A dose-response study of the bioavailability and lipid-lowering effects of grape seed proanthocyanidin metabolites in HepG2 cells

Maria Margalef [a1], Ligia Guerrero[ab1], Zara Pons[a], Francisca Isabel Bravo [a], Lluís Arola[ac], Begoña Muguerza[ac*], and Anna Arola-Arnal[a].

[a] Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona, 43007 Spain. [b] Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia. [c] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Reus, 43204, Spain.

[1]These authors contributed equally to this work.

Running title: Doses-response study and lipid-lowering effect of physiological metabolites from proanthocyanidins.

Corresponding author:

*Dra. Begoña Muguerza

Dpt. Bioquímica y Biotecnología, U. Rovira i Virgili, C/ Marcel.li Domingo s/n 43007 Tarragona, Spain.

Telephone: +34 977 55 95 66

Fax: +34 977 55 82 32

E-mail: begona.muguerza@urv.cat

Con formato: Centrado

Abstract

Hyperlipidemia is one of the principal causes of cardiovascular disease and proanthocyanidins (PAs) regulate lipid homeostasis. This study aims to evaluate the concentration~~bioavailability~~ of PAs in rat serum after the administration of different doses of PAs and to determine the capacity of these metabolites to reduce *de novo* lipid synthesis in HepG2 cells. Two hours after oral administration of different doses of a grape seed proanthocyanidin extract (GSPE) (1000, 375, 250 and 125mg/K~~k~~g), serum~~sera~~ was~~ere~~ semi-purified and characterised by HPLC-ESI-MS/MS before analysing the synthesis and secretion of lipids in HepG2 cells. Results showed a dose-dependent appearance of metabolised PAs in serum at doses up to 375mg/K~~k~~g and saturation at 1000mg/K~~k~~g of GSPE. A reduction in cholesterol esters (CE), free cholesterol (FC) and triglycerides (TG) synthesis was observed without dose-dependence when the cells were treated with PAs metabolites. Moreover, a low dose of metabolites (125mg/K~~k~~g) was sufficient to reduce FC and TG synthesis.

Keywords: cholesterol; HPLC-MS/MS; polyphenols; proanthocyanidins; triglycerides.

1. Introduction

Hyperlipidemia is a metabolic disorder that is characterised by increased blood levels of cholesterol and/or triglycerides (TG), both of which are correlated with the development of atherosclerosis, the underlying cause of cardiovascular disease (CVD) and stroke (Yang et al., 2012). Several lines of evidence indicate that lipid-lowering treatments can reduce the development of coronary atherosclerosis (Nissen et al., 2004); in fact, a primary goal of clinical treatments for CVD risk reduction is to achieve therapeutic target levels for all lipid parameters (Wang et al., 2011).

Polyphenols are among the most abundant phytochemicals present in the human diet, and increasing evidence points to the important health-promoting effects of select flavonoids (Hertog et al., 1995; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Inverse relationships between plant-derived food intake and coronary heart disease risk have been previously reported (Shivashankara & Acharya, 2010). One of the main contributors to polyphenol intake in humans are the flavanols or proanthocyanidins (PAs), which are found primarily in grapes, beans, nuts, cocoa, tea and wine (Bladé, Arola, & Salvadó, 2010; Borriello, Cucciolla, Della Ragione, & Galletti, 2010). Our group has previously shown that the oral administration of grape seed PAs reduces TG and cholesterol and modulates the hepatic expression of several related genes and microRNAs in fatty acid, TG, and cholesterol metabolism (Baselga-Escudero et al., 2013; Del Bas et al., 2008). However, the intake of large amounts of polyphenol-rich products is not directly linked to the concentration of these

