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Abstract: Red-fleshed apples have been previously described as a potential food source for different classes of polyphenols, with a special focus on their enhanced anthocyanins content. The aim of the study was to investigate comprehensively the metabolic pathways and bioavailability of individual red-fleshed apple derived metabolites, and to elucidate the potential intake biomarkers in humans. After the acute intake of a red-fleshed apple snack product, human plasma and urine were collected and analyzed by UPLC-MS/MS. A total of 37 phase-II and microbial phenolic metabolites were detected in the plasma and urine. Flavan-3-ol, hydroxyphenyl-□-valerolactone, dihydrochalcone and anthocyanin derivatives increased significantly in postprandial conditions. Among these, only phloretin glucuronide, cyanidin-3-O-galactoside and peonidin-3-O-galactoside were detected in the plasma and/or urine of all the volunteers. These three metabolites could be considered as specific food intake biomarkers, and might be used to establish the relationship between the intake and health benefits in human intervention studies.

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Vicenzo Fogliano, Editor-in-Chief
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Dear Dr. Fogliano,

Enclosed you will find the manuscript entitled "Metabolic pathways proposal and identification of potential intake biomarkers of red-fleshed apple (poly)phenols in an acute human intake" to be considered for publication in the Journal of Functional Foods.

Apples are an important source of bioactives and their diverse and high (poly)phenol content has been most consistently associated with reduced risk of cancer, heart disease, asthma, and type II diabetes. Recently, red fleshed apple cultivars have attracted the interest of apple producer and consumer due to their enhanced content of anthocyanins with high antioxidant activity and health-promoting effects.

In the present study we firstly aimed to comprehensively investigate the metabolic pathways and the bioavailability of individual red-fleshed apple derived metabolites in humans after the acute intake of a red-fleshed apple snack product. The results show that red-fleshed apple (poly)phenols are extensively metabolized resulting in the production a total of 37 phase II and microbial phenolic metabolites, detected in plasma and urine. Based on the diversity of phenolic metabolites whose concentration in plasma or urine increased after the apple snack intake, a complex picture of the metabolic pathways of the main apple phenolic compounds, as well as their interactions, has been proposed. The second objective was focused on the elucidation of potential biomarkers of red-fleshed apple consumption. The identification of the specific food intake biomarkers is of great importance to establish the relationship between (poly)phenols intake and health benefits in human intervention studies. Among the 37 detected metabolites, phloretin glucuronide, cyanidin-3-O-galactoside and peonidin-3-O-galactoside were selected as the best candidates as biomarkers of red-fleshed apple intake

The work has not been published elsewhere, either completely, in part, or in any other form and that the manuscript has not been submitted to another journal. The submitting author accepts the responsibility of having included as coauthors all appropriate persons. The submitting author certifies that all coauthors have seen a draft copy of the manuscript and agree with its publication.

Yours sincerely,

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## HIGHLIGHTS

- Red-flesh apples as a novel anthocyanin rich food
- Complete metabolic pathways for phenolics from red-flesh apples
- Phloretin glucuronide as specific apple intake biomarker
- Cyanidin galactoside and peonidin galactoside as red-flesh apple intake biomarkers

# Metabolic pathways proposal and identification of potential intake biomarkers of red-fleshed apple (poly)phenols in an acute human intake

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**Abstract**

Red-fleshed apples have been previously described as a potential food source for different classes of polyphenols, with a special focus on their enhanced anthocyanins content. The aim of the study was to investigate comprehensively the metabolic pathways and bioavailability of individual red-fleshed apple derived metabolites, and to elucidate the potential intake biomarkers in humans. After the acute intake of a red-fleshed apple snack product, human plasma and urine were collected and analyzed by UPLC-MS/MS. A total of 37 phase-II and microbial phenolic metabolites were detected in the plasma and urine. Flavan-3-ol, hydroxyphenyl-γ-valerolactone, dihydrochalcone and anthocyanin derivatives increased significantly in postprandial conditions. Among these, only phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside were detected in the plasma and/or urine of all the volunteers. These three metabolites could be considered as specific food intake biomarkers, and might be used to establish the relationship between the intake and health benefits in human intervention studies.

*Keywords:* anthocyanins, metabolic pathways, phenolic compounds, red-fleshed apple, UPLC-MS/MS.

## 1. INTRODUCTION

Apples are one of the most commonly consumed fruits and their diverse and high polyphenol content is considered one of the most important determinants of their health-promoting properties (Hyson, 2011; Bondonno et al., 2018). Some new genotypes of apple obtained by innovative breeding strategies have red flesh indicating high amounts of anthocyanins presumably with added healthy properties. Additionally, red-fleshed apples are a rich source of other (poly)phenols such as phenolic acids, dihydrochalcones, flavan-3-ols and flavonols (Bars-Cortina et al. 2017).

Before investigating the potential health benefits of red-fleshed apples for humans, the bioavailability and the complex metabolism of the different apple polyphenol classes, including anthocyanins, need to be established. One of the main limitations of the existing data from the bioavailability studies is that these studies are often restricted to a single class of polyphenols, and there are still very few human studies describing the metabolism and kinetics of the absorption of apple polyphenols (Trošt et al. 2018). Considering the complex transformations and overlapping metabolic pathways for different classes of polyphenols in the same food matrix, it is necessary to describe the human metabolic output of red-fleshed apple polyphenols comprehensively. Moreover, the measurement of dietary exposure and reliable intake biomarkers in interventional diet studies in humans is of crucial importance for the discovery of unbiased associations between the intake of bioactive compounds and the observed effects (Dragsted et al. 2018).

In order to investigate this, human postprandial studies that consider the food matrix in which the polyphenols are ingested are very useful and can

contribute to knowledge about the factors affecting polyphenol bioavailability (Motilva et al. 2015). Urine sampling is particularly useful in these studies because polyphenols are rapidly metabolized for excretion, and consequently, the quantification of phenolic metabolites circulating in the blood is limited over time. So, 24 h urine allows for total polyphenol absorption to be assessed more accurately and provides a better index of intake, as the total concentrations of both small- and large-intestinal metabolites may be monitored, without the need to take multiple blood samples (Motilva et al. 2015).

In the present work, we aimed to investigate comprehensively the metabolic pathways and the bioavailability of apple (poly)phenols in humans after the acute intake of a red-fleshed apple snack product. The second objective was focused on the accurate identification and quantification of individual red-fleshed apple phenol metabolites in urine and plasma samples, and among them, to elucidate the potential biomarkers of red-fleshed apple 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, *p*-coumaric acid, and caffeic acid were purchased from Extrasynthese (Genay, France). *p*-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)propionic acid, 3-(3',4'-dihydroxyphenyl)propionic acid (aka dihydrocaffeic acid), 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid (aka dihydroferulic acid), epicatechin, and chlorogenic acid were from Sigma-Aldrich

(St. Louis, MO, USA). Vanillic acid and ferulic acid were from Fluka (Buchs, Switzerland). Vanillic acid-4-*O*-sulphate, catechol-4-*O*-sulphate, and 4-methyl catechol 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), and acetic acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water used was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

Stock solutions of standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L, and stored in a dark flask at −30 ºC.

## 2.2. Apple snack

The red-fleshed 'Redlove' apple variety was provided by NUFRI SAT (Mollerussa, Lleida, Spain). To increase the useful life, obtaining good shelf-stability and, at the same time, minimize changes in the bioactive compounds of red-fleshed apples, the freeze-dried snack format was selected. Before drying, the apples were washed, dried and cut into 1 cm-sized cubes. The apple cubes were frozen in liquid nitrogen and lyophilization was then performed with a first drying at 0.6 bar with a temperature ramp of −20 to 0 °C for 25 hours, followed by a second complete vacuum drying with a temperature ramp of 0 to 20 °C for 40 hours (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain). The freeze-dried apple cubes were immediately transferred to airtight plastic containers and refrigerated (2 ºC) until the analysis of their phenolic composition and use in the acute intake study.

The analysis of the phenolic composition of the apple snack was based on the previous study by Bars-Cortina et al. (2017). Prior to the chromatographic analysis of the apple (poly)phenols, a fine powder of the freeze-dried samples was obtained with the aid of an analytical mill (A11, IKA, Germany). The ingested portion of the apple snack contained a total of 196 mg of phenolic compounds. The detailed phenolic composition of the apple snack is presented in the Supplementary Information, **Table 1S**.

### 2.3. Human intervention study and biological sample collection

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016). Ten healthy participants (five females and five males, mean age 37.3 ± 8.4 years) with a body mass index (BMI) of 18.5–24.9 kg/m$^2$ were enrolled. Exclusion criteria were pregnancy or lactation, any chronic medication, any antibiotic treatment during the 4 months prior to the study, cigarette smoking, alcohol intake > 80 g/day and use of dietary supplements. After two days of a diet low in phenolic compounds, the participants were invited to eat a portion of 80 g of red-fleshed apple snack after fasting overnight. Human blood samples were obtained by venipuncture before (0 h) and after the apple snack intake at 0.5, 1, 2, 4, 6, and 24 h using 6 mL Vacutainer™ tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing ethylenediamintetra acetic acid (EDTA) as an anticoagulant. To obtain the plasma samples, the blood tubes were centrifuged at 8784 *g* for 15 min (Hettich, Tuttlingen, Germany). Aliquots were stored at −80 ºC until the chromatographic analysis. On the other hand, urine samples were collected 12

h before and at the interval times of 0-2, 2-4, 4-8, and 8-24 h after the apple snack intake. The total volume of each sample was measured before storing the aliquots at −80 ºC until the chromatographic analysis.

