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TESI DOCTORAL

Red-fleshed apple as novel anthocyanin-biofortified food: Effect of food processing on the (poly)phenolic composition, bioavailability assessment and cardiometabolic effects of anthocyanins

Silvia Yuste Pérez

Memòria presentada per optar al grau de
Doctor per la Universitat de Lleida
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Dissertation presented by Silvia Yuste Pérez to obtain the PhD degree from the Universitat de Lleida. This work was carried out under the supervision of Laura Rubió Piqué and Iziar Amaia Ludwig Sanz-Orrio. The present work has been carried out in the Antioxidants Research Group in the Food Technology Department and is included in the "Ciència i Tecnologia Agrària i Alimentària" doctorate programme.

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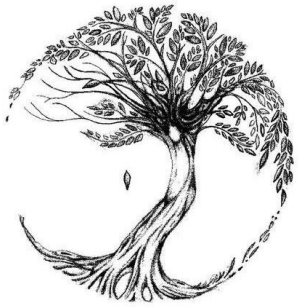

Cuando buscamos el tesoro,

nos damos cuenta de que el camino es el propio tesoro.

Paulo Coelho

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¡Qué afortunada soy de rodearme de gente como vosotros!

SUMMARY

The high prevalence of cardiovascular diseases (CVD) in the modern world due to unhealthy lifestyles requires new dietary prevention strategies. Enhancing traditional foods through biofortification with healthy compounds, such as flavonoids, has appeared as a potential strategy to increase food availability of bioactive compounds through affordable products and to guarantee their adequate intake. In this framework, a new hybrid apple variety with red pulp produced by traditional breeding methods with an enhanced content of anthocyanins has been poorly studied to date and represents an excellent chance to explore the potential of this new fruit variety in the control of CVD biomarkers. As a first step of the present Thesis, the impact of different thermal (hot air-drying, infrared-drying and purée pasteurization) and non-thermal (freeze-drying) processing technologies on the (poly)phenolic content of red-fleshed apple was evaluated for the development of a snack product suitable for consumption. The results showed that the processing that best preserved the anthocyanins and the rest of the (poly)phenols was freeze-drying. Among the thermal processes, hot air-drying and purée pasteurization presented (poly)phenol losses considerably lower than infrared-drying. The effect of the processing conditions on the apple (poly)phenol bioavailability was also evaluated in a human acute intake study and results showed that the pasteurized purée exhibited the highest (poly)phenolic bioavailability, providing evidence on how food processing plays a significant role in the absorption of (poly)phenols. Freeze-dried apple snack was finally selected as the one that best preserved the anthocyanins and allowed a feasible daily (poly)phenolic dose to be provided. To comprehensively investigate the (poly)phenolic metabolic pathways and the potential intake biomarkers of red-fleshed apple, a human postprandial study was performed as a first step prior to investigating the potential health benefits in human intervention studies. (Poly)phenols appeared to be extensively metabolized, resulting in the production of a large number of compounds based on several phase-II and microbial reactions. Among all the metabolites generated, phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside were proposed as the best candidates as intake biomarkers of red-fleshed apple. Results from this Thesis also revealed that a developed method based on dried blood spot cards could be useful as an alternative to classic venipuncture for future human postprandial studies, representing a minimally invasive, quick and easy strategy to determine the complete kinetic profile of the main (poly)phenolic metabolites. Finally, to establish the first insights into the cardiometabolic effects of red-fleshed apple compared to common white-fleshed apple, a sustained intake study was performed with hypercholesterolemic Wistar rats, observing a reduction of the aorta thickness, an improvement of the renal function and a reduction of insulin levels after administration of both apples. Moreover, an apple matrix effect was reported between red-fleshed apple and aronia infusion rich in anthocyanins, observing a higher absorption and excretion of (poly)phenols without the apple components. In this study, a gender effect was also reported in some cardiometabolic parameters related to gender differences in the metabolism, suggesting that (poly)phenols could influence the health outcomes differently depending on gender.

RESUMEN

La alta prevalencia de enfermedades cardiovasculares (ECV) en el mundo actual requiere del uso de nuevas estrategias de prevención dietéticas. La mejora de alimentos tradicionales a través de la biofortificación con compuestos saludables (como los flavonoides) ha emergido como una estrategia potencial para incrementar la disponibilidad de compuestos bioactivos en los alimentos a través de productos asequibles que garanticen su adecuada ingesta. En este contexto, representa una excelente oportunidad explorar el potencial en el control de biomarcadores de ECV de una nueva variedad poco estudiada de manzana híbrida de pulpa roja obtenida por métodos de cruce tradicionales y que posee un contenido mejorado de antocianinas. Como primer paso, en esta Tesis se evaluó el impacto de diferentes tecnologías de procesamiento térmico (secado por aire caliente, secado por infrarrojos y pasteurización de puré) y no térmico (liofilización) en el contenido (poli)fenólico de la manzana de pulpa roja para el desarrollo de un snack apropiado para su consumo. Los resultados mostraron que el procesamiento que mejor conservó las antocianinas y el resto de los (poli)fenoles presentes en esta manzana fue la liofilización. Respecto a los procesos térmicos, el secado con aire caliente y la pasteurización del puré mostraron pérdidas de (poli)fenoles considerablemente menores que el secado por infrarrojos. También se evaluó el efecto del procesamiento en la biodisponibilidad de los (poli)fenoles de dicha manzana tras una ingesta aguda en humanos. Los resultados mostraron que el puré pasteurizado exhibía la mayor biodisponibilidad (poli)fenólica, lo que evidencia el papel significativo que juega el procesamiento de los alimentos en la absorción de los (poli)fenoles. El snack de manzana liofilizado se seleccionó finalmente por ser el que mejor conservaba las antocianinas, y, porque además, permitía aportar una dosis (poli)fenólica diaria factible. Para investigar exhaustivamente las vías metabólicas (poli)fenólicas y los posibles biomarcadores de ingesta de la manzana de pulpa roja, y como primer paso antes de investigar los posibles efectos beneficiosos en el estudio de intervención humana, se realizó un estudio postprandial en humanos. Tras este estudio se observó que los (poli)fenoles se metabolizaron ampliamente, lo que resultó en la producción de un gran número de compuestos derivados de reacciones de fase-II y de reacciones microbianas. Entre todos los metabolitos generados, floretina glucurónido, cianidina-3-*O*-galactósido y peonidina-3-*O*-galactósido fueron propuestos como los biomarcadores de ingesta de la manzana de pulpa roja. Los resultados de esta Tesis también revelaron que el método desarrollado basado en el uso de tarjetas *"dried blood spot"* podría ser útil para futuros estudios postprandiales en humanos como alternativa a la punción venosa, representando una estrategia mínimamente invasiva, rápida y fácil para determinar el perfil cinético completo de los principales metabolitos (poli)fenólicos. Finalmente, para establecer los primeros conocimientos sobre los efectos cardiometabólicos de la manzana de pulpa roja (en comparación con la manzana de pulpa blanca común), se realizó una ingesta sostenida con ratas Wistar hipercolesterolémicas tras la que se observó una reducción del grosor de la aorta, la mejora de la función renal y la reducción de los niveles de insulina después de la administración de ambas manzanas. Además, se vio un efecto matriz entre la manzana de pulpa roja y una infusión de aronia rica en antocianinas, observándose una mayor absorción y excreción de los (poli)fenoles sin los componentes de la manzana. En este estudio, también se observó en algunos parámetros cardiometabólicos diferentes efectos dependiendo del género. Este hecho se relacionó con las diferencias observadas entre sexos en el metabolismo de los (poli)fenoles, lo que nos sugiere que los (poli)fenoles podrían influir de manera diferente en la salud en función del género.

RESUM

L'alta prevalença de malalties cardiovasculars (MCV) al món actual, requereix de l'ús de noves estratègies dietètiques per tal de prevenir-les. La millora d'aliments tradicionals a través de la biofortificació amb compostos saludables (com els flavonoides), ha emergit com una nova estratègia per incrementar la disponibilitat de compostos bioactius dels aliments a través de productes assequibles que garanteixin una ingesta adequada d'aquests. En aquest context, representa una excel•lent oportunitat l'explorar el potencial d'una nova varietat de poma híbrida de polpa vermella obtinguda per mètodes de creuament tradicionals, ja que posseeix un contingut incrementat d'antocianines i podria tenir beneficis afegits sobre marcadors de MCV. Com a primer pas en aquesta Tesi, es va avaluar l'impacte de diferents tecnologies de processament tèrmic (assecat amb aire calent, assecat amb infrarojos i pasteurització de puré) i processament no tèrmic (liofilització) sobre el contingut (poli)fenòlic de la poma de polpa vermella per al desenvolupament d'un snack. Els resultats van mostrar que el processament que millor va conservar les antocianines i la resta dels (poli)fenols presents a la poma estudiada, va ser la liofilització. Respecte als processaments tèrmics, l'assecat amb aire calent i la pasteurització van mostrar pèrdues de (poli)fenols considerablement menors que les produïdes durant l'assecat amb infrarojos. També, es va avaluar l'efecte del processament sobre la biodisponibilitat dels (poli)fenols de dita poma després d'una ingesta aguda en humans. Els resultats van mostrar que el puré pasteuritzat mostrava la major biodisponibilitat (poli)fenòlica, fet que evidencia la importància del paper que juga el processament dels aliments sobre l'absorció dels (poli)fenols. Finalment, l'snack de poma liofilitzada va ser el seleccionat per ser la millor forma de conservar les antocianines i, a més a més, perquè permetia aportar una dosi (poli)fenòlica diària assumible. Per investigar exhaustivament les vies metabòliques (poli)fenòliques i els possibles biomarcadors d'ingesta de poma de polpa vermella, i com pas previ a investigar els efectes beneficiosos en l'estudi d'intervenció, es va realitzar un estudi postprandial amb humans. Els resultats van mostrar que els (poli)fenols es metabolitzaven àmpliament, fet que va resultar en la producció d'un gran nombre de compostos derivats de reaccions metabòliques de fase-II i microbianes. Entre tots els metabòlits generats, la floretina glucurònid, cianidina-3-*O*-galactòsid i peonidina-3-*O*-galactòsid van ser els proposats com biomarcadors d'ingesta de poma de polpa vermella. Els resultats d'aquesta Tesi, també han revelat que el mètode desenvolupat basat en l'ús de targetes *"dried blood spot"* podria ser útil per futurs estudis postprandials en humans com a alternativa a la punció venosa, representant així una estratègia minimament invasiva, ràpida i fàcil per determinar el perfil cinètic complet dels principals metabòlits (poli)fenòlics. Finalment, per estudiar de forma preliminar els efectes cardiometabòlics de la poma de polpa vermella (en comparació amb la poma de polpa blanca comú), es va realitzar una ingesta sostinguda amb rates Wistar hipercolesterolèmiques. Els resultats van mostrar una reducció del gruix de l'aorta, una millora de la funció renal i una reducció dels nivells d'insulina després de suplementar les rates amb ambdós tipus de poma. Altrament, es va descriure un efecte matriu entre la poma de polpa vermella i la infusió d'aròmia rica en antocianines, observant una major absorció i excreció dels (poli)fenols quan aquests no anaven acompanyats de la matriu poma. En aquest estudi, també s'han observat diferents efectes sobre alguns paràmetres cardiometabòlics depenent del gènere. Aquest fet s'ha relacionat amb les diferències observades en el metabolisme dels (poli)fenols que es donen entre sexes, el que suggereix que els (poli)fenols podrien influenciar sobre la salut de manera distinta en funció del gènere.

CONTENT

INTRODUCTION

1. CARDIOVASCULAR DISEASES: A GLOBAL ISSUE

1.1. DATES AND PREVALENCE

Taking an estimated 17.9 million lives each year, cardiovascular diseases (CVD) are the number one cause of death globally representing 31% of all deaths. Another alarming fact is that, due to noncommunicable diseases (NCD), an estimated of 17 million premature deaths (under the age of 70) occur every year and it is estimated that 37% are caused by CVD. In addition to this total, 82% are in low- and middle-income countries, this high incidence in these countries being due to the fact that people have less access to effective and equitable health-care services which respond to their needs and they are therefore detected late in the course of the disease and die younger. CVD and other NCD contribute to poverty due to catastrophic health spending and high out-of-pocket expenditure (https://www.who.int/health-topics/cardiovascular-diseases/ accessed 20/04/2020).

CVD are a group of disorders of the heart and blood vessels and include coronary heart disease (disease of the blood vessels supplying the heart muscle), cerebrovascular disease (disease of the blood vessels supplying the brain), peripheral arterial disease (disease of blood vessels supplying the arms and legs), rheumatic heart disease (damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria) and others. Within these events, the most dangerous that usually occur are heart attacks and strokes, mainly caused by a blockage that prevents blood from flowing to the heart or brain. The common reason for this is a build-up of fat deposits on the inner walls of the blood vessels that supply the heart or brain, and this is mainly due to the presence of a combination of behavioural risk factors, such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol. The effects of behavioural risk factors may show up in individuals' physical factors, such as raised blood pressure, raised blood glucose, raised blood lipids, overweight and obesity. There are also a number of underlying determinants that participate to a lesser extent in the development of CVD, such as globalization, urbanization, population ageing, poverty, stress and hereditary factors (https://www.who.int/health-topics/cardiovascular-diseases/ accessed 20/04/2020).

1.2. FUTURE PERSPECTIVES AND NEW STRATEGIES

The WHO (World Health Organization) has identified effective interventions that are feasible to be implemented even in low-resource settings for prevention and control of CVD. They include two types of interventions which are recommended to be used in combination because, when used together, nearly 75% of recurrent vascular events may be prevented:

- Population-wide interventions, such as comprehensive tobacco control policies for cessation of tobacco use, taxation to reduce the intake of foods that are high in fat and/or sugar and increase the consumption of fruits and vegetables, building walking and cycle paths to increase physical activity, strategies to reduce harmful use of alcohol, and providing healthy school meals to children.

- Individual intervention: individual health-care interventions need to be targeted at those at high total cardiovascular risk or those with single risk factor levels above traditional thresholds, such as hypertension and hypercholesterolemia for prevention of heart attacks and strokes (https://www.who.int/health-topics/cardiovascular-diseases/ accessed 20/04/2020).

In this way the WHO, together with the support of all member states (194 countries), agreed in 2013 on global mechanisms to reduce avoidable NCD. The objective sought is to reduce the number of premature deaths from NCD by 25% by 2025 through nine voluntary global targets, and from among these, two targets are directly focused on preventing and controlling CVD. These targets are included in a "Global action plan for the prevention and control of NCD 2013-2020". Within these targets and to promote healthier life and eating styles, objective three is the creation of policy options to promote a healthy diet in the entire population. The main measures proposed are: (i) reduce the level of salt/sodium added to food (prepared or processed), (ii) reduce saturated fatty acids in food and replace them with unsaturated fatty acids, (iii) replace *trans*-fats with unsaturated fats, (iv) reduce the content of free and added sugars in food and non-alcoholic beverages, (v) limit excess calorie intake, (vi) reduce portion size and energy density of foods and (vii) increase availability, affordability and consumption of fruit and vegetables (https://www.who.int/health-topics/cardiovascular-diseases/ accessed 20/04/2020).

In this context, it is also necessary to develop policy to reinforce the measures aimed at food processors, retailers, caterers and public institutions. In addition, it is advisable to provide greater opportunities for utilization of healthy agricultural products and foods, and consequently for the development of functional foods that cover all the aspects previously set out and that could be consumed worldwide (WHO, 2013).

2. FUNCTIONAL FOODS

2.1. THE CONCEPT OF FUNCTIONAL FOOD

"Let food be thy medicine, thy medicine shall be thy food", ascribed to Hippocrates, the father of modern medicine, in around 400 BC was probably the first union between food and medicine that is known. Since then, this union has been investigated a great deal and many terms have been used to try to describe it, although it was not until the 1980s that the concept of functional food was established and introduced in Japan, because health authorities recognized the need for an improved quality of life and increasing life expectancy to control healthcare costs. The concept of foods developed specifically to promote health or reduce the risk of disease was called Foods for Specified Health Use (FOSHU) (Dalari & Lee, 2015).

Since then, this concept has evolved and a large number of definitions have been proposed. The variety of definitions range from those that suggest that any food, if marketed with the appropriate positioning, is a functional food (Hollingsworth, 1999) to others that argue that only fortified, enriched, or enhanced foods with a component having a health benefit beyond basic nutrition can be considered as functional food (Kleinschmidt, 2003), and including some definitions that indicate that if a health claim can be made it is a functional food (Weststrate et al., 2002) and other more complex ones such as "A functional food must remain food and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet: it is not a pill or a capsule, but part of the normal food pattern" (Diplock et al., 1999).

Given this variety of definitions, Doyon & Labrecque (2008) carried out a review of a hundred definitions, of which twenty-six were selected on the basis of their representation of the various types of existing definitions for functional foods and,

from among these, four key concepts were identified that appeared in most of them: i) health benefits, ii) the nature of the food, iii) the level of function and iv) the consumption pattern. Based on these keys that were repeated in most of the definitions, these authors defined a "functional food" as follows: "A functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions".

From this definition, the most important characteristic that defines a functional food is to produce physiological effects. The upper bound of the physiological effect dimension would be food that cures or heals and also from this definition it follows that a functional food should have benefits beyond its basic nutritional functions (a food that improves nutritional equilibrium should not be considered, on that single basis, a functional food). Another concept related to the functional food is functional intensity, measured through its physiological effects and its concentration of "active" components and, for this, a functional food must have a minimal degree of functional intensity. As illustrated in **Figure 1**, to define a functional food, physiological effects and functional intensity as two dimensions are required. Physiological effects could be positive or negative while functional intensity is positive. The dark portion does not exist or is undefined and drug-food is outside the boundaries of functional food. The functional food universe also includes foods that reduce risk of disease, enhance function and contribute to restoring health, all with a minimal level of intensity, and they would be considered as functional foods only if their physiological effects come within the boundaries (Doyon & Labrecque, 2008).

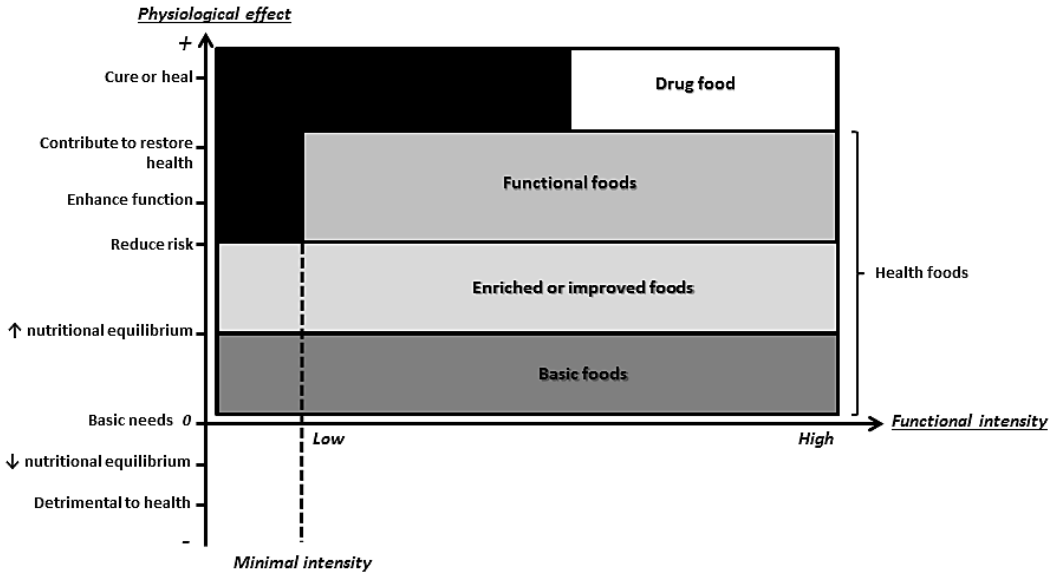


***Figure 1**. Limits of the functional food concept (Doyon & Labrecque, 2008).*

The beneficial effects on the body and functionality can be attributed to the molecules naturally present in foods and to the compounds added to industrial food matrices. These main molecules and compounds are vitamins, (poly)phenolic compounds, fibre, omega-3 fatty acids, minerals and bacterial cultures, among others (Keservani et al., 2010).

2.2. (POLY)PHENOLIC COMPOUNDS

The metabolism of plants can be divided into the primary and the secondary metabolism. The primary metabolism gives rise to lipids, proteins, carbohydrates and nucleic acids, in other words compounds that are common and essential to living organisms' cells maintenance. On the other hand, secondary metabolism gives rise to substances that are involved in many functions in plants, such as sensorial properties (colour, aroma, taste and astringency), structure, pollination, resistance to pests and predators, seed germinative processes after harvesting and growth as well as development and reproduction, among others (Vickery & Vickery, 1981). The major groups of secondary metabolites are alkaloids, terpenoids and (poly)phenolic compounds. Alkaloids are synthesized from L-amino acids such as tryptophan, tyrosine, phenylalanine, lysine and arginine, these compounds containing one or several nitrogen atoms as constituents of heterocycles (Buchanan et al., 2009; Heldt & Piechulla, 2011).

Regarding terpenoids (also known as isoprenoids), these compounds are the most abundant class of plant secondary metabolites (> 40.000) and are derived by repetitive fusion of branched five carbon isoprene units. Terpenes are classified with respect to the number of isoprene units present in the molecule as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), triterpenes (C30), tetraterpenes (C40), steroids, polyprenols, and polyterpenes (Heldt & Heldt, 2011). The third major and widely distributed groups of secondary metabolites are (poly)phenolic compounds, with more than 8.000 different (poly)phenolics identified in the plant kingdom (Scalbert & Williamson, 2000; Tomás-Barberán & Espín, 2001).

(Poly)phenolic compounds are an extensive and heterogeneous group of chemical components that have one (phenolic compounds) or more (polyphenolic compounds) aromatic benzene rings with a conjugated aromatic system and one or more hydroxyl groups (Bhattacharya et al., 2010; Cheynier, 2012).

The beneficial health effects attributed to (poly)phenolic compounds are due to the fact that these compounds can neutralize free radicals by donating an electron or hydrogen atom and thus suppress the generation of these radicals. They act as direct radical scavengers, for example in the lipid peroxidation chain reactions (chain breakers), by donating an electron to the free radical, neutralizing the radicals and becoming stable (less reactive) radicals themselves, thus stopping the chain reactions. They can also act as metal chelators by chelation, for example of transition metals such as Fe^{2+} that produce the Fenton reaction in which highly reactive hydroxyl radicals are produced (Guo et al., 2009; Perron & Brumaghim, 2009; Pietta, 2000).

The beneficial effects ascribed to (poly)phenolics are also due to the fact that they are involved in the regeneration of essential vitamins (Zhou et al., 2005) or can induce antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose hydroperoxides, hydrogen peroxide and superoxide anions, respectively, and inhibit the expression of enzymes such as xanthine oxidase (Du et al., 2007). Moreover, in recent years it has also been discovered that (poly)phenols may exert modulatory actions in cells through actions at protein kinase and lipid kinase signalling pathways (Williams et al., 2004).

Biogenetically, these phytochemicals are synthesized through the shikimic acid pathway where, mainly, phenylpropanoids are formed and the acetic acid pathway, in which the main products are phenols. The combination of both pathways leads to the formation of flavonoids that are the most numerous group of (poly)phenolic compounds in nature (Sánchez-Moreno, 2002).

There is discordance concerning how to classify (poly)phenolic compounds but the most adopted classification implies the subdivision of (poly)phenolics into two main groups: flavonoids and non-flavonoid (poly)phenols (**Figure 2**), based on the fact that at least 4.000 out of the 8.000 different (poly)phenolics that are known belong to the flavonoid subclass (Bravo, 1998; Cheynier, 2005; Harborne & Williams, 2000; Tsao, 2010).

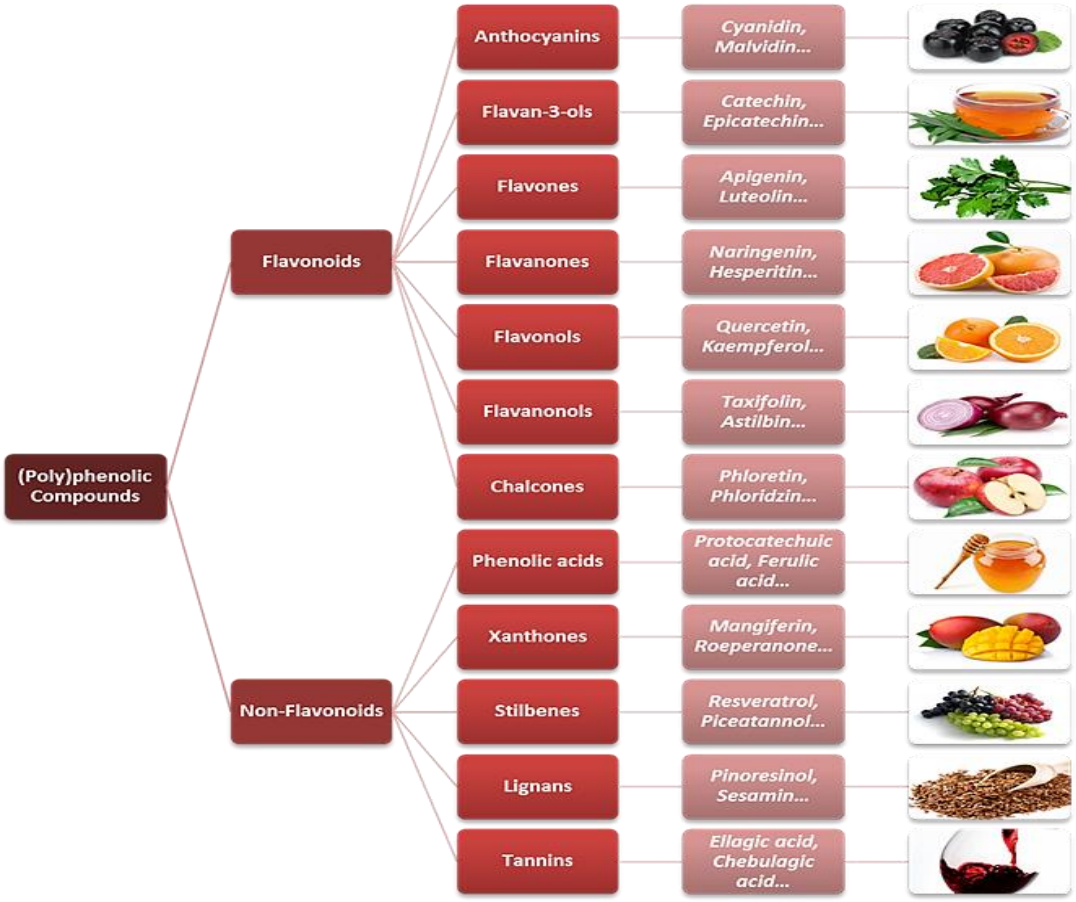


Figure 2. *Main flavonoid and non-flavonoid subclasses as well as (poly)phenols characteristic of each group and foods rich in these (Adlercreutz, 2007; Barreca et al., 2017; Boudjou et al., 2013; Chandrasekara & Shahidi, 2010; González-Laredo et al., 2018; Gutierrez-Orozco & Failla, 2013; Hostetler et al., 2017; Pollastri, & Tattini, 2011; Prior et al., 2001; Rozmer & Perjési, 2016; Si et al., 2006; Tsao, 2010).*

2.2.1. FLAVONOIDS

Flavonoids have the C6–C3–C6 general structural backbone (**Figure 3**) in which the two C6 units (Ring A and Ring B) are of phenolic nature and can be separated into different sub-groups due to the hydroxylation pattern and variations in the chromane ring (Ring C) (Durazzo et al., 2019).

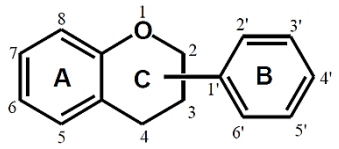


Figure 3. *Flavonoid backbone.*

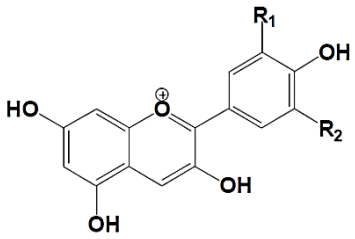
A wide range of pharmacological activities, including antioxidant, antibacterial, hepatoprotective, anti-inflammatory, and antihyperlipidemic effects, are attributed to these polyphenolic compounds (Belwal et al., 2017; D'Evoli et al., 2013; Farhadi et al., 2019; Farhat et al., 2017; Iriti et al., 2017; Ninfali et al., 2017; Rees et al., 2018; Riccio et al., 2018).

These principal subgroups are:

- ANTHOCYANINS

Anthocyanins are the glycosidic forms of anthocyanidins, the prevalent form in which they are present in plants. Chemically, anthocyanins are polyhydroxyl and polymethyl derivatives of flavynium salts, containing a flavylium cation on the C-ring, and are water-soluble compounds. More than 500 anthocyanins are known and differ depending on the hydroxylation, methoxylation patterns on the B ring, and glycosylation with different sugar units, and more than 30 anthocyanidins have been identified, but only six aglycones are common and widely distributed in foods: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (**Figure 4**). Anthocyanins most frequently occur as 3-monosides, 3-biosides and 3-triosides as well as 3,5-diglycosides and more rarely 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose, and xylose. These polyphenolics are the principal components of the red, blue and purple pigments of the majority of flower petals

(Greek anthos = flower and kyanos = blue), fruits and vegetables, and certain special varieties of grains. Some of the main food sources of these compounds are berries and red-skinned grapes, apples, and pears and various vegetables such as radishes and red/purple cabbage. Most of the anthocyanins in foods are cyanidin derivatives, cyanidin-3-glucoside being the most widely distributed anthocyanin in edible plants and, consequently, the most studied compound. Regarding biological effects, anthocyanins possess antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects, as well as prevention of cardiovascular diseases (CVD) (Bars-Cortina et al., 2020; Belwal et al., 2017; Blesso, 2019; Cimino et al., 2013; D'evoli et al., 2013; De la Rosa et al., 2009; Galvano et al., 2009; McCallum et al., 2007; Olivas-Aguirre et al., 2016; Seeram et al., 2003; Serraino et al., 2003; Wu et al.,2010).



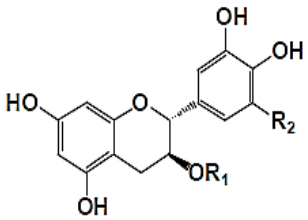
	R_1	R_2
Cyanidin	-OH	-H
Delphinidin	-OH	-OH
Pelargonidin	-H	-H
Malvidin	$-OCH_3$	$-OCH_3$
Peonidin	$-OCH_3$	-H
Petunidin	-OH	$-OCH_3$

Figure 4. *Major anthocyanins.*

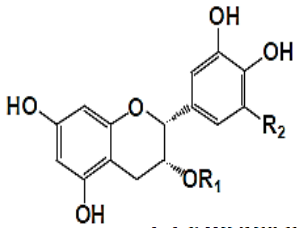
- FLAVAN-3-OLS

Flavanols or flavan-3-ols are commonly called catechins and are different from most flavonoids because there is no double bond between C2 and C3, and no C4 carbonyl in Ring C. This fact, together with the hydroxylation at C3, allows flavan-3-ols to have two chiral centres on the molecule (on C2 and C3), thus resulting in four possible diastereoisomers. Moreover, catechin and epicatechin (the principal monomeric flavan-3-ols) can form polymers, often referred to as proanthocyanidins because an acid-catalyzed cleavage of the polymeric chains produces anthocyanidins. The main food sources of these compounds are many fruits, particularly skins of grapes, apples, blueberries, tea leaves and cacao bean (chocolate) (Prior et al., 2001; Si et al., 2006; Tsao et al., 2003) and the major flavan-3-ols are shown in **Figure 5.** Flavan-3-ols have been reported to exhibit several beneficial health effects on being antioxidant,

antidiabetic, anticancer or cardiopreventive, among others (Márquez Campos et al., 2020; Raman et al., 2018; Seeram et al., 2003).



	R_1	R_2
(+)-Catechin	-H	-H
(+)-Catechin gallate	-gallyl	-H
(+)-Gallocatechin	-H	-OH
(+)-Gallocatechin gallate	-gallyl	-OH

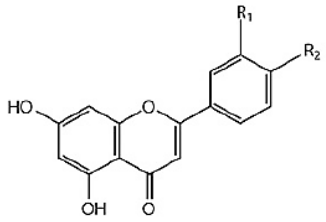


	R_1	R_2
(+)-Epicatechin	-H	-H
(+)-Epicatechin gallate	-gallyl	-H
(+)-Epigallocatechin	-H	-OH
(+)-Epigallocatechin gallate	-gallyl	-OH

Figure 5. *Major flavan-3-ols.*

- FLAVONES

Flavones have a double bond between C2 and C3 in the flavonoid skeleton, there is no substitution at the C3 position, and they are oxidized at the C4 position. Along with flavonols, they are the primary pigments in white- and cream-colored flowers and act as copigments with anthocyanins in blue flowers. **Figure 6** shows the structure of the main flavones. Chamomile and parsley have the highest flavone concentrations and are the main food sources of these compounds. Among the beneficial effects of these compounds, their anti-inflammatory properties stand out (Hostetler et al., 2017; Zhang et al., 2018).

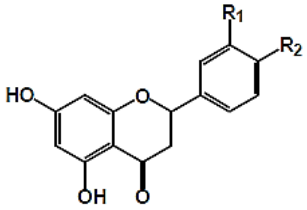


	R_1	R_2
Apigenin	-H	-OH
Acacetin	-H	$-OCH_3$
Luteolin	-OH	-OH

Figure 6*. Major flavones.*

- FLAVANONES

This group is structurally similar to flavones and only the lack of a double bond between C2 and C3 that gives them a chiral centre at the C2 position allows us to differentiate them. To date, about 350 flavanone aglycones and 87 flavanone glycosides have been identified in nature: **Figure 7** shows the structure of the most common. This variety is due to some of them having unique substitution patterns such as prenylated flavanones, furanoflavanones, pyranoflavanones, benzylated flavanones, giving a large number of substituted derivatives of this subgroup. The principal food sources of these compounds are citrus fruits (Barreca et al., 2017; Khan & Dangles, 2014). These compounds present potential beneficial effects related to anti-inflammatory, hypolipidemic, and vasoprotective properties (Crozier et al., 2009; Dalgård et al., 2008; Durazzo et al., 2019; Farhat et al., 2017).

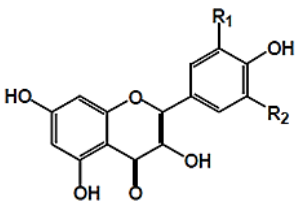


	R_1	R_2
Naringenin	-H	-OH
Hesperetin	-OH	$-OCH_3$
Eriodictyol	-OH	-OH

Figure 7. *Major flavanones.*

- FLAVONOLS

This subclass of flavonoids is also called 3-hydroxyflavones since they have a hydroxyl group attached to position-3 of the C-ring of the flavones. These compounds are extensively distributed in the outer parts as well as in the leaves of the higher plants, and about 450 different kinds of flavonol aglycones have been documented so far in higher plants (**Figure 8** shows the major ones). These compounds are present in glycosylated forms in nature, the associated sugar moiety is very often glucose or rhamnose, but other sugars might be involved (such as galactose, arabinose, xylose, glucuronic acid). The richest food sources are onions, curly kale, leeks, broccoli, and blueberries, among others (Crozier et al., 2000; Crozier et al., 2010; Pollastri, & Tattini, 2011). The intake of flavonols is inversely associated with subsequent coronary heart disease and with a protective effect on cancer (Hertog et al., 1997; Iriti et al., 2017).



	R_1	R_2
Kaempferol	-H	-H
Quercetin	-H	-OH
Myricetin	-OH	-OH
Isorhamnetin	$-OCH_3$	-H

Figure 8. *Major flavonols.*

- FLAVANONOLS

These compounds contain a flavan-3-one moiety, with a structure characterized by a 2-phenyl-3,4-dihydro-2H-1-benzopyran bearing a hydroxyl group and a ketone at the carbon C3 and C4, respectively, and are also called dihydroflavonols. This group is considered a minor flavonoid subgroup and taxifolin is the principal representative (**Figure 9**). The main food sources of these compounds are onions, black tea and citrus fruits, among others (Tsao, 2010). Among the effects of this group of compounds, its antimicrobial activity stands out (Kuspradini et al., 2009).

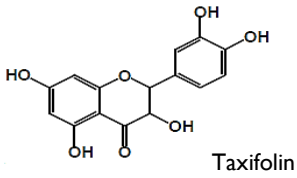


Figure 9. *Chemical structure of taxifolin, the principal flavanonol.*

- CHALCONES

Chalcones and dihydrochalcones have a linear C3 chain of the ring A connecting the two rings by a three-carbon α, β carbonyl system. The C3 chain of chalcones contains a double bond, whereas the C3 chain of dihydrochalcones is saturated. This chain makes the difference of these subclasses with respect to the other flavonoids. Chalcones are distributed in foods and beverages such as vegetables, fruits, tea, soy-based foodstuff and spices, and dihydrochalcones can be found principally in apples (**Figure 10**). Some of the main effects that have been ascribed to chalcones and

dihydrochalcones are antibacterial, antiviral, cardioprotective or neuroprotective, among others (Díaz-Tielas et al., 2016; Durazzo et al., 2019; Farhadi et al., 2019; Guo et al., 2017; Rozmer & Perjési, 2016; Vermerris & Nicholson, 2008).

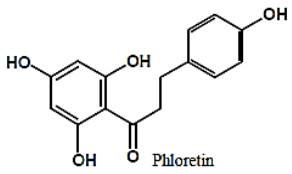


Figure 10. *Principal dihydrochalcone present in apples (phloretin).*

2.2.2. NON-FLAVONOIDS

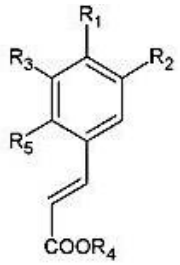
The following stand out among the main subclasses of phenolic compounds that are not flavonoids:

- PHENOLIC ACIDS

Phenolic acids are one of the most widely distributed plant non-flavonoid phenolic compounds present in free, conjugated-soluble and insoluble-bound forms. These phenolics are characterized by having one or more hydroxyl groups and a carboxylic acid function at the benzene ring. They are derived from two main phenolic compounds, benzoic acid (such as syringic acid, vanillic acid, gentisic acid...) and cinnamic acids (such as caffeic acid, ferulic acid, *p*-coumaric acid…) and they differ in the degree of hydroxylation and methoxylation of the aromatic ring (Lafay & Gil-Izquierdo, 2008) (**Figure 11**). Phenolic acids are predominantly found in the form of chlorogenic acids, where cinnamic acid moiety is attached to a quinic acid to form various isomers (Clifford, 1999). These phenolics are present at high concentrations in many food products, including fruits, vegetables, tea, cocoa, wine, honey, tea leaves, coffee, and whole grains. Free phenolic acids are mainly present in fruits and vegetables, whereas bound phenolic acids are the main representatives in grains and wholemeal products (Chandrasekara & Shahidi, 2010; Dueñas et al., 2016; Stuper-Szablewska & Perkowski, 2019). The biological effects due to the intake of these

compounds have been shown in cardiovascular diseases and obesity (Guo et al., 2017; Tresserra-Rimbau et al., 2014a; Tresserra-Rimbau et al., 2014b).

Hydroxybenzoic acids	R_1	R_2	R_3	R_4
p-Hydroxybenzoic acid	-OH	-H	-H	-H
Vanillic acid	-OH	-H	$-OCH_3$	-H
Gallic acid	-OH	-OH	-OH	-H
Syringic acid	-OH	$-OCH_3$	$-OCH_3$	-H



Hydroxycinnamic acids	R_1	R_2	R_3	R_4	R_5
p-Coumaric acid	-OH	-H	-H	-H	-H
Caffeic acid	-OH	-OH	-H	-H	-H
Sinapic acid	-OH	$-OCH_3$	$-OCH_3$	-H	-H
Ferulic acid	-OH	$-OCH_3$	-H	-H	-H

***Figure 11**. Major hydroxybenzoic acids and hydroxycinnamic acids.*

- XANTHONES

Xanthones comprise a family of O-heterocycle symmetrical compounds with a dibenzo-ɣ-pyrone scaffold and are known as xanthone, xanthene-9-one, or dibenzo-ɣ-pyrone. Depending on the chemical nature of the substituents in the dibenzo-ɣ-pirone scaffold, xanthones can be classified as simple oxygenated xanthones, glycosylated xanthones, and prenylated xanthones (Negi et al., 2013). The biological activities (anticancer, antibacterial, anti-inflammatory, and antidiabetic (Li et al., 2013; Miura et al., 2001; Wezeman et al., 2015; Yasunaka et al., 2005)) of this class of compounds are associated with their tricyclic scaffold (**Figure 12**) but vary depending on the nature and/or position of the different substituents. The principal natural sources of xanthones are mangosteen fruit and mango fruit (Gutierrez-Orozco & Failla, 2013).

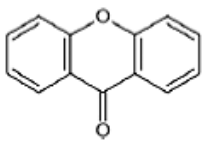


***Figure 12.** Structure backbone of xanthones subclass.*

- STILBENES

The basic chemical structure of stilbenes contains two benzene rings linked by a double bond, as shown in **Figure 13** (González-Laredo et al., 2018). The most studied and well-known stilbene is resveratrol, whose intake has been associated with a reduced risk of hypertension onset (Miranda et al., 2016), diabetes (Tresserra-Rimbau, et al., 2016) and obesity (Grosso et al., 2018). Stilbenes are reported to be present in grapes, almond, bean, blueberries, bilberries, peanuts, grapevine, cranberries, mulberries, plum, and wine (Arraki et al., 2017; Blaszczyk et al., 2019; González-Laredo et al., 2018).

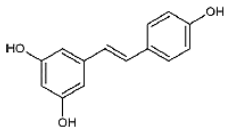


Figure 13. *Chemical structure of resveratrol.*

- LIGNANS

Lignans are diphenolic compounds derived from the combination of two phenylpropanoid C6-C3 units at the β and β' carbon atoms and can be linked to additional ether, lactone, or carbon bonds; they have a 1,4-diarylbutan like chemical structure (**Figure 14**) (Lewis & Davin, 1999). The intake of lignans has mostly been related to their possible cancer chemopreventive actions (due to their phytoestrogen properties) and to the prevention of cardiovascular diseases (Anandhi Senthilkumar et al., 2018; Xiao et al., 2018). The main sources of dietary lignans are oilseeds (flax, soy, rapeseed, and sesame), whole-grain cereals (wheat, oats, rye, and barley), legumes, various vegetables, and fruit (particularly berries) (Adlercreutz, 2007).

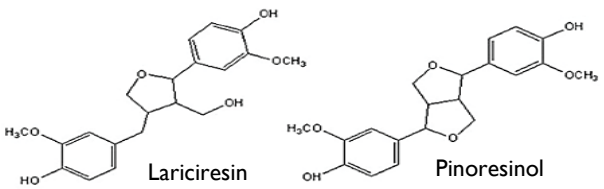


Figure 14. *Chemical structure of some dietary lignans.*

- TANNINS

Tannins are classified into two major groups: hydrolyzable and non-hydrolyzable tannins, also called condensed tannins or proanthocyanidins (Kabera et al., 2014; Smeriglio et al., 2017). Hydrolyzable tannins usually result from binding to sugar fragments (mainly to the d-glucose moiety) of gallic, meta-digallic, or hexahydroxydiphenic acid residues and can be hydrolyzed by weak acids or weak bases to produce carbohydrate and phenolic acids. Condensed tannins are formed by oxidative condensation of flavonoids (mainly flavan-3,4-diol monomers, less frequently catechins, stilbenes, and dihydrochalcones). They are polymers of 2–50 (or more) units, which are not susceptible to hydrolysis, and some of them are insoluble (Smeriglio et al., 2017). **Figure 15** shows the chemical structure of some tannins. These compounds are reported to be a constituent of legumes, such as beans, fruits, particularly berries, nuts and wine (Bittner et al., 2013; Boudjou et al., 2013; Campos-Vega et al., 2009). Tannins exert several pharmacological effects, including antioxidant and free radical scavenging activity, as well as antimicrobial, anticancer, and cardioprotective properties (Anderson et al., 2012; Gato et al., 2013; Smeriglio et al., 2017).

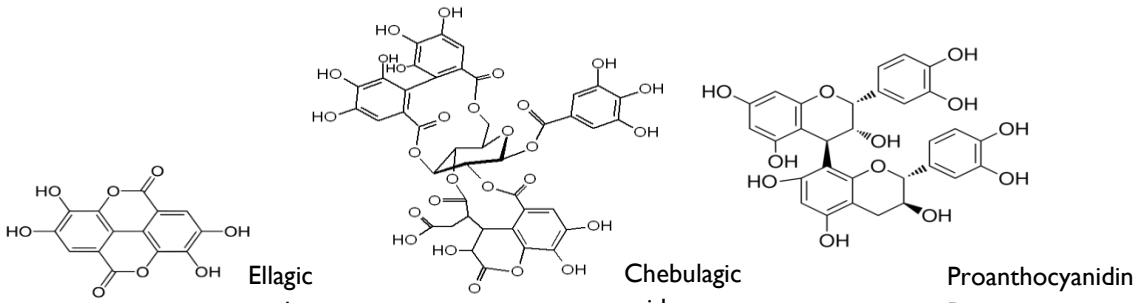


Figure 15. *Chemical structure of some dietary tannins.*

2.3. ONE OF THE MOST CONSUMED FRUITS: APPLES

The high prevalence of CVD requires lifestyle changes and new dietary prevention strategies based on an increment in intake of plant-based foods rich in bioactive compounds, such as (poly)phenolic compounds (WHO, 2013). The attractiveness of fruit or vegetables (main food sources of (poly)phenolic compounds) to consumers is

determined principally by visual attributes like appearance, size, uniformity, colour and freshness, as well as non-visual attributes such as taste, aroma, flavour, firmness (texture), nutritional value and healthiness (Petkovšek et al., 2009). In this context, apples (*Malus domestica*) are one of the most consumed fruits in the world, mainly being consumed fresh, while a lesser part is processed into juices, concentrates, and purées. Based on production quantities, in 2018 global apple production was 86.4 million tons and China was the main producing country. Regarding the consumption of this fruit, India, the United States and China are the main consumers. Apples can be grown under different climatic conditions, ranging from temperate climates like southern Siberia or the Mediterranean area to subtropical climates such as Brazil or South Africa (Shahbandeh, 2019). In Spain, the annual apple production corresponding to the average for the period 2015-2017 was 473.799 tons, which represents 4.1% of the production of the European Union (Iglesias & Carbó, 2018).

The popularity of apples is due to their low cost, beautiful appearance, crispy flesh, pleasant flavour, sweet taste and their good "health image", which proves the well-known ancient Welsh saying "An apple a day keeps the doctor away" (Harker et al., 2003; Wolfe et al., 2003).

This good "health image" is mainly due to their chemical composition that is very complex and consists of numerous organic and inorganic compounds, and macro and micro biogenic elements. The most represented organic compounds are sugars, acids, pectin, tannins, starch, cellulose, vitamins, enzymes, phytohormones, and (poly)phenolic compounds (principally flavonoids such as anthocyanins, dihydrochalcones, flavonols, flavanols and phenolic acids such as chlorogenic acid) (Lee et al., 2003). Meanwhile, the most represented chemical elements are nitrogen, phosphorus, potassium, calcium, sulphur, iron and magnesium.

The (poly)phenolic content in fruits depends on various factors, including physiological condition of the plant, soil, weather conditions, fruit cultivar, fruit ripeness, harvest period, storage conditions and fruit processing (Markuszewski & Kopytowski, 2008).

3. RED-FLESHED APPLES AS A SOURCE OF BIOACTIVE (POLY)-PHENOLIC COMPOUNDS

3.1. AN UNKNOWN BUT PROMISING APPLE VARIETY: BRIEF HISTORY AND GENETICS

The functional food market is expanding rapidly and represents one of the most promising and dynamically developing segments of the food industry (Siro et al., 2008). Among all the existing apple varieties, the preferred and most consumed in Europe are Goldchief, Rubens, Fuji and Pink lady, all characterized by their sweetness, hardness, juiciness, crispness, and all of them are red-peeled apples (Bonany et al., 2014). In addition to these commercial apple varieties, in the last decade new apple varieties with high commercial interest have been emerging.

As previously mentioned, apples are one of the most popular fruits, and much of this apparent value is probably due to the accumulations of anthocyanins in the skin of most cultivated apple varieties that gives them the red coloration and makes them better accepted by consumers (Bonany et al., 2014). Anthocyanin production in apple fruit skin is associated with fruit maturity and ripening, and is stimulated by light (Siegelman & Hendricks, 1958) and cool temperatures (Faragher, 1983). Moreover, numerous genotypes of *Malus* are known that synthesize anthocyanin in additional fruit tissues, including the core and the cortex (flesh) (**Figure 16**). These red-fleshed apple genotypes, together with the genetic variability within apple germplasm, represent a potential to improve the phytochemical content in apple fruit (Tsao et al., 2005; Wojdylo et al., 2008). Additionally, there is public interest in new crops for colouring food naturally (Stintzing & Carle, 2004) without transgenic or cysgenic programmes, because the attitude of consumers toward genetically modified foods is mainly negative (Costa-Font et al., 2008; Wunderlich & Gatto, 2015). All of the above, together with the health properties attributed to anthocyanins, justifies the research into the development, through the use of traditional breeding and biotechnology, of novel varieties of red-fleshed apples for consumption with health-promoting effects, which will generate a new market segment in the hypercompetitive fruit market (Espley et al., 2013).

Figure 16. *Red-fleshed apple.*

The first data about these apples date back to the 19th century when the Russian botanist Vladislav E. Niedzwetzky (1855–1918) began to show interest in *Malus niedzwetzkyana*, which was considered a wild rare colour mutant of *Malus domestica* and was found at very low frequency throughout the Tian Shan mountains (located in the border region between Kazakhstan, Kyrgyzstan and China) (Juniper & Mabberley, 2006). Since the discovery of this wild red-fleshed apple, a significant interest in this striking variety appeared and, in the years 1894 and 1897, it was introduced into the British Isles and the United States, respectively. The pioneering work on improvements to this wild, astringent, and sour variety was carried out by Niels Hansen and Albert Etter, who developed two main strains of red-fleshed apples in parallel. Hansen's type of red-fleshed apples is similar to Niedzwetzkyana in appearance but with improved flavour. On the other hand, Etter's type of red-fleshed apples presents the red pigment only in the fruit flesh and in the new leaves (Volz et al., 2007; Würdig et al., 2014) (**Figure 17**). Since then, work has continued by different apple breeders all over the world who are dedicated to continuing to improve the taste, flesh texture and appearance of these varieties through traditional breeding programmes. The first good-tasting red-fleshed apples have become available over the past decade and these are, for example, the 'Baya Marisa' (Germany), 'Rosette' (England), 'Redlove' (Switzerland), 'Weirouge' cultivars (Germany) and 'Kissabel' (France) (Deacon, 2012).

Figure 17. *Hansen's and Etter's type red-fleshed apple, respectively.*
(Retrieved from The diversity of redfleshed apples by Nigel Deacon, http://www.suttonelms.org.uk/apple104.html Accessed on 29th April 2020).

The characteristic red flesh of this apple is due to anthocyanin production in this part of the apple. At genetic level, the production of these flavonoids may principally be influenced by levels of MdMYB10, the transcription factor that promotes the synthesis of multiple key enzymes involved in their biosynthesis (Espley et al., 2007). This transcription factor may be over-expressed due to mutations (Espley et al., 2007; Espley et al., 2009) (Hansen's type) or due to a duplicated MYB gene (Etter's type) (Chagné et al., 2013; Umemura et al., 2013). Actually, the anthocyanin biosynthesis regulation in apples is continuously improving in order to fully elucidate the genetic mechanisms related to the production of these (poly)phenols (Liu et al., 2019; Wang et al., 2019).

3.2. CONSUMPTION AND ACCEPTANCE WORLDWIDE

Although red-fleshed apples have been consumed in countries like China for many years, in Europe it was not until the last decade that the first good-tasting red-fleshed apples became available, despite the fact that a fair number of red-fleshed cultivars were released in the early 1940s. The coloured flesh of the new apple varieties seemed to be an excessively radical innovation to European consumers and the rejection on believing it to be a genetically modified product meant that, in many countries like Spain, France or Italy, its consumption did not spread and this apple is still unknown to the majority of the population (Cirilli et al., 2014).

Work is still ongoing in crossing red-fleshed wild apples with modern white-fleshed commercial varieties to obtain red-fleshed apples with a good appearance (external fruit colour, shape, and aesthetic appeal, as well as the colour of the flesh) and a better taste (including flavour, texture, and mouth-feel attributes), and also with the over-expression of the transcription factor MYB10 to increase the concentrations of anthocyanins and consequently the potential health enhancement (Bulley et al., 2007; Espley et al., 2009; Espley et al., 2013).

Recent studies of consumer acceptance of these new varieties (Espley et al., 2013; Silvestri et al., 2018) have shown that, although people were not familiar with red-fleshed apples, they expressed a strong degree of interest in the innovative apple

product. The nutritional properties that are important for promoting health are the main motivations among consumers.

In recent years, the consumption of this new apple variety has been promoted and it is expected that in the not too distant future we will find these varieties in our markets (Fernández, 2018; Iglesias & Carbó, 2018).

3.3. Source of bioactives: Anthocyanins and others

Red-fleshed apples are predominantly made up of water (75-85%), carbohydrates (14-18%) and fibre (3-5%). The other main macronutrients and phytochemicals, although present in much lower quantities, are fats, proteins, organic acids, triterpenes and (poly)phenols (**Figure 18**) (Bars-Cortina et al., 2017; Bondonno et al., 2017).

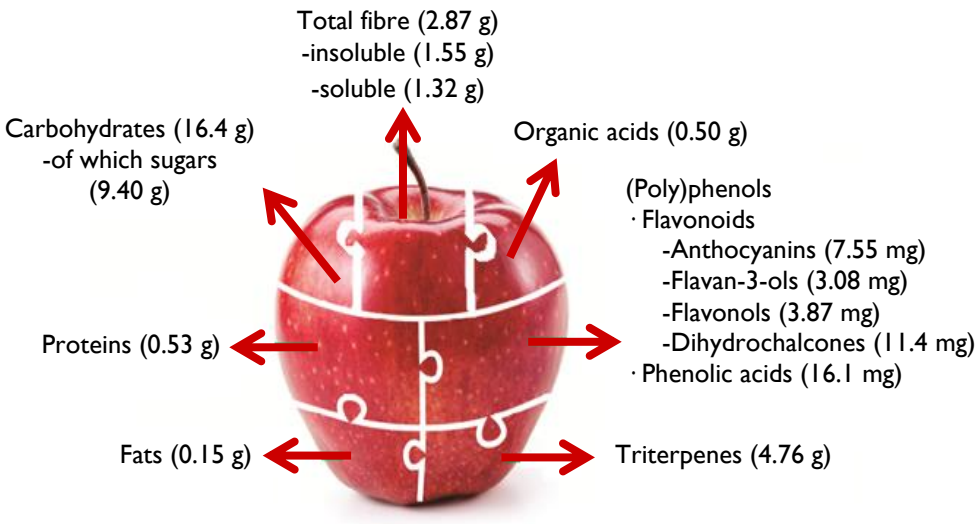


Figure 18. *Macronutrients and major classes of phytochemicals present in red-fleshed apples used in this Doctoral Thesis. This figure represents the average content of a whole, raw apple. Values were obtained from Bars-Cortina et al. (2017) and Bondonno et al. (2017) and are presented per 100 g fresh apple.*

Regarding (poly)phenols (one of the principal classes of bioactive compounds), one should keep in mind that, as with other fruits, the content of these compounds changes between cultivars and is affected by different ripening states of the apple, environmental factors, geographic regions, and also change between harvest period seasons. Therefore, these values will vary considerably from season to season, as well as from one apple variety to another (Bars-Cortina et al., 2018; Kevers et al., 2011; Laaksonen et al., 2017). However, generally speaking, the phenolic acids are the most abundant (poly)phenolic compounds in red-fleshed apples, representing >50% of the total (poly)phenol, chlorogenic acid being the most abundant (Bars-Cortina et al., 2017; Ceymann et al., 2012). Among flavonoids, the main groups present in red-fleshed apples are anthocyanins, dihydrochalcones, flavan-3-ols and flavonols, the first two groups being the most abundant. A wide range of anthocyanins have been identified and quantified in apples (in the flesh as well as in the peel), such as pelargonidin, peonidin, delphinidin, petunidin, and malvidin derivatives, although cyanidin-3-*O*-galactoside is the most abundant (around 95% of the total anthocyanin content). Furthermore, this anthocyanin (cyanidin-3-*O*-galactoside) is rarely found naturally in other fruits (Bars-Cortina et al., 2017; Guo et al., 2016). Dihydrochalcones are practically exclusive to apple fruit and it has been proven that the concentration of dihydrochalcones in red-fleshed apples is higher than in conventional cultivars (white-fleshed apples). Phloretin glucoside, phloretin xylosyl-glucoside jointly with hydroxyphloretin xylosil glucoside are the prevalent dihydrochalcones identified in red-fleshed apples (Bars-Cortina et al., 2017; Bars-Cortina et al., 2018). The main apple flavonols are quercetin derivatives, such as quercetin arabinoside, quercetin rhamnoside, and quercetin glucoside, and it has been proven that the concentrations of these polyphenolic compounds in the flesh are lower than in the peel. Moreover, no differences in the concentration of these polyphenolic compounds were observed between the red- and white-fleshed cultivars (Bars-Cortina et al., 2017; Feliciano et al., 2010; Wang et al., 2015).

With respect to the last main group of flavonoids, the flavan-3-ols, epicatechin and its polymerized forms (dimeric, trimeric, and tetrameric proanthocyanidins) are the main representatives. A lower concentration of these compounds has been quantified in red-fleshed apples (which contain high amounts of anthocyanins) in comparison with white-fleshed apples. This could be related to a competitive synthesis between

anthocyanins and flavan-3-ols because these polyphenolic subclasses are produced by closely related branches of the flavonoid pathway and utilize the same metabolic intermediates (Henry-Kirk et al., 2012; Kalinowska et al., 2014).

The last groups of flavonoids present at much lower concentration levels in this apple variety are flavones and flavanones (Alarcón-Flores et al., 2015). Luteolin glucoside and eriodictyol glucoside are the main flavones and flavanones, respectively, determined in red-fleshed apple (Bars-Cortina et al., 2017; Bars-Cortina et al., 2018).

Altogether, the main differences in (poly)phenolic content between these new varieties of red-fleshed apple *versus* the traditional white-fleshed apple are related to their higher content of dihydrochalcones and anthocyanins, whereas the white-fleshed apples are richer in flavan-3-ols (Bars-Cortina et al., 2017; Bars-Cortina et al., 2018; Wang et al., 2015).

Apart from (poly)phenolic compounds, red-fleshed apples also contain other phytochemicals such as triterpenes and organic acids, responsible for the acidity of the apples, which influences the perception of acidity and sweetness and, therefore, acceptance by the consumer (Bonany et al., 2013; Etienne et al., 2013). The principal terpenoid in red-fleshed apple is ursolic acid (the most abundant triterpene), together with other minor triterpenes such as hydroxyursolic acid, euscaphic acid, maslinic acid, and betulinic acid, while the main organic acids are malic acid, citric acid and quinic acid (Bars-Cortina et al., 2017; Bars-Cortina et al., 2018).

3.4. Development of new products: Apple snack

Since the production of apples is seasonal and, hence, apples may not be available in fresh conditions throughout the year, it is necessary to use alternative processing methods to obtain shelf-stability, increase their useful life while minimizing changes in apple quality attributes. In this respect, the development of new snacks represents an easy and promising way to promote apple consumption. In the industry, a great part of the fruits is processed as snacks by frying in oil. This type of thermal processing produces the degradation of antioxidants and (poly)phenolic compounds, directly affecting the nutritional quality of apple snacks (Vega-Gálvez et al., 2012). Further, due to the health concerns associated with consumption of high fat snack

foods, nutritionists emphasize the need to explore non-frying processes for the development of low- or no-fat snack foods (Hayman, 2020; Kavey et al., 2003; WHO, 2013). Thus, drying is an alternative process to produce healthy snacks from fruits such as apples, with retention of nutrients and bioactive compounds, and is currently a frequent practice, since dried apples are part of numerous prepared foods including snack preparations, whole-grain breakfast foods, as well as others (Akpinar et al., 2003).

Drying is one of the main and oldest techniques for preserving agricultural and food products and increases the shelf life. Dried food products also have the advantage of being easy to store and distribute, available throughout the year, and a healthier alternative to salty or sugary snacks. In addition, the reduction of moisture in the product greatly retards microbial and chemical deterioration and brings about a substantial volume reduction (Alasalvar & Shahidi, 2013; Doymaz & Pala, 2003). However, fruits are sensitive to drying conditions (temperature, air velocity and relative humidity), which can cause quality deterioration of products through oxidation, shrinkage, colour change, or loss of texture and nutritional-functional properties (Vega-Gálvez et al., 2009).

The drying process is complex, involving simultaneous heat and mass transfer requiring precise process control. Drying a fruit implies evaporation of both free and loosely bound water from inside the fruit into the atmosphere. The latent heat of vaporization may be supplied by convection, conduction and radiation or volumetrically *in situ* by placing the wet material in microwave or radiofrequency electromagnetic fields (Mujumdar, 2007). Some of the drying methods that can be used include drying by forced air, convection oven, microwaving, freeze-drying, fluidized-bed drying, osmotic dehydration, infrared-drying, and vacuum drying (Karam et al., 2016).

In most methods for dehydration of fruits, they are subjected to high temperatures. In the case of hot air-drying, the fruits are subjected to the action of a hot air stream (Kamiloglu et al., 2016) and, in the case of infrared-drying, fruits are subjected to radiation that is converted into heat, allowing the liquid to evaporate (Praveen Kumar et al., 2005). In the case of freeze-drying, the water is removed by freezing the product and subsequent sublimation of the ice under controlled vacuum and temperature conditions (Karam et al., 2016).

The main problems with these dehydration techniques are that thermal processing (such as hot air-drying or infrared-drying) often induces the degradation of (poly)phenolic compounds, because these compounds can be highly thermosensitive and unstable, while the main problem of freeze-drying is its excessive cost (Karam et al., 2016). All of this means that companies often use other formats to preserve and increase the shelf-life of these fruits, such as purée form (Guerra et al., 2012; Picouet et al., 2009). In typical industrial processes for apple purée production, raw apples are first diced and cooked at a temperature between 93 and 98°C for 4 to 5 min, then pulped and pasteurized at 90°C during 20 min to give, at 30°C, a shelf life of 6 months (Oszmiaski et al., 2008).

It has also been shown that food processing can induce modifications that may lead to an increased extractability of (poly)phenolic compounds during digestion, enhancing their bioaccessibility and bioavailability (Yang et al., 2011). Therefore, these parameters must be evaluated and studied to produce a "ready to eat healthy snack" from fresh red-fleshed apple that retains its nutrients and bioactive compounds and makes it a healthier alternative to salty or sugary snacks.

