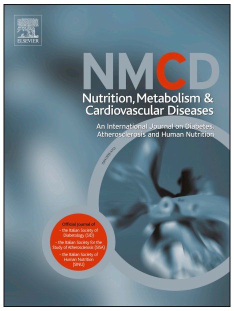

Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage

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PII: S0939-4753(20)30493-2

DOI: https://doi.org/10.1016/j.numecd.2020.11.016

Reference: NUMECD 2511

To appear in: *Nutrition, Metabolism and Cardiovascular Diseases*

Received Date: 12 August 2020

Revised Date: 22 October 2020

Accepted Date: 16 November 2020

Please cite this article as: Agulló V, Domínguez-Perles R, García-Viguera C, Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage, *Nutrition, Metabolism and Cardiovascular Diseases*, https://doi.org/10.1016/j.numecd.2020.11.016.

NUTRITION, METABOLISM, AND CARDIOVASCULAR DISEASES

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GRAPHICAL ABSTRACT

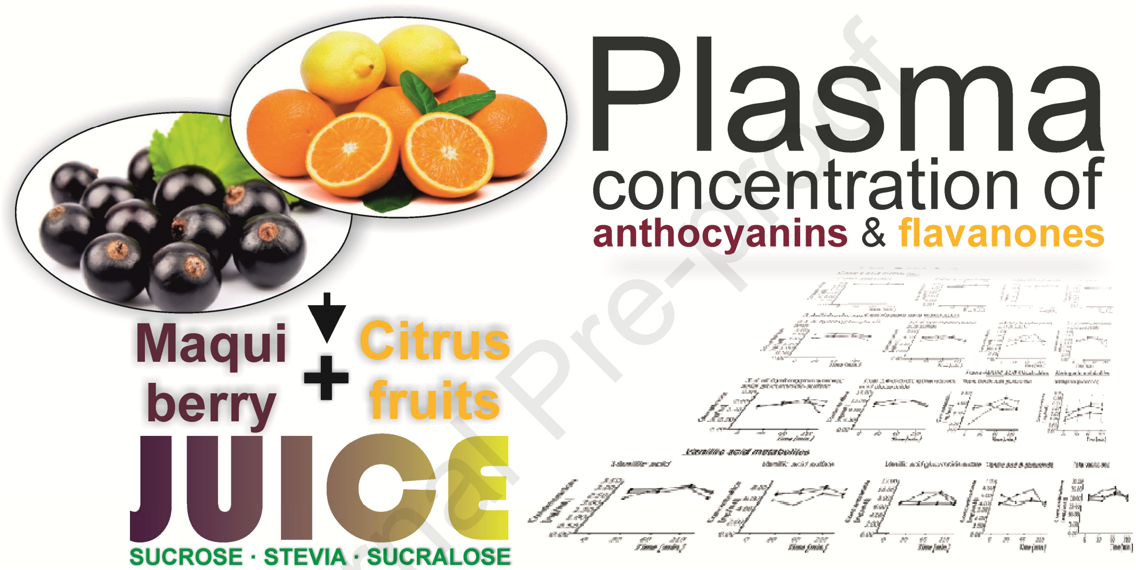

Figure caption: Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage

Research article

Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage

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Running title: Influence of sweeteners in the metabolism of flavonoids

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ABSTRACT

The overconsumption of sucrose is closely related to sugar-sweetened beverages and one of the main factors associated with the increase of metabolic diseases, such as type 2 diabetes, obesity, and insulin resistance. So, the addition of alternative sweeteners to new fruit-based drinks could contribute to minimizing the incidence or severity of these pathologies. Nevertheless, current knowledge on the influence of these additives on the bioactive compounds present in these beverages is still scarce. Hence, to contribute to the understanding of this issue, the plasma concentration of phenolic compounds (anthocyanins and flavanones), after the ingestion of a new maqui-citrus-based beverage, supplemented with sucrose (natural high caloric), stevia (natural non-caloric), or sucralose (artificial non-caloric), was evaluated as evidence of their intestinal absorption and metabolism previous to renal excretion. The beverages were ingested by volunteers (n=20) and the resulting phenolic metabolites in plasma were analyzed by UHPLC-ESI-MS/MS. A total of 13 metabolites were detected: caffeic acid sulfate, caffeic acid glucuronide, 3,4-dihydroxyfenylacetic, 3,4-dihydroxyfenylacetic sulfate. 3,4-dihydroxyfenylacetic acid di-sulfate, 3,4-dihydroxyfenylacetic di-glucuronide, 3,4-dihydroxyfenylacetic glucuronide-sulfate, *trans*-ferulic acid glucuronide, naringenin glucuronide, vanillic acid, vanillic acid sulfate, vanillic acid glucuronide-sulfate, and vanillic acid di-glucuronide, being recorded their maximum concentration after 30-60 minutes. In general, sucralose provided the greatest absorption value for most of these metabolites, followed by stevia. Due to this, the present study proposes sucralose and stevia (non-caloric sweeteners) as valuable alternatives to sucrose (high caloric sweetener), to avoid the augmented risk of several metabolic disorders.

Keywords: Anthocyanins; Flavanones; Stevia; sucralose; sucrose; Metabolites; UHPLC-ESI-MS/MS

Abbreviations: BMI, body mass index; CA, caffeic acid; CAT, catechol; Cy, Cyanidin; DHPAA, 3,4-dihydroxyphenylacetic acid; DM2, diabetes mellitus 2; Dp, delphinidin; E, eriodictyol; ESI, electrospray ionization; GA, gallic acid; Glc, glucoside; Glu, glucuronide; H, hesperetin; HA, hippuric acid; HE, homoeriodictyol; MRM, multiple reaction monitoring; N, naringenin; TFA, *trans*-ferulic acid; THBA, 2,4,6-trihydroxybenzaldehid; TIFA, *trans*-isoferulic acid; VA, vanillic acid; WHO, World Health Organization.

