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THÈSE réalisée en co-tutelle

pour le

DOCTORAT DE L'UNIVERSITÉ BORDEAUX 2 ET DE L'UNIVERSITÉ DE GLASGOW

Ecole doctorale des Sciences de la Vie et de la Santé

Mention : Sciences, Technologie, Santé

Option : Œnologie

Présentée et soutenue publiquement le 13 décembre 2013

KY Isabelle

Née le 27 avril 1986 à Woluwe St-Lambert, Belgique

Characterisation of Grape and Grape pomace Polyphenolics: Their Absorption and Metabolism and Potential Effects on Hypertension in a SHR Rat Model

Membres du Jury

Pr. J. RICARDO DA SILVA, Universidade de Lisbonne ..... Rapporteur
Dr. J. M. ROUANET, Université Montpellier 2 ..... Rapporteur
Pr. G. CROS, Université Montpellier 1 ..... Examinateur
Pr. P. L. TEISSEDRE, Université Victor Segalen Bordeaux 2 ..... Directeur de thèse
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Caractérisation d'Antioxydants Phénoliques de Raisins et de Marcs: leur Absorption, Métabolisme et Effets Potentiels sur l'Hypertension dans un Modèle de Rats SHR (Spontanément Hypertendus)

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To my parents,

Elisabeth and Anthony,

For their endless love, support and encouragement

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International publications list and patent

1. Ky, I., Lorrain, B., Crozier, A., Teissedre, P. L. (2013). Mediterranean wine by-products: phenolic characterisation and antioxidant evaluation of grapes and grape pomaces. Molecules, under submission.

2. Ky, I., Crozier, A., Cros, G., Teissedre, P. L. (2013). Polyphenols composition of wine and grape sub-products and potential effects on chronic diseases. Nutrition and Aging, article in press.

3. Ky, I., Lorrain, B., Pechamat, L. and Teissedre, P. L. (2013). Evolution of analysis of polyhenols from grapes, wines, and extracts. Molecules, **18** (1): 1076-1100.

4. Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., Gény, L., Rey, P., Doneche, B. and Teissedre, P. L. (2012). Assessment of grey mould (Botrytis cinerea) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Australian Journal of Grape and Wine Research, **18** (2): 215-226.

5. Lorrain, B., Ky, I., Pasquier, G., Jourdes, M., Dubrana, L. G., Gény, L., Rey, P., Donèche, B. and Teissedre, P. L. (2012). Effect of Esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines. Australian Journal of Grape and Wine Research, **18** (1): 64-72.

6. Silva, M. A., Ky, I., Jourdes, M. and Teissedre, P. L. (2012). Rapid and simple method for the quantification of flavan-3-ols in wine. European Food Research and Technology, **234** (2): 361-365.

7. Chira, K., Lorrain, B., Ky, I. and Teissedre, P. L. (2011). Tannin composition of cabernet-sauvignon and merlot grapes from the bordeaux area for different vintages (2006 to 2009) and comparison to tannin profile of five 2009 vintage mediterranean grapes varieties. Molecules, **16** (2): 1519-1532.

8. **Patent :**

   Authors: TEISSEDRE Pierre-Louis, KY lsabelle, CROS Gérard
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   Patent deposit date: 28 February 2013, Application number: 1351813, BFF12P0968/EG, Submission number: 1000183483.

International oral communications

1. Ky, I., Crozier, A., Cros, G., Teissedre, P. L. (2013). Polyphenols composition of wine and grapes sub-products and potential effects on chronic diseases. *Wine health*, Sydney, Australia.

2. Ky, I., Kolbas, N., Teissedre, P. L. (2011). Antioxidant capacity of grapes, pomaces and wines for five Mediterranean wines varieties. *WAC (Wine active compounds),* Beaune, France.

3. Ky, I., Pasquier, G., Jourdes, M., L'hyvernay, A., Geny, L., Donèche, B., Teissedre, P. L. (2010). Effect of *Botrytis cinerea* and esca on phenolic composition of berries and wines. *Macrowine,* Turin, Italy.

International poster communications

1. Ky, I., Crozier, A., Teissedre, P. L. (2013). Mediterranean wines by-products: flavan-3-ol and anthocyanin characterisations. *OIV,* Bucarest, Romania.

2. Ky, I., Crozier, A., Teissedre, P. L. (2012). *In vitro* characterisation of grape pomace extracts: polyphenolic content and antioxidant activity. *ICP*, Florence, Italie.

3. Ky, I., Crozier, A., Teissedre, P. L. (2012). Characterisation of grape and their respective pomace extracts: polyphenolic content and antioxidant activity. *Macrowine,* Bordeaux, France.

4. Ky, I., Favennec, M., Cros, G., Crozier, A., Teissedre, P. L. (2011). Polyphenolic content, *in vitro* and *in vivo* antioxidant capacity of 6 Mediterranean red grape pomace varieties. *ICPH,* Sitges, Spain.

5. Ky, I., Kolbas, N., Merillon, J. M., Teissedre, P. L. (2011). Polyphenolic content and *in vitro* antioxidant capacity of grapes, pomaces and wines of five Mediterranean wines varieties. *Oeno,* Bordeaux, France.

6. Lorrain, B., Ky, I., Pasquier, G., Jourdes, M., Fermaud, M., L'Hyvernay, A., Geny, L., Donèche, B., and Teissedre, P. L. (2011). *Botrytis cinerea* and esca impact on phenolic and sensory quality of Bordeaux grapes and their derived wines. *OIV,* Porto, Portugal.

7. Ky, I., Kolbas, N., Merillon, J. M., Teissedre, P. L. (2011). Valorization of Mediterranean wines by-products: phenolic characterisation and antioxidant activity. *1st Euro-Mediterranean Symposium,* Avignon, France.

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Contents

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Abbreviations list

%G	% of Galloylation
%P	% of Prodelphinidin
$\bullet O_2^-$	Superoxide anion
AAPH	Potassium persulfate
ABC	Adenosine triphosphate (ATP)-Binding Cassette
ABTS	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACE	Angiotensin-converting enzyme
ACE2	Angiotensin-converting enzyme 2
ALI	Alicante
Ang 1–7	Angiotensin(1–7)
Ang 1–9	Angiotensin(1–9)
Ang I	Angiotensin I
Ang II	Angiotensin II
ARB	Angiotensin receptor blocker
AT_1	Angiotensin receptor type 1
AT2	Angiotensin receptor type 2
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
BP	Blood pressure
C	Catechin
cAMP	Adenosine monophosphate
CAR	Carignan
CBG	Cytosolic-β-glucosidase
CD36	Cluster of differentiation 36
cGMP	Cyclic guanosine monophosphate
COMT	Catechol-*O*-methyltransferase
COU	Counoise
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAG	Diacyl glycerol
DBP	Diastolic blood pressure
DOCA	Deoxycorticosterone acetate
DPPH	2-Diphenyl-1-picrylhydrazyl
EA70	Hydro-alcoholic 70% extract
EAQ	Aqueous extract
EC	Epicatechin
ECG	Epicatechin gallate
EDCFs	Endothelium-derived contracting factors
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EGCG	Epigallocatechin gallate
eNOS	Endothelial NO synthase
ERα	α-isoform estrogen receptor
EtOH	Ethanol
FGF	Fibroblast growth factors
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRE1	Grenache Long du Coudoulet
GRE2	Grenache Face aux Pins
ICAM-	Intercellular adhesion molecule
IGF	Insulin-like growth factor
IL	Interleukin
INF-γ	Interferon-γ

IP_3	Inositol 1,4,5-triphosphate
LDL-c	Low-density lipoprotein cholesterol
LPH	Phloridzin hydrolase
LPO	Lipooxygenase
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
mDP	Mean degree of polymerization
MeOH	Methanol
MIP-2	Macrophage-inflammatory protein-2
MMPs	Matrix metalloproteinases
MOU	Mourvèdre
MRP	Including multidrug resistance protein
MRP2	Multidrug resistance associated protein 2
NADH/NADPH	Nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NEP	Non extractable polyphenols
NF-κB	Nuclear factor kappa beta
NO	Nitric oxide
ONOO-	Peroxynitrite anion
oxLDLs	Oxidized LDLs
p38 MAPK	P38 Mitogen-activated protein kinase
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PGI_2	Prostacyclin
P-gp	P-Glycoprotein
PPAR-γ	Peroxisome proliferator activated receptor γ
QTG	Quantitative trait gene
QTL	Quantitative trait loci
RAAS	Renin–angiotensin-aldosterone system
RAS	Renin–angiotensin system
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SGLT1	Active sodium-dependent glucose transporter
SHR	Spontaneously hypertensive rat
SOD	Superoxide dismutase
sP-selectin	Soluble P-selectin
SULT	Sulfotransferase
SYR1	Syrah Plantier
SYR2	Syrah Haut de Julien
TF	Tissue factor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor
TPTZ	2,4,6-tri(2-pyridyl)-*s*-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TXA2	Thromboxane A2
UGT	Uridine-5'-diphosphate glucuronosyltransferase
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor

General introduction and objective

Grape (*Vitis vinifera*) is one of the most cultivated fruit crops in the world, with an annual production of ~64 million metric tons in 2010 (OIV, 2011). During wine making the extract ability of grape (poly)phenols typically reaches only 30–40%, depending upon grape variety, vineyard location and the technological parameters during the wine making process including destemming, crushing, maceration and pressing (Kammerer et al. 2004). Therefore, grape pomace potentially constitutes a very abundant and relatively inexpensive source of a wide range of (poly)phenols including monomeric and oligomeric flavan-3-ols (proanthocyanidins) as well as anthocyanins (glucosides, acetylated glucosides and coumarylic glucosides) (Louli et al. 2004). Moreover, pomace has also been evaluated as a potential source of antioxidant (poly)phenols which could be used as nutraceuticals or food additives. Epidemiological studies have shown an inverse correlation between the consumption of (poly)phenol-enriched diets and a reduced risk of CVD (Mennen et al. 2004; He et al. 2007). (Poly)phenolic compounds are known to have some health benefits such as a chemopreventive role toward cardiovascular, cancer, and degenerative diseases related to the antioxidant activity conferred by (poly)phenols involving different processes and also different compounds (Huang et al. 2005).

Hypertension is the most important of cardiovascular risk factors worldwide. According to the World Health Organization 2013 data, hypertension accounted for approximately 9.4 million deaths a year, contributing to 45% of deaths due to heart disease and 51% of deaths due to stroke. The prevalence of hypertensive people under 25 years of age is low but steadily increases to ~40 % in 65 year olds and 90% at 85 years of age. It is estimated that by 2030, more than 23 million people will die annually from cardiovascular diseases (CVDs). The importance of oxidative stress, vascular inflammation and endothelial dysfunction has to be highlighted in the development of CVDs. The knowledge of the process has provided new perspectives to elaborate novel pharmaceutical or dietary strategies to control the development of vascular diseases.

Following this observation, Perrin & Fils society was willing to investigate wine by-products associated with the production of their wines at the Château de Beaucastel located in the Rhône Valley, appellation of Châteauneuf-du-Pape. In the first instance, the aims of our work was to characterize and select grape pomace (poly)phenolic extracts from different Rhône Valley grape varieties in order to develop food supplements preventing high blood pressure. Thereafter, grape pomace (poly)phenolic extracts will be associated with a common anti-hypertensive drug, verapamil, and will be further developed as a drug molecule "activator" of verapamil.

In the first part, different classes of phenolic compounds will be underlined also as their antioxidant capacities, their biological activities and their bioavailabilities. Their presence and content in grapes and wines will also be included in the discussion. Then, the current state of knowledge about high blood pressure will be presented.

In the second part, materials and methods used in grapes and grape pomaces characterisation and *in vivo* study will be presented.

In the third part, grape and their respective grape pomace samples were characterized for their phenolic contents (total phenol contents, tannin and anthocyanin contents - total and individuals; quantification of monomeric and oligomeric proanthocyanidins as well as some anthocyanins [glucosides, acetylated glucosides and coumarylic glucosides]) and for their degree of polymerization by HPLC-PDA-Fluo-MS method. The *in vitro* antioxidant capacities of all the extracts were evaluated by four different analytical methods: $ABTS^{\bullet+}$, DPPH, FRAP and ORAC. Ratio of initial phenolic compounds from grape to pomace was estimated. Correlation between antioxidant potential and phenolic composition was assessed. Grape pomace samples were selected for the *in vivo* study. This work was done at the Institut des Sciences de la Vigne et du Vin (ISVV), University of Bordeaux 2 Victor Segalen, supervised by Pr. Pierre-Louis TEISSEDRE.

In the fourth part, *in vivo* activities of grape pomace extracts and grape pomace extracts alone or in association with verapamil were investigated using a spontaneous hypertensive rat (SHR) model. Different experimental groups were made, blood pressure was follow and the effect of grape pomace on hypertension was determined. Some extracts were chosen for bioavailability studies in which urine, faeces, plasma and tissues (liver, heart and kidneys) were collected and analysed for metabolites by HPLC-PDA-MS^n and GC-MS. This work was carried out in collaboration with the Centre de Pharmacologie et d'Innovation dans

le Diabète (CPID), University of Montpellier I supervised by Pr. Gérard CROS and with the Plant products and Human Nutrition Group, University of Glasgow, supervised by Pr. Alan CROZIER.