3.5. BIOAVAILABILITY OF (POLY)PHENOLIC COMPOUNDS AND FOOD MATRIX EFFECT

The biological effects of (poly)phenolic compounds are ultimately determined by their bioavailability and their temporal and spatial distribution in the body. According to the U. S. Food and Drug Administration (FDA), the definition of bioavailability is "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action" (Food, 2014). To know the bioavailability, it is necessary to know the human metabolism of the compound(s) of interest. In the case of (poly)phenolic compounds, after ingestion (poly)phenol glycosides can be partially hydrolyzed in the oral cavity by saliva. Within the gastrointestinal tract, their absorption is associated with the hydrolyzing activity of a cascade of enzymes. First, in the small intestine the sugar unit of the glycosides is cleaved by the action of lactase phloridzin hydrolase (LPH), located in the brush border of epithelial cells, and the released aglycone may then enter the epithelial cells

by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane (Day et al., 2000). An alternative hydrolytic step is mediated by a cytosolic β-glucosidase (CBG). In this case, the active sodium-dependent glucose transporter, SGLT-1, is thought to be involved in the transport of flavonoid glycosides into epithelial cells, a necessary step to facilitate the action of CBG (Gee et al., 2000). Once absorbed, flavonoids and related phenolics follow the common metabolic pathway of exogenous organic substances and, like drugs and most xenobiotics, undergo phase II enzymatic metabolism. They can be conjugated with glucuronic acid, sulphate and methyl groups, in reactions catalyzed by UDP-glucuronosyltransferases (UGTs), sulphotransferases (SULTs) and catechol-*O*-methyltransferases (COMT), respectively (Del Rio et al., 2013). Phase II metabolism first occurs in the wall of the small intestine, after which metabolites pass through the portal vein to the liver, where they may undergo further conversions before entering the systemic circulation and eventually undergoing renal excretion. It is possible that some phase II metabolites recycle from the liver back to the small intestine through bile excretion and this is thought to involve members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters. This means that (poly)phenols and their metabolites are present for a longer time in the body, being able to exercise their protective functions (Donovan et al., 2006).

Substantial amounts of metabolites and the non-absorbed parent compounds travel down the gastrointestinal tract, reaching the large intestine where they are exposed to the resident colonic microflora (Borges et al., 2013; Erk et al., 2014). Gut bacteria can hydrolyze glycosides, glucuronides, sulphates, amides, esters and lactones, and the flavonoid skeleton undergoes ring fission, the products of which can then be subjected to reduction, decarboxylation, demethylation and dehydroxylation reactions (Selma et al., 2009). These complex modifications generate low molecular weight catabolites (including phenolic acids and hydroxycinnamates) that can be efficiently absorbed *in situ*, with some undergoing further phase II metabolism locally and/or in the liver before entering the circulation and being excreted in urine in substantial amounts that typically exceed the excretion of (poly)phenol metabolites absorbed in the upper gastrointestinal tract (Crozier et al., 2010). **Figure 19** shows this process schematically.

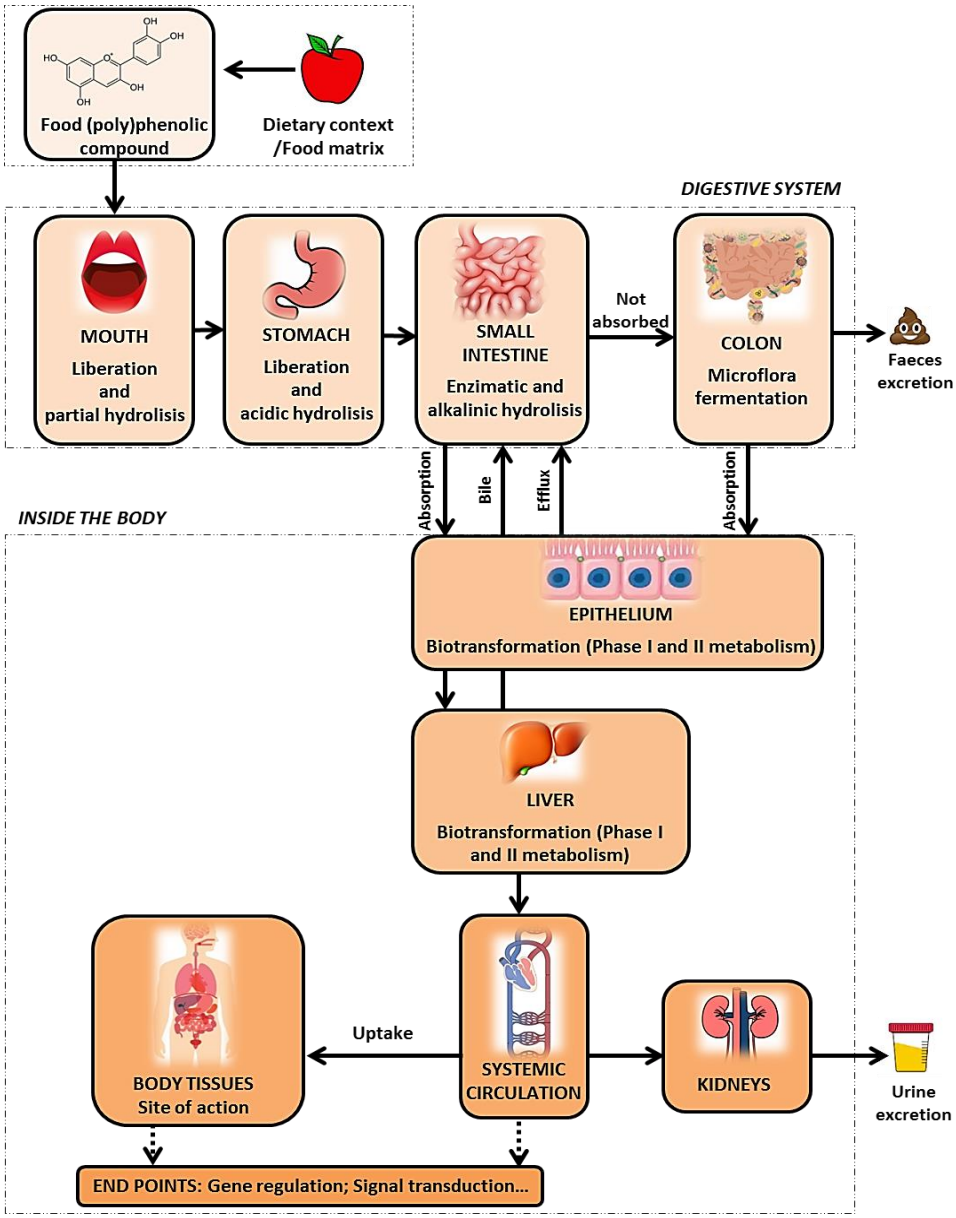


Figure 19. *Journey of (poly)phenolic compounds from food through the human body (Modified from Motilva et al., 2015).*

It is a known fact that the bioavailability of flavonoids is generally low and can vary drastically among different flavonoid classes, as well as individual compounds in a particular class. For example, Landete (2012) showed that relative urinary excretion of anthocyanins and daidzin intake was 0.3% and 43%, respectively. This bioavailability difference of (poly)phenols is due to several factors, the first of which is that most (poly)phenols are very unstable and their stability can be affected by factors such as temperature, concentration, light, solvents, presence of oxygen, enzymes, proteins, and metallic ions and, in the case of anthocyanins, the pH (Fang, 2014). The biochemistry of

anthocyanins is more complex than that of other flavonoid compounds and, in aqueous solutions at different pH, anthocyanins exist as 4 different molecular forms that are in dynamic equilibrium (**Figure 20**). This unique anthocyanin chemistry favours their absorption in their native glycosidic form, when compared with other flavonoids (Wang et al., 2012).

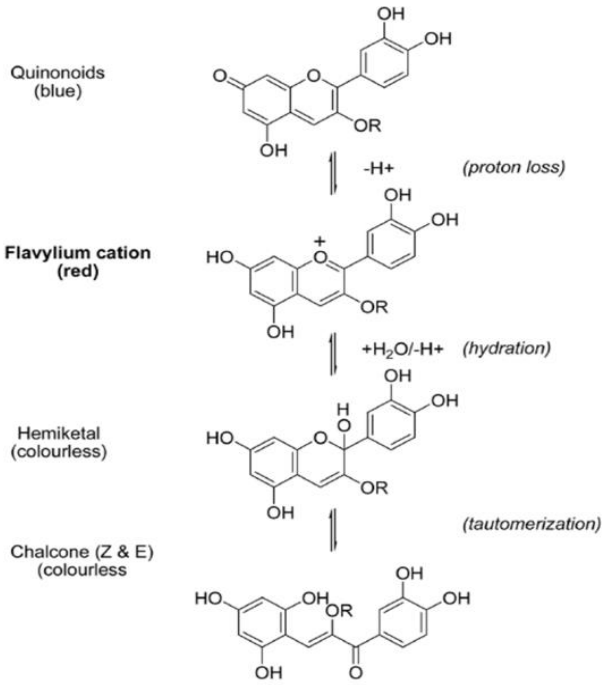


Figure 20. *Anthocyanin molecular structures that are known to be generated under different pH conditions (McGhie & Walton, 2007).*

Moreover, other factors such as molecular weight, molecular glycosylation or esterification, colonic microflora and food matrix, affect the bioavailability of ingested dietary flavonoids. Bioavailability is usually lower for flavonoids with complex structures and higher molecular weights (Landete, 2012; Scalbert et al., 2002). The presence of a sugar moiety had a great influence on flavonoid absorption. A study by Hollman et al. (1999) showed that quercetin glucosides were absorbed 10 times faster and their plasma concentration peaked 20 times higher than quercetin rutinosides in humans. Regarding metabolic conversion, this is an important factor affecting flavonoid bioavailability. Quercetin aglycone or its glycosides are found in lower concentrations in plasma compared to its glucuronic acid, sulfate or methyl conjugates (Ishizawa et al., 2011). The interaction of the flavonoid compounds with colonic microflora was reported to influence their bioavailability because the microbiota catabolizes

compounds to low molecular weight compounds that are readily absorbed (Moco et al., 2012). Finally, the food matrix composition where the flavonoids are found may also affect their bioavailability (Yang et al., 2011).

The co-ingestion of (poly)phenolic compounds with different foods, the structure of the (poly)phenolic compound, the degree and type of food processing, the interactions of (poly)phenolic compounds with each other and with other antioxidants have been shown to affect their content in food and also their bioavailability.

Respecting the structure of the (poly)phenolic compound, for example in anthocyanins, the presence or lack of glycosylation and hydroxylation of the basic flavylium structure, and the number, type, and acylation of bounded sugar molecules are linked to plant species and imply characteristic anthocyanin patterns. It has been shown that acylation increases anthocyanin stability and substantially reduces bioavailability (Charron et al., 2009; Novotny et al., 2012) and that anthocyanidin structure is determinant for bioavailability, with pelargonidin-based anthocyanins (3′-hydroxyanthocyanins) being more readily absorbed than anthocyanins with more substituents on the B-ring (Felgines et al., 2007).

The interaction between (poly)phenolic compounds and the other compounds co-ingested during a meal can also modify their bioavailability. For example, the ability of these compounds to interact with proteins that can modify or change their biological function and limit and/or interfere with both protein and (poly)phenolic absorption is well-known. Moreover, because the cell does not have specific mechanisms for (poly)phenolics entry, they use the cell machinery for other substances, interfering with the absorption of these molecules (such as organic cations or glucose, among others) (Faria et al., 2006; Keating et al., 2008). Besides, the interaction between (poly)phenolic compounds and antioxidants, such as in vitamin C, vitamin E, β-carotene and ascorbic acid, have been reported, and in many cases it has been suggested that the presence of these antioxidants that may prevent oxidation of (poly)phenols might enhance their bioavailability (Green et al., 2007). Further, (poly)phenolic compounds may interact among them synergistically or antagonistically. For example, a synergistic interaction was reported by Rossetto et al. (2002), who observed that malvidin-3-*O*-glucoside with high antioxidant potential can be regenerated by catechin, which was relatively ineffective in inhibiting linoleic acid oxidation in micelles. Another study by

Brand et al. (2010) demonstrated that the transport of the flavanone hesperetin toward the basolateral side of Caco-2 cells increased in the presence of other flavonoids, such as isoflavones, flavones, and flavonols. One antagonistic interaction was reported by Walton et al. (2006) who observed that quercetin-3-*O*-glucoside inhibited cyanidin-3-*O*-glucoside absorption in the mouse intestine.

Regarding the degree and type of food processing, including home food preparation practices, in general thermal processing results in a decrease in (poly)phenolic content but also in an increase in their bioaccessibility and bioavailability. Thermal treatments can produce degradation or modification of cell wall polysaccharides, molecular interactions and proteins, facilitating the release of the cytoplasmic content (including (poly)phenols) and making them more accessible for absorption (Celli & Brooks, 2017). For example, cooking cherry tomato tilted the balance towards an increased bioavailability of naringenin and caffeoylquinic acid (Bugianesi et al., 2004).

Finally, apart from all the above, the different bioavailability of (poly)phenols is also due to the host-related factors that result in different absorption between individuals. This individual physiological variability can be ascribed to intrinsic aspects such as genetics, age, sex, physiological or pathological states and the person's gut microbiota, all resulting in large differences in the expression of a functional enzyme. This means that ingested dietary (poly)phenolic compounds may be efficacious for one individual, but may not trigger the same effect for another (D'Archivio et al., 2010).

3.6. Dried blood spot cards: A novel tool for detection and quantification of anthocyanins and (poly)phenolic metabolites

Before investigating potential mechanisms of action, human postprandial studies in a small group of humans are necessary to test a food or a functional ingredient. These studies should be performed because metabolites in blood and/or in plasma samples in postprandial state need to be identified first and their absorption, distribution, metabolism, and excretion (ADME) established. These pharmacokinetic parameters in biological samples are modulated by the food matrix composition, because bioavailability results are not the same when the active compounds are tested as single

molecules or are tested in food (Donovan et al., 2006; Lund, 2003; Motilva et al., 2015).

Moreover, the determination of appropriate food intake biomarkers, including their pharmacokinetic profiles after ingestion, is essential to make accurate estimates of bioactive compounds intake and further relate them to the observed biological effects. This determination is also important to assess the optimal dose so as to maximize the potential health benefits of bioactive compounds. Data obtained from postprandial studies, considering the form in which the (poly)phenol is ingested, can contribute to knowledge of the factors affecting (poly)phenol bioavailability and can be of benefit for future design of functional foods and beverages (Patel et al., 2010).

Regarding the collection of biological samples, urine sampling is particularly useful for studies on bioavailability of (poly)phenols with short half-lives. The quantitative analysis of metabolite excretion in urine provides a measure of the total output of (poly)phenol metabolites over a 24 h period. It also provides a better index of intake, because 24 h-urine allows the total concentrations of both small- and large-intestinal metabolites excreted after (poly)phenol or food intake to be monitored (Motilva et al., 2015).

Other biological samples used extensively for pharmacokinetic analyses are blood or serum/plasma obtained after blood centrifugation. Many studies have analyzed plasma (poly)phenol levels 1-6 h postintake. Such sampling provides good data regarding the bioavailability of bioactive compounds and their metabolites of small-intestinal origin, but does not provide information regarding metabolic products formed in the large intestine. In this case, multiple blood sampling over a 24 h period should be considered (Motilva et al., 2015; Zamora-Ros et al., 2012). Nevertheless, in these longer postprandial studies, the collection of blood samples has serious limitations because, in general, it is necessary to insert a cannula into the vein for multiple blood sampling over a long time range, resulting in intense discomfort for the volunteers. Additionally, it requires staff qualified in blood extraction and special infrastructure where the volunteers must spend a long time. A strategy to overcome these limitations and simplify the blood sampling and sample pre-treatment procedure is the use of dried blood spot (DBS) cards. DBS are a minimally invasive method for the collection of small quantities of whole blood from finger or heel stick with application to specially prepared filter paper for drying (Hannon & Therrell, 2014). The DBS technique was

first introduced to diagnose phenylketonuria in newborns by measuring whole-blood phenylalanine concentration (Guthrie & Susi, 1963), and has since been applied to multiple applications such as preclinical drug development for lead validation, toxicokinetic and pharmacokinetic studies, clinical pharmacology, targeted and non-targeted metabolic profiling, therapeutic drug monitoring, forensic toxicology, doping or environmental contaminant control, microbiological and epidemiological disease surveillance, among others (Demirev et al., 2013).

Besides, DBS samples are compatible with a large number of bioanalytical methods, among them chromatography or immunoassays (Tanna & Lawson, 2011). More recently, the use of DBS cards together with liquid chromatography coupled with mass spectrometry (LC-MS) has gained significant interest as a potentially powerful tool for the analysis of small molecules in different areas, such as therapeutic drug monitoring, toxicology and pharmaceuticals (Hoeller et al., 2016; Li & Tse, 2010).

There are many advantages of using the DBS system over using fresh blood or plasma samples. These include the minimal volume of blood required (30-100 μL), ease of sample transport and storage. Further, adsorption and the solid nature of DBS cards typically make analytes less reactive than in (liquid) blood. In addition, the analytes often exhibit excellent stability in ambient conditions at least for several days once blood is dried, with only few precautions needed (samples packed in sealed bags with desiccant) (Chen & Hsieh, 2005; Li et al., 2011). This technique allows the storage of blood samples on the card and recovery of the target compounds following a series of preparation steps such as punching discs from cards, elution and extraction with a solvent prior to their chromatographic analysis. This is of great interest in postprandial nutritional studies, since DBS cards allow the easy collection of blood by finger prick (thus eliminating the need for venipuncture) anywhere and at any time to identify metabolites without causing inconvenience to the participants and thus obtain a complete picture of the metabolism of the (poly)phenols of interest (Demirev et al., 2013).

For these reasons, the use of DBS cards for blood collection is of great interest in postprandial studies in nutrition research, and more specifically for the evaluation of the pharmacokinetic parameters of food bioactive compounds, such as dietary (poly)phenols, in which their use has been poorly studied.

3.7. DIETARY ANTHOCYANINS AND CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVD) represent a major public health problem in the modern world, affecting millions of people worldwide. The major risk factors that can influence their development are genetic predispositions, age, smoking, high cholesterol levels, high blood pressure, physical inactivity, unhealthy diet and diabetes (WHO, 2013). These factors are related to increased oxidative stress in the body (with high production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)), which are the cause of the pathogenesis of cardiovascular diseases, increasing the damage of macromolecules such as DNA, proteins and cell lipids (Karbiner et al., 2013; Zafrilla et al., 2013).

In the cardiovascular system, the cellular damage is caused mainly by the action of ROS and RNS on lipid peroxidation of polyunsaturated fatty acids (PUFA), phospholipids, low-density lipoproteins (LDL), high density lipoproteins (HDL) and very low-density lipoproteins (VLDL) that leads to the activation of pro-inflammatory mediators which have a direct consequence in the development of atherogenesis (Tsimikas, 2006).

The protective mechanisms of the anthocyanins in the development of CVD can be direct or indirect. They can act directly by scavenging free radicals and thus reducing oxidative stress and/or they can indirectly modulate gene expression, cell signalling, and microRNA (miRNA) expression related to the development of this pathology.

In a direct way, it has been shown that anthocyanins capture free radicals and/or anions (Byrne et al., 2003), inhibit xanthine oxidase that generates reactive oxygen species, such as superoxide radicals and hydrogen peroxide (Borges et al., 2002), can chelate metal ions, like iron or copper, which contribute to the initiation and propagation of lipid peroxidation (Paixão et al., 2012), inhibit arachidonic acid, prostaglandines and leukotrienes that are biomarkers of inflammation and that lead to the development of CVD (Serhan, 2005). In addition, anthocyanins can inhibit the action of some vasodilators like prostacyclin and nitric oxide (NO) (Serraino et al., 2003).

This strong antioxidant activity is, first, the result of electron deficit in their chemical structure, and, second, due to the conjugated double-bond structure of anthocyanins,

allowing electronic delocalization which leads to more stable radical products (Byrne et al., 2003, Seeram et al., 2003).

In an indirect way, diet anthocyanins and their microbial metabolites can modulate the expression of genes coding for both anti- and pro-oxidant enzymes (such as heme oxygenase 1, NADPH quinone oxidoreductase 1, glutathione reductase, thioredoxin reductase 1, and superoxide dismutases 1 and 2, among others) or different genes implicated in processes such as inflammation, monocyte adhesion, and transendothelial migration (such as TNFα, interleukins vascular-cell-adhesion molecule 1 (VCAM-1), intercellular-adhesion molecule 1 (ICAM-1), and E-selectin, among others). This modulation carried out by anthocyanins and their metabolites is achieved by their direct binding to cell-signalling proteins, which induce changes in protein function and activation of downstream signalling proteins and transcription factors (Cimino et al., 2013; Krga et al., 2018; Sorrenti et al., 2007; Wu et al., 2010). Anthocyanin metabolites could affect cell-signalling pathways such as the NF-kB and MAPK pathways, which play essential roles in the initiation and regulation of inflammatory processes, proliferation and apoptosis (**Figure 21**) (Speciale et al., 2010; Yi et al., 2012). Moreover, the modulation of miRNA expression has recently emerged as a possible mechanism by which (poly)phenols (and anthocyanins), may exert their beneficial health effects. These endogenous, noncoding, single-stranded RNAs of around 22 nucleotides regulate gene expression on post-transcriptional level depending on the degree of complementarity with their targets. (Poly)phenols have been reported to modulate more than 100 miRNAs involved in the regulation of different cellular processes like inflammation and apoptosis (Milenkovic et al., 2012; Milenkovic et al., 2013) and specifically have shown that physiologically relevant concentrations of anthocyanins and their microbial metabolites can modulate miRNA expression in TNFα-activated endothelial cells in atherosclerosis development (Krga et al., 2018).

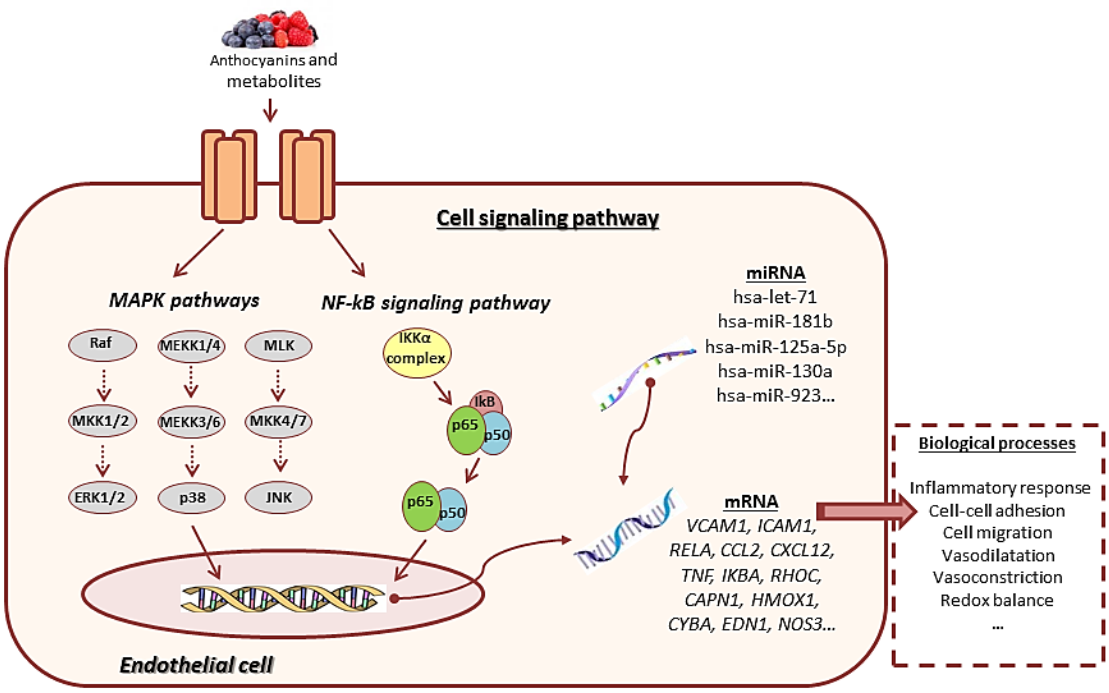


Figure 21. *Cell signalling pathway of anthocyanins in endothelial cells.*

Another remarkable fact is that the biological activity is different between the metabolites that they originate and the anthocyanins from which they come. There are studies that prove that the derivatives or metabolites may be more effective than the original anthocyanins (Sogo et al., 2015; Warner et al., 2017). However, other studies have showed that the metabolites are not always more effective than intact anthocyanins (Fernandes et al., 2013).

There is little information about the effects of metabolites from anthocyanins and metabolites originated from (poly)phenols in general and the underlying mechanism. More *in vitro* studies, with physiologically relevant designs and integrated holistic approaches investigating the effects of parent compounds and their metabolites together on gene, protein, and miRNA expression, are needed to completely understand the role of these compounds in the prevention of diseases like CVD.

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HYPOTHESIS AND OBJECTIVES

The high prevalence of CVD around the world requires lifestyle changes and new dietary prevention strategies based on an increased intake of plant-based foods rich in bioactive compounds that are considered as key mediators in the improvement of CVD factors. However, the diet of the majority of people does not appear to be sufficient to guarantee an adequate intake of fruits and vegetables and, consequently, the habitual intake in Europe of flavonoids such as anthocyanins (that has been inversely associated with CVD risk), is below the amounts found to have significant health effects. Moreover, buying fresh fruits and vegetables, for example, has become a luxury choice for families all over the world. In this context, research has turned its attention to improving the quality of foods, focusing on phytochemicals that have the ability to modulate physiological and pathophysiological molecular mechanisms, resulting in favourable health outcomes. In particular, enhancing traditional foods with healthy compounds, such as flavonoids, has appeared as a new solution to increase food availability of bioactive compounds to more people, thus helping to guarantee an adequate daily ingestion.

In this context, in recent years the interest in red-fleshed apples has grown. This is a new hybrid apple variety with red pulp produced by traditional breeding methods with an enhanced content of anthocyanins and probably other (poly)phenolic compounds that have not yet been explored. The hypothesis is that, by developing a red-fleshed snack that is ready to consume, some of the nutritional needs that are currently demanded could be met, also improving cardiometabolic biomarkers.

The present Doctoral Thesis is framed in the context of the AppleCOR project: *"Red-fleshed Apple as a novel Anthocyanin biofortified food to improve Cardiometabolic risk factors: Innovation in crop and application to health"*. The global aim of the project was to advance in the knowledge of the potential health effects and mechanism of action of anthocyanins in the improvement of the cardiometabolic risk (CMR) factors focused on the reduction of LDLc.

Prior to the human intervention study to evaluate the effect of diet supplementation of anthocyanins through red-fleshed apple on the reduction of several cardiometabolic biomarkers, several studies were required, which have been carried out in the framework of this Doctoral Thesis. The results are framed in Food Science and Technology, Chemistry, Clinical, Human Pharmacology and Biochemical expertise

(**Figure 22**). The added value of this Doctoral Thesis is its translational nature, which allows the transfer from Food Science and Technology innovations to possible health benefits and health claims for food developed products.

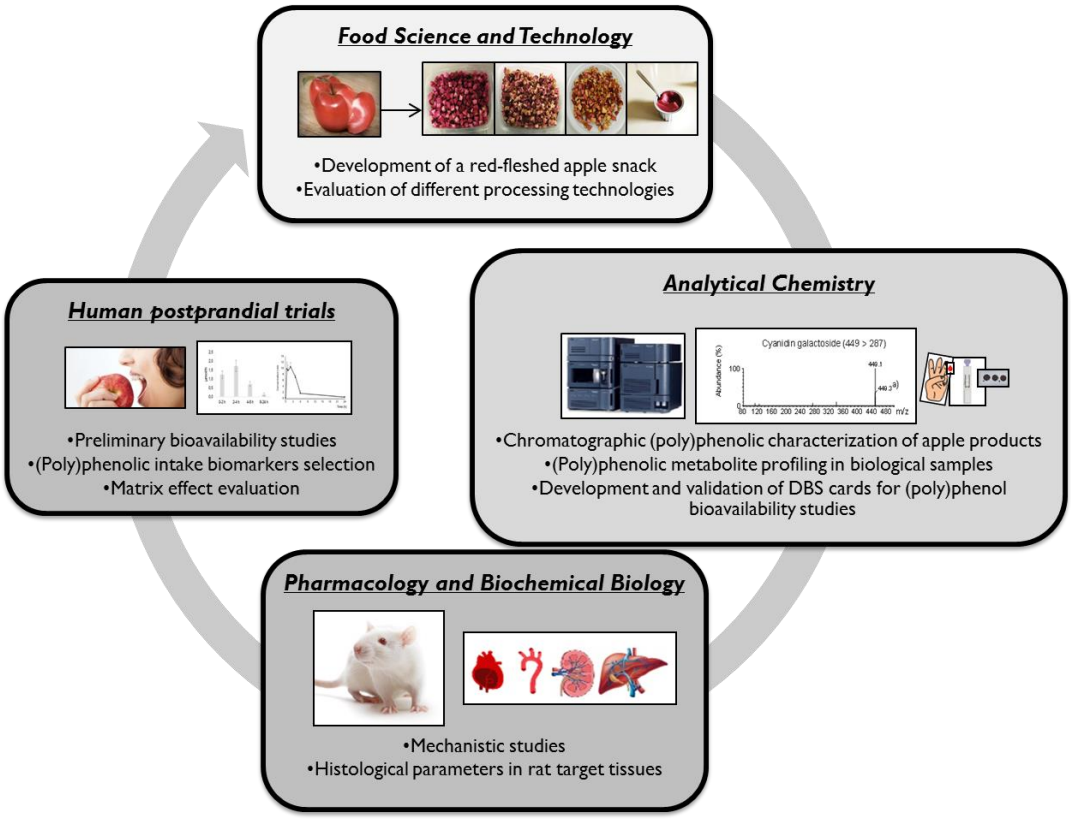


Figure 22. *Multidisciplinary approach of the present Doctoral Thesis.*

In this context, the global aim of the present Doctoral Thesis was to carry out the development of an easy-to-eat red-fleshed apple snack with a high content of (poly)phenolic compounds (mainly anthocyanins), and to assess the bioavailability and cardiometabolic effects of these bioactives as a previous step to human intervention studies.

To achieve this aim, three blocks with their respective objectives were defined:

BLOCK I: RED-FLESHED APPLE SNACK PRODUCT DEVELOPMENT.

- Objective 1. Evaluate the impact of different thermal (infrared-drying, hot air-drying and purée pasteurization) and non-thermal (freeze-drying) processing technologies on the (poly)phenolic content for the development of a red-fleshed apple snack product.

BLOCK II: IN VIVO ASSESSMENT OF THE BIOAVAILABILITY OF ANTHOCYANINS AND OTHER (POLY)PHENOLIC COMPOUNDS FROM RED-FLESHED APPLE AND THE EFFECT OF PROCESSING.

- Objective 2. Investigate the effect of different processing technologies (freeze-drying, hot air-drying and purée pasteurization) on the (poly)phenol bioavailability of red-fleshed apple in a human crossover pilot study.

- Objective 3. Develop and validate a method for detection and quantification of anthocyanins and (poly)phenolic metabolites in blood samples using dried blood spot cards (DBS), as an alternative blood sampling strategy, combined with ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) after an acute intake of red-fleshed apple snack in humans.

    3.1. Validate and apply a method to detect and quantify anthocyanins and (poly)phenolic metabolites in human blood samples using DBS cards.

    3.2. Assess the differences in metabolite profile and concentrations between venous and capillary blood sampling and between plasma and whole blood.

    3.3. Compare DBS cards with microElution solid phase extraction (μSPE) as the most common method for the analysis of circulating (poly)phenolic metabolites in plasma.

- Objective 4. Investigate the bioavailability and the metabolic pathways of anthocyanins and other (poly)phenols after an acute intake of red-fleshed apple snack in humans.

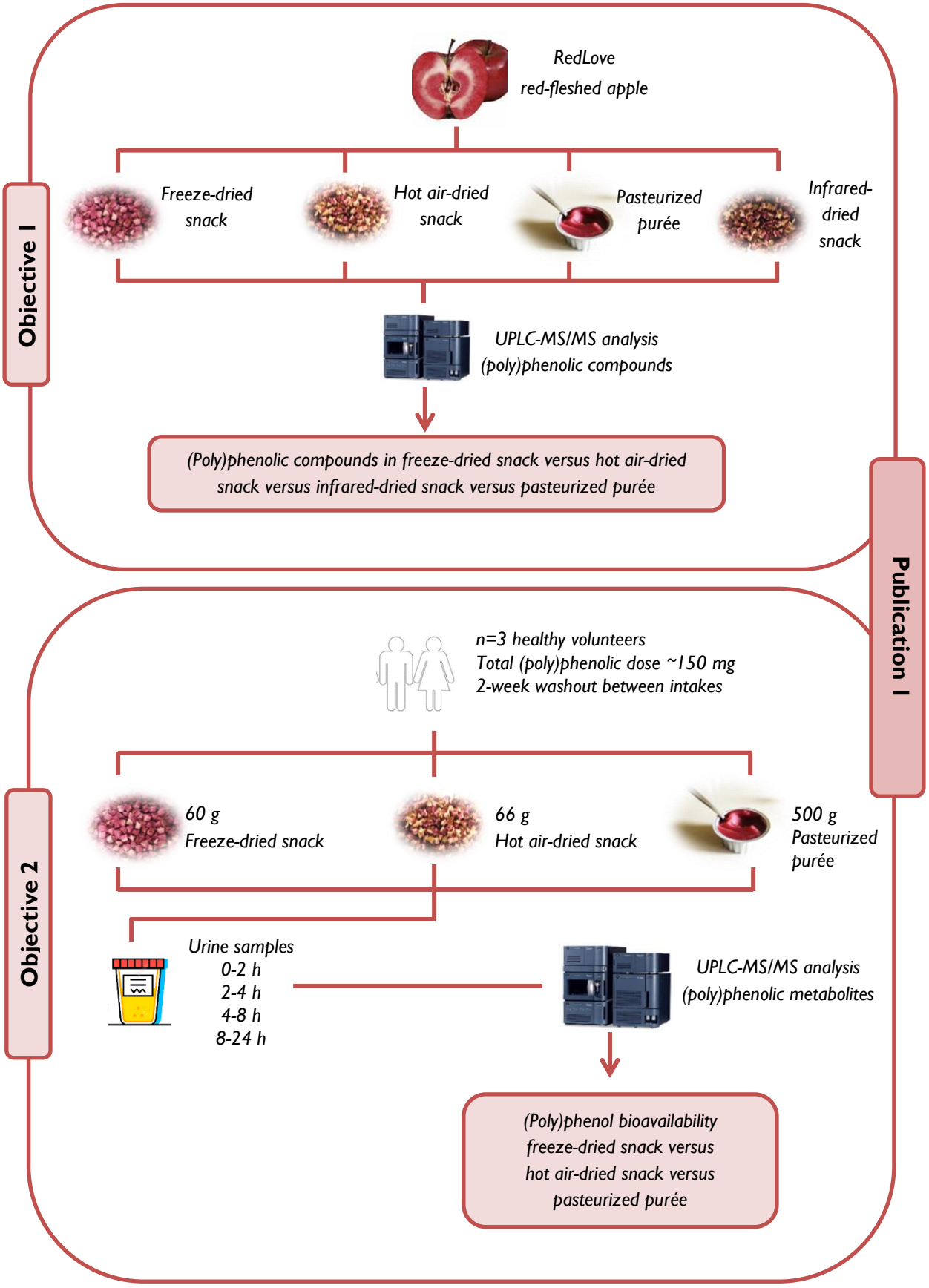
    4.1. Elucidate the *in vivo* biotransformation and the metabolic pathways of anthocyanins and other (poly)phenols from the red-fleshed apple.

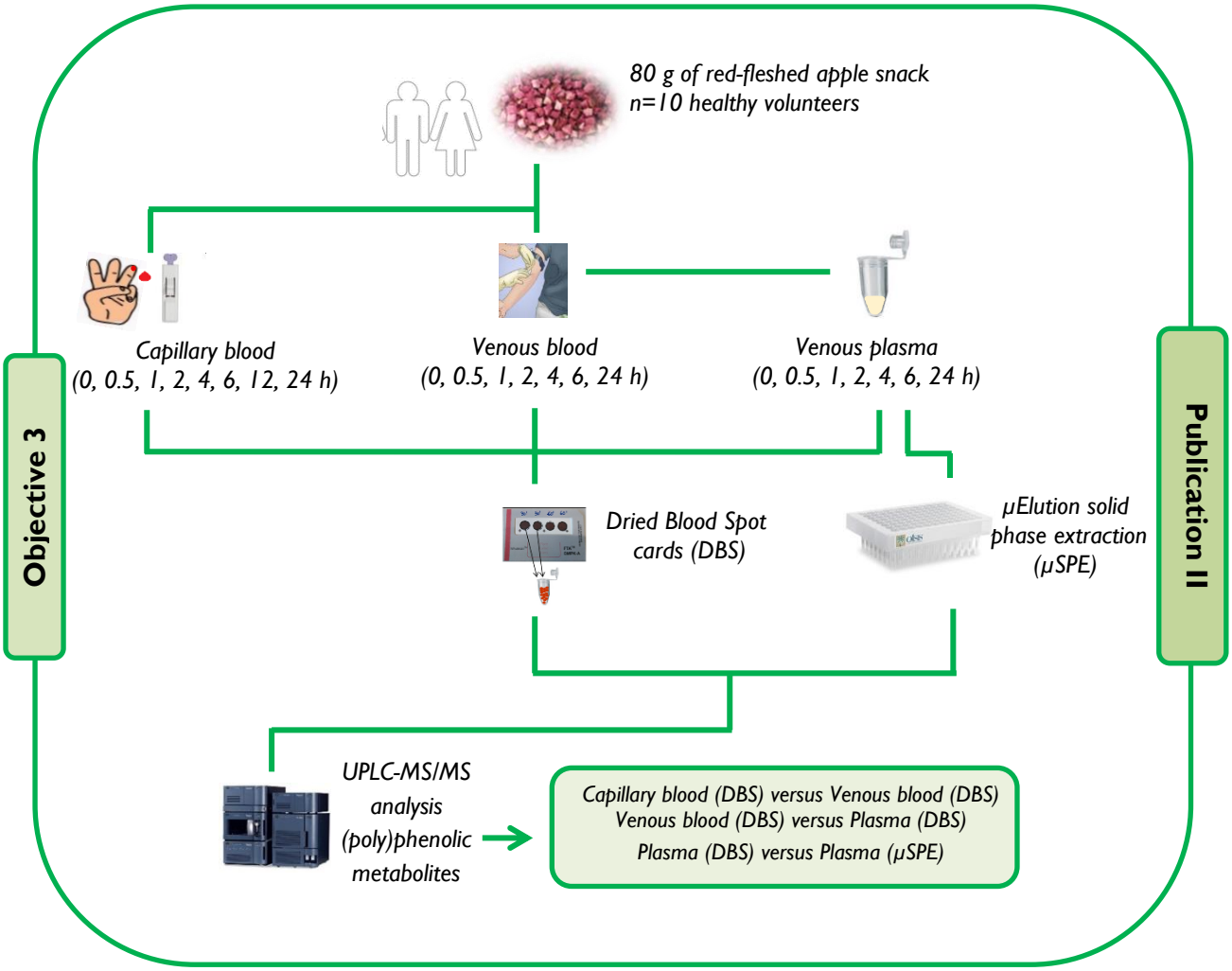
    4.2. Identify and select the potential (poly)phenolic intake biomarkers of red-fleshed apple in plasma and urine.

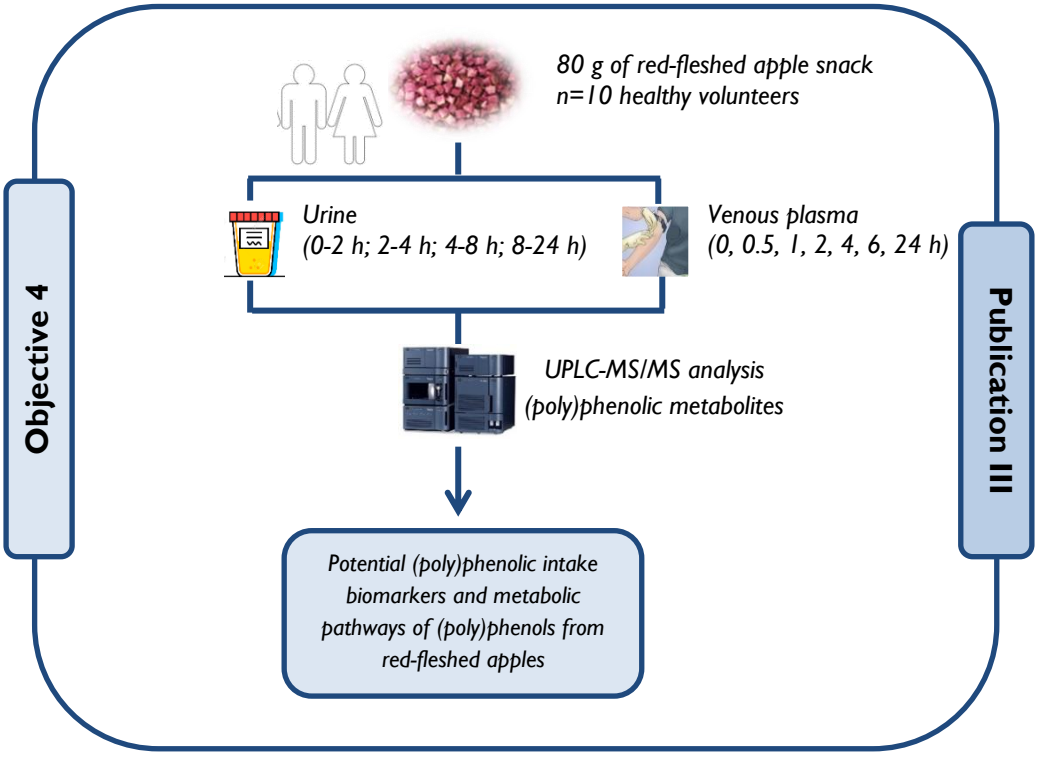
BLOCK III: ANIMAL STUDY: CARDIOMETABOLIC EFFECTS AND STUDY OF THE FOOD MATRIX EFFECT.

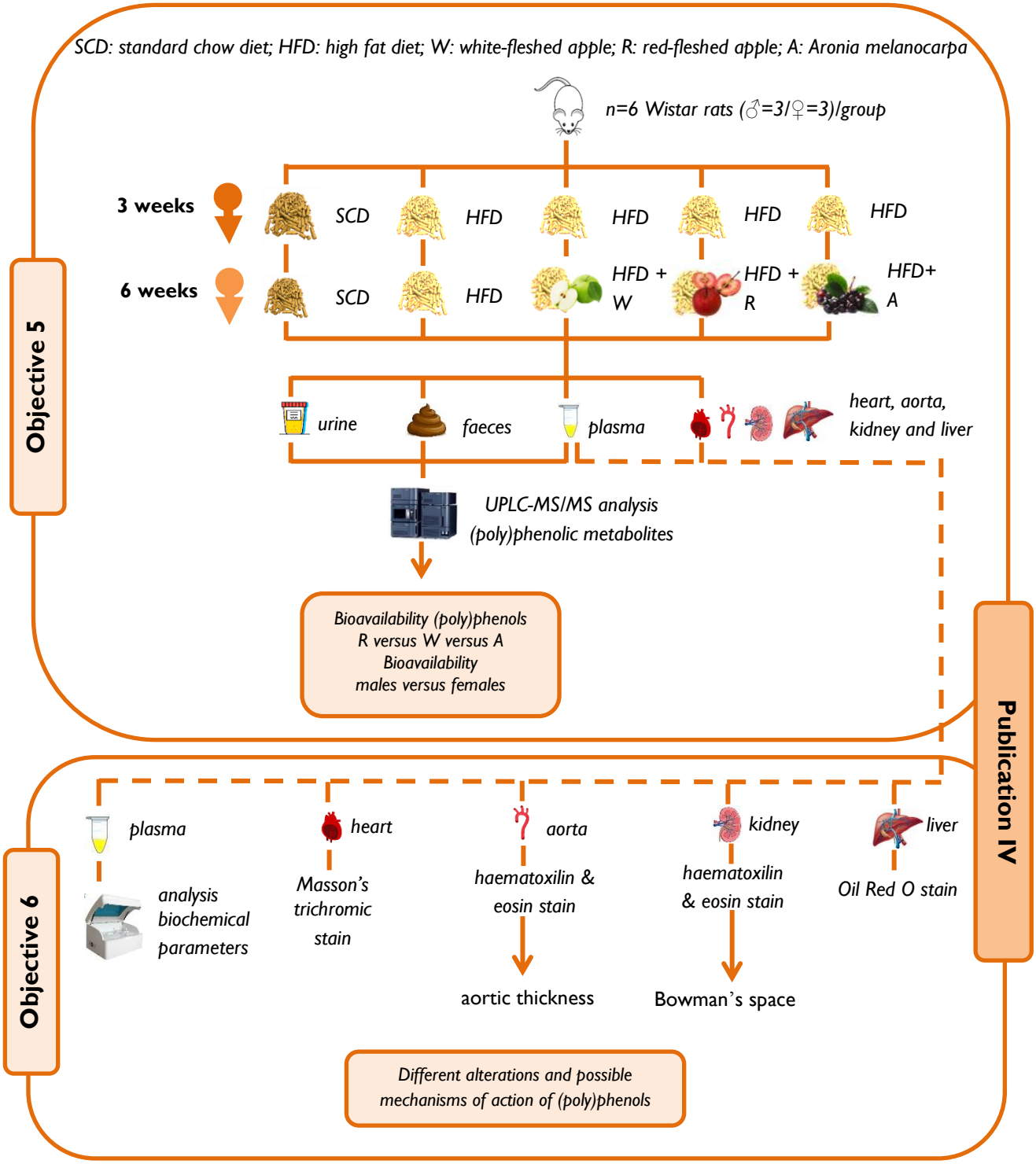
- Objective 5. Investigate the effect of the matrix (whole red-fleshed apple *versus* anthocyanin extract) on the anthocyanins and other (poly)phenols bioavailability after a sustained intake in Wistar rats.
- Objective 6. Provide first insights regarding the mechanisms of action of anthocyanins in the control of cardiometabolic risk factors after a sustained intake of red-fleshed apple snack *versus* anthocyanin-rich extract in hypercholesterolemic Wistar rats.

WORK PLAN: GENERAL METHODOLOGY USED


Objective 1
RedLove
red-fleshed apple
Freeze-dried
snack
Hot air-dried
snack
Pasteurized
purée
Infrared-
dried
snack
UPLC-MS/MS analysis
(poly)phenolic compounds
(Poly)phenolic compounds in freeze-dried snack versus hot air-dried snack versus infrared-dried snack versus pasteurized purée
Publication I
Objective 2
n=3 healthy volunteers
Total (poly)phenolic dose ~150 mg
2-week washout between intakes
60 g
Freeze-dried snack
66 g
Hot air-dried snack
500 g
Pasteurized
purée
Urine samples
0-2 h
2-4 h
4-8 h
8-24 h
UPLC-MS/MS analysis
(poly)phenolic metabolites
(Poly)phenol bioavailability
freeze-dried snack versus
hot air-dried snack versus
pasteurized purée


Objective 3
80 g of red-fleshed apple snack
n=10 healthy volunteers
Capillary blood
(0, 0.5, 1, 2, 4, 6, 12, 24 h)
Venous blood
(0, 0.5, 1, 2, 4, 6, 24 h)
Venous plasma
(0, 0.5, 1, 2, 4, 6, 24 h)
Dried Blood Spot cards (DBS)
µElution solid phase extraction (µSPE)
UPLC-MS/MS analysis (poly)phenolic metabolites
Capillary blood (DBS) versus Venous blood (DBS)
Venous blood (DBS) versus Plasma (DBS)
Plasma (DBS) versus Plasma (µSPE)
Publication II


Objective 4
80 g of red-fleshed apple snack
n=10 healthy volunteers
Urine
(0-2 h; 2-4 h; 4-8 h; 8-24 h)
Venous plasma
(0, 0.5, 1, 2, 4, 6, 24 h)
UPLC-MS/MS analysis
(poly)phenolic metabolites
Potential (poly)phenolic intake biomarkers and metabolic pathways of (poly)phenols from red-fleshed apples
Publication III


SCD: standard chow diet; HFD: high fat diet; W: white-fleshed apple; R: red-fleshed apple; A: Aronia melanocarpa
n=6 Wistar rats (♂=3/♀=3)/group
3 weeks
6 weeks
SCD
HFD
HFD
HFD
HFD
SCD
HFD
HFD + W
HFD + R
HFD+ A
Objective 5
urine
faeces
plasma
heart, aorta, kidney and liver
UPLC-MS/MS analysis (poly)phenolic metabolites
Bioavailability (poly)phenols
R versus W versus A
Bioavailability
males versus females
Publication IV
Objective 6
plasma
analysis biochemical parameters
heart
Masson's trichromic stain
aorta
haematoxilin & eosin stain
aortic thickness
kidney
haematoxilin & eosin stain
Bowman's space
liver
Oil Red O stain
Different alterations and possible mechanisms of action of (poly)phenols

RESULTS

OBJECTIVE 1 AND OBJECTIVE 2

PUBLICATION 1: THERMAL AND NON-THERMAL PROCESSING OF RED-FLESHED APPLE: HOW ARE (POLY)PHENOL COMPOSITION AND BIOAVAILABILITY AFFECTED?

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ROYAL SOCIETY OF CHEMISTRY

PAPER

Thermal and non-thermal processing of red-fleshed apple: how are (poly)phenol composition and bioavailability affected?

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Abstract: The present study evaluated the impact of different thermal (infrared-drying, hot air-drying and purée pasteurization) and non-thermal (freeze-drying) processing technologies on red-fleshed apple (poly)phenolic compounds. We further investigated the processing effect on the (poly)phenol bioavailability in a crossover postprandial study where three subjects consumed three apple products (freeze-dried snack, hot air-dried snack and pasteurized purée). (Poly)phenolic compounds present in the apple products and their biological metabolites in urine were analyzed using liquid chromatography coupled to mass spectrometry (UPLC-MS/MS). When comparing different processes, infrared-drying caused important losses in most of the apple (poly)phenolics, while hot air-drying and purée pasteurization maintained approximately 83% and 65% of total (poly)phenols compared with the freeze-dried snack, respectively. Anthocyanins in particular were degraded to a higher extend, and hot air-dried apple and pasteurized purée maintained respectively 26% and 9% compared with freeze-dried apple snack. The acute intake showed that pasteurized purée exhibited the highest (poly)phenol bioavailability, followed by hot air-drying and freeze-dried snack, highlighting the impact of processing on (poly)phenols absorption. In conclusion, for obtaining affordable new red-fleshed apple products with enhanced (poly)phenol bioavailability, purée pasteurization and hot air-drying represent viable techniques. However, to obtain a red-fleshed apple snack with high anthocyanin content, freeze-drying is the technique that best preserves them.

KEYWORDS: anthocyanins, bioavailability, (poly)phenolic compounds, red-fleshed apple, thermal-processing, UPLC-MS/MS

1. Introduction

Numerous *in vivo* and *in vitro* studies have demonstrated that (poly)phenol compounds exhibit a wide range of biological effects such as lowering cholesterol (Aprikian et al., 2002), antiproliferative activity on cancer cells (Feskanich et al., 2000), and reducing the risk of suffering heart diseases, asthma and type-2 diabetes (Knekt et al., 2002; Sesso et al., 2003; Woods et al., 2003), among others. However, the dietary habits of the majority of people do not guarantee an adequate intake of fruits and vegetables, and the intake of (poly)phenols is below the amounts found to have significant health effects (Mayer et al., 2008).

In recent years, due to the global interest in phytochemicals and in developing functional foods that are fortified, enriched, or enhanced with improved health-promoting effects, there has been growing interest in the development of commercial red-flesh apple cultivars. These apple cultivars have been obtained by traditional breeding methods from new hybrids with red pulp (Volz et al., 2009). The flesh of these singular apple varieties has an enhanced content of anthocyanins (red colour) with high antioxidant activity and potential health-promoting effects (Speer et al., 2020). Besides, through crossbreeding programs with good-flavoured white-fleshed apples, the poor taste of the wild red-flesh varieties has been improved (Bars-Cortina et al., 2017). So, these new apple varieties could make healthy eating easier and more available, thus satisfying the increasingly widespread needs for high food quality and diversity.

Since the production of apple fruit is seasonal, processing methods have been developed and applied to obtain apple derived products with shelf-stability and increase useful life while minimizing changes in the quality attributes (Vega-Gálvez et al., 2012). Dehydration is one of the main and oldest techniques for preserving agricultural and food products, and in recent years for producing ready-to-eat and healthy snacks from fresh fruit such as apples, while retaining their nutrients and bioactive compounds and thus, being a healthier alternative to salty or sugary snacks (Alasalvar & Shahidi, 2013; Kamiloglu et al., 2016). In the preparation of functional and ready-to-eat foods, freeze-drying method is commonly used as there is a minimum loss of flavour, aroma and bioactive compounds, with near-perfect preservation results. However, due to its expensive cost, other dehydration methods such as hot air-drying or infrared-drying are employed to produce high-quality dried fruits (Alasalvar & Shahidi, 2013; Doymaz & Pala, 2003; Karam et al., 2016). Pasteurization of fruit purée is another commercial and common thermal treatment used to increase shelf-life of fresh fruits. In addition fruit purée can be used as an intermediate product for the

production of other products such as nectars, juices with solid particles or smoothies (Guerra et al., 2012; Picouet et al., 2009; Tiwari, 2018). In most dehydration methods, such as hot air-drying or infrared-drying, vegetables are subjected to high temperatures at which highly thermosensitive and unstable (poly)phenols can be readily degraded (Julkunen-Tiitto & Sorsa, 2001; Katsube et al., 2009; Mohd Zainol et al., 2009; Mori et al., 2007; Sadilova et al., 2007). However, it has also been shown that food processing can induce chemical or physical modifications such as degradation or modification of cell wall polysaccharides or proteins, molecular interactions between components, and other food matrix factors that enhance (poly)phenol bioaccessibility and bioavailability during digestion (Bugianesi et al., 2004; Yang et al., 2011).

Therefore, the first aim of the present research was to evaluate the impact of four different processing conditions (infrared-drying, hot air-drying, freeze-drying and pasteurization of purée) on apple (poly)phenols stability. Secondly, we also investigated the effect of the processing conditions on the apple (poly)phenol bioavailability in a human pilot study in order to search the optimal conditions to obtain an apple snack product with a higher (poly)phenol bioavailability and, thus, enhancing its functional value.

2. *Materials and methods*

2.1. Chemicals and reagents

Pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, hydroxytyrosol, luteolin, kaempferol, eriodictyol, quercetin, luteolin-7-*O*-glucoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, procyanidin dimer B_2, quercetin-3-*O*-rutinoside (rutin), myricetin, and phloretin-2-*O*-glucoside were purchased from Extrasynthese (Genay, France). *p*-Hydroxybenzoic acid, 3,4-dihydroxy-benzoic acid (protocatechuic acid), *p*-coumaric acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid (5-caffeoylquinic acid), naringenin, catechin, and epicatechin were acquired from Sigma-Aldrich (St. Louis, MO, USA). The vanillic acid was from Fluka (Buchs, Switzerland). Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid, and formic acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water was of 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 1 g/L and storing it in dark flasks at -80°C.

2.2. Plant material

The red-fleshed apple variety used was 'Redlove Era'. The apples were provided by NUFRI SAT (Mollerussa, Lleida, Spain) and planted in the "La Rasa" experimental plot (La Rasa, Soria, Spain).

2.3. Non-thermal apple processing: freeze-drying

Before drying, the apples were washed, wiped with paper towels and cut into 1 cm-sized cubes. The apple cubes were frozen in liquid nitrogen and the freeze-drying was then performed with a first drying at 0.6 bar with a temperature ramp of -20 to 0°C over 25 hours, followed by a second complete vacuum drying with a temperature ramp of 0 to 20°C over 40 hours (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain).

2.4. Thermal apple processing

Hot air-drying: apples were washed, wiped with paper towels and cut into 1 cm-sized cubes. The apple cubes were immediately dried in a pilot dryer composed of a cylindrical stainless-steel basket where the sample was introduced, an electric resistance of 8 kW to heat the air to the desired temperature and a Casals model MA 26 M 2H fan (*Kv* = 0.865 Cv) (Casals, Girona, Spain) connected to the speed variation system. The drier was equipped with a dashboard with an Eliwell thermostat (Protherm controls, Atherstone, England) to control the air temperature and speed of the fan, and a Vaisala hygrometer equipped with a HMP 125B probe (Vaisale, Vantaa, Finland) that controlled the relative humidity of the air at the entrance and exit of the drying equipment. Finally, the control for the velocity of the air entering the basket containing the apple cubes consisted of a WM-DA 4000 turbine anemometer (Pacer instruments, Keene, United States) and several valves. The apple cubes were placed in the basket and hot air without recirculating was passed through it. Drying was carried out until a constant weight (weighing the basket with the apples on an electronic balance mod. EM-60 KAM, A&D Company, Tokyo) at 60°C for 80 minutes with an air velocity of 1.5 ms^{-1} and then the temperature was increased until 70°C and maintained for 40 minutes with the same air velocity.

***Infrared-drying*:** the infrared-drying process was carried out in an infrared moisture analyzer mod. IRCDi5 FIR dehydrator (Irconfort, Seville, Spain). A thin layer of the apple cubes was placed inside an aluminium support equipped with a precision balance to record the weight. The continuous weighing of the sample allowed the drying kinetics to be determined. The infrared-drying experiments were automatically stopped when the weight of the cubes remained constant. Several tests were performed for a range of infrared-drying temperatures

(35, 40, 50 and 60°C) and the sample mass *versus* time was recorded. In all the cases, the product weight, initial moisture content and dry matter content of the apple cubes were used to calculate the moisture content obtained at any drying time.

After the completion of the different drying processes, the dried apple cubes were immediately transferred to airtight plastic containers and kept at -80°C until the (poly)phenol chromatographic analysis. Prior to analysis, a fine powder of the dried apple samples was obtained with the aid of an analytical mill (A11, IKA, Germany).

Pasteurized apple purée: red-fleshed apples were supplied to a local company (Anela Fruits, Girona, Spain) to be processed to pasteurized purée. Briefly, apples were milled to a fine purée which was hermetically closed into sterile containers and submitted to continuous pasteurization in a tubular system (94°C for 10 min).

2.5. Apple (poly)phenol bioavailability human study

2.5.1. Subjects and study design

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). In this study the kinetics of (poly)phenolic metabolites in 24 h urine in different interval times (0-2, 2-4, 4-8, and 8-24 h) were monitored in healthy volunteers after the acute ingestion of freeze-dried apple, hot air-dried apple and pasteurized apple purée. The study was performed in a crossover design to reduce interpersonal variability. The volunteers comprised three healthy women, aged from 24 to 38 years, with body mass index between 19.4 and 25.1 kg/m^2. They declared no gastrointestinal alterations and reported no antibiotic use over the last months before the study. To standardize the baseline point, subjects were asked to follow a low-(poly)phenol diet during the 2 days preceding each dietary intervention and during the dietary intervention day. For this, subjects were asked to limit fruit and vegetables consumption, and to avoid cherries, strawberries, blueberries, tea, coffee, wine, beer, chocolate and all their derived products. Each volunteer received in random order the three apple products in a crossover design with a washout period of 14 days between interventions. The amount of each administered product was: 60 g of freeze-dried apple snack, 66 g of hot air apple snack and 500 g of apple pasteurized purée, which represented a similar (poly)phenolic dose (134±18 mg total (poly)phenols) (**Supplemental Table 1**).

Urine samples were collected 24 h before (basal conditions) and at the interval times of 0-2, 2-4, 4-8, and 8-24 h after the apple products intake. The volume of urine in

each interval was measured and aliquots were stored at -80°C prior to the (poly)phenol chromatographic analysis.

2.6. Analysis of (poly)phenolic compounds in apple products and (poly)phenolic metabolites in urine

2.6.1. Sample pre-treatment

The pre-treatment for the analyses of the (poly)phenolic compounds in apple products was carried out according to Bars-Cortina et al. (2017) with some modifications. Briefly, 0.4 g of dried apple powder or 1.6 g of lyophilized purée (the lyophilization parameters were the same as those detailed in section 2.3.) was weighed and extracted with 10 mL of methanol/Milli-Q water/formic acid (79.9:20:0.1, v/v/v). The samples were vortexed for 10 min and then centrifuged at 8784 *g* for 10 min. The extraction was repeated three times and the supernatants were collected, combined and filtered through a 0.22 μm PVDF filter (Scharlab, Barcelona, Spain) prior to the chromatographic analysis. Samples were analyzed in triplicate.

The urine samples were pre-treated by μSPE. The micro-cartridges and their conditioning and equilibration steps were the same as reported in our previous study (Yuste et al., 2019). In this case, 100 μL of phosphoric acid at 4% were added to 100 μL of the urine sample, and this solution was loaded into the micro-cartridge. The retained (poly)phenolic compounds were then eluted with 2 x 50 μL of methanol. Each sample was prepared in triplicate.

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

Liquid chromatography analyses were carried out on an AcQuity Ultra-Performance liquid chromatograph coupled to a tandem mass spectrometer from Waters (Milford, MA, USA). Two chromatographic methods using UPLC-MS/MS were used for the analysis of 1) anthocyanins and their metabolites, and 2) the rest of the (poly)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 study (Yuste et al., 2019). Briefly, for the analysis of (poly)phenolic compounds (including anthocyanins), the analytical column used was an AcQuity BEH C_{18} (100 mm × 2.1 mm i.d., 1.7 μm) equipped with a VanGuard PreColumn AcQuity BEH C_{18} (5 × 2.1 mm, 1.7 μm), also from Waters. For the analysis of anthocyanins and their metabolites, the mobile phase was 10% acetic acid (eluent A) and acetonitrile (eluent B). The elution gradient was 0−10 min, 3−25% B; 10−10.10 min, 25−80% B; 10.10−11 min, 80% B isocratic; 11−11.10 min, 80−3% B; 11.10−12.50 min, 3% B

isocratic. For the analysis of the rest of the (poly)phenolic compounds and their metabolites, the mobile phase was 0.2% acetic acid (eluent A) and acetonitrile (eluent B). The elution gradient for the analysis of these (poly)phenolic compounds was 0−5 min, 5−10% B; 5−12 min, 10−12.4% B; 12−18 min, 12.4−28% B; 18−23 min, 28−100% B; 23−25.5 min, 100% B isocratic; 25.5−27 min, 100−5% B; and 27−30 min, 5% B isocratic.

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. Ionization was achieved using the electrospray (ESI) interface operating in the positive mode $[M - H]^+$ for the analysis of anthocyanins and in the negative mode $[M - H]^-$ for the other compounds. The data were acquired through selected reaction monitoring (SRM). The ionization source parameters were as follows: capillary voltage, 3 kV; source temperature, 150°C; desolvation gas temperature, 400°C, with a flow rate of 800 L/h. Nitrogen (99.99% purity, N_2LCMS nitrogen generator, Claind, Lenno, Italy) and argon (≥99.99% purity, Aphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. The dwell time established for each transition was 30 ms. Data acquisition was carried out with MassLynx 4.1 software.

Due to the lack of commercial (poly)phenolic standards and their generated metabolites, some of the compounds were tentatively quantified by using the calibration curve of their precursor or of a (poly)phenolic compound with a similar structure. **Supplemental Table 2** shows the selected reaction monitoring (SRM) conditions as well as its cone voltage and collision energy used for the quantification of these (poly)phenolic compounds. This table also shows in which (poly)phenolic standard compound, these (poly)phenolics have been quantified.

2.7. Statistical analysis

Concentration values of the (poly)phenolic compounds and their metabolites studied were reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's test at a level of 0.05 were used to determine the significance of differences among the different apple processing techniques, among the urine excretion of the different (poly)phenolic families after the intake of the three red-fleshed apple products studied, and among intra- and inter-individual differences in this (poly)phenol excretion. Moreover, General Linear Model and One-way ANOVA at a level of 0.05 was used to determine the significance of the % absorption of total (poly)phenols after the intake of the three products derived from red-fleshed apple. All data were analyzed with the Minitab Statistical Software, version 17.2.1 (Minitab Inc., State College, Pennsylvania, United States).

