 60 days, by 138 healthy overweight adults, was followed. A total of 24 bioavailable metabolites derived from caffeic acid, 3,4-di-hydroxyphenylacetic acid, eriodictyol, homoeriodictyol, hippuric acid, naringenin, 2,4,6-tri-hydroxybenzaldehyde, and vanillic acid were detected in peripheral blood plasma. A similar augment of bioactive compounds in plasma concentrations were found for the three beverages, in the range 12.3% (day 0)- 85.3% (day 60), depending on the analyte considered. Due to this, the present study highlights sucralose and stevia as valuable alternatives to sucrose, providing and non-significantly different plasma concentration and cumulative effect in the plasma, thus contributing to prevent a diversity of metabolic disorders and health constraints.

## 1. Introduction

Nowadays, one of the greatest social challenges is represented by the climbing incidence of different metabolic diseases, namely type II diabetes, obesity, and metabolic syndrome (Stephens et al., 2020). These pathologies are mostly associated with unbalanced diets featured by a restricted intake of slowly digested carbohydrates and high consumption of added sugars of rapid absorption. This habit is strongly enclosed to the consumption of sweetened drinks, which play a central role in the connection of health disturbance and dietary habits (Ferreira et al., 2017; Prinz, 2019). In this regard, this kind of beverage has been associated with weight gain, hypertension, and cardiovascular diseases, among other pathophysiological conditions (Bernstein et al., 2012; Palmer et al., 2008; Pacheco et al., 2020).

Given the current scenario of incidence of metabolic diseases, to tackle health disturbances associated with dietary habits, global

strategies are focused on the use of alternative sweeteners for the production of new healthy beverages, rich in highly bioavailable bioactive compounds (Sloan, 2018). So, newly designed beverages, a source of bioactive phytochemicals with a positive impact on the referred medical situations, may contribute to reduce the consumption of sugar-based drinks and, consequently, diminish their harmful effects on metabolism (Domínguez-Perles et al., 2020). However, nowadays, there are controversial results on the bioavailability of the phytochemicals compounds present in plant-based foods and derived manufactured products. Indeed, it has been described as low values (up to 10% of the total intake), strongly conditioned by the physicochemical properties of the matrix (foods and beverages) and the variability between individuals. This low bioavailability is obtained when monitoring compounds in matching chemical forms relative to those present in plant-based foods, while when considering also phase II metabolites this rises up to 70% (Barreca et al., 2017).

**Abbreviations:** AMPK, AMP-activated protein kinase; CA, Caffeic acid; CAT, Catechol; CEIC, Ethical Committee of Clinical Studies; Cy, Cyanidin; Dp, Delphinidin; DHPAA, 3,4-Di-hydroxyphenylacetic acid; E, Eriodictyol; GA, Gallic acid; Glc, Glucoside; H, Hesperetin; HA, Hippuric acid; HE, Homoeriodictyol; mRNA, messenger ribonucleic acid; MRM, Multiple Reaction Monitoring; N, Naringenin; RP-HPLC-DAD, Reverse-Phase High-Performance Liquid Chromatography coupled to Diode Array Detector; Sam, Sambubioside; TFA, *trans*-Ferulic acid; THBA, 2,4,6-Trihydroxybenzaldehyde; TIFA, *trans*-IsoFerulic acid; UHPLC-ESI-MS/MS, Ultra-High Performance Liquid Chromatography ElectroSpray Ionization Mass Spectrometry; VA, Vanillic acid.

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When considering the relevance of the physicochemical properties of foods to modulate the intestinal absorption of phytochemicals, to date, it has been suggested that low weight polysaccharides, like sucrose, could play an important role, due to a positive interaction with phenolic compounds, which affects their intestinal absorption by increasing motility and secretion or by activating the membrane transporters that would inhibit the formation of insoluble protein–phenolic compounds complex. However, to date, it is not clear the extent to which this positive effect is related to modifications in the matrix interactions or to the stimulation of digestive processes that enhance intestinal uptake (Ribas-Agusti et al., 2018). In this regard, Agulló et al. recently studied the modulatory capacity of diverse sweeteners concerning the pharmacokinetics and bioavailability of phenolic compounds after an acute administration of polyphenol-rich beverages, proposing stevia and sucralose as alternatives to sucrose (Agulló, Domínguez-Perles, & García-Viguera, 2021a; Agulló, Domínguez-Perles, Moreno, Zafrilla, & García-Viguera, 2020a; Agulló, Villano, García-Viguera, & Domínguez-Perles, 2020b); however, despite the evidence retrieved, longitudinal trials are still required to demonstrate the potential benefits of alternative sweeteners on human health, after long-term ingestion, to double-check comprehensively the increased bioavailability associated with specific substitutes.

