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Phenol metabolic fingerprint and selection of intake biomarkers after acute and sustained consumption of red-fleshed apple versus common apple in humans. The AppleCOR study

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ABSTRACT

The present study aimed to evaluate the metabolism and bioavailability of anthocyanins (ACN) and other phenolics from red-fleshed apple (RFA) and to define the intake biomarkers compared to common white-fleshed apple (WFA). Acute and sustained (6-week) interventions were combined in a randomized, controlled and parallel study with 121 hypercholesterolemic subjects. Another arm consuming ACN-rich infusion from aronia fruit (ARO) provided matched content and profile of ACN. Plasma, urine and faeces samples were analyzed using UPLC-MS/MS. Results showed higher bioavailability of ACN after ARO compared to RFA, showing a clear apple matrix effect. The dihydrochalcone phloretin-2′-*O*-glucuronide was the most discriminant intake biomarker of both apples. The urinary peonidin-3-*O*-galactoside was a good biomarker after both ARO and RFA intakes, whereas peonidin-*O*-arabinoside was reported to be specific from ARO. The elucidation of the phenolic metabolism and the selection of intake biomarkers is a promising approach to relate phenolic compounds and human health.

1. Introduction

Different epidemiological studies have demonstrated that dietary phenolic compounds (PCs) could play an important role in the reduction of the risk of chronic diseases, including cardiovascular diseases (CVD) and age-related diseases (Potì et al. 2019). However, it has proved difficult to quantitatively establish the benefit afforded by PCs, in part due to the limited understanding regarding the extent of their absorption and metabolic fate (Tresserra-Rimbau et al., 2018).

Robust biological markers of dietary PCs exposure are essential in improving the understanding of the complex relationship between PCs and health outcomes, and most recent studies disclose the need of accurate methods, such as liquid chromatography coupled to mass spectrometry, to estimate and identify biological metabolites as markers of PCs intake in epidemiological studies (Charles et al., 2021). In general, food native PCs are poorly absorbed and are subjected to extensive metabolism following ingestion, both in the upper gastro-intestinal tract and in the large intestine, where the non-absorbed PCs reach the colon and are subjected to catabolic actions by the colonic microbiota (Spencer et al., 2008). A number of factors, including age, sex, genotype and gut microbiome may affect the metabolic processes. In addition, the bioaccessibility is a crucial factor in the phenol bioavailability and the food components such as proteins, lipids, carbohydrates and fiber can positively or negatively affect the subsequent bioavailability of the PCs (Velderrain-Rodríguez et al., 2014).

Apple (*Malus domestica*) is the most consumed fruit in Europe and has demonstrated to exert protective effects towards CVD and other chronic diseases (Hyson, 2011; Zhu et al. 2021). Apple is a well-known source of bioactive compounds, which are mainly represented by a wide range of PCs including hydroxycinnamic acids, flavan-3-ols, dihydrochalcones,

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and flavonols. Some apple varieties also contain small amounts of anthocyanins (ACN) in the peel, and the emerging biofortified red-fleshed apple (RFA) cultivars contain an enhanced content of ACN in its flesh, specifically of cyanidin-3-*O*-galactoside (Bars-Cortina et al., 2017). So the existence of ACN in RFA has added up in the list of health-promoting components in apple, as ACN have shown benefits in the prevention and management of cardiometabolic diseases (Yang et al. 2017). Preliminary *in vivo* studies after RFA supplementation reported cardiometabolic protective effects in hypercholesterolemic rats (Yuste et al. 2021) and a diminishing effect on the appearance of precancerous markers in a rat carcinogenesis model (Bars-Cortina et al., 2017).

The main aim of the present study was to unravel the metabolic pathways of ACN and the rest of PCs from RFA in front of a common white-fleshed apple (WFA) after acute and sustained dietary interventions in humans. Additionally, RFA was compared to an aronia fruit infusion (ARO), which provided matched content and profile of ACN but without the apple matrix components. Aside from proposing a comprehensive picture of the phenolic metabolome, we also aimed to select those metabolites that could be considered as potential intake biomarkers from RFA, WFA and ARO consumption.

2. Materials and methods

2.1. Chemicals and reagents

Cyanidin-3-*O*-galactoside, eriodictyol, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, dimer B_2, phloretin-2′-*O*-glucoside, 4′-hydroxycinnamic acid, and 3′,4′-dihydroxycinnamic acid (aka caffeic acid) were purchased from Extrasynthese (Genay, France). 4-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (aka protocatechuic acid), hippuric acid, 3-(4′-hydroxyphenyl) acetic acid, 3-(3′,4′-dihydroxyphenyl)acetic acid, 3-(3′-hydroxyphenyl)propanoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid (aka dihydrocaffeic acid), 3-(3′-hydroxy-4′-methoxyphenyl)propanoic acid (aka dihydroferulic acid), epicatechin, and 5-*O*-caffeoylquinic acid (aka chlorogenic acid) were from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxy-3-methoxybenzoic acid (aka vanillic acid) and 4′-hydroxy-3′-methoxycinnamic acid (aka ferulic acid) were from Fluka (Buchs, Switzerland). Hydroxy-3-methoxybenzoic acid-4-*O*-sulphate, benzene-1,2-diol-4-*O*-sulphate, and 4-methyl benzene-1,2-diol-*O*-sulphate were synthesized according to Pimpao et al. (2015) and were kindly supplied by Dr. Claudia N. Santos (Portugal).

Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid, and hydrochloric acid (HCl) were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water used was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

A stock solution of each phenolic compound was prepared by dissolving each standard compound in methanol at a concentration of 1000 mg/L and storing these in dark flasks at 4 °C. Stock dilutions were prepared daily.

2.2. Intervention products: red-fleshed apple and white-fleshed apple snacks and aronia infusion

In the study two different apple varieties were used, the red-fleshed “Redlove” apple variety, a new genotype naturally biofortified in ACN, and the common white-fleshed Granny Smith apple variety without ACN (both provided by NUFRI SAT, Mollerussa, Lleida, Spain). To preserve the phenolic compounds of the apples, a freeze-dried snack was prepared and provided to the volunteers. The detailed preparation process of the freeze-dried apple snacks is reported in our previous study (Yuste et al., 2019). The daily amount of apple snacks provided to participants was 80 g/day of RFA and 80 g/day of WFA snacks and were administered in individual daily plastic containers (Supplemental Fig. 1**).**

Additionally, an ACN-rich infusion from aronia fruit was also used in order to study the effect of ACN without the interaction of the apple matrix. Aronia fruit was selected as it is a rich source of cyanidin-3-*O*-galactoside and cyanidin-*O*-arabinoside, which are the main ACN in RFA. *Aronia melanocarpa* fruit powder (Aronia Pulver, BIOJOY, Nuremberg, Germany) was used to prepare a daily cold-water infusion by the volunteers. An infusion prepared with 50 g of Aronia fruit powder and 1 L of mineral water (Bezoya mineral water, Calidad Pascual, Aranda de Duero, Burgos, Spain) satisfactorily allowed us to provide the same ACN compounds present in RFA with an equivalent daily dose and without the fruit matrix. The volunteers were instructed to mix the Aronia fruit powder with water, to energetically homogenize the mixture in a glass bottle for 3 min and place the filtered in a light protected bottle ready to be consumed along the day. To prepare and consume 1 L/day ARO cold infusion, volunteers were provided with the required tools and instructions (Supplemental Fig. 1**).**

The detailed phenolic composition of the WFA and RFA snacks, and ARO infusion and their nutritional composition are shown in Supplemental Table 1, and in Supplemental Table 2, respectively.

2.3. Participants

Subjects from the general population were recruited by means of news in the newspapers, social networks, and tableaux advertisements in the Hospital Universitari Sant Joan (HUSJ)-Eurecat, Reus, Spain, between January 2019 and May 2019. From 179 subjects assessed for eligibility, 121 subjects were recruited and randomized.

Inclusion criteria were to be aged > 18, LDLc levels ≥ 115 mg/dL and willingness to provide informed consent before the initial screening visit. Exclusion criteria were: LDLc levels ≤ 115 and ≥ 190 mg/dL or with hypolipemiant treatment (drugs and functional foods); or with hypoglucaemiant treatment or type 1 and type 2 diabetes mellitus diagnosed; Body Mass Index (BMI) ≥ 35 kg/m^2; Triglyceride levels ≥ 350 mg/dL; Anaemia (hemoglobin ≤ 13 g/dL in men and ≤ 12 g/dL in women); Subjects diagnosed of intestinal disorders such as chronic disease, colitis ulcerous, celiac disease and irritable bowel syndrome; To present fructose and/or sorbitol and/or gluten intolerance; Use of antioxidants supplements; Pregnant or intending to become pregnant; To be in breast-feeding period; Chronic alcoholism; Smoking; Current or past participation in a clinical trial or consumption of a research product in the 30 days prior to inclusion in the study; Failure to follow the study guidelines.