3. Results and discussion

3.1. Effect of apple processing on the (poly)phenol stability

The aim of the present study was to explore the potential of new red-fleshed apple varieties, as anthocyanin enriched alternatives (biofortified) to common white-fleshed apples for the development of an apple snack product with an enhanced functional value. So, the first objective of this research was to evaluate the impact of different processing technologies on the (poly)phenols stability of red-fleshed apple and, therefore, to search the optimal conditions to develop an apple snack product with a high shelf-stability maintaining at the same time a high concentration of bioactive (poly)phenols. The different products are shown in **Figure 1**.

A total of 26 (poly)phenols were identified in the red-fleshed apple products using UPLC-MS/MS (**Table 1**) and included compounds from the following 6 (poly)phenol classes (**Figure 2**): i) Phenolic acids, ii) Flavan-3-ols, iii) Flavonols, iv) Anthocyanins, v) Flavanones, and vi) Dihydrochalcones.

The impact of the different processes on the stability of the (poly)phenols in red-fleshed apple products was compound-dependent and some (poly)phenols were degraded more than others (**Table 1**).

In general terms as seen in **Figure 2**, when comparing the different processes, infrared-drying caused important losses in most of the apple (poly)phenolics, while hot air-drying and purée pasteurization allowed the maintenance of approximately 83% and 65% of the (poly)phenols compared with the freeze-dried snack, respectively.

In accordance with the literature (Bars-Cortina et al., 2017; Ceymann et al., 2012; Ramírez-Ambrosi et al., 2013; Wang et al., 2015) 5-*O*-caffeoylquinic acid (chlorogenic acid) (**Table 1**) was the most abundant phenolic acid and the main (poly)phenolic compound detected in the apple samples (representing 90%-95% of the total phenolic acids and 60-70% of the total (poly)phenolic content).

The group of phenolic acids was significantly influenced by the dehydration technique used, freeze-drying and hot air-drying being the techniques that preserves them best (**Figure 2**). The total content of phenolic acids was 1661 mg/kg d.w. in freeze-dried apple samples, which was reduced to 1587 mg/kg d.w. in hot air-dried and followed by the values found in the pasteurized purée (1187 mg/kg d.w.), while infrared-dried samples presented the lowest amounts ranging from 474 to 690 mg/kg d.w. (**Table 1**).

Table 1. (Poly)phenolic composition of the red-fleshed apple products in relation to processing technology. Data are expressed in mg/kg dry weight apple as mean ± standard deviation (n=3).

	Treatment						
Compound (mg/kg dw apple)	**Freeze-dried**	**Hot air-dried**	**Infrared-dried 35°C**	**Infrared-dried 40°C**	**Infrared-dried 50°C**	**Infrared-dried 60°C**	**Pasteurized purée**
Protocatechuic acid	n.d.	14.6 ± 0.55 a	n.d.	n.d.	n.d.	n.d.	54.4 ± 0.98 b
Hydroxytyrosol	5.20 ± 0.20 b	5.60 ± 0.20 b	1.70 ± 0.40 a	3.01 ± 0.60 a	1.72 ± 0.30 a	1.71 ± 0.10 a	5.44 ± 0.62 b
Coumaric acid hexoside	13.7 ± 0.48 a	22.1 ± 1.40 c	n.d.	n.d.	n.d.	n.d.	16.9 ± 0.46 b
Coumaric acid derivate	6.13 ± 0.89 b	6.42 ± 1.53 b	1.93 ± 0.12 a	1.52 ± 0.10 a	1.83 ± 0.20 a	2.02 ± 0.30 a	8.04 ± 0.14 b
Vanillic acid hexoside	32.9 ± 1.20 b	26.1 ± 0.93 a	19.6 ± 1.82 a	25.3 ± 0.80 a	23.1 ± 1.51 a	22.4 ± 1.22 a	24.3 ± 4.34 a
Homogentisic acid	7.23 ± 0.62 c	5.38 ± 1.05 b	1.40 ± 0.20 a	1.32 ± 0.11 a	1.01 ± 0.10 a	1.32 ± 0.11 a	6.23 ± 0.09 bc
Ferulic acid	n.d.	2.42 ± 0.48 a	n.d.	n.d.	n.d.	n.d.	1.82 ± 0.23 a
Ferulic acid hexoside	18.3 ± 2.74 c	20.6 ± 0.44 c	4.41 ± 0.10 a	4.70 ± 0.30 a	2.91 ± 0.50 a	3.01 ± 0.40 a	12.3 ± 2.25 b
5-*O*-caffeoylquinic acid	1578 ± 59.9 d	1484 ± 39.0 d	661 ± 31.1 b	731 ± 0.40 b	510 ± 10.9 a	444 ± 47.0 a	1058 ± 12.6 c
Total Phenolic acids	*1661 ± 62.3 D*	*1587 ± 40.9 D*	*690 ± 33.6 B*	*767 ± 1.26 B*	*541 ± 11.6 A*	*474 ± 46.6 A*	*1187 ± 6.68 C*
Epicatechin	54.1 ± 8.32 c	27.4 ± 0.50 b	3.90 ± 0.11 a	3.81 ± 0.21 a	2.43 ± 0.41 a	2.10 ± 0.01 a	30.4 ± 0.73 b
Epigallocatechin	6.62 ± 0.61 a	5.31 ± 0.10 a	4.33 ± 0.11 a	5.61 ± 1.80 a	4.93 ± 1.21 a	4.33 ± 0.51 a	5.15 ± 0.32 a
Dimer	94.0 ± 8.76 c	43.9 ± 2.74 b	15.6 ± 0.10 a	9.93 ± 0.40 a	8.20 ± 1.40 a	7.42 ± 0.71 a	45.3 ± 2.61 b
Trimer	42.0 ± 1.93 b	24.2 ± 2.00 a	n.d.	n.d.	n.d.	n.d.	n.d.
Total Flavan-3-ols	*197 ± 7.24 D*	*101 ± 5.52 C*	*23.8 ± 0.15 A*	*19.3 ± 2.00 A*	*15.6 ± 3.00 A*	*13.9 ± 1.15 A*	*80.9 ± 0.75 B*
Quercetin	n.d.	1.34 ± 0.16 a	n.d.	n.d.	n.d.	n.d.	4.94 ± 0.48 b
Dihydroquercetin	2.08 ± 1.05 a	2.14 ± 0.66 a	2.44 ± 0.51 a	2.21 ± 0.10 a	1.75 ± 0.40 a	2.34 ± 0.20 a	1.87 ± 0.42 a
Quercetin arabinoside	6.55 ± 1.05 b	3.57 ± 0.81 a	5.70 ± 0.31 ab	7.33 ± 0.30 b	4.72 ± 1.11 ab	6.05 ± 0.80 ab	5.24 ± 0.13 ab
Quercetin rhamnoside	36.0 ± 1.39 d	36.5 ± 2.32 d	10.8 ± 0.50 b	11.6 ± 0.42 b	4.81 ± 0.50 a	6.72 ± 0.90 ab	27.8 ± 1.61 c
Quercetin glucoside	6.77 ± 1.44 b	1.87 ± 0.03 a	n.d.	n.d.	n.d.	n.d.	4.33 ± 0.47 ab
Dihydrokaempferol glucoside	12.6 ± 2.71 a	7.53 ± 0.39 a	n.d.	n.d.	n.d.	n.d.	n.d.
Total Flavonols	*64.4 ± 5.74 C*	*53.0 ± 2.51 B*	*18.9 ± 0.75 A*	*21.1 ± 0.23 A*	*11.3 ± 0.25 A*	*15.1 ± 1.49 A*	*44.2 ± 3.28 B*
Cyanidin arabinoside	29.4 ± 1.07 d	7.98 ± 0.50 c	0.08 ± 0.01 a	0.11 ± 0.01 a	0.05 ± 0.50 a	0.03 ± 0.01 a	4.10 ± 0.58 b
Cyanidin-3-*O*-galactoside	256 ± 3.57 d	65.2 ± 3.41 c	2.57 ± 0.62 a	3.12 ± 0.56 a	1.47 ± 0.17 a	0.85 ± 0.22 a	22.1 ± 0.97 b
Total Anthocyanins	*285 ± 4.41 D*	*73.2 ± 3.90 C*	*2.65 ± 0.62 A*	*3.23 ± 0.58 A*	*1.55 ± 0.22 A*	*0.88 ± 0.23 A*	*26.2 ± 1.41 B*
Naringenin glucoside	6.41 ± 0.89 b	4.08 ± 0.51 a	2.80 ± 0.10 a	3.03 ± 0.41 a	2.41 ± 0.10 a	3.23 ± 0.01 a	6.19 ± 1.05 b
Eriodictyol hexoside	9.24 ± 0.22 c	7.47 ± 1.02 b	1.40 ± 0.31 a	1.42 ± 0.10 a	1.01 ± 0.11 a	0.90 ± 0.20 a	8.98 ± 0.77 bc
Total Flavanones	*15.7 ± 1.07 C*	*11.6 ± 0.51 B*	*4.20 ± 0.24 A*	*4.45 ± 0.33 A*	*3.42 ± 0.04 A*	*4.13 ± 0.24 A*	*15.2 ± 1.58 C*
Phloretin glucoside	83.4 ± 18.6 abc	116 ± 7.80 c	59.1 ± 8.11 ab	60.4 ± 2.64 ab	39.0 ± 7.10 a	45.6 ± 3.61 ab	89.4 ± 21.9 bc
Phloretin xylosyl glucoside	202 ± 15.1 d	128±10.1 ab	151 ± 1.50 bc	196 ± 18.8 cd	172 ± 10.3 cd	90.1 ± 6.82 a	187 ± 16.8 cd
Hydroxyphloretin xylosyl glucoside	17.3 ± 3.26 ab	26.7± 5.87 b	14.7 ± 3.20 ab	12.2 ± 0.10 a	9.82 ± 1.81 a	9.00 ± 0.81 a	19.6 ± 3.84 ab
Total Dihydrochalcones	*303 ± 4.82 D*	*271 ± 1.74 D*	*225 ± 12.8 BC*	*269 ± 16.0 CD*	*221 ± 19.2 B*	*145 ± 4.01 A*	*296 ± 10.2 D*

For each row, values not displaying the same letter are significantly different (one-way ANOVA, Tukey's test between all means, $p < 0.05$). n.d.: not detected.

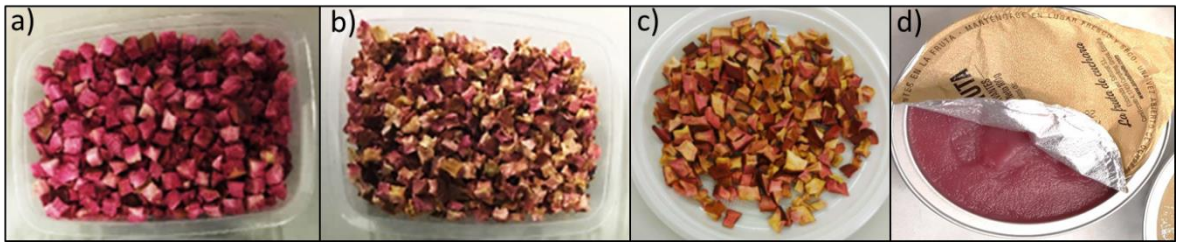

Figure 1. Different red-fleshed apple products used in this study obtained by: a) freeze-drying, b) hot air-drying, c) infrared-drying d) or by a process of purée pasteurization.

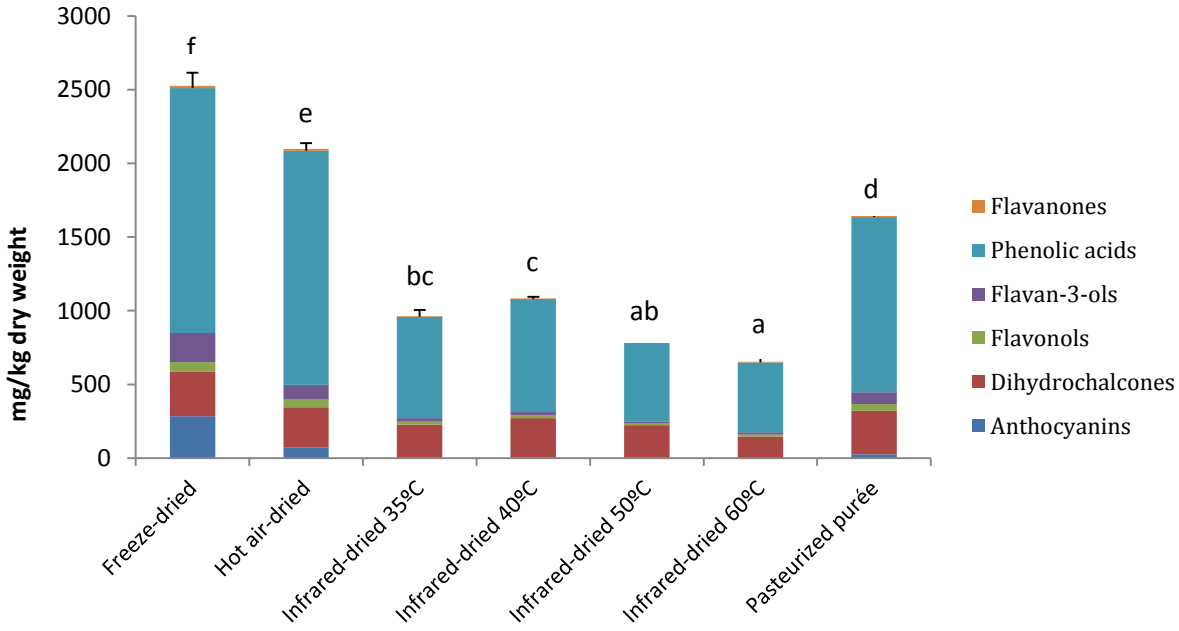

Figure 2. Impact of the different thermal and non-thermal processes on the (poly)phenolic groups in red-fleshed apple products. The letters express statistically significant differences between the content of the total (poly)phenol classes content among the processing technologies ($p < 0.05$).

In this study, four flavan-3-ols were detected and quantified in the apple samples. The freeze-dried samples contained the highest amounts (197 mg/kg d.w.), followed by the hot air-dried (101 mg/kg d.w.) and the pasteurized purée (80.9 mg/kg d.w.) (**Table 1**). In accordance with Bars-Cortina et al. (2017), the most abundant flavan-3-ol was a epicatechin dimer followed by epicatechin and a trimer. In the infrared-dried apple samples, all flavan-3-ols were found in significantly lower concentrations and the trimer was not detected in any of the infrared temperatures studied. Compared to phenolic acids, flavan-3-ols showed higher losses due to thermal treatment with losses of approximately 49% and 59% in hot air-dried apples and pasteurized purée, respectively and between 87% and 93% in

infrared-dried apples with respect to freeze-dried apples.

Regarding flavonols, the most abundant were those derived from quercetin, in accordance with the literature (Wang et al., 2015), being quercetin-rhamnoside the most abundant in all the apple products. Apart from these, other flavanols, such as dihydroquercetin and quercetin arabinoside were detected in all samples (**Table 1**). Quercetin was only detected in the hot air-dried and pasteurized purée samples. Furthermore, quercetin glucoside and dihydrokaempferol glucoside were not detected in the infrared-dried samples. As in the previous (poly)phenolic groups, the treatments that best preserved flavonol group, when comparing with the non-thermal freeze-drying method, were hot air-drying and purée pasteurization.

Regarding anthocyanins, the most distinctive (poly)phenolics in red-fleshed apple cultivars, cyanidin-3-*O*-galactoside and cyanidin arabinoside were detected in all the apple samples (**Table 1**). According to the literature (Bars-Cortina et al., 2017; Ceymann et al., 2012; Guo et al., 2016), cyanidin-3-*O*-galactoside was the most abundant anthocyanin detected and quantified in the red-flesh apple samples (around 90% of the total anthocyanin content). Concerning the impact of the different treatments, results showed that anthocyanins were degraded to a higher extend compared to other (poly)phenolic compounds. Results showed that freeze-drying is the technique that statistically best preserves these compounds (285 mg/kg d.w) followed by hot air-drying (73.2 mg/kg d.w.) and purée pasteurization (26.2 mg/kg d.w.). In the infrared-dried samples, these compounds were degraded almost completely with observed losses of > 98% when comparing with freeze-drying.

Other groups of (poly)phenols influenced by the processing technique applied were the flavanones and dihydrochalcones (**Table 1**). In the flavanone group, only naringenin-glucoside and eriodictyol-hexoside were detected as reported in the literature (Wang et al., 2015) and the processes that statistically best preserved them were freeze-drying and purée pasteurization with total values of 15.7 and 15.2 mg/kg d.w., respectively. Within the group of the dihydrochalcones, phloretin-xylosil-glucoside was the predominant compound.

At a general level, this polyphenol group is the least affected by the apple transformation, and the total values of non-thermal (freeze-drying), and some thermal (hot air-drying, infrared-drying at 40°C and pasteurized purée) treatments show no statistically significant differences. Our results are in accordance with a previous study (Fernández-Jalao et al., 2019), confirming that apple dihydrochalcones are more stable against the application of high temperatures than other (poly)phenol

classes, such as flavan-3-ols and anthocyanins.

Considering the freeze-dried samples as reference, infrared-drying (at all temperatures) caused significant ($p < 0.05$) losses in most of the apple (poly)phenols, while (poly)phenol losses by hot air-drying and by the purée pasteurization were considerably lower. This fact is probably a consequence of the intense time/ temperature treatment applied in the infrared processes and to a lesser extend in hot air-drying or in the production of the pasteurized purée, in which the treatment at high temperatures lasts only a few minutes. Thermal treatment can cause severe degradation of (poly)phenolic compounds as it is well known that these compounds are temperature sensitive (Julkunen-Tiitto & Sorsa, 2001; Katsube et al., 2009; Mohd Zainol et al., 2009; Mori et al., 2007; Sadilova et al., 2007). This fact is also reflected in infrared-dried samples where those subjected to higher temperatures (50°C and 60°C) showed the greatest (poly)phenol degradation/losses. Moreover, the losses could also be due to the presence of oxygen in the case of infrared-drying and hot air-drying producing an oxidative degradation of the (poly)phenols and consequently the browning of the apple samples (**Figure 1**). (Poly)phenols are the desirable substrates of oxidoreductive enzymes such as phenoloxidases, whose main function is to oxidized (poly)phenols (Julkunen-Tiitto & Sorsa, 2001; Ramírez-Ambrosi et al., 2013). These enzymes catalyze the oxidation of *o*-diphenols into quinones, which polymerize to form brown melanin pigment (Mathew & Parpia, 1971). In freeze-dried samples, these reactions do not occur because the freezing and subsequent sublimation of water under vacuum conditions prevents the action of these enzymes and consequently the browning of the samples (Kim et al., 2018). However, although this process efficiently preserves bioactives, freeze-drying costs can be 2-5 times higher than hot air-drying in order to achieve the same final moisture content. This fact justifies the need to find alternatives for the production of economically affordable healthy food for everyone (Normal & Joseph, 2001). In this sense, when comparing the different dehydration techniques for preparing healthy apple snacks preserving the content of (poly)phenols (**Figure 2**), infrared-drying showed high losses of 57-74% depending on the temperature. On the other hand, hot air-drying and purée pasteurization allow the maintenance of 83% and 65% respectively of (poly)phenols quantified in the freeze-dried snacks and may be good alternatives to the costly freeze-drying technique.

3.2. Effect of apple processing on the (poly)phenolic bioavailability: human pilot study

The second aim of the present work was to study how apple processing impacts on the release and absorption of (poly)phenols present in the apple products and determine the optimal processing technique that improves (poly)phenol bioavailability. For this, the apple products with the highest (poly)phenolic contents (freeze-dried apples, hot air-dried apples and pasteurized apple purée) were chosen to conduct a crossover acute intervention study with three subjects. The amounts of each product administered to the subjects were selected to match as best as possible the total content of (poly)phenolic and the contents of the different (poly)phenolic subclasses (**Supplemental Table 1**).

3.2.1. Effect of apple processing on (poly)phenolic compounds bioavailability

A total of 59 (poly)phenol metabolites were identified and quantified in urine samples after the intake of the three red-fleshed apple derived products, including derivatives of benzoic acids, phenyl-propionic acids, phenylvalerolactones and catechols (major groups) (**Figure 3 a, b, c and Supplemental Table 3**), and anthocyanins, flavan-3-ols, dihydrochalcones and hydroxycinnamic acids (minor groups) (**Figure 3 d, e, f and Supplemental Table 3**). The (poly)phenolic metabolites were mainly phase-II sulphated, glucuronided and/or methylated conjugates of parent compounds present in the apples as well as of microbial catabolites resulting from colonic degradation (**Supplemental Table 4**). Excretion kinetics for each of these (poly)phenolic groups expressed as µmol/h are shown in **Supplemental Figure 1 and Supplemental Figure 2.**

Our data show intra-individual, inter-individual and processing-derived differences among the three products. Large inter-individual differences in urine metabolite concentration were found between the three volunteers, with highest excretion found in volunteer 1 (V1) followed by volunteer 3 (V3) and volunteer 2 (V2) (**Figure 3 and Supplemental Table 3**). This trend was repeated in each of the three apple products (**Supplemental Table 3**) and for almost all (poly)phenolic groups (except for anthocyanins which are discussed separately).

Regarding the processing effect, pasteurized purée showed significantly higher total (poly)phenol bioavailability in all three volunteers, followed by the hot air-dried red-fleshed apple and finally, the freeze-dried red-fleshed apple showed the lowest. **Figure 4** shows the % excretion of (poly)phenols in urine, which have been calculated as the average between the three volunteers comparing the sum of (poly)phenols ingested and the sum of the

moles excreted in urine in 24 h. This was observed for almost all (poly)phenolic groups except for anthocyanin and flavan-3-ols derivatives. It is of interest to note that although freeze-drying is the method that best preserves (poly)phenolic compounds during processing, it was the product with the lowest % bioavailability, while pasteurized purée, with the highest losses during processing among the three products tested, showed the highest % bioavailability.

The results obtained corroborate the notion that the apple processing could enhance (poly)phenols bioavailability. Chemical structure, concentration and matrix interactions are the three basic pillars that govern bioaccessibility and bioavailability of (poly)phenolic compounds from fruits. It has been shown that food processing can influence and alter all these three factors (Ribas-Agustí et al., 2019). Regarding thermal processing, it has been reported that it can promote the (poly)phenol release disrupting intracellular barriers, thus modulating (poly)phenol bioaccessibility for example in tomatoes (Bugianesi et al., 2004), in which it was found that human plasma levels of naringenin and chlorogenic acid, increased several times after the intake of cooked tomatoes compared with levels observed after consumption of fresh tomatoes. This fact was also verified in a recent study (Monfoulet et al., 2020) in which authors observed that after the administration of similar doses of flavan-3-ols through three apple products (phenolic extract, raw apple or apple purée) in hypercholesterolemic pigs, higher number of genes were modulated by purée than raw apples, which suggests that the processing of apples into purée increased the bioavailability of some phytochemicals, as flavan-3-ols, that could contribute to the postprandial nutrigenomic response.

Apart from this, it has also been shown that liquid foods possess a lower viscosity and pass through the stomach more rapidly than solid food, this is due to a higher water content, and typically lower content in proteins or complex carbohydrates that may bind to (poly)phenols (Zhu et al., 2013). Several studies have reported rapid absorption of (poly)phenols from liquid foods such as in coffee (Erk et al., 2012) or in apple products (Hollman et al., 1997). This latter study reports a 3-fold higher quercetin plasma concentration after consumption of apple sauce than after vacuum-impregnated apple chips or apple peel extract capsules. Moreover, in this study (as observed in our pilot study) the different treatments resulted in a high inter-individual variability of all plasma pharmacokinetic parameter after equal intake of quercetin equivalents of the apple product types (Hollman et al., 1997).

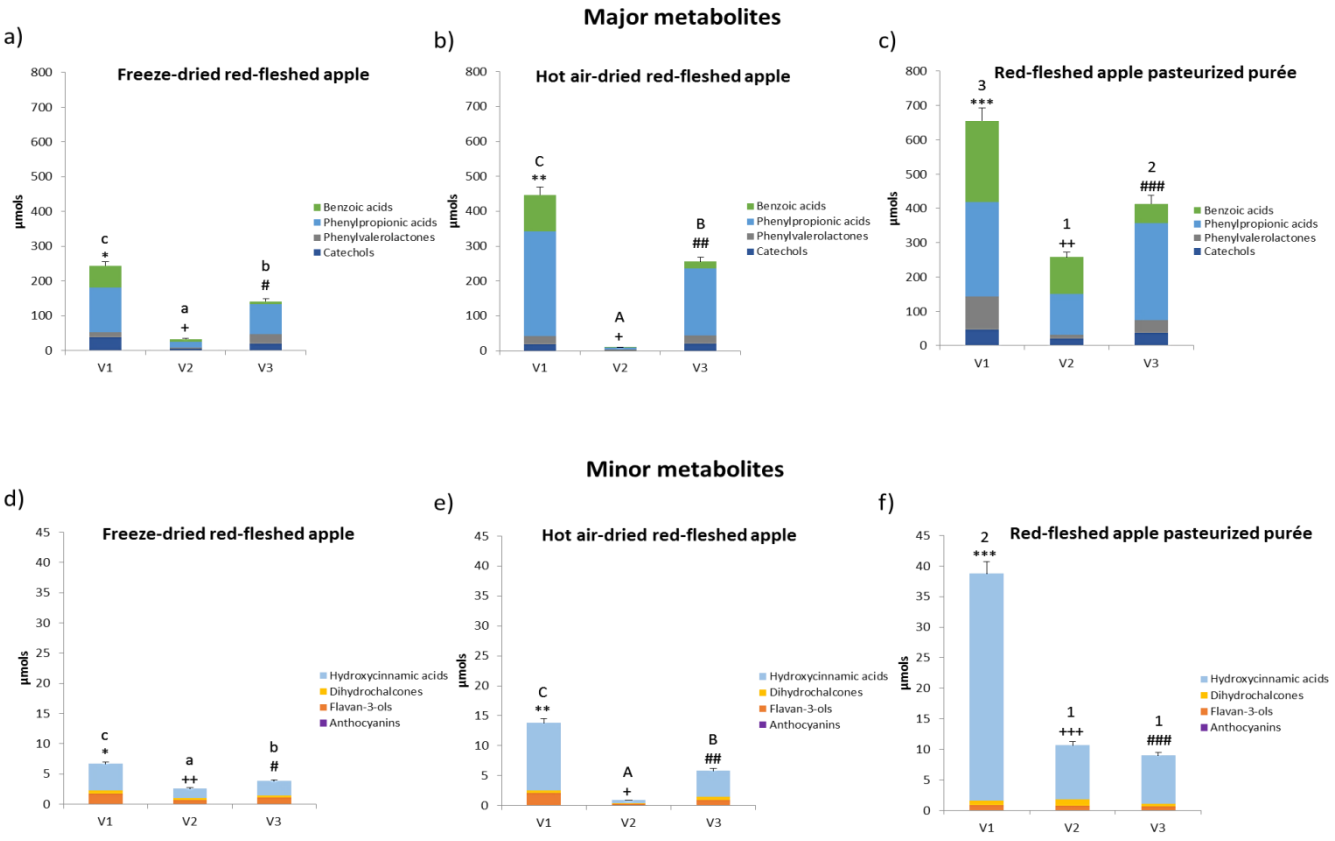

Figure 3. Total major (a-c) and minor (d-f) metabolite excretion after intake of freeze-dried apple snack, hot air- dried apple snack and apple pasteurized purée. Data expresed as mean values ± standard deviation. Different lowercase letters: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of freeze-dried red-fleshed apple. Different capital letters: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of hot air-dried red-fleshed apple. Different numbers: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of red-fleshed apple pasteurized purée. The simbols *, +, and # indicate differences between the three intakes for the same volunteer (One-way ANOVA, Tukey's test between all means, $p < 0.05$).

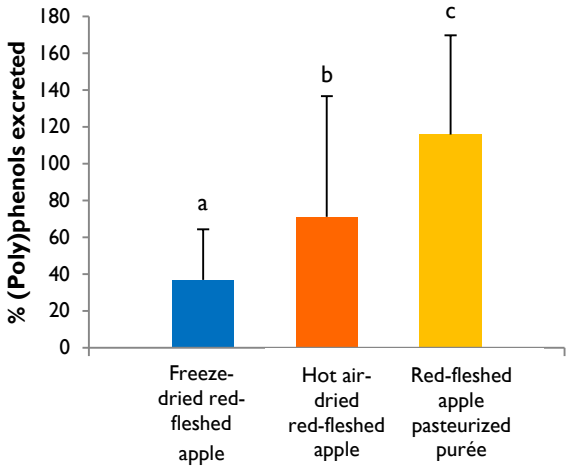

Figure 4. % Excretion in urine of total (poly)phenols after the intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée (n=3). This % was calculated as the ratio of total moles of excreted (poly)phenolic metabolites with respect to the total moles of ingested (poly)phenolic compounds. Different letters indicate significant differences in excretion between products (General Linear Model and One-way ANOVA, $p < 0.05$).

The high inter- and intra-individual variation in the response to (poly)phenolic intake, which in many cases leads to contradictory results in human trials, could result (in addition to the matrix effect already mentioned), from intrinsic aspects such as genetics, age, sex and physiological or pathological states. For example, it has been shown that genetic polymorphisms between individuals affect the effect of bioactive compounds (Miller et al., 2011), since they differentially affect genes that encode enzymes involved in the metabolism of these compounds.

Moreover, only small amounts of ingested (poly)phenols are absorbed in the upper gastrointestinal tract (GIT), while most compounds reach the lower GIT unmodified (Williamson & Clifford, 2017), where they undergo extensive metabolism mediated by the colonic microbiota. Thus, a key role is played by the gut microbiota, which may modify the structure of (poly)phenols, releasing lower molecular weight colonic catabolites that can be absorbed more easily (Williamson & Clifford, 2017). Each human has a unique gut microbiota that changes throughout life, and the environment, diet and lifestyle, all influence the microbiome (Rinninella et al., 2019; Zmora et al., 2019). In this sense, the existence of metabotypes (metabolic phenotype with specific gut microbiome-derived metabolites that characterize the metabolism of the parent compound) in the production of (poly)phenolic metabolites has been discussed almost exclusively in recent year. It was observed that the benefits associated with the ingestion of foods rich in ellagitannins, such as pomegranate and walnuts may be related to specific metabotypes (González-Sarrías et al., 2017; Selma et al., 2018; Tomás-Barberán et al., 2017). In our study, most of the apple (poly)phenol metabolites are of colonic origin while only a minority were parent compounds and their phase II conjugates from upper GIT absorption (**Supplemental Figure 3 and Supplemental Table 4**). Furthermore, despite having only three volunteers in our study, "low" (V2) and "high" (V1 and V3) excretors can be observed. It should be stressed that although V2 showed in all three products the lowest (poly)phenol bioavailability, this was greatly improved after the ingestion of apple purée.

Supplemental Figure 3 shows the representation of the (poly)phenolic groups detected after each ingestion for each volunteer, separated into those absorbed in the upper GIT (dihydrochal-cone, flavan-3-ol, and anthocyanin parent compounds and their phase II metabolites) and in the lower GIT (simple phenolic acids). The major groups of upper GIT absorption were dihydrochalcone and flavan-3-ol derivatives while phenylpro-pionic acid derivatives were the major ones among the metabolites that are absorbed in the lower GIT. Similar results were observed in our previous study in which

ten volunteers ingested 80 g of freeze-dried red-fleshed apple snack (Yuste et al., 2019).

It is of note that in the present study we observed intra-individual differences in the proportions of the different metabolite groups after the ingestion of the three apple products. If the observed differences depend on the apple processing or, more probable, on intrinsic aspects of each individual should be the focus of further studies.

The inter-individual differences are also shown here, since for V1 and V3 the (poly)phenolic groups that are absorbed in the upper GIT (dihydrochalcones, flavan-3-ols and anthocyanins) are greater in the freeze-dried snack, while for V2 it is greater in the hot air-dried red-fleshed snack (**Supplemental Figure 3**). Interestingly, in no case was the bioavailability of the (poly)phenols absorbed in the upper GIT greater after ingesting the purée as might be expected, since in the solid apple matrix of the freeze-dried snack and the hot air-dried snack these (poly)-phenolics are bound to cell walls by covalent bonds between (poly)phenolics and polysaccharides possibly restricting bioavailability in the small intestine (Padayachee et al., 2012). Besides, genetic variation between individuals for enzymes involved in the absorption and metabolism of these groups in the gut epithelium and/ or liver may result in large differences in the expression of a functional enzyme which might explain the observed inter-individual differences (Fernandes et al., 2014; Riches et al., 2009; Wu et al., 2011).

Regarding the metabolites found after the ingestion of the three apple products, the majority were sulphate and methyl-sulphate conjugates representing between 61%-83% and 7%-27% of the total (poly)phenol metabolites detected, respectively (**Figure 5 and Supplemental Table 4**). The rest of the (poly)phenol metabolites (glucuronide, methyl, glycine, sulphate-glucuronide and methyl-glucuronide conjugates, and free acids/parent compounds) varied considerable between volunteers and between the type of the ingested apple product showing again great inter- and intra-individual differences. The differences in human subjects' genetics regarding digestive enzymes, intestinal transporters, phase I and II metabolism or tissue carriers, and also differences in gut microbiota composition and functionality affecting the catabolism of the not absorbed (poly)-phenols in the small intestine being responsible of the differences observed (Morand et al., 2020).

Although it is a preliminary study, our bioavailability results show large differences in the concentrations of metabolites (inter-individual differences) as well as in the metabolic profile depending on the type of product ingested for each person (intra-

individual differences), which could result in different effects on health. This fact justifies the need to carry out future studies with a greater number of volunteers to be able to address these differences. Nevertheless, an important limitation of this study is the lack of some of the authentic standards to quantify more accurately the concentrations of some (poly)phenol metabolites and thus the real bioavailability % of red-fleshed apple products.

3.2.2. *Effect of apple processing on anthocyanin bioavailability*

In this study, the effect of apple processing on anthocyanin bioavailability was of special interest since this polyphenolic group is the most characteristic of the red-fleshed apple cultivars and is not found in the pulp of any common white-fleshed apple variety. This polyphenol group was however, the most affected by the processing treatments (**Table 1**) and, consequently, different amounts of anthocyanins were consumed (2, 4 and 16 mg) after the acute intake of pasteurized purée, hot air-dried and freeze-dried apple products, respectively (**Supplemental Table 1**). The results showed (contrary to what happens for the other (poly)phenolic groups, **Figure 4**) that the higher average excretion in urine of anthocyanins was observed after the intake of hot air-dried apple (0.07%) while for the freeze-dried apple and pasteurized purée it was 0.04% and 0.03%, respectively (data not shown).

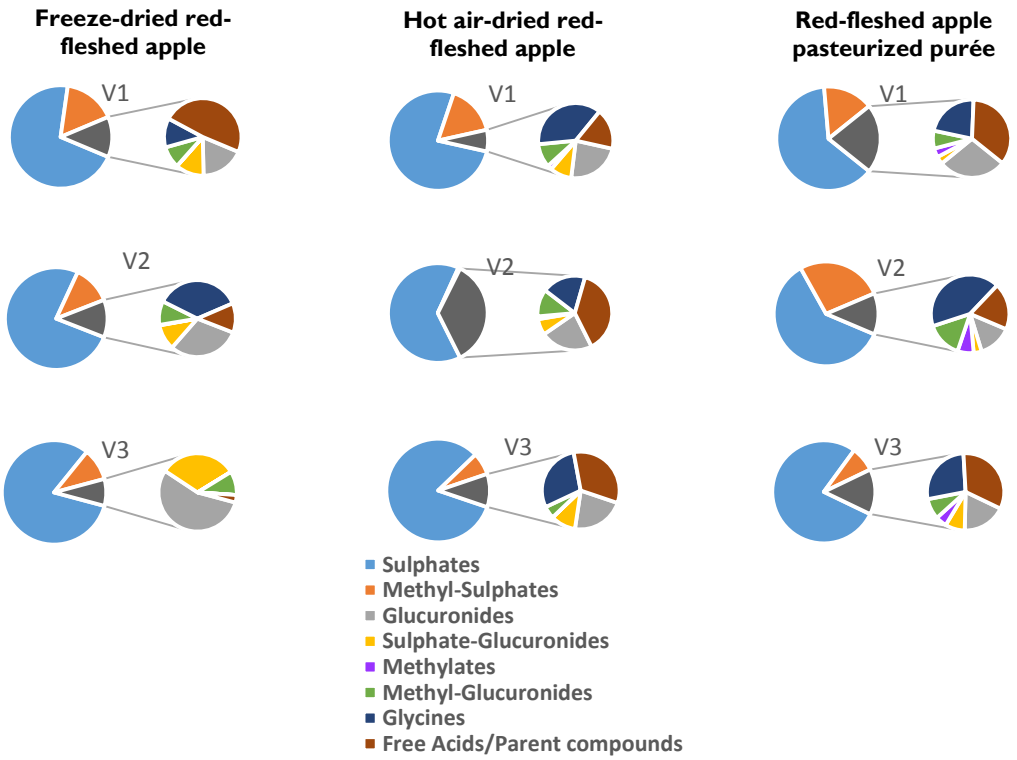

Figure 5. Schematic representation (% of each group over the total) of the main phase II metabolites, free acids and parent compounds found in urine in each volunteer after the intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée.

These excretion rates have been calculated as the average of the three volunteers and comparing the total ingested anthocyanins with the total anthocyanins and their phase II metabolites excreted in 24 h-urine. It should be noted that these observed differences are very small and probably, if more volunteers had participated in this study, the differences would had shown no significant differences between products.

Moreover, although the bioavailability of anthocyanins is very low compared to other (poly)phenolic groups, the major part of these compounds pass to the colon where they are degraded by the microbiota to simpler phenolic acids that are common to other (poly)phenol groups and once reabsorbed contribute to the pool of circulating (poly)phenolic metabolites in the body (Felgines et al., 2005; Fernandes et al., 2014; McDougall et al., 2005; Morand et al., 2020; Riches et al., 2009).

The fact that anthocyanins were more bioavailable in the solid matrices (hot air-dried and freeze-dried) than in the purée, may be due to the fact that these compounds are very unstable and more easily degraded. Thus, in the solid matrix, they could remain more attached to the fibre that may stabilize them or offer protection against further reactions until the site of absorption is reached. Our results could be in agreement with a previous study reporting that when raspberry extract was digested *in vitro* with foodstuffs (bread, breakfast cereals or ice cream) higher proportions of anthocyanins were bioaccessible compared to the extract digested alone (McDougall et al., 2005).

Finally, **Supplemental Figure 2** shows the urine excretion kinetics of anthocyanins after the apple products intake expressed as total nmols of anthocyanins and their phase II metabolites excreted per hour. The higher concentration observed in the freeze-dried format is due to the fact that the anthocyanin dose administered with this product was higher. In all cases, similar kinetics were observed with a maximum excretion between 2-4 hours, which is in agreement with previous *in vivo* studies reporting that anthocyanins are absorbed in the stomach and the small intestine with rapid detection of intact anthocyanin glycosides in urine and plasma within 30 to 60 min of ingestion (Felgines et al., 2005; Fernandes et al., 2014; McDougall et al., 2005; Morand et al., 2020; Riches et al., 2009).

4. Conclusions

Our findings revealed that, considering the freeze-drying as a reference technology to preserve food bioactives, infrared-drying at all temperatures caused significant losses in all the red-fleshed apple (poly)phenols, while (poly)phenol losses by hot air-drying

and by the purée pasteurization were considerably lower. Anthocyanins in particular were degraded to a higher extend after all thermal processing technologies. So, we conclude that for obtaining red-fleshed apple products affordable for the consumer, hot air-drying and purée pasteurization represent interesting technologies to obtain apple products with a high shelf-stability maintaining at the same time a high concentration of bioactive (poly)phenols. However, to obtain a product with the highest anthocyanin content, the extra cost of freeze-drying would have to be assumed.

Results obtained from the human postprandial crossover study showed that when comparing the total ingested (poly)phenol dose and the urinary excreted amount, the pasteurized apple purée proved to be the processing that resulted in the highest bioavailability, followed by the hot air-dried apple and the freeze-dried apple. Further, a great intra- and inter-individual variability between the metabolites was found, which highlights the importance of characterizing the metabotypes in future studies.

The present study is a proof of concept to select the most appropriate apple processing to preserve the apple (poly)phenolic compounds and provides further evidence on how food processing plays a significant role in the bioavailability of (poly)phenols, which is a step forward towards the design of healthier foods.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Supplemental Table 1. Concentration of (poly)phenolic compounds in different red-fleshed apple products expressed as mg/ingested portion ± SD (portions: red fleshed apple pasteurized purée=500 g, hot air-dried red fleshed apple snack = 66 g, freeze-dried red fleshed apple snack = 60 g).

Compound (mg/ingested portion)	Red-fleshed apple pasteurized purée	Hot air-dried red-fleshed apple	Freeze-dried red-fleshed apple
Cyanidin arabinoside	0.36 ± 0.05	0.47 ± 0.03	1.72 ± 0.06
Cyanidin-3-*O*-galactoside	1.93 ± 0.09	3.84 ± 0.20	14.9 ± 0.23
TOTAL Anthocyanins	***2.29 ± 0.12***	***4.31 ± 0.23***	***16.6 ± 0.26***
Protocatechuic acid	4.75 ± 0.09	0.86 ± 0.03	n.d.
Hydroxytyrosol	0.47 ± 0.05	0.33 ± 0.01	0.30 ± 0.01
Coumaric acid hexoside	1.47 ± 0.04	1.31 ± 0.08	0.80 ± 0.03
Coumaric acid derivate	0.70 ± 0.01	0.38 ± 0.09	0.36 ± 0.05
Vanillic acid hexoside	2.13 ± 0.38	1.54 ± 0.05	1.93 ± 0.07
Homogentisic acid	0.54 ± 0.01	0.32 ± 0.06	0.42 ± 0.04
Ferulic acid	0.16 ± 0.02	0.14 ± 0.03	n.d.
Ferulic acid hexoside	1.07 ± 0.19	0.08 ± 0.03	1.07 ± 0.16
5-*O*-caffeoylquinic acid	92.0 ± 1.10	87.4 ± 2.30	92.0 ± 3.50
TOTAL Phenolic acids	***115 ± 1.90***	***92.4 ± 2.90***	***96.9 ± 3.80***
Epicatechin	2.65 ± 0.06	1.61 ± 0.03	3.17 ± 0.49
Epigallocatechin	0.45 ± 0.03	0.31 ± 0.01	0.39 ± 0.04
Dimer	3.96 ± 0.23	2.59 ± 0.16	5.50 ± 0.51
Trimer	n.d.	1.43 ± 0.12	2.45 ± 0.11
TOTAL Flavan-3-ols	***7.10 ± 0.29***	***5.90 ± 0.44***	***11.5 ± 1.12***
Quercetin	0.43 ± 0.04	0.08 ± 0.01	n.d.
Dihydroquercetin	0.16 ± 0.03	0.01 ± 0.00	0.12 ± 0.06
Quercetin arabinoside	0.46 ± 0.01	0.21 ± 0.04	0.38 ± 0.06
Quercetin rhamnoside	2.43 ± 0.14	2.15 ± 0.14	2.11 ± 0.08
Quercetin glucoside	0.38 ± 0.04	0.11 ± 0.00	0.40 ± 0.08
Dihydrokaempferol glucoside	n.d.	0.44± 0.02	0.73 ± 0.16
TOTAL Flavonols	***3.86 ± 0.18***	***3.00 ± 0.20***	***3.74 ± 0.12***
Naringenin glucoside	0.54 ± 0.09	0.24 ± 0.03	0.37 ± 0.05
Eriodictyol hexoside	0.78 ± 0.06	0.44 ± 0.06	0.54 ± 0.01
TOTAL Flavanones	***1.32 ± 0.16***	***0.68 ± 0.09***	***0.91 ± 0.06***
Phloretin glucoside	7.82 ± 1.92	6.84 ± 0.46	4.88 ± 1.09
Phloretin xylosyl glucoside	16.4 ± 1.47	7.52 ± 0.59	11.8± 0.89
Hydroxyphloretin xylosyl glucoside	1.71 ± 0.33	1.58 ± 0.35	1.01 ± 0.20
TOTAL Dihydrochalcones	***25.9 ± 2.20***	***15.9 ± 0.58***	***17.7 ± 1.02***
TOTAL Rest (poly)phenolics	***153 ± 2.10***	***118 ± 2.30***	***131 ± 3.90***
TOTAL (POLY)PHENOLICS	***155 ± 2.20***	***122 ± 2.50***	***148 ± 4.00***

n.d.: not detected

Supplemental Table 2. SRM conditions used for the analysis of (poly)phenols and their generated metabolites.

(Poly)phenolic metabolites	SRM Quantification	CV (V) / CE (eV)	Standard in which has been quantified
Catechols			
Catechol sulphate	189 > 109	20 / 15	Catechol-4-*O*-sulphate
Methyl catechol sulphate	203 > 123	20 / 15	4-methyl catechol sulphate
Catechol glucuronide	285 > 109	20 /15	Catechol-4-*O*-sulphate
Methyl catechol glucuronide	299 > 123	20 / 15	4-methyl catechol sulphate
Benzoic acids			
Vanillic acid	167 > 123	30 / 10	Vanillic acid
Vanillic acid sulphate	247 > 167	30 / 25	Vanillic acid-4-*O*-sulphate
Vanillic acid glucuronide	343 > 167	30 / 25	Vanillic acid
Homovanillic acid	181 > 137	40 / 15	Vanillic acid
Homovanillic acid sulphate	261 > 181	40 / 15	Vanillic acid
Protocatechuic acid	153 > 109	45 / 15	Protocatechuic acid
Protocatechuic acid sulphate	233 > 153	35 / 15	Protocatechuic acid
Protocatechuic acid glucuronide	329 > 153	35 / 15	Protocatechuic acid
Hydroxytyrosol sulphate	233 > 123	35 / 15	Hydroxytyrosol
Hydroxytyrosol glucuronide	329 > 123	35 / 20	Hydroxytyrosol
Hydroxybenzoic acid	137 > 93	30 / 15	*p*-Hydroxybenzoic acid
Hydroxybenzoic acid sulphate	217 > 137	35 / 15	*p*-Hydroxybenzoic acid
Hydroxybenzoic acid glucuronide	313 > 137	35 / 15	*p*-Hydroxybenzoic acid
Hydroxyhippuric acid	194 > 100	40 / 10	*p*-Hydroxybenzoic acid
Phenylpropionic acids			
Hydroxyphenylpropionic acid	165 > 121	20 / 10	3-(3'-hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid sulphate	245 > 165	35 / 15	3-(3'-hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid glucuronide	341 > 165	40 / 25	3-(3'-hydroxyphenyl)propionic acid
Dihydroxyphenylpropionic acid	181 > 137	35 / 15	3-(3',4'-dihydroxyphenyl) propionic acid
Dihydroxyphenylpropionic acid sulphate	261 > 181	40 / 15	3-(3',4'-dihydroxyphenyl) propionic acid
Dihydrocaffeic acid glucuronide	357 > 181	40 / 10	3-(3',4'-dihydroxyphenyl) propionic acid
Hydroxycinnamic acid derivatives			
Coumaric acid	163 > 119	35 / 10	*p*-coumaric acid
Coumaric acid sulphate	243 > 163	35 / 15	*p*-coumaric acid
Caffeic acid sulphate	259 > 179	35 / 15	Caffeic acid
Caffeic acid glucuronide	355 > 179	35 / 15	Caffeic acid
Ferulic acid	193 > 134	30 / 15	Ferulic acid
Ferulic acid sulphate	273 > 193	35 / 15	Ferulic acid
Ferulic acid glucuronide	369 > 193	35 / 15	Ferulic acid
Dihydroferulic acid	195 > 136	35 / 10	Ferulic acid
Dihydroferulic acid sulphate	275 > 195	35 / 15	Ferulic acid
Dihydroferulic acid glucuronide	371 > 195	35 / 20	Ferulic acid
Phenyl-γ-valerolactone derivatives			
Hydroxyphenyl-γ-valerolactone sulphate	271 > 191	40 / 20	Epicatechin
Dihydroxyphenyl-γ-valerolactone sulphate	287 > 207	40 / 15	Epicatechin
Dihydroxyphenyl-γ-valerolactone glucuronide	383 > 207	40 / 20	Epicatechin
Dihydroxyphenyl-γ-valerolactone sulphate glucuronide	287 > 207	40 / 20	Epicatechin
Dihydroxyphenylvaleric acid	209 > 135	40/ 15	Epicatechin

Flavan-3-ol			
Catechin sulphate	369 > 289	40 / 15	Catechin
Epicatechin sulphate	369 > 289	40 / 15	Epicatehin
Methyl catechin sulphate	383 > 303	40 / 15	Catechin
Methyl epicatechin sulphate	383 > 303	40 / 15	Epicatehin
Catechin glucuronide	465 > 289	40 / 20	Catechin
Epicatechin glucuronide	465 > 289	40 / 20	Epicatechin
Methyl catechin glucuronide	383 > 303	40 / 15	Catechin
Methyl epicatechin glucuronide	383 > 303	40 / 15	Epicatechin
Dihydrochalcones			
Phloretin glucuronide	449 > 273	40 / 20	Phloretin-2'-*O*-gucoside
Phloretin sulphate	353 > 273	40 / 20	Phloretin-2'-*O*-gucoside
Phloretin sulphate glucuronide	529 > 353	40 / 20	Phloretin-2'-*O*-gucoside
Anthocyanins			
Cyanidin arabinoside	419 > 287	40 / 20	Cyanidin-3-*O*-galactoside
Cyanidin-3-*O*-galactoside	449 > 287	40 / 20	Cyanidin-3-*O*-galactoside
Cyanidin glucuronide	463 > 287	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin galactoside	463 > 301	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin glucuronide	477 > 301	40 / 20	Cyanidin-3-*O*-galactoside
Methyl peonidin glucuronide	491 > 315	40 / 20	Cyanidin-3-*O*-galactoside
Cyanidin sulphate glucuronide	543 > 367	40 / 20	Cyanidin-3-*O*-galactoside
Cyanidin galactoside glucuronide	625 > 463	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin galactoside glucuronide	639 > 463	40 / 20	Cyanidin-3-*O*-galactoside

CV: Cone voltage
CE: Collision energy

Supplemental Table 3. Amount of the main (poly)phenolic groups (mean ± SD) excreted in urine (0-24 h) after a intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée.

		Major metabolites			
		Benzoic acids	**Phenylpropionic acids derivatives**	**Phenylvalerolactones derivatives**	**Catechols derivatives**
Freeze-dried red-fleshed apple	V1	62.6 ± 2.51 ^{b*}	129 ± 5.73 ^{c*}	14.5 ± 0.74 ^{b*}	38.2 ± 1.94 ^{c**}
	V2	8.47 ± 0.40 $^{a+}$	16.5 ± 0.89 $^{a+}$	2.31 ± 0.12 $^{a+}$	5.87 ± 0.23 $^{a++}$
	V3	6.90 ± 0.36 $^{a\#}$	86.8 ± 5.01 $^{b\#}$	27.6 ± 1.15 $^{c\#}$	20.0 ± 0.90 $^{b\#}$
Hot air-dried red-fleshed apple	V1	105 ± 5.43 ^{C**}	300 ± 16.2 ^{C**}	23.9 ± 1.00 ^{B*}	17.8 ± 0.77 ^{B*}
	V2	1.19 ± 0.06 $^{A+}$	4.67 ± 0.22 $^{A+}$	3.21 ± 0.17 $^{A+}$	0.83 ± 0.05 $^{A+}$
	V3	18.5 ± 1.01 $^{B\#\#}$	192 ± 10.5 $^{B\#\#}$	24.4 ± 0.95 $^{B\#}$	20.4 ± 0.92 $^{B\#}$
Red-fleshed apple pasteurized purée	V1	236 ± 13.4 ^{3***}	276 ± 16.3 ^{2**}	97.7 ± 5.29 ^{3**}	45.7 ± 1.99 ^{3***}
	V2	107 ± 5.91 $^{2++}$	119 ± 8.12 $^{1++}$	11.4 ± 0.57 $^{1++}$	19.6 ± 0.55 $^{1+++}$
	V3	57.1 ± 2.82 $^{1\#\#\#}$	282 ± 18.4 $^{2\#\#\#}$	36.9± 1.89 $^{2\#\#}$	37.4 ± 1.59 $^{2\#\#}$

		Minor metabolites			
		Anthocyanins derivatives	**Flavan-3-ols derivatives**	**Dihydrochalcones derivatives**	**Hydroxycinnamic acids derivatives**
Freeze-dried red-fleshed apple	V1	0.02 ± 0.00 ^{a**}	1.70 ± 0.07 ^{c**}	0.55 ± 0.02 ^{b**}	4.45 ± 0.21 ^{c*}
	V2	0.01 ± 0.00 $^{a+++}$	0.70 ± 0.04 $^{a++}$	0.36 ± 0.02 $^{a++}$	1.53 ± 0.10 $^{a+}$
	V3	0.01 ± 0.00 $^{a\#\#}$	1.09 ± 0.07 $^{b\#\#}$	0.30 ± 0.01 $^{a\#}$	2.44 ± 0.14 $^{b\#}$
Hot air-dried red-fleshed apple	V1	0.00 ± 0.00 ^{A*}	2.14 ± 0.11 ^{C***}	0.42 ± 0.02 ^{B*}	11.2 ± 0.59 ^{C**}
	V2	0.01 ± 0.00 $^{B++}$	0.24 ± 0.01 $^{A+}$	0.16 ± 0.01 $^{A+}$	0.48 ± 0.02 $^{A+}$
	V3	0.01 ± 0.00 $^{C\#\#}$	0.95 ± 0.06 $^{B\#\#}$	0.48 ± 0.02 $^{B\#\#}$	4.39 ± 0.28 $^{B\#\#}$
Red-fleshed apple pasteurized purée	V1	0.00 ± 0.00 ^{1*}	0.92 ± 0.05 ^{2*}	0.68 ± 0.03 ^{2***}	37.1 ± 1.93 ^{2***}
	V2	0.00 ± 0.00 $^{1+}$	0.77 ± 0.05 $^{1,2++}$	1.03 ± 0.07 $^{3+++}$	8.84 ± 0.54 $^{1++}$
	V3	0.00 ± 0.00 $^{1\#}$	0.65 ± 0.04 $^{1\#}$	0.41 ± 0.02 $^{1\#\#}$	7.93 ± 0.40 $^{1\#\#\#}$

		Total metabolites (µmols)
Freeze-dried red-fleshed apple	V1	250 ± 11.5 ^{c*}
	V2	35.6 ± 2.04 $^{a+}$
	V3	145 ± 6.11 $^{b\#}$
Hot air-dried red-fleshed apple	V1	460 ± 24.1 ^{C**}
	V2	10.8 ± 0.54 $^{A+}$
	V3	261 ± 13.7 $^{B\#\#}$
Red-fleshed apple pasteurized purée	V1	694 ± 39.0 ^{3***}
	V2	268 ± 15.8 $^{1++}$
	V3	422 ± 25.2 $^{2\#\#\#}$

The values were compared inter-individual and intra-individual (One-way ANOVA, Tukey's test between all means, $p < 0.05$).

For each column, different lowercase letters indicate differences between volunteers in excretion of (poly)phenols after the intake of freeze-dried red-fleshed apple.

For each column, different capital letters indicate differences between volunteers in excretion of (poly)phenols after the intake of hot air-dried red-fleshed apple.

For each column, different numbers indicate differences between volunteers in excretion of (poly)phenols after the intake of red-fleshed apple pasteurized purée.

For each column, the simbols *, +, and # indicate differences between the 3 intakes for the same volunteer.

Supplemental Table 4. Amount of the (poly)phenolic compounds (mean ± SD) excreted in urine (0-24 h) after a intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée for each of the 3 volunteers.

	Freeze-dried red-fleshed apple		
(Poly)phenolic compound	**V1**	**V2**	**V3**
Anthocyanins derivatives (nmols)			
Cyanidin arabinoside	3.30 ± 0.14	0.06 ± 0.01	2.64 ± 0.42
Cyanidin galactoside	0.52 ± 0.30	1.10 ± 0.06	0.66 ± 0.05
Cyanidin glucuronide	6.69 ± 0.61	3.94 ± 0.26	4.52 ± 0.99
Peonidin galactoside	0.18 ± 0.12	1.24 ± 0.12	1.01 ± 0.13
Peonidin glucuronide	0.59 ± 0.09	2.68 ± 0.41	3.18 ± 0.07
Methyl peonidin glucuronide	2.52 ± 0.71	1.17 ± 0.12	1.68 ± 0.08
Cyanidin sulphate glucuronide	0.67 ± 0.13	0.85 ± 0.15	0.35 ± 0.06
Cyanidin galactoside glucuronide	n.d.	1.40 ± 0.21	n.d.
Peonidin galactoside glucuronide	0.61 ± 0.10	0.77 ± 0.03	0.86 ± 0.03
Catechols derivatives (µmols)			
Catechol sulphate	15.1 ± 0.55	4.67 ± 0.17	7.84 ± 0.30
Methyl catechol sulphate	19.9 ± 1.02	0.77 ± 0.04	11.1 ± 0.54
Catechol glucuronide	0.77 ± 0.04	0.30 ± 0.02	0.39 ± 0.02
Methyl catechol glucuronide	2.49 ± 0.34	0.14 ± 0.01	0.67 ± 0.04
Flavan-3-ols derivatives (µmols)			
Catechin sulphate	n.d.	0.09 ± 0.01	0.07 ± 0.00
Epicatechin sulphate	1.32 ± 0.05	0.24 ± 0.02	0.37 ± 0.02
Methyl catechin sulphate	n.d.	n.d.	0.17 ± 0.01
Methyl epicatechin sulphate	n.d.	0.25 ± 0.01	n.d.
Catechin glucuronide	0.20 ± 0.01	n.d.	0.39 ± 0.03
Epicatechin glucuronide	0.19 ± 0.01	0.10 ± 0.00	0.09 ± 0.01
Methyl catechin glucuronide	n.d.	n.d.	n.d.
Methyl epicatechin glucuronide	n.d.	n.d.	n.d.
Phenylvalerolactones derivatives (µmols)			
Hydroxyphenyl-ɣ-valerolactone sulphate	1.53 ± 0.05	n.d.	n.d.
Dihydroxyphenyl-ɣ-valerolactone sulphate	9.42 ± 0.48	1.57 ± 0.08	19.0 ± 0.69
Dihydroxyphenyl-ɣ-valerolactone glucuronide	n.d.	0.38 ± 0.02	4.84 ±0.24
Dihydroxyphenyl-ɣ-valerolactone sulphate glucuronide	3.57 ± 0.21	0.36 ± 0.02	3.75 ± 0.21
Dihydroxyphenylvaleric acid	n.d.	n.d.	n.d.
Dihydrochalcones derivatives (µmols)			
Phloretin sulphate	0.15 ± 0.01	0.10 ± 0.01	0.08 ± 0.00
Phloretin glucuronide	0.24 ± 0.01	0.16 ± 0.01	0.12 ± 0.01
Phloretin sulphate glucuronide	0.16 ± 0.01	0.09 ± 0.00	0.10 ± 0.00
Phenylpropionic acids derivatives (µmols)			
Hydroxyphenylpropionic acid	14.6 ± 0.52	n.d.	0.19 ± 0.01
Hydroxyphenylpropionic acid sulphate	75.0 ± 2.38	3.14 ± 0.11	35.0 ± 1.80

Hydroxyphenylpropionic acid glucuronide	3.62 ± 0.21	n.d.	0.25 ± 0.01
Dihydroxyphenylpropionic acid	0.38 ± 0.03	0.10 ± 0.00	n.d.
Dihydroxyphenylpropionic acid sulphate	35.0 ± 2.59	13.0 ± 0.76	50.9 ± 3.16
Dihidrocaffeic acid glucuronide	n.d.	0.21 ± 0.01	0.44 ± 0.03
Benzoic acids (µmols)			
Vanillic acid	n.d.	n.d.	n.d.
Vanillic acid sulphate	n.d.	2.34 ± 0.08	1.76 ± 0.06
Vanillic acid glucuronide	n.d.	0.22 ± 0.01	0.39 ± 0.02
Homovanillic acid	n.d.	n.d.	n.d.
Homovanillic ac sulphate	20.5 ± 0.75	n.d.	n.d.
Protocatechuic acid	n.d.	n.d.	n.d.
Protocatechuic acid sulphate	2.43 ± 0.13	0.45 ± 0.02	4.52 ± 0.27
Protocatechuic acid glucuronide	n.d.	0.01 ± 0.00	n.d.
Hydroxytyrosol sulphate	5.76 ± 0.30	n.d.	n.d.
Hydroxytyrosol glucuronide	0.07 ± 0.00	0.10 ± 0.01	0.09 ± 0.01
p-hydroxybenzoic acid	0.03 ± 0.00	0.28 ± 0.01	n.d.
Hydroxybenzoic acid sulphate	29.3 ± 1.11	3.47 ± 0.13	0.09 ± 0.01
Hydroxybenzoic acid glucuronide	0.64 ± 0.02	0.00 ± 0.00	0.05 ± 0.00
Hydroxyhippuric acid	3.85 ± 0.20	1.58 ± 0.14	n.d.
Hydroxycinnamic acids derivatives (µmols)			
Coumaric acid	0.43 ± 0.02	0.15 ± 0.01	0.18 ± 0.01
Coumaric acid sulphate	2.65 ± 0.13	n.d.	n.d.
Caffeic acid sulphate	0.27 ± 0.01	0.34 ± 0.02	0.59 ± 0.03
Caffeic acid glucuronide	0.04 ± 0.00	n.d.	n.d.
Ferulic acid	0.00 ± 0.00	0.01 ± 0.00	n.d.
Ferulic acid sulphate	0.80 ± 0.03	0.93 ± 0.07	1.54 ± 0.10
Ferulic acid glucuronide	0.15 ± 0.01	0.05 ± 0.00	0.13 ± 0.01
Dihydroferulic acid	0.02 ± 0.00	n.d.	n.d.
Dihydroferulic acid sulphate	n.d.	0.05 ± 0.00	n.d.
Dihydroferulic acid glucuronide	0.08 ± 0.00	0.01 ± 0.00	n.d.