Le raisin de vigne (*Vitis vinifera*) est l'un des fruits les plus cultivés dans le monde avec une production annuelle de 64 million de tonnes en 2010 (OIV 2011). Leur composition en polyphénols et l'extractibilité qui est loin d'être complète et n'atteint approximativement que 30-40%, en fonction de la variété, du terroir et des paramètres technologiques de vinification incluant le foulage, la macération et le pressurage (Kammerer et al. 2004). Suite à la vinification, les marcs sont produits en quantités abondantes et constitues une source peu coûteuse de polyphénols comprenant les monomères et oligomères de flavan-3-ols (proanthocyanidines) et des anthocyanes (glycosylées, acétylées et coumaroylées) (Louli et al. 2004). Plusieurs études ont démontré leurs richesses en antioxydants phénoliques qui pourraient être incluses dans les formulations nutraceutiques et compléments alimentaires dû à leurs effets bénéfiques tel que les maladies cardiovasculaires, le cancer et les maladies dégénératives (Huang et al. 2005)

En effet, l'hypertension artérielle (HTA) constitue l'un des problèmes majeurs de santé publique dans les pays développés et émergents. Il s'agit de la plus fréquente des affections cardio-vasculaires. Chez les personnes de 20 ans, le pourcentage d'hypertendus est très faible mais augmente ensuite régulièrement pour atteindre 40 % à 65 ans et 90 % à 85 ans. Selon l'OMS, le nombre d'individus hypertendus est en perpétuelle croissance et le nombre de décès dû aux maladies cardiovasculaires est estimé à 23 millions d'ici 2030. D'après une étude épidémiologique Mona Lisa établie entre 2005 et 2007 en France (Wagner et al. 2011), il a été démontré que 47% des hommes et 35% des femmes de 35 à 74 ans sont concernés par l'HTA qui se définit par une pression artérielle systolique supérieure à 140 mm Hg et/ou une pression diastolique supérieure à 90 mm Hg.

Suite à cette observation, la société Perrin&Fils, propriétaire du château de Beaucastel, situé en Vallée du Rhône dans l'appellation Châteauneuf-du-Pape est désireuse de valoriser les sous-produits de marc de raisin issus de l'élaboration de leur vins. L'objectif principal est

de procéder au développement d'extraits phénoliques de marcs issus des différents cépages caractéristiques de la Vallée du Rhône en tant que complément alimentaire préventif de l'hypertension artérielle. Un deuxième volet réside dans la mise en évidence de l'effet des extraits phénoliques de marcs issu de différents cépages en association avec le vérapamil en tant qu'extrait de raisin à portée thérapeutique agissant comme « activateur » du vérapamil dans la lutte contre l'hypertension artérielle.

Le premier chapitre de ce mémoire sera consacré à l'étude bibliographique. Les différentes classes de composés phénoliques seront présentées. La biodisponibilité des polyphénols, les métabolites générés ainsi que leurs effets potentiels sur la santé seront discutés. La présence de polyphénols dans les raisins et les vins seront aussi abordée dans cette étude bibliographique. L'état des connaissances actuelles concernant l'hypertension sera aussi étudiée.

Le deuxième chapitre traite des matériels et méthodes utilisés pour la caractérisation des raisins et des marcs de raisins ainsi que pour les expériences *in vivo* évaluant le potentiel et la biodisponibilité des extraits de marcs sur modèle de rat SHR.

Le troisième chapitre portera sur la caractérisation chimique des extraits phénoliques des pépins et pellicules des raisins et de leurs marcs respectifs ainsi qu'à l'évaluation de leurs activités antioxydantes. Dans un premier temps, le contenu en polyphénols des extraits a été dosé de façon globale (composés phénoliques totaux, tanins totaux et anthocyanes totales) et individuelles par HPLC-UV-Fluo-MS (tanins monomériques et condensés, degrés de polymérisation, anthocyanes glycosylées, acétylées et coumaroylées). Par la suite, l'activité antioxydante a été appréciée en utilisant quatre tests différents d'activités antioxydantes : $ABTS^{\bullet+}$, DPPH, FRAP et ORAC. Les ratios entre la quantité de polyphénols issus des raisins et la quantité finale retrouvée dans les marcs ont été estimés et la corrélation entre le contenu en composés phénoliques et l'activité antioxydante a été établie. Ce travail a été effectué à l'Institut des Sciences de la Vigne et du Vin (ISVV), Université Bordeaux 2 Victor Segalen, sous la direction du Pr. Pierre-Louis TEISSEDRE.

Le quatrième chapitre est dédié aux résultats des études *in vivo* sur un modèle de rat hypertendus (SHR, Spontaneously Hypertensive Rat). Différents groupes expérimentaux ont été mis en place et les extraits de pépins et pellicules de raisins et de marcs ont été administrés seuls ou en association avec le vérapamil aux SHR. La pression artérielle a été suivie et l'effet des extraits sur l'hypertension a été apprécié. Trois extraits ont été choisis afin d'étudier leur

biodisponibilité. Les urines, fèces, plasmas et organes (foie, reins et cœur) ont été récoltés. Les métabolites ont été identifiés et quantifiés par HPLC-UV-MS^n et GC-MS. Les expériences *in vivo* ont été effectuées en collaboration avec le Centre de pharmacologie et d'Innovation dans le Diabète (CPID), Université Montpellier I sous la direction du Pr. Gérard CROS. Les études de la biodisponibilité des polyphénols avec l'analyse de leurs métabolites ont été effectuées en collaboration avec l'Université de Glasgow dans le groupe « Plant products and Human Nutrition » sous la direction du Pr. Alan CROZIER.

Chapter 1:

Literature review

I. Phenolic compounds and classification

Phenolic compounds or (poly)phenols are the most abundant and ubiquitous secondary metabolites present in the plant kingdom with more than 8000 phenolic structures currently known. These compounds play an important role in plant growth and reproduction, providing protection against biotic and abiotic stress such as pathogen and insect attack, UV radiation and wounding (Weisshaar et al. 1998; Winkel-Shirley 2002). (Poly)phenols are widely distributed in the human diet mainly in plant-derived food and beverages (fruits, vegetables, nuts, seeds, herbs, spices, tea and red wine) and can influence multiple sensorial properties such as flavor and color, and contribute to the aroma and taste e.g., astringency and bitterness (Bautista-Ortín et al. 2013). Indeed, dietary intake of (poly)phenols has been estimated to be about 1g/day (Scalbert et al. 2000). Their intake is 10 times greater than that of vitamin C and 100 times that of vitamin E or the carotenoids (Scalbert et al. 2005). As a result, phenolic compounds are currently receiving much attention because of their beneficial health effect related to their antioxidant (Halliwell 1994; Landrault et al. 2001; Dröge 2002), anti-inflamatory (Delmas et al. 2005; Castilla et al. 2006), cardio-protective (Stoclet et al. 2004; Zern et al. 2005), cancer chemo-preventive (Castillo-Pichardo et al. 2009; Nichols et al. 2010) and neuro-protective properties (Halliwell 2001; Shukitt-Hale et al. 2006; Aquilano et al. 2008).

In the oenological field, a considerable number of studies on grape and wine phenolics have been published because of their complexity and importance in wine quality. Recently and from the clue of the "French paradox", interest are focused on the bioactive phenolic compounds in grape and wine and their role in beneficial health effects. The French paradox was based on epidemiological studies and showed a lower incidence of coronary heart disease in France despite high levels of saturated fat in the traditional French diet. A moderate daily consumption of red wine has been proposed to be responsible for this effect (Renaud et al. 1992). Another epidemiological study has revealed that individuals who habitually drink moderate amounts of wine experience a reduction in all-cause mortality, particularly those link to cardiovascular mortality, when compared with individuals who abstain or drink alcohol in excess (Morten et al. 1995; German et al. 2000). Some investigators believe that (poly)phenol therapeutic effects may actually be enhanced in wine, perhaps due to additive effects with the alcohol component of wine and/or to an increased bioavailability of wine

(poly)phenols as a result of the fermentation process. However, some authors argue that the average moderate wine drinkers are more likely to exercise, to be health conscious and to be of higher educational and socioeconomic class (Lindberg et al. 2008). Beside wines, there is also a large body of evidence that supports the health benefits derived from grapes, grape juices, grape seed/skin extracts and even grape by-products (Fitzpatrick et al. 1993; Bagchi et al. 2000; Castilla et al. 2006; Pérez-Jiménez et al. 2008). Although the chemical constituents of grapes and wines vary to some degree, similar therapeutic effects have been reported.

(Poly)phenols vary structurally from simple molecules such as phenolic acids with a C_6 ring structure to highly polymerized compounds such as tannins. Originating from the shikimic pathway and sharing at least one aromatic ring structure with one or more hydroxyl groups, phenolic compounds represent a large and diverse group of molecules including two main families: the flavonoids based on a common C_6-C_3-C_6 skeleton and the non-flavonoids. The majority of (poly)phenols have a sugar residue linked to the carbon skeleton. Glucose is a common sugar residue including different monosaccharides, disaccharides, or oligosaccharides. Other compounds including amines, organic acids, carboxylic acids, lipids, and other (poly)phenols may also be linked to the basic (poly)phenolic structure (Bravo 1998). Phenolic compounds can be classified by their sources of origin, biological function and chemical structure. To simplify, classification will be done according to the number and arrangement of their carbon atoms (Crozier et al. 2006) (Figure1).

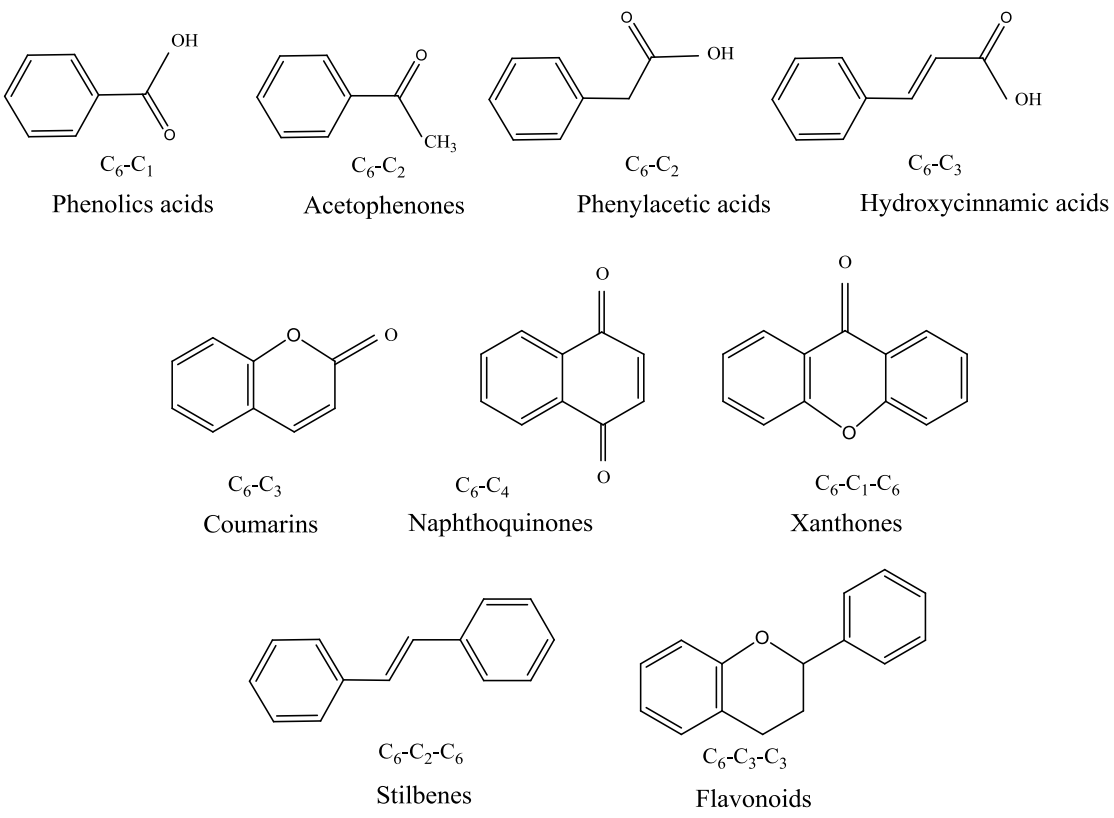


Figure 1: Structural skeleton of phenolic and (poly)phenolic compounds

I.1. Flavonoids

Flavonoids are the major constituents with more than 4000 compounds having been characterized (Bravo 1998; Harborne et al. 2000). They share a common flavan core consisting of fifteen carbons with 2 aromatic rings (A and B), bounded by 3 carbon atoms which form an oxygenated heterocycle (ring C) (Figure 2).

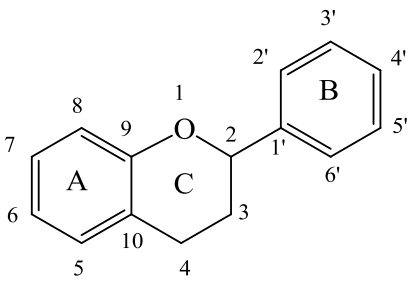


Figure 2: Flavonoid ring structure and numbering system

The flavonoid skeleton C_6-C_3-C_6 can have numerous substituents and occurs naturally as glyscosides rather than aglycones. Due to differences in the oxidation state and substitution on ring C, flavonoids can be further divided into different sub-groups comprising flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanins (Figure 3).

In grapes, flavonoid compounds constitute a significant portion of the phenolic material. Concentration of phenolic compounds differs extensively according to grape varieties, locations, viticultural and oenological practices and also the age of the wine (Makris et al. 2006).