*2.3.1. Plasma samples*

Before the chromatographic analyses, the plasma samples were pre-treated by micro-Elution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. The methodology used is the one reported in a previous study (Martí et al., 2010), but with some modifications. Briefly, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. 350 µL of 4% phosphoric acid was added to 350 µL of the plasma sample, and then this solution was loaded into the micro-cartridges. The loaded micro-cartridges were cleaned up with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained phenolic compounds were eluted with 2 x 50 µL of methanol. Each sample was prepared in triplicate.

*2.3.2. Urine samples*

The urine samples were also pre-treated by µSPE. The micro-cartridges and their conditioning and equilibration steps were the same as reported for plasma samples. In this case, 100 µL of phosphoric acid at 4% was added to 100 µL of the urine sample, and this solution was loaded into the micro-cartridge. The retained phenolic compounds were then eluted with 2 x 50 µL of methanol. Each sample was prepared in triplicate.

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

LC analyses were carried out on an AcQuity Ultra-Performance™ liquid chromatography and tandem mass spectrometry equipment from Waters (Milford, MA, USA). Two chromatographic methods were used for the analysis of 1) anthocyanins and their metabolites, and 2) the rest of the phenolic compounds and their metabolites. In both methods, the flow rate was 0.3 mL/min and the injection volume 2.5 µL. The UPLC.MS/MS conditions were the same used in our previous studies (Martí et al. 2010; Bars-Cortina et al. 2017; Yuste et al. 2018). Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. The selected reaction monitoring (SRM) transition for quantification and the cone voltage and collision energy for the analysis of the phenolic metabolites are shown in **Table 1**.

Due to the lack of commercial standards of phenolic metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another phenolic compound with a similar structure. Methyl catechol glucuronide was tentatively quantified by using the calibration curve of 4-methyl catechol sulphate. For hydroxybenzoic acid sulphate, the calibration curve of *p*-hydroxybenzoic acid was used; hydroxyhippuric acid with hippuric acid; protocatechuic acid sulphate with protocatechuic acid; vanillic acid glucuronide with vanillic acid; hydroxyphenylacetic acid sulphate and hydroxyphenylacetic acid glucuronide with 3-(4'-hydroxyphenyl)acetic acid; dihydroxyphenylacetic acid sulphate and dihydroxyphenylacetic acid glucuronide with 3-(3',4'-dihydroxyphenyl)acetic acid; hydroxyphenylpropionic

acid sulphate and hydroxyphenylpropionic acid glucuronide with 3-(3'-hydroxyphenyl)propionic acid; dihydroxyphenylpropionic acid sulphate with 3-(3',4'-hydroxyphenyl)propionic acid; hydroxymethoxyphenyl propionic acid sulphate (dihydroferulic acid sulphate) with 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid (dihydroferulic acid); coumaric acid sulphate with *p*-coumaric acid; caffeic acid sulphate with caffeic acid; ferulic acid sulphate with ferulic acid; hydroxyphenyl-γ-valerolactone sulphate, dihydroxyphenyl-γ-valerolactone, dihydroxyphenyl-γ-valerolactone glucuronide, dihydroxyphenyl-γ-valerolactone sulphate glucuronide, epicatechin sulphate, epicatechin glucuronide, and methyl epicatechin glucuronide with epicatechin; phloretin sulphate, phloretin glucuronide and phloretin sulphate glucuronide with phloretin-2'-*O*-glucoside; cyanidin arabinoside and peonidin-3-*O*-galactoside with cyanidin-3-*O*-galactoside.

## 2.5. Statistical analysis

The results are presented as mean values ± standard deviation (SD) for (poly)phenols in red-fleshed apple snacks, and as mean values ± standard error of the mean (SEM) for metabolites in the urine and plasma samples. For the 8 metabolite groups (catechol/pyrogallol derivatives, benzoic acid derivatives, phenylacetic/phenylpropionic acid derivatives, phenyl-γ-valerolactone derivatives, flavan-3-ol derivatives, phloretin derivatives, and anthocyanin derivatives), one-way repeated measures analysis of variance (ANOVA) was performed on the urine samples to compare the mean differences at the five defined time points. Post hoc analysis was conducted using pairwise comparisons with Bonferroni correction. Differences were considered significant

at $p < 0.05$. All statistical analyses were performed using the SPSS v 22.0 software package.

## 3. RESULTS AND DISCUSSION

The total amount of phenolic compounds in the snack portion (80 g) given to the volunteers accounted for 196 ± 10.7 mg. The analysis of the phenolic composition showed a wide range of phenolic groups, the more abundant being anthocyanins (cyanidin-3-*O*-galactoside and cyanidin arabinoside), five phenolic acids, with chlorogenic acid as the most abundant; three flavan-3-ols, mainly epicatechin and its dimer, three flavonols, all of them quercetin derivatives, such as quercetin rhamnoside, and three dihydrochalcones, with phloretin (xylosyl) glucoside as the main representative (Supporting Material **Table 1S**). Therefore, the red-fleshed apple snack used in the present study is a naturally rich source of phenolic compounds from different phenolic families. Considering the complex transformations and overlapping metabolic pathways for these different classes of polyphenols in the same food matrix, we aimed to describe the human metabolic output of red-fleshed apple polyphenols comprehensively.

### 3.1. Identification of the biological apple phenol metabolites by UPLC-MS/MS

In order to identify the phenolic metabolites generated after the acute intake of the red-fleshed apple snack, the detector system tandem MS was used due to its specificity, sensitivity and selectivity. The generated metabolites were determined and identified by the full scan mode in the MS mode, and in the daughter scan and SRM modes in the tandem MS mode. In addition to the

detector system (MS/MS), authentic standards were also used when they were available to determine their retention time and identify the phenolic metabolites generated in plasma and urine samples.

Although the volunteers spent two days on a diet low in phenolic compounds prior to the intervention day, some phenolic acids (phenylpropionic, phenylacetic, benzoic, and hydroxycinnamic acids) were detected and quantified in the analysis of the basal plasma and urine (fasting conditions) collected just before the apple snack intake (see **Table 2S** and **3S** in **Supporting Material**). After subtraction of these basal levels of phenolics, a total of 37 phenolic metabolites were detected in the urine and/or plasma samples in increased amounts after the red-fleshed apple snack intake (**Table 1,** and **Tables 2S** and **3S** in **Supporting Material**). These metabolites included four catechol and pyrogallol derivatives, six benzoic acid derivatives, five phenylacetic acid derivatives, six phenylpropionic acid derivatives, and four hydroxycinnamic acid derivatives. Four metabolites were hydroxyphenyl-γ-valerolactone derivatives, three epicatechin derivatives, two phloretin derivatives and three cyanidin derivatives.

The phenolic metabolites were mainly phase-II sulphated (18), glucuronided (11) and methylated (8) conjugates formed through the action of the enzyme sulphotransferases (SULFs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-*O*-methyltransferases (COMT), respectively. From all these phase-II metabolites, sulphation was the main transformation. The generation of simple phenolic acids, such as phenylpropionic, phenylacetic, and benzoic acid derivatives, is probably the result of microbial transformations occurring in the colon, which include ring fission, reduction, α-oxidation (one

decarboxylation), β-oxidation (two decarboxylations), dehydroxylation and demethylation. In addition, phase-I metabolism (dehydrogenation or reduction) may also be involved in the formation of these metabolites. These simple phenolic acids can then undergo phase-II metabolism at the colon level and/or be absorbed and reach the liver, where they would be subject to enzymatic metabolism before re-entering the systemic blood circulation and finally being excreted in the urine.

### 3.2. Proposed metabolic pathways of red-fleshed apple phenols

Based on the diversity of phenolic metabolites, whose concentration in plasma or urine increased after the apple snack intake (**Table 1**), a complex picture of the metabolic pathways of the main apple phenolic compounds, as well as their interactions, has been proposed. **Figure 1** shows the proposed metabolic routes to explain the phenolic metabolites generated from chlorogenic acid (in green), vanillic acid hexoside (in blue), cyanidin-3-*O*-galactoside (in orange), epicatechin and dimer $B_2$ (in lilac), quercetin derivatives (in pink), and phloretin (xylosyl) glucoside (in brown) determined in the red-fleshed apple snacks as the main phenolics. The name of the phase-II enzymes is shown in green, and the colonic catabolism in brown. In the next subsections, the metabolic pathways of the metabolites generated from the apple (poly)phenols are described.

### *3.2.1. Anthocyanins*

Cyanidin-3-*O*-galactoside, and cyanidin arabinoside (but at lower concentration levels), were the main anthocyanins present in the red-fleshed apple snack (Supplemental Information **Table 1S**). These cyanidin glycosides (galactoside and arabinoside) were also detected in both the plasma and urine samples in their native structure detected in apple snack. Other anthocyanin metabolites were also identified in the plasma and urine, derived from phase-II metabolism and microbial metabolism (**Figure 1).** Regarding phase-II metabolites, peonidin-3-*O*-galactoside was detected in the urine resulting from cyaniding-3-*O*-galactoside methylation by the action of COMT enzyme. Methylation, as one of the first metabolic reactions of cyanidin glycosides, was also reported by other authors in plasma and urine samples after the acute intake of aronia berry extract (Xie et al. 2016), and also after the oral ingestion of 500 mg of $^{13}$C-labelled cyanidin glucoside (De Ferrars et al. 2014).