compounds and their metabolites in blood and tissues (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). It is generally accepted that the bioavailability of polyphenol is relatively poor, although monomeric flavan-3-ols show higher bioavailability (Tomas-Barberan et al., 2007). It has been proposed that oligomeric and polymeric PAs are degraded into smaller units, especially monomers, by gastric juice (Ottaviani, Kwik-Uribe, Keen, & Schroeter, 2012; Prasain et al., 2009). In addition, after digestion, the metabolised compounds can lose their original properties or even acquire new activities (Gutierrez-Merino et al., 2011). In fact, the uptake and metabolism of polyphenols is usually associated with their methylation, sulphation, or glucuronidation. In addition, considerable quantities of ingested flavonoids are degraded by colonic microbiota upon reaching the large intestine, where they yield other, smaller molecules that are also absorbed into the body (Del Rio et al., 2013). Thus, in plasma, polyphenols occur more often in more diverse forms than are present in food (Rice-Evans, 2001). Therefore, the properties of polyphenol compounds or extracts differ depending on whether they are studied using *in vitro* or *in vivo* models (Del Bas, Laos, Caimari, Crescenti, & Arola, 2012; Kroon et al., 2004). Thereby, the development of bioactive *in vitro* models using physiologically appropriate conjugates and concentrations is an important requirement for establishing the flavonoid bioactivity mechanisms (Kay, 2010). Recently, we showed the bioactivity of physiological rat metabolites after the ingestion of grape seed polyphenols using an *in vivo* - *in vitro* system (Guerrero et al., 2013). This previous study was realized with a dose of 1000mg/Kg of a grape seed proanthocyanidin extract (GSPE) to obtain the metabolites maximum concentrations in serum. Hence, in the present study we aim to evaluate the

bioactivity of the rat metabolites that appear in serum after the ingestion of more physiological doses of GSPE (i.e. lower than 1000mg/Kg), or those with a well demonstrated *in vivo* effect (Quiñones et al., 2013) thus these lower doses could be extrapolated to human consumption. Specifically, in the present study, we evaluated the absorption and serum bioavailability of PAs at more physiological doses. Moreover, it is important to note that the lipid-lowering effects of PAs were evaluated in HepG2 cells using those products of their metabolism, which were obtained from purified rat serum after different administered doses of GSPE ~~of the physiological metabolites present in rat serum after the ingestion of the differing doses of GSPE was evaluated in HepG2 cells.~~

2. Materials and methods

2.1. Chemicals and reagents

~~*2.1.1. Chromatographic analysis*~~

A 200mg/L stock standard mixture of (+)-catechin, (-)-epicatechin, EGCG, and gallic acid in methanol, and 100mg/L of PAs B1 and B2 were prepared weekly and stored at -20°C. The stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

2.1.1~~2~~. Cell culture

Dulbecco´s modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-Glutamine, penicillin and streptomycin were purchased from Bio Whittaker Europe

(Verviers, Belgium). Bradford protein reagent was obtained from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA). ^{14}C-acetate was purchased from Amersham Biosciences (Buckinghamshire, England).

2.2. Grape seed rich-proanthocyanidin extract

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). Table 1 shows the total polyphenol, phenolic compounds (flavan-3-ols and phenolic acids) and the antioxidant capacity of the extract used in this study (adapted from (Quiñones et al., 2013).

2.3. Experimental procedure in rats

Seventeen- to twenty-week-old male Wistar rats (n=15) weighing 300-326 g were used for this study. The animals were obtained from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with 12h light/dark cycles (light from 9:00a.m. to 9:00p.m.). The animals consumed tap water and a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum* during the experiment. Rats were randomly divided into five groups, which were administered either 1mL water (control group) or different doses of GSPE dissolved in 1mL of water (125, 250, 375, and 1000mg/Kg groups). GSPE doses or water were administered by oral gavage between 9 and 10a.m. following overnight fasting. Two hours after treatment, rats were anesthetised with sodium pentobarbital (80mg/Kg) and blood was collected by cardiac puncture. To obtain serum samples, blood was left at room temperature for 30min to coagulate and was then centrifuged (2000 x g, 15min, 4°C). Serum were inactivated

at 56°C for 30min to avoid the risk of complement-mediated cell lysis and stored at -80°C until analysis. All methods were in accordance with the guidelines for care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain); procedure number 6777.