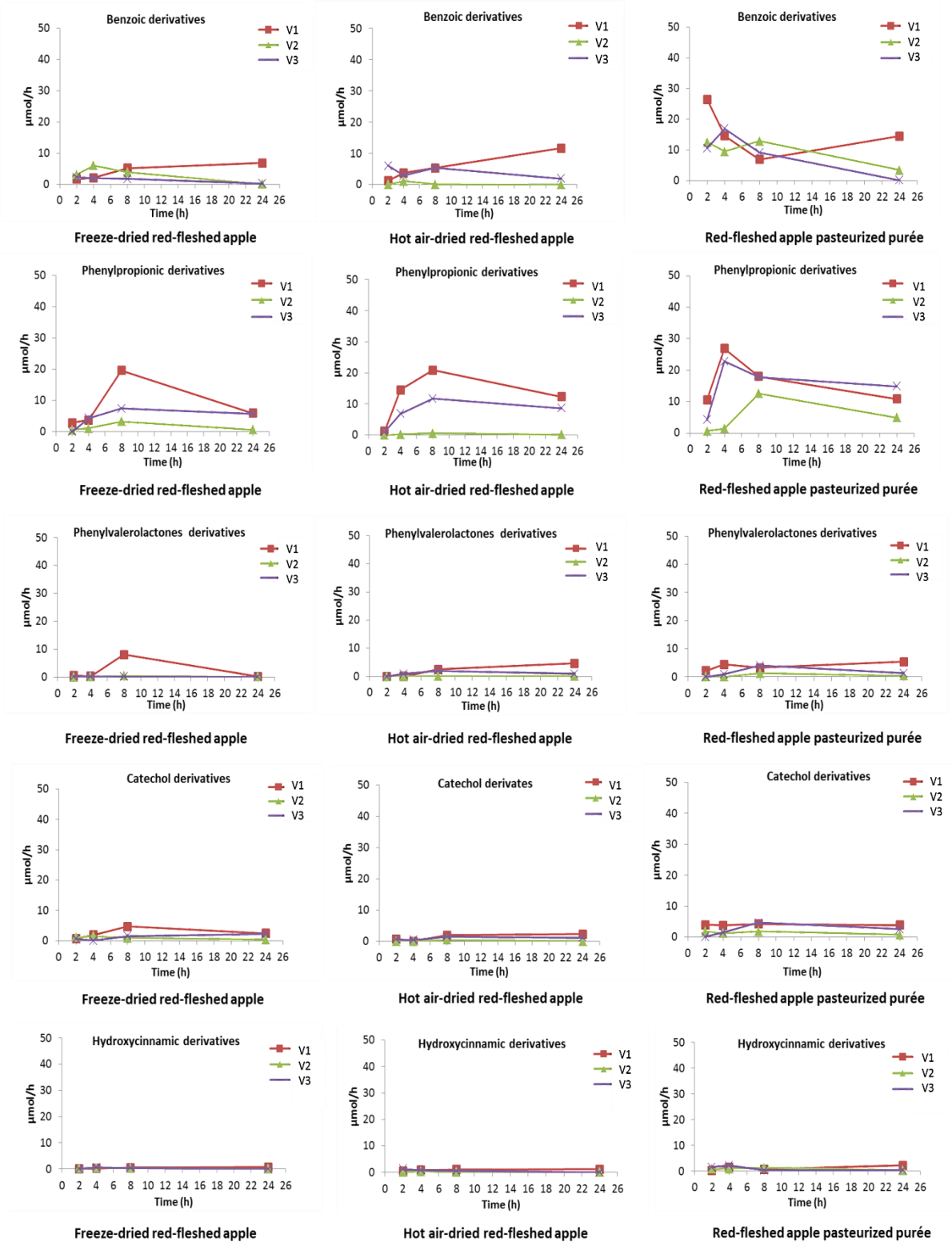
	Hot air-dried red-fleshed apple		
(Poly)phenolic compound	**V1**	**V2**	**V3**
Anthocyanins derivatives (nmols)			
Cyanidin arabinoside	0.48 ± 0.10	0.75 ± 0.36	1.58 ± 0.24
Cyanidin galactoside	0.14 ± 0.02	0.67 ± 0.06	0.83 ± 0.03
Cyanidin glucuronide	0.30 ± 0.06	1.83 ± 0.23	7.06 ± 0.38
Peonidin galactoside	1.56 ± 0.51	0.38 ± 0.10	0.93 ± 0.07
Peonidin glucuronide	0.65 ± 0.29	0.82 ± 0.10	0.02 ± 0.00
Methyl peonidin glucuronide	n.d.	n.d.	0.38 ± 0.06
Cyanidin sulphate glucuronide	0.07 ± 0.03	2.19 ± 0.10	0.83 ± 0.23
Cyanidin galactoside glucuronide	n.d.	0.03 ± 0.00	n.d.
Peonidin galactoside glucuronide	0.07 ± 0.01	0.11 ± 0.01	0.04 ± 0.00

Catechols derivatives (µmols)			
Catechol sulphate	10.2 ± 0.37	0.26 ± 0.02	9.76 ± 0.36
Methyl catechol sulphate	6.25 ± 0.32	n.d.	9.18 ± 0.47
Catechol glucuronide	n.d.	0.27 ± 0.02	0.46 ± 0.03
Methyl catechol glucuronide	1.37 ± 0.08	0.30 ± 0.01	0.94 ± 0.06
Flavan-3-ols derivatives (µmols)			
Catechin sulphate	n.d.	0.09 ± 0.00	n.d.
Epicatechin sulphate	0.20 ± 0.01	0.10 ± 0.00	0.40 ± 0.03
Methyl catechin sulphate	0.68 ± 0.03	n.d.	0.17 ± 0.01
Methyl epicatechin sulphate	0.36 ± 0.02	n.d.	n.d.
Catechin glucuronide	0.34 ± 0.02	n.d.	0.28 ± 0.01
Epicatechin glucuronide	0.05 ± 0.00	0.05 ± 0.00	0.09 ± 0.00
Methyl catechin glucuronide	0.22 ± 0.01	n.d.	n.d.
Methyl epicatechin glucuronide	0.29 ± 0.01	n.d.	n.d.
Phenylvalerolactones derivatives (µmols)			
Hydroxyphenyl-ɣ-valerolactone sulphate	n.d.	n.d.	n.d.
Dihydroxyphenyl-ɣ-valerolactone sulphate	16.4 ± 0.59	1.23 ± 0.09	18.0 ± 0.60
Dihydroxyphenyl-ɣ-valerolactone glucuronide	4.68 ± 0.24	0.37 ± 0.03	3.68 ± 0.19
Dihydroxyphenyl-ɣ-valerolactone sulphate glucuronide	2.89 ± 0.17	0.25 ± 0.01	2.73 ± 0.16
Dihydroxyphenylvaleric acid	n.d.	1.35 ± 0.05	n.d.
Dihydrochalcones derivatives (µmols)			
Phloretin sulphate	0.07 ± 0.00	0.02 ± 0.00	0.08 ± 0.00
Phloretin glucuronide	0.26 ± 0.01	0.12 ± 0.01	0.31 ± 0.02
Phloretin sulphate glucuronide	0.09 ± 0.01	0.01 ± 0.00	0.09 ± 0.01
Phenylpropionic acids derivatives (µmols)			
Hydroxyphenylpropionic acid	2.89 ± 0.11	n.d.	8.27 ± 0.48
Hydroxyphenylpropionic acid sulphate	27.0 ± 1.39	1.40 ± 0.05	110 ± 7.34
Hydroxyphenylpropionic acid glucuronide	1.05 ± 0.06	n.d.	1.11 ± 0.08
Dihydroxyphenylpropionic acid	2.27 ± 0.16	n.d.	0.11 ± 0.00
Dihydroxyphenylpropionic acid sulphate	266 ± 14.5	3.27 ± 0.17	72.5 ± 2.58
Dihidrocaffeic acid glucuronide	0.24 ± 0.01	n.d.	n.d.
Benzoic acids (µmols)			
Vanillic acid	n.d.	n.d.	n.d.
Vanillic acid sulphate	28.6 ± 1.06	n.d.	5.36 ± 0.20
Vanillic acid glucuronide	0.08 ± 0.00	0.12 ± 0.01	0.20 ± 0.01
Homovanillic acid	n.d.	n.d.	n.d.
Homovanillic ac sulphate	32.4 ± 1.94	n.d.	1.01 ± 0.06
Protocatechuic acid	n.d.	n.d.	n.d.
Protocatechuic acid sulphate	4.67 ± 0.30	0.31 ± 0.01	1.76 ± 0.12
Protocatechuic acid glucuronide	0.09 ± 0.01	n.d.	0.01 ± 0.00
Hydroxytyrosol sulphate	7.90 ± 0.35	n.d.	n.d.
Hydroxytyrosol glucuronide	n.d.	0.03 ± 0.00	0.03 ± 0.00
p-hydroxybenzoic acid	0.27 ± 0.01	n.d.	0.46 ± 0.02
Hydroxybenzoic acid sulphate	18.2 ± 0.93	n.d.	1.81 ± 0.09

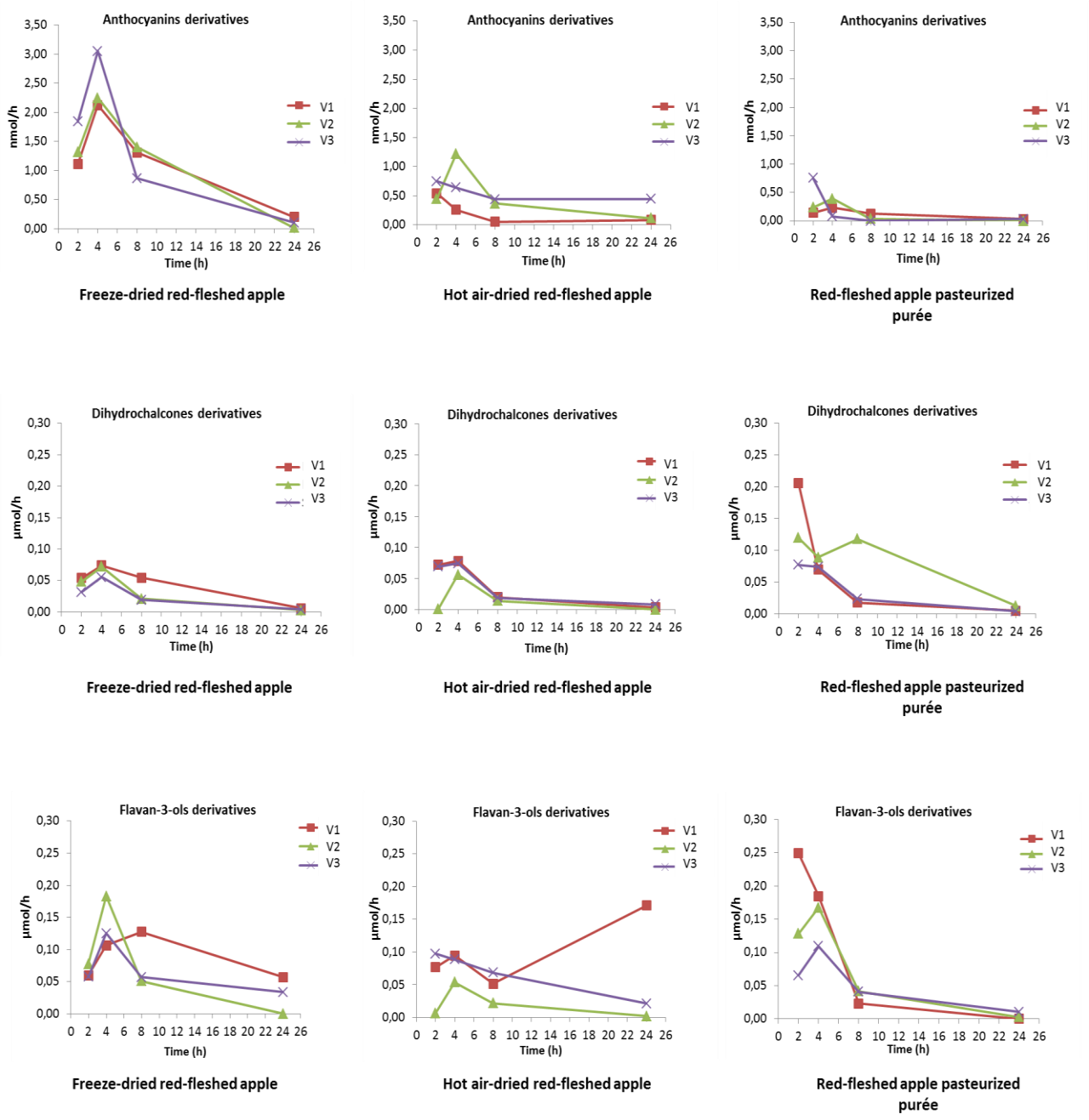
Hydroxybenzoic acid glucuronide	0.78 ± 0.05	n.d.	0.02 ± 0.00
Hydroxyhippuric acid	11.9 ± 0.79	0.72 ± 0.04	7.81 ± 0.52
Hydroxycinnamic acids derivatives (µmols)			
Coumaric acid	0.20 ± 0.01	0.11 ± 0.00	0.12 ± 0.00
Coumaric acid sulphate	0.89 ± 0.05	0.02 ± 0.00	0.62 ± 0.03
Caffeic acid sulphate	1.10 ± 0.06	0.22 ± 0.01	0.84 ± 0.05
Caffeic acid glucuronide	0.02 ± 0.00	n.d.	n.d.
Ferulic acid	0.11 ± 0.01	0.05 ± 0.00	0.02 ± 0.00
Ferulic acid sulphate	3.51 ± 0.15	0.07 ± 0.00	2.38 ± 0.18
Ferulic acid glucuronide	0.36 ± 0.01	0.03 ± 0.00	0.13 ± 0.00
Dihydroferulic acid	0.52 ± 0.03	n.d.	n.d.
Dihydroferulic acid sulphate	3.61 ± 0.21	n.d.	0.18 ± 0.00
Dihydroferulic acid glucuronide	0.93 ± 0.07	n.d.	0.11 ± 0.01

	Red-fleshed apple pasteurized purée		
(Poly)phenolic compound	**V1**	**V2**	**V3**
Anthocyanins derivatives (nmols)			
Cyanidin arabinoside	0.79 ± 0.15	1.75 ± 0.26	0.07 ± 0.01
Cyanidin galactoside	0.27 ± 0.01	n.d.	0.28 ± 0.01
Cyanidin glucuronide	0.74 ± 0.22	n.d.	0.60 ± 0.03
Peonidin galactoside	0.33 ± 0.01	n.d.	0.42 ± 0.06
Peonidin glucuronide	n.d.	n.d.	n.d.
Methyl peonidin glucuronide	n.d.	n.d.	n.d.
Cyanidin sulphate glucuronide	n.d.	n.d.	n.d.
Cyanidin galactoside glucuronide	n.d.	n.d.	n.d.
Peonidin galactoside glucuronide	n.d.	n.d.	n.d.
Catechols derivatives (µmols)			
Catechol sulphate	22.9 ± 0.65	17.2 ± 0.56	23.9 ± 0.87
Methyl catechol sulphate	18.8 ± 1.18	1.89 ± 0.02	11.5 ± 0.59
Catechol glucuronide	0.62 ± 0.09	0.24 ± 0.04	0.48 ± 0.03
Methyl catechol glucuronide	3.25 ± 0.07	0.31 ± 0.01	1.57 ± 0.10
Flavan-3-ols derivatives (µmols)			
Catechin sulphate	n.d.	0.09 ± 0.00	n.d.
Epicatechin sulphate	0.25 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
Methyl catechin sulphate	0.35 ± 0.02	n.d.	n.d.
Methyl epicatechin sulphate	0.05 ± 0.00	0.26 ± 0.02	0.15 ± 0.01
Catechin glucuronide	0.16 ± 0.01	0.13 ± 0.01	0.27 ± 0.02
Epicatechin glucuronide	0.12 ± 0.01	0.13 ± 0.01	0.08 ± 0.01
Methyl catechin glucuronide	n.d.	n.d.	n.d.
Methyl epicatechin glucuronide	n.d.	n.d.	n.d.
Phenylvalerolactones derivatives (µmols)			
Hydroxyphenyl-ɣ-valerolactone sulphate	4.88 ± 0.18	2.84 ± 0.10	0.86 ± 0.03
Dihydroxyphenyl-ɣ-valerolactone sulphate	50.7 ± 2.60	5.90 ± 0.30	24.1 ± 1.24

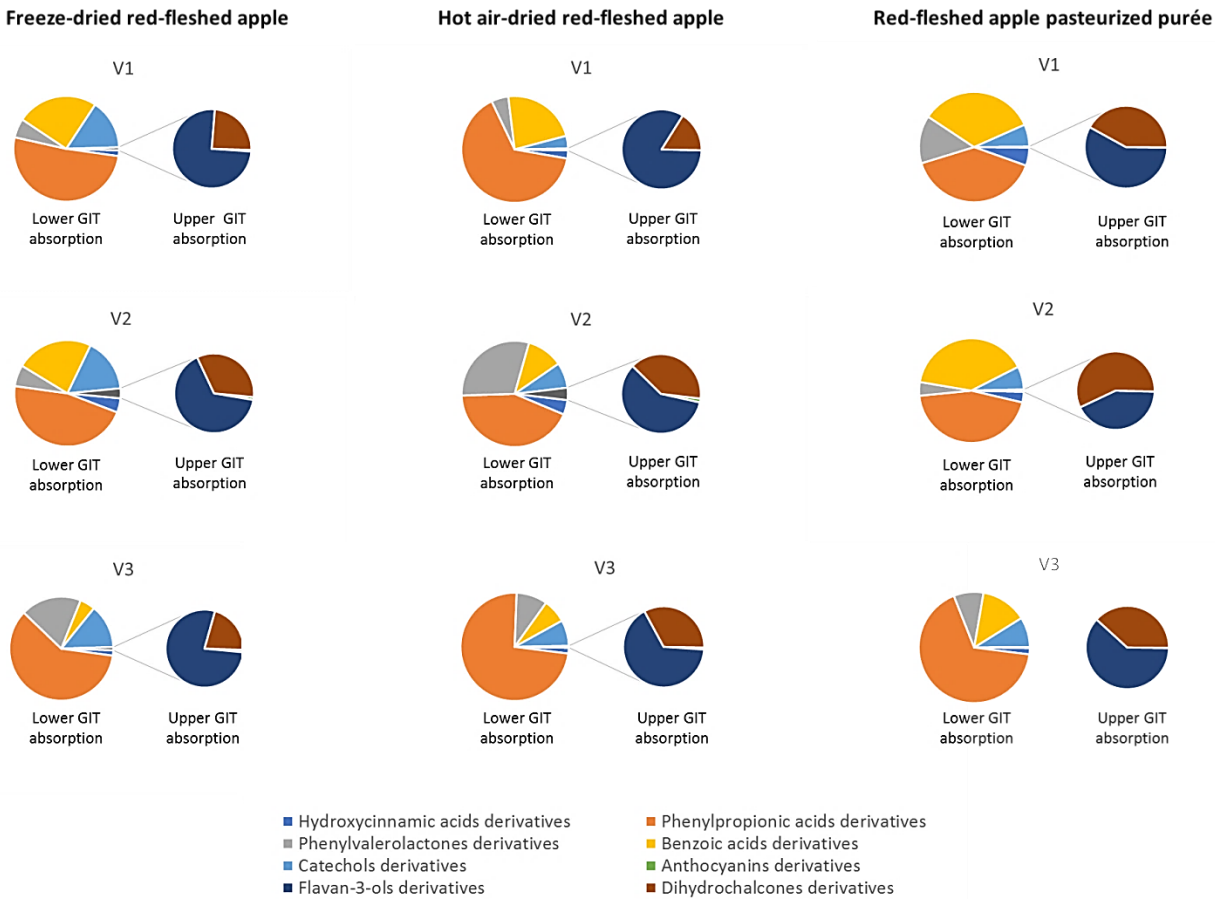
Dihydroxyphenyl-ɣ-valerolactone glucuronide	37.5 ± 2.21	1.63 ± 0.10	6.91 ± 0.41
Dihydroxyphenyl-ɣ-valerolactone sulphate glucuronide	4.53 ± 0.30	0.99 ± 0.07	4.95 ± 0.22
Dihydroxyphenylvaleric acid	n.d.	n.d.	n.d.
Dihydrochalcones derivatives (µmols)			
Phloretin sulphate	0.18 ± 0.01	0.11 ± 0.01	0.09 ± 0.00
Phloretin glucuronide	0.38 ± 0.02	0.80 ± 0.06	0.27 ± 0.01
Phloretin sulphate glucuronide	0.12 ± 0.01	0.13 ± 0.01	0.06 ± 0.00
Phenylpropionic acids derivatives (µmols)			
Hydroxyphenylpropionic acid	42.6 ± 1.55	1.40 ± 0.05	18.0 ± 0.65
Hydroxyphenylpropionic acid sulphate	108 ± 5.52	28.8 ± 1.48	77.7 ± 3.99
Hydroxyphenylpropionic acid glucuronide	1.49 ± 0.09	0.82 ± 0.05	2.09 ± 0.12
Dihydroxyphenylpropionic acid	4.68 ± 0.31	2.39 ± 0.16	0.95 ± 0.06
Dihydroxyphenylpropionic acid sulphate	119 ± 8.87	85.3 ± 6.35	183 ± 13.6
Dihidrocaffeic acid glucuronide	0.22 ± 0.01	0.76 ± 0.03	0.41 ± 0.02
Benzoic acids (µmols)			
Vanillic acid	3.13 ± 0.11	1.73 ± 0.06	n.d.
Vanillic acid sulphate	61.9 ± 3.17	57.2 ± 2.93	19.4 ± 0.70
Vanillic acid glucuronide	6.67 ± 0.39	4.32 ± 0.25	3.08 ± 0.16
Homovanillic acid	n.d.	n.d.	n.d.
Homovanillic ac sulphate	21.8 ± 1.45	7.22 ± 0.48	n.d.
Protocatechuic acid	n.d.	n.d.	n.d.
Protocatechuic acid sulphate	19.1 ± 1.42	13.8 ± 1.03	14.0 ± 0.82
Protocatechuic acid glucuronide	0.70 ± 0.03	0.04 ± 0.00	0.21 ± 0.01
Hydroxytyrosol sulphate	20.2 ± 0.73	0.66 ± 0.02	n.d.
Hydroxytyrosol glucuronide	0.11 ± 0.01	0.20 ± 0.01	0.09 ± 0.01
p-hydroxybenzoic acid	4.19 ± 0.25	2.30 ± 0.14	1.01 ± 0.04
Hydroxybenzoic acid sulphate	64.1 ± 4.27	5.08 ± 0.34	2.72 ± 0.10
Hydroxybenzoic acid glucuronide	0.86 ± 0.06	0.05 ± 0.00	0.18 ± 0.01
Hydroxyhippuric acid	33.6 ± 1.48	14.6 ± 0.64	16.4 ± 0.96
Hydroxycinnamic acids derivatives (µmols)			
Coumaric acid	1.42 ± 0.05	0.52 ± 0.02	0.24 ± 0.01
Coumaric acid sulphate	25.1 ± 1.29	1.02 ± 0.05	1.16 ± 0.06
Caffeic acid sulphate	1.44 ± 0.08	0.94 ± 0.05	0.73 ± 0.04
Caffeic acid glucuronide	n.d.	n.d.	n.d.
Ferulic acid	0.25 ± 0.02	0.14 ± 0.01	0.18 ± 0.01
Ferulic acid sulphate	2.78 ± 0.21	3.79 ± 0.28	1.21 ± 0.09
Ferulic acid glucuronide	0.29 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
Dihydroferulic acid	2.22 ± 0.08	0.44 ± 0.02	2.69 ± 0.10
Dihydroferulic acid sulphate	2.79 ± 0.14	1.55 ± 0.08	1.15 ± 0.06
Dihydroferulic acid glucuronide	0.83 ± 0.05	0.29 ± 0.02	0.41 ± 0.02



Supplemental Figure 1. Benzoic, phenylpropionic, phenylvalerolactones, catechol and hydroxy-cinnamic derivatives excretion kinetics (in µmol/h) in urine for each product derived from red-fleshed apple. Data expressed as mean values +/- SD of the sum of phase II methyl-, sulphate-, and glucuronide metabolites detected in urine 0-24 h post ingestion. Data of single compounds excretion kinetics are shown in Supplemental Table 4.



Supplemental Figure 2. Anthocyanins (in nmol/h), dihydrochalcones (in µmol/h) and flavan-3-ols derivatives (in µmol/h) excretion kinetics in urine for each product derived from red-fleshed apple. Data expressed as mean values +/- SD of the sum of glycosides and phase II methyl-, sulphate-, and glucuronide metabolites detected in urine 0-24 h post ingestion. Data of single compounds excretion kinetics are shown in Supplemental Table 4.



Supplemental Figure 3. Schematic representation (% of each group over the total) of the main (poly)phenolic groups excreted in urine of each volunteer after the intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée.

OBJECTIVE 3

PUBLICATION II: VALIDATION OF DRIED BLOOD SPOT CARDS TO DETERMINE APPLE (POLY)PHENOLIC METABOLITES IN HUMAN BLOOD AND PLASMA AFTER AN ACUTE INTAKE OF RED-FLESHED APPLE SNACK

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Validation of dried blood spot cards to determine apple (poly)phenolic metabolites in human blood and plasma after an acute intake of red-fleshed apple snack

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Abstract: The application of dried blood spot (DBS) cards for the study in human blood of dietary (poly)phenol bioavailability has been poorly studied. An analytical method based on blood sampling with DBS cards combined with LC-MS/MS has been developed and validated. To test the method validation, the (poly)phenolic metabolites are determined in human blood and plasma obtained after an acute intake of a red-fleshed apple snack in ten volunteers. Capillary blood by finger prick is compared to venous blood by venipuncture and whole blood is also compared to their corresponding venous plasma samples. Moreover, the venous plasma results using DBS cards are compared to those obtained by microElution solid phase extraction (μSPE). The main (poly)phenolic metabolites detected in blood and plasma samples are phloretin glucuronide, dihydroxyphenylpropionic acid sulphate, (methyl) catechol sulphate, catechol glucuronide, and hydroxyphenyl-γ-valerolactone glucuronide. No significant differences are observed between capillary blood, venous blood, and plasma samples using DBS, and neither between plasma samples analyzed by DBS or μSPE. Finger-prick blood sampling based on DBS appears to be a suitable alternative to the classic invasive venipuncture for the determination of circulating (poly)phenolic metabolites in nutritional postprandial studies.

KEYWORDS: dried blood spot cards, MS/MS, (poly)phenolic metabolites, plasma, whole blood

1. Introduction

Over recent years, there has been a large increase in the reporting of the use of dried blood spot (DBS) cards for therapeutic drug monitoring and quantitative biomarker assessment (Wagner et al., 2016). It has established itself as an innovative sampling technique where wet blood is spotted onto absorbent paper offering several potential benefits inherent to the technique, namely being less invasive than venous blood sampling, simplified blood sample collection, and convenient sample storage (Evans et al., 2015). The small volume of the blood sample and complexity of the matrix leads to analytical challenges in terms of sensitivity and selectivity that could have hampered the attractiveness of DBS cards. However, these limitations have been overcome by the significant improvement in the sensitivity of current LC-MS/MS instruments (Wagner et al., 2016).

One area that has received less attention in the application of DBS cards as a blood collection technique concerns the evaluation of the pharmacokinetic parameters of food bioactive compounds, such as dietary (poly)phenols. Red blood cells, particularly erythrocytes, represent a significant compartment for distribution of drugs and endogenous biological metabolites and it has been suggested that these could be factored into pharmacokinetic and pharmacodynamic evaluations (Hinderling, 1997). In fact, the binding of low concentrations of (poly)phenols to red blood cells has been previously reported (Biasutto et al., 2010; Kurlbaum et al., 2013; Rubió et al., 2014). Therefore, the possibility of direct blood sampling as the mainstay matrix instead of plasma might yield valuable information in *in vivo* bioavailability studies of dietary (poly)-phenols.

Moreover, as DBS sampling is based on arterial capillary blood and some amount of interstitial fluid, the concentrations of circulating metabolites could potentially be different from venous blood. As these differences are dependent on the characteristics of particular drugs or diet xenobiotics, case-to-case evaluation is necessary to validate the method (Antunes et al., 2016).

The analysis of (poly)phenolic metabolites in human blood was successfully applied in our previous studies after the acute intake of strawberry tree fruit (Mosele et al., 2016), and an olive oil phenolic extract (López de las Hazas et al., 2016). In these studies, the blood was collected with microcapillary blood collection tubes, and a fixed volume of blood was spotted onto the filter paper with a micropipette. However, a simplified strategy to quantify the (poly)phenolic metabolites by placing blood directly onto the DBS cards has not been tested.

The aim of the present study was to develop and validate an analytical method based on the combination of DBS cards as simple sampling procedure combined with a sensitive chromatographic method (ultra-performance LC coupled to MS/MS, UPLC-MS/MS) to analyze the main circulating (poly)phenolic metabolites in blood and plasma after a human acute intake of a red-fleshed apple snack. The method was validated in terms of linearity, reproducibility, method detection limits, method quantification limits, accuracy, and matrix effects. Specifically, the study focuses on three issues: 1) the comparison of venous and capillary blood sampling, 2) the assessment of the differences between plasma and whole blood, and 3) the comparison of DBS cards and microElution solid phase extraction (μSPE), as the most common method for the analysis of circulating (poly)phenolic metabolites in plasma.

2. *Experimental section*

2.1. *Chemicals and reagents*

The commercial standard 3-(2',4'-dihydroxyphenyl)propionic acid was purchased from Fluka (Buchs, Switzerland), epicatechin was from Sigma-Aldrich (St Louis, MO, USA), and phloretin-2'-*O*-glucoside from Extrasynthese (Genay, France). Catechol-4-*O*-sulphate and 4-methyl catechol sulphate were supplied by Dr. Claudia N. Santos (IBET, Oeiras, Portugal) and synthetized according to the method reported by Pimpao et al. (2015) Stock solutions of individual (poly)phenolic standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg L^{-1}, and storing these in dark flasks at 4°C. Acetonitrile (HPLC-grade) was from Romil (Teckno-kroma, Barcelona, Spain). Methanol (HPLC-grade), phosphoric acid (85%), and glacial acetic acid (99.8%) were from Scharlau S.L. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. *Red-fleshed apple snack*

The red-fleshed apple variety used was 'Redlove Era' and was provided by Nufri (Mollerussa, Lleida, Spain) and planted in the experimental plot at "La Rasa" (Soria, Spain). Immediately after the fresh apples arrived at the laboratory, they were washed, wiped with paper towels and cut into 1 cm cubes. The apple cubes were frozen in liquid nitrogen and freeze-drying was then performed with a first drying at 0.6 bar with a temperature ramp of -20 to 0°C for 25 h, followed by a second complete vacuum drying with a temperature ramp of 0 to 20°C for 40 h (Lyophilizer TELSTAR Lyobeta 15, Terra-ssa, Spain). After that, the apple snack was preserved in a desiccator protected from light.

For the quantification of (poly)phenolic compounds, 0.1 g of crushed apple snack

was analyzed according to previous study (Bars-Cortina et al., 2017). The phenolic dose ingested through a portion of 80 g of red-fleshed apple snack is shown in **Table 1**, where 46% were phenolic acids (mainly chlorogenic acid), 22% were anthocyanins (mainly cyanidin-3-*O*-galactoside), 17% dihydrochalcones (mainly phloretin glucoside derivatives), 9% flavonols (mainly quercetin derivatives), 7% flavan-3-ols (mainly epicatechin and dimer), and 0.2% flavanone (mainly eriodictyol derivatives).

Table 1. (Poly)phenolic compounds ingested in a dose of 80 g of red-fleshed apple snack.

(Poly)phenolic compound	mg/80 g red-fleshed apple snack
Cyanidin galactoside	39.7 ± 1.0
Cyanidin arabinoside	2.60 ± 0.24
Total Anthocyanins	**42.3 ± 1.18**
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
Total Phenolic acids	**88.03 ± 3.34**
Epicatechin	5.58 ± 0.78
Dimer	6.92 ± 0.29
Trimer	1.30 ± 0.12
Total Flavan-3-ols	**13.8 ± 1.18**
Quercetin arabinoside	3.67 ± 0.45
Quercetin rhamnoside	9.26 ± 0.94
Quercetin glucoside	4.41 ± 0.57
Total Flavonols	**17.3 ± 1.97**
Eriodictyol hexoside	0.42 ± 0.02
Total Flavanones	**0.42 ± 0.02**
Phloretin glucoside	21.7 ± 2.54
Phloretin xylosyl glucoside	11.7 ± 0.52
Hydroxyphloretin xylosil glucoside	0.32 ± 0.03
Total Dihydrochalcones	**33.7 ± 3.08**
Total (Poly)phenols	***195 ± 9.60***

2.3. Study design and blood sample collection

The protocol of the study was approved by the Ethical Committee of Human Clinical Research at the Arnau Vilanova University Hospital, Lleida, Spain (approval number: 13/2016). Ten heathy volunteers (five females and five males, mean age 37.3 ± 8.4 years) were recruited and exclusion criteria were age <25 or >50 years, body mass index <18.5 or >24.9 kg m^{-2}, pregnancy or lactation, any chronic medication, any antibiotic treatment during the 4 months prior to the study, cigarette smoking, alcohol intake >80 g d^{-1}, and use of dietary supplements. Subjects were asked to avoid the consumption of (poly)phenol-rich foods (e.g., coffee, fruit, vegetables, dark chocolate, green tea, and red wine) for the 3 days prior to the study. On the day of the study, the participants were invited to eat a portion of 80 g of red-fleshed apple snack after fasting overnight. Capillary blood, venous blood, and plasma samples were obtained at the baseline and at different time points after consumption of the apple snack (0 to 24 h). During this period, the participants avoided the consumption of (poly)phenol-rich foods. Capillary blood was obtained by fingerpricking and blood drops were directly applied to DBS cards at 0, 0.5, 1, 2, 4, 6, 12, and 24 h. Venous blood was collected by venipuncture at 0, 0.5, 1, 2, 4, 6, and 24 h in 6 mL Vacutainer tubes

(Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing EDTA as an anticoagulant. To obtain plasma samples, the blood tubes were centrifuged at 8784 *g* for 15 min (Hettich, Tuttlingen, Germany). The blood and plasma samples were aliquoted and stored at -80°C until further processing.

2.4. Blood and plasma samples pre-treatment

2.4.1. DBS cards

Capillary blood samples were obtained by pricking the volunteers' fingers with disposable lancets (Unistik, Owen Mumford Ltd, Woodstock, UK) and collected on FTA DMPK-A cards (DBS filter paper) (GB Healthcare, Buckinghamshire, UK). The first droplet was discarded, since the initial flow from the 'prick' can be contaminated with interstitial fluid, and good practice warrants discarding this first drop. After that, blood droplets were directly spotted on to two premarked circles on the filter papers until they were completely soaked, while avoiding direct contact between the finger and the card. Venous blood obtained by venipuncture and the corresponding plasma samples were also applied to DBS cards. Thirty microliters of venous blood or plasma was defined as the exact volume to completely fill each premarked circle. The pre-treatment procedure is depicted in **Figure 1A**.

In order to dry the spotted cards, they were maintained in the dark at room temperature for 2 h. Afterward, in order to extract and preconcentrate the target compounds, the whole surface of two blood or plasma-soaked circles were punched out using a 3-mm diameter Harris Uni-Core punch and a Cutting Mat (Whatman Inc., Sanford, ME, USA).

Different conditions were tested for extracting the analytes, and the optimal conditions appeared to be 150 µL of methanol/Milli-Q water (50:50, v/v) as the elution solvent, vortexed for 20 min and centrifuged at 8784 *g* for 10 min at room temperature. The supernatant was filtered with 0.22 µL Nylon 96-well filter microplate (Agilent technologies, Santa Clara, CA, USA), and 7.5 µL of the filtered solution was injected into the chromatographic system.

2.4.2. µSPE cartridges

µSPE was also applied for the analysis of the main circulating (poly)phenolic metabolites in venous plasma samples as it is the most common sample pre-treatment method used in previous studies, and the obtained results were compared to those achieved from plasma by using DBS cards. The µSPE methodology used was based on previous studies (Martí et al., 2010; Pérez-Ternero et al., 2017). Briefly, the µElution plates (OASIS HLB 30 µm, Waters,

Milford, MA, USA) were activated with 250 µL of methanol and equilibrated with 250 µL of 0.2% acetic acid. 350 µL of venous plasma and 350 µL of 4% phosphoric acid were centrifuged at 8784 *g* for 10 min at 4°C, and the supernatant was loaded into the micro-cartridge. 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 compounds were eluted with 2 × 50 µL of methanol. Eluted solution (2.5 µL) was directly injected into the chromatographic system (**Figure 1B**).

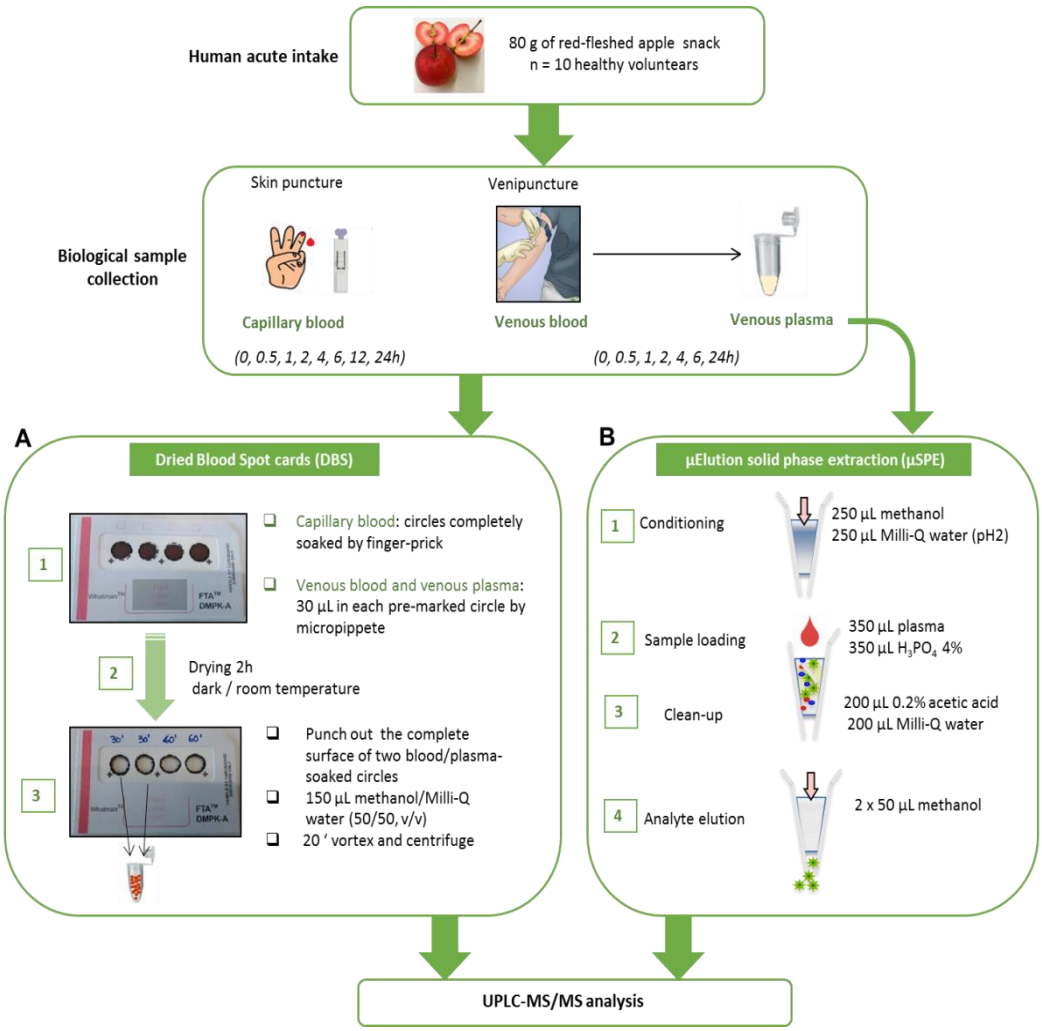

Figure 1. Schematic representation of the sample pre-treatment protocol developed based on DBS cards and µSPE.

2.5. (Poly)phenolic metabolites analysis by UPLC-MS/MS

The (poly)phenolic compounds and their generated metabolites were determined in blood and plasma samples by AcQuity UPLC coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford). The analytical column was an AcQuity BEH C_{18} column (100 mm × 2.1 mm id, 1.7 μm) equipped with a VanGuard Pre-Column AcQuity BEH C_{18} (2.1 × 5 mm, 1.7 μm), also from Waters. During the analysis, the column was kept at 30°C, and the flow rate was 0.3 mL min^{-1}. The mobile phase and elution gradient were the same as those reported in previous studies (Bars-Cortina et al., 2017; Mosele et al., 2016).

MS/MS analyses were carried out on a TQD mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. Ionization was achieved using the electrospray interface operating in the negative mode $[M-H]^-$ and the data were acquired through selected reaction monitoring (SRM). The ionization source parameters were the same as the ones reported in previous studies (Bars-Cortina et al., 2017; Mosele et al., 2016).

Two SRM transitions were selected, the most sensitive one was used for quantification, and the second for confirmation purposes. **Supplemental Table 1** shows the SRM transition for quantification and identification, as well as the cone voltage and collision energy for each (poly)phenolic metabolite. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software. Due to the lack of commercial standards of (poly)phenolic metabolites, some of these compounds were tentatively quantified by using the calibration curve of their native compound or another (poly)phenolic compound with a similar structure. Phloretin glucuronide was tentatively quantified by using the calibration curve of phloretin-2'-*O*-glucoside, dihydroxyphenylpropionic acid sulphate by using the calibration curve of 3-(2',4'-dihydroxyphenyl)propionic acid, and hydroxyphenyl-ɣ-valerolactone by using the calibration curve of epicatechin. Catechol sulphate and methyl catechol sulphate were quantified with their own calibration curves, and catechol glucuronide by using the calibration curve of catechol-4-*O*-sulphate.

2.6. Validation procedure

The instrumental quality parameters of the developed methods, such as linearity, precision, accuracy, detection limits (LODs), and quantification limits (LOQs), as well as the extraction recovery (%R) and matrix effect (%ME) for the determination of the main (poly)phenolic metabolites in blood and plasma samples by DBS cards, and plasma samples by μSPE, were determined by spiking blank biological

samples (venous blood and plasma samples obtained under fasting conditions) with known concentrations of standard (poly)phenolic compounds. Calibration curves were prepared with venous blood, since it was not possible to obtain large volumes of blank capillary blood. These instrumental quality parameters, as well as the %R and %ME, were determined as reported in previous study (Serra et al., 2013), and the results obtained are shown in **Table 2**.

2.7. Statistical analysis

The quantitative data were analyzed by Student's t-test in order to determine significant differences between mean values of the concentration of the main circulating apple (poly)phenolic metabolites in: 1) the capillary and venous blood at different post-intake times, 2) the venous blood (venipuncture) and plasma samples (venipuncture) at different times, and 3) the venous plasma analyzed by DBS cards and by μSPE at different times. Significant differences were considered at the level of $p < 0.05$. All the statistical analyses were carried out using STATGRAPHICS Plus 5.1 (Manugistics Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Optimization of DBS technique for (poly)phenolic metabolite analysis

This work was based on our previous studies, where phenolic metabolites were determined in plasma or blood samples after the acute intake of strawberry tree fruit (Mosele et al., 2016) and olive oil phenolic extract (López de las Hazas et al., 2016). In these previous studies, 20 μL of venous blood or plasma were spotted onto one premarked circle on the DBS and seven disks of 2 mm diameter were punched out from the card using 100 μL of methanol/Milli-Q water (50/50, v/v) for the phenolic extraction. Under these experimental conditions, only the most abundant circulating phenol metabolites were detected.

In the present study, in order to enhance the method sensitivity, different conditions were tested to improve the extraction and increase the preconcentration of the analytes. We also aimed to explore a strategy to quantify the (poly)phenolic metabolites directly by depositing blood droplets on DBS card in order to simplify the process.

Table 2. Instrumental quality parameters for the analysis by UPLC-MS/MS of the studied (poly)phenolic compounds in spiked venous blood and plasma samples by DBS cards, and venous plasma samples by μSPE.

(Poly)phenolic compound	RT (min)	%R	%ME	Linearity (μM)	%RSD (*n*=3), inter-day			Accuracy (%), *n*=3, (0.1 μM)	LOQ (nM)	LOD (nM)
					5 μM	0.1 μM	0.01 μM			
DBS cards										
Venous blood										
Catechol-4-*O*-sulphate	3.01	85 ± 0.1	4.52 ± 0.1	0.09-10	5.1	6.4	8.2	97	90	30
4-Methyl catechol sulphate	4.87	82 ± 0.3	9.50 ± 0.1	0.08-10	4.3	5.9	8.6	98	85	30
3-(2',4' dihydroxyphenyl)propionic acid	5.36	75 ± 0.2	7.91 ± 0.1	0.08-10	6.2	7.5	8.5	101	85	30
Epicatechin	7.05	90 ± 0.1	-5.83 ± 0.2	0.09-10	5.9	6.8	8.8	102	95	30
Phloretin-2'-*O*-glucoside	16.4	80 ± 0.2	-18.5 ± 0.1	0.04-10	7.8	8.9	10	101	45	15
Venous plasma										
Catechol-4-*O*-sulphate	3.01	83 ± 0.1	5.68 ± 0.1	0.07-10	4.8	6.1	8.3	103	70	25
4-Methyl catechol sulphate	4.87	80 ± 0.2	10.3 ± 0.2	0.07-10	4.6	6.4	8.3	101	73	25
3-(2',4'-dihydroxyphenyl)propionic acid	5.36	80 ± 0.1	-6.82 ± 0.1	0.07-10	6.6	7.3	8.8	100	75	25
Epicatechin	7.05	88 ± 0.2	-16.7 ± 0.2	0.05-10	5.8	7.4	9.4	99	50	20
Phloretin-2'-*O*-glucoside	16.4	80 ± 0.1	-17.2 ± 0.1	0.03-10	7.8	8.9	10	101	35	15
μSPE										
Venous plasma										
Catechol-4-*O*-sulphate	3.01	63 ± 0.1	-4.60 ± 0.1	0.0008-10	5.2	5.9	8.2	102	0.8	0.3
4-Methyl catechol sulphate	4.87	59 ± 0.1	-6.71± 0.2	0.001-10	3.9	5.2	8.0	100	1	0.3
3-(2',4'-dihydroxyphenyl)propionic acid	5.36	95 ± 0.2	-5.35 ± 0.1	0.007-10	5.8	6.2	8.1	98	7	2
Epicatechin	7.05	98 ± 0.2	-6.39 ± 0.2	0.05-10	5.4	6.8	8.5	101	50	20
Phloretin-2'-*O*-glucoside	16.4	90 ± 0.1	-9.99 ± 0.1	0.003-10	7.0	8.0	9.7	99	3	1

The factors studied were: (i) the blood volume spotted on the premarked circle (from 20 to 50 μL), (ii) the number of disks punched out from the card (from seven disks to the entire premarked circle) and the number of the premarked circles (1 or 2), (iii) the nature of the extraction solvent (methanol, methanol/Milli-Q water (50/50, v/v), and acetonitrile), and its volumen (from 100 to 200 μL), and (iv) the extraction time with vortex (from 5 to 30 min).

In the present study, six different phenolic metabolites derived from phase II and microbial metabolism were detected in both the blood and plasma human samples. Specifically, the main metabolites detected were phloretin glucuronide, dihydroxyphenylpropionic acid sulphate, (methyl) catechol sulphate, catechol glucuronide, and hydroxyphenyl-γ-valerolactone glucuronide. Therefore, to optimize the method different phenol standards as catechol-4-*O*-sulphate, 4-methyl catechol sulphate, 3-(2',4'-dihydroxy-phenyl)propionic acid, epicatechin, and phloretin-2'-*O*-glucoside were spiked at known concentrations into a pooled venous human blood obtained under fasting conditions.

3.1.1. Blood volume spotted on the premarked circle

Four different blood volumes applied to the cards were tested (20, 30, 40, and 50 μL) (data not shown). In our previous study, volumes from 5 to 20 μL were

tested and a greater instrumental response of the phenolic standards was reported as the blood volume increased (López de las Hazas et al., 2016). In the present study, 30 μL was selected as the optimum volume observing that, with this volume, one premarked circle was entirely soaked to its edge with all volunteers' blood samples. This fact denoted that the viscosity of the blood from all volunteers was similar as were its spreading properties on the DBS cards, thus indicating a similar hematocrit.

When droplet of capillary blood is directly deposited on the filter paper, the volume of this blood cannot be precisely controlled and inaccuracies in quantification values can be obtained due to differences in hematocrit. In the literature, different strategies to overcome this drawback have been proposed, such as the use of volumetric absorptive microsampling (VAMS) (Kok & Fillet, 2018) or the photometric measurement of the protein content (Riley et al., 2015). In the present study, a simplified strategy to quantify the (poly)phenolic metabolites was tested, assuming a linear relation between volume of blood applied and the area of the premarked DBS circle. In this sense, we asked volunteers to soak the entire surface of the premarked circles with capillary blood, estimating that the entire surface of one premarked circle contained 30 μL of capillary blood, as observed when a fixed volume of the volunteers' blood was dispensed on to the DBS.

3.1.2. Spot punch surface

In the present study, the fact of punching out the maxim number of disks (eight 2-mm diameter disks) or the entire surface of the premarked circle was tested and the instrumental response of all the phenolic compounds studied increased slightly when the entire premarked circle was analyzed (data not shown). Moreover, in a previous study, it was reported that the whole spot approach can effectively avoid any hematocrit effect in the analysis of a specific drug (apixaban) in human DBS samples compared to a partial spot-center punch (Zheng et al., 2015). Moreover, the fact of analyzing two instead of one premarked circles with reduced elution solvent volume was also tested, and a major preconcentration effect of the target compounds was observed.

3.1.3. Extraction solvent volume and nature

The tested elution volumes were 100, 150, and 200 μL. The use of 100 μL was discarded because this was not large enough to cover two entire premarked circles. Volumes of 150 and 200 μL allowed the (poly)phenolics to be extracted with good %R, but with 150 μL, a higher preconcentration rate was obtained and the %R and %ME were not significantly affected (data not shown). Different elution solvents were also tested in order to obtain the maximum sensitivity (peak

efficiency or narrow peaks) and maximum extraction recovery (%R). Among all the elution solvents tested, methanol/Milli-Q water (50/50, v/v) was the optimum in terms of peak efficiency, and %R, and therefore this elution solvent was chosen.

3.1.4. Extraction time

First, the extraction time (vortex) of the disks with the elution solvent was optimized, and 5, 10, 20, and 30 min were tested. It was observed that the extraction of the main (poly)phenolic metabolites increased with the extraction time and this fact was observed until 20 min with no differences between 20 and 30 min, so 20 min was selected as the optimum time (data not shown). So, the optimum pre-treatment conditions with DBS cards were defined as: 30 μL of venous blood in each premarked circle, punching out the entire surface of two premarked circles, 150 μL of methanol/Milli-Q water (50/50, v/v) solution as the extraction solvent and 20 min as the extraction time (vortex) (**Figure 1A**). The same conditions were used for the venous plasma and capillary blood. Under these experimental conditions, the %R of all the studied (poly)phenolics was above 75% and the %ME was lower than 18% (**Table 2**).

3.2. Analytical methods validation

The instrumental quality parameters of the developed methods using DBS cards for the analysis of the venous blood and plasma samples, and μSPE for the analysis of plasma samples, are shown in **Table 2**. In order to carry out these studies, catechol-4-*O*-sulphate, 4-methyl catechol sulphate,3-(2',4'-dihydroxyphenyl)propionic acid, epicatechin, and phloretin-2'-*O*-glucoside were spiked into blank venous blood samples and blank plasma samples at different known concentrations. The linearity range was from 0.04 to 10 μM for venous blood, and 0.03 to 10 μM for plasma samples when DBS cards were used; and 0.0008 to 10 μM for plasma samples when μSPE was used.

The calibration curves (based on the integrated peak area) were calculated by using five points at different concentration levels, and each concentration was injected three times. The determination coefficient (R^2) of the calibration curves was higher than 0.993. The precision of the analytical method (reproducibility) were determined by the %RSD in terms of concentration, and these were calculated at three concentration levels, these being 5, 0.1, and 0.01 μM for the analysis of venous blood and plasma samples (**Table 2**).

For all the (poly)phenolic compounds the %RSDs were lower than 10% in both DBS cards and μSPE methods. The accuracy was calculated from the ratio between the concentration found for the standard (poly)phenolic compounds studied compared with the spiked concentration.

This quotient was then multiplied by 100. This quality parameter was also studied at three concentration levels, the same as for the RSD%, and these ranged from 97% to 103%.

The LODs and LOQs were calculated using the S/N ratio criterion of 3 and 10, respectively. The respective values were in the 15-30 and 45-95 nM ranges for venous blood samples, and the 15-25 and 34-75 nM ranges for plasma samples when DBS cards were used. These results were 10-fold lower to our previous study where olive oil (poly)phenol metabolites were determined in blood samples using DBS cards (López de las Hazas et al., 2016). So, the fact of punching two entire premarked circles and decrease the elution solvent volume, improved the sensitivity of the method.

The LODs and LOQs in the analysis of plasma samples by μSPE were 0.3-20 and 0.8-50 nM, respectively. It is important to highlight the lower LODs and LOQs in comparison with DBS cards. This fact could be explained by the analyte preconcentration (3.5-fold) that is performed in μSPE (350 μL of plasma are loaded and 100 μL elution solvent are used to elute). Nevertheless when DBS cards are used, the analytes are diluted (2.5-fold). So, 60 μL of biological samples are directly deposited in the circles of the DBS card (two circles with 30 μL each one) and then 150 μL of extraction solvent are used to analyze the compounds of interest.

The %R for the analysis of the studied phenolics in venous blood and plasma samples (in both DBS cards and μSPE) were similar and these were higher than 75% (**Table 2**), except for (methyl) catechol sulphate in μSPE, being between 59% and 63%. The %ME for the analysis of the studied phenolics in these biological samples was lower than 15%, being lower in the μSPE pre-treatment (**Table 2**).

3.3. Capillary blood versus venous blood in DBS cards

Figure 2 shows the time course of the main generated (poly)phenolic metabolites determined in capillary blood, venous blood, and venous plasma using DBS cards at different time points (0-24 h) after the acute intake of red-fleshed apple snack. When comparing mean values of capillary blood *versus* venous blood, no significant differences ($p > 0.05$) were observed in any metabolite or at any time point. Previous studies with drugs found concentrations about 1.7 times higher in capillary than venous blood (Ashley et al., 2010), but these differences are dependent on the characteristics of particular compounds, so case-to-case evaluation is necessary. The results of the present study indicate that in the case of apple (poly)phenolic metabolites, values do not differ significantly between venous blood and capillary blood.

Regarding the time-course data, the maximum concentration of the circulating metabolites phloretin glucuronide and dihydroxyphenylpropionic acid sulphate was found between 2 and 4 h, indicating their metabolic transformation in the small intestine and liver (**Figure 2**). The other metabolites (catechol sulphate, methyl catechol sulphate, catechol glucuronide, and hydroxyphenyl-ɣ-valerolactone glucuronide) appeared to have their maximum concentrations 12 h after consumption of the apple snack, indicating that they could be products of the gut microbiota catabolism in the colon.

One of the most important advantages of using DBS cards reported in the present study is the possibility of analyzing more post-intake time points by self-sampling compared to venipuncture. Particularly, the possibility of taking sample at 12 h enabled the detection and quantification of two microbial metabolites (catechol glucuronide and hydroxyphenyl-ɣ-valerolactone glucuronide) (**Figure 2**). These results indicate the importance of collecting these time points to gain deeper insights into the *in vivo* distribution of metabolites produced by gut microbiota.

3.4. Venous blood versus venous plasma samples in DBS cards

The concentration values of the (poly)-phenolic compounds determined in venous blood samples after the acute intake of the red apple snack were also compared to those obtained for the analysis of their corresponding plasma samples and the results showed no significant differences in any metabolite at any time point (**Figure 2**). Pharmacokinetic studies are commonly carried out focusing on the levels of (poly)phenolic metabolites in plasma or serum. However, in the case of some (poly)phenols their affinity for human blood cells has been observed. For instance, quercetin (Chandhuri et al., 2007; Fiorani et al., 2003) and resveratrol (Blache et al., 1997) are known to partition into blood cells, associating with cell membranes, hemoglobin, and other proteins. The binding of low concentrations of (poly)phenols to erythrocytes was also reported for caffeic acid, taxifolin, and ferulic acid in a human study (Kurlbaum et al., 2013) and hydroxytyrosol phase-II metabolites in a rat model (Rubió et al., 2014). In a recent study, it was also reported a differential distribution of (poly)phenolic compounds between serum and blood cells depending on the characteristics of particular (poly)phenols (Mülek et al., 2017), confirming that case-to-case evaluation is necessary.

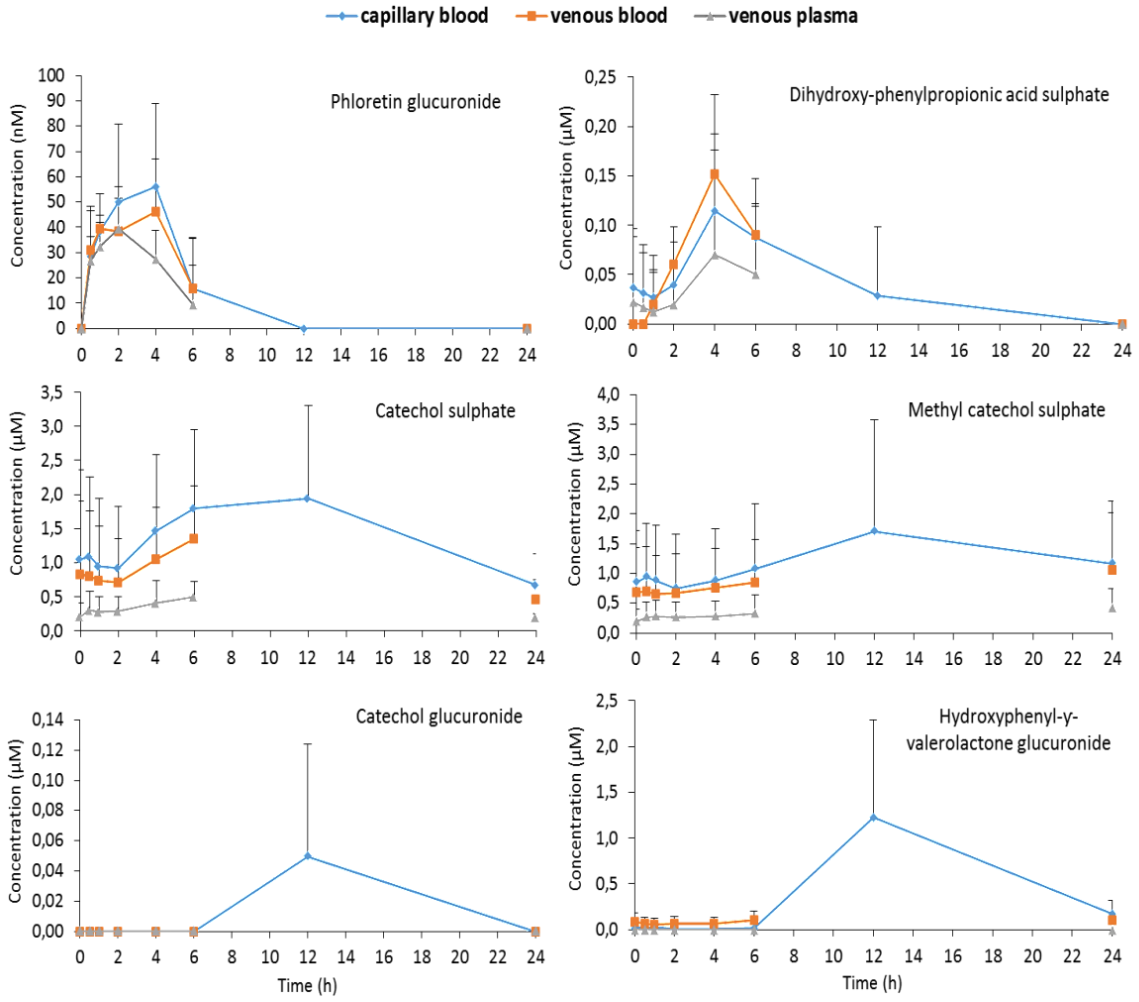

Figure 2. Time course of the main (poly)phenolic metabolites determined in capillary blood, venous blood, and venous plasma samples by DBS and UPLC-MS/MS after the acute intake of red-fleshed apple snack.

As shown in **Figure 2**, in the case of (poly)phenolic metabolites derived mainly from phenolic acids and anthocyanins from apple, plasma levels appear to be lower than blood concentration. However, no significant differences were found between blood and plasma, probably due to the large interindividual variability in the (poly)phenolic metabolism. Based on our results and also considering previous studies, the application of DBS cards with blood as the mainstay matrix might yield valuable information as a practical alternative to classic plasma analysis to determine the *in vivo* human absorption of (poly)phenolic metabolites.

3.5. DBS cards versus µSPE for venous plasma samples

µSPE is the sample pre-treatment technique most commonly used to extract (poly)phenolic metabolites from plasma samples. However, this technique requires venipuncture to obtain a sufficient volume of blood sample and subsequently of plasma sample. In order to evaluate

whether DBS cards can be an alternative strategy to μSPE, venous plasma samples were analyzed by using both methods.

Table 3 shows the average concentrations from the ten healthy volunteers of the main (poly)phenolic metabolites and no significant differences were detected among both methods. Although the sensitivity (LODs and LOQs) of DBS cards technique was lower than μSPE, the average concentrations of the main (poly)phenolic metabolites detected in plasma did not significantly differ between the two sample pre-treatment techniques. Therefore, we conclude that DBS cards has the limitation of its inability to accurately quantify low-level minor metabolites, but is still a suitable strategy for the analysis of the main (poly)phenolic intake biomarkers in human interventional studies, and also for urine analysis as we reported previously (Martí et al., 2010).

4. Concluding remarks

A method based on DBS cards combined with LC (UPLC-MS/MS) was developed and validated for the determination of (poly)phenolic metabolites in human blood and plasma samples. DBS cards has been reported as a rapid and easy blood-sampling strategy to determine the main circulating (poly)phenolic metabolites after an acute intake of red-fleshed apple snack. The comparison of capillary and venous sampling and also the assessment of the differences between whole blood and plasma samples showed no significant differences. In addition, there were no significant differences among DBS cards and μSPE.

Table 3. Mean concentration of the generated (poly)phenolic metabolites (μM or nM ± SD) in plasma samples, analyzed by DBS cards and μSPE combined with UPLC-MS/MS, after the acute intake of a red fleshed apple snack at different time points.

	0 h	0.5 h	1 h	2 h	4 h	6 h	24 h
Phloretin glucuronide (nM)							
DBS	n.d.	26.9 ± 9.46	32.5 ± 9.42	39.4 ± 16.8	27.6 ± 11.2	9.55 ± 15.4	n.d.
μSPE	n.d.	28.2 ± 11.2	42.9 ± 8.36	53.8 ± 16.2	54.0 ± 34.6	23.6 ± 16.4	n.d.
Dihydroxyphenylpropionic acid sulphate (nM)							
DBS	n.d.	17.3 ± 14.6	12.5 ± 19.4	19.5 ± 43.2	70.3 ± 105.6	50.6 ± 68.2	n.d.
μSPE	n.d.	14.4 ± 18.6	9.87 ± 10.2	10.6 ± 9.6	53.9 ± 63.1	33.0 ± 40.7	n.d.
Catechol sulphate (μM)							
DBS	0.22 ± 0.19	0.30 ± 0.28	0.27 ± 0.22	0.28 ± 0.21	0.40 ± 0.33	0.50 ± 0.23	0.20 ± 0.05
μSPE	0.42 ± 0.37	0.45 ± 0.36	0.42 ± 0.37	0.42 ± 0.29	0.55 ± 0.40	0.83 ± 0.71	0.29 ± 0.12
Methyl catechol sulphate (μM)							
DBS	0.19 ± 0.21	0.27 ± 0.25	0.28 ± 0.27	0.27 ± 0.25	0.28 ± 0.25	0.32 ± 0.31	0.42 ± 0.33
μSPE	0.41 ± 0.51	0.45 ± 0.47	0.45 ± 0.50	0.40 ± 0.46	0.35 ± 0.31	0.47 ± 0.44	0.47 ± 0.31

Catechol glucuronide and hydroxyphenyl-γ-valerolactone glucuronide were not detected in plasma samples at the collected time points; n.d. not detected.

The self-sampling of blood by the volunteers following simple instructions, allowed more time points to be collected during postprandial period, especially those late time points (12 h) to assess the intestinal microbial metabolism of (poly)phenolic compounds, which are normally missing with venipuncture. Our study reveals that finger-prick blood sampling based on DBS cards appears to be a suitable alternative to the classic invasive venipuncture for determining (poly)phenolic metabolites, including microbial fermentation catabolites, in human (poly)-phenol bioavailability and pharmacokinetic studies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Supplemental Table 1. Optimized SRM conditions for the analyses of the studied compounds and the main (poly)phenolic metabolites detected in blood and plasma samples by UPLC-MS/MS.