Other cyanidin metabolites, based on the B-ring fission and cleavage of the C-ring by the action of colonic enzymes (Mosele et al. 2015), were also detected in our study. As a result, protocatechuic acid and dihydroxyphenylpropionic acid (dihydrocaffeic acid) were respectively detected. Protocatechuic acid might also have been formed by β-oxidation of dihydroxyphenylpropionic acid. Then, as proposed in **Figure 1** (orange arrows), protocatechuic acid could either be further degraded by the action of the microbial flora to catechol metabolites (α-oxidation), pyrogallol metabolites (hydroxylation) and hydroxybenzoic acid (dehydroxylation), or methylated to vanillic acid.

Despite B-ring fission and C-ring cleavage, phloroglucinol sulphate could have been generated from A-ring fission. Nevertheless, this metabolite could not be differentiated from pyrogallol sulphate due to the lack of commercially available standards and because these two metabolites have the same precursor (*m/z* 205) and product ions (*m/z* 125 and 83) (See **Table 1**). Therefore, this metabolite could be tentatively identified as phloroglucinol sulphate due to the A-ring fission (metabolic pathways not shown), or as pyrogallol sulphate due to hydroxylation of catechol.

*3.2.2. Other phenolic compounds*

*Chlorogenic acid* (see **Figure 1** green arrows). Caffeic acid would be the first metabolite generated from chlorogenic acid by ester hydrolysis (**Figure 1**). From this metabolite (caffeic acid), different reactions based on microbial metabolism (dehydroxylation), and phase-I (dehydrogenation or reduction) and phase-II (COMT) metabolism could occur resulting in the generation of coumaric acid, dihydroxyphenylpropionic acid and ferulic acid, respectively. Then, dihydroxyphenylpropionic acid and ferulic acid could be further degraded to phenylpropionic acid, phenylacetic acid and vanillic acid. These metabolites could be further degraded to such simpler phenolic compounds as protocatechuic acid, *p*-hydroxybenzoic acid and catechol metabolites.

*Vanillic acid hexoside* (see **Figure 1** blue arrows). Vanillic acid hexoside was the second most abundant phenolic acid quantified in the red-fleshed apple snack (Supporting Material **Table S1**). After deglycosylation of this phenolic acid, vanillic acid could be formed and subsequently sulphated (vanillic acid

sulphate), glucuronided (vanillic acid glucuronide) and demethylated (protocatechuic acid). Then, as has been commented before, protocatechuic acid could also be further degraded by microbial activity to generate catechol and pyrogallol metabolites.

The presence of the metabolites derived from these two phenolic acids (chlorogenic and vanillic acids) was in agreement with the results reported in the literature for the bioavailability study after the consumption of foods rich in these phenolics, such as coffee (Monteiro et al. 2007; Renouf et al 2010; Ludwig et al 2013;), cereals (Calani et al. 2014), olive oil enriched with thyme phenols (Rubió et al. 2014) and apple juice (Kahle et al. 2011).

*Flavan-3-ols (epicatechin and dimer $B_2$)* (see **Figure 1** lilac arrows). Flavan-3-ol metabolites included both phase-II and microbial catabolites. The first metabolic step would be the hydrolysis of the proanthocyanidin dimer to catechin and epicatechin, and these monomers were found in urine as glucuronided ((epi)catechin glucuronide), sulphated ((epi)catechin sulphate), and further methylated (methyl (epi)catechin sulphate) conjugates. On the other hand, the flavan-3-ols monomers could also be metabolized by the gut microbiota to dihydroxyphenyl-γ-valerolactone, detected in urine samples. Similarly, other studies reported the valerolactones as specific flavan-3-ols metabolites (Aura, 2008; Hackman et al. 2008; Serra et al. 2011; Mosele et al. 2015). Then, dihydroxyphenyl-γ-valerolactone could also be glucuronided, and further sulphated and also dehydroxylated and further sulphated.

*Quercetin derivatives* (see **Figure 1** pink arrows). Quercetin galactoside/glucoside and arabinoside would firstly be deglycosylated, and then the generated aglycone (quercetin) could enter epithelial cells by passive diffusion and be absorbed. Nevertheless, in the present study, no phase-II metabolites of quercetin were identified. On the other hand, quercetin rhamnoside has been reported not to be metabolized in the small intestine and to reach the colon where this is metabolized to dihydroxyphenylpropionic acid (Arts et al. 2004; Aura, 2008; Serra et al. 2012; Mosele et al. 2015), and then progressively metabolized to generate simple phenols, such as phenylacetic and benzoic acids, down to catechol derivatives. In our study, only microbial metabolites from quercetin derivatives were observed.

*Phloretin (xylosyl) glucoside* (see **Figure 1** brown arrows). Phloretin (xylosyl) glucoside could be firstly deglycosylated to generate phloretin. Then, this dihydrochalcone could be glucuronided by UGT enzymes and further sulphated with SULF enzymes. Regarding the literature, phloretin glucuronide was also reported in plasma and urine samples after the oral consumption of apple cider (Marks et al. 2009) and apple fruit (Saenger et al. 2017). This compound could also be metabolized by the gut microbiota enzymes to dihydroxyphenylpropionic acid and further metabolized to generate simpler phenolic compounds, such as phenylacetic and benzoic acids down to catechol derivatives.

### 3.3. Biomarkers for apple phenol consumption

As evidenced in the metabolic pathways proposed in **Figure 1**, a large number of metabolites are generated from the five main phenolic groups (anthocyanins, phenolic acids, flavan-3-ols, flavonols and dihydrochalcones) quantified in the red-fleshed apple snack. Some of the metabolites identified in the plasma and urine samples after the snack intake are common to several phenolic groups. These compounds are mainly colonic metabolites, such as phenylpropionic, phenylacetic, and benzoic acids, and catechol derivatives. Additionally, these phenolic metabolites are common to other (poly)phenol-rich foods. That is why, more attention was paid in this study to specific phenolic compounds detected in plasma or urine that could be used as biomarkers for red-fleshed apple intake. The identification of the specific food intake biomarkers is of great importance to establish the relationship between (poly)phenols intake and health benefits in human intervention studies.

The 37 phenol metabolites detected in the urine and plasma samples after the red-fleshed apple snack intake (see **Table 1**, **and Tables 2S and 3S** in **Supporting Material)** could be classified into two groups according to their urine excretion (µmol) kinetic, as is shown in **Figure 2**. The first group would include derivatives from phenylpropionic and phenylacetic acids, benzoic acids, catechol and pyrogallol and hydroxycinnamic acids (see **Figure 2A**); and the second group the derivatives from flavan-3-ols, valerolactones, dihydrochalcones, and anthocyanins (see **Figure 2B**). The phenolic metabolites from the first group are excreted at high concentration levels (µmol) in the different interval times (0 to 24 h). However, these phenolic compounds presented low specificity to be considered as intake biomarkers, as they were

also quantified under basal conditions (before the apple intake), and only a slight but not significant increase in phenylpropionic/phenylacetic acids, benzoic acids and catechol/pyrogallol derivatives was observed after the apple intake in the urine excretion between 0 to 24 h. This fact was also observed in the plasma samples and their concentration was also only slightly enhanced after the intake of the red-fleshed apple snack (see Supplemental Information **Table 3S**). So, these compounds could be considered "endogenous" phenolic metabolites from the diet.

On the other hand, the phenolic metabolites from the second group were excreted in urine at high concentration levels (µmol) and significantly increased ($p$<0.05) after the red-fleshed apple intake at the different interval times studied (see **Figure 2B**). Phenolic metabolites from this group could be considered as potential biomarkers for red-fleshed apple consumption, since they were detected at trace levels or not detected in basal conditions.

**Figure 3** shows the individual profile of the urinary excretion (µmol) of the main phenols quantified after the red-fleshed apple snack intake. These urinary biomarkers include cyanidin galactoside (A1), cyanidin arabinoside (A2) and peonidin galactoside (A3) as cyanidin derivatives (anthocyanins); phloretin glucuronide (B1), phloretin sulphate glucuronide (B2) as phloretin derivatives from dihydrochalcones pathways, epicatechin sulphate (C1), methyl epicatechin sulphate (C2) and epicatechin glucuronide (C3) as epicatechin derivatives, and dihydroxyphenyl-γ-valerolactone (D1), dihydroxyphenyl-γ-valerolactone sulphate glucuronide (D2), dihydroxyphenyl-γ-valerolactone glucuronide (D3) and hydroxyphenyl-γ-valerolactone sulphate (D4) as phenyl-γ-valerolactone derivatives from flavan-3-ols pathways.

As shown in **Figure 3** and in **Table 2S (Supplemental Information)**, cyanidin (A1-A3), phloretin (B1 and B2) and epicatechin (C1-C3) derivatives were excreted in the first hours after the apple intake, their maximum excretion being detected from 2 to 4 h. Then, their urinary excretion decreased until 24 h. These results are in agreement with other studies after apple consumption (DuPont et al. 2002; Mennen et al. 2006; Kahle et al. 2007; Marks et al. 2009; Kristensen et al. 2012; Saenger et al. 2017).