2.4. Serum proanthocyanidin extraction

Prior to cell culture and chromatographic analysis, rat serum PAs were extracted and semi-purified by off-line micro-solid phase extraction (μSPE) following the previously described methodology (Guerrero et al., 2013), using 30μm OASIS HLB μ-Elution Plates (Waters, Barcelona, Spain). Briefly, micro-cartridges were conditioned sequentially with 250μL of methanol and 250μL of 0.2% acetic acid. Serum was centrifuged prior to extraction (2000 x g, 5min, 4°C). Two serum aliquots (350μL each) were individually mixed with 300μL of 4% phosphoric acid and50 μL of pyrocatechol (1000μg/L) and were then loaded onto two different plates. The two loaded plates were washed with 200μL of Milli-Q water and 200μL of 0.2% acetic acid. The retained flavanols were eluted with 2 x 50μL of acetone:Milli-Q water:acetic acid solution (70:29.5:0.5, v:v:v) for each plate. Finally, the two elutions were mixed to obtain a final volume of 200μL. Part of that solution (25μL) was evaporated to dryness using a SpeedVac Concentrator SPD 2010 SAVANT (Thermo Scientific, San Jose, CA, USA) at room temperature and redissolved with 25μL of an acetone:Milli-Q water:acetic acid solution (70:29.5:0.5, v:v:v). These samples were then directly injected in the HPLC tandem triple quadrupole mass spectrometer (HPLC-MS/MS) for chromatographic analysis; the sample volume was 2.5μL. The remaining 175μL of the semi-purified serum

was also evaporated to dryness using the same procedure described above and was then stored at -80°C until the cell culture experiment.

2.5. Chromatographic analysis

The chromatographic analysis was performed using a 1200 LC Series coupled to a 6410 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The separations were achieved using a Zorbax SB-Aq (150mm x 2.1mm i.d., 3.5µm particle size) as a chromatographic column from Agilent Technologies. The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4mL/min. The elution gradient was 0-10min, 5-55% B; 10-12min, 55-80% B; 12-15min, 80% B isocratic; 15-16min 80-5% B. A post run of 10min was applied. The electrospray ionisation (ESI) conditions were 350°C and 12L/min of drying gas temperature and flow, respectively, a nebuliser gas pressure of 45psi, and 4000V of capillary voltage. MS/MS was operated in negative mode. MS/MS acquisition was performed in multiple reaction monitoring (MRM) mode for PAs and their metabolites. Data acquisition was conducted using the MassHunter Software (Agilent Technologies, Palo Alto, CA, USA).

~~2.6. Method validation and samples quantification~~

The human hepatocellular carcinoma cell line HepG2 (ATCC code HB-8065, Manassas, VA, USA) was cultured in DMEM medium supplemented with 10% (v/v) foetal calf serum, 100U/mL of penicillin, 100µg/mL of streptomycin and 2mM of L-Glutamine in a cell culture flask at 37°C and a humidified atmosphere of 5% CO_2. The cells were fed every 2-3 days.

2.7. Lipid analysis

The HepG2 cells were seeded at $500x10^3$ cell/well in 12-well plates, and they were used upon reaching 80-90% confluence. Growth medium was replaced by supplemented culture media 12h before the treatments. HepG2 cells were cultivated with the PAs metabolites derived from semi-purified rat serum (Guerrero et al., 2013). Rat serum were obtained for *in vitro* use 2h after the administration of either water or increasing doses of GSPE (125, 250, or 375mg/Kg). The dried, semi-purified serum was redissolved in supplemented culture medium and was then added to the growth medium in the well (1:10, v/v). GSPE (25mg/L) and ethanol (1%) were used as a positive and negative control, respectively. ^{14}C-acetate (0.6µCi/mL) and the appropriate treatment were added simultaneously to the cell culture medium to evaluate lipid synthesis. Six hours after treatment with the purified serum, media and cells were collected and the lipid fraction was obtained via hexane:isopropanol (3:2, v:v) extraction and separated by Thin Layer Chromatography (TLC). In all experiments, the lipids evaluated were cholesterol esters (CE), free cholesterol (FC) and TG. TLC was performed as previously described (Pill, Aufenanger, Stegmeier, Schmidt, & Müller, 1987), with an additional separation using a Hexane:MTBE:NH_3 (30:20:0.1, v:v:v) solvent to obtain the TG fraction (Del Bas et al., 2008). The obtained lipid fractions were separated, and radioactivity was measured by scintillation counting. The values were normalised to milligrams of protein, determined using the Bradford methodology (Bradford, 1976).