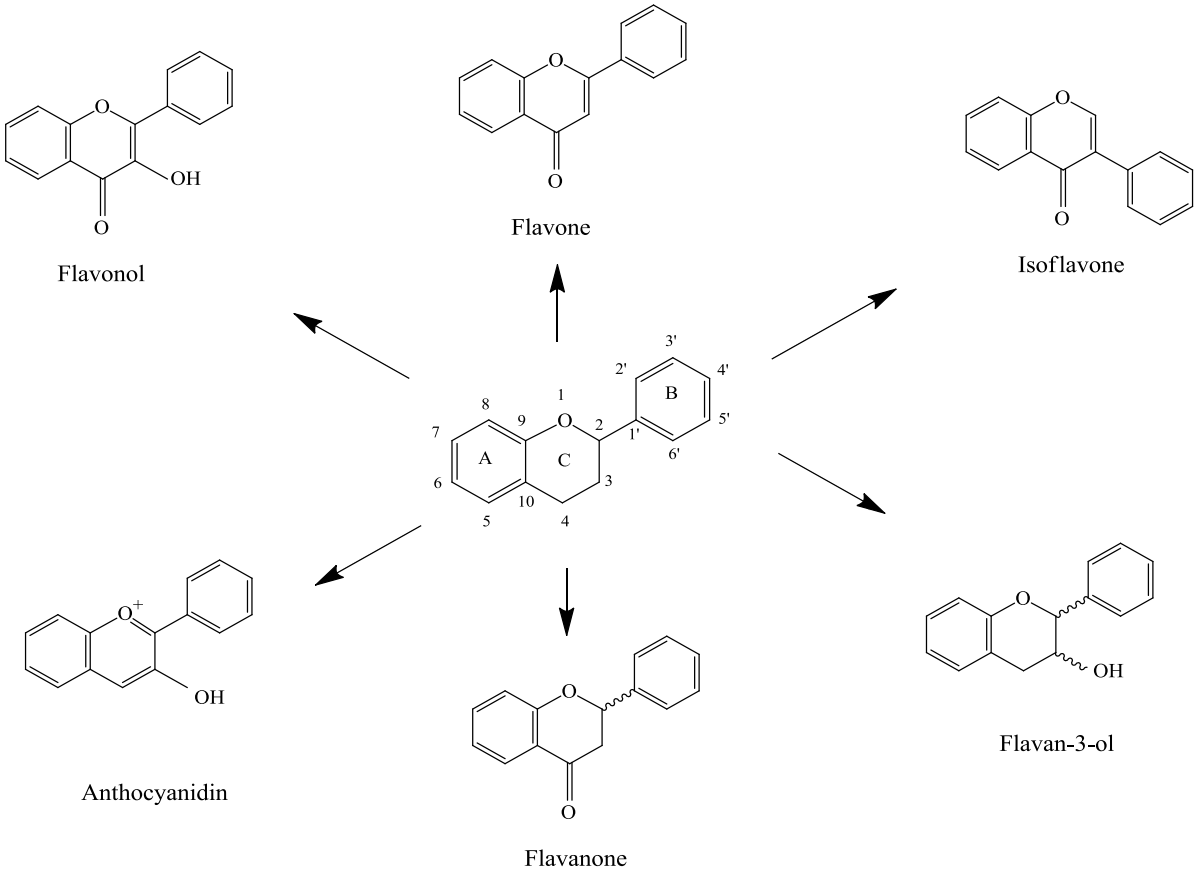


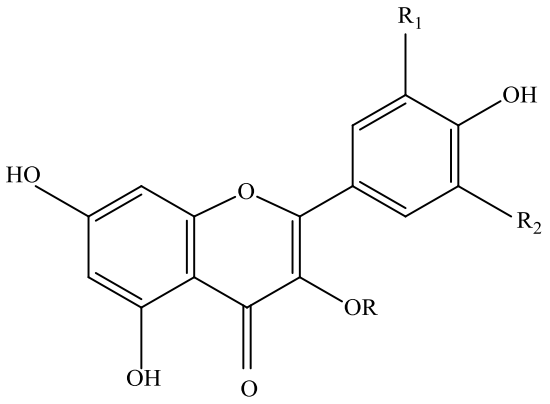
Figure 3: Structure of the flavonoid skeleton

I.1.1. Flavonols

Flavonols are the most widespread throughout the plant kingdom with the exception of algae and fungi. They can be found accumulated in the outer and aerial tissues (skins and leaves) with their biosynthesis being stimulated by light. Noticeable differences in concentration exist, depending on exposure to sunlight. Seasonal changes, exposure to

sunlight and varietal differences contribute to noticeable differences in flavonols concentration within fruits from the same tree and even between different sides of the same fruit (Hertog et al. 1992; Hertog et al. 1993b; Price et al. 1995; Crozier et al. 1997). As an example, Crozier et al (1997) found that cherry tomatoes have markedly higher flavonol content than either the traditional or beef varieties, with the Spanish-grown cherry tomatoes having higher content than the English one. Sizeable variations in flavonol content were also observed for lettuce with the commonly consumed small "round" lettuce which contained only 11 µg/g fresh weight compared to the outer leaves of "Lollo Rosso", a red variety of lettuce which contained 911 µg/g fresh weight.

The main representatives are quercetin, kaempferol, myricetin and isorhamnetin (Figure 4). They are characterized by the presence of a double bond between atoms C_2 and C_3 and a hydroxyl group in C_3. They are typically found as *O*-glycosides with conjugation occurring at the 5, 7, 3', 4' and 5' positions. The associated sugar moiety is very often glucose or rhamnose, but other sugars may also be involved such as galactose, arabinose, xylose and glucuronic acid. The 3-hydroxyl group can be glycosylated as well. Indeed, the most common flavonol aglycones, quercetin and kaempferol, alone have at least 279 and 347 different glycosidic combination respectively (Valant-Vetschera et al. 2005; Williams 2005; Tsao et al. 2009).



R = glucose; glucuronic acid

Kaempferol	$R_1 = H; R_2 = H$
Quercetin	$R_1 = OH; R_2 = H$
Myricetin	$R_1 = OH; R_2 = OH$
Isorhamnetin	$R_1 = H; R_2 = OCH_3$
Laricitrin	$R_1 = OH; R_2 = OCH_3$
Syringetin	$R_1 = OCH_3; R_2 = OCH_3$

Figure 4: The principal flavonols of grapes

In *Vitis vinifera* grapes, flavonols are yellow pigments mainly located in the vacuoles of the epidermal tissues. The principal are quercetin, kaempferol, and myricetin. They are primarily present in glycoside forms such as glucoside and glucuronide. Recently, also

isorhamnetin, laricitrin and syringetin were identified in grapes (Ribéreau-Gayon et al. 2006; Castillo-Muñoz et al. 2007; Jeffery et al. 2008; Hsu et al. 2009) (Figure 4). A recent study have reported for the first time the presence of quercetin-glucuronide-glucoside and malylonated kaempferol-glucoside based on their MS^n fragmentation profile in Sercial and Tinta Negra grape skins (white and red varieties respectively) (Perestrelo et al. 2012). In red wines, the concentration of flavonols ranges from approximately 50 to 200 mg/L and is primarily presented as quercetin glycosides. Levels in white wines, where fermentation takes place without grape solids, ranged from 1 to 3 mg/L according to grape varieties. Only quercetin, kaempferol and isorhamnetin have been detected. The flavonoids myricetin, laricitrin and syringetin were missing in all white varieties. This is due to the absence of the enzyme flavonoid 3′,5′-hydroxylase expression in white grape varieties (Mattivi et al. 2006; Jeffery et al. 2008).

I.1.2. Flavones

Flavones are much less common than flavonols in fruits and vegetables. The only important edible sources of flavones detected are celery (*Apium graveolens*), parsley (*Petroselinum hortense*) and some herbs. Apigenin and luteolin are the most widespread flavone aglycones. They structure are similar structurally to flavanols with a double bond between C_2 and C_3, but lack a hydroxyl group at C_3 (Figure 5).

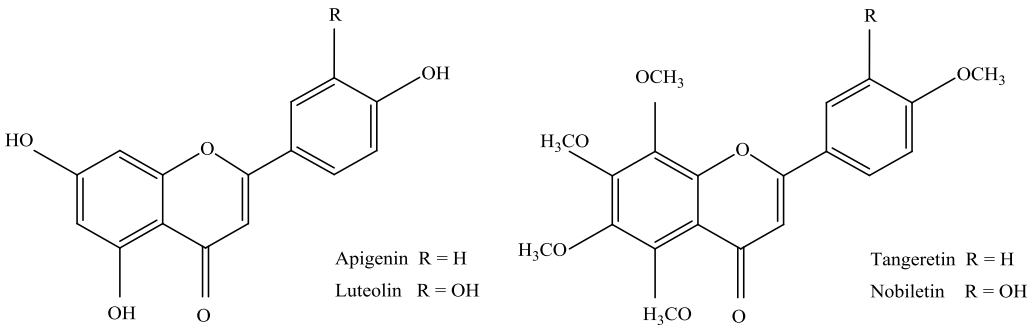


Figure 5: The flavone aglycones apigenin, luteolin and the polymethoxylated flavones tangeretin and nobiletin structure

A wide range of substitution including hydroxylation, methylation, *O*- and C-alkylation and glycosylation is possible. Glycosylation occur mostly at position C_5 and C_7 and methylation and acylation on hydroxyl groups of ring B. Polymethoxylated flavones such as tangeritin and nobiletin are found in citrus species.

In the leaves of *Vitis vinifera*, apigenin-8-*C*-glucoside, luteolin, as well as the 7-*O*-glucosides of apigenin and luteolin have been identified (Monagas et al. 2005). Data on grape flavones are scarce and there are not presented in significant amounts except for luteolin (Alarcón-Flores et al. 2013).

I.1.3. Isoflavones

Isoflavones are found almost exclusively within the leguminous plants with substantial quantities of daidzein and genistein in soybean. Isoflavones are isomers of flavones, displaying the aromatic ring B in the C_3 position rather than the C_2 position (Figure 6). They occur predominantly as β-glucosides or as acetyl-β-glucosides and malonyl-β-glucosides and are therefore polar, water soluble compounds. Isoflavones are structurally similar to estrogens. Although they are not steroids, they have hydroxyl groups in C_7 and $C_{4'}$ position in a configuration analogous to estrogens. This confers pseudo-hormonal properties and they are consequently classified as phytoestrogens.

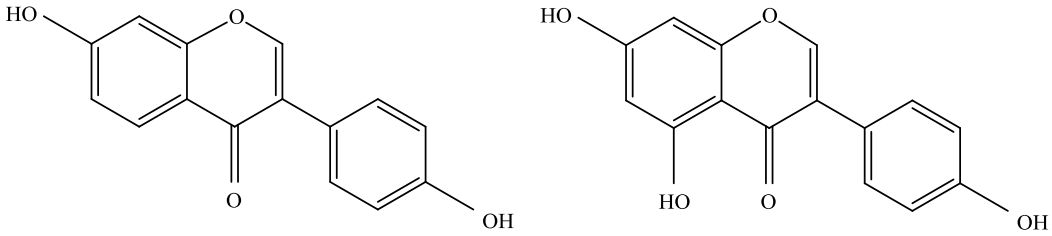


Figure 6: The isoflavone aglycones daidzein and genistein structure

A recent publication identified the presence of genistein and daidzein in grapes by a dehydration technique, an approach used to make dry and sweet wine in which the majority of phenolic compounds are concentrated. A control of temperature and weight loss intensity during the postharvest dehydration can help to increase the concentration of isoflavones especially for the daidzein which rose from 150 µg/kg DW to 1434 µg/kg DW in Grechetto white wine grape variety (De Sanctis et al. 2012).

I.1.4. Flavanones

Flavanones are found in tomatoes and certain aromatic plants such as mint but they are present in high concentrations only in citrus fruit (Manach et al. 2003). The structural features

of flavanones are basically the same as those of flavones. They are characterized by the absence of $\Delta^{2,3}$ double bond and the presence of a chiral center at C_2 (Figure 7).

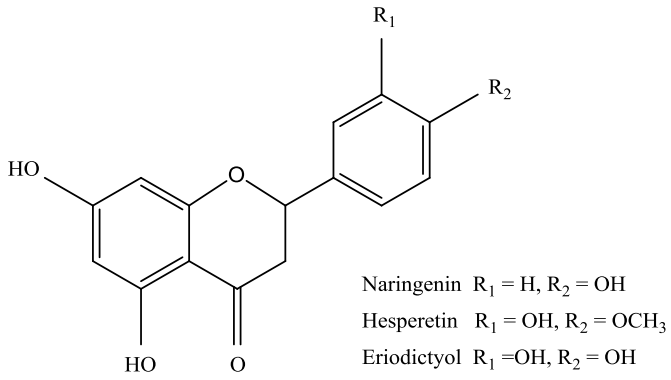


Figure 7: The flavanone aglycones naringenin, hesperetin and eriodictyol

In *planta*, flavanones occur predominantly as the *S*- or (–)-enantiomer with the C-ring attached to the B-ring at C_2 in the α-configuration (Crozier et al. 2009). Flavanones can be hydroxylated, methylated and/or glycosylated. Representative aglycones of this group are naringenin in grapefruits, hesperetin in oranges and eriodictyol in lemons. The most common flavanone glycosides are hesperetin-7-*O*-rutinoside (hesperidin) and narigenin-7-*O*-rutinoside (narirutin) in citrus peel. Flavanone rutinosides are tasteless. In contrast, flavanone neohesperidoside conjugates such as hesperetin-7-*O*-neohesperidoside (neohesperidin) from bitter orange (*Citrus aurantium*) and naringenin-7-*O*-neohesperidoside (naringin) from grapefruit peel (*Citrus paradisi*) are intensely bitter.

Flavanones are present in very low quantities in grapes and wine. Only 0.05 mg/100mL of wine were found for hesperetin and 0.75 mg/100mL of wine for naringin (Jandera et al. 2005). However, recent advances in equipment performance allowed Perestrelo et al. (2012) to identify in Sercial and Tinta Negra *V.vinifera* grape skins, the presence of naringenin, a naringenin hexose derivative and an eriodictyol-glucoside based on their MS^n fragmentation.

I.1.5. Flavanonols

Flavanonols can be considered as flavanones with a hydroxyl group on C_3 position. There are often referred to as dihydroflavonols (Figure 8). A well-known flavanonol is taxifolin or dihydroquercetin in citrus fruit.

Among the flavanonols found in the *Vitis vinifera* grapes, astilbin (dihydroquercetin-3-*O*-rhamnoside) and engeletin (dihydrokaempferol-3-*O*-rhamnoside) were firstly identified in skins and in wines from white grapes by Trousdale and Singleton (1983). Astilbin has also been found in grape pomaces (Lu et al. 1999), in grape stems (Souquet et al. 2000), and in red wines (Vitrac et al. 2001; Landrault et al. 2002). Both astilbin and engeletin have also been found in white wines (Baderschneider et al. 2001; Chamkha et al. 2003). Other flavanonols such as dihydromyricetin-3-*O*-rhamnoside, dihydrokaempferol, dihydroquercetin, dihydrokaempferol-3-*O*-glucoside, dihydroquercetin-3-*O*-glucoside, dihydroquercetin-3_-*O*-glucoside, and dihydroquercertin-3-*O*-xyloside have been reported in red and white wines (Baderschneider et al. 2001; Vitrac et al. 2001). Taxifolin-pentoside and taxifolin-deoxy hexoside have recently been evidenced (Perestrelo et al. 2012; Flamini 2013).