1. Introduction

Changes in dietary habits, set-up during the last decades, have been associated with an increased incidence of obesity and type 2 Diabetes Mellitus (DM2). One factor critically related to this metabolic disorder is the overconsumption of sucrose, which is closely linked to sugar-sweetened beverages [1].

In this scenario, the World Health Organization (WHO), jointly with other institutions, has called for regulations that could reduce the intake of sugar, including the regulation of the composition of marketable sweetened beverages. Due to this, the lookout for alternative sweeteners and the elaboration of healthy beverages has become a cornerstone to control risk factors associated with obesity and DM2 [2].

According to this growing awareness, fruit beverages fit the current trend of increasing the consumption of bioactive compounds that have an array of health benefits, contributing to prevent the incidence and severity of diverse diseases associated with sugar consumption [3]. However, the bioavailability of these phenolic compounds is generally low (up to 10% of the total intake) concerning non-esterified compounds, although when considering the derivatives formed as a result of the phase II reactions and the colonic metabolism, bioavailability ranges between 60% and 70%. Indeed, the variation of the bioavailability values is strongly conditioned by the inter-individual variability in terms of intestinal absorption and metabolism, as well as by the physicochemical properties of the food/beverage matrices [4]. In this regard, the health benefits associated to the consumption of these beverages have raised the incorporation in diets of natural sources of bioactive phytochemicals, being citrus fruits considered as a specially valuable ingredient in the development of functional beverages [3,5], as they are rich in flavanones (e.g. narirutin, eriocitrin, neohesperidin, and hesperidin), as well as other phenolic compounds.

Besides citrus fruits, Maqui berry (*Aristotelia chilensis* (Mol.) Stuntz), has been also widely used in the development of functional beverages, based on its value as a source of bioavailable and bioactive compounds, such as anthocyanins, represented by eight different derivatives of cyanidin and delphinidin [6].

Based on these antecedents, these two fruits (citrus juices and maqui berry) have been promoted as interesting ingredients for the development of new functional beverages [7–9]. However, additional information and a deeper understanding regarding the influence of alternative sweeteners on the pharmacokinetics of the bioactive compounds are still needed.

Therefore, the present article is aimed at uncovering the influence of diverse sweeteners, including sucrose (natural high caloric), stevia (natural non-caloric), and sucralose (artificial non-caloric), on the plasma concentration of flavanones and anthocyanins in healthy, overweight, humans, after the acute intake of (poly)phenol-rich beverages developed on the base of these fruits. The sweeteners assessed in this work were selected to compare a classical, natural, and high caloric sweetener (sucrose) and two non-caloric alternatives (sucralose, an artificial, non-caloric, and widely used sweetener and stevia, a natural emergent sweetener). The assessment of the concentration of these phenolic compounds and their metabolites in plasma is strongly motivated by the previous description of the urinary concentration in volunteers, which demonstrated critical differences between sweeteners [8,9]. Also, the analysis of the plasma concentration of these compounds will provide valuable information on the delay of their intestinal absorption after consumption, as well as about the identity of the circulating metabolites responsible for the biological attributions), which can differ from those excreted by the urine.

2. Material and methods

2.1. Chemicals and reagents

Cyanidin (Cy) 3-*O*-glucoside, delphinidin (Dp) 3-*O*-glucoside, eriodictyol (E), homoeriodictyol (HE), naringenin, and hesperetin were purchased from TransMIT (Geiben, Germany). Narirutin, hesperidin, eriocitrin, caffeic (CA; also known as 3,4-dihydroxycinnamic acid), gallic (GA; also known as 3,4,5-trihydroxybenzoic acid), 3,4-dihydroxyphenylacetic (DHPAA), hippuric (HA), trans-ferulic (TFA; also known as 4-hydroxy-3-methoxycinnamic acid), trans-isoferulic (TIFA; also known as 3-hydroxy-4-methoxycinnamic acid), and vanillic (VA; also known as 4-hydroxy-3-methoxybenzoic acid) acids, 2,4,6-trihydroxybenzaldehid (THBA), and catechol (CAT; also known as benzene-1,2-diol) were obtained from Sigma Aldrich (St. Louis, USA). Formic acid and acetonitrile of analytical grade were obtained from Fisher-Scientific (Loughborough, UK). 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 production and characterization on the (poly)phenolic content

Fresh dry organic maqui powder was provided by Maqui New Life S.A. (Santiago, Chile). Cítricos de Murcia S.L. (Murcia, Spain) and AMC Grupo Alimentación Fresco y Zumos S.A. (Murcia, Spain) supplied the citrus juices. Sucrose was obtained from AB Azucarera Iberia S.L. (Madrid, Spain), Stevia from AgriStevia S.L. (Murcia, Spain), and Sucralose from Zukan (Murcia, Spain).

Maqui-citrus beverages were processed as previously described [8,9]. Briefly, maqui powder was mixed with citrus juices to obtain the base drink. Then, the three selected sweeteners were added, to obtain the different beverages, and pasteurized by applying 85 °C during 58 seconds Afterward, the mixtures were bottled and stored at 5 ºC until being consumed by the volunteers.

Maqui-citrus drinks were characterized on their (poly)phenolic composition following the methodology previously described [7,10]. Beverages were centrifuged at 10500 rpm, during 5 min (Sigma 1-13, 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_{18}(2)100 Å column (250.0 x 4.6 mm), using Security Guard Cartridges PFD 4.0 x 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 auto-injector (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).