2.89. Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM) and analysed by Student's t-test and one-way ANOVA using the IBM SPSS Statistics software (Version 20.0.0). Differences between groups were assessed using the Bonferroni test (to correct for multiple comparisons). Differences between means were considered significant when $p<0.05$.

3. Results

3.1. GSPE serum metabolites determination

The HPLC-ESI-MS/MS analysis of rat serum PAs metabolites collected 2h after the ingestion of 1000, 375, 250, or 125mg/Kg GSPE is presented in Table 2 and Figure 1. Free forms of catechin, epicatechin, and dimeric PAs, in addition to phenolic acids such as gallic acid, were detected at low concentrations in serum (up to 0.80µM), in contrast to the high concentrations of these compounds found in GSPE (Quiñones et al., 2013). Moreover, other compounds abundant in GSPE were not detected in serum, such as monomeric and dimeric gallated conjugates or trimeric PAs (Table 2). However, the primary compounds detected in rat serum were conjugated forms of the monomeric flavan-3-ols (catechin and epicatechin). For all doses tested, the glucuronidated forms of flavanols were present in serum at substantially higher concentrations compared to methylated and sulphated conjugates (Figure 2). In addition, although the metabolite serum concentration was very compound-specific, the results corresponding to many physiological forms of PAs showed a dose-dependent effect on both metabolised and non-metabolised flavonoids up to a dose of

375mg/Kg. So that at the dose of 1000mg/Kg of GSPE, the metabolism of many compounds was reduced (Figures 2 and 3).

3.2. GSPE rat serum metabolites decrease lipid synthesis and excretion in HepG2 cells

3.2.1. Effect of different doses of GSPE rat serum metabolites on cholesterol ester synthesis in HepG2 cells

Treating HepG2 cells with semi-purified serum from GSPE-administered rats produced a dose-dependent decrease in CE synthesis relative to the cells treated with serum from water-administered rats (Figure 4A). However, only serum from the 375mg/Kg GSPE dose significantly reduced the CE synthesis compared to the control animals. The differences in CE synthesis were due to a decrease in the intracellular lipid content (69 ± 4.1%, after setting the CE synthesis of the control group to 100% for the dose of 375mg/Kg GSPE). For the three doses tested, the CE secretion into culture medium was similar to controls.

3.2.2. Effect of different doses of GSPE rat serum metabolites on free cholesterol synthesis in HepG2 cells

The total amount of intracellular FC was reduced when cells were incubated with the metabolites present in serum~~sera~~ (Figure 4B). No differences in the synthesised FC were observed for the three doses studied. The FC secreted by the cells into the culture medium was similar to that of the controls for all three tested doses.

3.2.3. Effect of different doses of GSPE rat metabolites on triglyceride *synthesis in HepG2 cells*

Although a decrease in TG synthesis and intracellular TG was observed for all doses, surprisingly, only treatment with a low dose of metabolites (GSPE intake of 125mg/Kkg) resulted in a statistically significant difference compared to the control (70 ± 4.0%, whereas the control was set to 100%). TG secretion into the cell culture medium was similar to that of the control metabolites for all three doses (Figure 4C).

4. Discussion

The regular consumption of flavonoids in the human diet has been associated with reduced mortality and morbidity of cardiovascular disease (CVD) (Crozier, Jaganath, & Clifford, 2009; Rasmussen et al., 2005). PAs are considered the most abundant flavonoids in the human diet (Bladé et al., 2010) and, similar to other flavonoids, their beneficial effects depend on both the amount consumed and their bioavailability (Manach et al., 2004). It has been shown that low molecular weight forms, especially monomeric flavan-3-ols and dimers, are absorbed in the small intestine and metabolized by the phase-II enzymes, whereas the polymeric forms are metabolized by the colonic microbiota (Aura, 2008; Monagas et al., 2010). It has also been demonstrated that at 2h after an acute PA administration, the main compounds that reach the systemic circulation and tissues are phase-II metabolites (Serra et al., 2010, 2013). In addition, the bioactive compounds that eventually reach tissues are substantially different from those that are initially