Participants signed informed consent prior to their participation in the study, which was approved by the Clinical Research Ethical Committee of Institut d’Investigació Sanitària Pere Virgili (S033/04Nov2016), Reus, Spain. The protocol and trial were conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (GCP ICH) and were reported as CONSORT criteria. The trial was registered at ClinicalTrials.gov Identifier: NCT03795324.

Assuming a 0.50 mmol/L (approximately 15%) post-intervention difference of LDLc and a 0.72 mmol/L standard deviation (SD), with $\alpha = 0.05$ and $1\text{-}\beta = 0.08$ a minimum of 22 participants were required. However, the sample size will be 40 participants for arm, in total 120 subjects.

2.4. Study design: Acute and sustained intake studies in humans

The present work is framed in the AppleCOR project, aimed to advance in the knowledge of the potential health effects and mechanisms of action of ACN in the improvement of the cardiometabolic risk factors focused on the reduction of LDL cholesterol. A parallel, randomized, controlled trial combining acute and sustained dietary interventions was executed in hypercholesterolemic subjects (Supplemental Fig. 2), where participants were assigned to one of the three intervention groups RFA, WFA, or ARO, to consume 80 g/day of the corresponding apple snack or 1 L/day of ARO infusion, which were freely consumed along the day for 6 weeks. Participants were randomly allocated to the three intervention groups by a computerized random number generator made by an independent statistician. PROC PLAN (SAS 9.2, Cary, NC: 83 SAS Institute Inc.) with a 1:1:1 allocation using random block sizes of 2, 4, and 6 was used.

At the beginning of the sustained study, an acute intake study was performed in a subsample of volunteers and the daily dose of the intervention products were administered all at once. After enrolment and following a 1-week run-in period on a control diet (maintained dietary habits based on nutritionist recommendations), volunteers started the intervention trial. During the intervention period participants were instructed to maintain their lifestyle, physical activity and dietary habits, to completely refrain from consuming ACN-rich foods (berries, grapefruit, plums, figs, pomegranate, green and red apples, avocado, black olives, red and black beans, red wine and mushrooms) and to avoid eating functional foods intended to improve cholesterol levels. Participant who performed the acute intake study were instructed to maintain a polyphenol-free diet two days previous to the acute intake. Participants attended 4 visits (every 2 weeks) at the Eurecat Human Nutrition Unit (Reus, Catalonia, Spain). The acute study was performed at Visit 1 and participants stay at Eurecat from 08:00 am to 02:00 pm, and received a light meal before leaving.

For the sustained intake study, blood, 24 h urine and faeces samples were obtained at visit 1 and visit 4. For the acute study, blood samples were obtained by traditional puncture at baseline, 2 h, 4 h, and 6 h and urine samples were collected 24 h before and after the intake at the intervals times of 0–3, 3–6, and 6–24 h. The total volume of urine of each volunteer in each interval time was measured and samples were stored at −80 °C in the central laboratory's Biobanc of HUSJ-Eurecat (biobanc.reus@iispv.cat) until required for batch analyses. Each biological sample was analysed and pre-treated in duplicate in the acute study and once in the sustained study.

The adherence of the participants to their dietary habits through the study was assessed by a 3-day food record at visit 1 and visit 4. At each visit, subjects also underwent a physical examination by a general practitioner, completed a Physical Activity Questionnaire Class AF (Vallbona et al., 2007), and anthropometric measurements were recorded.

2.5. Plasma, urine and faeces samples pre-treatment

Prior to the chromatographic analysis, plasma and urine samples were subjected to a clean-up with micro-Elution solid-phase extraction (μSPE) by using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges as reported in our previous studies (Yuste et al., 2019; Yuste et al., 2020). Regarding urine samples, the conditioning and equilibration steps were the same as reported for plasma samples. The methodology used for the analysis of faeces samples was the reported in our previous study (Mosele et al., 2015).

2.6. Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-ESI-MS/MS)

The analysis of PCs in the apple snacks and in the ARO infusion, and in biological samples was performed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) as the detection system (Waters, Milford, MA, USA). Tandem MS (MS/MS) analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The UPLC-MS/MS conditions were the reported in our previous studies (Martí et al., 2010; Bars-Cortina et al., 2017; Yuste et al., 2018; Yuste et al., 2019; Yuste et al., 2020). Supplemental Table 3 shows the SRM transition for quantification, as well as the cone voltage and collision energy for each phenolic compound. Due to the lack of commercial phenolic standards and their generated metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another PC with a similar structure. Supplemental Table 3 also shows in which phenolic standard compound these phenolics have been tentatively quantified. The phenolic metabolite nomenclature used was the recommended by Kay et al. (2020). When the position of the methyl, sulphate, or glucuronide group, as well as the hydroxyl position in phenylacetic acid and phenylpropanoic acid metabolites was unknown due to the lack of standard phenolic compounds, the general name was used.

2.7. Statistical analysis

The results are presented as mean values ± standard deviation (SD) for the PCs in WFA snack, RFA snack, and ARO infusion, and as mean values ± standard error of the mean (SEM) for the generated phenolic metabolites in the biological samples (plasma, urine and faeces).

In the acute study, a paired Student *t*-test (ANOVA-one way) was employed to compare the post-treatment and baseline values. Regarding the sustained study, the increasing concentration (day 6 w – day 0) for each volunteer after each treatment of all the phenolic metabolites were compared. Then, general Linear Model and with one-way repeated measures analysis of variance (ANOVA) followed by a Tukey's multiple-difference test was used. The statistical analyses and the principal components analysis (PCA) were performed with the Statgraphics plus version 5.1 software (Manugistics Inc., Rockville, MD, United States).

3. Results and discussion

3.1. Phenol content in red-fleshed and white-fleshed apple snacks, and aronia infusion

The daily dose of total PCs ingested through both apple snacks was very similar, 197 ± 17.5 mg/80 g WFA snack/day, and 193 ± 16.5 mg/80 g RFA snack/day (Supplemental Table 1). Both apple snacks had a similar phenolic profile of phenolic acids (benzoic and hydroxycinnamic acids), flavonols, flavones, flavanones, and dihydrochalcones, being the presence or absence of ACN the main difference between them. Both apple snacks also differed in the flavan-3-ol content, being its concentration in WFA snack around 8-fold higher than RFA snack. The diet supplementation with fresh fruit instead of a formulated food, did not allow a precise adjustment of the concentrations of different phenolic groups in both apple snacks. However, in this study one of the main focus was the evaluation of the ACN bioavailability and metabolism after the intake of RFA, using a WFA as a control, and ARO as an ACN-rich source without the apple matrix.

ARO infusion was selected to be a rich source of cyanidin-3-*O*-galactoside and cyanidin-*O*-arabinoside (Wicczkowski et al., 2010), which were the main ACN detected in RFA (Bars-Cortina et al., 2017). Although the daily dose of total PCs in ARO infusion (99.1 ± 10.9 mg/1L/day) was lower than in RFA snack (193 ± 16.5 mg/80 g snack/day), the daily ACN dose was nearly the same, around 35 mg/day. Half of the daily dose administered by ARO was ACN and the other half were phenolic acids, being caffeoylquinic acid and 3,4-dihydroxybenzoic acid the most abundant. It has been reported that ARO fruit is also rich in flavan-3-ols and their oligomeric forms of proanthocyanidins (Xie et al., 2016). In order to minimize their content, the volunteers ingested a cold infusion with aronia powder after a subsequent filtration to remove the solid residue which was rich in flavan-3-ols (data not shown).

3.2. Participants

From the 179 subjects assessed for eligibility, 58 did not meet the inclusion criteria and were excluded. The remaining 121 participants were randomly allocated to RFA (n = 40), WFA (n = 41), or ARO (n = 40) groups. Ultimately, 107 participants completed the study (32 in the RFA, 37 in the WFA, and 38 in the ARO groups). For the acute study, 29 participants were allocated (12 in the RFA, 8 in the WFA, and 9 in the ARO groups). See CONSORT Flowchart of the study in Supplemental

Fig. 3. No differences in baseline characteristics were observed among intervention groups (Supplemental Table 4). No differences in the level of physical activity or dietary habits were observed from the beginning to the end of the study in any of the groups.

3.3. Acute intake study: Time course of postprandial changes in phenol biological metabolites

In the following subsections, the time course and the pharmacokinetic parameters of the different phenolic metabolites detected in plasma and urine in the acute study is described. Based on those metabolites that presented significant differences between the tested meals, we performed an initial selection of those metabolites that could be considered as potential intake biomarkers.