The fruits selected for designing the new beverage (Agulló et al., 2021a) were chosen according to their known composition and bioactivity. In this aspect, maqui berry (*Aristotelia chilensis* (Mol.) Stuntz) is a source of natural colorants due to the presence of anthocyanins with positive biological effects, namely high antioxidant capacity, cardio-protection, and capacity to inhibit adipogenesis and diabetes symptoms (Girones-Vilaplana et al., 2014). Also, citrus fruits are rich in bioactive compounds, mainly represented by flavanones, which could provide additional health benefits against several chronic diseases, namely cancer, obesity, diabetes, and cardiovascular disease (Amiot et al., 2016; Gonzalez-Molina et al., 2010).

Hence, the present article aims at describing the contribution of non-caloric sweeteners (one natural (stevia) and other artificial (sucralose)) to the cumulative plasma concentration of phenolic compounds (anthocyanins and flavanones), on healthy overweighted humans, after a long-term intake (2 months) of maqui-citrus beverages relative to the classical caloric one (sucrose). This approach provides evidence on an equal or enhanced bioavailability achieved by replacing traditional caloric sweeteners with non-caloric ones. In this concern, this study increases the knowledge retrieved regarding the plasma level of circulating bioactive compounds after chronic ingestion of the proposed drinks, thus completing the information available on the accumulative effect, previously reported, where differences between the sweeteners were defined (Agulló et al., 2021b).

## 2. Material and methods

### 2.1. Chemicals and reagents

Cyanidin (Cy) 3-O-glucoside, delphinidin (Dp) 3-O-glucoside, eriodictyol (E), homoeriodictyol (HE), naringenin (N), and hesperetin (H) were obtained from TransMIT (Geiben, Germany). Hesperidin, eriocitrin, and narirutin were supplied from Merck (Darmstadt, Germany). Caffeic acid (CA; a.k.a. 3,4-di-hydroxycinnamic acid), gallic acid (GA; a.k.a. 3,4,5-tri-hydroxybenzoic acid), 3,4-di-hydroxyphenylacetic acid (DHPAA), hippuric acid (HA), *trans*-ferulic acid (TFA; a.k.a. 4-hydroxy-3-methoxycinnamic acid), *trans*-isoferulic acid (TIFA; a.k.a. 3-hydroxy-4-methoxycinnamic acid), vanillic acid (VA; a.k.a. 4-hydroxy-3-methoxybenzoic acid), 2,4,6-tri-hydroxybenzaldehyde (THBA), and catechol (CAT; a.k.a. benzene-1,2-diol) were obtained from Sigma-Aldrich (Steinheim, Germany). Fisher-Scientific (Loughborough, UK) provided acetonitrile and formic acid of analytical grade used for the chromatographic determinations. All solutions were prepared with ultrapure deionized water from a Milli-Q Advantage A10 ultrapure water

purification system (Millipore, Burlington, MA, USA).

### 2.2. Beverages preparation and characterization regarding polyphenol content

Maqui New Life S.A. (Santiago, Chile) and Cítricos de Murcia S.L. (Ceutí, Spain)/and AMC Grupo Alimentación Fresco y Zumos S.A. (Espinardo, Spain) supplied fresh dry organic maqui powder and the citrus juices, respectively. Sucrose, stevia, and sucralose were provided by AB Azucarera Iberia S.L. (Madrid, Spain), AgriStevia S.L. (Molina de Segura, Spain), and Zukan (Murcia, Spain), correspondingly.

Maqui-citrus beverages' performance was done according to the procedure described by Salar et al., and sweeteners were added in different proportions depending on the respective sweetening power (stevia and sucralose 4 mg per 100 mL and sucrose 7.5 g per 100 mL) (Salar et al., 2020). The maqui-citrus drinks were prepared every 15 days (during 2 months), as the beverages were provided in lots of 15 bottles to each volunteer, for their intake in the following 15 days, with instructions for preserving them in the fridge (at 5 °C) until being consumed as previously described (Agulló et al., 2021b). Each separate batch of juices was characterized from the polyphenolic quantitative profile to avoid deviations that would entail dispersion of the plasma concentration results and lack of significance when analyzing differences between sweeteners.

The type of sweetener was unknown for volunteers and researchers (who provided the beverages, and collected/processed the biological samples), as well as for researchers responsible for the results interpretation and statistical analyses.

The phenolic composition of the beverages was analyzed following the methodology previously described (González-Molina et al., 2012; Salar et al., 2020). In short, beverages were centrifuged at 10,500 rpm, for 5 min (Sigma 1e13, B. Braun Biotech International, Osterode, Germany). The supernatants were filtered through a 0.45 mm PVDF membrane (Millex HV13, Millipore, Bedford, Mass., USA) and analyzed by RT-HPLC-DAD. The chromatographic analyses were carried out on a Luna 5 µm C<sub>18</sub>(2)100 Å column (250.0 × 4.6 mm), using Security Guard Cartridges PFD 4.0 × 3.0 mm both supplied by Phenomenex (California, USA), using 5% formic acid in deionized Milli-Q water (solvent A) and 100% methanol (solvent B), upon the linear-gradient (time, %B) (0, 15%); (20, 30%); (30, 40%); (35, 60%); (40, 90%); (44, 90%); (45, 15%), and (50, 15%), using an Agilent Technologies 1220 Infinity Liquid Chromatograph, equipped with an autoinjector (G1313, Agilent Technologies) and a Diode Array Detector (1260, Agilent Technologies, California, USA). Chromatograms were recorded and processed on an Agilent ChemStation for LC 3D systems. The volume of injection and flow rate were 10 µL and 0.9 mL/min, respectively. The quantification of flavanones and anthocyanins was done on UV chromatograms recorded at 280 nm as hesperidin and 520 nm as cyanidin 3-O-glucoside, respectively, and expressed as mg per 100 mL of juice (mg/100 mL).