present in food (Kroon et al., 2004). In fact, the qualitative and quantitative PAs composition differs substantially between GSPE and the serum of animals administered a 1000mg/Kg dose of this same extract (Guerrero et al., 2013). As a result of these structural changes, many *in vitro* studies with no physiological forms of flavonoids have been questioned because their beneficial effects could be modulated by their metabolic conjugates (Kay, 2010; Kroon et al., 2004). In a previous study, we described a new methodology for evaluating the effects of bioactive forms of PAs on *de novo* lipid synthesis in cultured cells (Guerrero et al., 2013). Hence, the objective of the present work was to determine whether PAs are absorbed or metabolised differently depending on the dose administered to rats and whether the different absorption or amount of metabolites could affect the bioactivity of the PAs in regulation *de novo* lipid synthesis using the previously described methodology (Guerrero et al., 2013).

This study was conducted at 2h post GSPE administration and focused on flavanol-phase-II metabolites, since these compounds are known to peak serum concentration at 2h post PAs administration (Serra et al., 2010). Acute PAs bioavailability studies are usually conducted with high, non-physiological doses of PAs extracts, such as 1000mg/Kg of GSPE, to reach a serum or plasma metabolite concentration that is detectable by chromatographic analysis (Arola-Arnal et al., 2013; Guerrero et al., 2013; Serra et al., 2010). However, in this study is demonstrated that following treatment with low physiological doses of GSPE (i.e.; 125mg/Kg GSPE), PAs metabolites can be detected and quantified in serum and that a dose of 1000mg/Kg of GSPE saturates the system.

Moreover, a clear dose-response of both metabolised and non-metabolised PAs can be observed in rat serum 2h following the acute administration of low doses of GSPE (125, 250, and 375mg/Kg). These results indicate that the rat's ability to conjugate many flavonoids could be overwhelmed at high doses. Therefore, the administration of high doses of GSPE (from 375mg/Kg) does not result in a greater presence of serum PAs metabolites. In fact, the concentration of some metabolites at 1000mg/Kg is even decreased respect to lower doses. Only some minority aglycone forms, such as gallic acid and dimeric PAs, had greater serum concentrations at 1000mg/Kg than at 375mg/Kg. Similarly, when a 1000mg/Kg dose is administered to rats, the methyl-epicatechin glucuronide concentration is increased relative to 375mg/Kg. However, there are no differences between methyl glucuronidated metabolites (sum of methyl epicatechin glucuronide and methyl catechin glucuronide) when the doses of 375 and 1000mg/Kg of GSPE are compared. This observation could indicate that the enzyme O-methyl transferase has greater affinity for epicatechin than catechin (Figure 3).

On the other hand, several studies have shown the beneficial effects of flavonoids in reducing TG levels both *in vitro* (Pal et al., 2003) and *in vivo* (Auger et al., 2002; Vinson, Teufel, & Wu, 2001). Similarly, this is not the first demonstration of GSPE reducing lipid synthesis, especially for TG (Josep Maria Del Bas et al., 2008, 2009; Guerrero et al., 2013; Quesada et al., 2009). Furthermore, we have previously reported that a 1000mg/K~~k~~g dose of grape seed PAs metabolites in rat serum reduced *de novo* lipid synthesis in HepG2 cells (Guerrero et al., 2013). In

this study, the lipid-lowering effect of metabolites at lower doses of GSPE (125, 250, and 375mg/Kg) on *de novo* lipid synthesis were evaluated because the highest dose (1000mg/Kg) did not yield a higher concentration of serum metabolites than the 375mg/Kg dose (i.e. the system is saturated). The results showed a reduction in *de novo* lipid synthesis at all doses studied. However, although elevating the dose of GSPE to 375mg/Kg increased the metabolite concentrations appearing in serum, a dose-dependent effect was only observed on CE, but not TG or FC. Moreover, the lowest dose of 125mg/Kg showed the strongest effect on TG, indicating that a relatively moderate dose of 125mg/Kg is effective. The lack of dose-dependence effect of PAs has been previously reported by our group, indicating that lower doses of GSPE can be more efficient than higher doses (Quiñones et al., 2013).