Note that the native forms of some apple anthocyanins, such as cyaniding-3-*O*-galactoside, cyanidin arabinoside and peonidin-3-*O*-galactoside and also phloretin glucuronide (a phase-II metabolite) were detected in the urine samples from all the volunteers. Additionally, cyaniding-3-*O*-galactoside and phloretin glucuronide were also detected in all the plasma samples. Observing the plasma kinetic profile of these metabolites, they were rapidly absorbed in the small intestine showing a maximum concentration at 2-3 h. After that, their concentration decreased significantly until 24 h (see **Figure 4** and **Supplementary Information Table 3S**).

Regarding phenyl-γ-valerolactone derivatives (**Figure 3**, D1-D4) from flavan-3-ols metabolic pathways, their urinary excretion increased after the apple snack intake showing a maximum excretion from 8 to 24 h. This trend indicates intense colonic microbial metabolism, which could explain that these compounds were not detected in the plasma. Nevertheless, in our previous study in which we analyzed whole blood by sampling with dried blood spot (DBS) cards from the same volunteers, hydroxyphenyl-γ-valerolactone glucuronide was detected after 12 h (Yuste et al. 2018). These differences

could indicate higher sensitivity in the detection of circulating valerolactones from the analysis of whole blood instead of plasma.

From the results obtained in the present study, all the phenolic metabolites shown in **Figure 3** could be proposed as urinary markers for red-fleshed apple consumption. Nevertheless, epicatechin and phenyl-γ-valerolactone derivatives were not considered in the present study as biomarkers since these compounds are also present in other flavan-3-ol-rich foods, such as cocoa, wine and tea, which form part of a regular diet. Epicatechin phase-II metabolites, such as the sulphated, glucuronided and methylated derivatives found in the present study, as well as the microbial derived phenyl-γ-valerolactone metabolites, are certainly good biomarkers for the correct assessment of intake and health effects exerted by flavan-3-ol-rich diets (Van der Hooft et al. 2012; Urpi-Sardá et al. 2015). However, when proposing biomarkers for red-fleshed apple consumption, more specific metabolites must be sought. Therefore, phloretin glucuronide and cyanidin-3-*O*-galactoside may be the best candidates as biomarkers of red-fleshed apple snack intake. This proposal could be explained by different reasons: 1) phloretin glucuronide is a phase-II metabolite of the exclusively apple dihydrochalcones (phloretin glucoside and phloretin xylosyl glucoside) (Richling, 2012), while cyanidin-3-*O*-galactoside, the main anthocyanin of red-fleshed apples, is present in considerable amounts only in chokeberry (*Aronia melanocarpa*) and lingonberry (*Vaccinium vitis-idaea*) (Zheng et al. 2003) fruit that usually do not form part of a regular diet; 2) these metabolites were detected in all in the plasma and urine samples from all the volunteers; and 3) these metabolites were not detected under basal conditions, and their concentration increased significantly in the plasma and urine after the

apple intake. On the other hand, peonidin-3-*O*-galactoside was also quantified in the urine samples from all the volunteers, but at lower amounts than cyanidin-3-*O*-galactoside. This methyl conjugate of cyanidin-3-*O*-galactoside (peonidin-3-*O*-galactoside) was also selected as a urinary biomarker for red-fleshed apple consumption jointly with phloretin glucuronide and cyanidin-3-*O*-galactoside, as shown in **Figure 4.**

## Conclusions

In the present study, the different phenolic metabolites generated after the intake of red-fleshed apple snacks were identified and tentatively quantified in urine and plasma samples at different time intervals. Moreover, the metabolic pathways of the phenolic metabolites generated from red-fleshed apple phenolics were proposed, and these routes were based on phase-II and microbial reactions. The results show that after the consumption of red-fleshed apple snacks, (poly)phenols are extensively metabolized, resulting in the production of a large number of compounds with different structure, all of which should be considered when investigating the potential health effects of red-fleshed apples. From among the metabolites generated, phloretin glucuronide and cyanidin-3-*O*-galactoside were proposed as the best candidates as biomarkers of red-fleshed apple snack intake. The identification of these two phenolic metabolites could be exclusive to red-fleshed apple intake, therefore demonstrating their high specificity. These metabolites were not detected in basal samples (fasting conditions). Their concentration increased after the apple intake, and, in addition, these compounds were detected in the plasma and urine samples from all the volunteers. Jointly with phloretin glucuronide and

cyanidin-3-*O*-galactoside, peonidin-3-*O*-galactoside, which was also determined in the urine samples from all the volunteers, could also be considered a good urinary candidate as an intake biomarker for red-fleshed apple.


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## CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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## Figure captions

**Figure 1.** Proposed metabolic pathways for the generation of phenolic metabolites after the acute intake of red-fleshed apple snack. The metabolic route for chlorogenic acid is in green, for vanillic acid hexoside in blue, for anthocyanins in orange, from flavan-3-ols in lilac, from quercetin derivatives in pink and from dihydrochalcones in brown.

Reactions: *dH*, dehydrogenation; SULT, sulphotransferase; *UGT*, glucuronosyl-transferase; and *COMT*, catechol-O-methyltransferase; *dOH*, dehydroxylation; *dMe*, demethylation; *α-oxidation*, one decarboxylation; and *β-oxidation*, two decarboxylations.

Quercetin derivatives: quercetin glucoside, quercetin galactoside, quercetin arabinoside and quercetin rhamnoside.

**Figure 2.** Phenolic metabolite excretion rate in urine A) phenylpropionic/ phenylacetic acids, benzoic acids, catechol/pyrogallol and hydroxycinnamic acids derivatives; and B) flavan-3-ols, phenyl-γ-valerolactones, dihydrochalcones, and anthocyanins derivatives. Data expressed as µmol/h as mean values ± standard error of mean (*n*=10). Asterisks indicate significant differences ($p<0.05$) in excretion rate compared to basal conditions.

**Figure 3.** Urinary excretion of the proposed biomarkers for red-fleshed apple consumption 0-24 h after acute intake of 80 g apple snack. A1-A3: Cyanidin derivatives; B1-B2: Phloretin derivatives; C1-C3: Epicatechin derivatives; D1-

D4: Phenyl-γ-valerolactone derivatives. Data expressed as μmol/h as mean values ± standard error of mean (*n*=10).

**Figure 4.** Pharmacokinetic profile of the proposed biomarkers for red-fleshed apple snack intake. Data expressed as μmol as mean values ± standard error of mean (*n*=10).

**Table 1.** SRM conditions used for quantification and metabolites detected in plasma and urine after acute intake of red-fleshed apple snack.

| | Phenolic metabolites | SRM Quantification | Cone voltage (V) / Collision energy (eV) | Detected in[a] |
|---|---|---|---|---|
| | *Catechol and pyrogallol derivatives* | | | |
| 1 | Catechol sulphate | 189 > 109 | 20 / 15 | P (5), U (8) |
| 2 | Methyl catechol sulphate | 203 > 123 | 20 / 15 | P (5) U (7) |
| 3 | Methyl catechol glucuronide | 299 > 123 | 40 / 15 | U (6) |
| 4 | Pyrogallol sulphate or phloroglucinol sulphate | 205 > 125 | 40 / 15 | P (7) U (5) |
| | *Benzoic acid derivatives* | | | |
| 5 | Hydroxybenzoic acid | 137 > 93 | 30 / 15 | U (3) |
| 6 | Hydroxybenzoic acid sulphate | 217 > 137 | 35 / 15 | P (3), U (3) |
| 7 | Hydroxyhippuric acid | 194 > 100 | 40 / 10 | P (8) |
| 8 | Protocatechuic acid sulphate | 233 > 153 | 35 / 15 | P (4), U (5) |
| 9 | Vanillic acid sulphate | 247 > 167 | 30 / 25 | U (5) |
| 10 | Vanillic acid glucuronide | 343 > 167 | 30 / 25 | P (9) U (3) |
| | *Phenylacetic acid derivatives* | | | |
| 11 | Hydroxyphenylacetic acid | 151 > 107 | 20 / 10 | P (5), U (4) |
| 12 | Hydroxyphenylacetic acid sulphate | 231 > 151 | 20 / 15 | P (6), U (4) |
| 13 | Hydroxyphenylacetic acid glucuronide | 327 > 151 | 20 / 15 | U (2) |
| 14 | Dihydroxyphenylacetic acid sulphate | 247 > 167 | 30 / 15 | U (5) |
| 15 | Dihydroxyphenylacetic acid glucuronide | 343 > 167 | 30 / 15 | P (3), U (4) |
| | *Phenylpropionic acid derivatives* | | | |
| 16 | Hydroxyphenylpropionic acid | 165 > 121 | 20 / 10 | P (4), U (6) |
| 17 | Hydroxyphenylpropionic acid sulphate | 245 > 165 | 35 / 15 | U (7) |
| 18 | Hydroxyphenylpropionic acid glucuronide | 341 > 165 | 40 / 25 | U (4) |
| 19 | Dihydroxyphenylpropionic acid sulphate | 261 > 181 | 40 / 15 | P (2), U (5) |
| 20 | Hydroxymethoxyphenylpropionic acid | 195 > 136 | 30 / 15 | P (5), U (5) |
| 21 | Hydroxymethoxyphenylpropionic acid sulphate | 275 > 195 | 35 / 15 | P (2) U (2) |
| | *Hydroxycinnamic acid derivatives* | | | |
| 22 | Coumaric acid | 163 > 119 | 35 / 10 | P (2), U (5) |
| 23 | Coumaric acid sulphate | 243 > 163 | 35 / 15 | U (9) |
| 24 | Caffeic acid sulphate | 259 > 179 | 35 / 15 | P (2), U (2) |
| 25 | Ferulic acid sulphate | 273 > 193 | 35 / 15 | P (7), U (10) |
| | *Phenyl-γ-valerolactone derivatives* | | | |
| 26 | Hydroxyphenyl-γ-valerolactone sulphate | 271 > 191 | 40 / 20 | U (8) |
| 27 | Dihydroxyphenyl-γ-valerolactone | 207 > 163 | 40 / 15 | U (8) |
| 28 | Dihydroxyphenyl-γ-valerolactone glucuronide | 383 > 207 | 40 / 20 | U (8) |
| 29 | Dihydroxyphenyl-γ-valerolactone sulphate glucuronide | 463 > 287 | 40 / 20 | U (7) |
| | *Flavan-3-ol derivatives* | | | |
| 30 | Epicatechin sulphate | 369 > 289 | 40 / 20 | U (6) |
| 31 | Epicatechin glucuronide | 465 > 289 | 40 / 20 | U (6) |
| 32 | Methyl epicatechin glucuronide | 383 > 303 | 40 / 15 | U (7) |
| | *Dihydrochalcone derivatives* | | | |
| 33 | Phloretin glucuronide | 449 > 273 | 40 / 20 | P (10), U (10) |
| 34 | Phloretin sulphate glucuronide | 529 > 353 | 40 / 20 | U (8) |
| | *Anthocyanin derivatives* | | | |
| 35 | Cyanidin-3-*O*-galactoside | 449 > 287 | 40 / 20 | P (7), U (10) |
| 36 | Cyanidin arabinoside | 419 > 287 | 40 / 20 | P (1). U (10) |
| 37 | Peonidin-3-*O*-galactoside | 463 > 301 | 40 / 20 | U (10) |