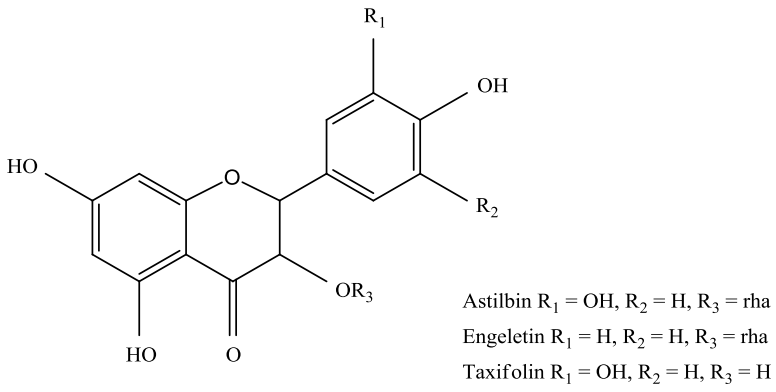
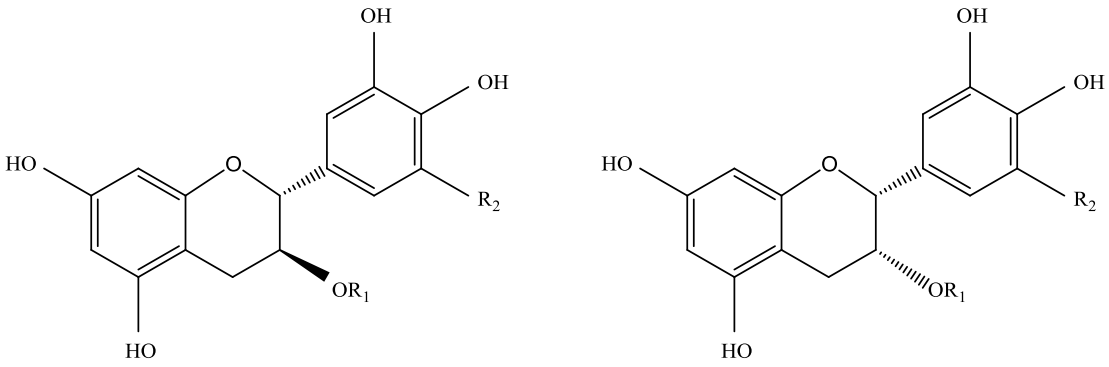


Figure 8: Flavanonols astilbin, engeletin and taxifolin structure

I.1.6. Flavan-3-ols

Flavan-3-ols are the most complex subclass of flavonoids ranging from monomeric form to the oligomeric and polymeric forms. Unlike flavones, flavonols, isoflavones and anthocyanidins, which are planar molecules, flavan-3-ols, proanthocyanidins and flavanones have a saturated C_3 element in the heterocyclic C-ring, and thus non-planar. The two chiral centres at C_2 and C_3 of the monomeric flavan-3-ol produce four isomers for each level of B-ring hydroxylation. (+)-catechin and (−)-epicatechin are widespread in nature whereas (−)-catechin and (+)-epicatechin are comparatively rare (Aron et al. 2008; Crozier et al. 2009) (Figure 9).



(+)-Catechin: $R_1 = R_2 = H$
(+)-Catechin gallate: R_1 = gallyl, $R_2 = H$
(+)-Gallocatechin: $R_1 = H$, $R_2 = OH$
(+)-Gallocatechin gallate: R_1 = gallyl, $R_2 = OH$

(-)-Epicatechin: $R_1 = R_2 = H$
(-)-Epicatechin gallate: R_1 = gallyl, $R_2 = H$
(-)-Epigallocatechin: $R_1 = H$, $R_2 = OH$
(-)-Epigallocatechin gallate: R_1 = gallyl, $R_2 = OH$

Figure 9: Flavan-3-ols structure

Another important feature of the flavan-3-ols is their ability to form polymers, which are often referred to as procyanidins, proanthocyanidins or even condensed tannins as opposed to hydrolysable tannins (gallotannins and ellagitannins). Oligomeric and polymeric proanthocyanidins have an additional chiral center at C_4 in the upper and lower units and their structures vary in the nature of their constitutive sub-units, mean degree of polymerization (mDP) and linkage position. Proanthocyanidins usually contain up to 50 units of monomeric flavan-3-ol with a molecular weight between 500 and 3000 Da (Chung et al. 1998) and the polymerization most often occurs via a carbon-carbon bond. Type B proanthocyanidins are dimers resulting from the condensation of two units of flavan-3-ols linked by a C_4-C_8 or C_4-C_6 bond (Figure 10). The four most common B-type proanthocyanidin dimers are the B_1, B_2, B_3 and B_4. Type A proanthocyanidins are dimers that, in addition to the C_4-C_8 or C_4-C_6 interflavan bond, also have an ether bond between the C_5 or C_7 carbons of the upper units. Two main groups of proanthocyanidins are distinguished depending on the nature of the liberated anthocyanidin in acidic conditions. Procyanidins which are composed of (+)-catechin and (-)-epicatechin releases cyanidins and prodelphinidins and those composed of (+)-gallocatechin and (-)-epigallocatechin liberates delphinidins.

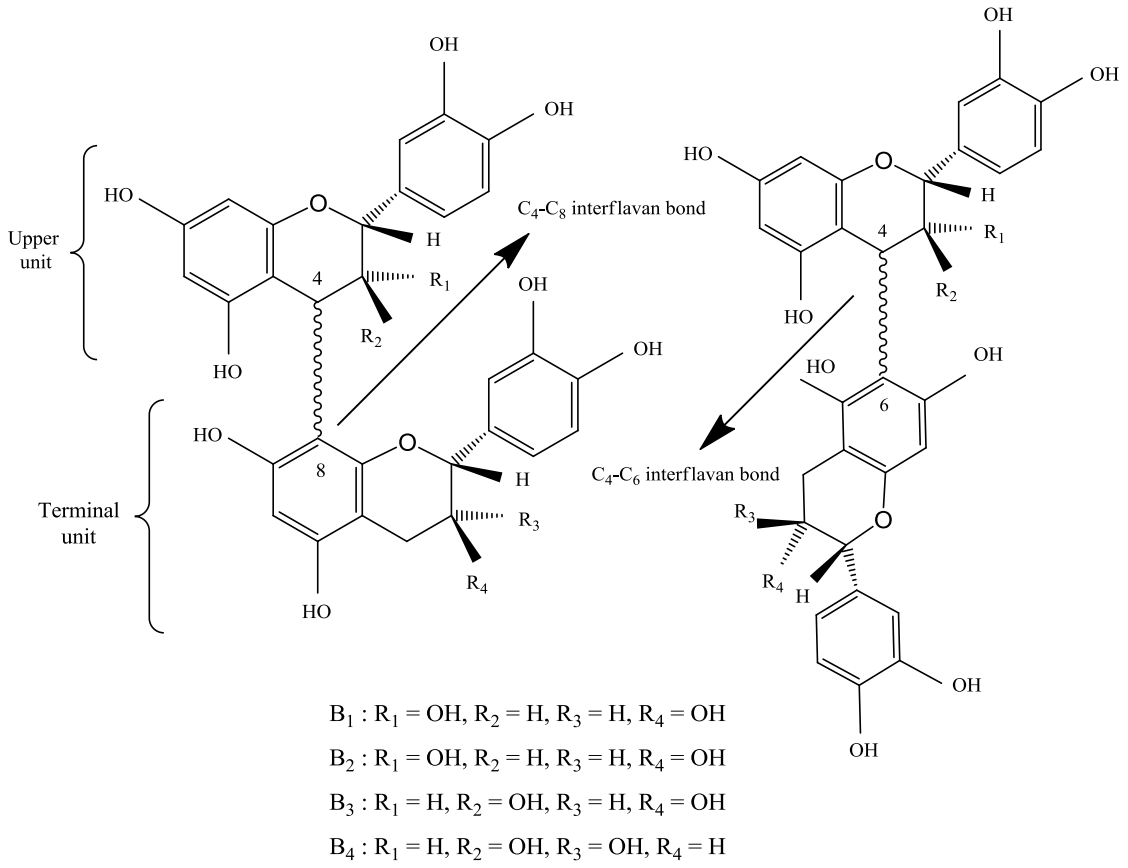


Figure 10: Type-B procyanidin structures and C_4-C_8/C_4-C_6 interflavan bonds

The content of proanthocyanidins in grape berries depends on climatic and geographical factors, cultural practices and stages of ripeness. They are mainly localize in the solid part of grape bunches (skins, seeds, stems). In the berry skins, they are found as a free form in the vacuolar sap or bound to proteins and polysaccharides in the internal face of the tonoplast and to the cell wall respectively (Amrani et al. 1994). Grape seeds are composed by an outer integument including a soft outer parenchymatous layer covered by a cuticle and an inner layer of hard, lignified, brown cells (hard, brown "hull"). The endosperm constitutes the white soft tissue inside the inner integument. The majority of both monomeric and polymeric flavan-3-ols are localized in the outer seed coat with 2 to 5 times more than the endosperm which is mainly constituted of monomeric flavan-3-ols. A considerable amount of proanthocyanidins can also be found in seed hull (Thorngate et al. 1994).

The flavan-3-ol monomeric units found in grapes are (+)-catechin, (–)-epicatechin, (+)-gallocatechin and (–)-epigallocatechin (Tsai Su et al. 1969). Moreover, (–)-epicatechin-3-*O*-gallate, (+)-gallocatechin, (–)-epigallocatechin, (+)-gallocatechin-3-*O*-gallate have also been identified in grapes (Figure 9).

Grape seed proanthocyanidins comprise only procyanidins whereas grape skin proanthocyanidins include both procyanidins and prodelphinidins (Prieur et al. 1994; Souquet et al. 1996). Skin proanthocyanidins have a higher mean degree of polymerization (mDP) and a lower proportion of galloylated sub-units than seed ones.

During red wine-making, grape proanthocyanidins are extracted from seeds and skins and concentration depend on the original grape profile but also on the extraction and winemaking techniques employed. The concentration of flavan-3-ols in red wines ranges from approximately 500 to 3000 mg/L and in white wines, between 100 to 300 mg/L. Proanthocyanidins are of great importance to sensory red wine quality due to their astringent and bitter properties (Gawel 1998; Peleg et al. 1999) and their role in the long-term colour stability via some chemical reactions with anthocyanins (copigmentation and/or condensation) (Somers 1971; Vivar-Quintana et al. 1999). Molecular size of proanthocyanidins reflected in the mDP affects their relative bitterness and astringency (Robichaud et al. 1990; Gawel 1998; Peleg et al. 1999; Vidal et al. 2003; Cheynier et al. 2006). Indeed, monomers are more bitter than astringent whereas the reverse is true for large molecular weight derivatives.

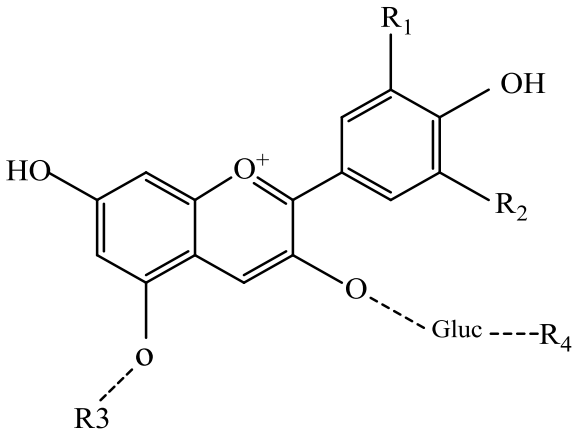
I.1.7. Anthocyanins

Anthocyanins are a unique subgroup of flavonoids that give plants distinctive colors ranging from pink, red, blue to purple pigments. They are involved in the protection of plant from excessive light and also have an important role in attracting pollinating insects. Anthocyanidins have a structure based on the flavylium cation (2-phenylbenzopyrylium). The most common are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. According to pH, they exist in different chemical forms, both colored and uncolored. They show colors from red (in very acidic conditions) to purple-blue (in intermediate pH conditions) and yellow-green (in alkaline conditions). The color can also be affected by acylation or methylation at the hydroxyl groups of ring A and B and glycosylation pattern (sugars or acylated sugars). In their unconjugated form, anthocyanidins are unstable. Degradation is prevented by glycosylation, generally with glucose at C_3 postion of the ring C and esterification with various organic acids such as citric and malic acids. Indeed, glycosylated molecules, generally known as anthocyanins, are stabilized by the formation of complexes with other flavonoids.

In grapes, anthocyanins are only localized in the grape skins except for “teinturier” grape varieties in which they can be found in the flesh (Amrani et al. 1994). Anthocyanin

concentrations in grapes vary not only with the ripening process (Pérez-Magariño et al. 2006) but also depend on climatic (Mori et al. 2007) and water stress condition (Roby et al. 2004; Koundouras et al. 2006), temperature (Mori et al. 2007), input of nutrients and plant growth regulators (Delgado et al. 2004), terroir and grape varieties (Vivas de Gaulejac et al. 2001). Anthocyanins firstly appear approximately three weeks before the first ripening stage in which berries change colour (véraison) (Darné 1991) and concentration increases substantially through full ripeness (maturity stage).

The structures for common anthocyanins in *Vitis vinifera* grapes and wines were determined in 1959 and malvidin-3-*O*-gucoside was found to be the major anthocyanins present along with its acylated form (Ribéreau-Gayon 1959). Moreover, Ribéreau-Gayon also showed that anthocyanins in *V. vinifera* differed in structure than those found in non-*vinifera* species such as *V. riparia* and *V. rupestris*. *Vitis vinifera* contained exclusively monoglucoside anthocyanins whereas non-*vinifera* species have also 3,5-diglucosides. Principal anthocyanins of *Vitis vinifera* are delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) and are present in the berry skin as 3-*O*-monoglucoside, 3-*O*-acetylmonoglucoside and 3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside. The Mv-3-*O*-(6-*O*-caffeoyl)monoglucoside is also often present. More recently, pelargonidin (Pg) 3-*O*-monoglucoside was found in grape (He et al. 2010) (Figure 11).