Given the high concentrations of conjugate forms present in serum, specifically the glucuronic acid conjugates, these metabolites seem to be involved in reducing the *de novo* synthesis of lipids in hepatic cells. However, these conjugated forms may not act directly at the cellular level. Previous studies have indicated that there is no direct relationship between the plasma concentration and the target tissue concentration of flavonoids, besides varying the distribution between blood and tissues depending on the concerned flavonoid (Hong, Kim, Kwon, Lee, & Chung, 2002; Maubach et al., 2003). In HepG2 cells, O'Leary *et al*. demonstrated that glucuronidated flavonoids are deconjugated following intact entry into cells by an unidentified transporter (O'Leary et al., 2003). Therefore, the deconjugation of glucuronidated metabolites to their bioactive forms inside HepG2

cells could explain the lack of a direct relationship between the serum concentration of these conjugated compounds and their biological functionality, including any effects on *de novo* lipid synthesis. Additionally, the lack of a relationship between the serum metabolite concentration and the regulation of lipid synthesis could be explained by the presence of other minority compounds in the serum that regulate lipid synthesis and have not been detected.

5. Conclusion

This study showed the dose-dependent appearance of both metabolised and non-metabolised PAs in rat serum 2h following the acute administration of low doses of GSPE (up to 375mg/Kkg) but a saturation of the system when a high dose of GSPE was administered. Moreover, the study demonstrated that these PAs metabolites exhibit no dose-dependent effects in reducing the *de novo* synthesis of lipids, especially TG, and showed that a relatively moderate dose of 125mg/Kkg is effective.

Acknowledgements

The authors have declared no conflict of interest.

This study was supported by grants from the Ministerio de Educación y Ciencia of the Spanish Government (AGL 2008-00387/ALI) and the Universitat Rovira i Virgili-Banco Santander (2011 LINE-12 and 2011 LINE-13).

References

Arola-Arnal, A., Oms-Oliu, G., Crescenti, A., Del Bas, J. M., Ras, M. R., Arola, L., & Caimari, A. (2013). Distribution of grape seed flavanols and their metabolites in pregnant rats and their fetuses. *Molecular Nutrition & Food Research*, *57*(10), 1741–1752.

Auger, C., Caporiccio, B., Landrault, N., Teissedre, P. L., Laurent, C., Cros, G., et al. (2002). Red Wine Phenolic Compounds Reduce Plasma Lipids and Apolipoprotein B and Prevent Early Aortic Atherosclerosis in Hypercholesterolemic Golden Syrian Hamsters (Mesocricetus auratus). *J. Nutr.*, *132*(6), 1207–1213.

Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds in the colon. *Phytochemistry Reviews*, *7*(3), 407–429.

Baselga-Escudero, L., Arola-Arnal, A., Pascual-Serrano, A., Ribas-Latre, A., Casanova, E., Salvadó, M.-J., et al. (2013). Chronic administration of proanthocyanidins or docosahexaenoic acid reverses the increase of miR-33a and miR-122 in dyslipidemic obese rats. *PloS One*, *8*(7), e69817.

Bladé, C., Arola, L., & Salvadó, M.J. (2010). Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Molecular Nutrition & Food Research*, *54*(1), 37–59.

Borriello, A., Cucciolla, V., Della Ragione, F., & Galletti, P. (2010). Dietary polyphenols: Focus on resveratrol, a promising agent in the prevention of cardiovascular diseases and control of glucose homeostasis. *Nutrition, Metabolism and Cardiovascular Diseases*, *20*(8), 618–625.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254.

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports*, *26*(8), 1001–1043.

Del Bas, J. M., Laos, S., Caimari, A., Crescenti, A., & Arola, L. (2012). *Detection of bioavailable peroxisome proliferator-activated receptor gamma modulators by a cell-based luciferase reporter system*. *Analytical Biochemistry* (Vol. 427, pp. 187–189).

Del Bas, J. M., Ricketts, M. L., Baiges, I., Quesada, H., Ardevol, A., Salvadó, M.J., et al. (2008). Dietary procyanidins lower triglyceride levels signaling through

the nuclear receptor small heterodimer partner. *Molecular Nutrition & Food Research*, *52*(10), 1172–1181.

Del Bas, J. M., Ricketts, M.L., Vaqué, M., Sala, E., Quesada, H., Ardevol, A., et al. (2009). Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. *Molecular Nutrition & Food Research*, *53*(7), 805–814.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*, *18*(14), 1818–1892.