2.3. Experimental design

A double-blind, randomized, cross-over clinical study has been conducted in overweight individuals (n=20), by the Catholic University of Murcia (Murcia, Spain). The study and protocol were approved by the Official Ethical Committee of Clinical

Studies (CEIC) of University Hospital ‘Morales Meseguer’ (Murcia) and registered at ClinicalTrials.gov (NCT04016337). The volunteers provided written consent to participate in this study. The criteria for volunteers’ selection to participate in the study were to be in good health, overweight (body mass index (BMI) between 24.9 and 29.9 kg/m^2 following WHO criteria), aged 40-60 years, non-smokers, non-affected by dyslipidemia, and normotensive, with no chronic illnesses and no taking any medication. After an initial phase of 3 days of wash-out with a (poly)phenols free and added sugar-free diet, 330 mL of the test drinks (stevia, sucralose, or sucrose added sweetener) were administered on fasting conditions. Plasma samples were collected before the beverage ingestion (0 min point), as well as in the following times: 30, 60, and 210 min. After 15 days, the process was repeated, for each volunteer ingesting another drink developed with remaining sweeteners until all types of drink were consumed by all the volunteers (3 rounds). Plasma samples collected were stored at -80°C until analyses that were performed once each period was finished and in the same batch to minimize analytical variations.

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

The plasma extraction procedure was applied according to the methodology previously described [8,9]. Briefly, plasma samples were defrosted and 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 15000 g for 10 min, at 5 °C (Sigma 1-16, B. Braun Biotech International, Osterode, Germany). Afterward, supernatants were concentrated in a speed vacuum concentrator and reconstituted in 200 µL methanol/Milli-Q-water 0.2% formic acid (v/v) (50:50, v/v). Later on, the samples were centrifuged at 15000 g for 10 min, at 5°C (Sigma 1-16, B. Braun Biotech International, Osterode, Germany), and stored at -20°C until analysis by UHPLC-ESI-MS/MS.

The identification and quantification of phenolic metabolites was performed by applying the methodology previously reported, with optimized injected volume, flow rate, and MS parameters, by comparison with freshly prepared standards curves of the different phenolics [8,9,11]. The analysis of the samples on the profile and concentration of phenolic metabolites was carried out on a chromatographic column Ascentis Express F5 (50 x 2.1 mm; 2.7 µm pore size) (Sigma, Osterode, Germany). The solvents used for the chromatographic separation were deionized Milli-Q water/formic acid (99.9:0.1, v/v) (solvent A) and acetonitrile/formic acid (99.9:0.1, v/v) (solvent B), upon the linear-gradient (time, %B) (0, 10%); (1, 10%); (10, 60%); (11, 80%); (13, 80%); (13.01, 10%), and (14.50, 10%); using an UHPLC system coupled with a triple quadrupole tandem mass spectrometer, model 6460 (Agilent Technologies, Waldbronn, Germany), operated in multiple reaction monitoring (MRM) and negative/positive electrospray ionization (ESI) modes. The volume injected and flow rate were 10 µL and 0.2 mL/min, respectively. The MS parameters, at the optimized conditions, were gas temperature 325 ºC; gas flow 10 L/min; nebulizer 40 psi; sheath gas heater 275 ºC; sheath gas flow 12; capillary voltage 4000–5000 V; Vcharging 1000–2000. Data acquisition and processing were performed by using MassHunter software version B.08.00 (Agilent Technologies, Walbronn, Germany) (Table 3). Regarding the quantification of the diverse compounds identified, values of area providing signal to noise ratio lower than 1/3, established as the consensus criteria for the limit of detection (LOD), were considered and in consequence values lower than LOD were neither identified nor quantified.

2.5. Statistical analysis

Quantitative data are presented as mean ± SD of 20 volunteers. Specific differences were examined by a Repeated Measure (RM) Multivariate analysis of variance (ANOVA) as the statistical analysis of election for a number of dependent variables higher than 2, measured as correlated, which is used when measuring the effect of an intervention (*e.g.*, a dietary intervention) at different time points. This statistical model allows testing the main effects within and between the subjects, interaction effects between factors, and covariate effects, as well as the effects of interactions between covariates and between subject factors. The data were processed using the SPSS 21.0 software package (SPSS Inc., Chicago, Ill., U.S.A.) and the level of significance was set-up at $p<0.05$.

3. Results and discussion

3.1. Flavanone and anthocyanin content of beverages

The quantitative profile of the juices regarding the phenolic compounds was set up, to establish the flavanones and anthocyanins present in the maqui-citrus beverages developed using three different sweeteners (stevia, sucralose, and sucrose) and thus, susceptible to be absorbed in the intestine after their dietary ingestion. In this regard, it was observed the presence of the four following flavanones: hesperidin (hesperetin 7-*O*-rutinoside) that presented the highest concentration (4.87 mg/100 mL, on average), followed by narirutin (naringenin 7-*O*-rutinoside; 1.31 mg/100 mL, on average), eriocitrin (eriodictyol 7-*O*-rutinoside, 0.32 mg/100 mL, on average), and *O*-triglycosyl-naringenin with the lowest concentrations (0.14 mg/100 mL, on average). On the other hand, anthocyanins were found in higher amounts, being Dp 3,5-*O*-di-glc the predominant (3.49 mg/100 mL, on average), followed by Dp 3-*O*-sam-5-*O*-glc (3.15 mg/100 mL, on average), Dp 3-*O*-glc (2.93 mg/100 mL, on average), Cy 3-*O*-sam-5-*O*-glc and Cy 3,5-*O*-di-glc (1.48 mg/100 mL, on average), Dp 3-*O*-sam (1.10 mg/100 mL, on average), Cy 3-*O*-glc (0.55 mg/100 mL, on average), and Cy 3-*O*-sam (0.40 mg/100 mL, on average). No statistically significant differences ($p>0.05$) were observed between the flavanone and anthocyanin contents of the different maqui-citrus drinks when considering individual or total flavanones (Tables 1 and 2).