[a]Metabolites detected in urine (U) and/or plasma (P). Figures in parenthesis indicate the number of samples (volunteers) in which the metabolite was detected.

# Metabolic pathways proposal for red-fleshed apple snack (poly)phenols and identification of potential biomarkers of apple consumption in human plasma and urine

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- Includes Supplementary Information on concentration of the main phenolic compounds in red fleshed apple snack. Data are expressed as mg/80 g portion dry weight (mean ± SD, *n*=3) (**Table S1**); total amounts of phenolic compounds excreted in urine (**Table S2**); as well concentrations of phenolic compounds detected in plasma (**Table S3**) after red-fleshed apple snack intake**.**

**Table S1.** Concentration of the main phenolic compounds in red fleshed apple snack. Data are expressed as mg/80 g portion dry weight (mean ± SD, *n*=3)

| Phenolic compound | Concentration (mg/80 g portion ) | |
|---|---|---|
| ***Anthocyanins*** | | *42.3 ± 1.18* |
| Cyanidin-3-*O*-galactoside | 39.7 ± 1.03 | |
| Cyanidin arabinoside | 2.60 ± 0.24 | |
| ***Phenolic acids*** | | *88.0 ± 3.34* |
| Protocatechuic acid | 1.71 ± 1.06 | |
| Coumaric acid hexoside | 0.77 ± 0.11 | |
| Ferulic acid hexoside | 2.12 ± 0.26 | |
| Vanillic acid hexoside | 4.28 ± 0.11 | |
| Chlorogenic acid | 79.1 ± 2.75 | |
| ***Flavan-3-ols*** | | *13.8 ± 1.18* |
| Epicatechin | 5.58 ± 0.78 | |
| Dimer | 6.92 ± 0.29 | |
| Trimer | 1.30 ± 0.12 | |
| ***Flavonol*** | | *17.3 ± 1.97* |
| Quercetin-3-*O*-arabinoside | 3.67 ± 0.45 | |
| Quercetin-3-*O*-rhamnoside | 9.26 ± 0.94 | |
| Quercetin-3-*O*-glucoside | 4.41 ± 0.57 | |
| ***Flavanone*** | | *0.42 ± 0.02* |
| Eriodictyol hexoside | 0.42 ± 0.02 | |
| ***Dihydrochalcones*** | | *33.7 ± 3.08* |
| Phloretin glucoside | 21.7 ± 2.54 | |
| Phloretin xylosyl glucoside | 11.7 ± 0.52 | |
| Hydroxyphloretin xylosil glucoside | 0.32 ± 0.03 | |
| **TOTAL Phenols** | | *196 ± 10.7* |

**Table S2.** Urinary excretion of phenolic compounds 0–24 h after acute intake of red-fleshed apple snack. Data expressed in µmol as mean values ± standard deviation after subtraction of baseline excretion from each volunteer.

| Phenolic metabolite[a] | Basal[b] | 0-2 h | 2-4 h | 4-8 h | 8-24 h |
|---|---|---|---|---|---|
| *Catechol and pyrogallol derivatives* | | | | | |
| Catechol sulphate (*n*=8) | 21.7 ± 3.60 | 2.10 ± 090 | 1.86 ± 0.80 | 18.2 ± 8.44 | 30.5 ± 4.28 |
| Methyl catechol sulphate (*n*=7) | 16.6 ± 4.80 | 0.82 ± 0.18 | 0.10 ± 0.05 | 1.64 ± 1.33 | 34.0 ± 21.9 |
| Methyl catechol glucuronide (*n*=6) | 0.27 ± 0.05 | 0.03 ± 0.01 | 0.00 ± 0.00 | 0.03 ± 0.02 | 1.09 ± 0.75 |
| Pyrogallol sulphate or phloroglucinol sulphate (*n*=5) | 1.53 ± 0.40 | 0.05 ± 0.03 | 0.15 ± 0.07 | 1.20 ± 0.89 | 1.67 ± 0.31 |
| *Benzoic acid derivative* | | | | | |
| Hydroxybenzoic acid (*n*=3) | 35.3 ± 10.3 | 18.3 ± 12.5 | 16.1 ± 8.01 | 15.4 ± 12.3 | 28.9 ± 21.1 |
| Hydroxybenzoic acid sulphate (*n*=3) | 125 ± 38.1 | 16.2 ± 9.17 | 8.17 ± 3.05 | 2.15 ± 2.15 | 131 ± .121 |
| Protocatechuic acid sulphate (*n*=5) | 16.0 ± 3.74 | 2.85 ± 0.88 | 11.5 ± 2.79 | 4.23 ± 1.83 | 8.85 ± 3.88 |
| Vanillic acid sulphate (*n*=5) | 36.3 ± 7.15 | 3.51 ± 1.74 | 14.5 ± 8.71 | 28.9± 28.9 | 90.3± 52.0 |
| Vanillic acid glucuronide (*n*=3) | 2.12± 0.74 | 0.15 ± 0.11 | 0.53 ± 0.15 | 1.65 ± 1.38 | 2.92 ± 0.91 |
| *Phenylacetic acid derivatives* | | | | | |
| Hydroxyphenylacetic acid (*n*=4) | 210 ± 26.3 | 8.59 ± 3.87 | 14.5 ± 5.59 | 73.5 ± 34.4 | 93.0 ± 37.9 |
| Hydroxyphenylacetic acid sulphate (*n*=4) | 52.0 ± 6.22 | 11.7 ± 5.32 | 6.20 ± 2.14 | 6.34 ± 3.68 | 47.4 ± 27.1 |
| Hydroxyphenylacetic acid glucuronide (*n*=2) | 2.03 ± 2.03 | 6.92 ± 0.66 | 3.69 ± 1.34 | 2.14 ± 0.18 | 4.53 ± 4.53 |
| Dihydroxyphenylacetic acid sulphate (*n*=5) | 24.6 ± 4.27 | 3.52 ± 1.30 | 2.01 ± 0.89 | 9.07 ± 4.16 | 20.7 ± 5.20 |
| Dihydroxyphenylacetic acid glucuronide (*n*=4) | 4.72 ± 0.26 | 0.90 ± 0.43 | 4.77 ± 2.62 | 5.37 ± 4.13 | 5.31 ± 2.81 |
| *Phenylpropionic acid derivatives* | | | | | |
| Hydroxyphenylpropionic acid (*n*=6) | 2.60 ± 0.88 | 1.86 ± 0.90 | 2.65 ± 0.98 | 3.79 ± 1.69 | 11.76 ± 4.35 |
| Hydroxyphenylpropionic acid sulphate (*n*=7) | 36.6 ± 11.1 | 4.35 ± 0.96 | 2.34 ± 0.52 | 13.4 ± 5.67 | 94.8 ± 42.2 |
| Hydroxyphenylpropionic acid glucuronide (*n*=4) | 1.57 ± 0.70 | 0.62 ± 0.41 | 0.13 ± 0.06 | 0.22 ± 0.06 | 3.40 ± 0.77 |
| Dihydroxyphenylpropionic acid sulphate (*n*=5) | 21.6 ± 2.83 | 2.86 ± 2.08 | 3.26 ± 1.61 | 22.4 ± 11.9 | 12.1 ± 3.75 |
| Hydroxymethoxyphenylpropionicacid (*n*=5) | 0.15 ± 0.04 | 0.02 ± 0.01 | 0.09 ± 0.04 | 0.29 ± 0.13 | 0.58 ± 0.22 |
| Hydroxymethoxyphenylpropionicacid sulphate (*n*=2) | 0.41± 0.16 | 0.09 ± 0.04 | 0.09 ± 0.01 | 0.48 ± 0.12 | 0.95 ± 0.42 |
| *Hydroxycinnamic acid derivatives* | | | | | |
| Coumaric acid (*n*=5) | 0.07 ± 0.05 | 0.32 ± 0.08 | 0.64 ± 0.12 | 1.28 ± 0.30 | 1.84 ± 0.43 |
| Coumaric acid sulphate (*n*=9) | 0.77 ± 0.26 | 0.49 ± 0.10 | 0.80 ± 0.17 | 1.48 ± 0.32 | 2.97 ± 0.63 |
| Caffeic acid sulphate (*n*=2) | 1.31 ± 0.91 | 0.51 ± 0.32 | 1.00 ± 0.74 | 1.30 ± 1.15 | 2.01 ± 0.92 |
| Ferulic acid sulphate (*n*=10) | 1.43 ± 0.41 | 0.29 ± 0.08 | 0.59 ± 0.11 | 0.74 ± 0.27 | 0.64 ± 0.24 |
| *Phenyl-γ-valerolactone derivatives* | | | | | |
| Hydroxyphenyl-γ-valerolactone sulphate (*n*=8) | 0.15 ± 0.15 | 0.02 ± 0.01 | 0.04 ± 0.02 | 1.21 ± 0.97 | 20.7 ± 17.1 |
| Dihydroxyphenyl-γ-valerolactone (*n*=8) | 3.51 ± 2.25 | 0.98 ± 0.63 | 2.96 ± 1.91 | 15.9 ± 4.61 | 81.9 ± 32.0 |
| Dihydroxyphenyl-γ-valerolactone glucuronide (*n*=8) | 0.01 ± 0.01 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.17 ± 0.07 | 1.12 ± 0.73 |
| Dihydroxyphenyl-γ-valerolactone sulphate glucuronide (*n*=7) | 0.07 ± 0.07 | 0.06 ± 0.03 | 0.16 ± 0.08 | 1.34 ± 0.47 | 9.47 ± 5.97 |
| *Flavan-3-ol derivatives* | | | | | |
| Epicatechin sulphate (*n*=6) | 0.14 ± 0.14 | 0.40 ± 0.10 | 0.71 ± 0.11 | 0.52 ± 0.13 | 0.49 ± 0.06 |
| Epicatechin glucuronide (*n*=6) | n.d. | 0.24 ± 0.07 | 0.37 ± 0.06 | 0.51 ± 0.28 | 0.32 ± 0.12 |
| Methyl epicatechin glucuronide (*n*=7) | 0.07 ± 0.07 | 0.23 ± 0.07 | 0.42 ± 0.08 | 0.36 ± 0.11 | 0.17 ± 0.06 |
| *Dihydrochalcone derivatives* | | | | | |
| Phloretin glucuronide (*n*=10) | n.d. | 0.71 ± 0.10 | 1.26 ± 0.32 | 1.06 ± 0.25 | 0.15 ± 0.11 |
| Phloretin sulphate glucuronide (*n*=8) | 0.03 ± 0.01 | 0.12 ± 0.03 | 0.11 ± 0.03 | 0.09 ± 0.02 | 0.13 ± 0.04 |
| *Anthocyanin derivatives* | | | | | |
| Cyanidin-3-*O*-galactoside (*n*=10) | n.d. | 2.49 ± 0.42 | 3.46 ± 0.63 | 2.88 ± 0.53 | 2.24 ± 1.38 |
| Cyanidin arabinoside (*n*=10) | 0.45 ± 0.07 | 0.74 ± 0.16 | 0.35 ± 0.11 | 0.35 ± 0.13 | 0.00 ± 0.00 |
| Peonidin-3-*O*-galactoside (*n*=10) | n.d. | 0.82 ± 0.15 | 1.61 ± 0.25 | 1.22 ± 0.29 | 0.35 ± 0.17 |