Guerrero, L., Margalef, M., Pons, Z., Quiñones, M., Arola, L., Arola-Arnal, A., & Muguerza, B. (2013). Serum metabolites of proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells. *The Journal of Nutritional Biochemistry*, *24*(12), 2092–2099.

Gutierrez-Merino, C., Lopez-Sanchez, C., Lagoa, R., K. Samhan-Arias, A., Bueno, C., & Garcia-Martinez, V. (2011). Neuroprotective Actions of Flavonoids. *Current Medicinal Chemistry*, *18*(8), 1195–1212.

Hertog, M. G., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., et al. (1995). Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of Internal Medicine*, *155*(4), 381–386.

Hong, S. J., Kim, S. Il, Kwon, S. M., Lee, J. R., & Chung, B. C. (2002). Comparative Study of Concentration of Isoflavones and Lignans in Plasma and Prostatic Tissues of Normal Control and Benign Prostatic Hyperplasia. *Yonsei Medical Journal*, *43*(2), 236–241.

Kay, C. D. (2010). The future of flavonoid research. *British Journal of Nutrition*, *104*, S91–S95.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., & Williamson, G. (2004). How should we assess the effects of exposure to dietary polyphenols in vitro? *Am J Clin Nutr*, *80*(1), 15–21.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727–747.

Maubach, J., Bracke, M. E., Heyerick, A., Depypere, H. T., Serreyn, R. F., Mareel, M. M., & De Keukeleire, D. (2003). Quantitation of soy-derived phytoestrogens

in human breast tissue and biological fluids by high-performance liquid chromatography. *Journal of Chromatography B*, *784*(1), 137–144.

Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., et al. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food & Function*, *1*(3), 233–253.

Nissen, S. E., Tuzcu, E. M., Schoenhagen, P., Brown, B. G., Ganz, P., Vogel, R. A., et al. (2004). Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. *JAMA : The Journal of the American Medical Association*, *291*(9), 1071–1080.

O'Leary, K. A., Day, A. J., Needs, P. W., Mellon, F. A., O'Brien, N. M., & Williamson, G. (2003). Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human β-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. *Biochemical Pharmacology*, *65*(3), 479–491.

Ottaviani, J. I., Kwik-Uribe, C., Keen, C. L., & Schroeter, H. (2012). Intake of dietary procyanidins does not contribute to the pool of circulating flavanols in humans. *The American Journal of Clinical Nutrition*, *95*(4), 851–858.

Pal, S., Ho, N., Santos, C., Dubois, P., Mamo, J., Croft, K., & Allister, E. (2003). Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *The Journal of Nutrition*, *133*(3), 700–706.

Pill, J., Aufenanger, J., Stegmeier, K., Schmidt, F. H., & Müller, D. (1987). Thin-layer chromatography of radioactively labelled cholesterol and precursors from biological material. *Fresenius' Zeitschrift Für Analytische Chemie*, *327*(5-6), 558–560.

Prasain, J. K., Peng, N., Dai, Y., Moore, R., Arabshahi, A., Wilson, L., et al. (2009). Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. *Phytomedicine*, *16*(2), 233–243.

Quesada, H., del Bas, J. M., Pajuelo, D., Díaz, S., Fernandez-Larrea, J., Pinent, M., et al. (2009). Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *International Journal of Obesity (2005)*, *33*, 1007–1012.

Quiñones, M., Guerrero, L., Suarez, M., Pons, Z., Aleixandre, A., Arola, L., & Muguerza, B. (2013). Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Research International*, *51*(2), 587–595.

Rasmussen, S. E., Frederiksen, H., Struntze Krogholm, K., & Poulsen, L. (2005). Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Molecular Nutrition & Food Research*, *49*(2), 159–174.

Rice-Evans, C. (2001). Flavonoid Antioxidants. *Current Medicinal Chemistry*, *8*(7), 797–807.

Serra, A., Macià, A., Romero, M. P., Valls, J., Bladé, C., Arola, L., & Motilva, M. J. (2010). Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *British Journal of Nutrition*, *103*(07), 944–952.