Supplemental Fig. 4 shows the time course of postprandial changes in plasma of the main phenolic metabolites generated over 6 h (area under the curve AUC, and maximum plasma concentration, C_{max}), and Supplemental Table 5 shows the concentration of the complete phenolic metabolites list detected in plasma. On the other hand, the complete urinary phenolic metabolites excreted in different time intervals are shown in Supplemental Table 6**.** Fig. 1 shows a selection of the main urinary phenolic metabolites (26 metabolites) presenting significant differences in 24 h urine between RFA and WFA, and between RFA and ARO.

3.3.1. Anthocyanin metabolites

The comparative analysis between RFA and ARO in plasma and urinary postprandial samples, revealed important differences in the bioavailability of ACN, although the ACN ingested dose was equivalent in both products (around 35 mg/day).

After the acute intake of both RFA snack and ARO infusion, three different plasma circulating ACN were detected, and these were the intact cyanidin-3-*O*-galactoside, its methylated metabolite peonidin-3-*O*-galactoside, and peonidin-*O*-glucuronide (Supplemental Fig. 4). Results showed that the concentration of these ACN metabolites was statistically higher after the intake of the ARO infusion compared to RFA snack in all plasma sampling times (Supplemental Table 5). In addition, three main simple phenolic acids, 3,4-dihydroxybenzoic acid-*O*-sulphate, 4-hydroxy-3-methoxybenzoic acid-*O*-glucuronide and 3-hydroxy-4-methoxybenzoic acid-*O*-glucuronide, which have been proposed as colonic metabolites of ACN (Vitaglione et al., 2007; Aura, 2008; Mosele et al., 2015), appeared also in significantly higher concentration after the ARO infusion intake compared to RFA. 3,4-Dihydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid could be generated after the microbial B-ring fission of cyanidin-*O*-glycosides and peonidin-*O*-glycosides, respectively, with the subsequent sulphation and glucuronidation. The pharmacokinetic parameters of ACN and their metabolites also showed that after the ARO infusion intake, the AUC and the C_{max} were statistically higher than RFA intake (Supplemental Fig. 4). Moreover, the total ACN excreted in 24 h urine after the RFA snack intake were 32.7 ± 3.35 nmols/24 h, in contrast to 129 ± 11.4 nmols/24 h after the ARO infusion intake (Fig. 1 and Supplemental Table 6).

It was also observed that the kinetics in the urinary ACN excretion was also different between RFA and ARO. For RFA snack, the maximum ACN excretion was between 6 and 24 h, in contrast for ARO infusion that was between 0 and 3 h. This delay in the ACN urine excretion could be due to the apple matrix complexity, in which the phenolics are mainly bound to fiber and they need to be released from the matrix to be absorbed in large intestine (Quirós-Sauceda et al., 2014; Pérez-Jiménez et al., 2013).

A matrix effect was also denoted in the higher bioavailability of ACN reported in both plasma and urine samples after ARO infusion intake. These results are in accordance with previous studies, reporting a strong influence of the food matrix on flavonoid bioavailability (Kamiloglu et al., 2020). Specifically, soluble fiber such as pectin, which is present in the apple, has shown to affect the flavonoid loss in serum and urine fractions due to the binding interactions with fiber (Padayachee et al., 2012; Viuda-Martos et al., 2018).

Regarding the metabolic profile of ACN metabolites, we observed that it was similar after the intake of RFA and ARO, with exception of peonidin-*O*-arabinoside that was only detected after ARO intake in urine samples. This fact could be the consequence of the higher concentration of cyanidin-*O*-arabinoside which was 3-fold higher in ARO infusion compared with RFA. Therefore, peonidin-*O*-arabinoside appeared to be a potential intake biomarker of ARO. The ACN metabolites detected in the present study were in agreement with the studies reported in the literature after the RFA (Yuste et al., 2019; Yuste et al., 2020) and ARO intakes (Wicczkowski et al., 2010; He et al., 2006). Also other berry fruits containing cyanidin-*O*-arabinoside, such as cranberries, have presented a similar metabolic profile in urine human samples after its acute ingestion (Ohnishi et al., 2006).

On the other hand, we reported that the ratio of peonidin-3-*O*-galactoside over cyanidin-3-*O*-galactoside (0.62 nmols) was practically identical to the ratio of peonidin-*O*-arabinoside over cyanidin-*O*-arabinoside (0.70 nmols) after ARO intake. These results are in accordance to a previous study supplementing rats with chokeberry rich in ACN (He et al., 2006). So our results confirm that methylation of ACN is not dependent of the sugar moiety.

3.3.2. Dihydrochalcone metabolites

In addition to ACN, the rest of metabolites derived from other phenolic groups also deserved attention. One of the most representative non-colored phenols quantified in both apple snacks were dihydrochalcones, a class of minor flavonoids. In the present study phloretin-2′-*O*-glucoside was identified as the main dihydrochalcone compound, which has been described in the literature as a specific compound present in apple fruit (Ulaszewska et al., 2018).

After the acute intake of both apples, phloretin-2′-*O*-glucuronide, and in minor amount phloretin-*O*-sulphate-*O*-glucuronide, were the main metabolites detected. Phloretin-2′-*O*-glycosides present in apple could undergo cleavage in the small intestine (deglycosylation), and the aglycone phloretin could be subjected to phase II metabolism, appearing rapidly in the circulatory system mainly as phloretin-2′-*O*-glucuronide.

Results showed that the plasma concentration values of phloretin-2′*O*-glucuronide after RFA were statistically higher (2-fold) than the values detected after the WFA in all time points (Supplemental Fig. 4). Also, the phloretin metabolites excreted in urine at all the interval times and their 24 h urinary excretion after the RFA snack (3.83 ± 0.54 μmols/24 h) appeared to be significantly higher than after the WFA snack (1.16 ± 0.14 μmols/24 h) **(**Supplemental Table 6 and Fig. 1), being the total excretion ratio of RFA *vs* WFA 3.3-fold. As observed in Supplemental Table 1, this relation was in accordance with the dihydrochalcone composition of RFA *vs* WFA (2.6-fold). So, phloretin-2′-*O*-glucuronide showed a dose–response relationship, similarly to that reported after apple consumption at different polyphenol doses in humans (Auclair et al., 2010; Saenger et al., 2017).

As it can be seen in Supplemental Fig. 4 and in Supplemental Table 4, the maximum plasma concentration of phloretin-2′-*O*-glucuronide was within the first hours post-intake. This early absorption peak was followed by a rapid decrease in plasma concentration, suggesting that the native dihydrochalcones from apple are quickly absorbed and metabolized in the upper gut with little or no contribution from the human colonic microbiota.

3.3.3. Flavan-3-ols metabolites

After the acute intake of both apple snacks, epicatechin-*O*-glucuronide was the main flavan-3-ol metabolite detected in plasma (Supplemental Fig. 4 and Supplemental Table 5). As it was expected, the AUC and the C_{max} of this metabolite was significantly higher after the intake of WFA compared with RFA, since WFA snack contained higher flavan-3-ol amounts. Similarly, a study by Actis-Goretta et al. 2013 reported that epicatechin-*O*-glucuronide was the most abundant metabolite