(Poly)phenolic compound	SRM 1 (Quantification)	Cone Voltage (V)	Collision energy (eV)	SRM 2 (Identification)	Cone Voltage (V)	Collision energy (eV)	Standard / metabolite
Catechol-4-*O*-sulphate	189 > 109	20	15	-	-	-	Standard and metabolite
Catechol glucuronide	285 > 109	20	15	-	-	-	Metabolite
4-Methyl catechol sulphate	203 > 123	20	15	-	-	-	Standard and metabolite
3-(2',4'-dihydroxyphenyl) propionic acid	181 > 137	35	15	181 > 121	40	25	Standard
Dihydroxyphenyl propionic acid sulphate	261 > 181	40	15	261 > 137	40	20	Metabolite
Epicatechin	289 > 245	40	15	289 > 205	40	20	Standard
Hydroxyphenyl-γ-valerolactone glucuronide	367 > 191	40	20	367 > 147	40	25	Metabolite
Phloretin-2'-*O*-glucoside	435 > 273	40	15	273 > 167	40	20	Standard
Phloretin glucuronide	479 > 273	40	20	479 > 167	40	25	Metabolite

OBJECTIVE 4

PUBLICATION III: *IN VIVO* BIOTRANSFORMATION OF (POLY)PHENOLS AND ANTHOCYANINS OF RED-FLESHED APPLE AND IDENTIFICATION OF INTAKE BIOMARKERS

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In vivo biotransformation of (poly)phenols and anthocyanins of red-fleshed apple and identification of intake biomarkers

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Abstract: The aim of this study was to investigate comprehensively the metabolic pathways and human bioavailability of anthocyanins and other (poly)phenols in an apple matrix, and to elucidate potential intake biomarkers. After the acute intake of a red-fleshed apple freeze-dried snack, plasma and urine were collected and analyzed by UPLC-MS/MS. A total of 37 phase-II and microbial (poly)phenolic metabolites were detected in plasma and urine. Among these, phloretin glucuronide, cyanidin-3-*O*-galactoside (plasma and urine) and peonidin-3-*O*-galactoside (urine) were the only metabolites detected in all the volunteers and not detected at basal conditions. The maximum urine excretion was detected at 2-4 h, and the main increase in plasma of phloretin glucuronide and cyanidin-3-*O*-galactoside was observed at 2 h post-intake (61.0 ± 6.82 and 10.3 ± 1.50 nM, respectively). These metabolites could be selected as the best intake biomarkers of red-fleshed apple and might be useful in human intervention studies when studying its health effects.

KEYWORDS: anthocyanins, metabolic pathways, (poly)phenolic compounds, red-fleshed apple, UPLC-MS/MS

1. Introduction

Apples are one of the most commonly consumed fruits and their diverse and high (poly)phenol content is considered one of the most important determinants of their health-promoting properties (Bondonno et al., 2018; Hyson, 2011). In the last few years, there has been a rapidly increasing interest in potential crops for coloring food naturally without transgenic or cysgenic programs. In order to obtain better-quality apples with added healthy properties, new genotypes of apple with red-flesh have been obtained by innovative breeding strategies through cross-breeding programs with wild red-fleshed apple varieties (with poor taste) and commercial good-flavored white-fleshed apples (Deacon, www.suttonelms.org.uk). The resulting red-fleshed apples contain a high amount of anthocyanin compounds in their flesh and have a good-tasting. Apart from anthocyanins, red-fleshed apples are also a rich source of other (poly)phenols that are also detected in common apple varieties such as phenolic acids, dihydrochalcones, flavan-3-ols, and flavonols (Bars-Cortina et al., 2017). Due to the enhanced content of anthocyanins reported in these red-fleshed apples, different studies have shown that the total phenolic content and antioxidant capacity were significantly higher in red-fleshed apples compared to traditional white-fleshed apples, which indicates that these apples could have presumably added healthy properties (Bars-Cortina et al., 2017; Rupasinghe et al., 2010).

Regarding the bioavailability of apple (poly)phenolic compounds, only a few studies have investigated the metabolism of these compounds in common varieties of apple and most of them were focused on the bioavailability after apple juice (Kahle et al., 2011; Trošt et al., 2018) or apple cider consumption (DuPont et al., 2002; Marks et al., 2009), with only one study reporting the (poly)phenolic metabolites after consumption of apple fruit (Saenger et al., 2017). Concerning the bioavailability of anthocyanins, there are plenty of studies reporting their human bioavailability and metabolism, however, they have been only studied in other food matrices such as blueberries, elderberries, blackcurrants, strawberries and red grapes or red wine (Bitsch et al., 2004; Kuntz et al., 2015; Stalmach et al., 2012; Wu et al., 2002; Zhong et al., 2017). So, to our knowledge, no study has been reported in the literature regarding the bioavailability of common apple (poly)phenolic compounds together with anthocyanidins in the same food matrix, which represents a specific characteristic of red-fleshed apple varieties. In the case of anthocyanins, various types of food samples have been used to determine the effects of food matrix on their bioavailability. For instance, anthocyanins in strawberries, blood oranges and red wine have been reported

to be highly bioavailable with their urinary levels varying between 1 and 5% of the ingested dose (Wallace et al., 2016). The differences reported in anthocyanin bioavailability from different food sources, to a large extent, is due to the presence of several structurally diverse anthocyanins in these foods, and the interactions between food matrix and these specific anthocyanins. Therefore, human postprandial studies are very useful and can contribute to knowledge about the food matrix affecting (poly)phenol bioavailability (Motilva et al., 2015).

Moreover, the measurement of dietary exposure and reliable intake biomarkers before investigating the potential health benefits of a new food product is of crucial importance for the discovery of unbiased associations between the intake of bioactive compounds and the observed effects (Dragsted et al., 2018).

So, considering the scarce data regarding the human bioavailability and metabolism of apple (poly)phenolic compounds, in the present work we aimed to investigate the bioavailability and the complex metabolic pathways of the red-fleshed apple as an innovative food source rich in different (poly)phenols, including anthocyanins. Among all the identified metabolites, we also aimed to identify and select those plasmatic and urinary metabolites that could be considered as potential intake biomarkers of red-fleshed apple consumption and might be used to establish the relationship between their intake and health benefits in future human intervention studies.

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°C.

2.2. Preparation of red-fleshed apple snack

The red-fleshed 'Redlove' apple variety was provided by NUFRI SAT (Mollerussa, Lleida, Spain), and planted in "La Rasa" experimental plot (La Rasa, Soria, 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, and dried. Then, the apple core was removed and the whole apple (with peel) was 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 h, followed by a second complete vacuum drying with a temperature ramp of 0 to 20°C for 40 h (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain). The freeze-dried apple cubes were immediately transferred to airtight plastic containers and refrigerated (2°C) until the analysis of their (poly)phenolic composition and use in the acute intake study.

The analysis of the (poly)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 mil (A11, IKA, Germany). The ingested portion of the apple snack contained a total of 196 mg of (poly)phenolic compounds. The nutritional composition and the detailed (poly)phenolic composition of the red-fleshed apple snack are presented in the **Supplemental Table 1 and Supplemental Table 2**, respectively.

2.3. Study design

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 (poly)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 ethylene diamine tetra-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°C 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 volumen of each sample was measured before storing the aliquots at -80°C until the chromatographic analysis.

2.4. Analysis of (poly)phenol metabolites in biological samples

2.4.1. Plasma samples pre-treatment

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 (poly)phenolic compounds were eluted with 2 × 50 μL of methanol. Each sample was prepared in triplicate.

2.4.2. Urine samples pre-treatment

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 (poly)phenolic compounds were then eluted with 2 × 50 μL of methanol. Each sample was prepared in triplicate.

2.4.3. 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 (poly)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 (Bars-Cortina et al., 2017; Martí et al., 2010; 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 (poly)phenolic metabolites are shown in **Table 1**.

Due to the lack of commercial standards of (poly)phenolic metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another (poly)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'-methoxy-phenyl)propionic acid (dihydroferulic acid); coumaric acid sulphate with *p*-coumaric acid; caffeic acid sulphate with caffeic acid; ferulic acid sulphate with ferulic acid; hydroxyphenyl-ɣ-valerolactone sulphate, dihydroxyphenyl-ɣ-valerolactone, dihydroxyphenyl-ɣ-valerolactone glucuronide, dihydroxyphenyl-ɣ-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/phenyl-propionic acid derivatives, phenyl-ɣ-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.

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

	(Poly)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.

3. Results and discussion

3.1. Red-fleshed apple snack (poly)phenolics characterization

The total amount of (poly)phenolic compounds in the apple snack portion (80 g) administered to the volunteers accounted for 196 ± 10.7 mg and the analysis of the (poly)phenolic composition showed a wide range of (poly)phenolic groups (**Supplemental Table 2**). The more abundant (poly)phenolic compounds were phenolic acids (45%), mainly chlorogenic acid; flavan-3-ols (7%), mainly epicatechin and its dimer; flavonols (9%), mainly quercetin derivatives, and dihydro-chalcones (17%), with phloretin glucoside being the main representative and a unique compound characteristic for apples.

Different from common apple varieties, red-fleshed apples have an added value as they also contain around 22% of anthocyanins. Anthocyanins in red-fleshed apple are located both in the peel and flesh (Bars-Cortina et al., 2017), and are mainly represented by cyanidin-3-*O*-galactoside. This specific anthocyanin has only been detected in considerable amounts in chokeberry (*Aronia melanocarpa*) and lingonberry (*Vaccinium vitis-idaea*) (Zheng & Wang, 2003), two fruits that are rarely consumed in a regular diet. Therefore, cyanidin-3-*O*-galactoside could be considered a very characteristic compound from red-fleshed apple and white-flesh red-skin apples.

Regarding the administered dose, anthocyanins have demonstrated beneficial effects at variable administered doses (7.35-640 mg/day) (Wallace et al., 2016). Considering this range described in the literature as effective in the prevention of chronic diseases, in the present study we selected a dose of anthocyanins around 50 mg/day (**Supplemental Table 2**) administered through a feasible amount of apple snack (80 g/day) that could be consumed daily by the volunteers without difficulty.

3.2. Identification of the biological apple (poly)phenol metabolites by UPLC-MS/MS

In order to identify the (poly)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 (poly)phenolic metabolites generated in plasma and urine samples. Their MS spectrum is shown in **Supplemental Figure 1**. Although the volunteers spent two days on a diet low in (poly)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 (**Supplemental Table 3 and Supplemental Table 4**). After subtraction of these basal levels of phenolics, a total of 37 (poly)phenolic metabolites were detected in the urine and/or plasma samples in increased amounts after the red-fleshed apple snack intake (**Table 1, Supplemental Table 3 and Supplemental Table 4**). 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-ɣ-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.

3.3. Proposed metabolic pathways of red-fleshed apple (poly)phenols

Based on the diversity of (poly)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 (poly)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 dark red) 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.

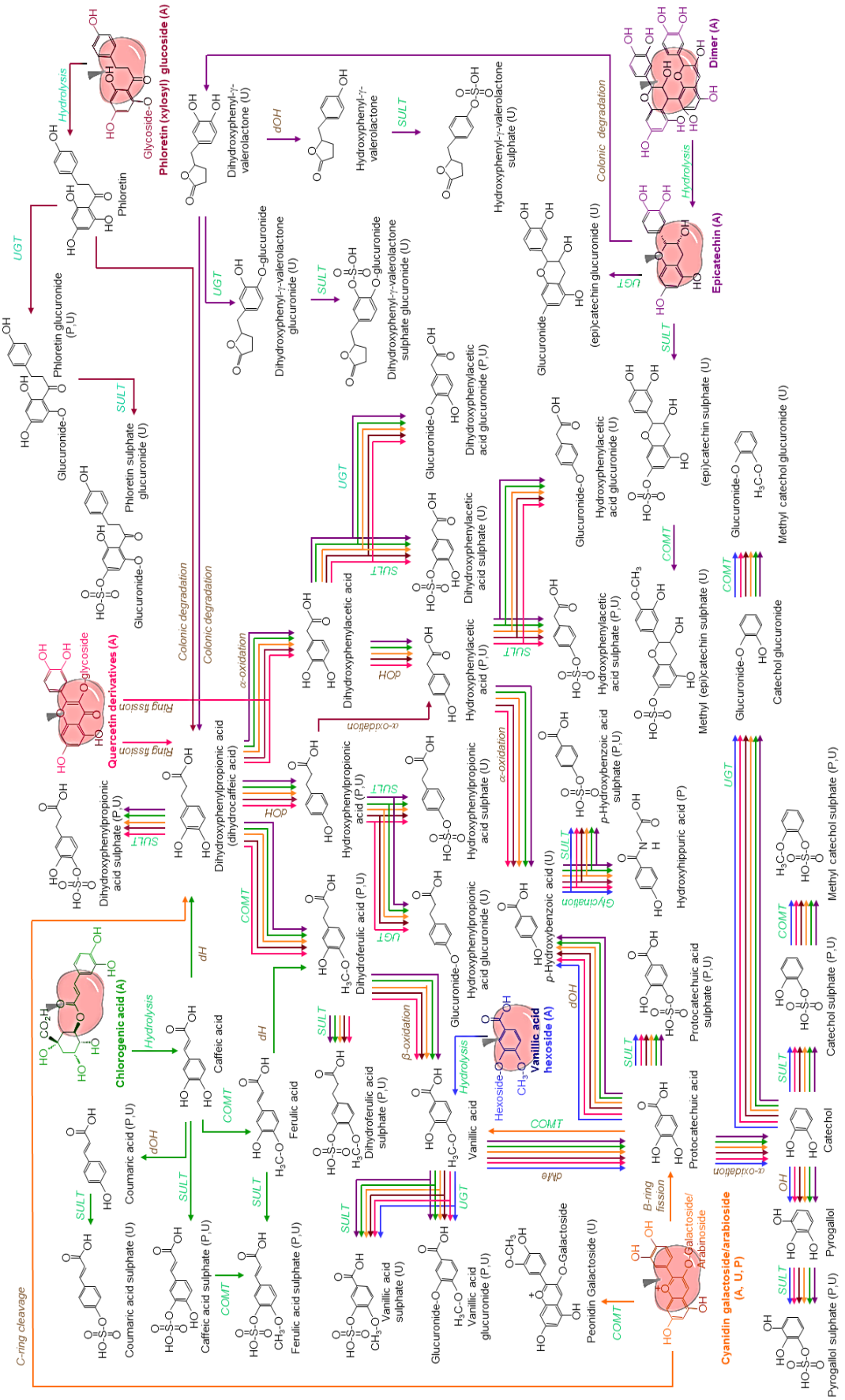

Figure 1. Proposed metabolic pathways for the generation of (poly)phenolic metabolites after the acute intake of red-fleshed apple snack. The metabolic route for chlorogenic acid is in green, for vanillic acid hexoside in blue, for anthocyanins in orange, from flavan-3-ols in lilac, from quercetin derivatives in pink and from dihydrochalcones in dark red. Reactions: *dH*, dehydrogenation; *SULT*, sulphotransferase; *UGT*, glucuronosyl-transferase; *COMT*, catechol-*O*-methyltransferase; *dOH*, dehydroxylation; *dMe*, demethylation; *α-oxidation*, one decarboxylation; and *β-oxidation*, two decarboxylations. Compounds detected in *A* (apple snack), *U* (urine) or *P* (plasma). Quercetin derivatives: quercetin glucoside, quercetin galactoside, quercetin arabinoside and quercetin rhamnoside.

In the next subsections, the metabolic pathways of the metabolites generated from the apple (poly)phenols are described.

3.3.1. 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 Table 2**). 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 cyanidin-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 gut microbiota 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) (**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.

3.3.2. Other phenolic compounds

Chlorogenic acid (**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, dihydroxy-phenylpropionic 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 (**Figure 1** blue arrows). Vanillic acid hexoside was the second most abundant phenolic acid quantified in the red-fleshed apple snack (**Supplemental Table 2**). 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 it 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 acid) 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 (Ludwig et al., 2013; Monteiro et al., 2007; Renouf et al., 2010), 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) (**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-ɣ-valerolactone, detected in urine samples. Similarly, other studies reported the valerolactones as specific flavan-3-ols metabolites (Aura, 2008; Hackman et al., 2008; Mosele et al., 2015; Serra et al., 2011). Then, dihydroxyphenyl-ɣ-valerolactone could also be glucuronided, and further sulphated and also dehydroxylated and further sulphated.

Quercetin derivatives (**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; Mosele et al., 2015; Serra et al., 2012), 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 (**Figure 1** dark red 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.

3.4. Biomarkers for red-fleshed apple (poly)phenol consumption

As evidenced in the metabolic pathways proposed in **Figure 1**, a large number of metabolites are generated from the five main (poly)phenolic groups (anthocyanins, phenolic acids, flavan-3-ols, flavonols and dihydrochalcones) present 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 (poly)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 (poly)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 (poly)phenol metabolites detected in the urine and plasma samples after the red-fleshed apple snack intake (**Table 1, Supplemental Table 3 and Supplemental Table 4**) could be classified into two groups according to their urine excretion 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 (**Figure 2A**); and the second group correspond to the derivatives from flavan-3-ols, valerolactones, dihydrochalcones, and anthocyanins (**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 and 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 (**Supplemental Table 3**). So, these compounds could be considered "endogenous" phenolic metabolites from the diet.

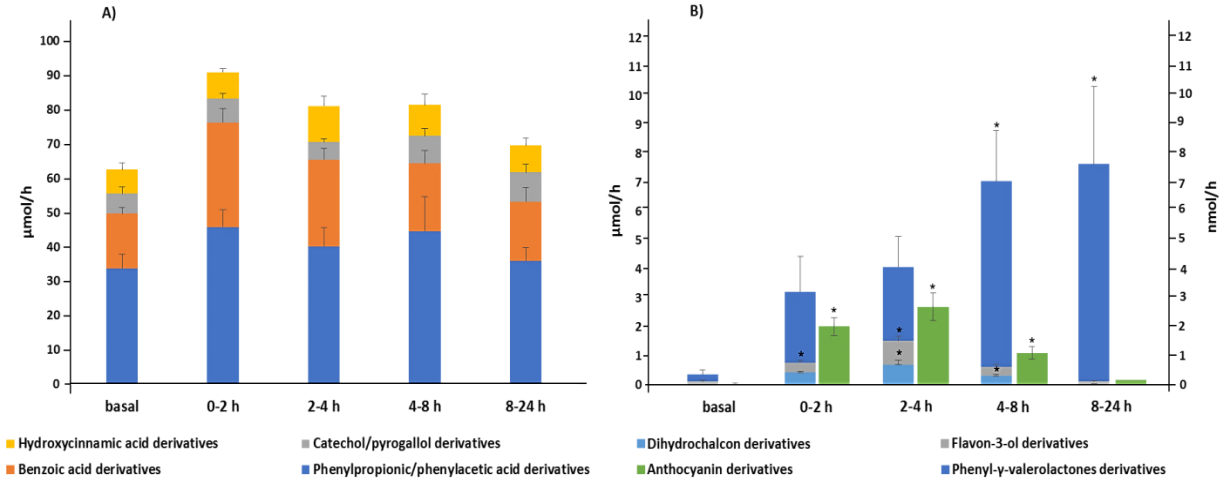

Figure 2. (Poly)phenolic metabolite excretion rate in urine (A) phenylpropionic/phenylacetic acids, benzoic acids, catechol/pyrogallol and hydroxycinnamic acids derivatives; and (B) flavan-3-ols, phenyl-γ-valerolactones, dihydrochalcones, and anthocyanins derivatives. Except for anthocyanin derivatives (nmol/h), 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.

On the other hand, the (poly)phenolic metabolites from the second group significantly increased ($p < 0.05$) after the red-fleshed apple intake at the different interval times studied (**Figure 2B**). (Poly)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 and nmols for anthocyanins) of the potential biomarkers 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), and 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-ɣ-valerolactone (D1), dihydroxyphenyl-ɣ-valerolactone sulphate glucuronide (D2), dihydroxyphenyl-ɣ-valerolactone glucuronide (D3) and hydroxyphenyl-ɣ-valerolactone sulphate (D4) as phenyl-ɣ-valerolactone derivatives from flavan-3-ols pathways.

As shown in **Figure 3** and in **Supplemental Table 3**, 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; Kahle et al., 2007; Kristensen et al., 2012; Marks et al., 2009; Mennen et al., 2006; Saenger et al., 2017).

Note that the native forms of some apple anthocyanins, such as cyanidin-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, cyanidin-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 (**Figure 4 and Supplemental Table 4**).

Regarding phenyl-ɣ-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.

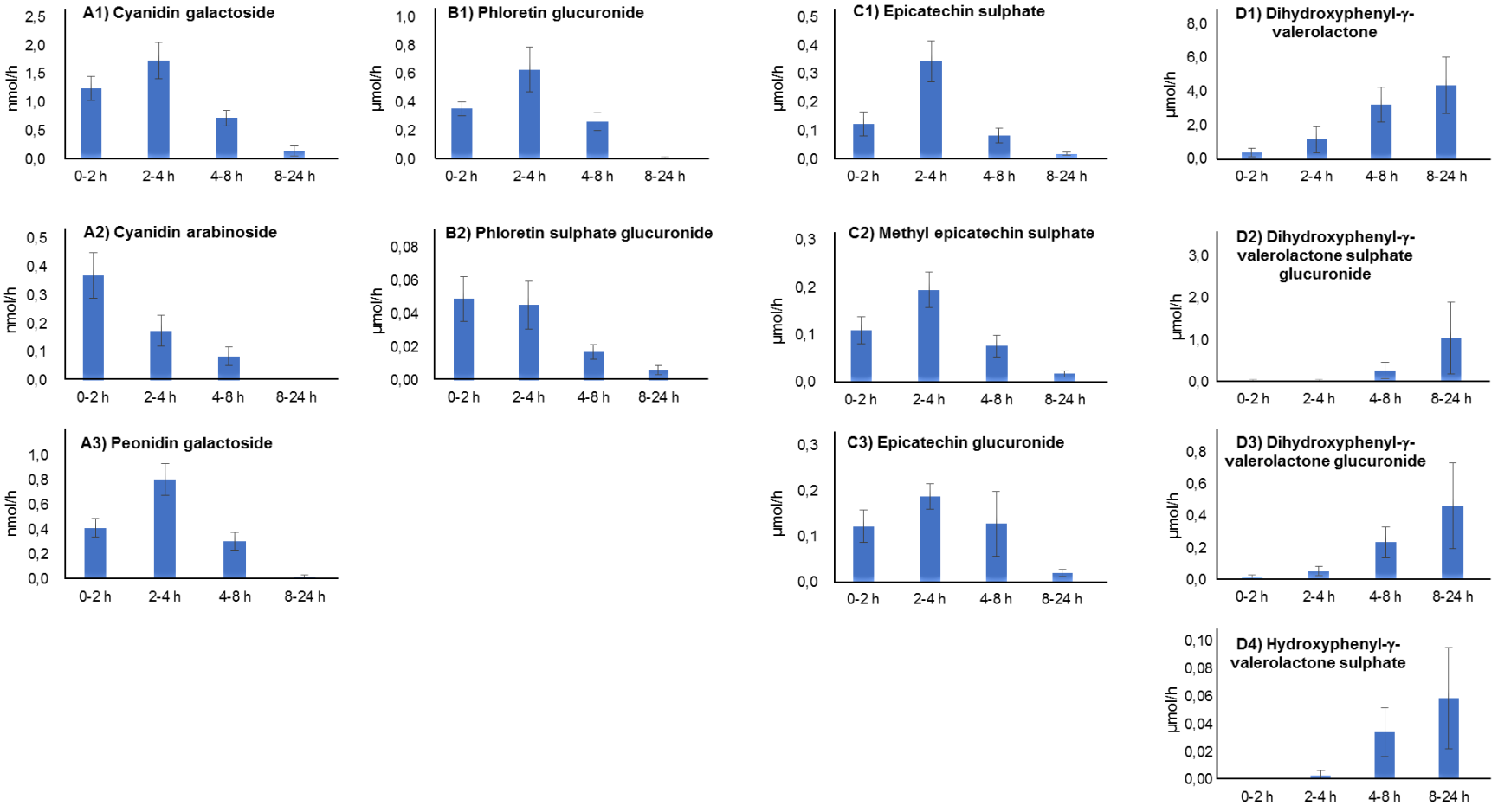

Figure 3. Urinary excretion of the proposed biomarkers for red-fleshed apple consumption 0–24 h after acute intake of 80 g apple snack. A1-A3: Cyanidin derivatives; B1-B2: Phloretin derivatives; C1-C3: Epicatechin derivatives; D1-D4: Phenyl-ɣ-valerolactone derivatives. Except for anthocyanin derivatives (nmol/h), data expressed as µmol/h as mean values ± standard error of mean (n=10).

Nevertheless, in our previous study in which we analyzed whole blood by sampling with dried blood spot (DBS) cards from the same volunteers, hydroxyphenyl-ɣ-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 (poly)phenolic metabolites shown in **Figure 3** could be proposed as urinary markers for red-fleshed apple consumption. Nevertheless, epicatechin and phenyl-ɣ-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 are consumed in 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-ɣ-valerolactone metabolites, are certainly good biomarkers for the correct assessment of intake and health effects exerted by flavan-3-ol-rich diets (Urpi-Sardá et al., 2015; Van der Hooft et al., 2012). However, when proposing intake biomarkers, more specific metabolites must be sought.

On the one hand, as reported in previous studies (Mennen et al., 2006; Saenger et al., 2017), we corroborate that phloretin derived metabolites are good biomarkers for apple intake, as phloretin glucoside is an exclusively apple dihydrochalcone (Richling, 2012). In the present study, phloretin glucuronide was considered to be a good biomarker as it was detected in all the plasma and urine samples from all the volunteers and was not detected under basal conditions.

On the other hand, cyanidin-3-*O*-galactoside, detected in plasma and urine, was considered also a good intake biomarker as it is the main anthocyanin of red-fleshed apples (Bars-Cortina et al., 2017; Guo et al., 2016), and apart from these apple varieties, it can be only found in considerable amounts in chokeberry and lingonberry fruits. Therefore, cyanidin-3-*O*-galactoside could be considered as good intake biomarker for red-fleshed apple, and probably also for apples with white flesh and red skin, although no data has been found in the literature. This metabolite was also detected in all the plasma and urine samples from all the volunteers and was not detected under basal conditions. 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 could also be selected as a good urinary biomarker for red-fleshed apple consumption jointly with phloretin glucuronide and cyanidin-3-*O*-galactoside (**Figure 4**).

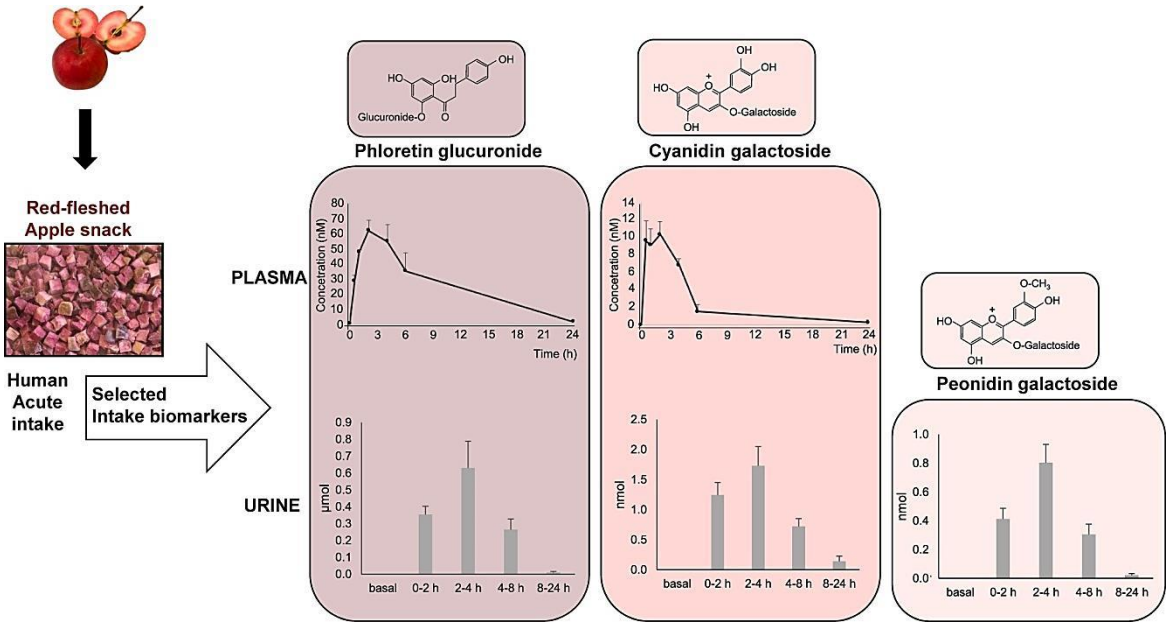

Figure 4. Pharmacokinetic profile of the proposed biomarkers for red-fleshed apple snack intake. In urine samples, data expressed as µmol as mean values ± standard error of mean (n=10), except for anthocyanin derivatives (nmol/h).

As pointed out, these three polyphenolic metabolites (phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside) are good intake biomarkers although not strictly specific for red-fleshed apples. However, the presence of both phloretin glucuronide and cyanidin galactoside metabolites (cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside) could be considered as more specific intake biomarkers from apples with red-flesh.

Further, it is important to remark, as we previously reported (Bars-Cortina et al., 2017), that the red-fleshed apples contains a higher concentration of anthocyanins but a lower concentration of flavan-3-ols in its flesh in comparison to the traditional white-fleshed varieties with red skin. This fact could be explained by a competitive synthesis between anthocyanins and proanthocyanidins in the flesh. Presumably this different ratio of anthocyanins/flavan-3-ols reported in red-flesh apples, might be reflected in the concentrations of the generated metabolites. So, in quantitative terms, after a red-fleshed apples intake a lower amount of flavan-3-ols metabolites would be expected in comparison to white-fleshed apples with red skin intake.

To support this hypothesis, the generated metabolites after an intake of red-fleshed and traditional white-fleshed snack apples are being analyzed in an ongoing study.

4. Conclusions

In the present study, the different (poly)phenolic metabolites generated after the intake of red-fleshed apple snack were identified and tentatively quantified in urine and plasma samples at different time intervals. Moreover, the metabolic pathways of the (poly)phenolic metabolites generated from red-fleshed apple (poly)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 snack, (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. Among all the metabolites generated, phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside were proposed as the best candidates as biomarkers after the intake of red-fleshed apple. These three polyphenolic metabolites were not detected in basal samples and were detected in the urine and/or plasma samples from all the volunteers. It is important to highlight that these three polyphenolic metabolites are not strictly exclusive to red-fleshed apple intake, since phloretin glucuronide is a common biomarker for all apple fruits, and cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside could appear, not only after the intake of red-flesh apple, but also after white-flesh with red-skin apples or other fruits such as chokeberry and lingonberry. However, the presence of these three metabolites could be useful as intake biomarkers in human intervention studies when studying the biological effects of red-fleshed apple.

5. 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).

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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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Supplemental Table 1. Nutritional facts of red-fleshed apple snack.

Nutritional composition	Per 100g	Per portion (80g)
Calories (KJ)	982.6	786.1
(Kcal)	234.8	187.9
Fat (g)	0.9	0.7
Total Carbohydrates (g)	93.6	74.9
- of which Sugars (g)	53.8	43.0
Protein (g)	3.0	2.4 g
Fibre (total) (g)	16.5	13.2
- Soluble fibre (g)	7.6	6.1
- Insoluble fibre (g)	8.9	7.1
Minerals (g)	1.7	1.3

Nutritional facts. The samples were analysed for moisture, fat, protein, fibre (soluble and insoluble), sugars (reducing and non-reducing sugars) and ash content. All reagents used were of analytical grade. Moisture was estimated by weight difference after drying. Total protein content was estimated by the Dumas method and total fat was extracted with hexane from previously dried samples using a Soxhlet extractor. Insoluble fibre was determined following the method described by Van Soest et al. (1991), while soluble fibre was calculated gravimetrically as the alcohol insoluble residue according to Maran (2015). Sugar quantification (reducing and non-reducing sugars) was carried out by titrimetry based on the Fehling reaction in alkaline media after acid hydrolysis of non-reducing sugars. Ash content was determined by incineration at 550°C ± 10°C. Finally, total carbohydrates were calculated by difference. Results were expressed as grams of each compound per 100 grams of sample (g/100 g).

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Supplemental Table 2. Concentration of the main (poly)phenolic compounds in red fleshed apple snack. Data are expressed as mg/80 g portion dry weight (mean ± SD, n=3).

(Poly)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	0.42 ± 0.02	
Dihydrochalcones		*33.7 ± 3.08*
Phloretin glucoside	21.7 ± 2.54	
Phloretin xylosyl glucoside	11.7 ± 0.52	
Hydroxyphloretin xylosyl glucoside	0.32 ± 0.03	
TOTAL (Poly)phenols		***196 ± 10.7***

Supplemental Table 3. Urinary excretion of phenolic compounds 0–24 h after acute intake of red-fleshed apple snack. Except for anthocyanin derivatives (nmol), data expressed in μmol as mean values ± standard deviation after subtraction of baseline excretion from each volunteer.

(Poly)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 ± 0.90	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 derivatives					
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	11.8 ± 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
Hydroxymethoxyphenylpropionic acid (*n*=5)	0.15 ± 0.04	0.02 ± 0.01	0.09 ± 0.04	0.29 ± 0.13	0.58 ± 0.22
Hydroxymethoxyphenylpropionic acid 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					
Hydroxylphenyl-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
Dihydroxylphenyl-γ-valerolactone (*n*=8)	3.51 ± 2.25	0.98 ± 0.63	2.96 ± 1.91	15.9 ± 4.61	81.9 ± 32.0
Dihydroxylphenyl-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
Dihydroxylphenyl-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.

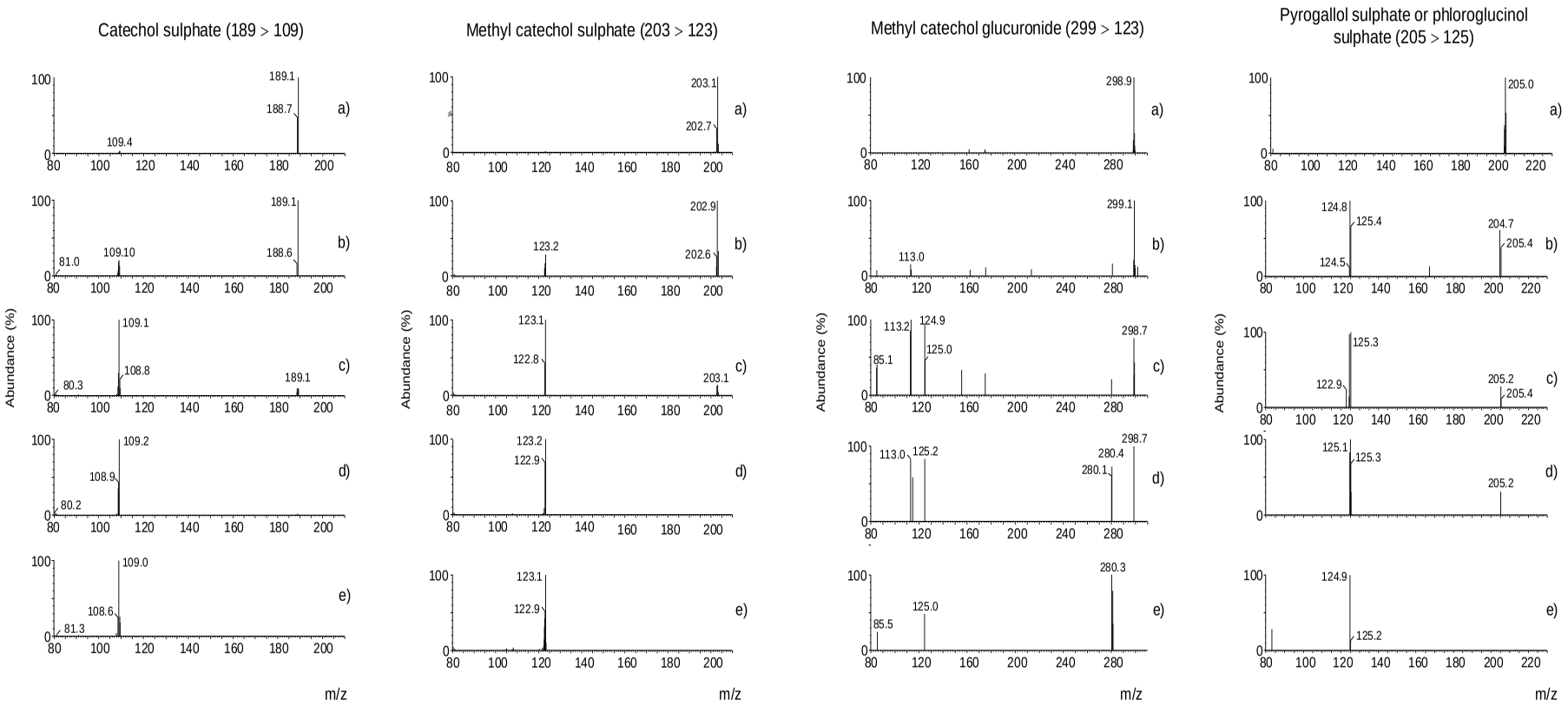
[b] Content of urine collected for 12 h prior to supplementation and on an excretion per hour basis used to subtract from excretion values obtained after red-fleshed apple snack consumption to obtain the values cited in the Table.

n.d.: not detected.

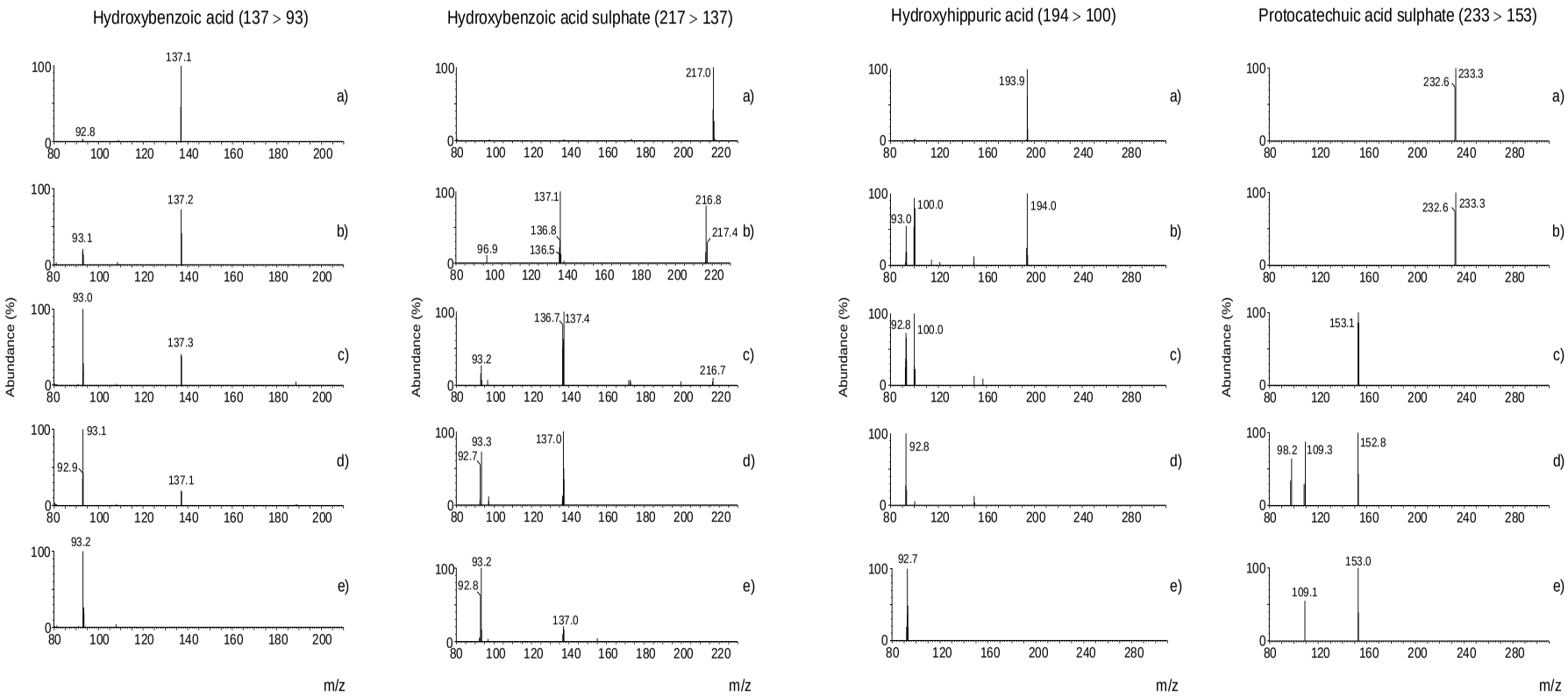
Supplemental Table 4. (Poly)phenolic compounds concentrations in plasma 0–24 h after acute intake of red-fleshed apple snack. Data expressed in nmol/L as mean values ± standard deviation.

(Poly)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.

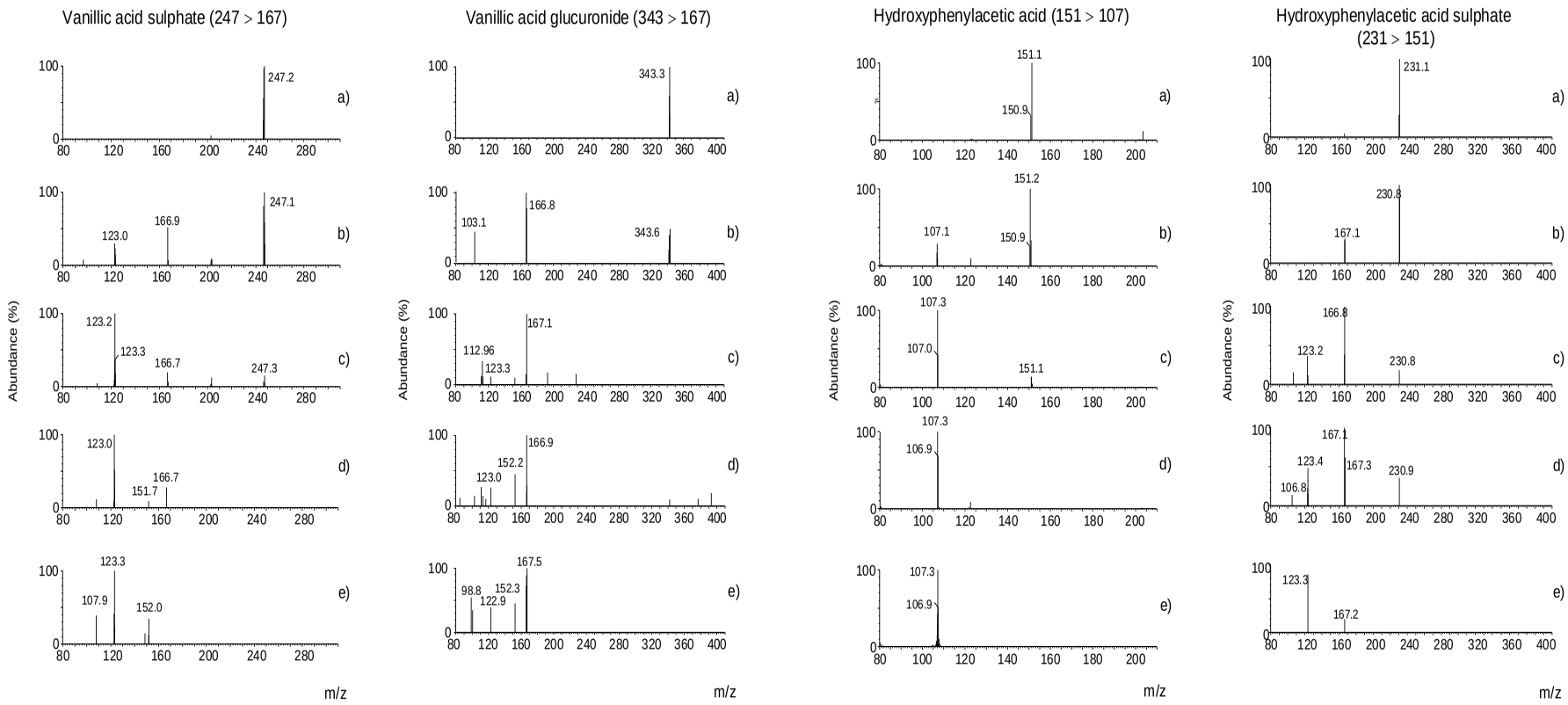
[a]Figures in parenthesis next to compound names indicate the number of samples (volunteers) in which the metabolite was detected.
n.d.: not detected.



Supplemental Figure 1. MS spectrum of the (poly)phenolic compounds and generated metabolites after the acute intake of the red-fleshed apple snack. Collision energy applied was: a) 5 eV, b) 10 eV, c) 15 eV, d) 20 eV, and e) 25 eV.



Supplemental Figure 1. Continuation.



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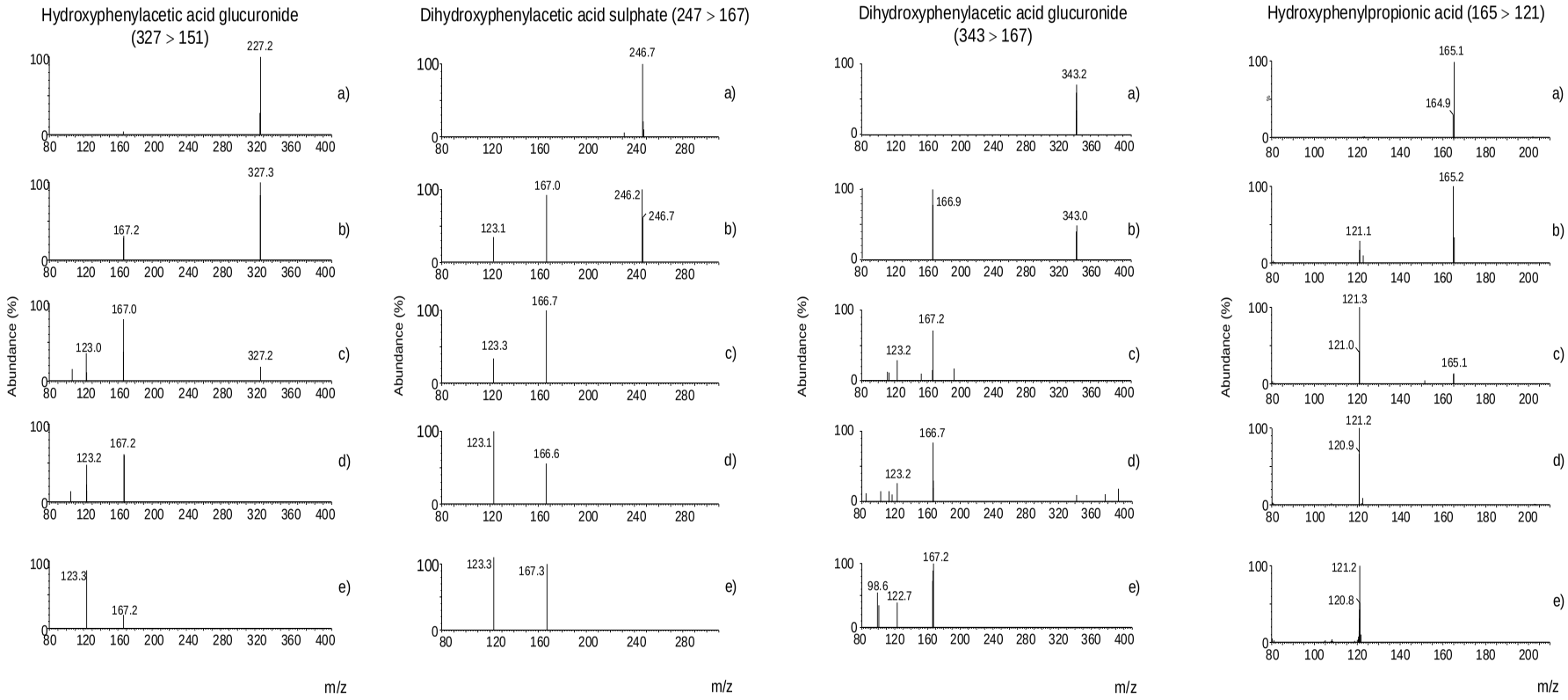

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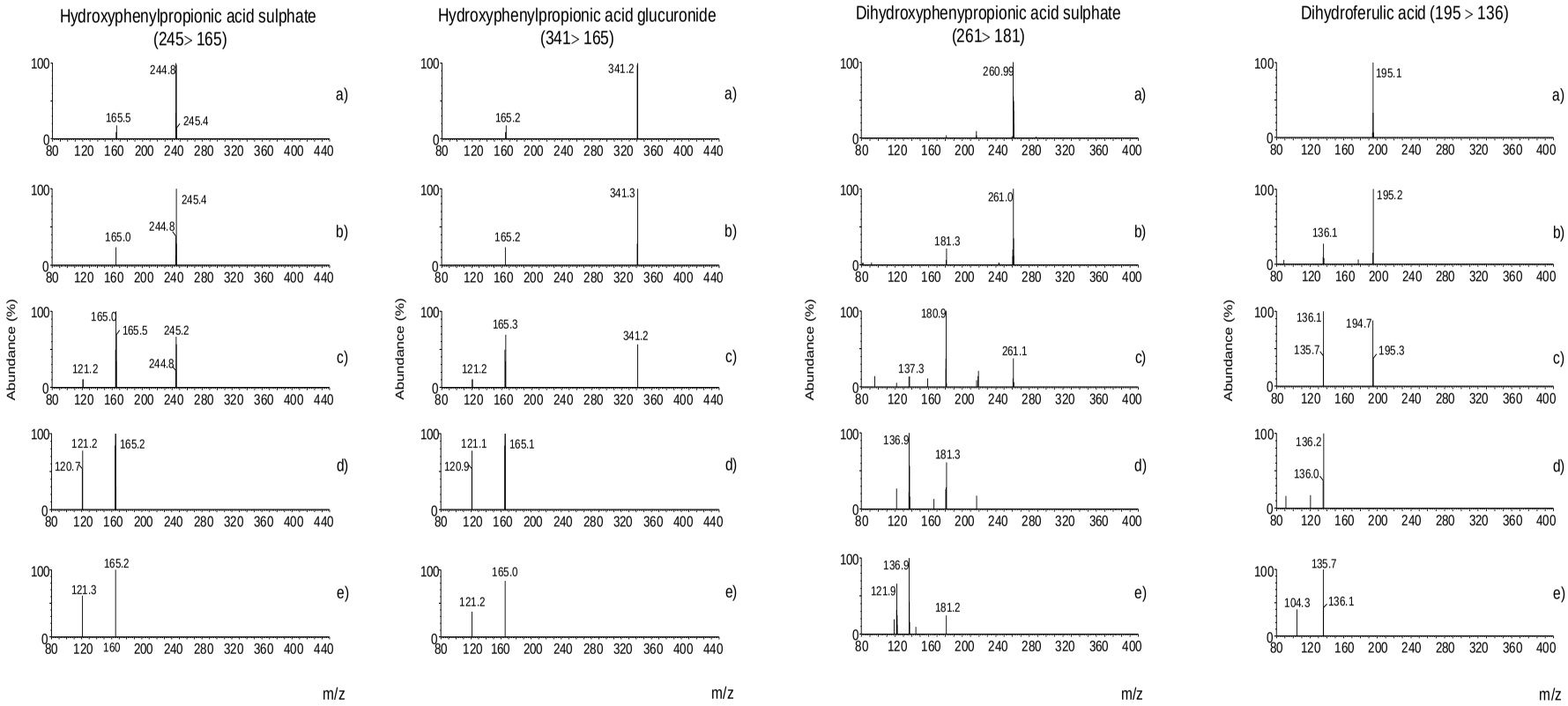

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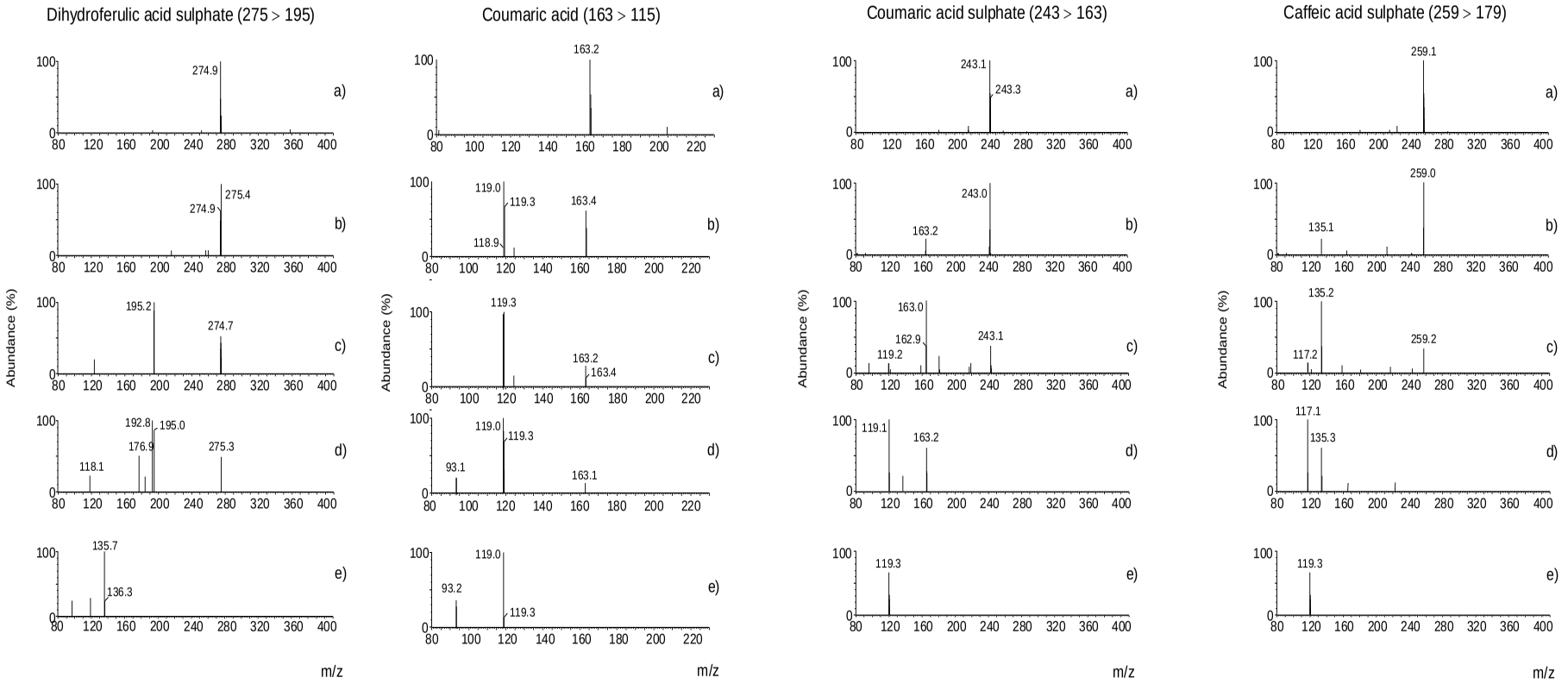

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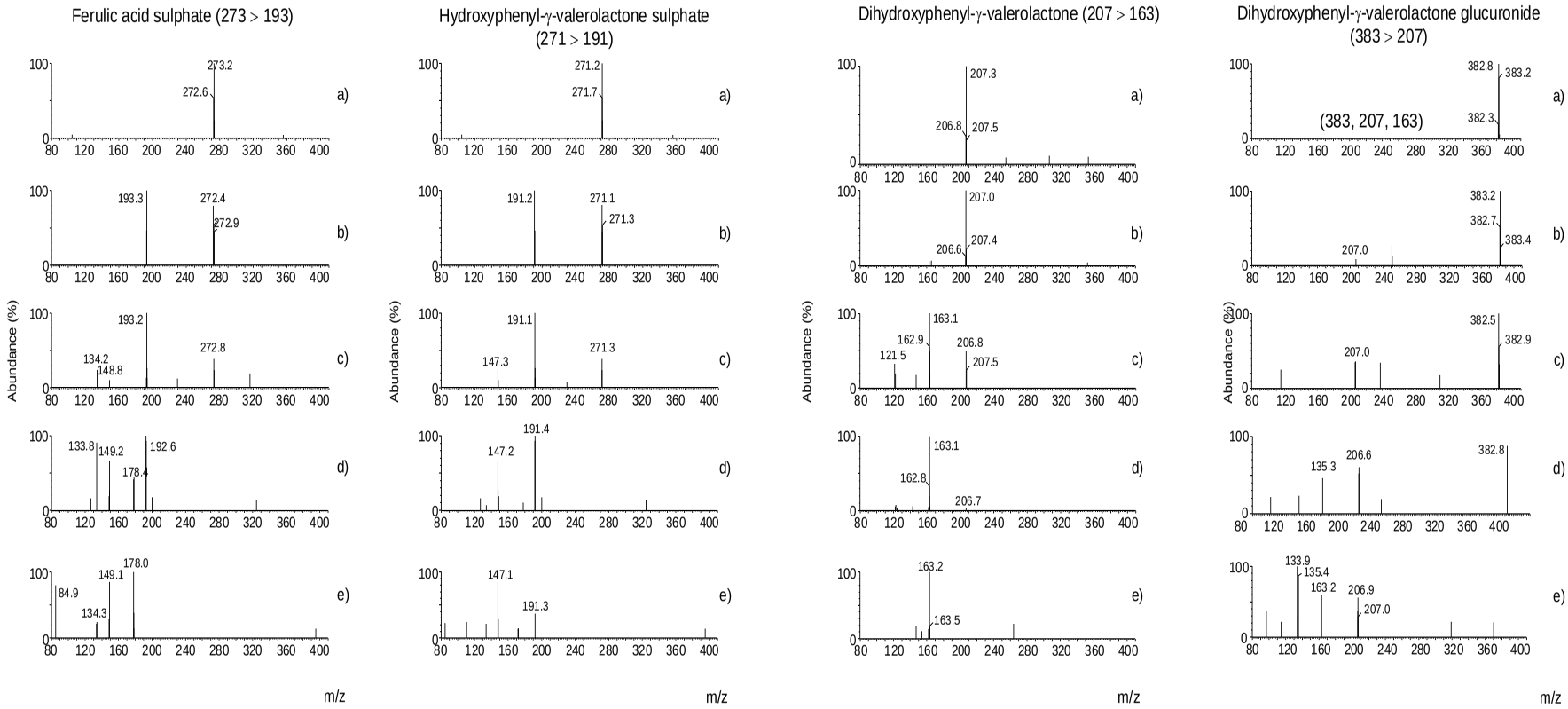

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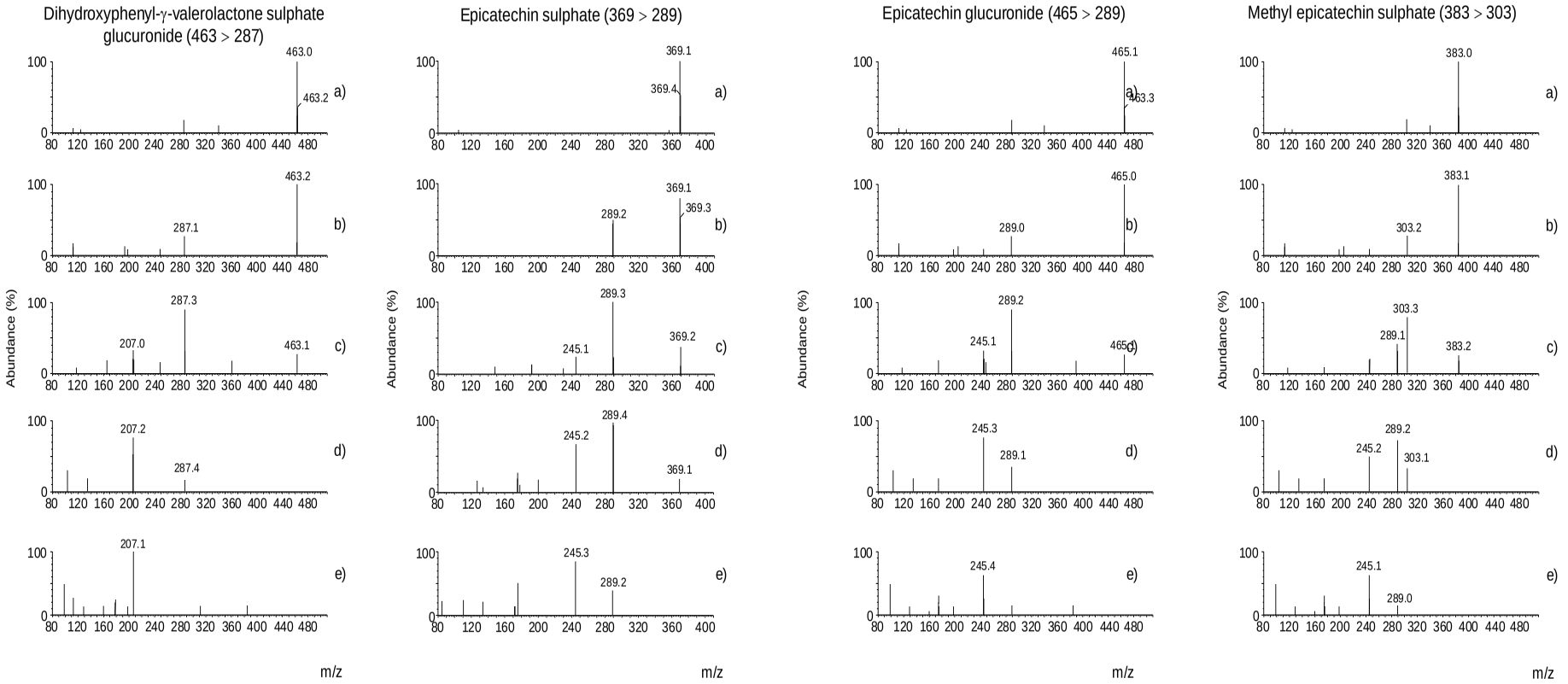

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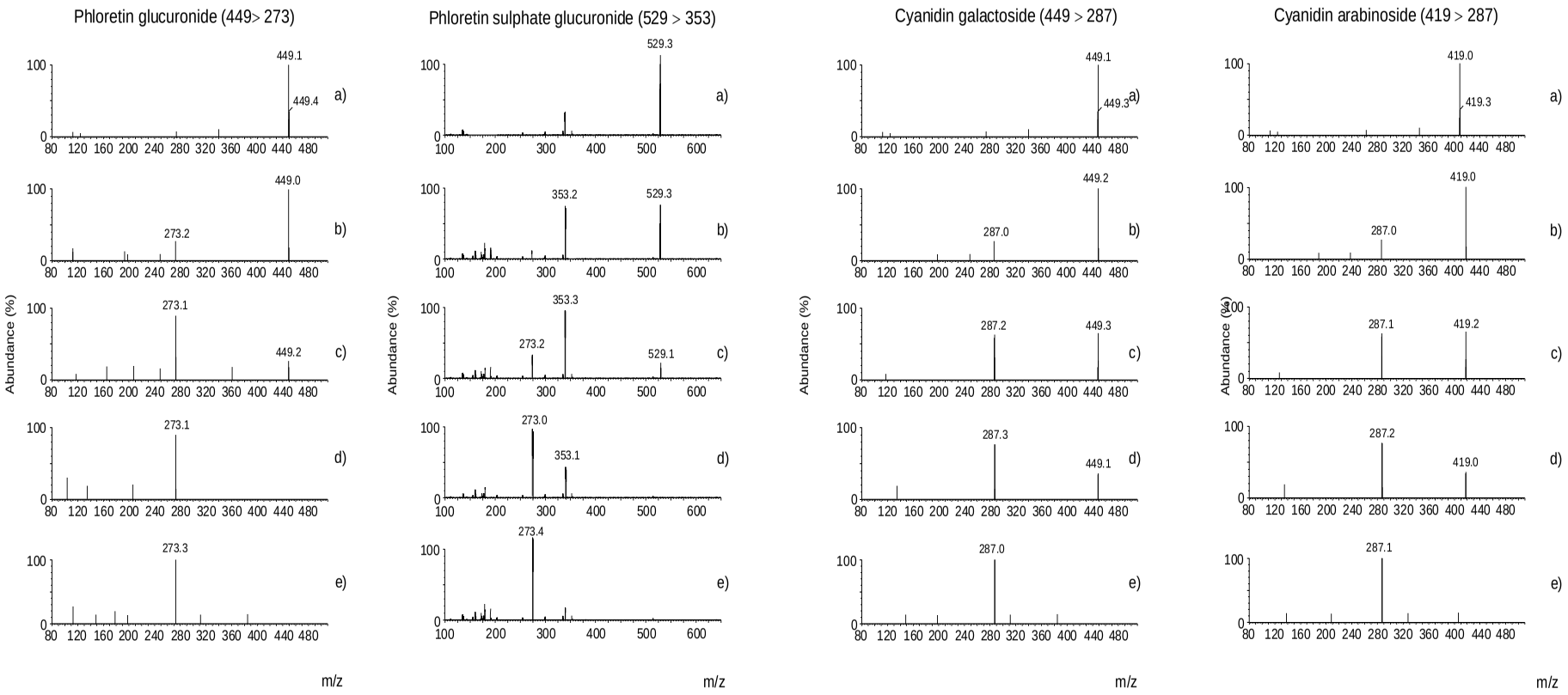

Supplemental Figure 1. Continuation.