[a]Figures in parenthesis next to compound names indicate the number of samples (volunteers) in which the metabolite was detected.
[b]Content of urine collected for 12 h prior to supplementation and on an excretion per hour basis used to subtract from excretion values obtained after red-fleshed apple snack consumption to obtain the values cited in the Table.
n.d.: not detected

**Table S3.** Phenolic compounds concentrations in plasma 0–24 h after acute intake of red-fleshed apple snack. Data expressed in nmol/L as mean values ± standard deviation

| Phenolic compound [a] | Basal (0 h) | 0.5 h | 1 h | 2 h | 4 h | 6 h | 24 h |
|---|---|---|---|---|---|---|---|
| *Catechol and pyrogallol derivatives* | | | | | | | |
| Catechol sulphate (*n*=5) | 310 ± 59.2 | 367 ± 77.7 | 329 ± 62.2 | 389 ± 84.7 | 743 ± 243 | 1220 ± 434 | 373 ± 70.8 |
| Methyl catechol sulphate (*n*=5) | 133 ± 67.6 | 167 ± 79.3 | 195 ± 1075 | 159 ± 93.0 | 151 ± 64.5 | 260 ± 124 | 243 ± 71.3 |
| Pyrogallol sulphate (*n*=7) | 17.4 ±6.19 | 11.8 ± 4.84 | 11.6 ± 5.12 | 11.5 ± 5.26 | 17.1 ± 8.28 | 67.6 ± 21.0 | 16.3 ± 5.67 |
| *Benzoic acid derivative* | | | | | | | |
| Hydroxybenzoic acid sulphate (*n*=3) | 133 ± 44.1 | 179 ± 37.0 | 212 ± 52.4 | 132 ± 20.1 | 110 ± 21.3 | 144 ± 38.3 | 146 ± 52.5 |
| Hydroxyhippuric acid (*n*=8) | 13.5 ± 3.50 | 15.9 ± 5.68 | 21.3 ± 5.83 | 26.7 ± 7.57 | 27.8 ± 4.84 | 29.4 ± 7.33 | 15.8 ± 7.00 |
| Protocatechuic acid sulphate (*n*=4) | n.d. | 19.5 ± 11.5 | 39.3 ± 3.15 | 29.2 ± 4.46 | 20.3 ± 10.7 | 16.4 ± 16.4 | n.d. |
| Vanillic acid glucuronide (*n*=9) | 6.18 ± 2.52 | 10.7 ± 3.10 | 16.1 ± 2.61 | 23.6 ± 4.17 | 27.3 ± 3.25 | 23.9 ± 3.41 | 10.1 ± 2.54 |
| *Phenylacetic acid derivatives* | | | | | | | |
| Hydroxyphenylacetic acid (*n*=5) | 2178 ± 148 | 1946 ± 131 | 2049 ± 133 | 2577 ± 262 | 2788 ± 137 | 2993 ± 195 | 1820 ± 157 |
| Hydroxyphenylacetic acid sulphate (*n*=6) | 1016 ± 711 | 2047 ± 1120 | 2169 ± 1310 | 1398 ± 857 | 1607 ± 1166 | 1946 ± 1453 | 1523 ± 918 |
| Dihydroxyphenylacetic acid glucuronide (*n*=3) | 13.0 ± 13.0 | 23.3 ± 23.3 | 39.1 ± 20.3 | 63.2 ± 44.5 | 86.1 ± 17.6 | 84.6 ± 31.3 | 13.2 ± 13.2 |
| *Phenylpropionic acid derivatives* | | | | | | | |
| Hydroxyphenylpropionic acid (*n*=4) | 48.3 ± 48.3 | 55.4 ± 55.4 | 69.8 ± 69.8 | 265 ± 134 | 357 ± 186 | 897 ± 393 | 852 ± 432 |
| Dihydroxyphenylpropionic acid sulphate (*n*=2) | n.d. | 8.52 ± 0.95 | 9.94 ± 1.10 | 18.3 ± 2.04 | 38.1 ± 4.23 | 30.4 ± 3.37 | 10.4 ± 1.16 |
| Hydroxymethoxyphenylpropionic acid (*n*=5) | 11.1 ± 3.58 | 12.9 ± 2.71 | 12.8 ± 2.17 | 25.5 ± 9.89 | 41.6 ± 8.77 | 40.5 ± 8.01 | 37.9 ± 17.3 |
| Hydroxymethoxyphenylpropionic acid sulphate (*n*=2) | n.d. | 1.26 ± 1.26 | 0.99 ± 0.99 | 6.67 ± 0.98 | 11.2 ± 1.07 | 3.20 ± 3.20 | 2.87 ± 2.87 |
| *Hydroxycinnamic acid derivatives* | | | | | | | |
| Coumaric acid (*n*=2) | 143 ± 10.8 | 148 ± 24.8 | 171 ± 0.47 | 194 ± 24.2 | 165 ± 18.6 | 292 ± 30.5 | 157 ± 16.4 |
| Caffeic acid sulphate (*n*=2) | 8.21 ± 1.99 | 29.1 ± 14.3 | 37.5 ± 13.8 | 14.3 ± 8.06 | 11.8 ± 4.59 | 18.7 ± 4.78 | 13.0 ± 1.05 |
| Ferulic acid sulphate (*n*=7) | 18.2 ± 4.57 | 25.9 ± 4.76 | 25.9 ± 3.64 | 20.6 ± 3.09 | 24.8 ± 6.01 | 30.0 ± 3.45 | 17.7 ± 2.82 |
| *Dihydrochalcone derivatives* | | | | | | | |
| Phloretin sulphate (*n*=2) | n.d. | 20.7 ± 16.3 | 18.4 ± 12.4 | 8.35 ± 5.01 | 8.43 ± 5.00 | 24.0 ± 4.18 | 10.9 ± 6.81 |
| Phloretin glucuronide (*n*=10) | n.d. | 28.1 ± 3.54 | 46.7 ± 1.57 | 61.0 ± 6.82 | 53.9 ± 11.0 | 34.5 ± 11.7 | 1.16 ± 1.16 |
| *Anthocyanin derivatives* | | | | | | | |
| Cyanidin-3-*O*-galactoside (*n*=7) | n.d. | 9.66 ± 2.26 | 9.15 ± 1.81 | 10.3 ± 1.50 | 6.86 ± 0.74 | 1.52 ± 0.82 | 0.30 ± 0.30 |
| Cyanidin arabinoside (*n*=1) | n.d. | 2.60 | n.d. | n.d. | n.d. | n.d. | n.d. |