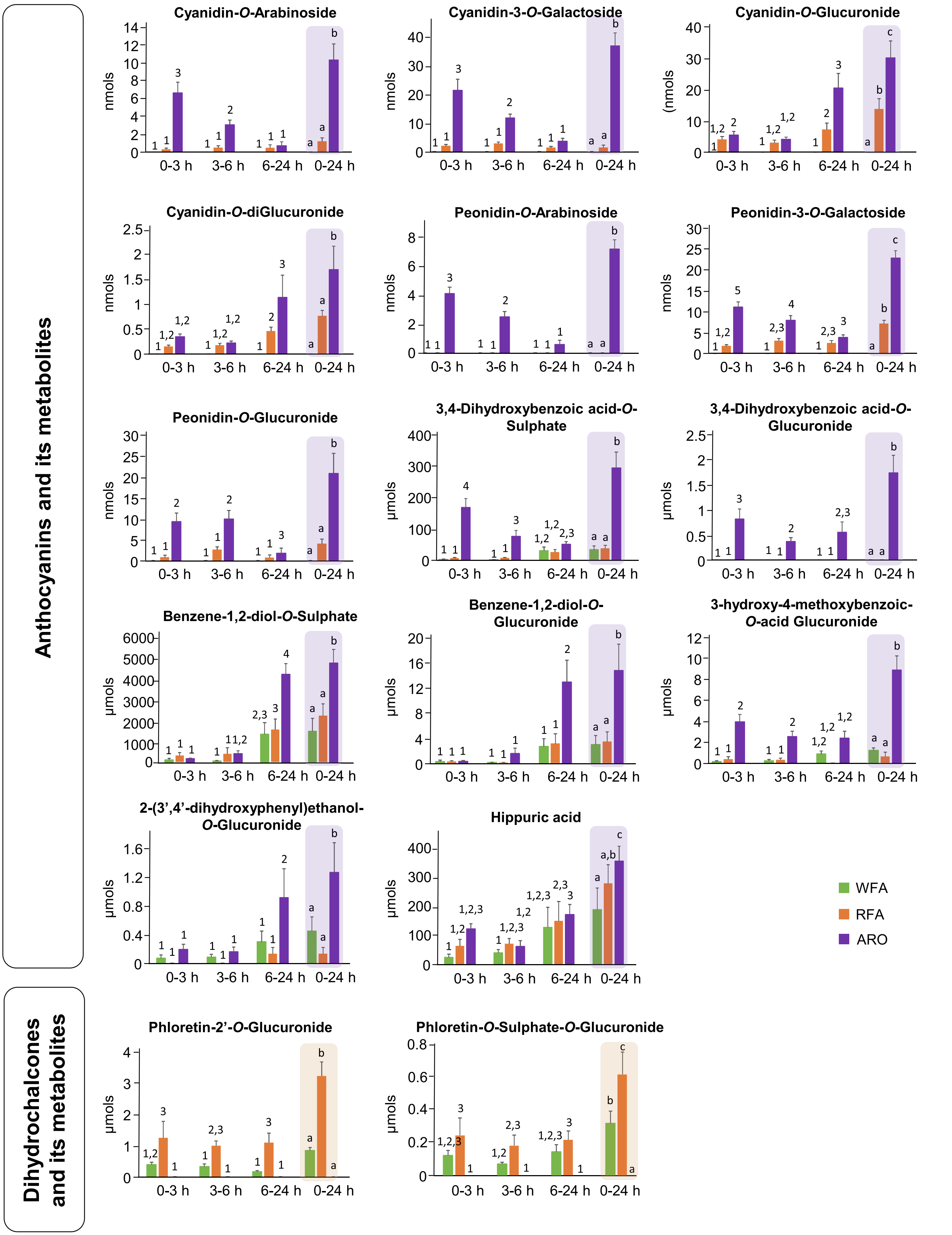

Fig. 1. Kinetics of the urinary phenolic metabolites excreted in the different time intervals and 24 h after the intake of WFA and RFA snacks, and ARO infusion. Different numbers (1, 2, 3) means significant differences among the different time intervals, $p < 0.05$. Different letters (a, b, c) means significant differences in 24 h, $p < 0.05$.

found in plasma after a single ingestion of a rich-flavan-3-ol meal.

In urine postprandial samples, ten flavan-3-ol metabolites (both phase II and colonic metabolites) were detected. In line with the plasma results, the total flavan-3-ol excretion after the WFA snack (3725 ± 594 μmols/24 h) was statistically higher than the detected after RFA snack (1429 ± 483 μmols/24 h) intakes (Fig. 1 and Supplemental Table 6). Among all the flavan-3-ol metabolites, (methyl) epicatechin-*O*-sulphate, epicatechin-*O*-sulphate-*O*-glucuronide, 5-(4′-hydroxyphenyl)-γ-valerolactone, and 5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-glucuronide, presented significant differences in the total urinary excretion between the two apple snack intakes (Fig. 1). Therefore, these five flavan-3-ol metabolites appeared to be potential intake biomarkers for WFA snack.

5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone-*O*-sulphate appeared to be the flavan-3-ol metabolite most abundant in 24 h-urine after the intake of WFA and RFA snacks and ARO infusion being the 76%, 89% and 86%, respectively, in relation to the total flavan-3-ol metabolites excreted. Our results are in accordance with previous studies, that reported this metabolite to be one of the most important microbial catabolites after the apple intake generated by colonic catabolism of polymeric flavan-3-ols (Appeldoorn et al., 2009; Serra, 2011).

3.3.4. Phenylpropanoic and phenylacetic acids metabolites

After the acute intake of the apple snacks and ARO infusion, four phenylpropanoic acid derivatives were detected in urine, and these were hydroxyphenylpropanoic acid-*O*-sulphate, 3-(2′,4′-dihydroxyphenyl) propanoic acid and its sulphate and glucuronide forms. The urine excretion of these metabolites at different intervals, and the total 24 h-urine excretion were statistically similar between the three intakes. An exception was for 3-(2′,4′-dihydroxyphenyl)propanoic acid, whose urine excretion was statistically higher after the WFA snack > ARO infusion > RFA snack (Fig. 1 and Supplemental Table 6).

We also observed that the phenylpropanoic acid derivatives in WFA and 3-(2′,4′-dihydroxyphenyl)propanoic acid in ARO infusion were mainly excreted in urine at the interval of 6–24 h. These phenylpropanoic acid metabolites are simple PCs generated in the large intestine by colonic degradation of flavan-3-ols, abundant in WFA snack (Velderrain-Rodríguez et al., 2014; Xie et al., 2016; Vitaglione et al., 2007). So, flavan-3-ols that are not absorbed in the small intestine could be transformed into lower molecular weight compounds by the colonic microbiota. These metabolites have also been previously reported in urine after the intake of flavan-3-ols rich sources such as coffee, grape juice or almonds (Del Rio et al., 2013; Aura 2008; Mosele et al., 2015). 3-(2′,4′-Dihydroxyphenyl)propanoic acid has also been reported to be a microbial metabolite generated from colonic degradation of B-ring fission of cyanidin derivative anthocyanins, which could explain the detection of this metabolite in ARO and RFA containing ACN.

Apart from phenylpropanoic acids, five phenylacetic acid metabolites were detected in urine after the three acute intakes, and these were phenylacetic acid, hydroxyphenylacetic acid (4′-, 3′-), and (di)hydroxyphenylacetic acid-*O*-sulphate. These hydroxyphenylacetic acids were also reported as colonic metabolites mainly from flavan-3-ols (Del Rio et al., 2013; Aura, 2008; Appeldoorn et al., 2009; Serra, 2011). When the different urine time intervals between the three intakes were compared, only significant differences were observed in the interval time of 6–24 h (Supplemental Table 6), observing higher values of hydroxyphenylacetic acid (4′-, 3′-), and total phenylacetic acid derivatives after WFA intake, compared to RFA and ARO infusion. However, the concentration of dihydroxyphenylacetic acid-*O*-sulphate was statistically higher after the ARO infusion intake. This could be explained because this colonic metabolite can be generated from cyanidin derivatives *via* 3-(2′,4′-dihydroxyphenyl)propanoic acid after B-ring fission of ACN by microbial α-oxidation (Del Rio et al., 2013; Aura 2008; Mosele., 2015a,b). Moreover, the concentration of hydroxyphenylacetic acid-*O*-sulphate was statistically similar after the WFA snack and ARO infusion intakes, but lower than RFA snack. This could be explained because this metabolite is a common colonic metabolite from flavan-3-ols and ACN (cyanidin-*O*-glycosides) (Yuste et al., 2019; Aura 2008; Mosele et al., 2015).

3.3.5. Phenolic acid metabolites

The three tested meals contained an important amount of phenolic acids (benzoic and hydroxycinnamic acids), and accordingly, a wide range of phenolic acids metabolites were detected, predominantly after ARO infusion intake (Supplemental Table 6). These metabolites have also been described as colonic metabolites from ACN.

Regarding benzoic acids, the total urinary (24 h) excretion was statistically higher after the ARO infusion, specifically, 3,4-dihydroxybenzoic acid-*O*-sulphate, 3,4-dihydroxybenzoic acid-*O*-glucuronide, and hippuric acid. The main hydroxycinnamic acids detected in urine were 3′,4′-dihydroxycinnamic acid-*O*-sulphate, 3′-hydroxy-4′-methoxycinnamic acid, 3′-hydroxy-4′-methoxycinnamic acid-*O*-sulphate, 4′-hydroxy-3′-methoxycinnamic acid-*O*-sulphate, and 3′-hydroxy-4′-methoxycinnamic acid-*O*-glucuronide. The concentrations of the sulphated forms of 3′,4′-dihydroxycinnamic acid and 3′-hydroxy-4′-methoxycinnamic acid after the ARO infusion in 24 h-urine were statistically similar than after WFA snack and higher than RFA snack. Regarding the interval excretion time, the maximum concentration of these compounds was mainly at the interval time of 6–24 h.

3.3.6. Benzene-1,2-diol metabolites

Benzene-1,2-diol derivatives, such as (methyl) benzene-1,2-diol-*O*-sulphate and (methyl) benzene-1,2-diol-*O*-glucuronide, were also determined after the three intakes and their urinary concentration was significant higher after ARO infusion intake at the interval time of 6–24 h (Supplemental Table 6). These metabolites have been described as colonic metabolites from ACN, but they can also come from other phenolic families (Fig. 1). Similar results were reported in the literature after the intake of berries (Gomes et al., 2019).