Quality and safety tests were performed, as well as shelf-life evaluations, to ensure the harmless feature of the beverages from the toxicological and microbiological point of view, while the nutritional and phytochemical composition of the drinks remained intact (Salar et al., 2020).

### 2.3. Experimental design

A double-blind, randomized, longitudinal, crossover clinical study was performed in overweight individuals (n = 138), by the Catholic University of Murcia (UCAM, Murcia, Spain), under the supervision of Dr. Villano and the principles of the Declaration of Helsinki. The criteria for the volunteers' selection for the study were to be in good health, overweight (between 24.9 and 29.9 kg/m<sup>2</sup> following World Health Organization (WHO) criteria), aged 40–60 years, non-smokers, non-dyslipidemic and normotensive, with no chronic illnesses, and not taking any medication. The protocol was approved by the Official Ethical

Committee of Clinical Studies (CEIC) of the University Hospital “Morales Meseguer” (Murcia, Spain) and registered at <https://ClinicalTrials.gov> (NCT04016337), as previously referred to concerning the characteristics of the dietary intervention (Agulló et al., 2021a; Agulló et al., 2020a; Agulló et al., 2021b; Agulló et al., 2020b) that briefly consisted of the daily ingestion of 330 mL of the maqui-citrus beverage, over 60 days. To reach the analytical objective, peripheral blood was collected at the initial (day 0) and the end of the intervention (day 60), and centrifuged to obtain plasma according to the methodology described (Agulló et al., 2021a), and stored at  $-80^{\circ}\text{C}$ . In this scenario, the control (reference for establishing comparisons) considered in the present work is represented by the consumption of beverages developed using the caloric sweetener (sucrose). Samples processing and analyses were conducted when intervention tasks were completed, once the intervention tasks were finished, in the same batch, to minimize errors due to those variables. To obtain realistic information on the effect of the sweetener on the plasma concentration in the frame of a standard diet, this was not controlled, by monitored by an exhaustive nutrition survey. Besides the faithful perspective of the experimental design implemented, this enhanced the adherence of the volunteers to the dietary patterns needed for the successful conclusion of the study.

#### 2.4. Plasma samples collection, processing, and analysis by UHPLC-ESI-MS/MS

The plasma collected was thawed and further processed according to the methodology described by Agulló et al. on each day of analysis (Agulló et al., 2021a). This source of bioavailable polyphenols was selected instead serum to avoid the degradation of the target metabolites as a result of the time-lapse required for clotting (generally ranging from 30 to 60 min), as well as to allow comparisons with data available in the literature (most of them characterizing the occurrence of polyphenols in peripheral blood through plasma assessment). Before UHPLC-analysis, the plasma samples were diluted in acetonitrile/formic acid (98:2, v/v) 1:2,5 (v/v), vortex for 1 min, sonicated for 10 min, and centrifuged at 15,000 g for 10 min, at  $5^{\circ}\text{C}$  (Sigma 1e16, B. Braun Biotech International, Osterode, Germany). Afterward, supernatants were concentrated in a speed vacuum concentrator and reconstituted in 200 mL methanol/Milli-Q-water 0.2% formic acid (v/v) (50:50, v/v). Later on, the samples were centrifuged at 15,000 g for 10 min, at  $5^{\circ}\text{C}$  (Sigma 1e16, B. Braun Biotech International, Osterode, Germany), and stored at  $-20^{\circ}\text{C}$  until analysis by UHPLC-ESI-MS/MS. The identification and quantification of phenolic metabolites were achieved by UHPLC-ESI-QQ-MS/MS, applying the targeted metabolomics method previously reported (Agulló et al., 2021a; Agulló et al., 2020b).

#### 2.5. Statistical analysis

Quantitative data are presented as mean  $\pm$  SD of 46 volunteers per sweetener ( $n = 138$  interventions). Differences between basal and final concentrations were examined by a paired sample *t*-test using the SPSS 21.0 software package (SPSS Inc., Chicago, Ill., U.S.A.). The level of significance was set at  $p < 0.05$ .