3.2. Qualitative analysis of plasma metabolites of flavanones and anthocyanins of the maqui-citrus beverage

To profile the diversity of citrus flavanones and maqui-berry anthocyanins absorbed at the intestinal level and subsequently present in plasma, samples were collected before and after the ingestion of 330 mL of the diverse beverages at different times, and processed to identify possible differences between the three beverages (with stevia, sucralose, or sucrose added). Based on previous researches [8,9], metabolites already described in urine after the ingestion of similar juices were looked for. Upon this strategy, 30 phenolic metabolites derived from flavanones and anthocyanins, were detected in plasma (Table 3). More specifically, the compounds identified in plasma were eriodictyol (E), eriodictyol glucuronide (E glu), eriodictyol sulfate (E sulfate), homoeriodictyol glucuronide (HE glu), homoeriodictyol glucuronide-sulfate (HE glu-sulfate), naringenin (N), *O*-triglycosyl-naringenin and naringenin glucuronide (N glu), regarding flavanones. In addition, it was also identified the presence of caffeic acid glucuronide (CA glu), caffeic acid sulfate (CA sulfate), caffeic acid glucuronide-sulfate (CA glu-sulfate), 3,4-dihydroxyphenylacetic acid (DHPAA), 3,4-dihydroxyphenylacetic acid glucuronide (DHPAA glu), 3,4-dihydroxyphenylacetic acid di-glucuronide

(DHPAA di-glu), 3,4-dihydroxyphenylacetic acid sulfate (DHPAA sulfate), 3,4-dihydroxyphenylacetic acid glucuronide-sulfate (DHPAA glu-sulfate), 3,4-dihydroxyphenylacetic acid di-sulfate (DHPAA di-sulfate), hippuric acid (HA), hippuric acid sulfate (HA sulfate), hippuric acid glucuronide-sulfate (HA glu-sulfate), trans-ferulic acid (TFA), trans-ferulic acid glucuronide (TFA glu), trans-ferulic acid di-sulfate (TFA di-sulfate), 2,4,6-trihydroxybenzaldehide (THBA), 2,4,6-trihydroxybenzaldehide sulfate (THBA sulfate), trans-isoferulic acid (TIFA), vanillic acid (VA), vanillic acid di-glucuronide (VA di-glu), vanillic acid sulfate (VA sulfate) and vanillic acid glucuronide-sulfate (VA glu-sulfate), concerning anthocyanins. These phenolic compounds were tentatively identified according to their retention time, molecular masses, and fragmentation pattern according to the information available in the literature [11].

Although hesperidin (hesperetin 7-rutinoside) was the most abundant flavanone in the beverages, accounting for 73.3% of total flavanones, neither this flavanone nor its phase II derivatives (H glu, H sulfate, H glu-sulfate, H di-glu and H di-sulfate) were observed in plasma in concentrations higher than the limit of detection of the method. Also, none of the precursor anthocyanins (Dp and Cy aglycones) were found in the plasma samples analyzed. The lack of a proper identification of these anthocyanins in plasma could be attributed to their degradation during the gastrointestinal digestion process, as well as due to their low absorption rate at the intestinal level, especially because of the intestinal absorption mechanisms of anthocyanin glycosides are still speculative. Moreover, in some extent the fraction of these compounds absorbed at intestinal level could suffer the metabolism of the epithelial cells and hepatocytes giving rise to phase II derivatives [12]. In this aspect, it has been recently demonstrated that the metabolism of the precursor, towards different degradation metabolites, depends on the metabolic traits of the volunteers and the inter-individual variation [13]. So that, most metabolites detected (E, E glu, E sulfate, HE glu, HE glu-sulfate, N, N glc, CA glu-sulfate, DHPAA glu, HA sulfate, HA glu-sulfate, TFA, TFA di-sulfate, THBA, THBA sulfate and TIFA) were found in a reduced number of volunteers in a quantifiable amount, turning them into non-representative, and reinforcing the relevance of the biological features inherent to each volunteer for the final bioavailability of (poly)phenols. Moreover, the dispersion detected in the quantitative profile of plasma metabolites, enclosed to the inter-individual variation, would make difficult to find significant differences between beverages.

On the other hand, N glu, CA glu, CA sulfate, DHPAA, DHPAA di-glu, DHPAA sulfate, DHPAA glu-sulfate, DHPAA di-sulfate, HA, TFA glu, VA, VA di-glu, VA sulfate, and VA glu-sulfate were identified and quantified in plasma of all volunteers. However, HA was not considered because of its high background levels, probably originated from other dietary and endogenous sources, making the determination of the amounts coming from the metabolism of anthocyanins difficult [14].

3.3. Quantitative profile of flavanone and anthocyanin metabolites in plasma

The quantification of circulating metabolites was developed on basal plasma (0 minutes), as well as on plasma at 30, 60, and 210 minutes. The kinetic for the above-referred metabolites exhibited the highest concentration at 30-60 min in plasma, after the intake of the beverages, according to previous studies [15], suggesting that sweeteners do not change the absorption kinetic of flavonoids. For this reason, all concentrations described are referred to as that time-points (Fig. 1).

The content of N glu in plasma was 0.10 µg/mL, for those volunteers that ingested stevia as sweetener. This concentration was higher than the reached when using sucralose and sucrose, which displayed concentrations ~25% lower, on average, in comparison with beverages done using sucralose (Fig. 1). Despite these differences between sweeteners, the application of the RM ANOVA allowed identifying significant augments regarding the concentration of N glu at min 30 ($p<0.05$) for juices developed using stevia as sweetener.