3.4. Sustained intake study: changes in phenol biological metabolites concentration

Bioavailability of PCs following acute intake has been deeply studied, however, there are only limited data on the effects of repeated and combined exposure to specific PCs food sources in their bioavailability and metabolic fate.

Changes in the phenolic metabolome in plasma, urine and faeces samples (Tables 1–3, respectively) after sustained diet supplementations during 6 weeks were studied. As it was done in the acute study, and as a previous step to determine the most discriminant biomarkers from each product, we selected the phenolic metabolites whose concentration was significantly increased between day 0 and day 42 and presented significant differences between WFA, RFA and ARO dietary supplementations. The complete metabolic data from the sustained study is included in Supplemental Tables 7–9 for WFA, RFA and ARO, respectively.

When comparing the acute and sustained studies, about 65% of acute consumption metabolites were also detected in samples from the sustained study. Similarly to the acute study, after the sustained intake of WFA snack, the main generated phenolic metabolites were from flavan-3-ols and dihydrochalcones, whereas the main phenolic metabolites after RFA intake were from ACN and dihydrochalcones phenolic groups. After the prolonged consumption of ARO infusion, a wider range of PCs were detected compared to the acute study, mainly from ACN and from hydroxycinnamic acids.

In plasma samples, 14 phenolic metabolites increased their concentration significantly and presented differences between the three studied products (Table 1), in front of 8 metabolites in the acute study. This could be due to the fact that we only collected plasma samples until 6 h post-intake, whereas, in the sustained study 24 h urine was collected, allowing detecting more colonic metabolites.

Regarding the urine samples (Table 2), 38 phenolic metabolites presented significant differences post-intake, and among them, 35

Table 1
Increase in plasma concentration (nM ± SEM) of the main phenolic metabolites generated after the sustained intake for 6 weeks of WFA and RFA snacks, and ARO infusion.

	△ Concentration (day 6 w – day 0)		
	WFA snack	RFA snack	ARO infusion
Anthocyanins			
Peonidin-3-*O*-galactoside	n.d. [1]	0.73 ± 0.03 [2]	0.63 ± 0.02 [2]
Peonidin-*O*-glucuronide	n.d. [1]	0.23 ± 0.10 [1]	1.90 ± 0.08 [2]
Flavan-3-ols			
5-(4'-Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	45.6 ± 2.21 [2]	n.d. [1]	10.2 ± 2.04 [1,2]
Dihydrochalcones			
Phloretin-2'-*O*-glucuronide	0.36 ± 0.34 [1]	8.83 ± 0.37 [2]	n.d. [1]
Phenylacetic acids			
4'-Hydroxyphenylacetic acid	-54.7 ± 27.9 [1]	569 ± 30.5 [1,2]	122 ± 25.6 [2]
3'-Hydroxyphenylacetic acid	919 ± 29.0 [2]	695 ± 31.8 [1,2]	226 ± 26.6 [1]
Dihydroxyphenylacetic acid-*O*-sulphate	−15.8 ± 5.16 [1]	138 ± 5.66 [2]	49.8 ± 4.73 [1]
Benzoic acids			
Hippuric acid	−1100 ± 176 [1]	3238 ± 193 [2]	1541 ± 162 [1,2]
Hydroxycinnamic acids			
3'-Hydroxy-4'-methoxycinnamic acid	4.43 ± 1.63 [1]	−11.2 ± 1.79[1]	33.0 ± 1.49 [2]
4'-Hydroxy-3'-methoxycinnamic acid-*O*-glucuronide	−2.26 ± 1.84 [1]	−8.45 ± 2.01 [1]	34.0 ± 1.68 [2]
3'-Hydroxy-4'-methoxycinnamic acid-*O*-glucuronide	-4.53 ± 2.59 [1]	−10.7 ± 2.84 [1]	44.8 ± 2.37 [2]
3-(4'-Hydroxy-3'-methoxyphenyl) propanoic acid-*O*-sulphate	−0.89 ± 1.37 [1]	−2.63 ± 1.50 [1]	24.3 ± 1.25 [2]
Catechol derivatives			
Benzene−1,2-diol-*O*-sulphate	-6914 ± 1245 [1]	14444 ± 1366 [2]	24063 ± 1144 [2]

Different number means significant differences among tested products ($p < 0.05$) by ANOVA-one way.
n.d.: not detected.
Volunteers analyzed: WFA (n=34), RFA (n=31), and ARO (n=37).

metabolites allowed to discriminate WFA *vs* RFA, and RFA *vs* ARO intakes, in front of 26 metabolites in urine samples from the acute study.

After the sustained intake of WFA snack, the concentration of 10 flavan-3-ol metabolites, both phase II and colonic metabolites, was statistically higher compared to RFA and ARO intakes, similarly as in the acute intake. The phase II metabolites were methyl epicatechin-*O*-sulphate, catechin-*O*-glucuronide, epicatechin-*O*-sulphate-*O*-glucuronide, and dimer; and the colonic metabolites were 5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-sulphate, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in its sulphate, glucuronide, and sulphate-*O*-glucuronide forms, and phenylacetic acid, 3′-hydroxyphenylacetic acid, which have been reported to be microbial metabolites from the oligomer forms from flavan-3-ols, such as dimer, and trimer (Appeldoorn et al., 2009; Serra, 2011).

Another phenolic family that allowed differentiating the three intakes was dihydrochalcones. After both apple snack intakes, apart from phloretin-2′-*O*-glucuronide and phloretin-*O*-sulphate-*O*-glucuronide also detected after the acute intake, the metabolite phloretin-2′-*O*-sulphate and the intact phloretin-2′-*O*-xylosylglucoside were also detected,

Table 2
Increase in the 24 h-urine excretion (µmols ± SEM) of the main phenolic metabolites generated after the sustained intake for 6 weeks of WFA and RFA snacks, and ARO infusion.

	△ Concentration (day 6 w – day 0)		
	WFA snack	**RFA snack**	**ARO infusion**
Anthocyanins *			
Cyanidin-3-*O*-galactoside	n.d. [1]	13.4 ± 1.09 [1]	36.8 ± 0.93 [2]
Peonidin-*O*-arabinoside	n.d. [1]	n.d. [1]	7.98 ± 1.00 [2]
Peonidin-3-*O*-galactoside	n.d. [1]	4.94 ± 0.36 [2]	18.4 ± 0.22 [3]
Peonidin-*O*-glucuronide	n.d. [1]	5.60 ± 0.79 [1]	48.9 ± 0.69 [2]
Flavan-3-ols			
Methyl epicatechin-*O*-sulphate	11.6 ± 0.33 [2]	1.76 ± 0.38 [1]	0.89 ± 0.33 [1]
Catechin-*O*-glucuronide	2.57 ± 0.07 [2]	0.19 ± 0.08 [1]	−0.18 ± 0.07 [1]
Epicatechin-*O*-sulphate-*O*-glucuronide	0.45 ± 0.02 [2]	0.04 ± 0.02 [1]	0.01 ± 0.02 [1]
Dimer	0.09 ± 0.00 [2]	0.01 ± 0.00 [1]	0.00 ± 0.00 [1]
5-(3',4'-Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	94.2 ± 4.70 [2]	1.08 ± 5.35 [1]	14.0 ± 4.70 [1]
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-*O*-sulphate	1372 ± 36.6 [2]	53.0 ± 41.6 [1]	276 ± 36.5 [1]
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-*O*-glucuronide	43.0 ± 1.54 [2]	−0.07 ± 1.75 [1]	14.3 ± 1.54 [1]
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone-*O*-sulphate-*O*-glucuronide	37.1 ± 0.97 [2]	3.94 ± 1.10 [1]	3.90 ± 0.97 [1]
Dihydrochalcones			
Phloretin−2'-*O*-xylosylglucoside	n.d. [1]	0.00 ± 0.00 [2]	n.d. [1]
Phloretin−2'-*O*-glucuronide	0.63 ± 0.03 [2]	1.50 ± 0.03 [3]	−0.06 ± 0.03 [1]
Phloretin−2'-*O*-sulphate	0.19 ± 0.00 [2]	0.28 ± 0.00 [3]	n.d. [1]
Phloretin-*O*-sulphate-*O*-glucuronide	0.11 ± 0.00 [2]	0.17 ± 0.00 [3]	0.02 ± 0.00 [1]
Phenylacetic acids			
Phenylacetic acid	20.7 ± 0.97 [2]	−0.09 ± 1.10 [1]	2.52 ± 0.97 [1]
4'-Hydroxyphenylacetic acid	10.7 ± 4.46 [1]	-38.9 ± 5.09 [1]	94.3 ± 4.46 [2]
3'-Hydroxyphenylacetic acid	589 ± 20.9 [2]	108 ± 23.8 [1]	204 ± 20.9 [1]
2'-Hydroxyphenylacetic acid	-3.28 ± 5.51 [1]	−12.7 ± 2.86 [1]	41.9 ± 2.51 [2]
Dihydroxyphenylacetic acid-*O*-sulphate	−131 ± 24.4 [1,2]	−284 ± 27.7 [1]	232 ± 24.4 [2]
Dihydroxyphenylacetic acid-*O*-sulphate	−25.5 ± 2.98 [1]	-6.78 ± 3.40 [1]	133 ± 2.98 [2]
Dihydroxyphenylacetic acid-*O*-glucuronide	−21.7 ± 4.32 [1]	-7.13 ± 4.90 [1]	121 ± 4.32 [2]
Benzoic acids			
4-Hydroxy-3-methoxybenzoic acid-*O*-sulphate	−12.8 ± 82.3 [1]	-57.6 ± 46.3 [1]	220 ± 63.7 [2]
3-Hydroxy-4-methoxybenzoic acid-*O*-sulphate	-54.5 ± 41.8 [1]	4.96 ± 8.93 [1]	89.2 ± 20.5 [2]
4-Hydroxy-3-methoxybenzoic acid-*O*-glucuronide	-6.75 ± 7.17 [1]	−0.40 ± 2.23 [1]	24.0 ± 6.04 [2]
3-Hydroxy-4-methoxybenzoic acid-*O*-glucuronide	−1.65 ± 1.69 [1]	0.47 ± 0.70 [1]	9.34 ± 1.28 [2]
3,4-Dihydroxybenzoic acid-*O*-sulphate	3.59 ± 2.51 [1]	5.00 ± 2.86 [1]	206 ± 2.51 [2]
	n.d. [1]		