Pelargonidin $R_1 = H, R_2 = H$
Cyanidin $R_1 = OH, R_2 = H$
Delphinidin $R_1 = OH, R_2 = OH$
Peonidin $R_1 = OCH_3, R_2 = H$
Malvidin $R_1 = OCH_3, R_2 = OCH_3$
Petunidin $R_1 = OCH_3, R_2 = OH$
R_3 = H, glucose
R_4 = H, acetyl, coumaroyl, caffeoyl

Figure 11: The principal monomer anthocyanins of grape

Indeed, among the five principal anthocyanins, malvidin is the dominant molecule in all grape varieties, varying from 90% (Grenache) to less than 50% (Sangiovese) whereas the quantity of acylated monoglucoside is highly variable according to grape variety. The

concentration of anthocyanins varies between 350 and 500 mg/L in red wine from *Vitis vinifera* varieties.

The wine anthocyanins composition depends on the original grape profiles but also on the extraction and winemaking techniques used. Maceration, which allows the diffusion of anthocyanins and other phenolic compounds from the solid part of the grape to the must, can occur before fermentation, as in the case of thermovinification, or during the alcoholic fermentation using crushed (traditional vinification) or whole (carbonic maceration) grapes. After reaching a maximum level after a few days of fermentation, the concentration of anthocyanins decreases as a consequence of their adsorption on yeast cell walls, precipitation in the form of colloidal material together with tartaric salts and elimination during filtration and fining. Hydrolysis reactions, as well as condensation reactions with other phenols during winemaking, also modify the anthocyanin composition of wines.

It has been demonstrated that in acidic or neutral medium, four different anthocyanin structures exist in equilibrium: the flavyluim cation A^+ (red), the quinoidal base AO (blue), the carbinol pseudo-base AOH (colorless), and the chalcone C (pale yellow) (Brouillard et al. 1978) (Figure 12).

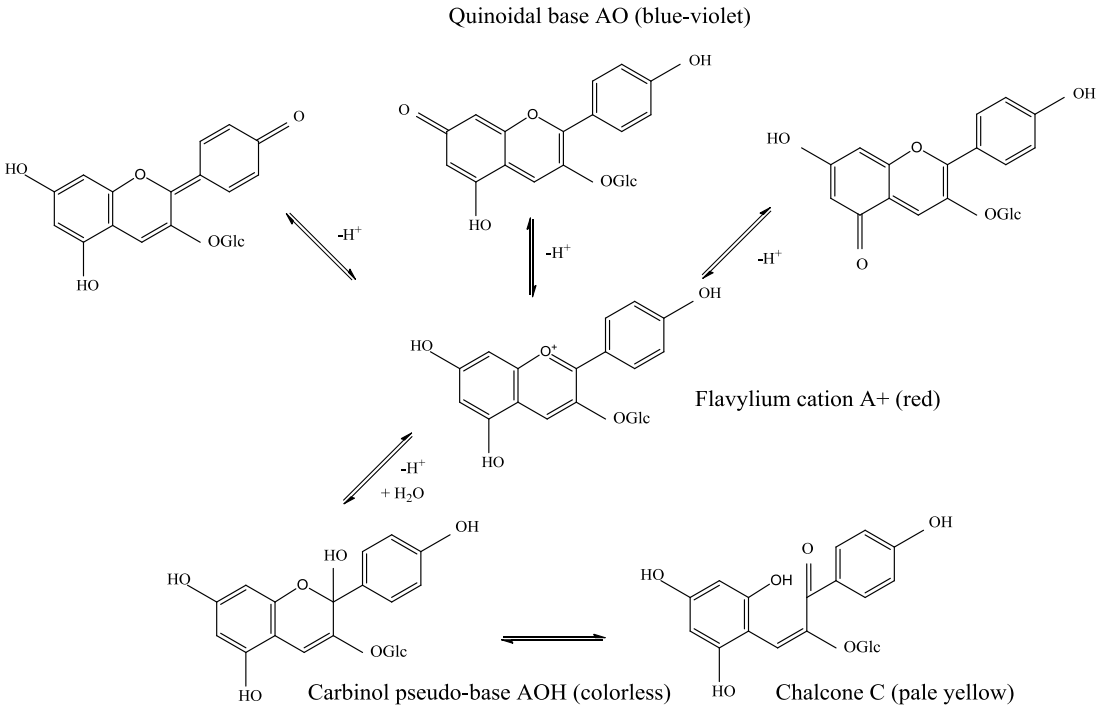


Figure 12: Structural transformation of anthocyanins according to Brouillard et al. (1978)

In a young red wine (pH ~ 3.5) approximately 12.2 % of free anthocyanins are in the red flavylium form, 45.2% in carbinol pseudo-base form, 27.6% in chalcone form and 15.0% in quinoidal base form (Glories 1984) (Figure 13).

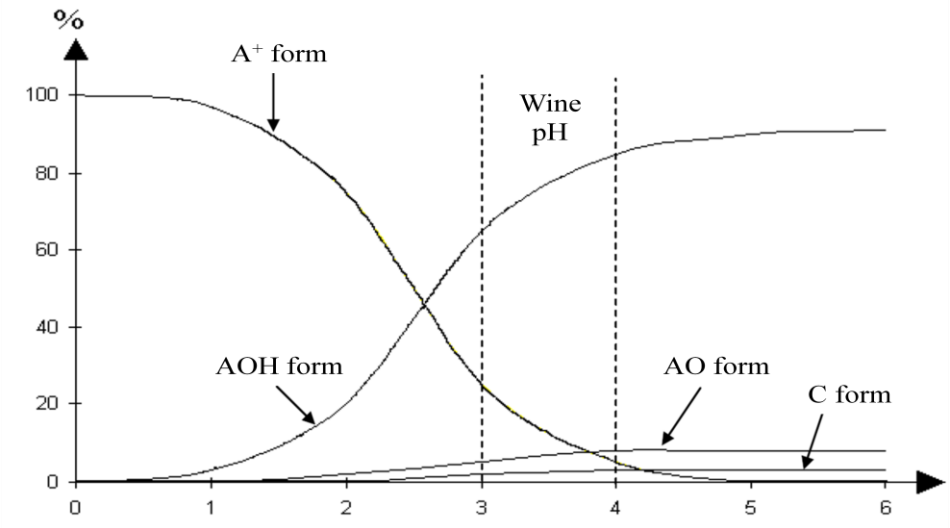


Figure 13: The distribution of the different form of anthocyanins according to pH. A^+, Flavylium form; AOH, carbinol pseudo-form; AO, quinoidal base form; C; chalcone form.

However wine anthocyanins, and thus color, can be stabilized by copigmentation or through their conversion into more stable pigments by different condensation reactions that occur during the winemaking process. Copigmentation consists of the hydrophobic interaction of the colored form of anthocyanins (flavylium cation and quinoidal base) with another molecule or copigment (intermolecular copigmentation) or with an aromatic residue linked to the pigment (intramolecular copigmentation). The complex is stabilized by hydrogen bonding and hydrophobic interaction (Brouillard et al. 1994). This interaction is responsible for a bathochromic shift in absorbance (anthocyanins change from red to more blue) and a hyperchromic effect, in which the intensity of the anthocyanins color is fortified (Robinson et al. 1933; Asen et al. 1975). According to Boulton (2001), copigmentation can account for between 30 to 50% of the color of young wines .

Moreover anthocyanins can undergo reactions such as oxidation, hydrolysis and condensation reactions with other matrix compounds and form new molecules with different chromatic characteristics. As a result, reactions of anthocyanins with monomers, oligomers and polymers of flavan-3-ol shift the wine from purple-red to brick-red hue, and the formation of stable structures (i.e. pyroanthocyanins) formed by reaction between anthocyanins and

acetaldehyde, pyruvic acid, vinylphenols, vinylcatechol, vinylguaiacol or vinyl(epi)catechin shift the wine toward orange hue (Somers 1971; Timberlake et al. 1976; Fulcrand et al. 1998; Hayasaka et al. 2002; Asenstorfer et al. 2003; Alcalde-Eon et al. 2007; Marquez et al. 2012). Indeed, more than hundred structures derivate from these reactions were identified in wines (Vivar-Quintana et al. 1999; Marquez et al. 2012; Flamini 2013).

The direct reaction between free anthocyanins and certain yeast by-products, such as pyruvic acid, acetaldehyde and vinylphenols can lead to the formation of stabilized pigments, the pyranoanthocyanins (Asenstorfer et al. 2003; Marquez et al. 2012). They constitute one of the most important classes of anthocyanin-derived pigments occurring naturally in red wine (Mateus et al. 2001). Resulting from cycloaddition, pyroanthocyanins have an additional pyran ring between the C_4 position of the C ring and the hydroxyl group on the C_5 position of the A ring. Pyranoanthocyanin structures can be formed through the reaction of an anthocyanin molecule with a compound containing a polarizable double-bound. Thus, compared to the free anthocyanins, pyranoanthocyanins have two heteroaromatic rings, and they have a dynamic equilibrium among different flavylium cation forms. These new pigments are mainly formed from grape anthocyanins during the fermentation of must and later during the maturation and aging of red wines (Schwarz et al. 2003; Freitas et al. 2011).

To date, a great number of pyranoanthocyanins have been identified from red wines, especially in the aged red wines, including carboxy-pyranoanthocyanins (A type vitisins), B type vitisins, methylpyranoanthocyanins, hydroxyphenyl-pyranoanthocyanins (pinotins), flavanyl-pyranoanthocyanins, and their second generated pigments, such as flavanyl/phenyl-vinylpyranoanthocyanins (portisins), pyranone-anthocyanins (oxovitisins), pyranoanthocyanin dimers and others (Ribéreau-Gayon et al. 2006; Freitas et al. 2011). Some structures are presented in Figure 14. Such pyranoanthocyanins are highly stable and resistant to sulfur dioxide bleaching and oxidative degradation, therefore they can significantly contribute to the color stability of red wines (Fulcrand et al. 1998; Boido et al. 2006; Freitas et al. 2011). However, most pyranoanthocyanins possess yellow to orange color and contribute to the tawny color shift associated with red wine aging, except for the newly found A type portisins, which are blue (Bakker et al. 1997; Pozo-Bayón et al. 2004; Rentzsch et al. 2007).

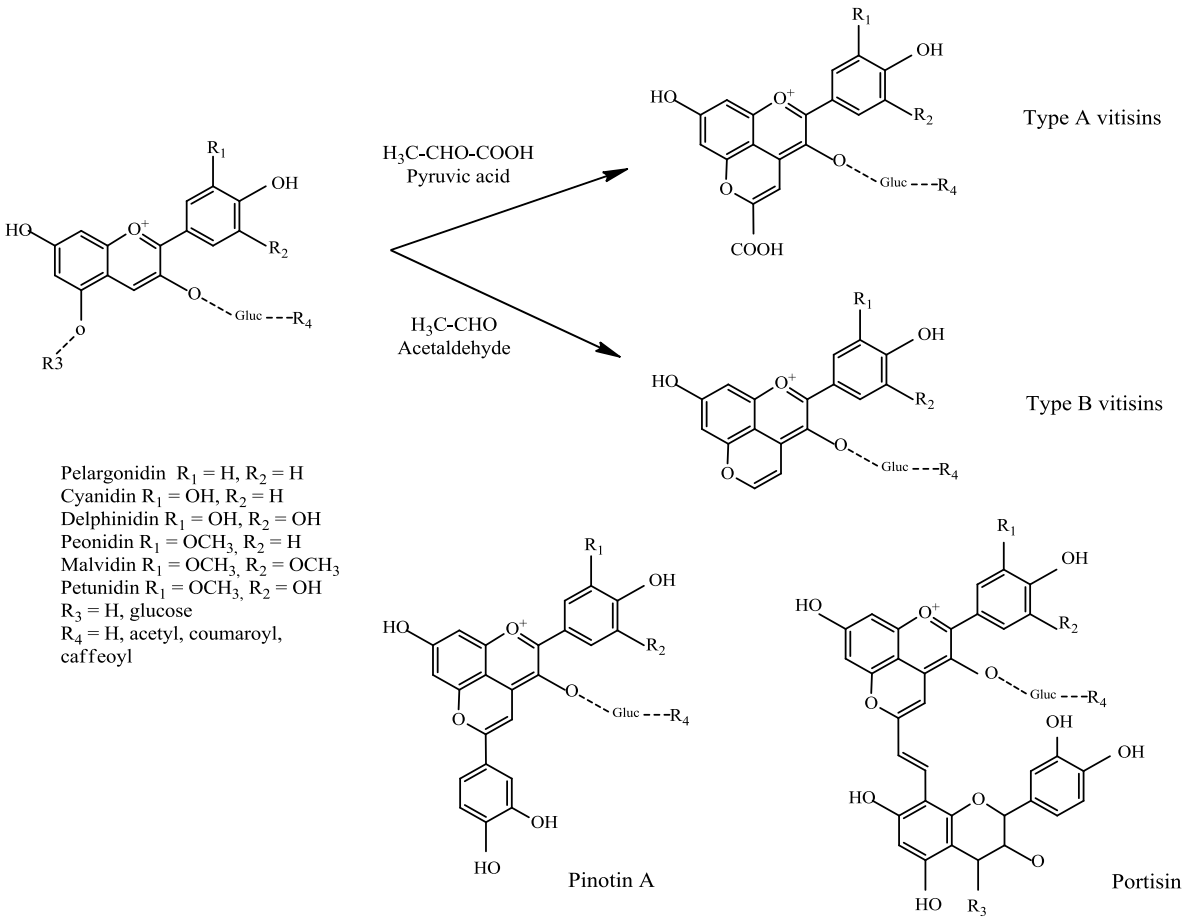


Figure 14: Type A vitisins, type B vitisins, pinotin A and portisin structure

I.2. Non-flavonoids

The main non-flavonoid are the phenolic acids which can be further divided into two main groups, benzoic acid and cinnamic acid derivatives based on C_6-C_1 and C_6-C_3 backbones and the polyphenolic C_6-C_2-C_6 stilbenes (Figure 1). Non-flavonoids are structurally simpler than the flavonoids.