[a]Figures in parenthesis next to compound names indicate the number of samples (volunteers) in which the metabolite was detected.
n.d.: not detected

**Figure 1**

**Click here to download Figure: Figure 1_Yuste_et_al_JFF.ppt**

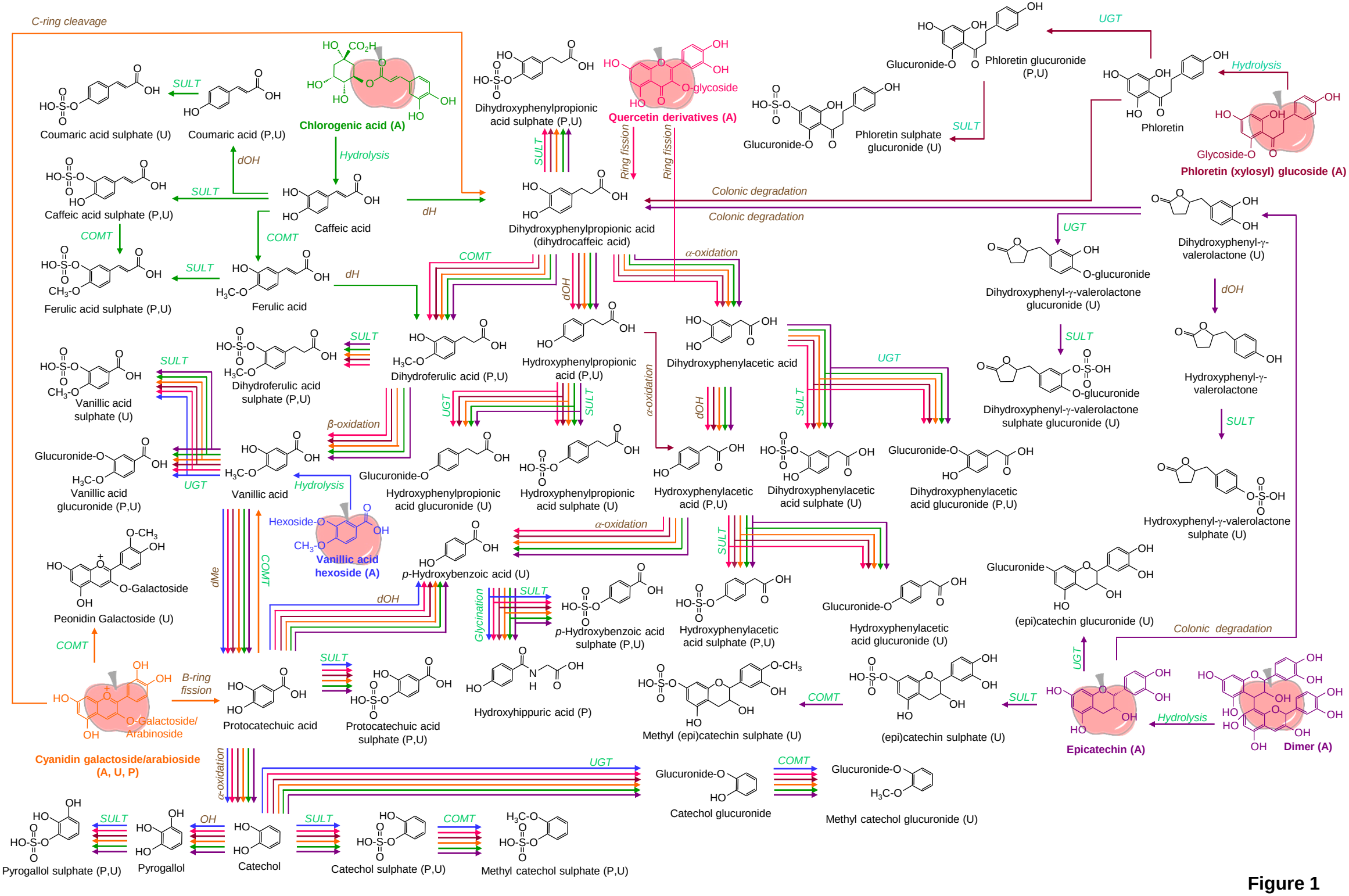


**Figure 1**

Figure 2

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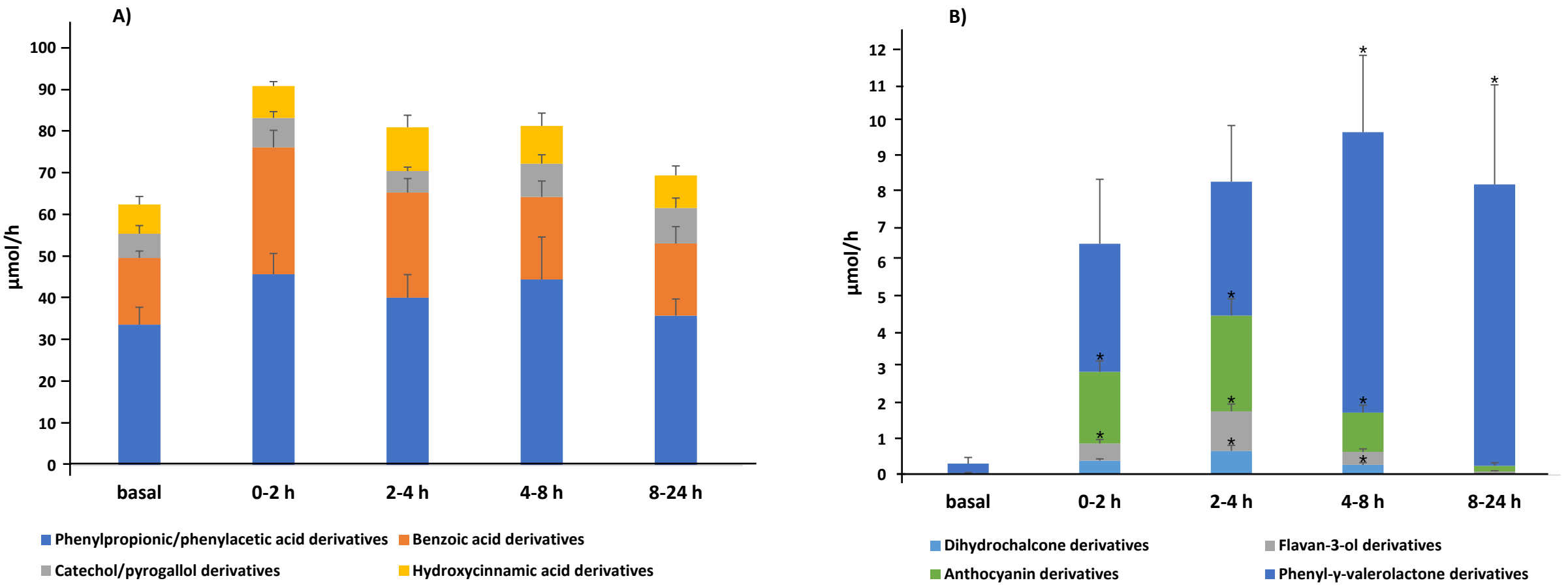