(*continued on next page*)

Table 2 (*continued*)

	△ Concentration (day 6 w – day 0)		
	WFA snack	RFA snack	ARO infusion
3,4-Dihydroxybenzoic acid-*O*-glucuronide		0.20 ± 0.03 [1]	1.44 ± 0.02 [2]
Hippuric acid	−109 ± 9.44 [1]	45.6 ± 10.7 [1]	405 ± 9.44 [2]
Hydroxycinnamic acids			
3'-Hydroxy-4'-methoxycinnamic acid-*O*-sulphate	0.87 ± 0.70 [1,2]	−0.38 ± 0.39 [1]	2.27 ± 0.63 [2]
4'-Hydroxy-3'-methoxycinnamic acid-*O*-glucuronide	−0.14 ± 0.30 [1]	−0.01 ± 0.13 [1,2]	0.86 ± 0.33 [2]
3'-Hydroxy-4'-methoxycinnamic acid-*O*-glucuronide	−0.36 ± 0.41 [1]	0.01 ± 0.22 [1]	1.76 ± 0.53 [2]
3-(4'-Hydroxy-3'-methoxyphenyl) propanoic acid-*O*-sulphate	−2.08 ± 0.87 [1]	0.98 ± 0.99 [1,2]	15.1 ± 0.87 [2]
3-(4'-Hydroxy-3'-methoxyphenyl) propanoic acid-*O*-glucuronide	−1.26 ± 0.08 [1]	0.03 ± 0.09 [1,2]	0.91 ± 0.08 [2]
Catechol derivatives			
Benzene−1,2-diol-*O*-sulphate	−292 ± 68.3 [1]	435 ± 77.6 [1]	4003 ± 68.3 [2]
Methyl Benzene−1,2-diol-*O*-sulphate	224 ± 32.2 [1]	178 ± 36.8 [1]	906 ± 32.2 [2]
Benzene−1,2-diol-*O*-glucuronide	−1.70 ± 0.28 [1]	−0.02 ± 0.31 [1]	8.33 ± 0.28[2]

* Increase concentration expressed in nmols
Different number means significant differences among tested products ($p < 0.05$) by ANOVA-one way
n.d.: not detected.
Volunteers analyzed: WFA (n=33), RFA (n=29), and ARO (n=33).

being this last an exclusive metabolite from RFA. Consistently with the acute study, phloretin metabolites showed a dose–response pharmacokinetics after the sustained consumption. From these results we conclude that flavan-3-ols together with dihydrochalcones could differentiate apple intake from other foods rich in flavan-3-ols, such as almonds (nuts), tea or cocoa, since dihydrochalcones have been reported to be selective from apple products (Ulaszewska et al., 2018).

Regarding the urinary metabolites derived from ACN, peonidin-*O*-arabinoside, peonidin-3-*O*-galactoside and peonidin-*O*-glucuronide appeared to be significantly increased after the sustained intake of RFA and ARO (Table 2). It is important to note that as in the acute study, peonidin-*O*-arabinoside was only detected after the ARO infusion, which reinforces that this metabolite could be an exclusive potential intake biomarker of ARO.

Regarding faeces, 11 phenolic metabolites were significantly increased and presented differences between tested meals (Table 3). Some of these metabolites were from the colonic degradation of flavan-3-ols (5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-sulphate, 3′,4′-dihydroxyphenylacetic acid, 3-(2′,4′-dihydroxyphenyl) propanoic acid, and 3′,4′-dihydroxycinnamic acid), as previously reported (Aura, 2008; Mosele et al., 2015). Moreover, the higher concentrations of cyanidin-*O*-arabinoside and cyanidin-3-*O*-galactoside, and its colonic metabolite 3,4-dihydroxybenzoic acid, allowed distinguishing ARO from RFA. Similar results were previously observed in rat urine samples after a sustained consumption of ARO infusion (Yuste et al., 2021).

The high inter-individual variability observed in the concentration of phenolic metabolites could arise due to variances in absorption, metabolism and gut microbiota catabolic action, which has been consistently described in previous human studies targeting PCs bioavailability (Manach et al., 2017).

Table 3
Increase in the faeces concentration (μmols/kg dried feaces ± SEM) of the main phenolic metabolites generated after the sustained intake for 6 weeks of WFA and RFA snacks, and ARO infusion.

	△ Concentration (day 6 w – day 0)		
	WFA snack	RFA snack	ARO infusion
Anthocyanins *			
Cyanidin-*O*-arabinoside	−0.01 ± 0.02 [1]	0.01 ± 0.02 [1]	0.65 ± 0.02 [2]
Cyanidin-3-*O*-galactoside	n.d. [1]	0.06 ± 0.03 [1]	0.58 ± 0.03 [2]
Flavan-3-ols			
5-(4'-Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	3.24 ± 0.19 [2]	n.d. [1]	n.d. [1]
4-(4'-Hydroxyphenyl)valeric acid	317± 18.9 [2]	-95.7 ± 18.9 [1]	87.0 ± 15.3 [1,2]
Phenylacetic acids			
3'-Hydroxyphenylacetic-*O*-acid	2187 ± 87.8 [2]	915 ± 87.8 [1,2]	466 ± 71.2 [1]
3',4'-Dihydroxyphenylacetic-*O*-acid	240 ± 14.9 [2]	−1.10 ± 14.9 [1]	-3.32 ± 12.1 [1]
Phenylpropanoic acids			
3-(2',4'-Dihydroxyphenyl) propanoic acid	178 ± 13.0 [2]	-51.5 ± 13.0 [1]	-30.2 ± 10.6 [1]
Hydroxybenzoic acids			
3,4-Dihydroxybenzoic acid	6.54 ± 1.00 [1]	0.64 ± 0.82 [1]	22.0 ± 0.81 [2]
Hydroxycinnamic acids			
3',4'-Dihydroxycinnamic acid	10.0 ± 0.37 [2]	3.16 ± 0.37 [1]	0.93 ± 0.30 [1]
4'-Hydroxy-3'-methoxycinnamic acid	8.41 ± 0.56 [2]	−2.29 ± 0.56 [1]	1.09 ± 0.45 [1,2]
Catechol and its derivatives			
Benzene−1,2-diol	0.36 ± 0.50 [1]	3.37 ± 0.50 [1,2]	10.7 ± 0.41 [2]

* Increase concentration expressed in nmols.
Different number means significant differences among tested products ($p < 0.05$) by ANOVA-one way.
n.d.: not detected.
Volunteers analyzed: WFA (n=25), RFA (n=34), and ARO (n=36).

3.5. *Selection of phenol intake biomarkers due to acute and sustained interventions*

In order to identify the most discriminant biomarkers common in acute and sustained studies, only the urinary phenol metabolites previously selected were used to perform a PCA, considering that, urine allowed to collect the same time interval of 24 h in both studies.