### 3. Results and discussion

#### 3.1. Polyphenol content of maqui-citrus beverages

The stevia, sucralose, and sucrose-sweetened beverages presented the same composition regarding the quantitative profile of anthocyanins, with the following decreasing concentration order (values are the average of the three sweetened beverages): Dp 3,5-*O*-di-glc (5.09 mg/100 mL) > Dp 3-*O*-sam-5-*O*-glc  $\approx$  Dp 3-*O*-glc (both at the average concentration 4.48 mg/100 mL) > co-eluting Cy 3-*O*-sam-5-*O*-glc and Cy 3,5-*O*-di-glc (2.09 mg/100 mL) > Dp 3-*O*-sam (1.51 mg/100 mL) > Cy 3-*O*-glc (0.81 mg/100 mL) > Cy 3-*O*-sam (0.51 mg/100 mL)

(Supplementary Table 1 exhibits the average concentration of individual anthocyanins of all batches of juices elaborated).

With respect to flavanones, the same individual compounds were found in the three drinks analyzed, in the following decreasing order of concentration (values are the average of the three): hesperidin (hesperetin 7-*O*-rut) (9.11 mg/100 mL) > eriocitrin (eriodictyol 7-*O*-rut) (1.91 mg/100 mL) > narirutin (naringenin 7-*O*-rut) (1.73 mg/100 mL) > *O*-tri-glycosyl-naringenin (0.19 mg/100 mL) (Supplementary Table 2). The differences observed regarding the quantitative profile of the beverages developed using distinct sweeteners were not statistically different ( $p > 0.05$ ) (Supplementary Table 2 exhibits the average concentration of individual anthocyanins of all batches of juices elaborated).

These quantitative polyphenolic profiles, together with the antioxidant capacity, and the  $\alpha$ -glucuronidase and lipase inhibitory activities, described by Gironés et al. (Gironés-Vilaplana et al., 2014; Gironés-Vilaplana et al., 2012), would turn these drinks into good candidates contributing to preventing metabolic disorders, such as obesity and type II diabetes mellitus. However, the biological benefits derived from polyphenols are closely dependent on the efficiency of intestinal absorption after acute and/or long-term ingestion. Thereby, in the present work, the cumulative plasma concentration of the metabolites referred to from maqui-citrus beverages, after 60-days of dietary intervention, was explored to cover the existing gap of knowledge on how an extended intake of dietary sources of phenolic compounds could modify the concentration of circulating metabolites, especially for those already identified as responsible for health benefits, which is the aim of the present work.

#### 3.2. Profiling plasma metabolites of maqui-citrus beverage's polyphenols

To set up the quantitative profile of circulating compounds and the possible augment of concentration, induced by the intake of the drinks, identifying the metabolic derivatives, as a result of phase II reactions of polyphenols, becomes essential. Besides, the possible influence of the sweetener added to the beverages should be determined. To accomplish these objectives, the analysis of the metabolites of maqui's anthocyanins and citrus' flavanones, in plasma, was done in healthy volunteers ( $n = 46$  per beverage type of added sweetener) after the intake of 330 mL of maqui-citrus beverages each day, for 60 days. In this regard, the starting hypothesis was that, although no significant differences in the bioavailability of the phytochemicals were found when comparing the diverse beverages analyzed in the present work (as referred to above), the use of different sweeteners could modify the absorption of polyphenols at the intestinal level, due to the participation of specific transport mechanisms that could promote a competition activity, as it has been observed that alternative sweeteners, depending on sweetness intensity, could increase glucose transport via SGLT1 and GLUT2, two transporters that participate in polyphenol absorption (Bilal Hussain et al., 2019; O'Brien et al., 2016; Xie et al., 2020). In consequence, it would be expected that this fact is reflected in the quantitative plasma profile after long-term intake of maqui-citrus drinks, depending, on the sweetener added.

Based on previous results obtained, metabolites described in plasma after acute ingestion of the beverages (Agulló et al., 2020b) were searched, evidencing the presence of 24 phenolic derivatives (Supplementary Table 3). Specifically, the compounds identified were caffeic acid (CA), CA glucuronide, CA sulfate, CA glucuronide-sulfate, CA di-sulfate, 3,4-di-hydroxyphenylacetic acid (DHPAA), DHPAA glucuronide, DHPAA di-glucuronide, DHPAA glucuronide-sulfate, DHPAA di-sulfate, eriodictyol (E), E glucuronide, E sulfate, homoeriodictyol (HE) glucuronide, hippuric acid (HA), HA sulfate, naringenin (N) glucuronide, 2,4,6-tri-hydroxybenzaldehyde (THBA) glucuronide, THBA sulfate, vanillic acid (VA), VA di-glucuronide, VA sulfate, VA glucuronide-sulfate, and VA di-sulfate. Besides, 21 out of these 24 metabolites, accounting for  $\approx 88\%$  of the total, were excreted by urine (Agulló et al.,

2021b), which suggests that, to some extent, they are metabolized before excretion.

Interestingly, regarding the anthocyanins and the flavanone hesperidin, their phase II derivatives were not detected in plasma, which is in close agreement with the results retrieved from the acute intervention (Agulló et al., 2021a). This fact could be attributed to the degradation during digestion, as well as to their transformation towards phase II derivatives (glucuronide-, sulfate-, or methyl-derivatives) upon specific metabolic reactions in the epithelial cells of the proximal gastrointestinal tract, as reported for anthocyanins (Kay et al., 2017). In this regard, some flavanones could be affected by similar metabolic routes, thus giving rise to matching types of derivatives (Agulló et al., 2021b).