Supplemental Figure 1. Continuation.

OBJECTIVE 5 AND OBJECTIVE 6

PUBLICATION IV: METABOLIC FATE AND CARDIOMETABOLIC EFFECTS OF (POLY)PHENOLIC COMPOUNDS FROM NEW CULTIVARS OF RED-FLESHED APPLE IN HYPERCHOLESTEROLEMIC RATS: A COMPARATIVE STUDY WITH COMMON WHITE-FLESHED APPLE. THE APPLECOR STUDY

MOLECULAR NUTRITION AND FOOD RESEARCH (UNDER REVIEW)

Metabolic fate and cardiometabolic effects of (poly)phenolic compounds from red-fleshed apple in hypercholesterolemic rats: A comparative study with common white-fleshed apple. The AppleCOR Study

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***Abstract*:** The present study aimed to investigate the metabolic fate and the cardiometabolic effects of (poly)phenolic compounds provided by a red-fleshed apple variety biofortified in anthocyanins. Wistar rats were feed with high fat diet (HFD) to induce hypercholesterolemia and supplemented with red-fleshed apple (HFD+R), white-fleshed apple (HFD+W) or an anthocyanin-rich infusion from aronia fruit (HFD+A) providing matched content and profile of anthocyanins. Plasma biochemical parameters, histological analysis and (poly)phenol biological metabolites were determined. Plasma, urine and faeces showed a significant increase of anthocyanin metabolites after HFD+R and HFD+A, while flavan-3-ols were significantly increased after HFD+W and dihydrochalcones derivatives increased after both apples supplementation. A cardioprotective effect was observed after both apples and aronia infusion supplementation in the reduction of aortic thickness. The kidney function was improved after all supplementations but only in females, probably related to the higher (poly)phenol bioavailability reported in females. A decrease in insulin plasma concentration after both apples supplementation was also observed only in males. Our findings support that anthocyanins without apple matrix can induce cardioprotective effects. Anthocyanins or flavan-3-ols, together with dihydrochalcones, compose a polyphenolic phytocomplex in red and white-fleshed apples, respectively, that could act synergistically in the attenuation of cardiovascular outcomes in hypercholesterolemic rats.

KEYWORDS: anthocyanins, red-fleshed apple, (poly)phenolic compounds, aortic thickness, Bowman's space, sustained intake, UPLC-MS/MS

1. Introduction

(Poly)phenolic compounds are among the most abundant phytochemicals present in the human diet, and increasing evidence highlights their important health-promoting effects (Abdel-Rahim & El-Beltagi, 2010; Setorki et al., 2009). At present, (poly)-phenols garner much attention due to their protection against cardiovascular diseases (CVD) (Shivashankara & Acharya, 2010), which are the first cause of death globally (17.9 million people died from CVD in 2016) (https://www.who.int/en/news-room-/fact-sheets/detail/cardiovascular-diseases-cvds). Understanding the bioavailability of (poly)phenols, their metabolism and tissue distribution is critical since their physiological impact depend on their delivery to target tissues (D'Antuono et al., 2015; McDougall et al., 2005).

Once the (poly)phenolic compounds are absorbed in the upper part of the gastrointestinal tract, they are mainly metabolized in the small intestine and liver by phase-II enzymes to their sulphate, methyl, and glucuronide conjugates. However, it is estimated that only 5-10% of the total (poly)phenol intake is absorbed in the small intestine. The remaining 90-95% may accumulate in the large intestinal lumen where they are subjected to the enzymatic activities of the gut microbial community (Lin et al., 2019). All these metabolites are those that reach circulation and target tissues and may be responsible for the health effects derived from the consumption of (poly)phenol-rich foods.

Another fact to consider in the study of (poly)phenols bioavailability and their health effects is the food matrix in which these compounds are found, since in most fruits and vegetables many of these compounds are linked to carbohydrates, fibre, proteins and cell walls as well as to other (poly)phenols by covalent bonds, hydrogen bonding, and hydrophobic and hydrophilic interactions (Saura-Calixto, 2012). During gastrointestinal digestion, the binding of (poly)phenols to these molecules affects their release in the gut as well as the efficacy by which they are transported across the mucosal epithelium and this may lead to a decrease in the (poly)phenol bioaccessibility and, in consequence in their bioactivity (Stanisavljević et al., 2015; Tagliazucchi et al., 2010).

Anthocyanins have been extensively reported to manifest therapeutic properties against hyperlipidaemia and CVD, among others (Qin & Anderson, 2012; Rouanet et al., 2010; Yanni et al., 2015). Some authors reported that after oral intake short-term experiments in animals, intact anthocyanins were mainly detected in tissues like heart, liver, kidney, lung (Marczylo et al., 2009) or brain (Fornasaro et al., 2016). In the last few years, there has been an increasing interest in potential crops for coloring food

naturally, such as red-fleshed apples cultivars. Due to their enhanced content of anthocyanins, different studies have shown that the total (poly)phenolic content and antioxidant capacity of red-fleshed apples were significantly higher compared to common white-fleshed apple cultivars, which indicates that they could have presumably added healthy properties (Bars-Cortina et al., 2017). Indeed, red-fleshed apple supplementation in rats has already shown protective effects against colon carcinogenesis retarding/diminishing the appearance of the precancerous markers and the expression of genes related to this cancer (Bars-Cortina et al., 2020). The emerging potential of red-fleshed apples as a novel anthocyanin-rich fruit along with the differences reported in the (poly)phenol bioavailability and bioactivity depending on the food matrix, substantiates the present research focused on the posible health benefits of red-fleshed apple.

The main objective of the present study was to investigate the cardiometabolic effects of the diet supplementations with red-fleshed apple and compared to common white-fleshed apple in hypercholesterolemic rats through the analysis of histological parameters in target tissues (liver, heart, kidney and aorta). To study the apple matrix effect, rats were also supplemented with an anthocyanin-rich infusion from aronia fruit (*Aronia melanocarpa*). To fully understand the observed effects, we also performed a comprehensive analysis of the metabolic fate and metabolic pathways of anthocyanins and other (poly)phenols from apple in rat plasma, urine and faeces.

2. *Materials and methods*

2.1. *Chemicals and reagents*

Cyanidin-3-*O*-galactoside, eriodictyol, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, procyanidin 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 5-*O*-caffeoylquinic acid (aka 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 synthetized according to Pimpao et al. (2015), and were kindly supplied by Dr. Claudia N. Santos (University of Lisbon, 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). 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°C.

2.2. *Red- and white-fleshed apples and aronia fruit infusion*

To study and compare the cardio-protective effects, two different apple varieties were selected: i) the red-fleshed "Redlove" apple variety, a new genotype naturally biofortified in anthocyanins, and ii) the common white-fleshed Granny Smith apple variety (both provided by NUFRI SAT, Mollerussa, Lleida, Spain) without anthocyanins. Additionally, and to study the effect of anthocyanins without the possible interaction of the apple matrix, an anthocyanin-rich infusion from aronia fruit was selected.

To minimize changes in the bioactive compounds of the apples, a freeze-dried format was selected to prepare the supplemented diets. Briefly, the apple core was removed and the whole apple (with peel) was cut into 1 cm-sized cubes. The apple cubes were immediately frozen in liquid nitrogen and then lyophilized on a Lyobeta 15 TELSTAR Lyophilizer (Terrassa, Spain). The freeze-dried apple cubes were immediately transferred to airtight plastic containers and stored in refrigeration (2°C) until the analysis of their (poly)phenolic composition, and the preparation of the supplemented diets. To obtain the anthocyanin-rich extract, a cold water infusion of *Aronia melanocarpa* fruit powder (Aronia Pulver, BIOJOY, Nuremberg, Germany) was prepared, which was equivalent in dose and type of anthocyanins to the red-fleshed apple. Briefly, distilled water was added to aronia fruit powder (in proportion 1:1), and the mixture was homogenized (Kinematica Polytron, Polytron Corporation, Montreal, Canada) for 60 seconds. The resulting mixture was centrifuged (5403 *g* for 5 min), and the supernatant was analyzed and added to the drinking water of the rats. The (poly)-phenolic composition of the freeze-dried apples, and the aronia infusion is shown in **Table 1**.

2.3. Animals and experimental procedure

Thirty Wistar rats weighing between 300 and 350 g were purchased from Charles River Laboratories (Barcelona, Spain). They were divided into 5 groups of 6 animals each one (3 males and 3 females) as follows, Group 1: standard chow-diet (SCD) (Teklad 2014, rodent maintenance diet, Envigo, Huntingdon, Cambridgeshire, United Kingdom); Group 2: high fat diet (HFD) (Atherogenic Rodent Diet TD. 02028, Envigo, Huntingdon, Cambridgeshire, United Kingdom) to induce hypercholesterolemia; Group 3: HFD supplemented with white-fleshed apple (HFD+W), Group 4: HFD supplemented

with red-fleshed apple (HFD+R), and Group 5: HFD supplemented with anthocyanin extract from aronia (HFD+A).

The design of the study is shown in **Supplemental Figure 1.** Group 1 was fed with chow diet for 9 weeks. The other groups were fed during 3 weeks with a HFD and the following 6 weeks with the HFD supplemented with the different products. For HFD+R and HFD+W (Groups 3 and 4), HFD pellets were crushed in a mill along with the freeze-dried apples. For HFD+A (Group 5), the aronia extract was dissolved daily in the drinking water. Moreover, diets from Group 2 (HFD) and Group 5 (HFD+A) were modified by adding 25% of chow diet in the same proportion as apples, so that all groups except Group 1, would take the same proportion of HFD during the supplementation period.

To prepare the supplemented diets, HFD pellets and lyophilized apples (red- or white-fleshed) were crushed in a mill (MC300132, Moulinex, Alençon, France) until a homogeneous powder was obtained and mixed. Then distilled water (10%) was added, the mixture was homogenized and new apple-enriched pellets were prepared and dried in an oven (JA Selecta, Barcelona, Spain) at 25°C for 3 days (at darkness).

The supplementation of anthocyanin through red-fleshed apple or aronia was based on the human equivalent dose of 70 mg/day of anthocyanins, according to Reagan-Shaw et al. (2008). So, the quantity of red-fleshed apple administered and the aronia fruit extract in water was adjusted to a dose of 1.8 and 1.9 mg/day/rat of anthocyanins, respectively (**Table 1**). The quantity of white-fleshed apple to prepare HFD+W was equivalent to HFD+R.

During the 9 weeks of the experiment, the rats were housed in cages on a 12 h light-12 h dark schedule at controlled temperature (21 ± 1°C), and humidity (55 ± 10%). Food and water were available *ad libitum*. The body weight, food and water intakes were recorded every 3 days. The day before sacrifice, the rats were caged in metabolic cages for 24 h to collect urine and faeces. The rats were sacrificed by an intracardiac puncture after isoflurane anesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). Blood was collected in EDTA tubes, and plasma samples were obtained by centrifugation (3000 *g*, 10 min at 4°C) and stored at -80°C until chromatographic analysis of (poly)phenolic metabolites. After blood collection, the rats were perfused with an isotonic solution of sodium chloride (0.9%) to remove the remaining blood in tissues. The heart, aorta, liver and kidneys were excised and immediately frozen in liquid nitrogen. A part of the tissue samples were stored at -80°C until analysis and the other part were fixed in 10% (v/v) formalin for a minimum of 24 h. The animal procedures were conducted in accordance with the guidelines of the European Communities

Directive 2010/63/EU regulating animal research. The protocols were approved by the Animal Ethical Committee of the University of Lleida (CEEA 01-10/17), and performed under a Generalitat de Catalunya Project License (10038). The study complies with the ARRIVE guidelines developed by the NC3Rs (Kilkenny et al., 2010).

2.4. Plasma, urine and faeces analysis of anthocyanin and (poly)phenolic metabolites by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

2.4.1. Pre-treatment of plasma samples

Before the chromatographic analysis, the rat plasma samples were pre-treated by microElution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. The methodology used is reported in our previous study (Yuste et al., 2019). Briefly, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. Then, 350 µL of 4% phosphoric acid solution was added to 350 µL of the rat 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. Afterwards, the retained (poly)phenolic compounds were eluted with 2 x 50 µL of methanol. Each sample was prepared in duplicate.

2.4.2. Pre-treatment of 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 (poly)phenolic compounds were then eluted with 2 x 50 µL of methanol. Each sample was prepared in duplicate.

2.4.3. Pre-treatment of faeces samples

The faeces were pre-treated as it was reported in our previous study (Mosele et al., 2015a). Briefly, 100 mg of lyophilized faeces were mixed in 1 ml of the solution methanol/HCl/Milli-Q water (79.9/0.1/20, v/v/v), and shaken in a vortex (Multi vortex, VWR, Franklin, MA, USA) for 15 min. After that, the sample was centrifuged at 8784 *g* for 10 min at 4°C, and the supernatant was collected, and centrifuged under the same conditions. Finally, the supernatant was filtered with a 0.22 µm syringe Nylon filter and transferred into chromatographic vials until the chromatographic analysis.

2.4.4. Chromatographic analysis (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 (poly)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 (Bars-Cortina et al., 2017; Yuste et al., 2018; Yuste et al., 2019). 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. Due to the lack of commercial (poly)phenolic standards and their generated metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another (poly)phenolic compound with a similar structure. **Supplemental Table 1** shows the selected reaction monitoring (SRM) conditions as well as its cone voltage and collision energy used for the quantification of these (poly)phenolic compounds. This table also shows in which (poly)phenolic standard compound, these (poly)phenolics have been quantified.

2.5. Histological analysis

To investigate the possible protective effects against the high fat diet of red-fleshed apple, white-fleshed apple or aronia infusion, different histological stains of aorta, kidney, heart, and liver tissues were performed and different parameters were assessed.

2.5.1. Aorta and kidney haematoxylin-eosin staining

Aorta and kidney samples were fixed in 10% formaldehyde, dehydrated in a graded alcohol series and cleared in xylene. Later, the samples were embedded in paraffin (Panreac Quimica Slu, Castellar del Vallès, Spain) and the different sections were cut using a microtome (Microm HM 340E, Barcelona, Spain). Each paraffin block was cut to 4-µm thickness. The sections were stained with haematoxylin and eosin for light microscopic examination according to standard procedures. Microscopic tissue images were taken in an Olimpus BX50 microscopic system (Olympus Corporation, Shinjuku, Tokyo, Japan) at x10 magnification.

To evaluate possible deposition of lipids in aorta, which can lead to the formation of atherosclerotic plaques, its thickness was measured in the stained sections. To assess possible protective effects in kidney, the renal structure was evaluated through the analysis of the Bowman's space, which can be altered after the chronic administration of dietary lipids.

The aortic thickness and the Bowman's space were determined using the program CellSens Entry (Microscopy Imaging Software by Olympus Life Science, Olympus Corporation, Shinjuku, Tokyo,

Japan). To evaluate the thickness of the aorta and the Bowman's space 60 and 30 measurements were taken per animal, respectively.

2.5.2. Liver Oil Red O staining

A frozen preserved portion of the liver of each rat was subjected to cryostat (Microm HM 505N, Barcelona, Spain) section to a thickness of 8 µm. The stock solutions of Oil Red O were prepared by completely dissolving 0.5 g of Oil Red O (Merck, Darmstadt, Germany) with 100 ml 2-isopropanol 60% (Sigma Aldrich, St Louis, Missouri, United States) using a magnetic stirrer and later it was boiled and filtered using a Whatman No. 2 filter paper (GE Healthcare Life Sciences). The samples were stained following the procedure recommended by the manufacturer. Finally, the nuclei were lightly stained by dipping the slides into haematoxylin solution (Casa Álvarez, Madrid, Spain) for 5 min and rinsing with distilled water for examination using light microscopy (Olympus BX50 microscopic system).

2.5.3. Heart Masson's trichome staining

To assess possible protective effects on heart fibrosis, heart samples were stained with Masson's Trichrome stain to observe collagen deposition. Heart samples were embedded in paraffin and cut following the same procedure as kidney and aorta. The sections were stained with Masson's trichrome staining. Briefly, the paraffin samples were deparaffinized by submerging into xylol and rehydrated by submerging in ethanol 100%, 95% and 70% in this order. The slides were submerged in Bouin's solution, in haematoxilin by Weigert, in trichromic solution and in green light solution in this order (with distilled water washes between each step). After that, slides were washed with distillated water and put in ethanol 100%. Before observation, slides were dipped into xylol and xylol eucalyptol and finally mounted with cover slip for examination using light microscopy (Olympus BX50 microscopic system).

2.6. Plasma biochemical parameters

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDLc), non-high-density lipoprotein cholesterol (non-HDLc), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glucose were measured in the Wistar rat plasma by standardized methods using the Cobas Mira Plus autoanalyzer (Roche Diagnostics, Spain). Insulin was measured by Mercodia Rat Insulin Enzyme-linked Immuno Sorbent Assay (ELISA; reference 10-1250-10) from AD Bioinstruments S.L. (Barcelona, Spain). TC, TG, HDLc, non-HDLc, and Glucose are expressed as mg/dL, insulin is expressed as µg/L, and ALT and AST are expressed as U/L. Non-HDLc was calculated by subtracting the HDLc value from the TC value, for each case.

Table 1. Daily dose (mean ± standard deviation) of the main (poly)phenolics (µg (poly)phenol / day / rat) ingested through the diet supplementation with white-fleshed apple, red-fleshed apple, and aronia infusion by LC-MS/MS.

(Poly)phenolic compounds	White-fleshed apple (µg / 5 g / day)	Red-fleshed apple (µg / 5 g / day)	Aronia (µg / 20 ml infusion / day)
Cyanidin arabinoside	6.88 ± 6.12	167 ± 6.00	480 ± 18.1
Cyanidin galactoside	21.8 ± 18.1	1690 ± 36.0	1426 ± 48.0
Total Anthocyanins	***28.6 ± 24.2***	***1857 ± 42.0***	***1906 ± 67.0***
Protocatechuic acid	n.d.	108 ± 67.3	462 ± 65.2
Coumaric acid hexoside	48.7 ± 7.03	48.7 ± 7.03	2.00 ± 0.03
Ferulic acid hexoside	67.3 ± 7.44	134 ± 16.4	5.00 ± 0.02
Vanillic acid	n.d.	n.d.	14.0 ± 1.01
Vanillic acid hexoside	58.2 ± 4.05	271 ± 7.24	138 ± 22.0
5-*O*-caffeoylquinic acid	1386 ± 28.08	5004 ± 174	814 ± 434
3-*O*-caffeoylquinic acid	n.d.	n.d.	362 ± 97.0
Gallic acid	n.d.	n.d.	36.0 ± 5.03
Gallic acid hexoside	n.d.	n.d.	25.0 ± 6.02
Caffeic acid	n.d.	n.d.	32.0 ± 1.04
Homogentisic acid	n.d.	n.d.	21.0 ± 7.12
Total Phenolic acids	***1837 ± 56.2***	***5566 ± 211***	***1911 ± 638***
Catechin	377 ± 25.3	n.d.	n.d.
Epicatechin	1867 ± 27.1	353 ± 49.3	12.0 ± 1.12
Dimer	3663 ± 121	438± 18.1	32.0 ± 7.15
Trimer	350 ± 51.6	82.0± 7.34	8.00 ± 1.06
Total Flavan-3-ols	***6259 ± 226***	***875 ± 74.9***	***52.0 ± 9.00***
Quercetin arabinoside	166 ± 24.3	232 ± 28.7	5.00 ± 1.07
Quercetin rhamnoside	230 ± 26.6	587 ± 59.7	n.d.
Quercetin glucoside	541 ± 81.0	279± 35.9	133 ± 11.2
Quercetin rutinoside	n.d.	n.d	87.0 ± 12.3
Total Flavonols	***938 ± 132***	***1098 ± 125***	***225 ± 24.0***
Eriodictyol hexoside	n.d.	26.4 ± 1.42	7.00 ± 2.05
Naringenin	n.d.	n.d.	n.d.
Total Flavanones	***n.d.***	***26.4 ± 1.42***	***7.00 ± 2.00***
Phloretin glucoside	247 ± 34.5	1371 ± 160	n.d.
Phloretin xylosyl glucoside	276 ± 17.6	739 ± 32.7	n.d.
Hydroxyphloretin xylosyl glucoside	14.2 ± 1.01	20.5 ± 1.52	n.d.
Total Dihydrochalcones	***536 ± 53.1***	***2130 ± 195***	***n.d.***
TOTAL (POLY)PHENOLICS	***9598 ± 487***	***11552 ± 1420***	***4101 ± 740***

The number of replicates was three. (*n*=3). n.d.: not detected.

2.7. Statistical analysis

The results are presented as mean values ± standard deviation (SD) for (poly)phenolics in SCD, HFD, HFD+W, HFD+R, and HFD+A. The concentration of the anthocyanins and (poly)phenolic metabolites in plasma, urine, and faeces samples are presented as mean values ± standard error of the mean (SEM). For the concentration of the (poly)phenolic metabolites, the values of males and females, and the sum of males and females were compared intra-groups and inter-day with one-way repeated measures analysis of variance General Linear Model and ANOVA one-way.

The results of the plasma biochemical parameters were presented as mean values ± standard deviation (SD), and were analyzed using Student t-test comparing each treatment *versus* HFD.

The results of the histological analysis were presented as mean values ± SEM. The mean of Bowman's space and the mean of the aorta thickness were compared intra-groups with Student t-test (between males and females) and compared between the males of the different groups and between the females of different groups with analysis of variance General Linear Model and ANOVA one-way.

Differences were considered significant at $p < 0.05$. All data were analyzed with Minitab Statistical Software, version 17.2.1 (Minitab Inc., State College, Pennsylvania, United States).

3. Results

3.1. Daily dose of (poly)phenolics administered

A complete (poly)phenolic characterization and quantification of the administered products (red-fleshed apple, white-fleshed apple and aronia infusion) were performed in order to study their metabolic fate after their supplementation in rats and to evaluate their possible biological activities depending on the product administered. **Table 1** shows the daily dose of (poly)-phenols administered to the rats through the supplemented diets.

The daily dose of total (poly)phenols administered through white-fleshed and red-fleshed apples was very similar, 9.60 and 11.6 mg/day/rat, respectively. Regarding the (poly)phenolic composition, both apples had a similar (poly)phenolic profile and content of phenolic acids, flavonols, flavanones, and dihydrochalcones, being the daily dose of anthocyanins the main difference between them. Both apples were also different in the flavan-3-ols content, observing that white-fleshed apple contained around 8-fold higher amounts of flavan-3-ols than red-fleshed apple (**Table 1**).

Aronia fruit administered as an infusion was selected as a rich source of anthocyanins without the components of the apple matrix, containing cyanidin-3-*O*-galactoside and cyanidin arabinoside, the main anthocyanin present in red-fleshed apple. Although, the daily dose of total (poly)phenolic compounds through aronia infusion (4.10 mg/day/rat) was lower than the red-fleshed apple (11.6 mg/day/rat), the anthocyanin dose was nearly the same (around 2 mg/day/rat). Half of the (poly)phenolic dose in aronia were anthocyanins (46.5%), and the other half were phenolic acids, being caffeoylquinic acid and protocatechuic acid the most abundant (**Table 1**).

3.2. (Poly)phenolic profile in plasma, urine and faeces, and their metabolic pathways

To study the metabolic fate of the (poly)phenols administered through both apples and the aronia infusion, we analyzed the (poly)phenol biological metabolites in plasma, urine and faeces, and different metabolic pathways were proposed. **Supplemental Tables 2, 3 and 4** show the concentration of the individual (poly)-phenolic metabolites that presented a concentration significantly higher than the control diets (SCD or HFD) in plasma, urine and faeces, respectively. These data is presented independently for males and females and also as the sum of both. For a better understanding of the results, data from plasma, urine and faeces have been summarized and represented by the sum of compounds of the main (poly)phenolic groups in **Figure 1.** The detected metabolites were derived from the (poly)phenolic families of anthocyanins, phenolic acids (benzoic, phenylacetic and phenylpropionic acids), flavan-3-ols, and dihydrochalcones. The (poly)phenolic metabolites were mainly phase-II sulphated, glucuronided and/or methylated conjugates, and also microbial catabolites from colonic degradation. In order to elucidate how each (poly)phenolic metabolite was generated from its precursor present in apples and aronia infusion, respectively, in **Figure 2** we propose the main metabolic pathways after the intake of the three administered products.

Additionally, in the present study, qualitative and quantitative differences on the metabolic fate of (poly)phenols depending on the gender have been reported, which can be observed in **Figure 1 and in Suplemental Tables 2, 3 and 4**. The most significant differences between males and females are commented in sections below and also reflected in the metabolic pathways represented in **Supplemental Figures 2, 3, and 4,** which refer to urinary metabolites detected after HFD+W, HFD+R, and HFD+A, respectively. In addition, the significant differences between males and females observed in faeces samples are shown in **Supplemental Figure 5**.

3.2.1. Anthocyanin metabolites

As expected, anthocyanins and its metabolites were higher after HFD+R and HFD+A than HFD+W, specially reflected in urine samples (**Figure 1b**)**.** Although the supplemented diets HFD+R and HFD+A provided the same daily amount and type of anthocyanins (2 mg/day/rat), qualitative and quantitative differences were observed in the biological samples studied.

As observed in **Figures 1a and 1b**, the concentration of anthocyanins in plasma and urine were significantly higher after HFD+A compared with HFD+R. In plasma samples, cyanidin-3-*O*-galactoside was the main anthocyanin detected after red-fleshed apple and aronia supplementation. Remarkably, peonidin galactoside was only detected in HFD+A group (**Supplemental Table 2**).

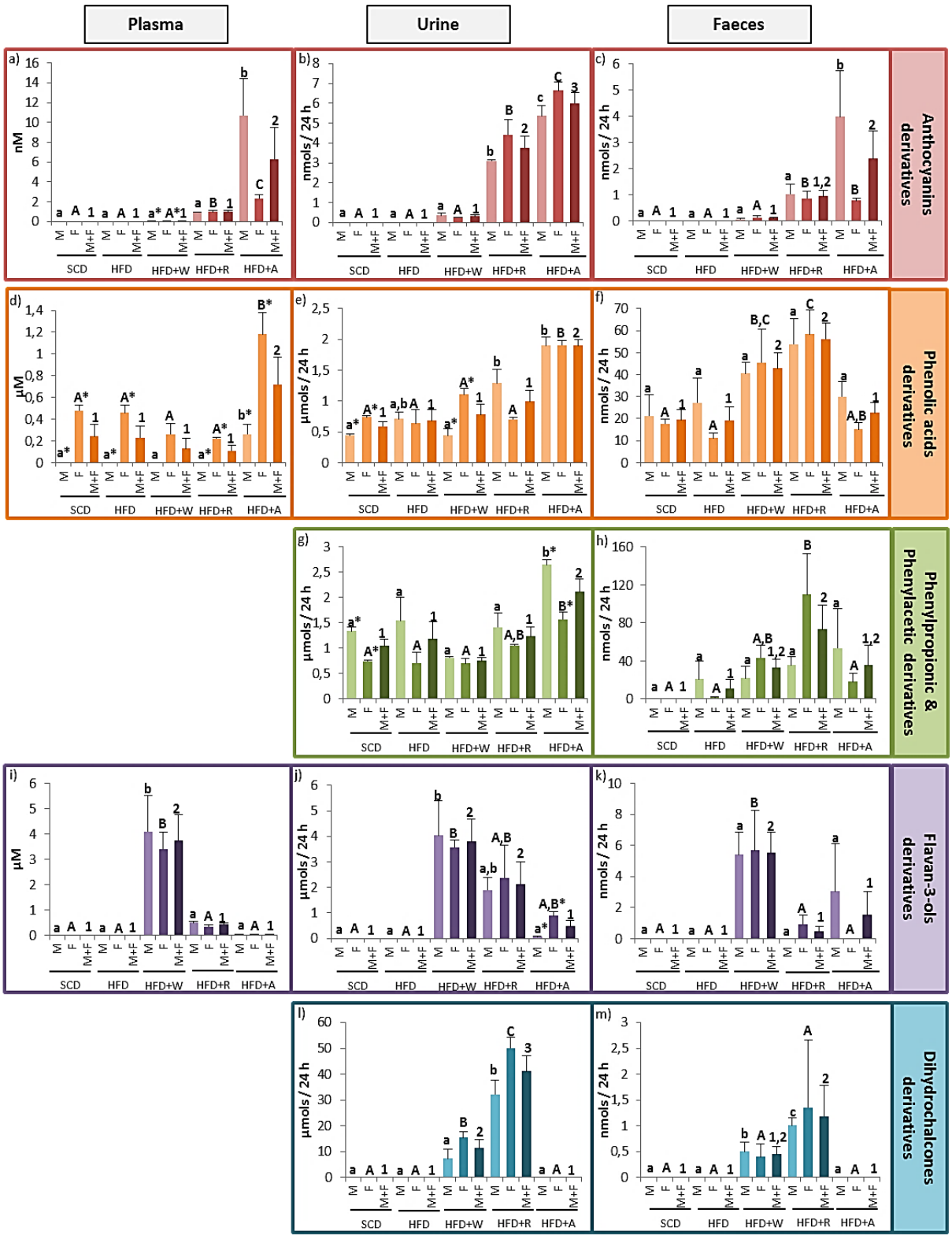

Figure 1. Anthocyanin, phenolic acids, phenylpropionic & phenylacetic, flavan-3-ols and dihydrochalcones derivatives content in plasma, urine and faeces of rats fed with SCD, HFD, HFD+W, HFD+R and HFD+A. Data expressed as mean values ± standard error of mean. Different lowercase letters: indicates differences between males (M) in the different groups; different capital letters: indicates differences between females (F) in the different groups; and different numbers indicates differences between males + females (M+F) in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$). * indicate differences between males & females of the same group (t-student test, $p < 0.05$).

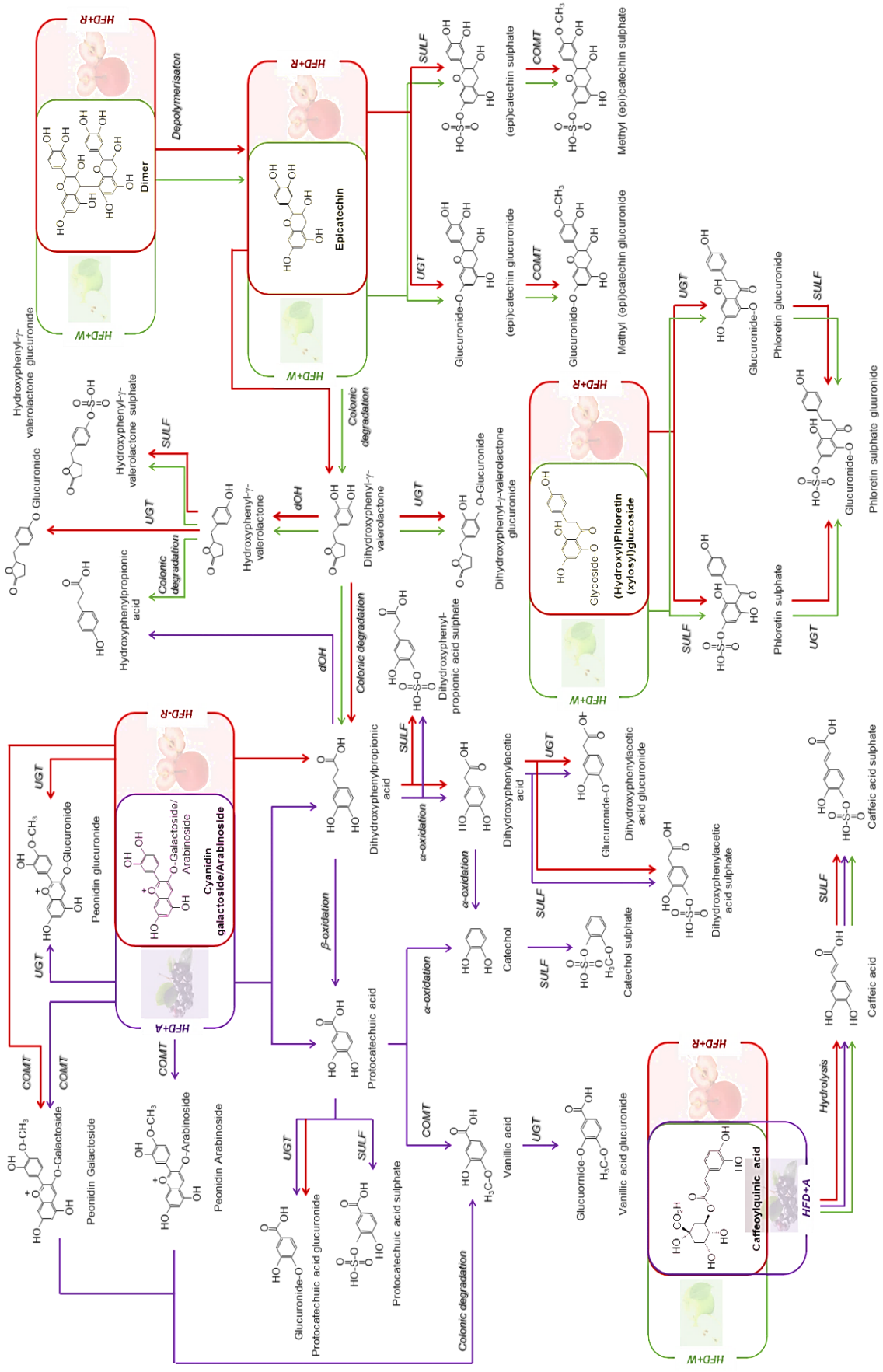

Figure 2. Schematic representation of the main metabolic pathways of the generated (poly)phenolic metabolites after the HFD+W, HFD+R, and HFD+A intakes in M+F and in urine samples.

In urine samples, cyanidin-3-*O*-galactoside was also the main anthocyanin detected in HFD+R, however, peonidin galactoside appeared to be the most abundant in HFD+A. Urinary peonidin arabinoside and peonidin galactoside in HFD+A appeared to be significantly higher than in HFD+R, whereas peonidin glucuronide was significantly higher in HFD+R (**Supplemental Table 3**).

It is noteworthy, that in faeces only the anthocyanins parent compounds were detected, being cyanidin-3-*O*-galactoside the main anthocyanin detected in HFD+R, and cyanidin arabinoside in HFD+A (**Supplemental Table 4**).

Figure 2, also shows some other differences observed in the anthocyanin metabolic pathways between red-fleshed apple (red arrows) or aronia infusion (purple arrows). Protocatechuic acid and dihydroxyphenylpropionic acid are the phenolic acids generated after the fission of B-ring (colonic degradation) of cyanidin glycosides (Mosele et al., 2015b). Our results show that after HFD+R and HFD+A, colonic catabolites from protocatechuic acid and dihydroxyphenyl-propionic acid route were generated. Although similar metabolites were observed between HFD+R and HFD+A, a higher amount of metabolites were detected in urine and plasma after HFD+A, such as protocatechuic acid sulphate, vanillic acid glucuronide and catechol sulphate (**Supplemental Tables 2 and 3**). It must be noted that the metabolites derived from dihydroxyphenyl-propionic acid could also be generated by colonic degradation *via* valerolactones of flavan-3-ols.

No notable differences in anthocyanins metabolism were observed between males and females ($p < 0.05$).

3.2.2. Phenolic acid metabolites

The phenolic acid metabolites detected in plasma were protocatechuic acid sulphate and vanillic acid glucuronide, and together with caffeic acid sulphate, these were also the main excreted metabolites in urine after the three diet supplementations (**Supplemental Tables 2 and 3**). As seen in **Figure 1**, the total phenolic acids concentration only appeared to be significantly higher after HFD+A intake compared to other groups, in plasma and urine samples. Our results are in accordance with a previous study in rats that reported phase II metabolites from protocatechuic acid and vanillic acid in plasma and urine after the intake of an anthocyanin-rich extract (Gomes et al., 2019).

In faeces, the metabolites detected were protocatechuic acid and hippuric acid. Although in plasma and urine phenolic acids were only detected in higher amounts after aronia supplementation, in faeces, these metabolites appeared in higher

concentration after both apple supplementations compared to aronia (**Figure 1d, 1e, 1f**).

It is remarkable that hippuric acid was only detected in faeces samples. Regarding the possible metabolic routes of hippuric acid, we hypothesize three different pathways: 1) by protocatechuic acid derived from the colonic metabolism of cyanidin glycoside; 2) by hydroxyphenylpropionic acid, which has been reported to be a colonic metabolite of dihydrochalcones (Monge et al., 1984; Skjevrak et al., 1986; Tomás-Barberán & Clifford, 2000); and 3) by dihydroxyphenylpropionic acid, which is a colonic metabolite of flavan-3-ols (**Supplemental Figure 5).**

Regarding the gender differences, we observed that in general terms total phenolic acid metabolites were detected in significantly higher amounts in females compared to males, mainly observed in plasma (**Figure 1**). Specifically, after HFD+W, females excreted in urine 6-fold more vanillic acid glucuronide than males (**Supplemental Table 3**), and in plasma this metabolite also appeared 7-fold higher in females than in males after HFD+A, and was only detected in females after HFD+R (**Supplemental Table 2**).

As observed in **Figure 1**, phenolic acid metabolites were also detected in significant amounts in SCD and HFD (control diets). Although protocatechuic and vanillic acid metabolites have been reported as colonic metabolites from cyanidin and peonidin glycosides (B-ring fission), respectively (Mosele et al., 2015b), vanillic acid glucuronide could also be generated by β-oxidation from ferulic acid, which was present in significant amounts in SCD and HFD (control diets) (**Supplemental Table 5 and Supplemental Figure 6)**.

3.2.3. Phenylacetic and phenylpropionic acid metabolites

After the three supplemented diets (HFD+W, HFD+R and HFD+A), significant differences in phenylacetic acids (PAA) and phenylpropionic acids (PPA) were only observed in urine and faeces.

In urine samples, the total concentration of PPA and PAA was only significantly higher after HFD+A (**Figure 1g**), being dihydroxyphenylacetic acid sulphate the most abundant metabolite. PPA and PAA metabolites could be generated mainly from anthocyanins by B-ring fission (colonic degradation) in HFD+A, but also from flavan-3-ols in diets supplemented with both apples (**Figure 2**). Similarly to phenolic acids, PAA and PPA presented a different response in faeces compared to urine, being significantly higher in urine after HFD+A and higher in faeces after HFD+R (**Figure 1g, 1h**). Specifically, *m*-hydroxyphenylacetic was the predominant metabolite in faeces significantly incremented after HFD+R and was only

detected in females (**Supplemental Table 4 and Supplemental Figure 5b**).

3.2.4. Flavan-3-ol metabolites

Flavan-3-ols were more abundant in the white-fleshed apple (**Table 1**), which was clearly reflected in biological samples, specially in plasma, observing significant higher levels of all the derived metabolites after HFD+W (**Figure 1i**). The sum of flavan-3-ol metabolites includes both phase II metabolites and colonic metabolites (valerolactones). These monomers (catechin and epicatechin) can also be generated from dimer by depolymerization (**Figure 2**).

In plasma, all the detected metabolites were significantly higher after HFD+W than in other groups, being catechin glucuronide the most abundant (**Supplemental Table 2**).

Similarly, in urine samples, the concentrations of all the individual metabolites were significantly higher in the HFD+W than other groups (**Supplemental Table 3**). In this case, hydroxyphenyl-ɣ-valerolactone sulphate was the most abundant metabolite. It is noteworthy that significant gender differences were reported in urine in some flavan-3-ols metabolites in the HFD+W group. For instance, we observed that after HFD+R females excreted almost 3-fold more methyl epicatechin suphate than males ($p < 0.05$). Also, after HFD+A intake, females presented almost 60-fold more hydroxy-phenyl-ɣ-valerolactone sulphate compared to males ($p < 0.05$).

In faeces samples, (epi)catechin, dimer, trimer and methyl catechin sulphate were detected, being all metabolites also significantly higher after HFD+W compared to other groups (**Supplemental Table 4**).

3.2.5. Dihydrochalcone metabolites

Dihydrochalcones, and particularly phloretin, are a specific polyphenolic group from apples, and this was reflected in our results, observing that their derived metabolites were only detected after both apples supplementation. It was also noted that dihydrochalcones were the most abundant excreted in urine after the supplementation with both apples (HFD+R and HFD+W), representing around the 90% of total metabolites excreted in the HFD+R group. The concentration of dihydrochalcones in red-fleshed apple was around 4-fold higher than in white-fleshed apple (**Table 1**), and this fact was reflected in urine and in faeces observing that the total urine dihydrochalcone concentration in HFD+R was significantly higher than in HFD+W (**Figures 1l, 1m**). In urine samples, phloretin sulphate was the most abundant metabolite. In faeces, only the parent compounds (hydroxyl)phloretin and (xylosyl)glucoside were detected after supplementation of both apples. The total concentration in HFD+R was similar to

HFD+W and the concentrations of the three compounds followed the same trend (**Supplemental Table 4**).

3.3. Aorta, kidney, liver and heart histological analysis

3.3.1. Aorta samples: differences in thickness

To evaluate possible deposition of lipids in the aorta, which can lead to the formation of atherosclerotic plaques, its thickness was measured in sections stained with haematoxylin-eosin. Results showed significant differences between males and females in all diet groups, therefore, we analyzed the results independently (**Figures 3a and 3b)**. Regarding rat males, the aortic thickness of the HFD group (136 ± 10.1 μm) was significantly higher than the other groups (**Figure 3a and Supplemental Figures 7a-e)**. Male aortic thickness of HFD+W (112 ± 8.38 μm), HFD+R (109 ± 8.17 μm) and HFD+A (107 ± 8.00 μm) groups were similar to that of SCD control group (112 ± 8.32 μm).

Unlike the males, the females had different response depending on the administered diet (**Figure 3b and Supplemental Figures 7f-j**). The aortic thickness in HFD+W (106 ± 7.92 μm) was similar to those in SCD group (102 ± 7.62 μm) and lower compared to males in the same group. In the HFD+R group (115 ± 8.64 μm) a significant improvement was observed compared to the HFD group (127 ± 9.44 μm), although the effect was not as effective as HFD+W. By contrast, the HFD+A diet (128 ± 9.50 μm) did not show a significant reduction in aortic thickness compared with HFD control group.

3.3.2. Kidney samples: Bowman's space

To assess possible protective effects against a fatty diet on the kidneys, differences in Bowman's space were evaluated. Since significant differences were observed between males and females of the same group, results were presented separately (**Figures 3c and 3d**). It has been proven that the chronic administration of dietary lipids alters the renal structure and one of the parameters that is altered is the Bowman's space that increases and leads to glomerular atrophy and functional loss of glomeruli and tubules (Altunkaynak et al., 2008; Hazarika et al., 2016).

In males, no significant differences were observed in the Bowman's space area among groups (**Figure 3c**). In contrast, female samples showed that after HFD+W (1675 ± 177 μm^2), HFD+R (1560 ± 164 μm^2) and HFD+A (1578 ± 166 μm^2), the Bowman's space area was significantly reduced compared to the HFD group (2388 ± 251 μm^2) ($p < 0.05$), but significantly higher than SCD (889 ± 93.8 μm^2).

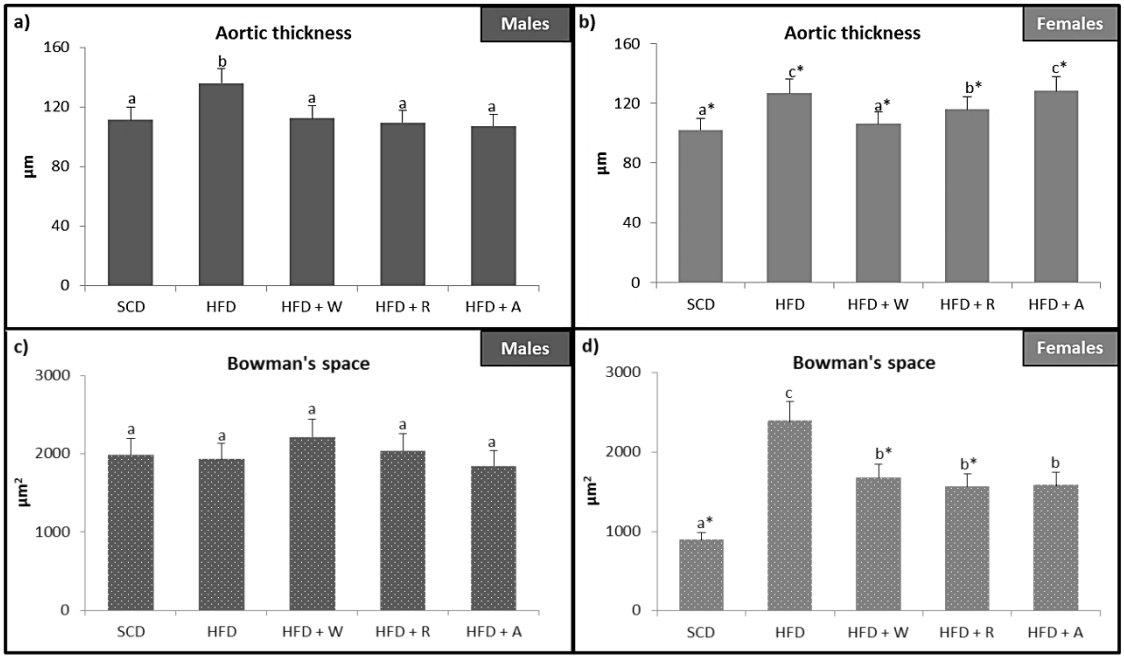

Figure 3. Aortic thickness (µm) in males (a) and in females (b); and Bowman's space (µm²) in males (c) and in females (d) of the groups SCD, HFD, HFD+W, HFD+R and HFD+A.
Data expressed as mean values ± standard error of mean. Values not displaying the same letter are significantly different (one-way ANOVA, Tukey's test between all means, $p < 0.05$). *indicates differences between males and females of the same group (t-student test $p < 0.05$).

3.4. Fatty liver development by Oil Red O staining

To evaluate possible attenuation effects of (poly)phenols on fatty liver development, samples were stained with Oil Red O staining to assess the lipid accumulation. As it can be observed in **Figure 4**, HFD led to an increase in cellular lipid accumulation compared to SCD. However, the diet supplementation with either apples or aronia did not reduce the lipid accumulation, inflammation or modified hepatocytes, in comparison to the HFD group.

3.5. Heart Masson's trichrome staining

To assess possible protective effects of (poly)phenols on the development of heart fibrosis that occurs in a chronic inflammatory process, heart samples were stained with Masson's trichrome stain to observe collagen deposition. As seen in **Figure 4**, this staining revealed that widths of cardiac myocytes and the collagen deposition (blue color) in the perivascular area that leads to cardiac fibrosis increased similarly after HFD, HFD+R, HFD+W and HFD+A compared to the SCD group. No differences were observed between the groups supplemented with apples or aronia infusion.

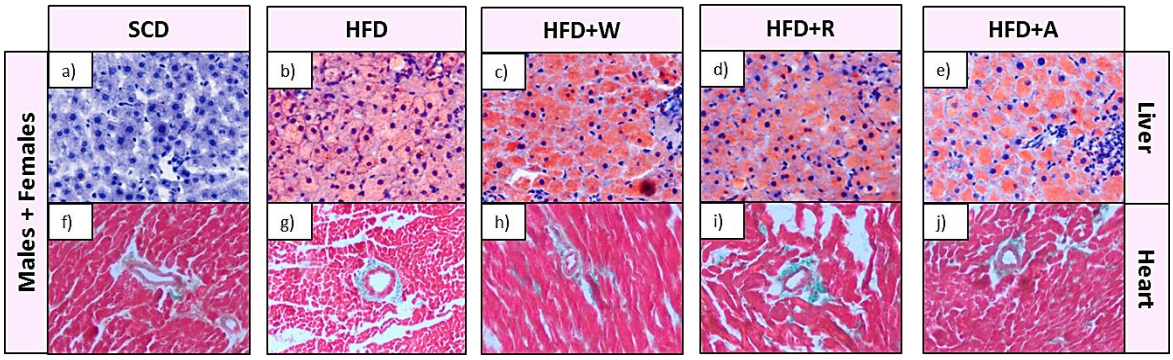

Figure 4. Representative images (at a magnification of 20x) of Oil Red O-stained livers of females and males from: a) SCD, b) HFD, c) HFD+W, d) HFD+R, and e) HFD+A. Representative images (at a magnification of 20x) of Masson's trichrome stained hearts of females and males from: f) SCD, g) HFD, h) HFD+W, i) HFD+R, and j) HFD+A.

Table 2. Biochemical parameters of rat plasma from the different studied groups.

		TC (mg/dL)	TG (mg/dL)	HDLc (mg/dL)	Non-HDLc (mg/dL)
HFD	F (n=3)	426 ± 147	130 ± 51.4	47.4 ± 12.4	379 ± 147
	M (n=3)	224 ± 62.0	151 ± 43.0	36.3 ± 11.9	188 ± 52.8
	M&F (n=6)	325 ± 150	141 ± 44.0	41.8 ± 12.5	283 ± 144
SCD	F (n=3)	69.2 ± 7.72 (ns)	56.4 ± 29.3 (ns)	52.5 ± 2.83 (ns)	16.8 ± 6.14 (0.013*)
	M (n=3)	73.5 ± 13.3 (0.046*)	141 ± 90.9 (ns)	54.6 ± 7.27 (ns)	18.9 ± 7.09 (0.029*)
	M&F (n=6)	71.4 ± 10.0 (0.009)	96.4 ± 76.0 (ns)	53.5 ± 5.07 (ns)	17.8 ± 6.05 (0.006*)
HFD+W	F (n=3)	477 ± 190 (ns)	115 ± 7.97 (ns)	33.5 ± 15.6 (ns)	443 ± 174 (ns)
	M (n=3)	227 ± 41.0 (ns)	91.5 ± 29.8 (ns)	55.3 ± 27.9 (ns)	172 ± 39.6 (ns)
	M&F (n=6)	352 ± 184 (ns)	103 ± 23.4 (ns)	44.4 ± 23.5 (ns)	307 ± 187 (ns)
HFD+R	F (n=3)	548 ± 307 (ns)	216 ± 144 (ns)	44.8 ± 23.5 (ns)	503 ± 289 (ns)
	M (n=3)	233 ± 33.6 (ns)	88.3 ± 27.3 (ns)	40.6 ± 9.37 (ns)	192 ± 24.4 (ns)
	M&F (n=6)	390 ± 261 (ns)	152 ± 116 (ns)	42.7 ± 16.2 (ns)	347 ± 250 (ns)
HFD+A	F (n=3)	536 ± 157 (ns)	212 ± 68.6 (ns)	40.3 ± 2.60 (ns)	495 ± 154 (ns)
	M (n=3)	236 ± 66.8 (ns)	137 ± 84.1 (ns)	43.4 ± 13.4 (ns)	193 ± 72.7 (ns)
	M&F (n=6)	386 ± 196 (ns)	174 ± 80.0 (ns)	41.8 ± 8.78 (ns)	344 ± 198 (ns)

		ALT (U/L)	AST (U/L)	Insulin (µg/L)	Glucose (mg/L)
HFD	F (n=3)	110 ± 50.8	447 ± 146	1.24 ± 0.66	225 ± 23.0
	M (n=3)	189 ± 70.0	314 ± 76.8	5.27 ± 0.34	211 ± 11.2
	M&F (n=6)	149 ± 70.0	381 ± 127	3.26 ± 2.25	218 ± 18.0
SCD	F (n=3)	43.3 ± 16.2 (ns)	170 ± 88.6 (0.048*)	0.46 ± 0.10 (ns)	222 ± 18.6 (ns)
	M (n=3)	44.3 ± 10.1 (0.024*)	179 ± 1.73 (ns)	0.84 ± 0.13 (<0.001*)	264 ± 39.9 (ns)
	M&F (n=6)	43.8 ± 12.1 (0.013*)	175 ± 56.3 (0.005*)	0.61 ± 0.23 (0.035*)	243 ± 36.2 (ns)
HFD+W	F (n=3)	107 ± 61.4 (ns)	410 ± 280 (ns)	1.07 ± 0.74 (ns)	228 ± 13.2 (ns)
	M (n=3)	241 ± 102 (ns)	494 ± 219 (ns)	3.49 ± 0.63 (0.013*)	228 ± 13.7 (ns)
	M&F (n=6)	174 ± 105 (ns)	452 ± 229 (ns)	2.52 ± 1.44 (ns)	228 ± 12.0 (ns)
HFD+R	F (n=3)	101 ± 48.4 (ns)	380 ± 93.7 (ns)	1.33 ± 0.94 (ns)	278 ± 62.3 (ns)
	M (n=3)	210 ± 40.4 (ns)	318 ± 61.8 (ns)	3.22 ± 0.34 (0.002*)	236 ± 14.5 (ns)
	M&F (n=6)	156 ± 71.5 (ns)	349 ± 78.8 (ns)	2.28 ± 1.21 (ns)	257 ± 46.7 (ns)
HFD+A	F (n=3)	88.7 ± 61.7 (ns)	358 ± 197 (ns)	1.39 ± 1.03 (ns)	260 ± 44.3 (ns)
	M (n=3)	246 ± 23.5 (ns)	450 ± 83.3 (ns)	2.08 ± 2.24 (ns)	209 ± 42.3 (ns)
	M&F (n=6)	167 ± 95.8 (ns)	404 ± 144 (ns)	1.73 ± 1.60 (ns)	235 ± 47.8 (ns)

Concentration is expressed as mean ± standard deviation. Data were analyzed using Student t-test comparing each treatment *versus* HFD. * $p < 0.05$ each treatment *versus* HFD.

3.6. Plasma biochemical parameters

Data of various biochemical parameters including insulin, glucose, lipid profile and liver enzymes are presented as mean ± SD values in **Table 2**. In general, high intra-individual variability was observed in all parameters**.** The effects of the HFD were reflected in a significant increase of TC, non-HDLc, ALT, AST, and insulin values ($p < 0.05$) compared to SCD. Results showed that the supplemented diets with apples or aronia did not attenuate these effects, observing no significant reductions compared to HFD in all parameters, except for insulin. Remarkably, this depletion of insulin levels was only observed in males in the three supplemented HFD diets, being statistically significant ($p < 0.05$) in HFD+R and HFD+W diets.

4. Discussion

In the present study, we investigate the response to a sustained intake of red-fleshed apple biofortified in anthocyanins on the (poly)phenolic metabolism and their cardiovascular effects associated. For this purpose, we supplemented rats that fed a HFD to induce hypercholesterolemia with red-fleshed apple and compared results to a common white-fleshed apple without anthocyanins. To evaluate the impact of the components of the apple matrix, a group supplemented with anthocyanin-rich infusion was included.

Regarding the results on absorption and metabolic fate of anthocyanins, although the amount and profile of anthocyanins in red-fleshed apple and aronia infusion were the same, the metabolic profile detected in biological samples differed quantitatively and qualitatively. The differences observed could be related to the effect of the apple matrix. In apple fruit, the anthocyanins are bound to fibre and saccharides, whereas in the aronia infusion the anthocyanins are in their free forms more available, which favour their gastrointestinal absorption and metabolism. The metabolites observed in the present study were in agreement with previous studies describing the metabolic fate of anthocyanin after the intake of red-fleshed apple in a postprandial study in humans (Yuste et al., 2019), and aronia in rats (He et al., 2006; Wiczkowski et al., 2010). The apple matrix effect was also observed in phenolic acids, PPA and PAA metabolites, which appeared in higher concentration in urine after HFD+A, and in faeces after HFD+R. This fact indicates that anthocyanins and phenolic compounds linked to apple fibre are absorbed more slowly compared to aronia infusion, and therefore, they reach the large intestine where they are catabolized by the colonic microbiota appearing in higher amounts in faeces. Regarding the other (poly)phenolic metabolites, our results from flavan-3-ols are in agreement with previous studies, reporting similar phase II metabolites and

valerolactones after the intake of flavan-3-ol rich extracts, such as apple (Yuste et al., 2019), or wine (Motilva et al., 2016). The detected dihydrochalcone metabolites were also reported by other authors in biological samples after the intake of cider (Marks et al., 2009), and red-fleshed apple snack in humans (Yuste et al., 2018; Yuste et al., 2019). To our knowledge, the colonic metabolism of dihydrochalcones has been scarcely studied, and the obtained results were in agreement with previous studies (Monge et al., 1984; Skjevrak et al., 1986; Tomás-Barberán & Clifford, 2000).

When studying the metabolic fate of (poly)phenols after apple and aronia supplementation, a gender effect was also reported. In general terms, the differences between males and females detected in urine samples could be summarized as: i) the methylated, glucuronided, methyl-glucuronided and methylsulphate conjugates were found in higher concentrations or predominantly in females; and ii) the sulphate conjugates in males. These results were in agreement with those reported in previous studies in rat model (Soukup et al., 2016; Yang et al., 2011) and in humans (Dellinger et al., 2014) showing that the sulphatation and glucuronidation were more intense in males than in females (Dellinger et al., 2014). Contrary to our results, several studies have reported that some isoforms of catechol-*O*-methyltransferases (COMT) enzymes may be more active in males than in females (Margalef et al., 2016; Piskula, 2000; Yang et al., 2011). In quantitative terms, the most significant differences between genders were observed in phenolic acid metabolites, observing that females absorbed and excreted significant higher amounts of these metabolites after all the supplemented diets. These differences could be explained by the sex-dependent expression of many isoforms of gastrointestinal enzymes or/and transporters that participate in the absorption of (poly)phenolics (Cassidy & Minihane, 2017). For example, in rats, β-glucosidase activity is greater in females than in males (Wang & Trumbo, 1996).

Concerning the cardioprotective parameters studied, a significant reduction was reported in aortic thickness as a consequence of apple (red- and white-fleshed) and aronia intake compared with HFD, indicating a protective atherogenic effect of the three administered products.

Particularly, the effect observed in HFD+W group could be mainly due to flavan-3-ols metabolites, being catechin glucuronide and hydroxyphenyl-ɣ-valerolactone sulphate the most abundant metabolites detected in plasma and urine samples, respectively (**Supplemental Tables 2 and 3**). The anti-atherosclerotic potential of flavan-3-ols from apples has already been demonstrated in mice, after the administration of cider apple extracts in apo-E deficient mice (Auclair et al., 2008). Similarly, the diet

supplementation with an apple (poly)phenolic extract rich in flavan-3-ols for 12 weeks in hypercholesterolemic male mice (Xu et al., 2015) produced a reduction of the mean atherosclerotic lesion area in the aortic sinus. Similar effects were also observed in male hypercholesterolemic rabbits after the sustained intake of apple juices producing a significant reduction of the atherosclerotic lesions of coronary arteries (Setorki et al., 2009). Also, in agreement with our results, after the chronic administration in Sprague Dawley rats of a persimmon fruit extract rich in catechin derivatives, a significant decrease in aortic thickness was observed (Yuwanda et al., 2019).

Regarding the HFD+R group, anthocyanins and dihydrochalcones were the most abundant polyphenolic compounds and also their metabolites in plasma and urine, so we hypothesize that the reduction of the aortic thickness at levels of SCD group could be mainly related to anthocyanins and dihydrochalcones, with a minor contribution of flavan-3-ols. Similar effects have been reported in rabbits and mice studies supplementing with apples in which dihydrochalcones were studied (Auclair et al., 2008; Setorki et al., 2009; Xu et al., 2015). The potential anti-atherosclerotic role of anthocyanins was also consistent with other studies in which lower fatty deposition in the arteries of hypercho-lesterolemic male rabbits was observed after the intake of sun-dried berries for 8 weeks (Yanni et al., 2015), or after the administration of red-fruit juices like raspberry in hypercholesterolemic hamsters (Rouanet et al., 2010).

In the case of aronia supplementation, the attenuation effects observed in the aorta can be attributed to anthocyanins, as these were the main (poly)phenols administered through the aronia infusion and was also reflected in a higher amount of their derived metabolites in plasma and urine. These results are in agreement with a previous study (Qin & Anderson, 2012) in which it was observed a decrease in epididymal fat accumulation in hypercholesterolemic Wistar rats after the administration of aronia fruit (at two different concentration, 3 or 6 mg daily) for 6 weeks.

Unlike the males, the females had different response on the aortic thickness depending on the administered diet, observing a lower attenuation effect and no effect after aronia intake in females. In relation to this, contradictory results are described in the literature regarding the anti-atherogenic effects of anthocyanins. Some studies with males of different species such as rabbits (Yanni et al., 2015), hamsters (Rouanet et al., 2010) or Wistar rats (Qin & Anderson, 2012) have reported the anti-atherogenic effects of anthocyanins, whereas other studies (Valcheva-Kuzmanova et al., 2007a; Valcheva-Kuzmanova et al., 2007b) did not report histological differences in aorta after

the sustained administration of *Aronia melanocarpa* (106.8 mg anthocyanins per 100 ml water) in males Wistar rats. Regarding the HFD+W group, a reduction of aortic thickness was detected in both males and females, which corroborates the protective effect of flavan-3-ol in the development of atherosclerotic lesions.

The attenuation effects in the reduction of aorta thickness were not related with the plasma lipid profile, since no significant differences were observed in TC, TG, HDL, non-HDL after the three supplemented diets compared with HFD group. This fact could indicate that the absolute cholesterolemia is not an indicative parameter of the aortic fatty streak deposition (Andrews et al., 1995; Breinholt et al., 1999).

Regarding the studied effects on kidney, an attenuation effect in the renal structure was only observed in females after the three supplemented diets compared to HFD. This fact could probably be related to the gender-related differences in the (poly)phenol bioavailability, as we observed higher amounts of urinary (poly)phenolic metabolites in females compared to males. Although the differences were not significant, the total excretion of (poly)phenolic metabolites in urine was greater in females after the three supplementation diets, suggesting that an accumulation of (poly)phenolic metabolites in the kidney could lead to major effects in female kidneys. This higher excretion of (poly)phenolic metabolites observed in females is consistent with studies in which a greater excretion of isoflavone conjugates (Lu & Anderson, 1998) or hydroxytyrosol derivatives (Domínguez-Perles et al., 2017) was observed in females after the administration of soy milk or high doses of hydroxytyrosol in humans and in Sprague Dawley rats, respectively.

As it was discussed for the aorta, the attenuation effects reported in kidney after the supplemented diets could be mainly related to anthocyanins, flavan-3-ols, and dihydrochalcones. The effect observed after HFD+W on the accumulation of fat in the kidney could be mainly attributed to the flavan-3-ol metabolites, being hydroxyplenyl-ɣ-valerolactone sulphate (urine) and catechin glucuronide (plasma and urine) the main metabolites detected.