From the acute intake, 26 urinary metabolites were selected to perform a PCA considered as putative biomarkers since their concentration in 24 h-urine were statistically different when comparing RFA *vs* WFA or RFA *vs* ARO (Fig. 1 and Fig. 2A and B). Results from this PCA showed that principal component (PC) 1 (41.5%) and 2 (13.8%) explained about 55.3% of the variation and the three products were completely separated between them, being the WFA the most disperse. The loading plot (Fig. 2B) shows the distribution of the urinary excreted metabolites, confirming the reported results and observing that the concentration of the ACN metabolites was higher after the ARO intake, dihydrochalcones metabolites after the RFA snack intake, and flavan-3-ol metabolites after the WFA snack intake. In Fig. 2B it is also shown how strongly each metabolite influenced the PC, and the first PC showed

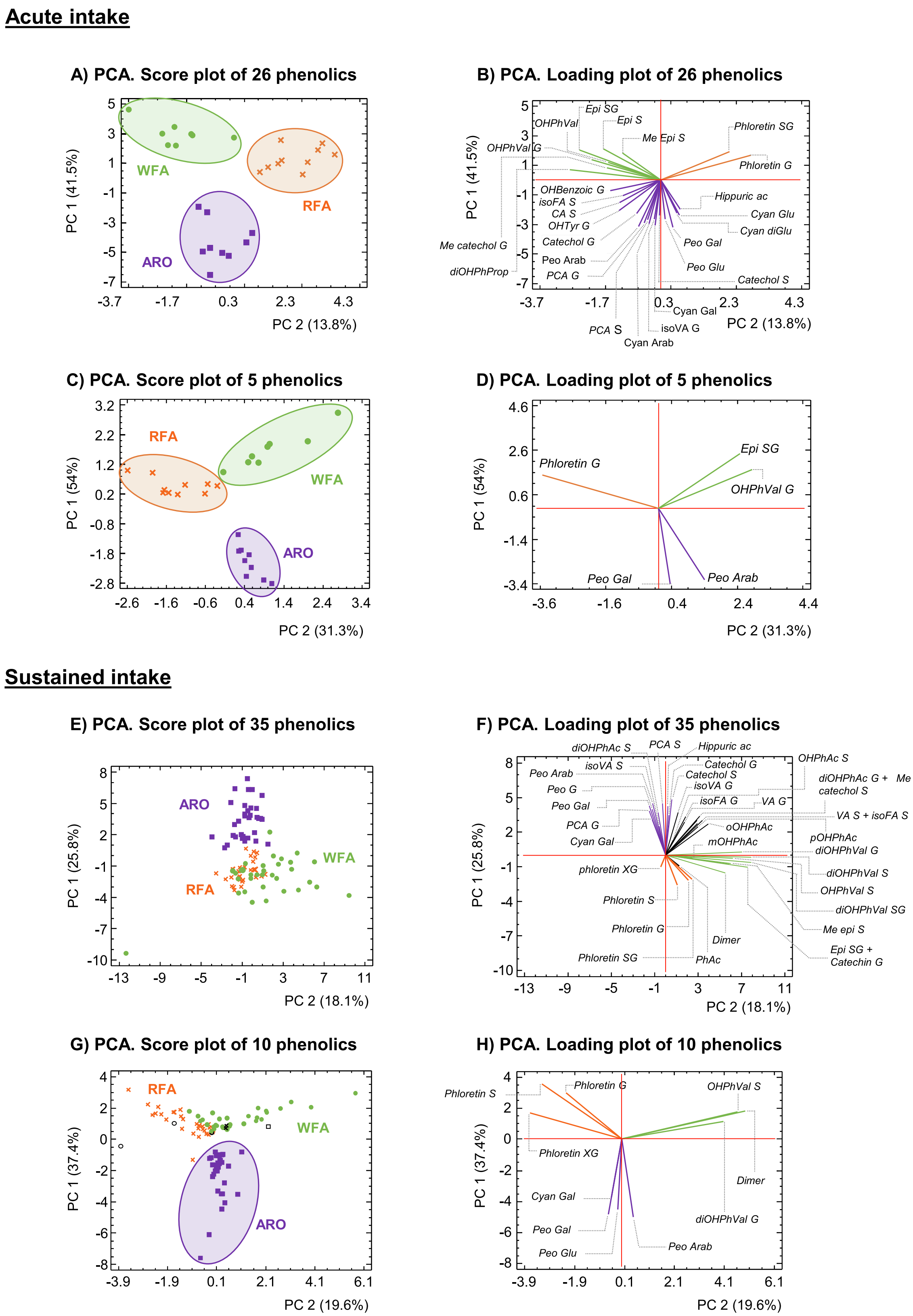

Fig. 2. Principal component analysis (PCA) of the three tested products (WFA and RFA snacks, and ARO infusion) after the acute (A–D) and sustained (E–H) intakes. Score plot (A, C, E, G), and loading plot (B, D, F, H). Cyan: cyanidin; Peo: peonidin¸ isoVA: 3-hydroxy-4-methoxybenzoic acid: PCA: 3,4-dihydroxybenzoic acid; OHTyr: 2-(3′,4′-dihydroxyphenyl)ethanol; diOHPhPr: 3-(2′,4′-dihydroxyphenyl)propanoic acid; OHPhVal: 5-(4′-hydroxyphenyl)-γ-valerolactone; *Epi*: epicatechin; PhAc Ac: phenylacetic acid; cat: catechin; DiOHPhVal: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; OHPhAc Ac: hydroxyphenylacetic acid; VA: 4-hydroxy-3-methoxybenzoic acid; isoFA: 3′-hydroxy-4′-methoxycinnamic acid; Gal: galactoside; Arab: arabinoside; XG: xylosylglucoside; G: glucuronide; S: sulphate; SG: sulphate glucuronide; Me: methyl; 3′-: meta; 2′-: ortho; 4′-: para.

large negative associations with ACN metabolites, whereas the second component presented large negative associations with flavan-3-ol metabolites. In addition, the second PC presented positive association with dihydrochalcone metabolites. From the loading plot, it could be also reported that the main colonic metabolites of cyanidin-*O*-arabinoside and peonidin-*O*-arabinoside were 3,4-dihydroxybenzoic acid-*O*-sulphate, and 3,4-dihydroxybenzoic acid-*O*-glucuronide, respectively, due to its strong relationship. This fact could be important to understand the metabolic pathways of ACN.

Five metabolites from the acute intake were chosen as the most selective biomarkers to perform a second PCA (Fig. 2D). The criterion to select them was to have the lines strongly influenced by the PC. In addition, from the metabolites that were positively correlated between them, it was only selected one, whose line was heavily influenced by the PC. Based on this, phloretin-2′-*O*-glucuronide was selected as the most representative dihydrochalcone metabolite; epicatechin-*O*-sulphate-*O*-glucuronide and 5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-glucuronide as flavan-3-ol metabolites; and peonidin-3-*O*-galactoside, and peonidin-*O*-arabinoside as ACN metabolites. As it is shown in the score plot (Fig. 2C), the three tested products were completely separated between them when these 5 urinary biomarkers were selected. Epicatechin-*O*-sulphate-*O*-glucuronide and 5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-glucuronide allowed differentiating WFA and RFA intakes; and peonidin-*O*-arabinoside ARO infusion from RFA intakes. In the case of RFA intake, peonidin-3-*O*-galactoside and phloretin-2′-*O*-glucuronide were identified as RFA biomarkers, but these phenolics were not specific for RFA, as they were also common to ARO and WFA intake, respectively. Based on the selection of these biomarkers from the acute intake, the metabolic pathways from the native phenols have been proposed in Fig. 3A.

On the other hand, another PCA was performed with the results obtained after the sustained intake including the 35 metabolites whose urinary increase after 6-week consumption was statistically different when comparing RFA *vs* WFA or RFA *vs* ARO (Table 2). PC 1 and 2 only explained about 43% of the variation (PC1 was 25.8 % and PC2 was 18.1 %). It was necessary 4 PCs to explain 62% (data not shown). As it is observed in the score plot (Fig. 2E), the apple groups were partially overlapped.

From this initial PCA analysis, it was observed that the metabolic profiles were clearly associated with metabolites from a microbial origin in comparison to the acute study. These colonic metabolites are intermediate and/or end-product of the microbial catabolism of several phenolic families present in the apples, and ARO infusion, such as flavan-3-ols and ACN, respectively. This fact could explain the overlapping of the apple groups.