Some phenolic metabolites referred to above were found in the plasma of  $\approx 15\%$  of the volunteers (CA di-glucuronide, CA glucuronide-sulfate, E glucuronide, THBA glucuronide, and THBA sulfate). Although these compounds were found in quantifiable concentrations, the limited number of volunteers exhibiting these molecules in plasma turns them into no representative. As broadly discussed in the literature, this fact could be due to inter-individual differences of metabolic traits (Réveillon et al., 2019), as evidenced by the results retrieved in the present work, for the concentration of the different metabolites. On the other hand, CA, CA glucuronide, CA sulfate, DHPAA, DHPAA glucuronide, DHPAA di-glucuronide, DHPAA glucuronide-sulfate, DHPAA di-sulfate, E, E sulfate, HE glucuronide, Hippuric acid (HA), HA sulfate, N glucuronide, VA, VA di-glucuronide, VA sulfate, VA glucuronide-sulfate, and VA di-sulfate were identified and quantified in plasma of all volunteers. Nevertheless, HA was not taken into consideration

because of its high basal levels, as a result of its endogenous production, as well as the widespread occurrence of this compound in a broad diversity of dietary sources, which does not allow discriminating the amount obtained from the metabolism of polyphenols of the beverages ingested during this dietary intervention (Ludwig et al., 2015).

### 3.3. Quantitative analysis of plasma metabolites of polyphenols of maqui-citrus beverages

In peripheral blood plasma, 19 circulating metabolites were quantified under basal conditions (before the beginning of the dietary intervention, which was considered as an authentic internal control) and in samples collected after 60 days ingestion of the experimental beverages (Fig. 1). Although almost all of them followed the trend concerning plasma concentration in comparison with basal values, the differences observed were statistically significant only for 14 compounds. The lack of statistical differences for some of the polyphenolic derivatives identified and quantified seems to be strongly influenced by the dispersion of the data collected between volunteers within the same experimental group, which is attributable to inter-individual variations (Bento-Silva et al., 2020).

Caffeic acid (CA) is a natural polyphenolic compound and a common degradation product of both anthocyanins and flavanones (Ludwig et al., 2015). Upon the described intervention, a significant increase ( $p < 0.05$ ) of the plasma concentration of unesterified CA and CA glucuronide was observed after 60 days of consumption of the maqui-citrus beverages, which was closely dependent on the sweetener used (Fig. 1). The