The effect observed in kidney structure after both apple supplementation, especially after HFD+R, could be also attributed to the dihydrochalcone metabolites detected in high concentration in urine, being phloretin sulphate the main metabolite with a higher concentration in females. The effects observed in kidney after the apple consumption are consistent with the studies reported in the literature. After the intake of a dried apple rich in flavan-3-ols and dihydrochalcones, kidney function improved, and the concentration of uric acid, urea, and creatinine decreased

in 8 Sprague Dawley hyperlipidemic rats (Abdel-Rahim & El-Beltagi, 2010). In addition, after apple supplementation (approximately 20% of the daily intake) of obese Zucker rats, the glomerulopathy with the consequent proteinuria was reduced (Aprikian et al., 2002).

In addition, anthocyanins could be also responsible for the effects observed in the HFD+A group, and to a lesser extent in the HFD+R group as commented for aorta results, which could be in agreement with previous studies. After the administration of black soybean in hypercholesterolemic Sprague Dawley rats, anthocyanins reversed the effects of HFD on body weight, serum lipids and decreased the weights of epididymal and perirenal fat pats (Kwon et al., 2007). In another study in Wistar rats, after the supplementation with *Aronia melanocarpa* extract (with (poly)phenol doses approximately 8-fold higher than those administered in our study), the antioxidant status was improved, especially, the concentration of a lipid peroxidation indicator, the thiobarbituric acid reactive substances (TBARS) in the kidneys (Jurgoński et al., 2008).

It is noteworthy that, apart from the effects observed in aortas and kidneys, a significant decrease in insulin values was also observed in the male plasma from the supplemented groups with apples (3.49 ± 0.63 and 3.22 ± 0.34 μg/L in HFD+W and HFD+R, respectively) compared with HFD group (5.27 ± 0.34 μg/L). These results are in agreement with the results reported in the literature (Anhê et al., 2015; Qin & Anderson, 2012). After the administration of cranberry extract rich in anthocyanins and flavan-3-ols (Anhê et al., 2015) or aronia extract (Qin & Anderson, 2012) in obese mice, the insulin values decreased with respect to the control HFD. One of the effects of HFD diets is their induction of adipose tissue dysfunction. This can alter diverse factors, inducing to systemic insulin resistance, which is a major contributor to the development of type 2 diabetes (Lackey et al., 2016). The anti-insulin resistance effect of (poly)phenols is partly due to their ability to reverse this dysregulation. It has also been reported that after chokeberry extract consumption rich in anthocyanins, the Ppargamma mRNA expression increased, the Fabp4, Fas and Lpl mRNA levels were suppressed, a decrease of mRNA expression of TNF-α and IL-b and IL-6 was induced and in consequence, the plasma levels of TNF-α and IL-6 were decreased (Qin & Anderson, 2012). In other studies in diet-induced insulin-resistant animals, after the administration of (poly)phenols from cinnamon (Qin et al., 2010), and green tea (Lee et al., 2009), Fas and Lpl mRNA expression and other genes related with lipogenesis were inhibited leading to improve systemic insulin sensitivity and dyslipidemia by enhancing insulin signalling.

The obtained results in liver and heart stainings are in agreement with plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values, since no significant differences were observed concerning HFD after diet supplementations. These results may be due to the fact that the HFD diet was so powerful that the attenuation effect of (poly)phenols administered in relatively low doses could not be appreciated at the macromolecular level. These results contrast with another study (Aprikian et al., 2002) performed in another rat model (obese Zuckers rats) in which after the administration of a similar dose of lyophilized apple, a cardioprotective (decreasing TG in the heart) and hepatoprotective (limiting liver steatosis) effects were observed.

The main limitation of the study is the low number of animals per group, so future studies with higher *n* are necessary to corroborate the observed effects and also to determine the molecular mechanisms.

5. Conclusions

Our study showed the *in vivo* cardiometabolic protective effects of both red-fleshed and white-fleshed apples and aronia infusion supplementation in hypercholesterolemic rats, specifically in the reduction of the aorta thickness, the improvement of the renal function and the reduction of insulin levels. Our findings support that anthocyanins without apple matrix through aronia infusion can induce cardioprotective effects. In the case of apples, anthocyanins or flavan-3-ols, together with dihydrochalcones, compose a polyphenolic phytocomplex in red and white-fleshed apples, respectively, that could act synergistically in the attenuation of cardiovascular outcomes.

A gender effect was also reported probably related with the differences observed in the metabolic fate of (poly)phenols. These differences in (poly)phenol metabolism were specially noted in the kidney function which was improved only in females, and may be related to the higher (poly)phenol bioavailability observed in females. So our results suggest that differences in the metabolic fate of (poly)phenolic compounds underlie the possibility that (poly)phenols can differently influence the health outcomes of males and females.

Moreover, an apple matrix effect was reported between red-fleshed apple and aronia infusion observing a higher absorption and excretion of anthocyanins after aronia supplementation without the apple components.

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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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Supplemental Table 1. SRM conditions used for the analysis of phenolic and their generated metabolites.

(Poly)phenolic metabolites	SRM Quantification	CV (V) / CE (eV)	Standard in which has been quantified
Catechols			
Catechol sulphate	189 > 109	20 / 15	Catechol-4-*O*-sulphate
Methyl catechol sulphate	203 > 123	20 / 15	4-methyl catechol sulphate
Methyl catechol glucuronide	299 > 123	20 / 15	4-methyl catechol sulphate
Benzoic acids			
Hydroxybenzoic acid	137 > 93	30 / 15	*p*-Hydroxybenzoic acid
Hydroxybenzoic acid sulphate	217 > 137	35 / 15	*p*-Hydroxybenzoic acid
Hippuric acid	178 > 134	40 / 10	*p*-Hydroxybenzoic acid
Hydroxyhippuric acid	194 > 100	40 / 10	*p*-Hydroxybenzoic acid
Gallic acid	169 > 125	35 / 10	Gallic acid
Gallic acid hexoside	331 > 169	40 / 15	Gallic acid
Protocatechuic acid	153 > 109	45 /15	Protocatechuic acid
Homogentisic acid	153 > 109	45 /15	Protocatechuic acid
Protocatechuic acid sulphate	233 > 153	35 / 15	Protocatechuic acid
Protocatechuic acid glucuronide	329 > 153	35 / 15	Protocatechuic acid
Vanillic acid	167 > 123	30 / 10	Vanillic acid
Vanillic acid hexoside	329 > 167	30 / 15	Vanillic acid
Vanillic acid sulphate	247 > 167	30 / 25	Vanillic acid-4-*O*-sulphate
Vanillic acid glucuronide	343 > 167	30 / 25	Vanillic acid
Phenylacetic acids			
p-Hydroxyphenylacetic acid	151 > 107	20 / 10	3-(4'-hydroxyphenyl)acetic acid
m-Hydroxyphenylacetic acid	151 > 107	20 / 10	3-(4'-hydroxyphenyl)acetic acid
o-Hydroxyphenylacetic acid	151 > 107	20 / 10	3-(4'-hydroxyphenyl)acetic acid
Hydroxyphenylacetic acid sulphate	231 > 151	20 / 15	3-(4'-hydroxyphenyl)acetic acid
Hydroxyphenylacetic acid glucuronide	327 > 151	20 / 15	3-(4'-hydroxyphenyl)acetic acid
Dihydroxyphenylacetic acid sulphate	247 > 167	30 / 15	3-(3',4'-dihydroxyphenyl) acetic acid
Dihydroxyphenylacetic acid glucuronide	343 > 167	30 / 15	3-(3',4'-dihydroxyphenyl) acetic acid
Phenylpropionic acids			
Phenylpropionic acid	149 > 105	20 / 10	Phenylpropionic acid
Hydroxyphenylpropionic acid	165 > 121	20 / 10	3-(3'-hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid sulphate	245 > 165	35 / 15	3-(3'-hydroxyphenyl)propionic acid
Hydroxyphenylpropionic acid glucuronide	341 > 165	40 / 25	3-(3'-hydroxyphenyl)propionic acid
Dihydroxyphenylpropionic acid	181 > 137	35 / 15	3-(3',4'-dihydroxyphenyl) propionic acid
Dihydroxyphenylpropionic acid sulphate	261 > 181	40 / 15	3-(3',4'-dihydroxyphenyl) propionic acid
Dihydroxyphenylpropionic acid glucuronide	357 > 181	40 / 20	3-(3',4'-dihydroxyphenyl) propionic acid
Hydroxymethoxyphenylpropionic acid	195 > 136	30 / 15	3-(3'-hydroxy-4'-methoxyphenyl)propionic acid
Hydroxymethoxyphenylpropionic acid sulphate	275 > 195	35 / 15	3-(3'-hydroxy-4'-methoxyphenyl)propionic acid

Hydroxycinnamic acid derivatives			
Coumaric acid	163 > 119	35 / 10	*p*-coumaric acid
Coumaric acid hexoside	325 > 163	35 / 15	*p*-coumaric acid
Caffeic acid	179 > 135	35 / 15	Caffeic acid
3-*O*-caffeoylquinic acid	353 > 191	30 / 10	5-*O*-caffeoylquinic acid
5-*O*-caffeoylquinic acid	353 > 191	30 / 10	5-*O*-caffeoylquinic acid
Caffeic acid sulphate	259 > 179	35 / 15	Caffeic acid
Ferulic acid	193 > 134	30 / 15	Ferulic acid
Ferulic acid hexoside	355 > 193	35 / 15	Ferulic acid
Phenyl-ɣ-valerolactone derivatives			
Hydroxyphenyl-ɣ-valerolactone	191 > 147	40 / 10	Epicatechin
Hydroxyphenyl-ɣ-valerolactone sulphate	271 > 191	40 / 20	Epicatechin
Hydroxyphenyl-ɣ-valerolactone glucuronide	367 > 191	40 / 20	Epicatechin
Dihydroxyphenyl-ɣ-valerolactone sulphate	287 > 207	40 / 15	Epicatechin
Dihydroxyphenyl-ɣ-valerolactone glucuronide	383 > 207	40 / 20	Epicatechin
Flavan-3-ol			
Catechin	289 > 245	45 / 15	Catechin
Epicatechin	289 > 245	45 /15	Epicatechin
Catechin glucuronide	465 > 289	40 / 20	Catechin
Epicatechin glucuronide	465 > 289	40 / 20	Epicatechin
Methyl catechin sulphate	383 > 303	40 / 15	Catehin
Methyl epicatechin sulphate	383 > 303	40 / 15	Epicatehin
Methyl catechin glucuronide	383 > 303	40 / 15	Catechin
Methyl epicatechin glucuronide	383 > 303	40 / 15	Epicatechin
Dimer	577 > 289	45 / 20	Dimer B_2
Trimer	865 > 287	60 / 30	Dimer B_2
Dihydrochalcones			
Phloretin glucoside	435 > 273	40 / 15	Phloretin-2'-*O*-gucoside
Phloretin xylosylglucoside	567 > 273	45 / 15	Phloretin-2'-*O*-gucoside
Hydroxylphloretin xylosylglucoside	583 > 289	45 / 15	Phloretin-2'-*O*-gucoside
Phloretin glucuronide	449 > 273	40 / 20	Phloretin-2'-*O*-gucoside
Phloretin sulphate	353 > 273	40 / 20	Phloretin-2'-*O*-gucoside
Phloretin sulphate glucuronide	529 > 353	40 / 20	Phloretin-2'-*O*-gucoside
Anthocyanins			
Cyanidin arabinoside	419 > 287	40 / 20	Cyanidin-3-*O*-galactoside
Cyanidin-3-*O*-galactoside	449 > 287	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin arabinoside	433 > 301	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin galactoside	463 > 301	40 / 20	Cyanidin-3-*O*-galactoside
Peonidin glucuronide	477 > 301	40 / 20	Cyanidin-3-*O*-galactoside
Flavonols			
Quercetin arabinoside	433 > 301	45 / 15	Quercetin-3-*O*-glucoside
Quercetin rhamnoside	447 > 300	45 / 25	Quercetin-3-*O*-rhamnoside
Quercetin glucoside	463 > 301	40 / 25	Quercetin-3-*O*-glucoside
Quercetin rutinoside	609 > 301	45 / 25	Quercetin-3-*O*-rutinoside
Flavanones			
Eriodictyol hexoside	287 > 151	45 / 10	Eriodictyol
Naringenin	271 > 301	40 / 15	Naringenin

Supplemental Table 2. Concentration of the main (poly)phenolic metabolites (mean ± SEM) generated in rat plasma after a sustained intake of SCD, HFD, HFD+W, HFD+R and HFD+A (3 male and 3 female per group).

(Poly)phenolic metabolites	Males (M)				
	SCD	HFD	HFD+W	HFD+R	HFD+A
Cyanidin arabinoside	n.d. ^{a}	n.d. ^{a}	0.02 ± 0.00 ^{a}	0.15 ± 0.01 ^{a}	2.65 ± 0.98 ^{b}
Cyanidin galactoside	n.d. ^{a}	n.d. ^{a}	0.03 ± 0.00 ^{*a}	0.69 ± 0.01 ^{a}	7.74 ± 2.69 ^{b}
Peonidin galactoside	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	0.26 ± 0.03 ^{b}
Peonidin glucuronide	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	0.08 ± 0.04 ^{b}	n.d. ^{a}
Anthocyanins (nM)	**n.d. ^{a}**	**n.d. ^{a}**	**0.05 ± 0.00 ^{*a}**	**0.92 ± 0.02 ^{a}**	**10.7 ± 3.71 ^{b}**
Protocatechuic acid glucuronide	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	0.10 ± 0.03 ^{b}
Vanillic acid glucuronide	n.d. ^{*a}	n.d. ^{*a}	n.d.^{a}	n.d. ^{*a}	0.17 ± 0.06 ^{*b}
Phenolic acids (µM)	**n.d. ^{*a}**	**n.d. ^{*a}**	**n.d. ^{a}**	**n.d. ^{*a}**	**0.26 ± 0.09 ^{*b}**
Catechin glucuronide	n.d. ^{a}	n.d. ^{a}	1.31 ± 0.62 ^{b}	0.18 ± 0.02 ^{*ab}	0.02 ± 0.01 ^{a}
Methyl catechin glucuronide	n.d. ^{a}	n.d. ^{a}	0.47 ± 0.11 ^{b}	0.03 ± 0.01 ^{a}	n.d. ^{a}
Methyl epicatechin glucuronide	n.d. ^{a}	n.d. ^{a}	1.21 ± 0.38 ^{b}	0.20 ± 0.05 ^{a}	n.d. ^{a}
Hydroxyphenyl-γ-valerolactone sulphate	n.d. ^{a}	n.d. ^{a}	0.32 ± 0.12 ^{b}	n.d. ^{a}	n.d. ^{a}
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. ^{a}	n.d. ^{a}	0.79 ± 0.34 ^{b}	0.09 ± 0.01 ^{a}	n.d. ^{a}
Flavan-3-ols (µM)	**n.d. ^{a}**	**n.d. ^{a}**	**4.10 ± 1.42 ^{b}**	**0.50 ± 0.07 ^{a}**	**0.02 ± 0.01 ^{a}**
TOTAL (POLY)PHENOLICS (µM)	***n.d. ^{*a}***	***n.d. ^{*a}***	***4.10 ± 1.42 ^{*b}***	***0.50 ± 0.07 ^{a}***	***0.61 ± 0.13 ^{*a}***

(Poly)phenolic metabolites	Females (F)				
	SCD	HFD	HFD+W	HFD+R	HFD+A
Cyanidin arabinoside	n.d. ^{A}	n.d. ^{A}	0.02 ± 0.00 ^{A}	0.22 ± 0.05 ^{B}	0.33 ± 0.04 ^{B}
Cyanidin galactoside	n.d. ^{A}	n.d. ^{A}	0.07 ± 0.01 ^{*A}	0.71 ± 0.14 ^{B}	1.60 ± 0.27 ^{C}
Peonidin galactoside	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.34 ± 0.03 ^{B}
Peonidin glucuronide	n.d.	n.d.	n.d.	0.05 ± 0.05	0.08 ± 0.08
Anthocyanins (nM)	**n.d. ^{A}**	**n.d. ^{A}**	**0.09 ± 0.01 ^{*A}**	**0.98 ± 0.18 ^{B}**	**2.35 ± 0.35 ^{C}**
Protocatechuic acid glucuronide	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.07 ± 0.05 ^{B}
Vanillic acid glucuronide	0.48 ± 0.05 ^{*A}	0.46 ± 0.07 ^{*A}	0.26 ± 0.10 ^{A}	0.22 ± 0.01 ^{*A}	1.11 ± 0.22 ^{*B}
Phenolic acids (µM)	**0.48 ± 0.05 ^{*A}**	**0.46 ± 0.07 ^{*A}**	**0.26 ± 0.10 ^{A}**	**0.22 ± 0.01 ^{*A}**	**1.18 ± 0.20 ^{*B}**
Catechin glucuronide	n.d. ^{A}	n.d. ^{A}	1.24 ± 0.39 ^{B}	0.09 ± 0.01 ^{*A}	0.02 ± 0.01 ^{A}
Methyl catechin glucuronide	n.d. ^{A}	n.d. ^{A}	0.32 ± 0.02 ^{B}	0.02 ± 0.02 ^{A}	n.d. ^{A}
Methyl epicatechin glucuronide	n.d. ^{A}	n.d. ^{A}	0.80 ± 0.03 ^{C}	0.18 ± 0.04 ^{B}	n.d. ^{A}
Hydroxyphenyl-γ-valerolactone sulphate	n.d.	n.d.	0.30 ± 0.21	n.d.	n.d.
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. ^{A}	n.d. ^{A}	0.75 ± 0.17 ^{B}	0.05 ± 0.03 ^{A}	n.d. ^{A}
Flavan-3-ols (µM)	**n.d. ^{A}**	**n.d. ^{A}**	**3.41 ± 0.65 ^{B}**	**0.33 ± 0.09 ^{A}**	**0.03 ± 0.01 ^{A}**
TOTAL (POLY)PHENOLICS (µM)	***0.48 ± 0.05 ^{*A}***	***0.46 ± 0.07 ^{*A}***	***3.67 ± 0.74 ^{B}***	***0.56 ± 0.10 ^{A}***	***1.21 ± 0.28 ^{*A}***

n.d: not detected.
* indicate differences between males & females of the same group (t-student test, $p < 0.05$).
For each row, different lowercase letters indicate differences between males in the different groups and different capital letters indicate differences between females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 2. Continuation.

(Poly)phenolic metabolites	SCD	HFD	HFD+W	HFD+R	HFD+A
	M + F	M + F	M + F	M + F	M + F
Cyanidin arabinoside	n.d. [1]	n.d. [1]	0.02 ± 0.00 [1]	0.19 ± 0.04 [1]	1.49 ± 0.80 [2]
Cyanidin galactoside	n.d. [1]	n.d. [1]	0.05 ± 0.01 [1]	0.70 ± 0.09 [1]	4.52 ± 2.24 [2]
Peonidin galactoside	n.d. [1]	n.d. [1]	n.d. [1]	n.d. [1]	0.24 ± 0.06 [2]
Peonidin glucuronide	n.d.	n.d.	n.d.	0.06 ± 0.03	0.04 ± 0.04
Anthocyanins (nM)	**n.d. [1]**	**n.d. [1]**	**0.07 ± 0.01 [1]**	**0.95 ± 0.15 [1]**	**6.31 ± 3.16 [2]**
Protocatechuic acid glucuronide	n.d. [1]	n.d. [1]	n.d. [1]	n.d. [1]	0.08 ± 0.04 [2]
Vanillic acid glucuronide	0.24 ± 0.11 [1,2]	0.23 ± 0.11 [1,2]	0.13 ± 0.09 [1]	0.11 ± 0.05 [1]	0.64 ± 0.26 [2]
Phenolic acids (µM)	**0.24 ± 0.11 [1]**	**0.23 ± 0.11 [1]**	**0.13 ± 0.09 [1]**	**0.11 ± 0.05 [1]**	**0.72 ± 0.25 [2]**
Catechin glucuronide	n.d. [1]	n.d. [1]	1.28 ± 0.46 [2]	0.14 ± 0.02 [1]	0.02 ± 0.01 [1]
Methyl catechin glucuronide	n.d. [1]	n.d. [1]	0.40 ± 0.07 [2]	0.03 ± 0.01 [1]	n.d. [1]
Methyl epicatechin glucuronide	n.d. [1]	n.d. [1]	1.00 ± 0.26 [2]	0.19 ± 0.04 [1]	n.d. [1]
Hydroxyphenyl-γ-valerolactone sulphate	n.d. [1]	n.d. [1]	0.31 ± 0.16 [2]	n.d. [1]	n.d. [1]
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. [1]	n.d. [1]	0.77 ± 0.24 [2]	0.07 ± 0.02 [1]	n.d. [1]
Flavan-3-ols (µM)	**n.d. [1]**	**n.d. [1]**	**3.75 ± 1.00 [2]**	**0.42 ± 0.08 [1]**	**0.02 ± 0.01 [1]**
TOTAL (POLY)PHENOLICS (µM)	***0.24 ± 0.11 [1]***	***0.23 ± 0.11 [1]***	***3.89 ± 1.02 [2]***	***0.53 ± 0.07 [1]***	***0.74 ± 0.25 [1]***

n.d: not detected.

For each row, different numbers indicate differences between males + females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 3. Concentration of the main (poly)phenolic metabolites (mean ± SEM) excreted in rat urine after a sustained intake of SCD, HFD, HFD+W, HFD+R and HFD+A (3 male and 3 female per group).

(Poly)phenolic metabolites	Males (M)				
	SCD	HFD	HFD+W	HFD+R	HFD+A
Cyanidin arabinoside	n.d. [a]	n.d. [a]	0.32 ± 0.09 [b]	0.60 ± 0.02 [c]	1.02 ± 0.04 [d]
Cyanidin galactoside	n.d. [a]	n.d. [a]	0.03 ± 0.02 [a]	1.59 ± 0.13 [b]	1.26 ± 0.06 [b]
Peonidin arabinoside	n.d. [a]	n.d. [a]	n.d. [a]	0.05 ± 0.01 [a]	0.81 ± 0.18 [b]
Peonidin galactoside	n.d. [a]	n.d. [a]	n.d. [a]	0.60 ± 0.03 [a]	2.18 ± 0.34 [b]
Peonidin glucuronide	n.d. [a]	n.d. [a]	n.d. [a]	0.26 ± 0.05 [b]	0.09 ± 0.03 [a]
Anthocyanins (nmols/24h)	**n.d. [a]**	**n.d. [a]**	**0.35 ± 0.10 [a]**	**3.10 ± 0.07 [b]**	**5.36 ± 0.54 [c]**
Protocatechuic acid sulphate	0.27 ± 0.02 *[a,b]	0.50 ± 0.09 [b]	0.15 ± 0.04 [a]	0.82 ± 0.05 *[c]	0.92 ± 0.09 [c]
Protocatechuic acid glucuronide	0.01 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.04 ± 0.01 [b]	0.08 ± 0.00 *[c]
Vanillic acid glucuronide	0.16 ± 0.01 [c]	0.04 ± 0.01 [a,b]	0.01 ± 0.00 *[a]	0.04 ± 0.01 [a]	0.08 ± 0.01 *[b]
Caffeic acid sulphate	0.01 ± 0.00 [a]	0.17 ± 0.03 [a]	0.28 ± 0.10 [a]	0.39 ± 0.19 [a,b]	0.82 ± 0.09 *[b]
Phenolic acids (µmols/24h)	**0.44 ± 0.03 *[a]**	**0.71 ± 0.11 [a,b]**	**0.44 ± 0.11 *[a]**	**1.29 ± 0.22 [b]**	**1.90 ± 0.14 [b]**
Dihydroxyphenylpropionic acid sulphate	0.23 ± 0.02 [a]	0.57 ± 0.10 [a]	0.51 ± 0.09 [a]	0.56 ± 0.09 [a]	1.44 ± 0.15 *[b]
Dihydroxyphenylacetic acid sulphate	1.11 ± 0.08 *[b]	0.91 ± 0.16 [b]	0.29 ± 0.07 [a]	0.76 ± 0.16 [a,b]	1.17 ± 0.08 [b]
Dihydroxyphenylacetic acid glucuronide	0.00 ± 0.00	0.06 ± 0.04	0.01 ± 0.00	0.09 ± 0.03	0.04 ± 0.01
Phenylpropionic and phenylacetic acids (µmols/24h)	**1.34 ± 0.07 *[a]**	**1.54 ± 0.46 [a]**	**0.81 ± 0.03 [a]**	**1.41 ± 0.28 [a]**	**2.65 ± 0.09 *[b]**
Catechol sulphate	1.00 ± 0.03 *[c]	0.66 ± 0.09 [b]	0.09 ± 0.02 [a]	0.60 ± 0.08 [b]	0.86 ± 0.05 *[b,c]
Catechols (µmols/24h)	**1.00 ± 0.03 *[c]**	**0.66 ± 0.09 [b]**	**0.09 ± 0.02 [a]**	**0.60 ± 0.08 [b]**	**0.86 ± 0.05 *[b,c]**
Catechin glucuronide	n.d.	n.d.	223 ± 72.1	297 ± 159	7.67 ± 1.55
Methyl epicatechin sulphate	n.d.	n.d.	150 ± 91.3	12.9 ± 4.68 *	3.00 ± 1.08
Methyl catechin glucuronide	n.d. [a]	n.d. [a]	115 ± 14.6 [b]	44.3 ± 20.7 [a]	9.00 ± 3.24 [a]
Methyl epicatechin glucuronide	n.d.	n.d.	4.67 ± 3.30	3.33 ± 2.36	0.33 ± 0.24
Hydroxyphenyl-γ-valerolactone	n.d. [a]	n.d. [a]	157 ± 61.3 [b]	56.7 ± 18.4 [a,b]	10.0 ± 0.00 [a]
Hydroxyphenyl-γ-valerolactone sulphate	n.d. [a]	n.d. [a]	3183 ± 1305 [b]	1173 ± 466 [a,b]	13.3 ± 2.36 *[a]
Hydroxyphenyl-γ-valerolactone glucuronide	n.d. [a]	n.d. [a]	40.0 ± 28.3 [a]	276 ± 103 [b]	10.0 ± 7.07 [a]
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. [a]	n.d. [a]	167 ± 41.7 [b]	33.7 ± 14.6 [a,b]	17.7 ± 4.78 [a]
Flavan-3-ols (nmols/24h)	**n.d. [a]**	**n.d. [a]**	**4040 ± 1349 [b]**	**1897 ± 506 [a,b]**	**71.0 ± 9.19 *[a]**
Phloretin glucoside	n.d. [a]	n.d. [a]	0.60 ± 0.10 [a]	11.1 ± 3.73 [b]	n.d. [a]
Phloretin xylosylglucoside	n.d. [a]	n.d. [a]	0.48 ± 0.06 [a]	8.19 ± 1.16 [b]	n.d. [a]
Hydroxylphloretin xylosylglucoside	n.d. [a]	n.d. [a]	0.48 ± 0.17 [a]	5.83 ± 2.08 [b]	n.d. [a]
Phloretin glucuronide	n.d. [a]	n.d. [a]	0.45 ± 0.16 *[a]	3.16 ± 1.15 [b]	n.d. [a]
Phloretin sulphate	n.d.	n.d.	5.02 ± 3.15	2.76 ± 0.16	n.d.
Phloretin sulphate glucuronide	n.d. [a]	n.d. [a]	0.26 ± 0.13 [a]	1.18 ± 0.21 [b]	n.d. [a]
Dihydrochalcones (µmols/24h)	**n.d. [a]**	**n.d. [a]**	**7.30 ± 3.69 [a]**	**32.2 ± 5.67 [b]**	**n.d. [a]**
TOTAL (POLY)PHENOLICS (µmols/24h)	***2.78 ± 0.14 [a]***	***2.91 ± 0.39 [a]***	***12.5 ± 3.77 [a]***	***37.4 ± 6.10 [b]***	***5.49 ± 0.60 [a]***

n.d: not detected.
* indicate differences between males & females of the same group (t-student test, $p < 0.05$).
For each row, different lowercase letters indicate differences between males in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 3. Continuation.

(Poly)phenolic metabolites	Females (F)				
	SCD	HFD	HFD+W	HFD+R	HFD+A
Cyanidin arabinoside	n.d. ^{A}	n.d. ^{A}	0.23 ± 0.05 ^{B}	0.72 ± 0.06 ^{C}	0.87 ± 0.07 ^{C}
Cyanidin galactoside	n.d. ^{A}	n.d. ^{A}	0.03 ± 0.03 ^{A}	2.40 ± 0.53 ^{B}	1.80 ± 0.21 ^{B}
Peonidin arabinoside	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.09 ± 0.03 ^{A}	0.99 ± 0.04 ^{B}
Peonidin galactoside	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.74 ± 0.10 ^{B}	2.74 ± 0.15 ^{C}
Peonidin glucuronide	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.48 ± 0.10 ^{B}	0.24 ± 0.03 ^{A,B}
Anthocyanins (nmols/24h)	**n.d. ^{A}**	**n.d. ^{A}**	**0.26 ± 0.01 ^{A}**	**4.43 ± 0.76 ^{B}**	**6.64 ± 0.44 ^{C}**
Protocatechuic acid sulphate	0.52 ± 0.02 ^{*A}	0.48 ± 0.19 ^{A}	0.62 ± 0.12 ^{A}	0.47 ± 0.04 ^{*A}	1.22 ± 0.09 ^{B}
Protocatechuic acid glucuronide	0.01 ± 0.00 ^{A}	0.00 ± 0.00 ^{A}	0.01 ± 0.00 ^{A}	0.03 ± 0.01 ^{B}	0.16 ± 0.01 ^{*C}
Vanillic acid glucuronide	0.20 ± 0.01 ^{B}	0.09 ± 0.03 ^{A}	0.05 ± 0.01 ^{*A}	0.08 ± 0.02 ^{A}	0.33 ± 0.02 ^{*C}
Caffeic acid sulphate	0.01 ± 0.00	0.06 ± 0.02	0.44 ± 0.05	0.11 ± 0.05	0.21 ± 0.05 *
Phenolic acids (µmols/24h)	**0.74 ± 0.02 ^{*A}**	**0.64 ± 0.22 ^{A}**	**1.11 ± 0.09 ^{*A}**	**0.70 ± 0.04 ^{A}**	**1.91 ± 0.07 ^{B}**
Dihydroxyphenylpropionic acid sulphate	0.19 ± 0.01	0.34 ± 0.11	0.23 ± 0.05	0.34 ± 0.03	0.30 ± 0.06 *
Dihydroxyphenylacetic acid sulphate	0.55 ± 0.03 ^{*A}	0.37 ± 0.11 ^{A}	0.45 ± 0.05 ^{A}	0.60 ± 0.03 ^{A}	1.24 ± 0.09 ^{B}
Dihydroxyphenylacetic acid glucuronide	0.01 ± 0.00 ^{A}	0.01 ± 0.00 ^{A}	0.02 ± 0.00 ^{A}	0.11 ± 0.04 ^{B}	0.03 ± 0.01 ^{A}
Phenylpropionic and phenylacetic acids (µmols/24h)	**0.74 ± 0.03 ^{*A}**	**0.71 ± 0.21 ^{A}**	**0.70 ± 0.09 ^{A}**	**1.05 ± 0.03 ^{A,B}**	**1.57 ± 0.14 ^{*B}**
Catechol sulphate	1.61 ± 0.07 ^{*B}	0.52 ± 0.19 ^{A}	0.29 ± 0.06 ^{A}	0.75 ± 0.06 ^{A}	2.55 ± 0.32 ^{*C}
Catechols (µmols/24h)	**1.61± 0.07 ^{*B}**	**0.52 ± 0.19 ^{A}**	**0.29 ± 0.06 ^{*A}**	**0.75 ± 0.06 ^{A}**	**2.55 ± 0.32 ^{*C}**
Catechin glucuronide	n.d. ^{A}	n.d. ^{A}	1040 ± 228 ^{B}	204 ± 72.1 ^{A}	15.0 ± 2.27 ^{A}
Methyl epicatechin sulphate	n.d. ^{A}	n.d. ^{A}	118 ± 14.6 ^{C}	50.4 ± 3.73 ^{*B}	2.61 ± 0.50 ^{A}
Methyl catechin glucuronide	n.d. ^{A}	n.d. ^{A}	336 ± 75.4 ^{B}	71.0 ± 30.3 ^{A}	8.00 ± 0.41 ^{A}
Methyl epicatechin glucuronide	n.d. ^{A}	n.d. ^{A}	25.3 ± 5.95 ^{B}	7.67 ± 3.06 ^{A}	1.00 ± 0.71 ^{A}
Hydroxyphenyl-γ-valerolactone	n.d.	n.d.	93.3 ± 8.50	100 ± 63.6	40.0 ± 8.16
Hydroxyphenyl-γ-valerolactone sulphate	n.d.	n.d.	1723 ± 210	1887 ± 1292	664 ± 164 *
Hydroxyphenyl-γ-valerolactone glucuronide	n.d.	n.d.	n.d.	n.d.	n.d.
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. ^{A}	n.d. ^{A}	231 ± 17.3 ^{B}	62.4 ± 6.46 ^{A}	153 ± 39.1 ^{B}
Flavan-3-ols (nmols/24h)	**n.d. ^{A}**	**n.d. ^{A}**	**3567 ± 277 ^{B}**	**2382 ± 1260 ^{A,B}**	**880 ± 179 ^{*A,B}**
Phloretin glucoside	n.d. ^{A}	n.d. ^{A}	0.97 ± 0.37 ^{A}	7.12 ± 2.54 ^{B}	n.d. ^{A}
Phloretin xylosylglucoside	n.d. ^{A}	n.d. ^{A}	1.18 ± 0.18 ^{A}	10.8 ± 3.05 ^{B}	n.d. ^{A}
Hydroxylphloretin xylosylglucoside	n.d. ^{A}	n.d. ^{A}	1.33 ± 0.23 ^{A}	9.01 ± 2.28 ^{B}	n.d. ^{A}
Phloretin glucuronide	n.d.	n.d.	3.51 ± 0.64 *	3.71 ± 2.16	n.d.
Phloretin sulphate	n.d.	n.d.	8.00 ± 2.44	18.8 ± 9.63	n.d.
Phloretin sulphate glucuronide	n.d. ^{A}	n.d. ^{A}	0.54 ± 0.07 ^{B}	0.86 ± 0.07 ^{C}	n.d. ^{A}
Dihydrochalcones (µmols/24h)	**n.d. ^{A}**	**n.d. ^{A}**	**15.5 ± 2.34 ^{B}**	**50.3 ± 4.15 ^{C}**	**n.d. ^{A}**
TOTAL (POLY)PHENOLICS (µmols/24h)	***3.09 ± 0.11 ^{A}***	***1.87 ± 0.61 ^{A}***	***21.0 ± 2.41 ^{B}***	***55.1 ± 4.14 ^{C}***	***6.92 ± 0.51 ^{A}***

n.d: not detected.

* indicate differences between males & females of the same group (t-student test, $p < 0.05$).

For each row, different capital letters indicate differences between females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 3. Continuation.

(Poly)phenolic metabolites	SCD	HFD	HFD+W	HFD+R	HFD+A
	M + F	M + F	M + F	M + F	M + F
Cyanidin arabinoside	n.d. [1]	n.d. [1]	0.28 ± 0.07 [2]	0.66 ± 0.04 [3]	0.94 ± 0.06 [4]
Cyanidin galactoside	n.d. [1]	n.d. [1]	0.03 ± 0.01 [1]	2.00 ± 0.39 [2]	1.53 ± 0.18 [2]
Peonidin arabinoside	n.d. [1]	n.d. [1]	n.d. [1]	0.07 ± 0.01 [1]	0.90 ± 0.12 [2]
Peonidin galactoside	n.d. [1]	n.d. [1]	n.d. [1]	0.67 ± 0.07 [2]	2.46 ± 0.27 [3]
Peonidin glucuronide	n.d. [1]	n.d. [1]	n.d. [1]	0.37 ± 0.09 [3]	0.16 ± 0.04 [2]
Anthocyanins (nmols/24h)	**n.d. [1]**	**n.d. [1]**	**0.30 ± 0.06 [1]**	**3.76 ± 0.57 [2]**	**6.00 ± 0.53 [3]**
Protocatechuic acid sulphate	0.40 ± 0.06 [1]	0.49 ± 0.13 [1]	0.38 ± 0.13 [1]	0.65 ± 0.09 [1]	1.07 ± 0.11 [2]
Protocatechuic acid glucuronide	0.01 ± 0.00 [1]	0.00 ± 0.00 [1]	0.00 ± 0.00 [1]	0.04 ± 0.01 [2]	0.12 ± 0.02 [3]
Vanillic acid glucuronide	0.18 ± 0.01 [2]	0.07 ± 0.02 [1]	0.03 ± 0.01 [1]	0.06 ± 0.02 [1]	0.20 ± 0.06 [2]
Caffeic acid sulphate	0.01 ± 0.00 [1,2]	0.11 ± 0.03 [1]	0.36 ± 0.08 [1,2]	0.25 ± 0.14 [1,2]	0.51 ± 0.15 [2]
Phenolic acids (µmols/24h)	**0.59 ± 0.07 [1]**	**0.68 ± 0.18 [1]**	**0.78 ± 0.17 [1]**	**0.99 ± 0.19 [1]**	**1.90 ± 0.10 [2]**
Dihydroxyphenylpropionic acid sulphate	0.21 ± 0.02 [1]	0.48 ± 0.11 [1,2]	0.37 ± 0.09 [1]	0.45 ± 0.08 [1,2]	0.87 ± 0.27 [2]
Dihydroxyphenylacetic acid sulphate	0.83 ± 0.14 [2]	0.67 ± 0.19 [1,2]	0.37 ± 0.06 [1]	0.68 ± 0.11 [1,2]	1.21 ± 0.07 [3]
Dihydroxyphenylacetic acid glucuronide	0.00 ± 0.00 [1]	0.03 ± 0.03 [1]	0.01 ± 0.00 [1]	0.10 ± 0.03 [2]	0.03 ± 0.01 [1]
Phenylpropionic and phenylacetic acids (µmols/24h)	**1.04 ± 0.14 [1]**	**1.18 ± 0.34 [1]**	**0.75 ± 0.07 [1]**	**1.23 ± 0.19 [1]**	**2.11 ± 0.26 [2]**
Catechol sulphate	1.35 ± 0.13 [2]	0.59 ± 0.13 [1]	0.19 ± 0.06 [1]	0.68 ± 0.07 [1]	1.71 ± 0.43 [2]
Catechol sulphate (µmols/24h)	**1.35 ± 0.13 [2]**	**0.59 ± 0.13 [1]**	**0.19 ± 0.06 [1]**	**0.68 ± 0.07 [1]**	**1.71 ± 0.43 [2]**
Catechin glucuronide	n.d. [1]	n.d. [1]	0.63 ± 0.24 [2]	0.25 ± 0.11 [1]	0.01 ± 0.00 [1]
Methyl catechin glucuronide	n.d. [1]	n.d. [1]	0.23 ± 0.07 [2]	0.06 ± 0.02 [1]	0.01 ± 0.00 [1]
Methyl epicatechin sulphate	n.d. [1]	n.d. [1]	0.13 ± 0.06 [2]	0.03 ± 0.01 [1]	0.00 ± 0.00 [1]
Methyl epicatechin glucuronide	n.d. [1]	n.d. [1]	0.02 ± 0.01 [2]	0.01 ± 0.00 [1]	0.00 ± 0.00 [1]
Hydroxyphenyl-γ-valerolactone	n.d. [1]	n.d. [1]	0.13 ± 0.04 [2]	0.08 ± 0.04 [1,2]	0.03 ± 0.01 [1]
Hydroxyphenyl-γ-valerolactone sulphate	n.d. [1]	n.d. [1]	2.45 ± 0.90 [2]	1.53 ± 0.88 [1,2]	0.34 ± 0.18 [1]
Hydroxyphenyl-γ-valerolactone glucuronide	n.d. [1]	n.d. [1]	0.02 ± 0.02 [1,2]	0.14 ± 0.09 [2]	0.00 ± 0.00 [1]
Dihydroxyphenyl-γ-valerolactone glucuronide	n.d. [1]	n.d. [1]	0.20 ± 0.03 [3]	0.05 ± 0.01 [1,2]	0.09 ± 0.03 [2]
Flavan-3-ols (µmols/24h)	**n.d. [1]**	**n.d. [1]**	**3.80 ± 0.88 [2]**	**2.14 ± 0.87 [2]**	**0.48 ± 0.21 [1]**
Phloretin glucoside	n.d. [1]	n.d. [1]	0.79 ± 0.26 [1]	9.09 ± 2.52 [2]	n.d. [1]
Phloretin xylosylglucoside	n.d. [1]	n.d. [1]	0.83 ± 0.20 [1]	9.47 ± 2.14 [2]	n.d. [1]
Hydroxyphloretin xylosylglucoside	n.d. [1]	n.d. [1]	0.90 ± 0.26 [1]	7.42 ± 2.08 [2]	n.d. [1]
Phloretin glucuronide	n.d. [1]	n.d. [1]	1.98 ± 0.80 [1,2]	3.43 ± 1.55 [2]	n.d. [1]
Phloretin sulphate	n.d. [1]	n.d. [1]	6.51 ± 2.61 [1,2]	10.8 ± 7.08 [2]	n.d. [1]
Phloretin sulphate glucuronide	n.d. [1]	n.d. [1]	0.40 ± 0.11 [2]	1.02 ± 0.16 [3]	n.d. [1]
Dihydrochalcones (µmols/24h)	**n.d. [1]**	**n.d. [1]**	**11.4 ± 3.32 [2]**	**41.3 ± 6.01 [3]**	**n.d. [1]**
TOTAL (POLY)PHENOLICS (µmols/24h)	***2.99 ± 0.12 [1]***	***2.46 ± 0.61 [1]***	***16.9 ± 3.35 [2]***	***46.3 ± 6.08 [3]***	***6.20 ± 1.02 [1,2]***

n.d: not detected.

For each row, different numbers indicate differences between males + females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 4. Concentration of the main (poly)phenolic metabolites (mean ± SEM) excreted in rat faeces after a sustained intake of SCD, HFD, HFD+W, HFD+R and HFD+A (3 male and 3 female per group).

(Poly)phenolic metabolites (nmols/24h)	Males (M)				
	SCD	**HFD**	**HFD+W**	**HFD+R**	**HFD+A**
Cyanidin arabinoside	n.d. [a]	n.d. [a]	0.01 ± 0.01 [a]	0.07 ± 0.02 [a]	3.27 ± 1.65 [b]
Cyanidin galactoside	n.d. [a]	n.d. [a]	0.05 ± 0.03 [a]	0.96 ± 0.37 [b]	0.71 ± 0.15 [b]
Anthocyanins	**n.d. [a]**	**n.d. [a]**	**0.06 ± 0.03 [a]**	**1.02 ± 0.39 [a]**	**3.98 ± 1.76 [b]**
Protocatechuic acid	n.d.	0.20 ± 0.20	n.d.	2.72 ± 1.62	2.60 ± 2.11
Hippuric acid	21.3 ± 9.57	27.1 ± 11.3	40.5 ± 5.13	51.1 ± 11.3	27.4 ± 7.03
Phenolic acids	**21.3 ± 9.57**	**27.3 ± 11.2**	**40.5 ± 5.13**	**53.8 ± 11.8**	**30.0 ± 6.64**
m-hydroxyphenylacetic acid	n.d.	0.94 ± 0.67	13.3 ± 6.90	23.5 ± 13.6	n.d.
Phenylpropionic acid	n.d.	n.d.	n.d.	n.d.	n.d.
Dihydroxyphenylpropionic acid	n.d.	19.7 ± 18.3	8.39 ± 5.90	12.1 ± 6.07	53.1 ± 41.5
Phenylacetic and phenylpropionic acids	**n.d.**	**20.7 ± 19.0**	**21.7 ± 12.4**	**35.6 ± 8.57**	**53.1 ± 41.5**
Catechin	n.d. [a]	n.d. [a]	0.79 ± 0.24 [b]	n.d. [a]	n.d. [a]
Epicatechin	n.d.	n.d.	2.91 ± 0.99	n.d.	3.06 ± 3.06
Dimer	n.d. [a]	n.d. [a]	1.57 ± 0.16 [b]	n.d. [a]	n.d. [a]
Trimer	n.d. [a]	n.d. [a]	0.16 ± 0.08 [b]	n.d. [a]	n.d. [a]
Methyl catechin sulphate	n.d.	n.d.	n.d.	n.d.	n.d.
Flavan-3-ols	**n.d.**	**n.d.**	**5.42 ± 1.42**	**n.d.**	**3.06 ± 3.06**
Phloretin glucoside	n.d. [a]	n.d. [a]	0.12 ± 0.04 [a]	0.39 ± 0.11 [b]	n.d. [a]
Phloretin xylosylglucoside	n.d. [a]	n.d. [a]	0.17 ± 0.07 [b]	0.29 ± 0.04 [b]	n.d. [a]
Hydroxyphloretin xylosylglucoside	n.d. [a]	n.d. [a]	0.21 ± 0.08 [b]	0.33 ± 0.03 [b]	n.d. [a]
Dihydrochalcones	**n.d. [a]**	**n.d. [a]**	**0.50 ± 0.19 [b]**	**1.01 ± 0.15 [c]**	**n.d. [a]**
TOTAL (POLY)PHENOLICS (nmols/24h)	***21.3 ± 9.57 [a]***	***48.0 ± 30.2 [a,b]***	***68.2 ± 9.83 [a,b]***	***91.4 ± 2.86 [b]***	***90.1 ± 35.1 [b]***

n.d: not detected.

* indicate differences between males & females of the same group (t-student test, $p < 0.05$).

For each row, different lowercase letters indicate differences between males in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 4. Continuation.

(Poly)phenolic metabolites (nmols/24h)	Females (F)				
	SCD	**HFD**	**HFD+W**	**HFD+R**	**HFD+A**
Cyanidin arabinoside	n.d. ^{A}	n.d. ^{A}	0.03 ± 0.02 ^{A}	0.05 ± 0.01 ^{A}	0.35 ± 0.05 ^{B}
Cyanidin galactoside	n.d. ^{A}	n.d. ^{A}	0.10 ± 0.06 ^{A}	0.81 ± 0.27 ^{B}	0.43 ± 0.02 ^{A,B}
Anthocyanins	**n.d.** ^{A}	**n.d.** ^{A}	**0.13 ± 0.08** ^{A}	**0.85 ± 0.28** ^{B}	**0.78 ± 0.07** ^{B}
Protocatechuic acid	n.d. ^{A}	n.d. ^{A}	0.22 ± 0.23 ^{A}	1.67 ± 0.69 ^{B}	0.58 ± 0.31 ^{A,B}
Hippuric acid	17.7 ± 2.20 ^{A}	11.1 ± 2.30 ^{A}	45.1 ± 15.1 ^{B}	56.8 ± 10.4 ^{B}	14.6 ± 2.96 ^{A}
Phenolic acids	**17.7 ± 2.20** ^{A}	**11.1 ± 2.30** ^{A}	**45.4 ± 15.1** ^{B,C}	**58.4 ± 11.1** ^{C}	**15.2 ± 2.83** ^{A,B}
m-hydroxyphenylacetic acid	n.d. ^{A}	0.49 ± 0.49 ^{A}	27.7 ± 8.11 ^{A}	106 ± 43.5 ^{B}	16.1 ± 9.35 ^{A}
Phenylpropionic acid	n.d. ^{A}	n.d. ^{A}	n.d. ^{A}	0.35 ± 0.35 ^{A}	1.72 ± 0.16 ^{B}
Dihydroxyphenylpropionic acid	n.d. ^{A}	1.06 ± 1.06 ^{A}	15.7 ± 4.79 ^{B}	3.81 ± 2.45 ^{A}	n.d. ^{A}
Phenylacetic and phenylpropionic acids	**n.d.** ^{A}	**1.55 ± 0.92** ^{A}	**43.4 ± 12.7** ^{A,B}	**110 ± 43.0** ^{B}	**17.8 ± 9.51** ^{A}
Catechin	n.d.	n.d.	0.68 ± 0.44	n.d.	n.d.
Epicatechin	n.d. ^{A}	n.d. ^{A}	2.62 ± 0.96 ^{B}	0.75 ± 0.42 ^{A}	n.d. ^{A}
Dimer	n.d. ^{A}	n.d. ^{A}	1.19 ± 0.48 ^{B}	0.19 ± 0.19 ^{A}	n.d. ^{A}
Trimer	n.d. ^{A}	n.d. ^{A}	0.16 ± 0.09 ^{B}	n.d. ^{A}	n.d. ^{A}
Methyl catechin sulphate	n.d. ^{A}	n.d. ^{A}	1.05 ± 0.60 ^{B}	n.d. ^{A}	n.d. ^{A}
Flavan-3-ols	**n.d.** ^{A}	**n.d.** ^{A}	**5.70 ± 2.57** ^{B}	**0.95 ± 0.59** ^{A}	**n.d.** ^{A}
Phloretin glucoside	n.d.	n.d.	0.10 ± 0.06	0.63 ± 0.59	n.d.
Phloretin xylosylglucoside	n.d.	n.d.	0.16 ± 0.13	0.39 ± 0.39	n.d.
Hydroxyphloretin xylosylglucoside	n.d.	n.d.	0.14 ± 0.08	0.35 ± 0.34	n.d.
Dihydrochalcones	**n.d.**	**n.d.**	**0.40 ± 0.26**	**1.36 ± 1.31**	**n.d.**
TOTAL (POLY)PHENOLICS (nmols/24h)	***17.7 ± 2.20*** ^{A}	***12.7 ± 1.54*** ^{A}	***94.9 ± 29.5*** ^{B}	***172 ± 44.1*** ^{C}	***33.8 ± 12.3*** ^{A,B}

n.d: not detected.

* indicate differences between males & females of the same group (t-student test, $p < 0.05$).

For each row, different capital letters indicate differences between females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 4. Continuation.

(Poly)phenolic metabolites (nmols/24h)	SCD	HFD	HFD+W	HFD+R	HFD+A
	M + F	M + F	M + F	M + F	M + F
Cyanidin arabinoside	n.d. [1]	n.d. [1]	0.02 ± 0.01 [1]	0.06 ± 0.01 [1]	1.81 ± 0.99 [2]
Cyanidin galactoside	n.d. [1]	n.d. [1]	0.07 ± 0.03 [1]	0.88 ± 0.21 [2]	0.57 ± 0.10 [2]
Anthocyanins	**n.d. [1]**	**n.d. [1]**	**0.10 ± 0.04 [1]**	**0.94 ± 0.22 [1,2]**	**2.38 ± 1.07 [2]**
Protocatechuic acid	n.d. [1]	0.10 ± 0.10 [1]	0.11 ± 0.11 [1]	2.20 ± 0.82 [2]	1.59 ± 1.06 [1,2]
Hippuric acid	19.5 ± 4.46 [1]	19.1 ± 6.29 [1]	42.8 ± 7.22 [2]	53.9 ± 6.99 [2]	21.0 ± 4.35 [1]
Phenolic acids	**n.d. [1]**	**19.2 ± 6.28 [1]**	**42.9 ± 7.20 [2]**	**56.1 ± 7.32 [2]**	**22.6 ± 4.53 [1]**
m-hydroxyphenylacetic acid	n.d. [1]	0.71 ± 0.38 [1]	20.5 ± 5.75 [1]	64.9 ± 27.5 [2]	8.04 ± 5.38 [1]
Phenylpropionic acid	n.d. [1]	n.d. [1]	n.d. [1]	0.18 ± 0.18 [1]	0.86 ± 0.39 [2]
Dihydroxyphenylpropionic acid	n.d.	10.4 ± 9.23	12.0 ± 3.77	7.94 ± 3.46	26.6 ± 22.0
Phenylacetic and phenylpropionic acids	**n.d. [1]**	**11.1 ± 9.53 [1]**	**32.5 ± 9.29 [1,2]**	**73.0 ± 25.8 [2]**	**35.5 ± 20.6 [1,2]**
Catechin	n.d. [1]	n.d. [1]	0.74 ± 0.23 [2]	n.d. [1]	n.d. [1]
Epicatechin	n.d. [1]	n.d. [1]	2.76 ± 0.62 [2]	0.38 ± 0.25 [1,2]	1.53 ± 1.53 [1,2]
Dimer	n.d. [1]	n.d. [1]	1.38 ± 0.24 [2]	0.10 ± 0.10 [1]	n.d. [1]
Trimer	n.d. [1]	n.d. [1]	0.16 ± 0.06 [2]	n.d. [1]	n.d. [1]
Methyl catechin sulphate	n.d.	n.d.	0.53 ± 0.36	n.d.	n.d.
Flavan-3-ols	**n.d. [1]**	**n.d. [1]**	**5.56 ± 1.31 [2]**	**0.47 ± 0.34 [1]**	**1.53 ± 1.53 [1]**
Phloretin glucoside	n.d. [1]	n.d. [1]	0.11 ± 0.03 [1,2]	0.51 ± 0.27 [2]	n.d. [1]
Phloretin xylosyl glucoside	n.d. [1]	n.d. [1]	0.17 ± 0.07 [1,2]	0.34 ± 0.17 [2]	n.d. [1]
Hydroxyphloretin xylosyl glucoside	n.d. [1]	n.d. [1]	0.18 ± 0.05 [1,2]	0.34 ± 0.15 [2]	n.d. [1]
Dihydrochalcones	**n.d. [1]**	**n.d. [1]**	**0.45 ± 0.15 [1,2]**	**1.19 ± 0.60 [2]**	**n.d. [1]**
TOTAL (POLY)PHENOLICS (nmols/24h)	***19.5 ± 4.46 [1]***	***30.3 ± 15.7 [1,2]***	***81.6 ± 15.1 [2,3]***	***132 ± 26.8 [3]***	***62.0 ± 20.7 [1,2]***

n.d: not detected.

For each row, different numbers indicate differences between males + females in the different groups (General Linear Model and One-way ANOVA, $p < 0.05$).

Supplemental Table 5. Concentration of the main phenolic compounds (mg compound / kg feed ± standard deviation) in standard chow diet and high fat diet by LC-MS/MS.

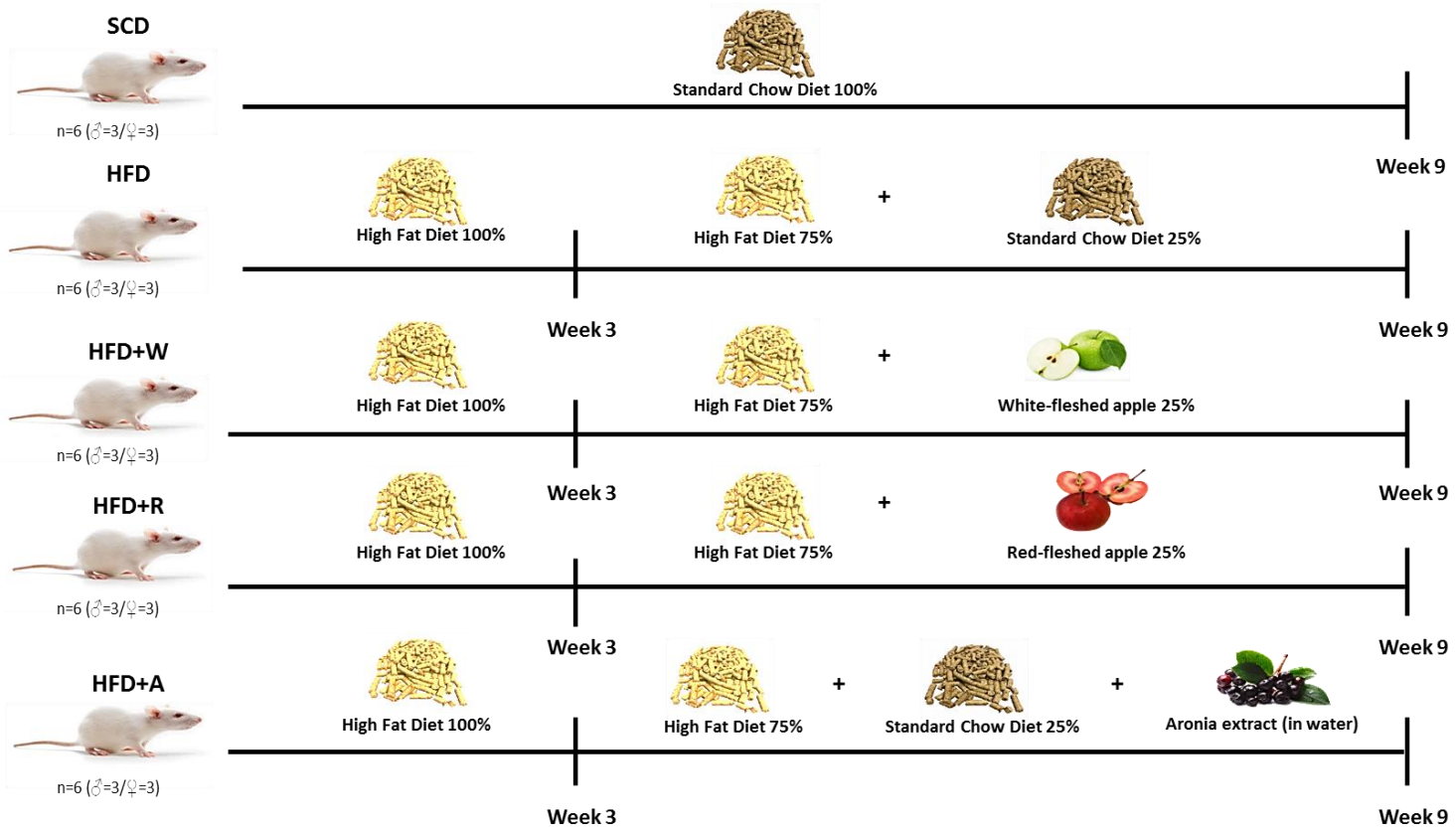
Phenolic compound	Standard chow diet	High fat diet
Benzoic acids		
Protocatechuic acid	10.7 ± 1.58	n.d.
Homogentisic acid	9.10 ± 1.35	n.d.
Vanillic acid	4.33 ± 0.46	0.35 ± 0.04
p-hydroxybenzoic acid	6.40 ± 0.31	1.95 ± 0.20
Hydroxycinnamic acids		
Coumaric acid	13.8 ± 0.21	3.04 ± 0.18
Caffeic acid	7.23 ± 1.00	3.11 ± 0.38
Ferulic acid	16.9 ± 0.21	1.63 ± 0.04
5-*O*-caffeoylquinic acid	1.75 ± 0.26	n.d.
Phenylacetic acids		
p-hydroxyphenylacetic acid	2.18 ± 1.93	1.71 ± 0.18
TOTAL PHENOLICS	***72.4 ± 4.18***	***11.8 ± 0.84***

The number of replicates was three, (*n*=3). n.d.: not detected.

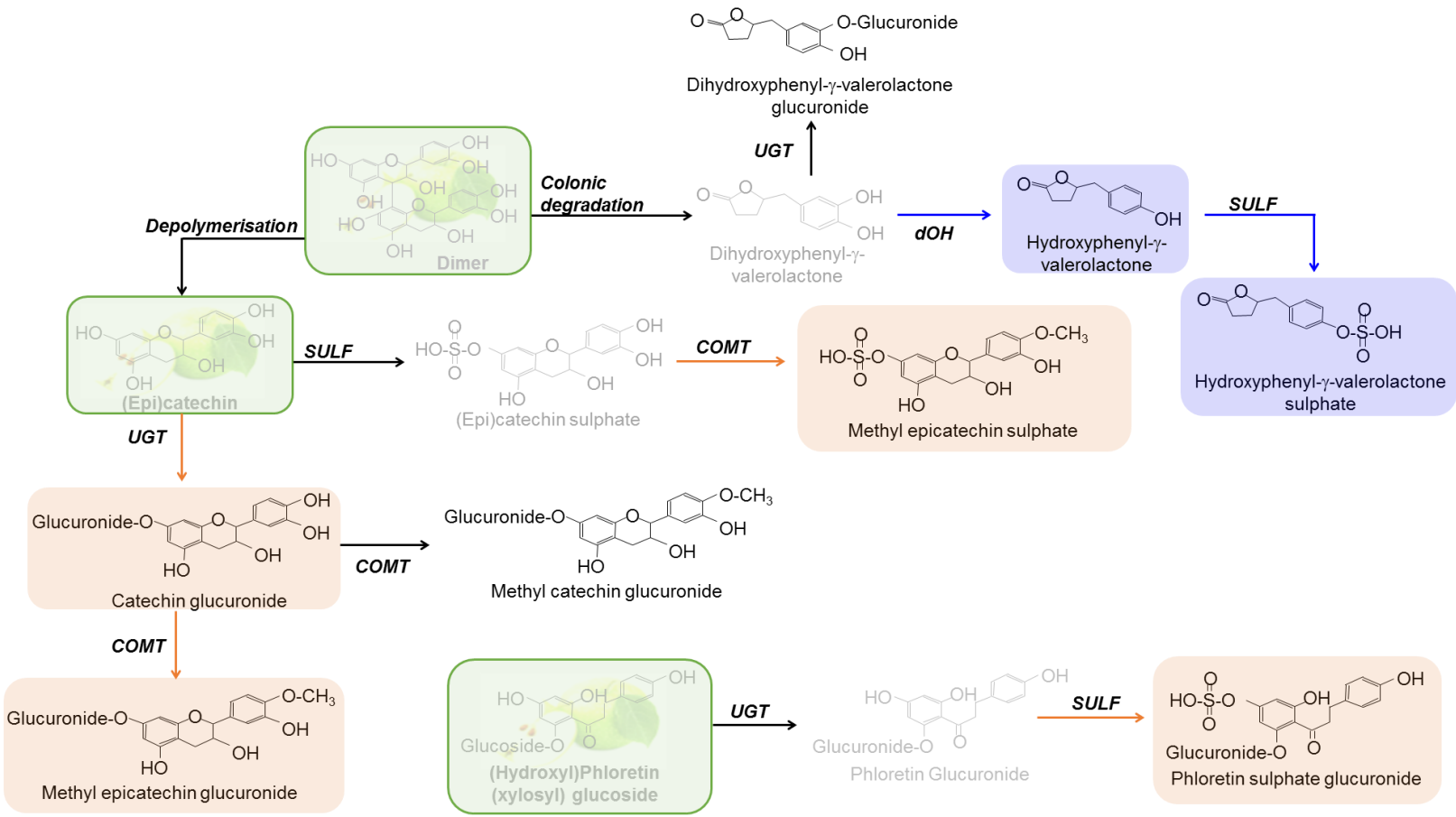
Supplemental Table 6. Animal performance (mean ± standard deviation) according to diet treatment and rat gender.