In order to distinguish the two apple groups, the metabolites that could distinguish the two apple intakes were selected, such as flavan-3-ols, ACN and dihydrochalcones. The microbial metabolites were discarded because they were not specific from these phenolic groups; an exception was for valerolactone metabolites, which are exclusive from flavan-3-ols. As it can be seen in Fig. 2F, some metabolites were correlated with others but they were not strongly influenced. Then, these metabolites were also discarded. Although phloretin-2′-*O*-xylosylglucoside was not strongly influenced, this metabolite was not discarded because it could distinguish the two apple groups. The best results were obtained when the anthocyanins cyanidin-3-*O*-galactoside, and

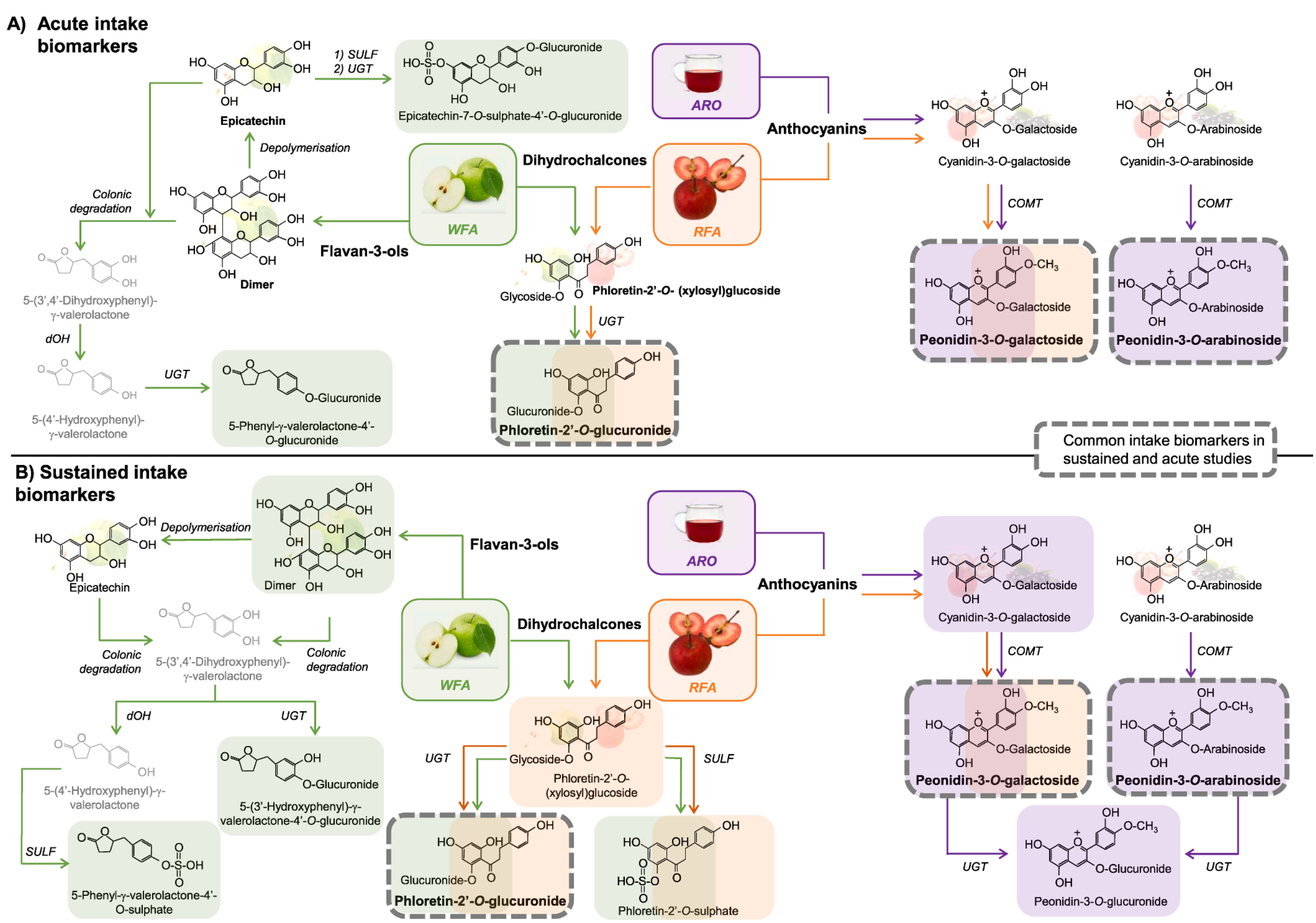

Fig. 3. Metabolic pathways of the phenolic biomarkers selected to distinguish the three tested meals (WFA and RFA snacks, and ARO infusion) after the acute and sustained studies.

peonidin derivatives (peonidin-*O*-arabinoside, peonidin-3-*O*-galactoside, and peonidin-*O*-glucuronide), phloretin derivatives (phloretin-2′-*O*-xylosylglucoside, phloretin-2′-*O*-glucuronide and phloretin-2′-*O*-sulphate) and the flavan-3-ols dimer, 5-(4′-hydroxyphenyl)-γ-valerolactone-*O*-sulphate and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-*O*-glucuronide were selected (Fig. 2G).

As observed, the PCA score plot (Fig. 2G) and loading plot (Fig. 2H), after the selection of theses 10 metabolites, showed that the separation among groups was significantly improved. In this case, PC 1 (37.4%) and 2 (19.6%) explained about 57.0% of the variation.

It is important to note that 5 volunteers from the sustained study, showed outlier values, which appear highlighted in black in Fig. 2G. The outlier volunteer from ARO infusion group was the only one that peonidin-*O*-arabinoside was not detected. Additionally, cyanidin-3-*O*-galactoside and peonidin-*O*-glucuronide were also not detected. The 2 outlier volunteers from RFA group were the only ones that ACNs and phloretin-2′-*O*-glucuronide were not detected. Regarding to the 2 outlier volunteers from WFA group, these were the only ones that the concentration of flavan-3-ol metabolites (phase II and valerolactones) were not increased.

Based on this selection process, Fig. 3B summarizes the proposed metabolic pathways with the 10 metabolites selected from the sustained study. The dihydrochalcone phloretin-2′-*O*-glucuronide appeared to be in acute and sustained studies, so it appeared to be the most promising phenolic intake biomarker of both WFA and RFA. Also, several urinary microbial flavan-3-ols metabolites were selected as specific biomarkers of WFA intake and phloretin-2′-*O*-xylosylglucoside was selected as a good and exclusive RFA biomarker after its sustained intake. Peonidin derivatives (peonidin-*O*-arabinoside and peonidin-*O*-glucuronide), and cyanidin-3-*O*-galactoside were reported to be good biomarkers after ARO sustained intake, being peonidin-*O*-arabinoside a common ARO biomarker in both acute and sustained studies. Regarding ARO and RFA, the ACN peonidin-3-*O*-galactoside appeared to be the optimum urinary biomarker after both intakes under sustained and acute ingestions.

4. Conclusions

The present study provides new results regarding human phenolic metabolome facing the challenge of complete food consumption with its phytochemical composition complexity. Acute and sustained intakes of two types of apple snacks (with and without ACN) together with an ACN-rich source without the fruit matrix, promoted significant changes in the profiles of plasmatic, urinary and fecal phenolic metabolites.

Results showed that the main generated phenolic metabolites from WFA were flavan-3-ols and dihydrochalcones, whereas the main phenolic metabolites after RFA intake were from ACN and dihydrochalcones phenolic groups. The main differences between the phenolic metabolome in acute and sustained studies were clearly associated with the presence of microbial metabolites after the chronic study due to the phenolic continuous exposition, denoting a strong involvement of the intestinal microbiota in the metabolism of complex plant polyphenols.

A special emphasis was made on ACN bioavailability, reporting a strong apple matrix effect. This was reflected in the delay of ACN urinary excretion after RFA intake and also in the higher bioavailability of ACN after ARO intake, although the same ACN dose was provided.

A selection of the most discriminatory phenolic metabolites was performed, being peonidin-3-*O*-galactoside the finest urinary intake biomarker after both ACN tested products (RFA and ARO infusion), whereas peonidin-O-arabinoside was reported to be specific from ARO. Phloretin-2′-*O*-glucuronide was also selected as a promising phenolic intake biomarker of both apple snacks.

We conclude that a complete elucidation of the overall phenolic metabolome and the selection of the consumption biomarkers combining acute and sustained dietary studies might be a promising approach for understanding the complex relationship between PCs and their impact on human health.

CRediT authorship contribution statement

Alba Macià: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Maria-Paz Romero:** Resources, Funding acquisition. **Silvia Yuste:** Investigation. **Iziar Ludwig:** Formal analysis. **Anna Pedret:** Resources, Investigation. **Rosa Maria Valls:** Resources, Investigation. **Patricia Salamanca:** Resources. **Rosa Solà:** Project administration. **Maria José Motilva:** Visualization, Project administration, Funding acquisition, Writing – review & editing. **Laura Rubió:** Supervision, Investigation, Writing – 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.2022.132612.

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