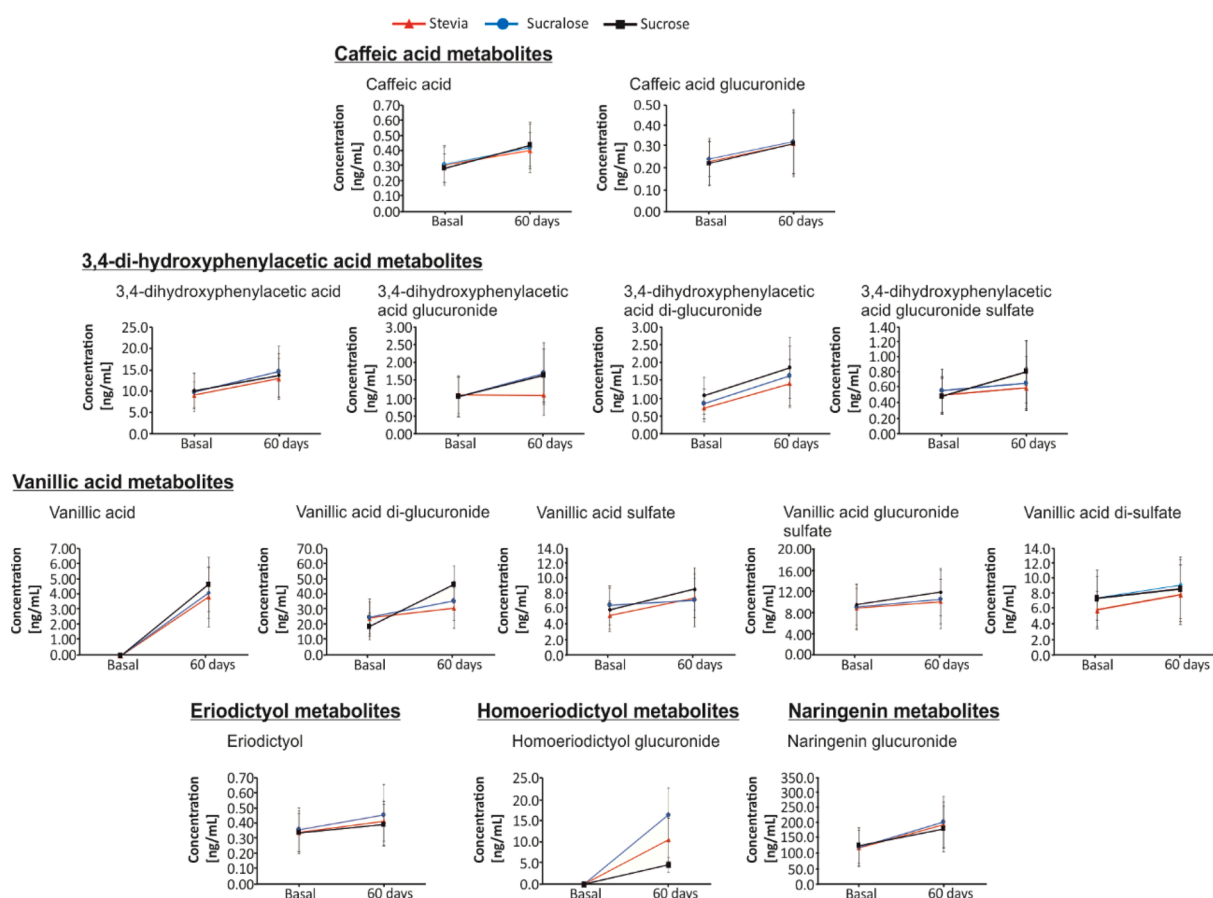


Fig. 1. Content of single anthocyanins and flavanones metabolites (caffeic acid, caffeic acid glucuronide, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid glucuronide, 3,4-dihydroxyphenylacetic acid di-glucuronide, 3,4-dihydroxyphenylacetic acid glucuronide-sulfate, eriodictyol, homoeriodictyol glucuronide, naringenin glucuronide, vanillic acid, vanillic acid di-glucuronide, vanillic acid glucuronide-sulfate, vanillic acid di-sulfate) in basal urine and 60 days plasma of healthy volunteers after daily ingestion of 330 mL of maqui-citrus drinks developed using as sweeteners stevia ( $\Delta$ ), sucralose (O), and sucrose ( $\square$ ). Significantly different bioavailability according to an analysis of variance (ANOVA) and Duncan's multiple rank test was found at  $p < 0.001$  (\*\*\*)



transport efficiency of CA across the intestinal barrier depends on the nature of derivatives. The highest values of 0.44 and 0.33 ng/mL, respectively, were recorded for unesterified CA and CA glucuronide, when ingesting sucrose- and sucralose-based drinks.

However, it is important to assess the magnitude of the concentration changes caused by the different drinks. The paired *t*-test done indicated that beverages developed with sucrose increased to a higher extent the plasma concentration for both CA and CA glucuronide compared to the basal level (53% and 42%, respectively) (Fig. 1). Moreover, concerning the plasma circulating CA, it was observed that this phenolic compound augmented significantly, as a result of the intake of stevia and sucralose-based beverages ( $p < 0.01$ ), although to a lesser extent when compared with sucrose-based drinks ( $p < 0.001$ ). Besides, for CA glucuronide, a significant increase ( $p < 0.01$ ) was observed independently of the sweetener used (Fig. 1).

This outcome is of special relevance since CA derivatives are featured by an array of biological attributions related to health, such as a protective effect against angiopathy and type II diabetes mellitus (Abduljawad et al., 2013). Moreover, recently, Xu et al. provided evidence on the association of these compounds with a reduction in body weight in mice, which has been attributed to their contribution to the regulation of the gut microbiota (Xu et al., 2020). Regarding this, the increased bioavailability observed for these metabolites especially referred to as non-caloric sweeteners could connect the exploration of alternative sweeteners with a positive effect on health, derived from an enhanced bioavailability (Agulló et al., 2021b). The benefits derived from the biological potential of bioavailable polyphenols would provide pathophysiological advantages beyond the health benefits derived from the reduction of the dietary intake of sucrose and its undesirable metabolic effects (Prinz, 2019).

Related to 3,4-dihydroxyphenylacetic acid (DHPAA), a colonic metabolite of flavonoids, all the derivatives identified augmented significantly after the intake of maqui-citrus beverages, exception made of DHPAA di-sulfate. The long-term consumption of sucralose-based drinks gave rise to the highest values for unesterified DHPAA (14.56 ng/mL) and DHPAA glucuronide (1.70 ng/mL), while sucrose-based beverages improved significantly the plasma concentration of DHPAA di-glucuronide (1.86 ng/mL) and DHPAA glucuronide-sulfate (0.81 ng/mL). In terms of efficiency of transport through the intestinal barrier, the results obtained suggested that stevia and sucralose-based drinks trigger the highest augments of the plasma concentration of unesterified DHPAA (42% and 48%, respectively, both significant at  $p < 0.001$ ). Sucrose-based ones produced also a significant increase of 35% ( $p < 0.01$ ). Therefore, it is important to notice that stevia and sucralose-based drinks were responsible for the highest increase of the plasma concentration of total DHPAA di-glucuronide (by 93% and 91%, respectively). According to the results obtained, independently of the sweetener employed, all drinks increased significantly the plasma level of DHPAA di-glucuronide, while no significantly different plasma concentrations were achieved depending on the sweetener applied ( $p < 0.001$ ) (Fig. 1).