Figure 2

Figure 3

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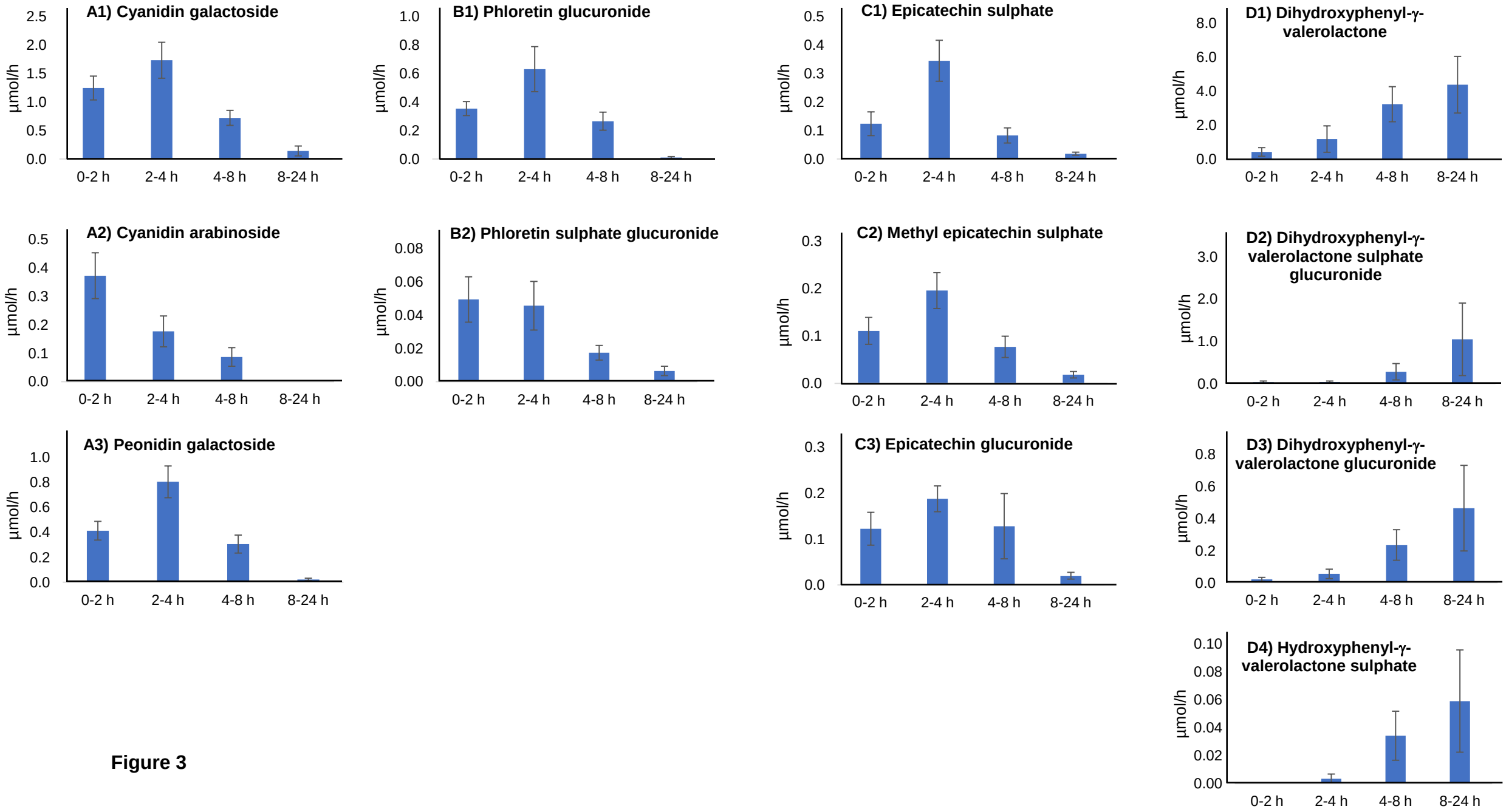


Figure 3

Figure 4

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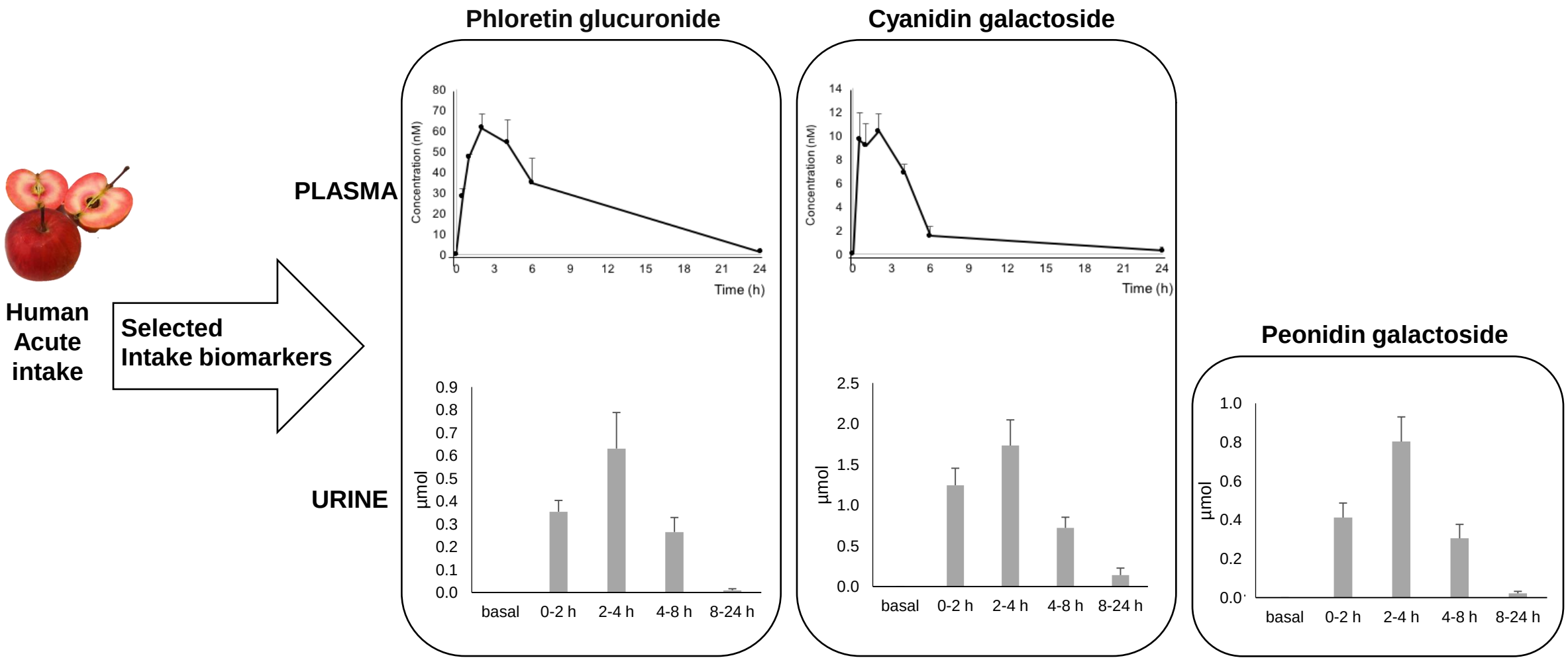


*Graphical Abstract

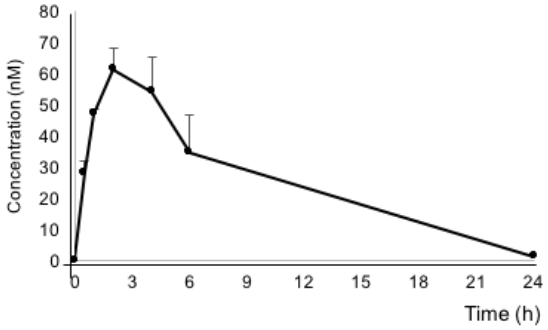
Human
Acute
intake

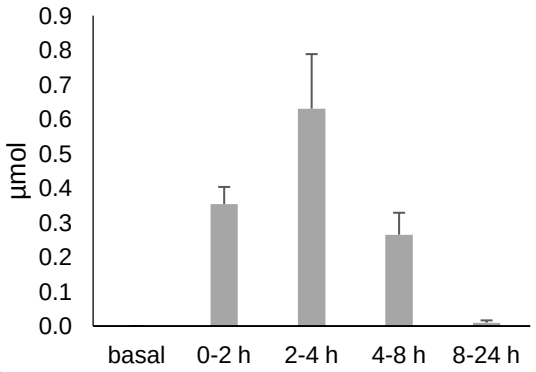
Selected
Intake biomarkers

PLASMA

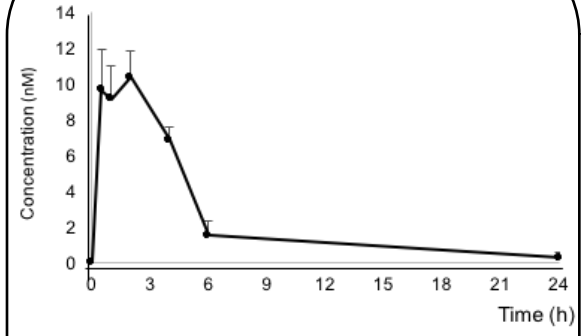
URINE

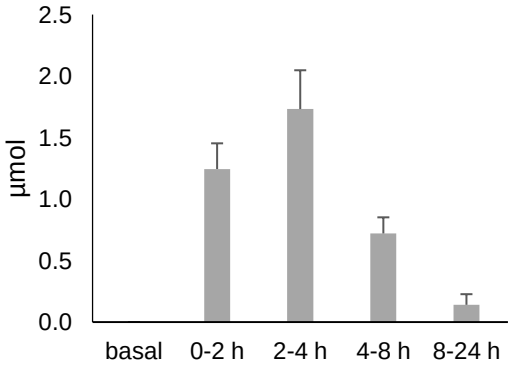
Phloretin glucuronide



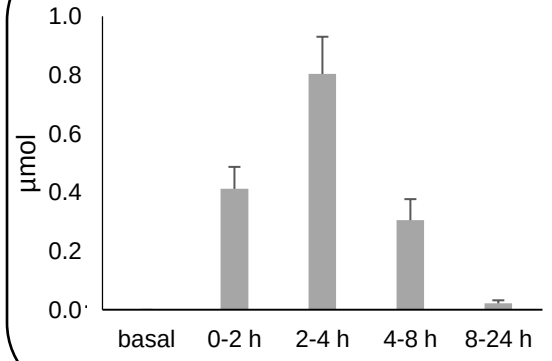




Cyanidin galactoside




Peonidin galactoside



## CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

## ETHICS STATEMENT

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016).