The amount of whole phase II derivatives of CA recorded was 0.37 ng/mL after the intake of sucralose-sweetened beverages, being a 29% higher, on average, than the provided after the intake of beverages prepared using stevia and sucrose as sweeteners. When analyzing the effect of the sweetener regarding the individual compounds, for CA glu and CA sulfate, the highest values corresponded to juices developed using sucralose as sweetener, which gave rise to plasma concentrations of 0.22 and 0.07 ng/mL, respectively. These concentrations were ~36% higher than those reached when using stevia and sucrose. (Fig. 1). Summarizing the effect of consuming the juices under evaluation, the only phase II derivative of CA that increased significantly its concentration in peripheral blood plasma 30 and 60 min after the dietary intervention, according with the RM ANOVA was CA glu ($p<0.05$ and $p<0.01$, respectively for stevia and both time points significant at $p<0.05$ for sucrose and sucralose).

Moreover, sucrose was the sweetener that provided the highest plasma concentration for the sum of DHPAA and their phase II metabolites (2.44 ng/mL), which was ~36% higher than the reached when using sucralose and stevia. For DHPAA di-glu, DHPAA glu-sulfate and DHPAA di-sulfate, the highest value corresponded to the juices developed with added stevia (0.58; 0.19 and 0.29 ng/mL, respectively). Nevertheless, for DHPAA sulfate, the beverages elaborated with sucrose were the ones that provided the highest concentration in plasma, 1.42 ng/mL. The intake of sucralose sweetened juices rendered a higher plasma concentration than the other sweeteners for

DHPAA, 0.33 ng/mL. In this case, when comparing the sweetened beverage which provided the highest bioavailability for each compound against the remaining ones, the intestinal absorption rates were ~34% lower, for DHPAA and their phase II metabolites considered individually (Fig. 1). The only significant increase of the basal plasma concentration was retrieved for DHPAA di-sulfate at 30 and 60 min after the ingestion of juices elaborated using sucrose and sucralose as sweeteners (p<0.05).

Regarding TFA glu, stevia-sweetened juices provided the highest plasma concentration (0.81 ng/mL), which resulted in a 75% higher, on average, than the obtained after the ingestion of sucrose- and stevia-sweetened beverages.

The sum of the plasma concentration of VA and its phase II derivatives provided values of 6.13 ng/mL, in volunteers ingesting sucralose-sweetened juices, while the beverages developed based on stevia and sucrose gave rise to plasma concentrations 57% lower values, on average. When considering individual metabolites, for VA di-glu, VA sulfate, and VA glu-sulfate, the highest value corresponded to sucralose-sweetened juices, with the average plasma concentration values 1.93, 1.45 and 2.38 ng/mL, respectively. However, for VA, stevia-based drinks displayed the highest concentrations (0.81 ng/mL) (Fig. 1). Although, the dispersion of the values recorded as a result of inter-individual variability did not allow retrieving significant differences between sweeteners when applying the one-way ANOVA and Duncan's multiple range tests [16], when analyzing the increase caused by the separate sweeteners using MR ANOVA, it was observed that non-esterified VA and total VA experienced a significant augment after 60 min (p<0.05) for stevia-sweetened beverages.

As overall, results, described on the intestinal absorption of flavanones and anthocyanins after the intake of the developed beverages, indicated that stevia and sucralose were the most efficient sweeteners, regarding the plasma concentrations achieved for most flavanone and anthocyanin metabolites (N glu, DHPAA di-glu, DHPAA glu-sulfate, DHPAA di-sulfate, TFA glu and VA, for stevia; CA glu, CA sulfate, DHPAA, VA di-glu, VA sulfate and VA glu-sulfate, for sucralose), while sucrose only provided significantly higher concentrations of DHPAA sulfate. These differences could be attributable to the central role of intestinal sugar transporters in the absorption of flavonoids, as well as to the competence events that could be established between the separate sweeteners used and the phenolic compounds found [12]. In this regard, the central role of the sugar transporters in the absorption of phenolic compounds has been established on the base of characterizing the influence of the attached sugar in esterified phenolics that also condition strongly their solubility in the intestinal mucus as part of the mechanism of polyphenols absorption, as described for catechins, flavanones or phenolic acids [17]. However, the gap of knowledge on the effects of other components of foods, such as sweeteners present in manufactured products, on the bioavailability of polyphenols are underexplored, being required additional human studies to set-up the general principles affecting absorption *in vivo*.

Thus, these results suggest that both stevia and sucralose were better than sucrose in terms of intestinal absorption of citrus and maqui phenolics. In this aspect, several studies, describing the effects on human health and metabolic diseases of stevia and sucralose, have reported contradictory results as extensively reviewed by Daher et al. [18]. In this case, the majority of these interventional studies are focused on non-nutritional sweeteners isolated, the reason why the interaction of sweeteners with diet remains underexplored [18].

The interest of establishing the pharmacokinetic features of (poly)phenols in functional beverages is supported by their widely recognized biological benefits in humans, as a result of their dietary ingestion. In this regard, to the present date, several studies have suggested the cardioprotective activity of hesperidin and its aglycone, hesperetin (H) [4,19,20]. Besides, a study carried out on adults affected by metabolic syndrome revealed that the dietary ingestion of 500 mg hesperidin per day, for three weeks, improves the endothelial function and reduces the level of inflammatory markers [21]. On the other hand, anthocyanins are featured by valuable biological properties, including high radical scavenging activity, and the capacity to protect humans against risk factors for cardiovascular diseases and to inhibit adipogenesis, inflammation, and diabetes symptoms [6,22]. Also, recent meta-analyses of prospective cohort studies have evidenced that the dietary intake of anthocyanins reduces the risk of DM2 and cardiovascular disease, thus providing vascular benefits [23].