When evaluating the evolution of the plasma level of DHPAA glucuronide, it was found that sucralose-based drinks produced the highest increase of 61% respect basal values ( $p < 0.001$ ) followed by the sucrose-based ones with 56% ( $p < 0.01$ ), while the use of stevia as sweetener did not show a significant advantage in this regard. Concerning DHPAA glucuronide-sulfate, the beverages elaborated with sucrose were the only ones that increased significantly (66%) the basal plasma values ( $p < 0.001$ ).

The relevance of DHPAA is based on its capacity to prevent or delay the damage caused by glucotoxicity, a major cause in the pathogenesis of type II diabetes (Álvarez-Cilleros et al., 2018), and protect against pancreatic  $\beta$ -cells dysfunction developed in the frame of hypercholesterolemia (Carrasco-Pozo et al., 2015). Again, according to the results retrieved in the present work, the chemical and phytochemical characteristics of maqui-citrus beverages affect positively the bioavailability of phenolic compounds, e.g., DHPAA molecules, and thus, the potential

biological effects referred to before.

On the other hand, eriodictyol (E) and naringenin (N) derivatives that showed a significant increase, after the consumption of the beverages, were unesterified E and N glucuronide. In this regard, stevia and sucralose-based drinks caused the highest augment for E glucuronide, by 22% and 27%, respectively ( $p < 0.01$ ), and by 63% and 67% for N glucuronide, respectively ( $p < 0.001$ ). These percentages are mirrored in the achievement of high plasma concentrations (Fig. 1). This effect would be tentatively due to the use of sucralose as a sweetener, which would be responsible for the highest increase of plasma concentration after the intake of maqui-citrus beverages (0.46 ng/mL for unesterified E, and 204.29 ng/mL for N glucuronide). Regarding sucrose, this sweetener only augmented significantly the basal plasma concentration of N glucuronide (by 43%,  $p < 0.01$ ). Besides, homoeriodictyol (HE) glucuronide was not detected in basal plasma samples, but after the ingestion of the drinks, the highest values were achieved as a result of the intake of sucralose-sweetened beverages (16.50 ng/mL) (Fig. 1).

Results concerning the plasma concentration of flavanones reached after 60 days of dietary intervention should be analyzed in the frame of the raising interest in the biological power of flavanones in the last years, mainly eriodictyol, homoeriodictyol, and naringenin. The gathered evidence on their healthy attributions in humans, most of them retrieved from clinical trials, have allowed stating namely cardioprotective, anticancer, antidiabetic, anti-obesity, neuroprotective, anti-inflammatory, and/or hepatoprotective activities, among others (Islam et al., 2020; Nguyen-Ngo et al., 2019). Specifically, the administration of naringenin presented various effects in different diabetic rat models, upon which have been demonstrated the capacity to decrease the plasma level of glucose (in streptozotocin-induced diabetic rats), an improvement of insulin sensitivity (in fructose-fed insulin resistance rats), and the potential to lower insulin resistance (in the high-fat diet-fed mice) (Al-Ishaq et al., 2019). On the other hand, eriodictin has been associated with several benefits against obesity (the leading risk factor for type II diabetes), as its consumption increase fatty acid oxidation in adipocytes, energy expenditure, and the transcription thermogenesis-related genes towards messenger ribonucleic acid (mRNA) encoding essential proteins in brown adipose tissue and skeletal muscle, while decreases lipogenesis-related gene expression in white adipose tissue (Kwon & Choi, 2020). Furthermore, lately, Liu et al. suggested that eriodictyol and naringenin derivatives contribute to inhibit the formation of advanced glycation end products, closely related to Alzheimer's disease, retinopathy, neuropathy, or nephropathy (Liu et al., 2020). Thereby, according to the increased amount of these flavonoid derivatives described in this work, their daily intake could enhance boost the occurrence of these bioactivities, even though the specific health benefits should be further demonstrated in the frame of additional clinical trials and nutritional interventions.

Finally, vanillic acid (VA) derivatives, specifically VA di-glucuronide, VA sulfate, VA glucuronide-sulfate, and VA di-sulfate also experienced a significant increase (Fig. 1). For VA di-glucuronide and VA glucuronide-sulfate, sucrose-sweetened drinks gave rise to the highest plasma concentrations, significantly higher than the values corresponding to basal plasma ( $p < 0.001$  and  $p < 0.01$ , respectively). Consequently, sucrose-sweetened maqui-citrus beverages caused an average increase of 76% and 38% higher than sucralose- and stevia-based drinks, correspondingly. Moreover, the sucrose-based drinks provided also the highest values for these metabolites (46.46 and 12.13 ng/mL, respectively). For VA di-glucuronide, sucralose- and stevia-based beverages showed also a significant increase ( $p < 0.001$  and  $p < 0.01$ , respectively), while for VA sulfate, the beverages sweetened with stevia and sucrose boosted a significant increase ( $p < 0.01$ ) by 44% and 45%, correspondingly. For VA di-sulfate, sucralose sweetened drinks allowed achieving the highest plasma concentration (7.79 ng/mL), while stevia was the only sweetener that allowed maqui-citrus beverages to cause a significant increase (by 35%) relative to the basal level ( $p < 0.01$ ). Furthermore, unesterified VA was only detected and

quantified after the ingestion of the drinks, reaching the highest values, during the intake of sucrose-sweetened beverages (4.63 ng/mL).