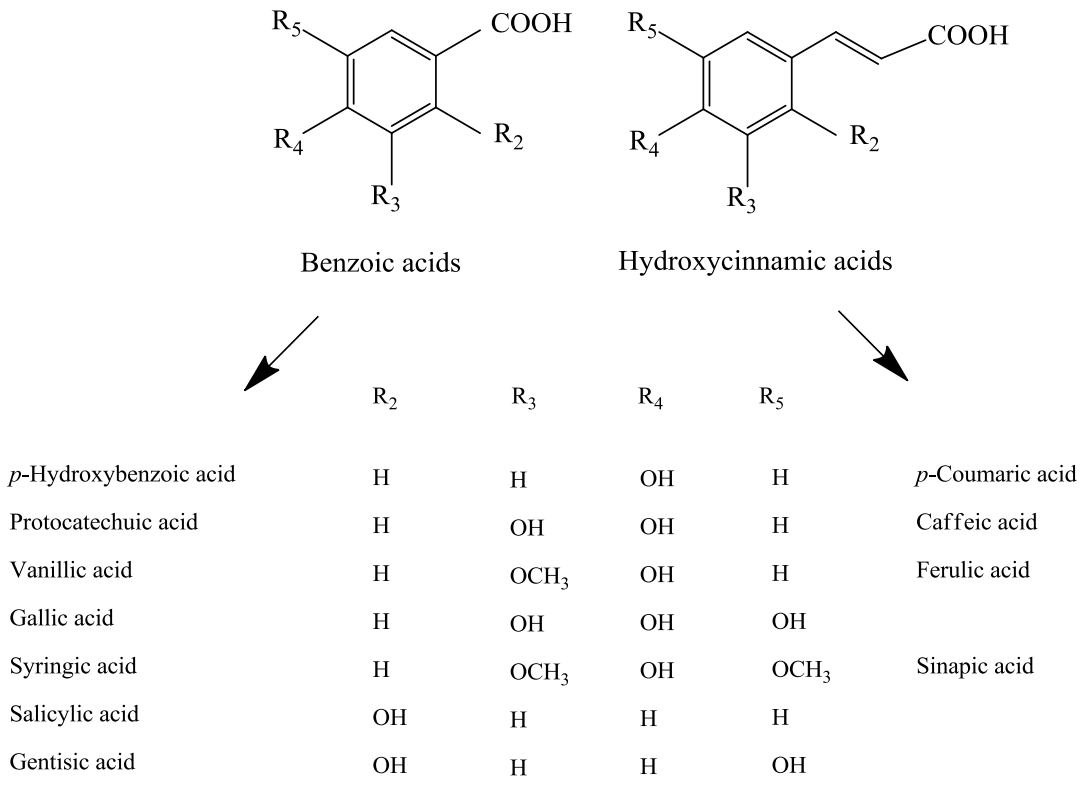
I.2.1. Phenolic acids

Phenolic acids are one of the other main phenolic classes of the plant kingdom. These compounds exist predominantly as hydroxybenzoic and hydroxycinnamic acid derivatives. The average concentration of phenolic acids ranged from 100 to 200 mg/L and 10 to 20 mg/L in red and white wine respectively (Ribéreau-Gayon et al. 2006; Chira et al. 2008).

I.2.1.a. Hydroxybenzoic acids

Hydroxybenzoic acids are characterized by a C_6-C_1 skeleton and the most commonly found is the gallic acid. It can be considered as the most important since it is the precursor of all hydrolyzable tannins. Indeed, gallic acid and hexahydroxydiphenic and its derivative (e.g., ellagic acid) compose the base unit. These acids are usually esterified with D-glucose. These hydrolysable tannins are known as gallotannins (hydrolysable to gallic acid) and ellagitannins (hydrolysable to ellagic acid). Hydrolyzable tannins are not found in *V. vinfera*, but can be found in other fruits such as raspberries or muscadine grapes.

The most common derivatives found in wine are gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, syringic acid, salicylic acid, and vanillic acid (Figure 15). In wine, the different hydroxybenzoic acids can be mainly found in their free form (Salagoïty-Auguste et al. 1984; Fernandez de Simon et al. 1992; Garcia-Viguera et al. 1995; Vanhoenacker et al. 2001; Pozo-Bayón et al. 2003). Gallic acid is found in a high concentration in wine. It not only originates from the grapes itself but gallic acid is also formed by hydrolysis of hydrolyzable and condensed tannins, i.e. the gallic acid esters of flavan-3-ols. Moreover, several hydroxybenzoic acid derivatives have also been identified in red and white wine (Riesling wine) such as ethyl vanillate, ethyl *p*-hydroxybenzoate, ethyl *p*-hydroxyphenylacetate, ethyl protocatechuate, methyl ester of protocatechuic and vanillic acids, glucose ester of vanillic acid, protocatechuic acid-glucoside and *p*-hydroxybenzoyl glucoside (Güntert et al. 1986; Baderschneider et al. 2001; Perestrelo et al. 2012).



	R_2	R_3	R_4	R_5	
p-Hydroxybenzoic acid	H	H	OH	H	*p*-Coumaric acid
Protocatechuic acid	H	OH	OH	H	Caffeic acid
Vanillic acid	H	OCH_3	OH	H	Ferulic acid
Gallic acid	H	OH	OH	OH	
Syringic acid	H	OCH_3	OH	OCH_3	Sinapic acid
Salicylic acid	OH	H	H	H	
Gentisic acid	OH	H	H	OH	

Figure 15: The benzoic acids and hydroxycinamic acids structure

I.2.1.b. Hydroxycinnamic acids

Hydroxycinnamic acids possess a C_6-C_3 structure and formally belong to the group of phenylpropanoids. In grapes, they are located in the vacuoles of the skins and pulp cells in the form of tartaric esters (Ribéreau-Gayon 1965). The compounds present in wine mainly derived from the hydroxycinnamic acids are caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid (Figure 15). These derivatives can be present in *cis*- and *trans*-configured forms, while the *trans* forms are more stable and therefore more prevalent (Singleton et al. 1978).

The concentration levels of hydroxycinnamic acid derivatives in wine depend on many factors like grape variety, growing conditions, climate, etc. Among the hydroxycinnamic acids, caftaric acid predominates with up to 50% of total hydroxycinnamic acids in both red and white grapes and wines from *Vitis vinifera*. Other important substances are the tartaric esters of *p*-coumaric acid and ferulic acid, glucose derivatives of caffeic, sinapic, *trans*-*p*-coumaric, *trans*-ferulic and cinnamic acids (Somers et al. 1987). Other compounds with

phenylpropanoid structure can be found in wine, such as *p*-hydroxyphenylpropionic acid and 2′-hydroxy-3′-phenylpropionic acid (Gómez-Cordovés et al. 1997; Baltenweck-Guyot et al. 2000; Baderschneider et al. 2001) Concentrations of some hydroxycinnamic acids are given in Table 1. Perestrelo et al., (2012) have recently identified but did not quantified novel hydroxycinnamic acids in grapes: the caftaric acid vanilloyl pentoside and the *p*-coumaric acid-erythroside.

I.2.2. Stilbenes

Stilbenes are phytoalexins structurally characterized by a C_6-C_2-C_6 skeleton with microbial inhibitory activity and are produce by plants as a defense response to some exogenous stimuli such as UV radiation, fungal, bacterial and viral pathogen attacks (Langcake et al. 1977; Jeandet et al. 1991; Korhammer et al. 1995).

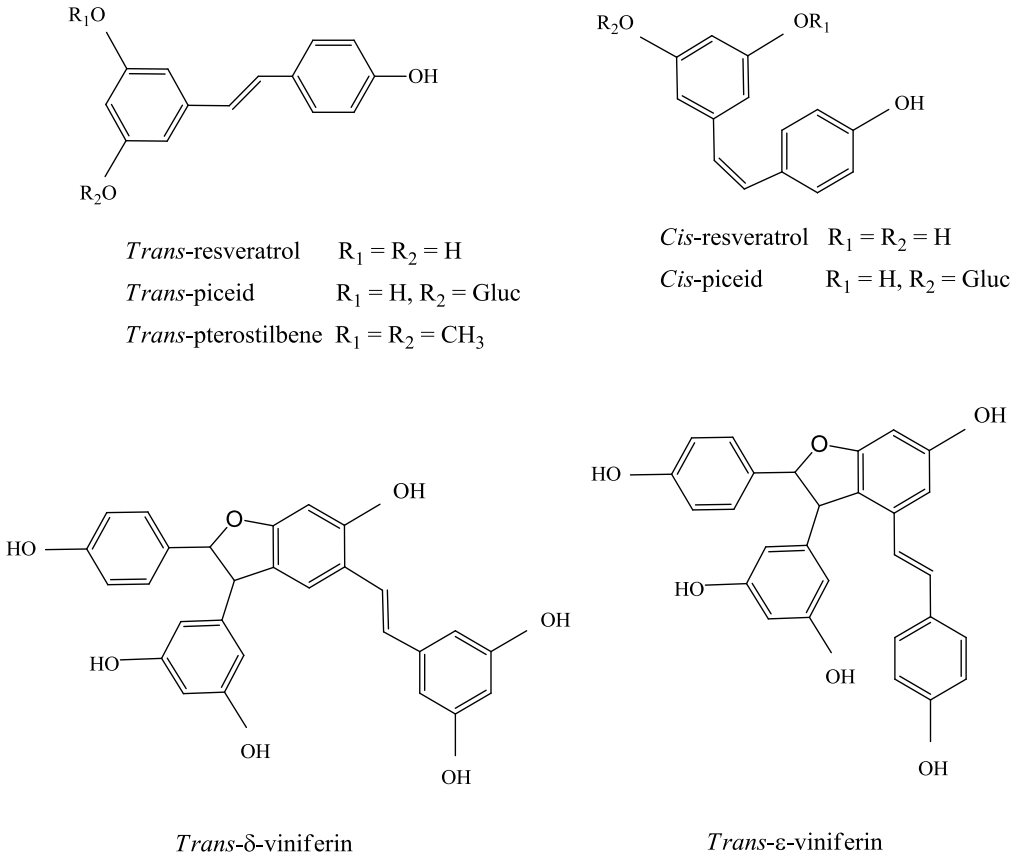


Figure 16: Structures of Stilbenes and their derivatives

One of the most extensively studied stilbene is resveratrol (3,5,4'-trihydroxystilbene) which exists in both *cis* and *trans* forms as well as conjugated derivatives, including *cis* and *trans* piceid (i.e. *trans*-resveratrol-3-*O*-glucoside). *Cis*-resveratrol is known to be produced by UV irradiation of the *trans*-isomer (Goldberg et al. 1995; Jeandet et al. 1995a; Trela et al.

1996). Stilbenes can also occur in oligomeric and polymeric forms, (i.e. viniferins) which are induced by oxidative polymerization of the monomer resveratrol (Chong et al. 2009) (Figure 16).

In grapes, stilbenes (especially the *trans*-resveratrol) are produce by the skin cells and by the leaves in response to *Botrytis cinerea* and other fungal infections on grapevines (Jeandet et al. 1995b) and can be transferred into musts and wines during the winemaking process. Resveratrol is presented in both *cis* and *trans* form in wine, but *trans*-resveratrol predominates and may be further methylated, glycosylated and polymerized (Chong et al. 2009). In recent years, numerous resveratrol derivatives have been reported in wine including the dehydrodimers, *ε*-viniferin (Landrault et al. 2002), *δ*-viniferin (Vitrac et al. 2005), parthenocissin A, quadrangularin A (Chen et al. 2009), and ampelopsin D (Mattivi et al. 2011), the dimer pallidol (Vitrac et al. 2001), *cis*- and *trans*-scirpusin A (Mattivi et al. 2011), the trimer *α*-viniferin (Guebailia et al. 2006) and ε-miyabenol C (Flamini et al. 2013), and the tetramer hopeaphenol (Guebailia et al. 2006). In addition, some oligomeric glucosides like *cis*- and *trans*-*ε*-viniferin diglucosides, *cis*- and *trans*-pallidol-3-*O*-glucoside and pallidol-3,3''-diglucoside have also been detected (Baderschneider et al. 2000). Concentrations of some stilbenes are given in Table 1.

Red wines usually contain higher stilbene concentrations than rosé or white wines because of the more prolonged skin contact of the must during fermentation and the high phenolic content of red grape cultivars (Perrone et al. 2007).The average concentration of resveratrol in red wine is 7 mg/L , 2 mg/L in rosé and 0.5 mg/L in white wine (Romero-Pérez et al. 1996).

I.3. Phenolic compounds in grapes and wines

Grapes (*Vitis vinifera* L.) are considered the world's most prevalent fruit crop and their large amounts of phenolic compounds have been extensively studied. Phenolic compounds reside mainly in the skins and seeds. Figure 17 shows phenolic compounds distribution in grape berry (Kennedy 2008). Polyphenols from grapes are mainly concentrated in seeds (60%) and skins (30%), and to a lower extent in the pulp and stems (less than 10%).

The main polyphenols present in wine are phenolic acids, anthocyanins, flavonols, dihydroflavonols, flavan-3-ol monomers (catechins), flavan-3-ol polymers (proanthocyanidins) and stilbenes. Phenolic acids are largely present in the pulp, anthocyanins

and stilbenes in the skin, and other polyphenols (flavonols, flavan-3-ols and proanthocyanidins) in the skin and the seeds (Chira et al. 2008). The proportion of the different polyphenols in wines will therefore vary according to variety, vineyard location and type of vinification.