4. Conclusions

The results obtained in the present work shed light on the absorption ratio for berry anthocyanins and citrus flavanones, as well as a variety of phase II derivatives, resulting from the gastrointestinal process on these phenolic compounds. This is of special relevance because of the protective attributions of these compounds against cardiovascular diseases, and to decrease the severity of inflammatory processes and diabetes symptoms. The results obtained suggested that the greatest bioavailability for most of these metabolites was provided by stevia and sucralose, while, sucrose showed higher bioavailability only in 1 out of the 13 metabolites analyzed. So, considering the significantly different bioavailability achieved when ingesting beverages developed using the three sweeteners, this study proposes sucralose and stevia (non-caloric sweeteners) as valuable alternatives to sucrose (high caloric sweetener), which consumption has been associated to an augmented risk of DM2, obesity, and other metabolic disorders. Moreover, since this work shows promising results based in an acute intervention study, the development of an intervention assay addressed to set-up the effect of the chronic ingestion of the best juices according to our results could be interesting to understand more about the effects on human health of the two alternatives.

Acknowledgements

This work was funded by the Spanish MINECO through Research Project AGL2016-75332-C2-1-R (AEI/FEDER, UE). V.A. was supported by FPI (BES-2017-079754) grant of the Fellowship Program from the Spanish Ministry of Science, Innovation, and Universities (MICIU).

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FIGURE CAPTIONS

Fig. 1. Content (mean ± SD, n=20) of single flavonoid metabolites in basal peripheral blood plasma and plasma obtained, from healthy overweight volunteers, 30, 60, and 210 minutes after ingesting 330 mL of maqui-citrus beverages developed using stevia (▲), sucralose (●), and sucrose (■), as sweeteners.

TABLES

Table 1. Flavanone composition of the maqui-citrus juices.

Beverages	**Flavanones ^{Z} (mg/100mL)**				
	O-triglycosyl-N	**E 7-*O*-rutinoside**	**N 7-*O*-rutinoside**	**H 7-*O*-rutinoside**	**TOTAL**
Stevia	0.15 ± 0.02	0.32 ± 0.04	1.30 ± 0.01	4.87 ± 0.01	6.64 ± 0.2
Sucralose	0.14 ± 0.02	0.32 ± 0.01	1.31 ± 0.01	4.86 ± 0.01	6.63 ± 0.1
Sucrose	0.14 ± 0.01	0.31 ± 0.03	1.31 ± 0.01	4.88 ± 0.01	6.64 ± 0.1
P-value	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$

^{Z} N, naringenin; E, eriodictyol; H, hesperetin.

Table 2. Anthocyanins composition of the maqui-citrus juices.

Beverages	**Anthocyanins ^{Z} (mg/100mL)**							
	Dp 3-*O*-sam-5-*O*-glc	**Dp 3,5-*O*-diglc**	**Cy 3-*O*-sam-5-*O*-glc + Cy 3,5-*O*-di-glc**	**Dp 3-*O*-sam**	**Dp 3-*O*-glc**	**Cy 3-*O*-sam**	**Cy 3-*O*-glc**	**TOTAL**
Stevia	3.06 ± 0.12	3.59 ± 0.02	1.54 ± 0.02	1.09 ± 0.01	2.87 ± 0.02	0.40 ± 0.01	0.54 ± 0.01	13.1 ± 0.2
Sucralose	3.19 ± 0.05	3.51 ± 0.01	1.51 ± 0.01	1.11 ± 0.01	3.02 ± 0.01	0.41 ± 0.01	0.57 ± 0.01	13.3 ± 0.1
Sucrose	3.19 ± 0.01	3.36 ± 0.09	1.38 ± 0.01	1.09 ± 0.01	2.90 ± 0.01	0.40 ± 0.01	0.55 ± 0.01	12.9 ± 0.2
P-value	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$	>0.05 $^{N.s.}$

^{Z} Cy, cyanidin; Dp, delphinidin; Glc, glucoside; Sam, sambubioside.

Table 3. Qualitative analysis of anthocyanin and flavanone metabolites in plasma after the ingestion of maqui-citrus juices determined by UHLC-ESI-QqQ-MS/MS operated in multiple reaction monitoring, in negative and positive ionization modes, respectively.