These results are of special relevance, as vanillic acid has been associated with anti-cancer, anti-obesity, anti-inflammatory, and cardioprotective properties (Baniahmad et al., 2020; Park et al., 2020; Ziadlou et al., 2020). The anti-obesity effects of this compound have been associated with the increase of thermogenesis and AMP-activated protein kinase (AMPK) activation in white adipose tissue after its intake (Park et al., 2020), while its cardio-protective benefits are related to the suppression of toll-like receptor 4 and consequently, inflammation pathway, decreasing oxidative stress and biomarkers of cardio-toxicity (Baniahmad et al., 2020).

To sum up, the results related to the bioavailability of flavonoid metabolites, after two months, intake of the beverages with different added sweeteners (sucrose, sucralose and stevia), were similar for the three drinks evaluated, regarding the capacity of the maqui-citrus beverage to raise the basal plasma concentrations. However, this absence is a remarkable result, as it demonstrates that different sweeteners do not affect negatively to the accumulative effect observed due to the long-term intake of the beverages. Thereby, sweeteners can be chosen based on the number and concentration of the generated circulating metabolites in plasma. Regarding this, stevia can be placed in the first position (eriodictyol, naringenin glucuronide, 3,4-di-hydroxyphenylacetic acid, 3,4-di-hydroxyphenylacetic acid di-glucuronide, vanillic acid sulfate, and vanillic acid di-sulfate), followed by sucrose (caffeic acid, caffeic acid glucuronide, 3,4-di-hydroxyphenylacetic acid glucuronide-sulfate, vanillic acid di-glucuronide, vanillic acid glucuronide-sulfate, and vanillic acid sulfate), and sucralose (eriodictyol, naringenin glucuronide, 3,4-di-hydroxyphenylacetic acid, 3,4-di-hydroxyphenylacetic acid glucuronide, and 3,4-di-hydroxyphenylacetic acid di-glucuronide). As the efficiency of the non-caloric sweeteners was similar to the caloric one (even improving the plasma concentration for specific polyphenolic derivatives), the obtained results suggested stevia and sucralose as valuable alternatives to sucrose, thus reducing sugar intake and preventing the onset of metabolic disorders (Agulló et al., 2021a; Agulló et al., 2020a; Agulló et al., 2021b; Agulló et al., 2020b).

Overall results indicate an increased plasma concentration along with the time-consuming maqui-citrus drinks for most metabolites resulting from the phenolic compounds present in the beverages, regardless of the significance of the augment and the sweetener employed for their development. Moreover, this suggests an accumulative effect due to the chronic consumption of the newly designed beverages, that somehow, could allow an extended presence of such molecules in peripheral blood. Besides, the biological properties of the metabolites detected are of special interest due to their protective attributions against an array of metabolic diseases.

#### 4. Conclusions

The results described in the present work show the bioavailability and the diversity of metabolites, including phase II derivatives, formed as a result of the degradation of precursor flavanones and anthocyanins present in the maqui-citrus sweetened beverages. However, no parental anthocyanidins neither hesperetin were found. The major outcomes described evidence a significant increase in their concentration, after the long-term intake of the beverage with low caloric sweeteners (sucralose and stevia) added, specifically 3,4-di-hydroxyphenylacetic acid glucuronide for sucralose, vanillic acid sulfate, and vanillic acid di-sulfate for stevia, and eriodictyol, naringenin glucuronide, 3,4-di-hydroxyphenylacetic acid, and 3,4-di-hydroxyphenylacetic acid di-glucuronide for both of them.

Even more, as the efficiency of the non-caloric sweeteners was similar to sucrose, this study proposes stevia and sucralose, as alternatives to sucrose, which consumption is directly related to type II diabetes, obesity, and cardiovascular diseases, among other pathological

conditions. In this regard, although no characterization was done on the effect of the beverages intake relative to the markers of these pathological processes, the major outcomes retrieved, strongly encourage to develop further dietary interventions that allows unravelling how the bioavailable compounds identified in the present work could modulate in a health fashion the markers and the clinical status of patients affected by these diseases.

Complementarity, to gain further insights in the biological traits described in the present work, it would be required to implement model *in vitro* systems and metabolomic workflows that help to address the mechanisms and interactions between proximate and phytochemical component of the newly developed beverages, and how these interactions could modulate the bioavailability of polyphenols identified.

#### CRedit authorship contribution statement

Agulló has participated in the formal analysis, data curation, investigation and writing the original draft. Dominguez-Perles has participated in the conceptualization, investigation, methodology, supervision, writing- review and editing Garcia- Viguera in the conceptualization, funding acquisition, project administration, resources, supervision and review- editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131051>.

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