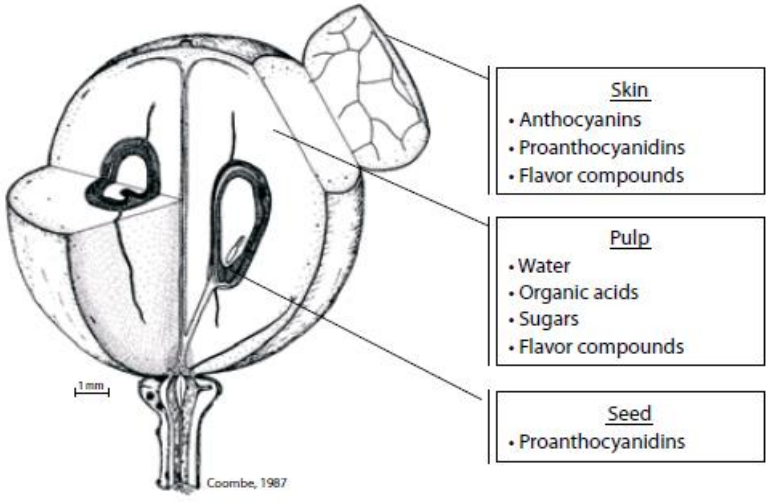


Figure 17: Phenolic compounds distribution in the grape berry

Average total polyphenol contents measured by the Folin-Ciocalteu method is 216 mg/100 ml for red wine and 32 mg/100 ml for white wine (Frankel et al. 1995; Carando et al. 1999b; Teissedre et al. 2000). The content of polyphenols in rosé wine (82 mg/100 ml) is intermediate between red and white wines (Sato et al. 1996; Arnous et al. 2002). Table 1 showed the mean concentration of principal polyphenols obtained by reverse phase HPLC and normal phase for procyanidins.

Table 1: Mean concentration of principal polyphenols in red and white wines. Adapted from the online database Phenol-Explorer (Neveu et al. 2010).

	Red wine (mg/100 mL)			White wine (mg/100mL)		
	Mean content[a]	**Min[a]**	**Max[a]**	**Mean content[a]**	**Min[a]**	**Max[a]**
Anthocyanins						
Cyanidin-3-*O*-glucoside	0.21	0.01	0.9			
Peonidin-3-*O*-glucoside	0.82	0.15	5.97			
Delphinidin-3-*O*-glucoside	1.06	0.17	2.50			
Petunidin-3-*O*-glucoside	1.40	0.26	3.44			
Malvidin-3-*O*-glucoside	9.97	0.00	38.2			
Delphinidin-3-*O*-(6″-acetyl-glucoside)	0.42	0.06	1.24			
Cyanidin-3-*O*-(6″-acetyl-glucoside)	0.08	0.05	0.29			
Petunidin-3-*O*-(6″-acetyl-glucoside)	0.57	0.07	1.58			
Malvidin-3-*O*-(6″-acetyl-glucoside)	3.52	0.48	11.29			
Peonidin-3-*O*-(6″-acetyl-glucoside)	0.47	0.08	1.10			
Malvidin-3-*O*-(6″-p-coumaroyl-glucoside)	1.95	0.58	4.47			
Peonidin-3-*O*-(6″-p-coumaroyl-glucoside)	0.52	0.02	1.02			
Petunidin-3-*O*-(6″-p-coumaroyl-glucoside)	0.39	0.01	1.16			
Delphinidin-3-*O*-(6″-p-coumaroyl-glucoside)	0.18	0.01	0.27			
Malvidin-3-*O*-(6″-caffeoyl-glucoside)	0.18	0.18	0.18			
Vitisin A	0.31	0.15	0.98			
Pinotin A	0.22	0.01	1.79			
Dihydroflavonols						
Dihydroquercetin-3-*O*-rhamnoside	0.97	0.11	1.51	0.27	0.07	1.29
Dihydromyricetin-3-*O*-rhamnoside	4.47	4.47	4.47	0.30	0.30	0.30
Flavanols						
(+)-Catechin	6.81	1.38	39	1.08	0.00	4.60
(–)-Epicatechin	3.78	0.00	16.5	0.95	0.00	6.00
(+)-Gallocatechin	0.08	0.00	0.42	3.33e-03	0.00	0.01
(–)-Epigallocatechin	0.06	0.00	0.28	0.02	0.00	0.13
(–)-Epicatechin-3-*O*-gallate	0.77	0.00	0.93			
Procyanidin dimer B_1	4.14	2.15	14.0	8.43e-03	0.00	0.05
Procyanidin dimer B_2	4.97	0.43	9.00	4.71e-03	0.00	0.03
Procyanidin dimer B_3	9.47	0.00	11.96	2.86e-03	0.00	0.02
Procyanidin dimer B_4	7.29	0.08	11.3	7.14e-03	0.00	0.05
Procyanidin dimer B_7	0.27	0.27	0.27			
Prodelphinidin dimer B_3	0.11	0.11	0.11			
Procyanidin trimer C_1	2.56	0.22	2.63			
Procyanidin trimer T_2	6.71	6.71	6.71			
Flavanols (normal phase)						
Monomers	2.00	2.00	2.00			
Dimers	4.00	4.00	4.00			
Trimers	2.70	2.70	2.70			
Tetra-hexamers	6.70	6.70	6.70			
Hepta-decamers	5.00	5.00	5.00			
Polymers (>10 mers)	11.00	11.0	11.0			
Flavonols						
Kaempferol	0.23	0.00	0.36	0.02	0.00	0.26
Quercetin	0.83	0.00	3.16	0.04	0.00	0.83
Quercetin-3-*O*-glucoside	1.14	0.79	2.32			
Quercetin-3-*O*-rhamnoside	1.15	0.00	1.82			
Quercetin-3-*O*-rutinoside	0.81	0.00	3.17	0.19	0.00	0.92
Quercetin-3-*O*-arabinoside	0.49	0.44	0.54	0.22	0.13	0.32
Myricetin	0.83	0.00	1.79			
Isorhamnetin	0.33	0.006	0.65			
Kaempferol-3-*O*-glucoside	0.79	0.57	1.08			
Isorhamnetin-3-*O*-glucoside	0.26	0.16	0.51			
Hydroxybenzoic acids						
Protocatechuic acid	0.17	0.00	0.96	0.33	0.01	1.30
Gallic acid	3.59	0.00	12.6	0.22	0.00	1.10
Vanillic acid	0.32	0.00	0.75	0.04	0.01	0.12
Gentisic acid	0.46	0.00	0.80	1.82	0.00	2.00
4-Hydroxybenzoic acid	0.55	0.00	2.18	0.02	0.00	0.04
Syringic acid	0.27	0.00	2.33	5.43e-03	0.00	0.02

2-Hydroxybenzoic acid	0.04	0.00	0.09	0.04	0.02	0.10
2,3-Dihydroxybenzoic acid	0.08	0.00	0.64			
Gallic acid ethyl ester	1.53	1.37	1.70			
Hydroxycinnamic acids						
p-Coumaric acid	0.55	0.00	4.00	0.15	0.00	0.49
Caffeic acid	1.88	0.00	7.70	0.24	0.00	0.70
Ferulic acid	0.08	0.00	1.04	0.09	0.03	0.21
Caffeoyl tartaric acid	3.35	0.14	17.94	2.15	2.14	2.20
o-Coumaric acid	0.03	0.02	0.04	0.03	0.00	0.07
Sinapic acid	0.07	0.00	0.54	0.06	0.00	0.28
p-Coumaroyl tartaric acid	1.18	0.21	1.79			
2,5-di-S-Glutathionyl caftaric acid	2.86	1.12	4.71			
Stilbenes						
Trans-Resveratrol	0.18	0.00	1.05	0.03	0	0.08
Trans-Resveratrol-3-*O*-glucoside	0.41	0.00	2.92	0.17	0.00	0.45
Piceatannol	0.58	0.00	2.57			
Cis-Resveratrol	0.13	0.00	2.32	0.02	0.00	0.07
ε-Viniferin	0.15	0.01	0.43	5.57e-03	0.00	0.01
δ-Viniferin	0.64	0.00	2.24			
Cis-Resveratrol-3-*O*-glucoside	0.22	0.00	1.48	0.08	0.00	0.25
Pallidol	0.20	0.00	0.25	6.82e-04	0.00	0.03
Piceatannol-3-*O*-glucoside	0.95	0.63	1.31	0.46	0.14	0.80
Resveratrol	0.27	0.00	2.78	0.04	0.00	0.17
Resveratrol-3-*O*-glucoside	0.62	0.00	4.40	0.25	0.00	0.61
Hydroxybenzaldehydes						
Syringaldehyde	0.66	0.00	4.45			
Protocatechuic aldehyde	0.05	0.00	0.11	0.10	0.10	0.10
Tyrosols						
Tyrosol	3.12	0.59	4.47	0.21	0.11	0.30
Hydroxytyrosol	0.53	0.05	0.96	0.21	0.16	0.27

[a]Min, minimum concentration detected, Max, maximum concentration detected. Data are expressed as mg/100 mL.

II. Phenolic compounds: intake, bioavailability and health effects

II.1. Dietary intake and sources of (poly)phenols

The reference data of dietary (poly)phenols intake was firstly given by Kuhnau (1976) who estimated the intake in the United States at ~ 1 g/day with the following distribution: 16 % of flavonols, flavones and flavanones, 17% of anthocyanins and 20 % of catechins.

Similar to Kuhnau, Scalbert and Williamson (2000) estimated dietary intake of (poly)phenols at 1 g/day in which phenolic acids account for about one third of the total intake and flavonoids for the remaining two third. Their intake is 10 times greater than that of vitamin C (70-100 mg/day) and 100 times that of vitamin E (7-10 mg/day) or the carotenoids (2-3 mg/day) (Hertog et al. 1993a; Scalbert et al. 2005). Hertog et al. (1993a) calculated the intake of flavonols and flavones in the Dutch diet among 4,112 adults and found it to be averagely 23 mg/day with quercetin being the most important flavonoid ingested (16 mg/day). A similar flavonol intake was found in other studies in the United States and Denmark (ca. 20-22 mg/day and 26 mg/day respectively) (Justesen et al. 1997; Sampson et al. 2002). Considering both extractable and non-extractable (poly)phenols, Saura-Calixto et al., (2007) estimated the mean daily intake of (poly)phenols in the Spanish diet to be somewhat larger at 2590–3016 mg/day.

In the framework of the PREDIMED study, using a total of 7200 participants, the mean total (poly)phenol intake was estimated at 820 ± 323 mg/day, in which 443 ± 218 mg/day were flavonoids, 304 ± 156 mg/day phenolic acids and 73 mg belonged to other (poly)phenol groups. Fruits were the main source of (poly)phenols (44% of the total, with more than half of total flavonoids and 23% of total phenolic acids) (Tresserra-Rimbau et al. 2013). In another Spanish investigation, the EPIC study, including 40,683 subjects aged from 35 to 64 years from northern and southern regions of Spain, the mean total flavonoid intakes were 313 mg/day and the most abundant subgroup was proanthocyanidins (60.1%), followed by flavanones (16.9%), flavan-3-ols (10.3%), flavonols (5.9%), anthocyanidins (5.8%), flavones (1.1%), and isoflavones (<0.01%) (Zamora-Ros et al. 2010). In the USA, a study with 8809 subjects over 19 years of age revealed the mean daily total flavonoid intake was

189.7 mg/day. Flavan-3-ols accounted for 83.5% followed by flavanones (7.6%), flavonols (6.8%), anthocyanins (1.6%), flavones (0.8%) and isoflavones (0.6%) (Chun et al. 2007).

The consumption of anthocyanins in the USA was recently estimated at 12.5 mg/day (Wu et al. 2006) compared to early paper published in the 1970s in which an intake of 185-215 mg/day was estimated (Kuhnau 1976) and this figure is still widely quoted. According to Wu et al., these data have limitations due to the lack of comprehensive data on anthocyanin content, daily food intake data and individual anthocyanins. The authors estimated that cyanidin, delphinidin and malvidin contributed for 45%, 21% and 15 % of the total anthocyanins intake, respectively. Non-acylated accounted for 77% compared to 23% of acylated anthocyanins.

Concerning flavan-3-ols, a recent publication surveyed 15,371 subjects in Germany aged between 14-80 years and the mean intake of total flavan-3-ols, flavan-3-ol monomers and procyanidins were 386, 120 and 196 mg/day respectively (Vogiatzoglou et al. 2013).

With the "5-a-day for better Health Program" which was initiated in 1991 in the United States which encouraged people to eat at least five servings of fruits and vegetables every day, it can be seen that an individual who consumes 5-a-day could take in average > 500 mg of (poly)phenols daily. When beverages such as cocoa, tea, coffee and wine are consumed on a regular basis this can easily increase the daily intake by 500-1000 mg (Carando et al. 1999a). Subjects who eat small quantities of fruits and vegetables and who do not drink coffee ingest less than 25 mg/day of (poly)phenols. (Clifford 1999; Pulido et al. 2003; Williamson et al. 2008).

All these different data illustrate the difficulties to achieve an accurate estimation of dietary intake of (poly)phenols because of the poor characterisation in foods (raw and process food) and the great variability within foods due to cultivar, year, growing conditions and seasons, etc. (Manach et al. 2004). Furthermore, the high variability in (poly)phenols intake differs from one country to another because of different diets, ethnicity, the availability of fruits and vegetables and individual food preferences. Cultural habits, lifestyle factor, age and gender should also be also taken into consideration.

Significant dietary sources of (poly)phenols are provided by plant foods (fruits, vegetables, cereals, nuts, etc.) and beverages (wine, cider, cocoa, tea, coffee etc.). The phenolic content of all these products can vary substantially even between cultivars of the

same species and are greatly influence by genetic factors and environmental conditions (soil type, sun exposure, culture in greenhouse or fields, conventional or biological culture, fruit yield per tree etc.). In addition, the content can also be influence by factors such as degree of ripeness, variety, processing, storage and culinary preparation (Kuhnau 1976; Herrmann 1988; Mazza et al. 1995). For instance, simple peeling can eliminate a significant portion of the (poly)phenols because of their predominant presence in the outer parts of fruits and vegetables. Cooking may also have a major effect. Onions and tomatoes can lose up to 75%-80% of their initial quercetin content after boiling for 15 min, 65% after cooking in a microwave oven and 30% after frying (Manach et al. 2004).

Oxidation, which leads to the formation of polymerized compounds during processing or storage, results in either beneficial or undesirable characteristics of food products. For example, oxidative change such as browning of cocoa or the oxidative polymerization of tea (poly)phenols during the processing and manufacture result in the development of distinctive and desirable organoleptic properties. Conversely, the enzymatic browning reactions, typically mediated by polyphenol oxidases, result in the formation of undesirable color and flavor on fruits and vegetables.