Compound	RT (min)	Precursor ion [M-H]$^-$ *m/z*	Product ion MS2 [M-H]$^-$ *m/z*	Fragmentation (V)	CE (eV)	Polarity
Cyanidin metabolites						
Cyanidin (Cy)	8.81 (<LOD)	287.0	137.0	100	20	Positive
Cy 3-*O*-glucoside	<LOD	449.0	287.0	100	20	Positive
Cy 3,5-*O*-di-glucoside	<LOD	743.0	287.0	100	20	Positive
Cy 3-*O*-sambubioside	<LOD	581.0	287.0	100	20	Positive
Cy 3-*O*-sambubioside-5-*O*-glucoside	<LOD	611.0	287.0	100	20	Positive
Delphinidin metabolites						
Delphinidin (Dp)	5.18 (<LOD)	303.0	229.0/257.0	100	20	Positive
Dp 3-*O*-glucoside	<LOD	465.0	303.0	100	20	Positive
Dp 3,5-*O*-di-glucoside	<LOD	627.0	303.0	100	20	Positive
Dp 3-*O*-sambubioside	<LOD	597.0	303.0	100	20	Positive
Dp 3-*O*-sambubioside-5-*O*-glucoside	<LOD	759.0	303.0	100	20	Positive
Eriodictyol metabolites						
Eriodictyol (E)	6.49	287.0	151.0	70	10	Negative
Eriocitrin	<LOD	449.0	287.0	70	10	Negative
E glucuronide	4.87	463.0	287.0	70	10	Negative
E di-glucuronide	<LOD	639.0	287.0	70	10	Negative
E sulfate	5.53	367.0	287.0	70	10	Negative
E di-sulfate	<LOD	447.0	287.0	70	10	Negative
E glucuronide-sulfate	<LOD	543.0	287.0	70	10	Negative
Hesperetin metabolites						
Hesperetin (H)	7.30 (<LOD)	302.0	151.0	70	20	Negative
Hesperidin	<LOD	609.0	302.0	70	20	Negative
H glucuronide	<LOD	478.0	302.0	70	20	Negative
H di-glucuronide	<LOD	664.0	302.0	70	20	Negative
H sulfate	<LOD	382.0	302.0	70	20	Negative
H di-sulfate	<LOD	462.0	302.0	70	20	Negative
H glucuronide-sulfate	<LOD	558.0	302.0	70	20	Negative
Homoeriodictyol metabolites						
Homoeriodictyol (HE)	7.30 (<LOD)	301.0	151.0	110	15	Negative
HE glucuronide	5.50	477.0	301.0	110	15	Negative
HE di-glucuronide	<LOD	653.0	301.0	110	15	Negative
HE sulfate	<LOD	381.0	301.0	110	15	Negative
HE di-sulfate	<LOD	461.0	301.0	110	15	Negative
HE glucuronide-sulfate	4.67	557.0	301.0	110	15	Negative
Naringenin metabolites						
Naringenin (N)	7.26	271.0	119.0	130	20	Negative
O-triglycosyl-N	4.63	433.0	271.0	130	20	Negative
Narirutin	<LOD	579.0	271.0	130	20	Negative
N glucuronide	5.07	433.0	271.0	130	20	Negative
N di-glucuronide	<LOD	623.0	271.0	130	20	Negative
N sulfate	<LOD	351.0	271.0	130	20	Negative
N di-sulfate	<LOD	431.0	271.0	130	20	Negative
N glucuronide-sulfate	<LOD	527.0	271.0	130	20	Negative

<LOD, lower than the limit of detection.

Table 3. Qualitative analysis of anthocyanin and flavanone metabolites in plasma after the ingestion of maqui-citrus juices determined by UHLC-ESI-QqQ-MS/MS operated in multiple reaction monitoring, in negative and positive ionization modes, respectively (*Cont.*).

Caffeic acid metabolites						
Caffeic acid (CA)	3.25 (<LOD)	179.1	135.0	70	15	Negative
CA glucuronide	2.40	355.1	179.1	70	15	Negative
CA di-glucuronide	1.67	531.1	179.1	70	15	Negative
CA sulfate	2.99	259.1	179.1	70	15	Negative
CA glucuronide-sulfate	1.95	435.1	179.1	70	15	Negative
CA di-Sulfate	<LOD	339.1	179.1	70	15	Negative
Catechol metabolites						
Catechol (CAT)	5.04 (<LOD)	109.0	67.0	80	6	Negative
CAT glucuronide	<LOD	286.0	109.0	80	6	Negative
CAT di glucuronide	2.83 (<LOD)	461.0	109.0	80	6	Negative
CAT sulfate	1.59 (<LOD)	189.0	109.0	80	6	Negative
CAT glucuronide-sulfate	1.38 (<LOD)	365.0	109.0	80	6	Negative
CAT di-sulfate	<LOD	269.0	109.0	80	6	Negative
3,4-Dihydroxyphenylacetic acid metabolites						
3,4-Dihydroxyphenylacetic acid (DHPAA)	1.80	166.8	123.2	70	5	Negative
DHPAA glucuronide	1.58	342.8	166.8	70	5	Negative
DHPAA di-glucuronide	1.04	518.8	166.8	70	5	Negative
DHPAA sulfate	1.14	246.8	166.8	70	5	Negative
DHPAA glucuronide-sulfate	0.74	422.8	166.8	70	5	Negative
DHPAA di-sulfate	1.07	326.8	166.8	70	5	Negative
Hippuric acid metabolites						
Hippuric acid (HA)	2.55	178.0	134.4	80	5	Negative
HA glucuronide	1.70 (<LOD)	354.0	178.0	80	5	Negative
HA di-glucuronide	0.59 (<LOD)	530.0	178.0	80	5	Negative
HA sulfate	1.78	258.0	178.0	80	5	Negative
HA glucuronide-sulfate	1.50	434.0	178.0	80	5	Negative
HA di-sulfate	<LOD	338.0	178.0	80	5	Negative
Gallic acid metabolites						
Gallic acid (GA)	0.71 (<LOD)	169.0	125.0	70	10	Negative
GA glucuronide	<LOD	345.0	169.0	70	10	Negative
GA di-glucuronide	<LOD	521.0	169.0	70	10	Negative
GA sulfate	<LOD	249.0	169.0	70	10	Negative
GA glucuronide-sulfate	<LOD	425.0	169.0	70	10	Negative
GA di-sulfate	<LOD	329.0	169.0	70	10	Negative
Trans-*ferulic acid metabolites*						
Trans-ferulic acid (TFA)	4.46	192.8	133.8	20	5	Negative
TFA glucuronide	4.25	368.8	192.8	20	5	Negative
TFA di-glucuronide	1.74 (<LOD)	544.8	192.8	20	5	Negative
TFA sulfate	3.56 (<LOD)	272.8	192.8	20	5	Negative
TFA glucuronide-sulfate	<LOD	448.8	192.8	20	5	Negative
TFA di-sulfate	1.32	352.8	192.8	20	5	Negative

<LOD, lower than the limit of detection.