Serra, A., Macià, A., Rubió, L., Anglès, N., Ortega, N., Morelló, J. R., et al. (2013). Distribution of procyanidins and their metabolites in rat plasma and tissues in relation to ingestion of procyanidin-enriched or procyanidin-rich cocoa creams. *European Journal of Nutrition*, *52*(3), 1029–1038.

Shivashankara, K. S., & Acharya, S. N. (2010). Bioavailability of Dietary Polyphenols and the Cardiovascular Diseases. *The Open Nutraceuticals Journal*, *3*, 227–241.

Tomas-Barberan, F. A., Cienfuegos-Jovellanos, E., Marín, A., Muguerza, B., Gil-Izquierdo, A., Cerda, B., et al. (2007). A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of Agricultural and Food Chemistry*, *55*(10), 3926–3935.

Vinson, J. A., Teufel, K., & Wu, N. (2001). Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in a hamster model. *Atherosclerosis*, *156*(1), 67–72.

Wang, X., Hasegawa, J., Kitamura, Y., Wang, Z., Matsuda, A., Shinoda, W., et al. (2011). Effects of hesperidin on the progression of hypercholesterolemia and fatty liver induced by high-cholesterol diet in rats. *Journal of Pharmacological Sciences*, *117*(3), 129–138.

Yang, Y., Piao, X., Zhang, M., Wang, X., Xu, B., Zhu, J., et al. (2012). Bioactivity-guided fractionation of the triglyceride-lowering component and in vivo and in vitro evaluation of hypolipidemic effects of Calyx seu Fructus Physalis. *Lipids in Health and Disease*, *11*(1), 38–45.

Figure legends

Figure 1. Extracted ion chromatogram of serum flavonoids 2h after the administration of 1000mg GSPE /Kkg (in green), 375mg GSPE /Kkg (in black), 250mg GSPE /Kkg (in red), and 125mg GSPE /Kkg (in blue). (1)Gallic Acid; (2)Dimer B1; (3)Dimer B3; (4)Dimer B2; (5)Methyl-catechin-glucuronide; (6)Methyl-epicatechin-glucuronide; (7)Catechin glucuronide; (8)Epicatechin glucuronide; (9)Catechin; (10)Epicatechin; (11)CEpicatechin sulphate; (12)Methyl-catechin-O-sulphate; (13)Methyl-epicatechin-O-sulphate; (14)3-methyl-epicatechin; (15)4-methyl-epicatechin.

Figure 2. Rat serum concentrations (μM) of gallic acid and non-metabolised GSPE compounds and their flavan-3-ols glucuronidated, methyl-glucuronidated, sulphated, and methylated metabolites 2h after the administration of 1000, 375, 250, and 125mg GSPE/Kkg. The results are expressed as the mean ± standard error (SEM). Different letters indicate statisticallysadistically significant differences compared to the control ($p < 0.05$).

Figure 3. Rat serum concentrations (μM) of non-metabolised and metabolised GSPE compounds at 2h after the administration of 1000, 375, 250, and 125mg GSPE /Kg. The results are expressed as the mean ± standard error (SEM). A) catechin and epicatechin, B) dimeric proanthocyanidins, C) gallic acid, D)

(epi)catechin-glucueronide, E) methyl-(epi)catechin-glucueronide, F) (epi)catechin-sulphate, G) methyl-(epi)catechin-Oe-sulphate, H) 3- and 4-methyl-epicatechins.

Figure 4. Effect of rat semi-purified serum obtained 2h after the administration of GSPE (375, 250, and 125mg/Kkg) on HepG2 cells. Cells were simultaneously incubated with ^{14}C-labelled acetate and rat semi-purified serum. Six hours after the treatment, radioactivity incorporated into media (□) and cellular (■) lipids was measured. The total synthesis represents the radioactivity present in the cells and culture medium (■). All values are the mean ± SEM of triplicates of three independent experiments. **A.** Results related to cholesterol ester synthesis and secretion in HepG2 cells. **B.** Results related to free cholesterol synthesis and secretion in HepG2 cells. **C.** Results related to triglycerides synthesis and secretion in HepG2 cells. Different letters indicate statisticallysadistically significant differences compared to the control ($p < 0.05$).