		Parameter		
		Increased weight (g)	**Water intake (ml/day)**	**Food intake (g/day)**
Group 1 SCD	M	270 ± 40 ^{a*}	34 ± 2.1 ^{a}	25 ± 2.4 ^{a*}
	F	104 ± 6.1 ^{A*}	28 ± 3.9 ^{B}	18 ± 0.5 ^{AB*}
	M+F	187 ± 95 ^{1}	31 ± 4.3 ^{1}	22 ± 3.8 ^{1}
Group 2 HFD	M	288 ± 12 ^{a*}	39 ± 10 ^{a}	22 ± 0.8 ^{a*}
	F	131 ± 18 ^{A*}	33 ± 5.3 ^{AB}	18 ± 1.2 ^{AB*}
	M+F	209 ± 87 ^{1}	36 ± 7.8 ^{1}	20 ± 2.7 ^{1}
Group 3 HFD+W	M	304 ± 48 ^{a*}	35 ± 5.7 ^{a}	23 ± 2.7 ^{a}
	F	123 ± 7.1 ^{A*}	40 ± 3.3 ^{A}	19 ± 1.0 ^{A}
	M+F	213 ± 104 ^{1}	38 ± 5.1 ^{1}	21 ± 2.9 ^{1}
Group 4 HFD+R	M	281 ± 25 ^{a*}	39 ± 13 ^{a}	22 ± 0.3 ^{a*}
	F	110 ± 15 ^{A*}	27 ± 1.6 ^{B}	16 ± 1.2 ^{BC*}
	M+F	196 ± 95 ^{1}	33 ± 11 ^{1}	19 ± 3.3 ^{1}
Group 5 HFD+A	M	320 ± 20 ^{a*}	43 ± 4.7 ^{a}	22 ± 1.4 ^{a*}
	F	134 ± 20 ^{A*}	40 ± 2.7 ^{A}	15 ± 0.8 ^{C*}
	M+F	227 ± 103 ^{1}	42 ± 3.8 ^{1}	18 ± 4.1 ^{1}

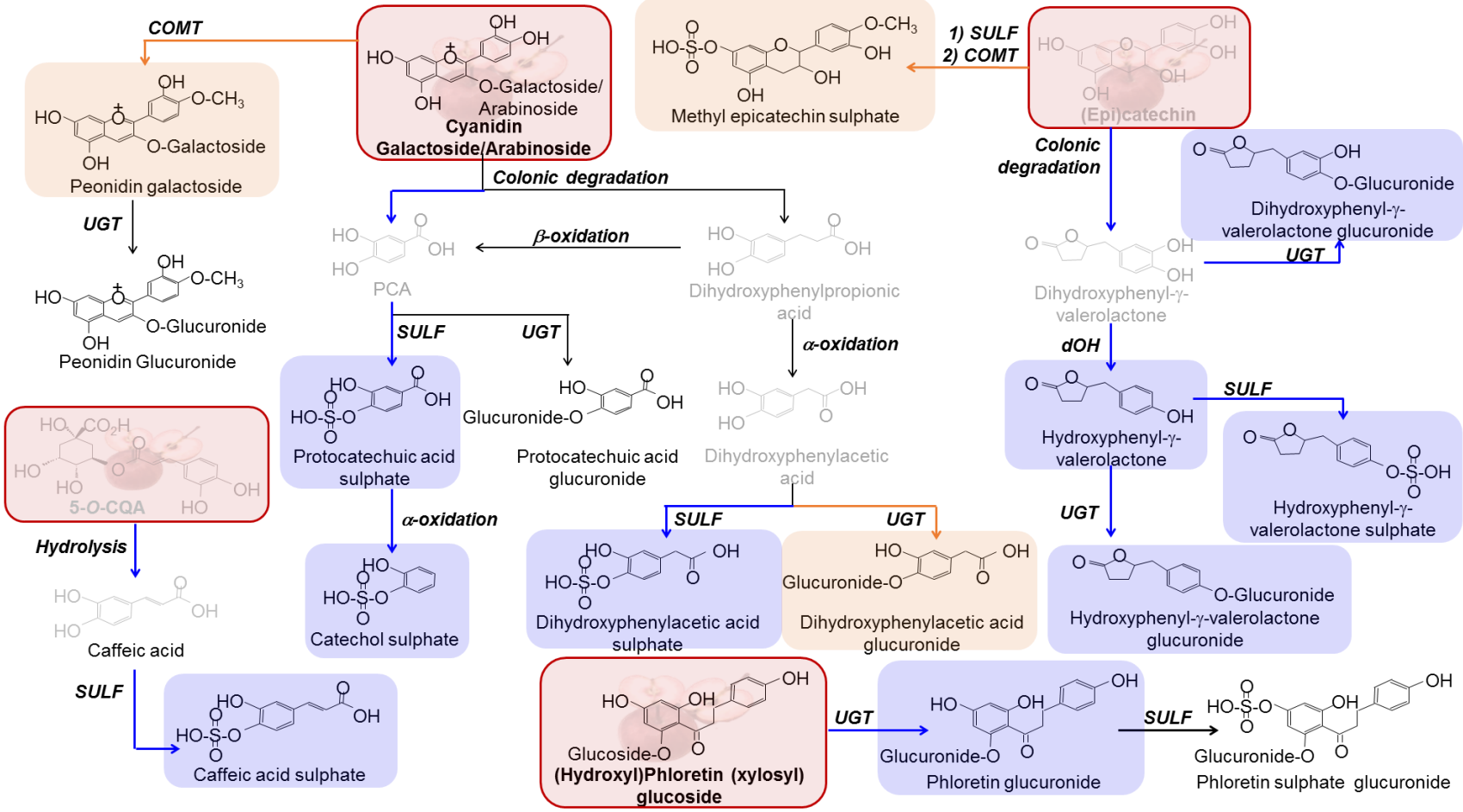
For each parameter, the values were compared between the males (lowercase letters) of the different groups, between the females (capital letters) of different groups and between males + females of the different groups (numbers).
For each column, values not displaying the same letter or number are significantly different (one-way ANOVA, Tukey's test between all means, $p < 0.05$).
* indicate differences between males and males of the same group (t-student test, $p < 0.05$).



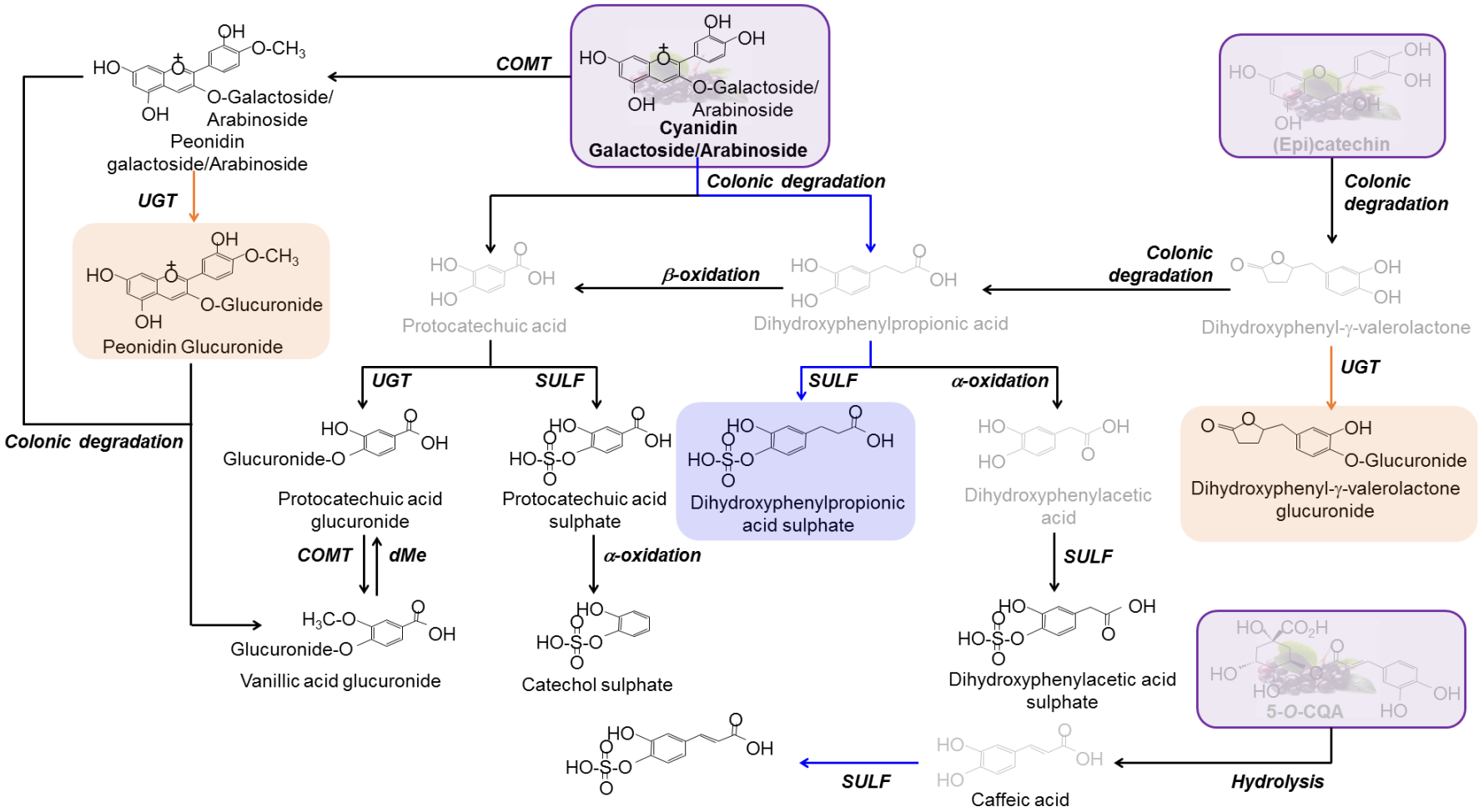
Supplemental Figure 1. Design of the study. 30 Wistar rats were divided into 5 groups of 6 animals each (3 males and 3 females). Group 1: standard chow-diet (SCD), Group 2: high fat diet (HFD), Group 3: high fat diet + white-fleshed apple (HFD+W), Group 4: high fat diet + red-fleshed apple (HFD+R) and Group 5: high fat diet + Aronia (HFD+A).



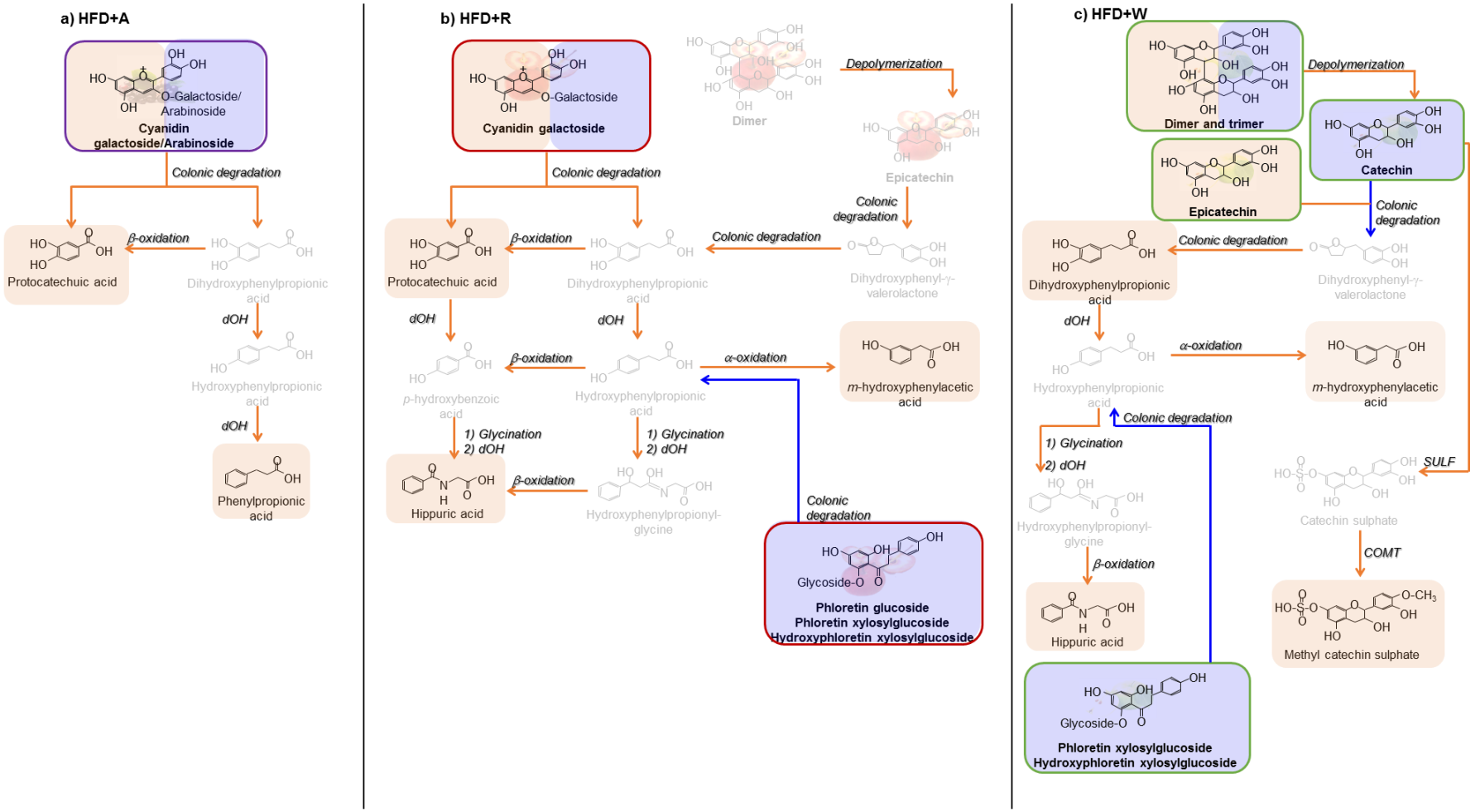
Supplemental Figure 2. Schematic representation of the metabolic pathways of the generated (poly)phenolic metabolites after the fed of HFD+W in urine samples. The common (poly)phenolic metabolites in males and in females are in black color, the metabolites detected only in males are in blue color, and the only in females in orange color. The intermediate metabolites, and the parent compounds from the intake that have not been detected are shown in grey color.



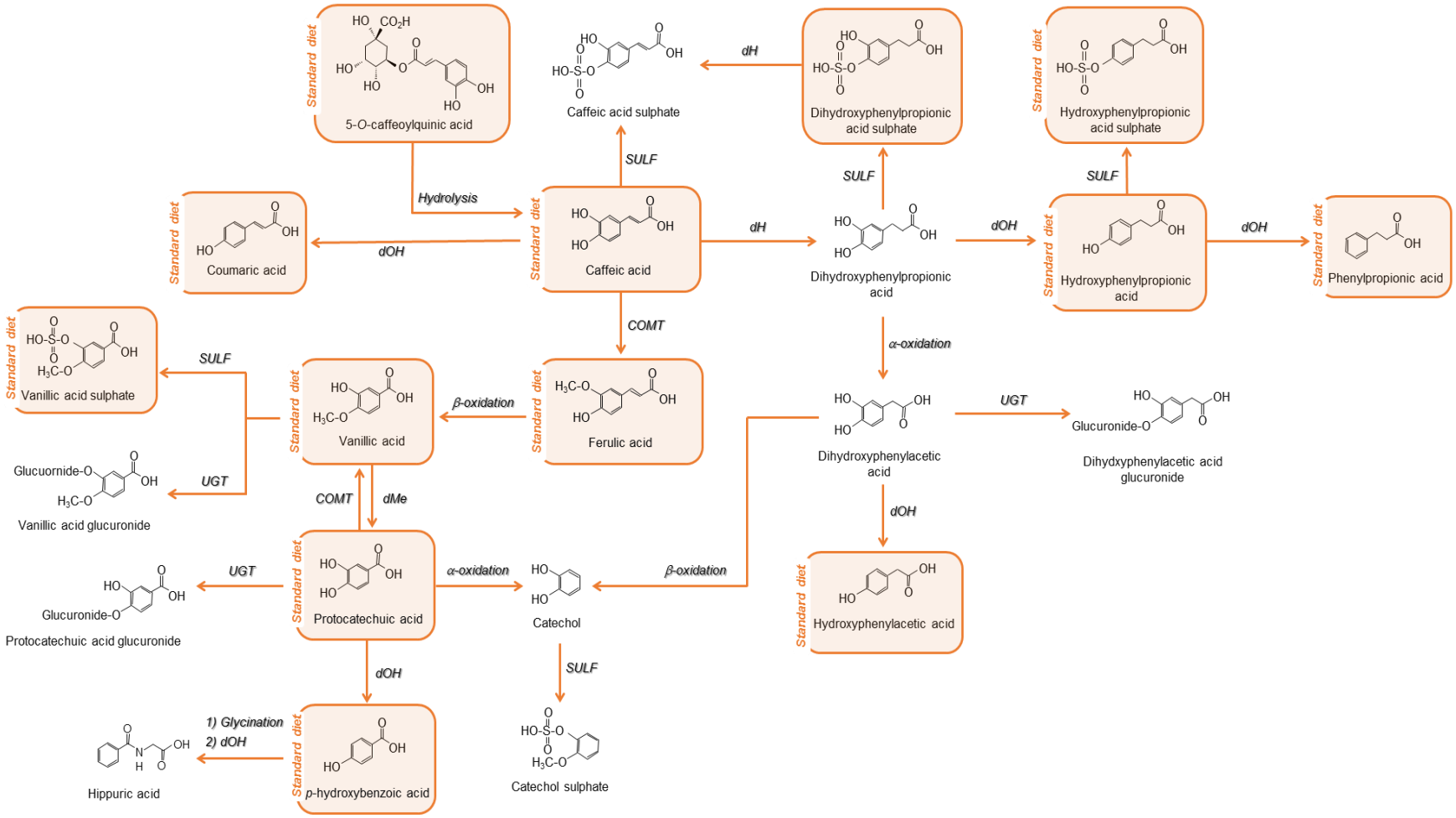
Supplemental Figure 3. Schematic representation of the metabolic pathways of the generated (poly)phenolic metabolites after the fed of HFD+R in urine samples. The common (poly)phenolic metabolites in males and in females are in black color, the metabolites detected only in males are in blue color, and the only in females in orange color. The intermediate metabolites, and the parent compounds from the intake that have not been detected are shown in grey color.



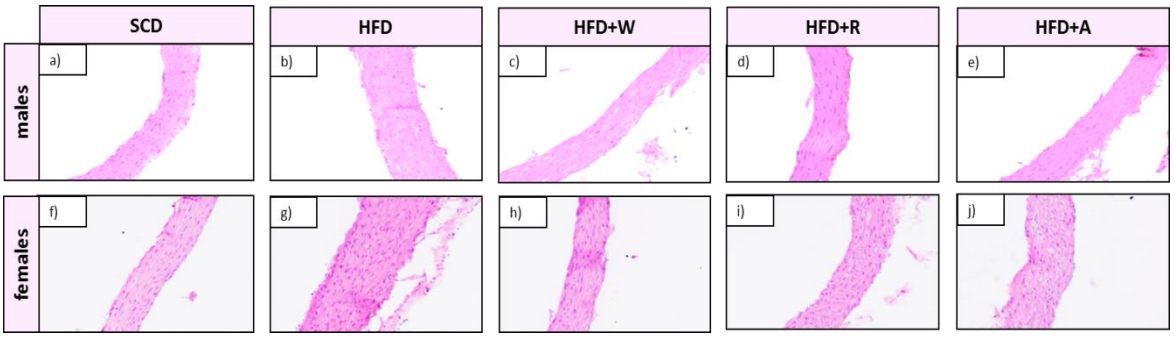
Supplemental Figure 4. Schematic representation of the metabolic pathways of the generated (poly)phenolic metabolites after the fed of HFD+A in urine samples. The common (poly)phenolic metabolites in males and in females are in black color, the metabolites detected only in males are in blue color, and the only in females in orange color. The intermediate metabolites, and the parent compounds from the intake that have not been detected are shown in grey color.



Supplemental Figure 5. Schematic representation of the metabolic pathways of the generated (poly)phenolic metabolites in faeces samples after the fed of a) HFD+A, b) HFD+R, and c) HFD+W. The metabolites detected only in males are in blue color, and the only in females in orange color. The intermediate metabolites, and the parent compounds from the intake that have not been detected are shown in grey color.



Supplemental Figure 6. Schematic representation of the metabolic pathways of the generated phenolic metabolites after the standard diet (SCD) and high fat diet (HFD) in urine samples.



Supplemental Figure 7. Representative images (at a magnification of 4x) of Haematoxilin-Eosin stained aortas of males from: a) SCD, b) HFD, c) HFD+W, d) HFD+R, and e) HFD+A; and of females from: f) SCD, g) HFD, h) HFD+W, i) HFD+R, and j) HFD+A.

GENERAL DISCUSSION

As presented in the objectives section, the present Doctoral Thesis is included in the context of a coordinated project with the title "*Red-fleshed Apple as a novel Anthocyanin-biofortified food to improve Cardiometabolic risk factors: Innovation in crop and application to health*" (AppleCOR Project), whose main objective is to advance in the knowledge of the potential health effects and mechanism of action of anthocyanins coming from the new biofortified red-fleshed apples in the improvement of cardiometabolic risk factors.

The activities carried out during this Thesis have mainly focused on the development and characterization of a red-fleshed apple snack product to preserve its bioavailable bioactive compounds, exploring different processing technologies. As a first step prior to human clinical trials, a human postprandial study was carried out to investigate the *in vivo* biotransformation of the (poly)phenolic compounds present in the red-fleshed apple and also to study the effect of the food matrix on their absorption based on the analysis of the circulating metabolites in plasma and urine samples. Furthermore, to facilitate the taking of blood sampling, an analytical method based on DBS cards was developed and validated as a suitable alternative to the classic venipuncture for the determination of circulating (poly)phenolic metabolites. Finally, to establish first insights into the anthocyanin mechanisms in the control of cardiometabolic risk factors prior to the human clinical trial, we conducted an animal study based on a sustained intake of red-fleshed apple snack in hypercholesterolemic Wistar rats.

In this section, an overview of the principal results obtained from the studies conducted as part of this Doctoral Thesis work is presented, based on the proposed objectives and discussed in relation to other recently published works.

1. RED-FLESHED APPLE SNACK PRODUCT DEVELOPMENT.

Implicated research articles:

Publication 1: Thermal and non-thermal processing of red-fleshed apple: how are (poly)phenol composition and bioavailability affected? Food and Function, DOI: 10.1039/D0FO02631J, 2020.

Nowadays, there is a growing demand for snack products that not only provide pleasant sensory attributes but also nutritional and health benefits. In this context, red-

fleshed apples have potential for use in value-added snack product manufacturing due to the presence of higher concentration of red pigments of anthocyanins in the flesh when compared with regular apples (Bars-Cortina et al., 2017).

In this Thesis, several thermal processing (hot air-drying, infrared-drying and purée pasteurization) and non-thermal (freeze-drying) technologies were compared for the development of a red-fleshed apple snack.

The highest values in all the (poly)phenolic groups present in the red-fleshed apple were found in the freeze-dried snack, followed by the hot air-dried snack and the pasteurized purée in which 83% and 65% of total (poly)phenols were preserved in comparison to the freeze-dried snack, respectively (**Table 1 in Publication 1**). The lowest values were found in the infrared-dried snacks. These (poly)phenolic losses that occur in thermal treatments are mainly due to the high temperatures to which apples are subjected and to which, as is known, (poly)phenols are sensitive (Zielinska et al., 2013). Apart from the high temperatures, the presence of oxygen also contributes to (poly)phenolic degradation by promoting the action of phenoloxidases that degrade (poly)phenols to *O*-quinones and whose rapid polymerization produces the known browning of plant tissues. This fact, for example, was already observed by Mejia-Meza et al. (2008) and Zielinska & Michalska (2016), who demonstrated that the prolonged exposure to oxygen influenced the content of total (poly)phenols of blueberries during drying processes such as hot air-drying or microwave-vacuum-drying.

The (poly)phenolic group most affected by the thermal treatments was the anthocyanins, 25%, 9.2% and less than 1%, respectively, remaining in the hot air-dried snack, pasteurized purée and infrared-dried snack with respect to the freeze-dried snack. These data corroborate that this polyphenol group is extremely sensitive to temperature, oxygen and light, among many other factors that cause its degradation. Regarding these factors, temperature was a major factor affecting the content of anthocyanins, as confirmed by Wojdyło et al. (2014). In this study the reduction in the anthocyanin content was higher at increased air-drying temperatures, especially those above 50°C. Other studies, such as Kwok et al. (2004) also showed that anthocyanins content decreased 85% in Saskatoon berries subjected to hot air convective drying at 75°C. In contrast, the only (poly)phenolic group in which no significant losses were observed between thermal and non-thermal (freeze-drying) treatments was the group

of dihydrochalcones. This polyphenolic group is characteristic for apples and has been shown to exhibit a wide range of biological and pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, cytotoxic, and antispasmodic effects (Fernández-Jalao et al., 2019; Rivière, 2016).

Therefore, we concluded that hot air-drying and purée pasteurization represent viable techniques with a lower cost compared to freeze-drying to obtain red-fleshed apple products preserving a high amount of most of the (poly)phenolic classes in red-fleshed apple. However, if the aim is to obtain a product with a high content of anthocyanins, the polyphenolic group most characteristic of this apple, freeze-drying appears to be the best option. For this reason, the freeze-dried apple was selected as the most suitable snack for the sustained human clinical trial performed in the last stage of the AppleCOR Study.

2. *IN VIVO ASSESSMENT OF THE BIOAVAILABILITY OF ANTHOCYANINS AND OTHER (POLY)PHENOLIC COMPOUNDS FROM RED-FLESHED APPLE AND THE EFFECT OF PROCESSING.*

Implicated research articles:

Publication I: Thermal and non-thermal processing of red-fleshed apple: how are (poly)phenol composition and bioavailability affected? Food and Function, DOI: 10.1039/D0FO02631J, 2020.

Publication II: Validation of dried blood spot cards to determine apple (poly)phenolic metabolites in human blood and plasma after an acute intake of red-fleshed apple snack. Molecular Nutrition and Food Research, 62(23), 1800623, 2018.

Publication III: In vivo biotransformation of (poly)phenols and anthocyanins of red-fleshed apple and identification of intake biomarkers. Journal of Functional Foods, 55, 146-155, 2019.

For plant (poly)phenolic compounds, bioavailability is often defined as the amount of (poly)phenols metabolized through host metabolic pathways and requires knowledge of the absorption, distribution, metabolism, and, ultimately, excretion (ADME) of each

individual (poly)phenolic compound. This is considered to be essential information for all those involved in food production and nutritional assessment to understand (poly)phenolic compound health effects (Motilva et al., 2015). Nevertheless, while many epidemiological studies have associated the consumption of (poly)phenols within fruits and vegetables with a decreased risk of developing several chronic diseases, intervention studies have generally not confirmed these beneficial effects. The reasons for these discrepancies are not fully understood but include potential differences in dosing, in interaction with the food matrix and in endogenous factors, such as microbiota and digestive enzymes that can also considerably affect bioaccessibility, uptake, and further metabolism of (poly)phenols (Bohn, 2014).

In order to be bioavailable, dietary (poly)phenolic compounds must be bioaccessible, in other words they must be released from the food matrix during small intestinal digestion or as a result of the colon microbiota metabolism. Within the food matrix, (poly)phenols are mostly linked to carbohydrates, organic acids, or to one another, and for later bioavailability, disruption of the cell walls and cellular compartments and cleavage from carbohydrates would be required. In this respect, it has been proven that some food processing conditions, such as the application of heat, can favour the release of (poly)phenols from the food matrix, thus modulating their bioaccessibility and bioavailability and consequently their health effects (Ribas-Agustí et al., 2018).

In this Thesis, the effect of different thermal and non-thermal processing conditions on the bioavailability of apple (poly)phenolic compounds was investigated. We tested the three products derived from the red-fleshed apple that best retained (poly)phenols (hot air-dried snack, pasteurized purée and freeze-dried snack) based on the results of the first objective of the Doctoral Thesis. For this, a human crossover pilot study was carried out as a preliminary study, in which three healthy volunteers ingested doses close to 150 mg of total (poly)phenols through the intake of 66 g of hot air-dried snack, 500 g of pasteurized purée and 60 g of freeze-dried snack. The kinetics of (poly)phenolic metabolites in 24 h urine at different interval times (0-2, 2-4, 4-8, and 8-24 h) was monitored. The intakes were separated by 15 days of wash-out period and the volunteers followed a diet low in (poly)phenols during the three days before each intervention. Although the sample size was small, the crossover design allowed us to reduce interpersonal variability.

The results showed great inter- and intra-individual differences among volunteers, demonstrating the influence of endogenous factors, such as the microbiota and digestive enzymes, on the metabolism of (poly)phenols (Miller et al., 2011; Rinninella et al., 2019; Williamson & Clifford, 2017; Zmora et al., 2019). A processing effect was also detected, observing that for almost all the (poly)phenolic groups their bioavailability was greater in all three volunteers after ingesting the pasteurized purée (**Figure 3 in Publication 1**). This may be mainly due to two factors discussed in publication 1. On the one hand, heat treatment can break the cells, facilitating the release and absorption of (poly)phenols during digestion. On the other hand, the more liquid matrices that are less viscous and generally poorer in fibre, could favour (poly)phenol absorption, because the fibre can 'arrest' (poly)phenols within the food matrix, restricting their absorption (Monfoulet et al., 2020; Palafox-Carlos et al., 2011). These results agree with previous studies, in which an increased naringenin bioavailability was reported for cooked tomato sauce (Martínez-Huélamo et al., 2015) or epicatechin administered from whole apple was less bioavailable compared to an apple extract (Monfoulet et al., 2020).

However, the bioavailability of flavan-3-ols showed important variability. Thus, for volunteer 1 maximum polyphenol bioavailability was observed after the hot air-dried snack intake; for volunteer 2 the maximum was after the freeze-dried snack and pasteurized purée intake, and for volunteer 3 it was after the freeze-dried snack and hot air-dried snack (**Supplemental Table 3 in Publication 1**). This demonstrates that inter-individual variability represents a great difficulty in nutritional studies and highlights the importance of studying individual bioavailability. Recent meta-analyses have already shown the importance of inter-individual variability in the cardiometabolic response. In this way, González-Sarrías et al. (2017a) found that the beneficial effects of flavonoids on modulating several cardiometabolic risk factors, such as plasma lipids or body mass, were higher in participants with a body mass index ≥ 25 kg/m^2 and in non-medicated individuals.

In this respect, in recent years metabotypes (metabolic phenotype with specific gut microbiome-derived metabolites that characterize the metabolism of the parent compound) have begun to be characterized for each volunteer in order to address the results obtained in a more accurate and precise way. Mena et al. (2019) investigated

the existence of three possible metabotypes in the production of flavan-3-ol colonic metabolites in humans after the daily intake of both green tea and coffee bean extracts for eight weeks. Although much work remains to be done in this field, recent studies have shown that the benefits associated with the ingestion of some foods rich in (poly)phenols may be related to each specific metabotype (González-Sarrías et al., 2017b; Selma et al., 2018; Tomás-Barberán et al., 2017).

The other polyphenolic group which did not show the highest bioavailability in the pasteurized purée was the anthocyanins, which demonstrated a slightly greater bioavailability after the intake of the hot air-dried snack in all volunteers. Although a better bioavailability of anthocyanins was observed in the hot air-dried snack, the differences were small with respect to the other treatments (0.07% absorption in hot air-dried snack *versus* 0.04% and 0.03% absorption in freeze-dried snack and pasteurized purée, respectively, comparing the average of the three volunteers' total ingested anthocyanins with the total anthocyanins and their phase II metabolites excreted in 24 h-urine). These slight differences suggest, however, that with a higher sample size with more volunteers no significant differences would probably have been observed. On the other hand, it should be noted that most studies of anthocyanin bioavailability only take into account the parent compounds that are mainly absorbed in the upper tract (Landete, 2012; Manach et al., 2005; Prior & Wu, 2006), indicating very low absorption (less than 1%). In this regard, the main colonic metabolites derived from anthocyanins (such as protocatechuic acid) are common to other (poly)phenol metabolic pathways, and they should not therefore be considered as anthocyanin derivatives (Mosele et al., 2015).

In general terms, the results bore out the strong impact of thermal processing on the degradation of food (poly)phenols, observing that the processing that best preserved (poly)phenols (freeze-dried) was the one with the lowest bioavailability rates. These results were consistent with Kamiloglu et al. (2015), who showed that marmalade and jam processing of black carrots drastically decreased the total (poly)phenolic (89-90%) and phenolic acids (49-97%) contents, while the bioaccessibility of the remaining compounds was higher when compared to unprocessed carrots (7-13% increase for total (poly)phenolic content and 5-31% increase for phenolic acids). However, it must be taken into account that the amounts of apple-derived products, necessary to reach

a final (poly)phenol dose of approximately 150 mg, were very different among products, requiring an intake of 500 g of pasteurized purée compared to 60 g of freeze-dried snack. Therefore, the pasteurized purée and the hot air-dried snack were shown to be affordable products containing high amounts of bioavailable (poly)phenols. However, to obtain a red-fleshed apple product with a high anthocyanin content that could be consumed in reasonable amounts in the frame of a balanced diet, the freeze-dried format would be the best option. This confirms the importance of carrying out these studies during the process of developing a functional food, since bioavailability of (poly)phenolic compounds in processed food is a balance between the preservation of these compounds during processing and those finally absorbed into the organism.

Once the impact of the apple processing conditions on the content and bioavailability of (poly)phenols has been determined, as a first step prior to human clinical trials, postprandial feasibility studies should be performed as a proof-of-concept to explore the effect of the dose as well as to determine biotransformation of (poly)phenols and their circulating metabolites in biological samples in order to be able to assign future biological effects (Motilva et al., 2015). Nevertheless, to perform these postprandial studies, the collection of blood samples by venipuncture presents several limitations, such as intense discomfort for volunteers, the fact that it requires trained staff to collect samples and also special infrastructures where volunteers must spend a long time. To solve these limitations, the use of dried blood spot (DBS) cards was proposed for the first time by researchers from our group as a suitable alternative applied to the study of pharmacokinetics and bioavailability of (poly)phenols. Previous studies have applied DBS cards combined with UPLC-MS/MS to determine nutritional biomarkers in human biological samples with satisfactory results, such as olive oil polyphenol metabolites in urine, plasma and blood samples (López de las Hazas et al., 2016; Serra et al., 2013), phenolics from *Arbutus unedo* fruit in blood samples (Mosele et al., 2016) and more recently, fat-soluble micronutrients in blood samples (Rubió et al., 2020). In recent years, there has been an increase in the use of DBS cards as a useful and quick tool to analyze health markers in human blood samples such as riboflavin (Lin et al., 2020), thiamine, or pyridoxal phosphate (which are useful indices to assess the status of vitamins B1, B2, and B6 in the body) (Zhang et al., 2019).

In this Thesis, a method was developed and validated using DBS cards, as a blood sampling procedure, coupled with UPLC-MS/MS to determine the (poly)phenolic metabolites originated after intake of the freeze-dried red-fleshed apple snack in a postprandial study carried out with ten healthy volunteers (**Publication II**). The method developed allowed us to detect six (poly)phenolic metabolites derived from phase II and microbial catabolism in capillary blood, venous blood and venous plasma human samples. These metabolites were phloretin glucuronide, dihydroxy-phenylpropionic acid sulphate, catechol sulphate, methyl catechol sulphate, catechol glucuronide and hydroxyphenyl-ɣ-valerolactone glucuronide. The number of metabolites detected using this method is far from the number of metabolites detected in this same study in urine or plasma obtained by venipuncture (**Publication III**). However, a great deal of information is lost in nutritional studies in which venipuncture is used for blood sampling, since colonic metabolites that originate after between 8 and 24 hours are not usually detected. Thus, for (poly)phenol metabolism pathway studies, venipuncture should be selected as the preferred blood sampling procedure, while DBS card sampling allows a more complete view of the metabolism of main metabolites such as specific biomarkers between 0 and 24 h after food intake, representing an alternative method of monitoring metabolites in nutritional studies.

In the frame of this study, we also aimed to compare capillary blood (obtained by fingerprick) *versus* venous blood (obtained by venipuncture and placed manually on DBS cards), since capillary blood can contain some amount of interstitial fluid and can cause metabolite concentrations that could differ from venous blood (Antunes et al., 2016). Our results showed that there were no significant differences between the two kinds of samples.

The fact that no differences were observed between capillary blood and venous blood represents an advantage as more post-intake time points by self-sampling can be analyzed, allowing the detection of colonic metabolites and obtaining the entire spectrum of food (poly)phenolic metabolites.

We also evaluated the differences between venous blood and venous plasma, since the affinity and binding of some (poly)phenols to red blood cells has already been reported (Chaudhuri et al., 2007; Fiorani et al., 2003) and could change their concentration in plasma compared to venous blood. In our study, although (poly)phenolic metabolites

were detected at lower levels in plasma, the differences compared to venous blood were not significant. Finally, we performed a comparative study between plasma samples analyzed with DBS cards *versus* microElution solid phase extraction (μSPE), as the most common technique used to preconcentrate (poly)phenolic metabolites which requires venipuncture and presents the previously mentioned drawbacks (Walker & Mills, 2002). Results showed that no differences were observed between the two methods (**Table 3 in Publication II**).

Therefore, our overall results revealed that the blood sampling method developed, based on DBS cards, could be useful for future studies in human dietary supplementation studies with red-fleshed apple, especially in postprandial studies, representing a quick and easy strategy to determine the complete kinetic profile of the main circulating (poly)phenolic metabolites derived from apple intake.

Amongst all dietary flavonoids, anthocyanin bioavailability is especially difficult to evaluate, due to its particular physico-chemical features. Several *in vivo* and *in vitro* approaches have been undertaken on this issue. However, much remains to be done to elucidate the mechanisms by which dietary anthocyanins are absorbed and metabolized *in vivo* (Fernandes et al., 2015). In this respect, the human biotransformation of anthocyanins through red-fleshed apple supplementation has not previously been studied. Therefore, the elucidation of the metabolic pathways and human bioavailability of anthocyanins and other (poly)phenols from red-fleshed apple and the identification of potential intake biomarkers are justified prior to investigating its potential health benefits in human intervention studies.

To study (poly)phenol metabolic pathways and the potential intake biomarkers of red-fleshed apple, we analyzed plasma and urine samples taken from the postprandial study performed with the ten healthy volunteers. After ingestion of 80 g of freeze-dried red-fleshed apple snack, blood and urine samples were collected under postprandial conditions at different times to determine the kinetics of the metabolites.

The concentration of 37 (poly)phenolic metabolites, mainly sulphated, glucuronided and methylated phase II-conjugates, increased significantly in the plasma and urine samples after ingestion of the snack. The metabolites detected were derivatives of hydroxyphenyl-γ-valerolactone, epicatechin, phloretin and anthocyanin, as well as

simple phenolic acids such as catechol and pyrogallol derivatives, benzoic acids, phenylacetic, phenylpropionic, and hydroxycinnamic acids. These simple phenolic acids are the result of the transformations carried out by the microbiota in the colon through ring fission, reduction, α-oxidation, β-oxidation, dehydroxylation and demethylation reactions, and also dehydrogenation and reduction reactions. The metabolic pathways proposed were set up from the major (poly)phenols detected in red-fleshed apple. These (poly)phenols are chlorogenic acid, vanillic acid hexoside, cyanidin-3-*O*-galactoside, epicatechin and dimer B_2, quercetin derivatives and phloretin glucoside (**Figure 1 in Publication III**).

Briefly, according to Mosele et al. (2015), from cyanidin-3-*O*-galactoside its methylated form peonidin-3-*O*-galactoside was formed, in addition to protocatechuic acid which, after the action of the microbiota, can be degraded to catechols, pyrogallols and hydroxybenzoic acid, or methylated to give vanillic acid. From chlorogenic acid, mainly caffeic acid was formed whose further degradation gives rise to protocatechuic acid, *p*-hydrozybenzoic acids and catechol metabolites. From vanillic acid hexoside, protocatechuic acid was formed which, as already explained, can be degraded to simpler phenols. The formation of these compounds after the intake of apple juice has also been described by Kahle et al. (2011). From the flavan-3-ols epicatechin and dimer B_2 (proanthocyanidin), apart from their glucuronidated, sulphated and methylated forms, dihydroxyphenyl-ɣ-valerolactones were formed by the action of gut microbiota which have been described as specific metabolites of the degradation of flavan-3-ols (Serra et al., 2011). Quercetin derivatives, such as quercetin rhamnoside, were metabolized in the colon to dihydroxyphenylpropionic acid which was further metabolized to generate simpler phenols, such as phenylacetic and benzoic acids, which can further degrade to give catechol derivatives. Finally, phloretin glucoside was degraded to simpler phenols such as phenylacetics and benzoics and consequently to catechols as a consequence of the action of the gastrointestinal tract bacteria (Serra et al., 2012). This study showed that a great variety of metabolites with different structures are generated after the intake of the red-fleshed apple. All of them may have possible health effects and, therefore, future research on all of them is necessary.

The second objective of this study was to identify specific markers of red-fleshed apple consumption, based on the main (poly)phenol metabolites, in order to establish the

possible relationships between intake of (poly)phenols and the health effects in future human intervention studies. The proposed metabolites were phloretin glucuronide and cyanidin-3-*O*-galactoside in plasma and urine samples and peonidin-3-*O*-galactoside in urine. These proposed biomarkers were not detected in basal conditions and were detected in the samples of all the volunteers after ingestion of the red-fleshed apple snack. The three metabolites separately are not strictly exclusive to red-fleshed apple, as cyanidin-3-*O*-galactoside is found in other, although not so commonly consumed fruits, such as chokeberry (Zheng & Wang, 2003), and phloretin metabolites have already been reported as good markers of apple intake (of any variety) (Saenger et al., 2017). However, the presence of the three markers together is specific to the intake of red-fleshed apple. Along the same lines, in our study we observed, in hypercholesterolemic Wistar rats (**Publication IV**), that phloretins in urine (especially phloretin sulphate, phloretin xylosyl glucoside and phloretin glucoside), together with the methylated forms of cyanidins (peonidin glucuronide in plasma and urine and peonidin glucuronide, peonidin arabinoside and peonidin galactoside in plasma), are reliable markers of red-fleshed apple consumption.

In summary, results from the (human) *in vivo* studies performed in the frame of this Thesis regarding how processing affects the bioavailability of anthocyanins and other (poly)phenols from red-fleshed apple, together with the elucidation of the metabolic pathways and the potential intake biomarkers, represent a considerable development in knowledge about the metabolism of bioactive compounds present in this novel apple cultivar.

3. *ANIMAL STUDY: CARDIOMETABOLIC EFFECTS AND STUDY OF THE FOOD MATRIX EFFECT.*

Implicated research articles:

Publication IV: Metabolic fate and cardiometabolic effects of (poly)phenolic compounds from new cultivars of red-fleshed apple in hypercholesterolemic rats: A comparative study with common white-fleshed apple. Molecular Nutrition and Food Research (under review).

The last part of this Doctoral Thesis focused on a preliminary animal study, prior to the human clinical trial, concerning the evaluation of the biological effects of dietary supplementation with red-fleshed apple on the improvement of cardiometabolic risk factors. To accomplish this aim, Wistar rats were divided into five groups which were fed with a high fat diet (HFD) to induce hypercholesterolemia and supplemented with red-fleshed apple (HFD+R), white-fleshed apple (HFD+W) or an anthocyanin-rich infusion from aronia fruit (HFD+A), providing matched content and profile of anthocyanins. Also, two control groups that took standard chow diet (SCD) or HFD were included in the experiment. Although it is a preliminary study with a small sample size, we included both male and female rats and this allowed us to observe different biological effects depending on gender that could be further related to different metabolic profiles of (poly)phenols in males and females.

A previous study by the "Antioxidants Group" reported protective effects of red-fleshed apple against certain markers of colon carcinogenesis (Bars-Cortina et al., 2020), and, together with the advances in the knowledge of the metabolism and bioavailability of anthocyanins and the rest of the (poly)phenols present in this apple (**Publication I, Publication II and Publication III**), the present study was performed to provide broader evidence of the potential beneficial effects of this novel fruit variety.

The selection of Wistar rat as the animal model used in the present study is due to the fact that these animals have the advantage of easy handling and housing (Xiangdong et al., 2011). Moreover, hyperlipidemia and atherogenesis can be easily induced with high cholesterol/high fat diets (Joris et al., 1983). All these advantages make this animal one of the most widely used animal models for atherosclerosis research (Abu-Elsaad & El-Karef, 2018; Altunkaynak et al., 2008; Fernandes et al., 2017).

In order to evaluate the control of the development of cardiovascular disease derived from diet supplementation with red-fleshed apple, histological stains were performed to assess the possible effects on different target tissues, together with the analysis of several biochemical parameters. In rat aorta samples, the development of the atherosclerosis process was evaluated, because it is the main underlying cause of cardiovascular diseases and it is characterized by the build-up of modified fat, carbohydrate complexes, blood, and fibrous tissue deposit on the wall of large- and

medium-sized arteries. This deposition culminates in the formation of atherosclerotic plaques and is accompanied by a severe immunological response. The formation of these plaques in the arteries can lead to their occlusion, causing ischemia at that specific point, or come off in the form of an embolus, blocking any other artery in the body, which can lead to its most critical manifestation: an acute myocardial infarction or a cerebral infarction (Galkina & Ley, 2009; Ross, 1999).

In our study, it is important to highlight that we did not observe the presence of atherosclerotic plaques or the presence of large lipid deposits in the aortas of rats after supplementing with HFD. This could be explained because the duration of the study was insufficient for the formation of atherosclerotic plaques. We therefore measured the aortic wall thickness, a valuable biomarker of early atherosclerosis (Ludwig et al., 2003; Skilton et al., 2019). Arterial stiffness (produced among other reasons by small lipid deposits) is accepted as a main determinant of increased systolic pressure and pulse and, therefore, is an important determinant of stroke and heart attack myocardium (Mattace-Raso et al., 2006). Our results showed significant differences between SCD and HFD in aortic wall thickness, which indicates that nine weeks of intake of an HFD is enough to observe these first signs of atherosclerosis.

When comparing the HFD group with the supplemented diets, we observed significant reductions in aortic thickness, indicating that both apples and aronia supplementation appeared to be effective in the attenuation of this cardiovascular parameter. However, these reductions were significantly higher after both apples compared to aronia (data not shown). These findings support, on the one hand, that anthocyanins through aronia infusion without apple matrix can induce cardioprotective effects. On the other hand, the similar and higher attenuation effects observed after both apples supplementation suggests that anthocyanins or flavan-3-ols, together with dihydrochalcones (the main polyphenolic groups in apples), constitute a polyphenolic phytocomplex in red and white-fleshed apples, respectively, that could act synergistically in the attenuation of this cardiovascular outcome. Also, it cannot be ruled out that other bioactive compounds present in the apple matrix, such as fibre, could be in part responsible for this observed effect in apples. While anthocyanins could play an important role in the protective atherogenic effect observed in red-fleshed apple, the effects observed in the aortas of the common white-fleshed apple group may be mainly attributed to flavan-3-

ols, the most abundant polyphenolic group in this apple. The high anti-atherosclerotic activity of flavan-3-ols has been widely demonstrated in other foods such as tea (Greyling et al., 2014; Ivey at al., 2013), cocoa-based products (Berends et al., 2015; Shrime et al., 2011), and particularly after the administration in mice of apple extracts (Auclair et al., 2008; Xu et al., 2015).

The potential cardioprotective effect of anthocyanins in apple is consistent with other animal studies such as New Zealand rabbits or Syrian Golden hamsters (all of them hypercholesterolemic) after the administration of red fruits such as raspberry, bilberry and strawberry juices or currants (Rouanet et al., 2010; Yanni et al., 2015). The attenuation effect observed in aronia infusion could be attributed to anthocyanins, as already commented, and our results agree with a previous study in which they observed that aronia administration produced a decrease in epididymal fat accumulation in hypercholesterolemic Wistar rats (Qin & Anderson, 2012).

Regarding anthocyanins, the matrix effect in (poly)phenol bioavailability was also studied, comparing the (poly)phenol absorption and excretion from red-fleshed apple and anthocyanin-rich infusion from aronia fruit. Results showed significantly higher amounts of anthocyanin derivatives, phenolic acids, phenylacetic and phenylpropionic acids after HFD+A than HFD+R in plasma and urine samples. This fact shows that anthocyanins and (poly)phenolic compounds linked to apple fibre are absorbed less and more slowly. These compounds reach the large intestine where they are catabolized by colonic microbiota to simpler phenols, observing more elimination through the faeces of these simpler phenols in the HFD+R group. This might be due to the aforementioned fact that liquid matrices such as aronia infusion do not contain fibre and other food components; therefore, the apple matrix could be responsible for the (poly)phenols' retention, restricting their absorption. This could indicate that the (poly)phenols present in the red-fleshed apple (and not in the aronia) could exert beneficial effects on the gastrointestinal tract, mainly on the colon. Our preliminary results indicate that future research in this regard is necessary to verify these possible differences.

It has been proven that the sustained administration of dietary lipids alters the renal structure of rats, based on the alteration of the Bowman's space that increases and leads to glomerular atrophy and functional loss of glomeruli and tubules (Altunkaynak

et al., 2008; Hazarika et al., 2016). Regarding the effects on kidney, an attenuation effect in the renal structure (Bowman's space) was observed only in females after the three supplemented diets compared to HFD. This could probably be related to the gender differences observed in (poly)phenol bioavailability between females and males. These differences were found in urine and faeces samples. In urine samples we found that the methylated, glucuronided, methylglucuronided and methylsulphate metabolites were higher in females, while the sulphate forms were more prevalent in males. Moreover, we observed that the total excretion of (poly)phenolic metabolites in urine was higher in females after the three supplementation diets than in males, suggesting that an accumulation of (poly)phenolic metabolites in the kidney could lead to the protective effects observed in female rats (**Supplemental Table 3 in Publication IV**). These differences between genders in the metabolic fate of (poly)phenols has been previously described in Wistar rats (Margalef et al., 2016; Piskula, 2000; Soukup et al., 2016; Yang et al., 2011) and in humans (Dellinger et al., 2014) and are due to a differential expression of the enzymes that catalyze the transformations of (poly)phenols. In faeces samples, we also found that a significant increase in hippuric acid and protocatechuic acid was only detected in females. The sex-dependent expression of many isoforms of gastrointestinal enzymes and/or transporters that participate in the absorption of (poly)phenolics could explain these differences (Cassidy & Minihane, 2017), causing less absorption of some (poly)phenolics and appearing in faeces in significantly higher amounts. In this respect, it has been observed in Wistar rats that the expression of some enzymes or transporters that participate in the metabolism of (poly)phenols, such as intestinal p-glycoprotein (p-gp), is lower in females than in males (Lifschitz et al., 2006) and organic anion transporter (AOT) is greater in females, while some isoforms of the cytochrome P450, such as cytochrome 3A1 and cytochrome 32, are greater in males (Kawase et al., 2015).

These effects on the kidney's Bowman's space may be due to the same polyphenolic groups as discussed for aorta: flavan-3-ols, dihydrochalcones and anthocyanins. The kidney protective effects of apple (poly)phenols (principally flavan-3-ols and dihydrochalcones) have been observed in Sprague-Dawley rats (Abdel-Rahim & El-Beltagi, 2010) and in obese Zucker rats (Aprikian et al., 2002). Moreover, the protective effects of anthocyanins in the kidney in situations of hypercholesterolemia observed in our study agree with other studies in Sprague-Dawley or Wistar rats that

consumed black chokeberry or black soybean (Jurgoński et al., 2008; Kwon et al., 2007).

The effects observed in both aorta and kidney by flavan-3-ols, anthocyanins and dihydrochalcones historically would have been attributed only to their free radical-scavenging property. Nowadays, it is commonly accepted that (poly)phenols express their antioxidant activity mainly through modulation of gene transcription involved in antioxidant defence and detoxification. Apart from their antioxidant activity, (poly)phenols also contribute to cardiometabolic health by modulating a set of cellular processes that are not directly associated with the antioxidant enzymes, through nutri(epi)genomic mechanisms subjected to extensive research, for example regulating pro-inflammatory transcription factor NF-kB that is established as a key mediator of inflammation in several diseases like cardiovascular diseases (Monfoulet et al., 2017), or modulate the expressions of some microRNAs in endothelial cells (Milenkovic et al., 2018).

Specifically in the case of CVD, flavonoids improve the catabolism of lipoproteins rich in triglycerides in rats (Cignarella et al., 1996), and inhibit the enzymes 3-hydroxy-3-methylglutaryl-CoA reductase and acyl-CoA, leading to a reduction in the cholesterol synthesis and cholesterol esterification and a subsequent decrease in its absorption and inclusion in lipoproteins, also in rats (Bok et al., 2002).

Apart from the described protective effects in aorta and kidney, a significant decrease in plasmatic insulin concentration was also reported in males from the groups supplemented with apples compared with control HFD group. These results agree with the results obtained by Anhê et al. (2015) and Qin & Anderson (2012) in which, after the administration of different extracts rich in anthocyanins and flavan-3-ols in obese male mice or in obese male Wistar rats, a reduction in the insulin levels was observed, thus enhancing insulin sensitivity. One of the effects of HFD is the induction of adipose tissue dysfunction that can alter diverse factors, which in turn induce systemic insulin resistance, a major contributor to the development of type 2 diabetes. One of the alterations derived from an HFD is the lower peroxisome proliferator-activated receptor gamma (PPARrɣ) messenger RNA (mRNA) expression. PPARɣ regulates multiple genes in the adipose tissue regulating adipogenesis, including those encoding the adipocyte fatty acid-binding protein 4 (FABP4) or fatty acid synthase

(FAS) that is a key enzyme in de novo lipogenesis (Tso et al., 2007; Xu et al., 2007). HFD also induces the overexpression of lipoprotein lipase (LPL) that provides triacylglycerol-derived fatty acids to adipose tissue for storage. This increases the production of pro-inflammatory cytokines by adipose tissue and the overexpression of plasma tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which both contribute to the development of CVD by promoting insulin resistance, dyslipidaemia and endothelial dysfunction (Wilson, 2008). The anti-insulin resistance effects of (poly)phenols are partly due to the fact that they are able to reverse this dysregulation. In this respect, and in line with our results, a previous study has demonstrated that aronia extract consumption rich in anthocyanins increased PPARɣ mRNA expression (Qin & Anderson, 2012). Also (poly)phenols from cinnamon and green tea inhibit FAS mRNA expression, LPL mRNA expression and other genes of lipogenesis in diet-induced insulin-resistant animals (Lee et al., 2009; Qin et al., 2010). Growing evidence indicates that various dietary (poly)phenols may influence diabetes management at different levels. However, we still need more research in this field to determine the effects of (poly)phenol-rich foods, their effective dose, and mechanisms in managing diabetes.

To conclude, this study showed for the first time preliminary *in vivo* cardiometabolic effects of red-fleshed apples, which could probably be attributed to the synergistic effect of anthocyanins and dihydrochalcones, specifically in reduction of aorta thickness, improvement of renal function and reduction of insulin levels. These results need to be confirmed through the analysis of other cardiometabolic parameters in the human clinical trial included in the AppleCor project. Moreover, a gender effect was also reported in the kidney function, which suggests that differences in the metabolism of the bioactives of red-fleshed apple could differently influence the health outcomes of males and females.

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CONCLUSIONS

The main conclusions drawn from the results of this Doctoral Thesis are presented below. The structure of the Conclusion section has been organized based on the proposed objectives.

Objective 1. Evaluate the impact of different thermal (infrared-drying, hot air-drying and purée pasteurization) and non-thermal (freeze-drying) processing technologies on the (poly)phenolic content for the development of a red-fleshed apple snack product.

Conclusion I. When comparing different thermal and non-thermal processes in the development of a red-fleshed apple snack, the freeze-drying technique proved to be the one that best preserved the apple (poly)phenols.

Conclusion II. Anthocyanins, the most distinctive (poly)phenols in red-fleshed apple, were the (poly)phenolic class that was better retained in the freeze-dried apple compared to other processing technologies.

Conclusion III. Hot air-drying as well as purée pasteurization proved to be viable alternatives to the expensive method of freeze-drying, observing a good retention of most of the apple (poly)phenols compared to infrared-drying.

Objective 2. Investigate the effect of different processing technologies (freeze-drying, hot air-drying and purée pasteurization) on the (poly)phenol bioavailability of red-fleshed apple in a human crossover pilot study.

Conclusion IV. The human postprandial study revealed that apple purée proved to be the processing that resulted in the highest bioavailability of most of the (poly)phenolic groups, corroborating the fact that thermal processing can enhance (poly)phenol absorption.

Conclusion V. A great intra and inter-individual variability between (poly)phenolic metabolites was found after acute intake of apple snacks, highlighting the importance of studying the individual bioavailability of (poly)phenols and related health effects in future studies.

Conclusion VI. Purée pasteurization and hot air-drying represent viable techniques to obtain affordable red-fleshed apple products with a high content of (poly)phenols combined with an enhanced bioavailability.

Objective 3. Develop and validate a method for detection and quantification of anthocyanins and (poly)phenolic metabolites in blood samples using dried blood spot cards (DBS), as an alternative blood sampling strategy, combined with ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) after an acute intake of red-fleshed apple snack in humans.

Objective 3.1. Validate and apply a method to detect and quantify anthocyanins and (poly)phenolic metabolites in human blood samples using DBS cards.

Conclusion VII. Application of DBS cards proved to be a rapid and easy blood-sampling strategy and a suitable alternative to the classic invasive venipuncture to determine the main circulating (poly)phenolic metabolites after an acute intake of red-fleshed apple snack in humans. It allowed more time points to be collected for the determination of the microbial fermentation catabolites, enabling a better elucidation of the apple (poly)phenols metabolism.

Objective 3.2. Assess the differences in metabolite profile and concentrations between venous and capillary blood sampling and between plasma and whole blood.

Conclusion VIII. The comparison of capillary and venous sampling and also the assessment of the differences between whole blood and plasma samples showed no significant differences.

Objective 3.3. Compare DBS cards with microElution solid phase extraction (µSPE) as the most common method for the analysis of circulating (poly)phenolic metabolites in plasma.

Conclusion IX. There were no significant differences between the use of DBS cards and µSPE, the commonly used method for the determination of (poly)phenols in biological samples that requires venipuncture.

Objective 4. Investigate the bioavailability and the metabolic pathways of anthocyanins and other (poly)phenols after an acute intake of red-fleshed apple snack in humans.

Objective 4.1. Elucidate the *in vivo* biotransformation and the metabolic pathways of anthocyanins and other (poly)phenols from the red-fleshed apple.

Conclusion X. Red-fleshed apple (poly)phenols are extensively metabolized, resulting in the production of a large number of phase-II and microbial metabolites. Based on these metabolites, metabolic pathways of the main apple (poly)phenolic compounds were proposed. All these metabolites should be considered when investigating the potential health effects of red-fleshed apples.

Objective 4.2. Identify and select the potential (poly)phenolic intake biomarkers of red-fleshed apple in plasma and urine.

Conclusion XI. Among all the metabolites generated in the human postprandial study, phloretin glucuronide, cyanidin-3-*O*-galactoside and peonidin-3-*O*-galactoside were proposed as the best candidates as biomarkers after the intake of red-fleshed apple, as they were not detected in basal samples and were detected in the urine and/or plasma samples from all the volunteers.

Objective 5. Investigate the effect of the matrix (whole red-fleshed apple *versus* anthocyanin extract) on the anthocyanins and other (poly)phenols bioavailability after a sustained intake in Wistar rats.

Conclusion XII. In Wistar rats a matrix effect was observed between red-fleshed apple and an anthocyanin-rich infusion from aronia fruit, observing a higher absorption and excretion of anthocyanins after aronia supplementation.

Conclusion XIII. A matrix effect was also observed in phenolic acids, phenylacetic acids and phenylpropionic acid metabolites, which appeared in higher concentration in urine after aronia infusion and in higher amounts in faeces after red-fleshed apple supplementation. This indicates that (poly)phenols linked to apple fibre are absorbed more slowly and, therefore, reach the large intestine where they are catabolized by the colonic microbiota.

Conclusion XIV. Qualitative and quantitative differences were observed between male and female rats in the metabolic fate of red-fleshed apple (poly)phenols. Specifically, total phenolic acids and some flavon-3-ol metabolites were detected in significantly higher amounts in urine and plasma of females, suggesting that (poly)phenols could differently influence the health outcomes depending on the gender (poly)phenolic metabolism.

Objective 6. Provide first insights regarding the mechanisms of action of anthocyanins in the control of cardiometabolic risk factors after a sustained intake of red-fleshed apple snack *versus* anthocyanin-rich extract in hypercholesterolemic Wistar rats.

Conclusion XV. A cardioprotective effect was observed after the supplementation of red-fleshed apple, white-fleshed apple and aronia infusion in the reduction of aortic thickness in hypercholesterolemic Wistar rats.

Conclusion XVI. The kidney function was improved after the apple and aronia infusion supplementations but only in females, which could be related to the higher (poly)phenol bioavailability observed in females.

Conclusion XVII. A decrease in insulin plasma concentration after both apples supplementation was observed in males.

Conclusion XVIII. Anthocyanins in aronia, flavan-3-ols and dihydrochalcones in white-fleshed apple and anthocyanins and dihydrochalcones in red-fleshed apple constitute a polyphenolic phytocomplex that could act synergistically in the attenuation of cardiovascular outcomes in hypercholesterolemic rats.

OTHER MERITS

i) International scientific stage during PhD studies (November 1st 2019-February 1st 2020) in Teagasc centre (Moorepark, Cork, Ireland) under the supervision of Dr. Orla O'Sullivan. During this stay, the PhD candidate upskilled on the analysis of next generation sequencing data through various bioinformatic tools and R based statistic.

ii) During the PhD studies in the Antioxidant's group of the Department of Food Technology in the Universitat de Lleida (May 1st 2017-October 1st 2020), the PhD candidate participated in the coauthorship of several research group publications. These publications are:

- Calderón-Pérez, L., Gosalbes, M. J., **Yuste, S.**, Valls, R. M., Pedret, A., Llauradó, E., Jiménez-Hernández, N., Artacho, A., Pla-Pagà, L., Companys, J., Ludwig, I. A., Romero, M. P., Rubió, L., & Solà R. (2020). Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study. *Scientific Reports, 10(1),* 6436.

- Rubió, L., **Yuste, S.**, Ludwig, I. A., Romero, M. P., Motilva, M. J., Calderón, L., Pla-Pagà, L., Companys, J., & Macià, A. (2020). Application of Dried Blood Spot Cards combined with liquid chromatography-tandem mass spectrometry to determine eight fat-soluble micronutrients in human blood. *Journal of Chromatography B, 1152,* 122247.

- Calderón-Pérez, L., Llauradó, E., Companys, J., Pla-Pagà, L., Pedret, A., Rubió, L., Gosalbes, M. J., **Yuste, S.**, Solà, R., & Valls, R. M. (2020). Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study. *Food Chemistry*, 128567.

- Catalán, Ú., Pedret, A., **Yuste, S.**, Rubió, L., Piñol-Felis, C., Sandoval-Ramírez, B. A., Companys, J., Foguet, E., Herrero, P., Canela, N., Motilva, M. J., & Solà, R. (2020). Red-fleshed apples rich in anthocyanins and white-fleshed apples modulate aorta and heart proteome in hypercholesterolemic rats. The AppleCOR study. *Food Chemistry* (under review).

- Sandoval-Ramírez, B. A., Catalán, Ú., Llauradó, E., Salamanca, P., Rubió, L., **Yuste, S.**, & Solà, R. (2020). The health benefits of anthocyanins: an umbrella review of systematic reviews and meta-analysis of observational studies and controlled clinical trials. *Critical Reviews in Food Science and Nutrition* (under review).

iii) The PhD candidate participated in the following international and national conferences:

- Poster presented in 8th International Conference on Polyphenols and Health (ICPH). Quebec, Canada, 2017. Title: Phenolic Compounds in Barley Based Food Products and their Behaviour during *in vitro* Gastrointestinal Digestion combined with *in vitro* Colonic Fermentation. Authors: Mosele, J. I., **Yuste, S.**, Ludwig, I. A., Romero, M. P., & Motilva, M. J.

- Poster presented in the Conference of the Sociedad Española de Nutrición (SEÑ) and the X Meeting of the Associació Catalana de Ciències de l' Alimentació (ACCA). Barcelona, Spain, 2018. Title: Validation of dried blood spot cards to determine apple phenolic metabolites after a human acute intake of red-fleshed apple snack. Authors: **Yuste, S.**, Macià, A., Ludwig, I. A., Romero, M. P., Fernández-Castillejo, S., Catalán, Ú., Motilva, M. J., & Rubió, L.

iv) The PhD candidate performed 52 hours (5.2 ECTS, European Credit Transfer System) of teaching lab tasks in the Degree in Food Science and Technology at the Universitat de Lleida.