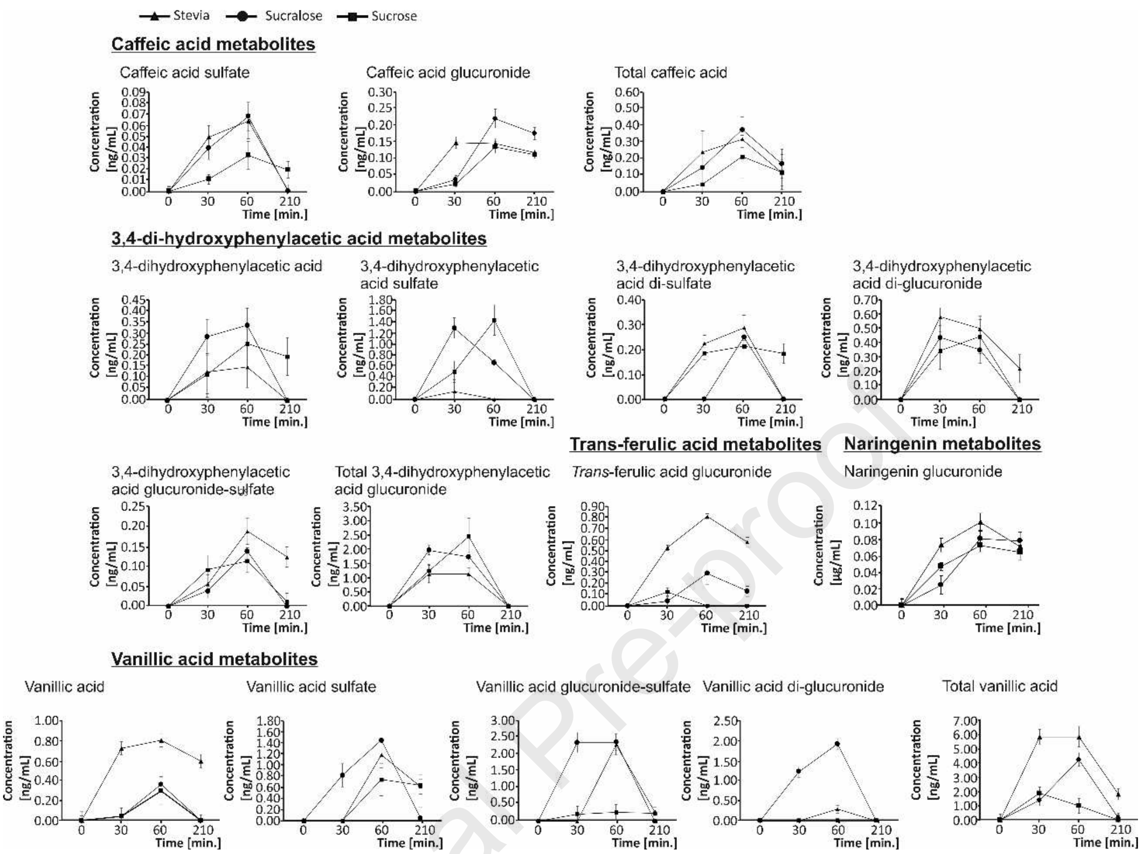
Table 3. Qualitative analysis of anthocyanin and flavanone metabolites in plasma after the ingestion of maqui-citrus juices determined by UHLC-ESI-QqQ-MS/MS operated in multiple reaction monitoring, in negative and positive ionization modes, respectively (*Cont.*).

2,4,6-Trihydrobenzaldehid metabolites						
2,4,6-Trihydrobenzaldehid (THBA)	5.10	153.1	106.8	90	18	Negative
THBA glucuronide	5.08 (<LOD)	329.1	153.1	90	18	Negative
THBA di-glucuronide	<LOD	505.1	153.1	90	18	Negative
THBA sulfate	1.46	233.1	153.1	90	18	Negative
THBA glucuronide-sulfate	<LOD	409.1	153.1	90	18	Negative
THBA di-sulfate	<LOD	313.1	153.1	90	18	Negative
Trans-isoferulic acid metabolites						
Trans-isoferulic acid (TIFA)	1.46	193.7	134.7	70	5	Negative
TIFA glucuronide	<LOD	366.7	193.7	70	5	Negative
TIFA di-glucuronide	<LOD	545.7	193.7	70	5	Negative
TIFA sulfate	1.45 (<LOD)	273.7	193.7	70	5	Negative
TIFA glucuronide-sulfate	<LOD	449.7	193.7	70	5	Negative
TIFA di-sulfate	<LOD	353.7	193.7	70	5	Negative
Vanillic acid metabolites						
Vanillic acid (VA)	3.18	167.0	151.8	100	15	Negative
VA glucuronide	1.57 (<LOD)	343.0	167.0	100	15	Negative
VA di-glucuronide	1.01	519.0	167.0	100	15	Negative
VA sulfate	1.14	247.0	167.0	100	15	Negative
VA glucuronide-sulfate	0.93	423.0	167.0	100	15	Negative
VA di-sulfate	1.13 (<LOD)	327.0	167.0	100	15	Negative

<LOD, lower than the limit of detection.

FIGURES

Fig. 1.



NUTRITION, METABOLISM, AND CARDIOVASCULAR DISEASES

Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage

HIGHLIGHTS

- Plasma concentration of bioavailable anthocyanins and flavanones was determined
- Sweetener were assessed on their capacity to influence the bioavailability of anthocyanins and flavanones
- The greatest bioavailability for these metabolites was provided by sucralose and stevia
- Sucralose and stevia are valuable alternatives to sucrose

NUTRITION, METABOLISM, AND CARDIOVASCULAR DISEASES

Sweetener influences plasma concentration of flavonoids in humans after an acute intake of a new (poly)phenol-rich beverage

CONFLICT OF INTEREST FORM

The authors declare neither financial nor other relationships that might lead to a conflict of interest.