However, beneficial health effects of these food constituents is highly depend upon their uptake into the body, and their metabolism and disposition in target tissues and cells. Once in the body, (poly)phenols can be poorly absorbed from the gastrointestinal tract, highly metabolized and/or rapidly eliminated. A further complication is that the biological activity of metabolites resulting from digestive or hepatic activity is likely to differ from that of the parent molecules that are ingested.

II.2. Bioavailability of (poly)phenols

Bioavailability differs greatly between (poly)phenols. The absorption of food phenolics is determined primarily by their chemical structure, which depends on factors such as the degree of glycosylation/acylation, their basic structure (i.e., benzene or flavones derivatives), conjugation with other phenolics, molecular size, degree of polymerization, and solubility (Karakaya 2004). In addition, each (poly)phenols may be present in foods in different forms and can affect the intestinal absorption. The bioavailability may be even lower when the food (poly)phenols have a large molecular weight, which is the case for hydrolysable and condensed tannins and complex flavonoid conjugates with several sugars or acylated with hydroxycinnamic acids. Food contains usually more complex phenolics as

opposed to simpler derivatives and these complex molecules might be underestimated due to analytical problems. Food matrix can also have an impact on bioavailability. Direct interaction between (poly)phenols and some components of food such as binding proteins and polysaccharides can occur and this may affect absorption. In addition, the diet may influence diverse parameters of gut physiology (e.g., pH, intestinal fermentations, biliary excretion, transit time) and consequently alter the absorption of (poly)phenols. Other components such as some micronutrients or xenobiotics may also induce or inhibit enzymes involved in this process.

It is generally accepted that the bioavailability of phenolics is low and the relative urinary excretion of the intake range from 0.1% for anthocyanins to 20-50 % for isoflavones such as daidzein (Crozier et al. 2009). This demonstrates the great variability in the bioavailability of the different (poly)phenols. Moreover, the gut microbiota composition and metabolism, which can vary between individuals, has to be taken into account because it can alter absorption, bioavailability and biological activity of dietary phenolics.

II.2.1 Absorption, metabolism and excretion

II.2.1.a. Composition of the intestinal microbiome

Apart from the role of digestion, the gut microbiota has been associated with diverse body functions such as gastrointestinal tract permeability, vitamin synthesis, detoxification of xenobiotics and immune system homeostasis (Kau et al. 2011; Littman et al. 2011). The composition of the microbial community in the gut is governed by age, diet and environment (Ley et al. 2008). The gut is mainly populated by bacteria accounting for about 100 trillion cells representing hundreds of species and thousand of subspecies, with the largest community residing in the colon. The concentration is estimated at 10^{12} microorganisms per gram of gut content (Whitman et al. 1998). Over 50 bacterial phyla have been identified in the human gut but four main phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* are reported to be dominant (Arumugam et al. 2011; Moco et al. 2012). The bacterial communities composition varies along the digestive tract and have the capability to adapt through life according to lifestyle and nutrition of the host, conferring humans the capacity to live on widely diverse diets (Xu et al. 2007). This capacity of adaptation is possible because of the 2-4 million genes of the microbial genome in the gut (Qin et al. 2010). All these genes are responsible for considerable processes such as substrate breakdown, protein synthesis, biomass production and production of signaling molecules. The intestinal microbiota genome

also encodes biochemical pathways that human have not evolved (Egert et al. 2006). Therefore, the gastrointestinal tract microbiota constitutes a very important element in the absorption and bioavailability and their further roles will be discussed below.

II.2.1.b. Intestinal and colonic absorption

After ingestion, native (poly)phenols have to pass a multitude of barriers and are exposed to extensive metabolism prior to entry into the systemic circulation. With the exception of flavan-3-ols and proanthocyanins, almost all flavonoids are present in the diet as glycosides, The type of sugar attached to the flavonoid is a determinant for the site and extent of absorption whereas the position of the sugar moiety on the flavonoid skeleton mostly affects the mechanisms involved in intestinal uptake.

Phenolic compounds are released from the matrix after mastication. Flavonoid glycosides can be already deglycosylated in the mouth by microbiota and/or oral epithelial cells (Walle et al. 2005). The stomach reduces the particle size of food and enhances further the release of phenolic compounds. Absorption occurs mostly in the small intestine with multiple factors appearing to influence the rate and extent of the process. In general, aglycones can readily be absorbed in the small intestine after hydrolysis by lactase phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells (Day et al. 2000; Sesink et al. 2003). LPH shows broad substrate specificity for flavonoid-*O*-β-D-glucosides and the resulting aglycones may enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane. Within the epithelial cell, an alternative way of hydrolysis is possible via the action of the cytosolic-β-glucosidase (CBG). The polar glucosides must be transported into the epithelial cell by the active sodium-dependent glucose transporter SGLT1 in order for CBG-catalysed hydrolysis to occur (Gee et al. 2000). Thus, there are two possible routes by which the glycoside conjugates are hydrolysed and the resultant aglycones appear in the epithelial cells, namely “LPH/diffusion” and “transport/CBG”. However, in a recent investigation, contradicting data indicated that once SLGT1 was expressed in *Xenopus laevis* oocytes, at least in this model system, SLGT1 does not transport flavonoids and that glycosylated flavonoids, and some aglycones, have the capability to inhibit the glucose transporter (Kottra et al. 2007). Moreover, numerous studies have shown that the relative contributions of “LPH/diffusion” and “transport/CBG” depend on the position of glycosylation (Day et al. 1998; Gee et al. 2000; Day et al. 2003).

Prior the passage into the blood stream, the aglycones undergo extensive metabolism forming sulphate, glucuronide and/or methylated metabolites through the respective action of sulfotransferases (SULT), uridine-5′-diphosphate glucuronosyltransferases (UGTs) and catechol-*O*-methyltransferases (COMT). This mechanism reflects a metabolic detoxification process common to many xenobiotics that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity. Glucuronidation occurs on the luminal side of the endoplasmic reticulum whereas sulphation and methylation both occur in the cytosol. The conjugation reactions occur within various tissues and cells. The intestine and the liver appear to be the most important organs involved in flavonoid metabolism, although other organs such as kidney may also contribute. Some of the conjugated metabolites are actively effluxed back into the lumen of the small intestinal involving members of the adenosine triphosphate (ATP)-binding cassette (ABC) family transporters, including multidrug resistance protein (MRP) and P-glycoprotein (P-gp) (Walgren et al. 2000; Vaidyanathan et al. 2003). MRP-3 and the glucose transporter GLUT2 are also implicate in the efflux of metabolites from the basolateral membrane in the enterocytes (van de Wetering et al. 2009; Manzano et al. 2010). Once in the portal bloodstream, metabolites can be subjected to phase II metabolism with further conversions occurring in the liver.

After an extensive phase I and phase II metabolism, a fraction of the absorbed (poly)phenols is excreted back into the small intestine by enterohepatic recirculation via bile excretion as glucuronides and/or sulphates and reach the colon where bacterial β-glucuronidases and sulfatases can release the aglycones (Donovan et al. 2007). The fraction of the (poly)phenols that reaches the colon directly or indirectly can subsequently be absorbed from the colon or subjected to the action of colonic microflora with their extensive metabolic potential. Investigations with ileal fluid collected from ileostomists after ingestion of various foodstuffs revealed that even when dietary flavonoids are absorbed in the proximal gastrointestinal tract, considerable quantities pass from the small to the large intestine where a wide array of low molecular weight aromatic acids are produced by breakdown due to microbial activity (Kahle et al. 2005; Jaganath et al. 2006; Marks et al. 2009; Crozier et al. 2010). Gut microbiota can hydrolyse glycosides, glucuronides, sulphates, amides, esters and lactones via esterase, glucosidase, reduction, demethylation, dehydroxylation and decarboxylation activities (Simons et al. 2005; Ávila et al. 2009; Selma et al. 2009). The degree of degradation of many phenolic compounds is significantly influenced by the

substrate concentration in the diet as well as individual variations in the composition of the colonic microbiota. The dissimilarities in the populations of colonic microbiota can explain the large inter-individual variability in the health benefits.

The resultant aglycones can be absorbed and may be subjected to phase II metabolism in the liver before being excreted in urine in substantial quantities that, in most instances, are well in excess of the flavonoid metabolites that entered the circulatory system via the small intestine. (Jaganath et al. 2006; Roowi et al. 2010; Stalmach et al. 2013).

II.2.1.c. Elimination of phenolic compounds

Seen as xenobiotics by the body, (poly)phenol metabolites are rapidly eliminated by biliary and urinary excretion. Extensively conjugated metabolites are more likely to be eliminated in the bile whereas small conjugated such as monosulphates are preferentially excreted in urine where only a small fraction containing the intact flavonoid ring is recovered. An investigation showed that the relative biliary and urinary excretion varies from one (poly)phenol to another (Crespy et al. 2003).

Several authors published detailed reviews on the bioavailability of (poly)phenols in human and their potential health effect (Manach et al. 2005; Williamson et al. 2005). Kinetics and extent of (poly)phenols absorption among adults, after ingestion of a single dose of (poly)phenols provided as pure compound, plant extract or whole food/beverage were described. Urinary excretion is extensively studied in human and the total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentration. Isoflavones were highly excreted (16-66% of intake for daidzein and 10-24% of intake for genistein) (Watanabe et al. 1998; Richelle et al. 2002; Zhang et al. 2003), followed by flavanones citrus fruit (4-30% of intake) and especially for naringenin in grapefruit juice (Fuhr et al. 1995; Ishii et al. 1999; Erlund et al. 2001; Manach et al. 2003). Concerning anthocyanins, the urinary excretion was very low with only 0.005-0.1% of intake (Wu et al. 2002; Frank et al. 2003; Milbury et al. 2010) but up to 5% were found by Lapidot et al. after red wine consumption (Lapidot et al. 1998). Urinary recovery of flavonols accounted for 0.3-1.4% of the ingested dose for quercetin and its glycosides (Hollman et al. 1995; Hollman et al. 1997a; Graefe et al. 2001), 0.5-6% of intake for some tea catechins (Lee et al. 1995; Yang et al. 2000), 2-10% for red wine catechin (Donovan et al. 2002a), and up to 30% for cocoa epicatechin (Baba et al. 2000). Some of these data were obtained using enzyme treatments such as glucuronidases/sulfatases releasing aglycones quantifiable by HPLC using

absorbance, fluorescence or electrochemical detection. More recently, a review by Crozier et al., (2010) concentrates principally post-2005 human bioavailability studies where metabolites and related compounds were identified by mass spectrometric-based methods without recourse to the use of enzyme hydrolysis prior to analysis.

II.2.2. (Poly)phenol metabolites

In this paragraph, only flavan-3ols, proanthocyanins, anthocyanins and resveratrol metabolites will be emphasize.

II.2.2.a. Flavan-3-ols

The absorption and metabolism of flavan-3-ol monomers was investigated extensively. Both human and animal studies indicated that (+)-catechin and (−)-epicatechin were rapidly absorbed from the upper portion of the small intestine. During digestion and transfer across the small intestine and the liver, flavan-3-ols undergoes phase II metabolism forming *O*-sulphated, *O*-glucuronidated and *O*-methylated form conjugated (Kuhnle et al. 2000; Vaidyanathan et al. 2002). The methylated metabolites of catechin, epicatechin and epicatechin gallate predominated over the original unmethylated forms in plasma (García-Ramírez et al. 2006). A maximum level of (+)-catechin at 76.7 nmol/L was detected in human plasma at 1.4 h after intake of 121 µmol (+)-catechin in dealcoholized red wine (Bell et al. 2000). The presence or absence of alcohol does not necessary influence the absorption and/or metabolism of flavan-3-ols. Moreover, a peak plasma (−)-epicatechin level of 260 nmol/L was detected within 2 h in humans after the ingestion of 557 mg of a procyanidin-rich chocolate containing 137 mg of (−)-epicatechin (Rein et al. 2000). High recovery was also observed in other investigations using green tea and cocoa products (Manach et al. 2005; Auger et al. 2008), confirming that (−)-epicatechin and (+)-catechin are highly bioavailable especially for the (−)-epicatechin. Stalmach et al. (2009) reported a lower concentration of green tea flavan-3-ol metabolites in plasma than in urine showing that flavan-3-ols are rapidly turned over the circulatory system, and, rather than accumulating, are excreted via the kidneys.

In a paper publish in 1971 (Das 1971), eleven metabolites were identified in human urine after (+)-catechin ingestion. The major ones were 3-(3-hydroxyphenyl)propionic acid and 5-δ-(3′-hydroxyphenyl)-γ-valerolactone. More recently, the main metabolites detected in urine after the intake of catechin and epicatechin were 3-(3′-hydroxyphenyl)propionic acid, 5-

(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone and 3-hydroxyhippuric acid (Selma et al. 2009). An *in vitro* study by Tzounis et al. (2008b) in which (−)-epicatechin and (+)-catechin were incubated with faecal bacteria led to the formation of several metabolites summarize in Table 2. The formation of these metabolites from (+)-catechin needed to be converted into (+)-epicatechin first.

Table 2: Metabolites of dietary flavan-3-ols and proanthocyanindins from *in vitro* microbial conversion experiments

	Metabolites *in vitro*	Microbiota	References
(+)-Catechin	(−)-5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (M6) 5-Phenyl-γ-valerolactone 3-Phenylpropionic acid	Human feacal microbiota	(Tzounis et al. 2008b)
	3-(3′,4′-Dihydroxyphenyl)propionic acid 3-(3′-Hydroxyphenyl)propionic acid 3-Phenylpropionic acid 5-(3′,4′-Dihydroxyphenyl)valeric acid	Human feacal microbiota	(Aura et al. 2008)
(−)-Epicatechin	(−)-5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (M6) 5-Phenyl-γ-valerolactone Phenylpropionic acid	Human feacal microbiota	(Tzounis et al. 2008b)
	3-(3′-Hydroxyphenyl)propionic acid 3-Phenylpropionic acid 5-(3′,4′-Dihydroxyphenyl)valeric acid 5-(3′-Hydroxyphenyl)valeric acid	Human feacal microbiota	(Aura et al. 2008)
(+)-Catechin and/or (−)-epicatechin	4-Hydroxy-5-(3′-hydroxyphenyl)valeric acid 4-Oxo-5-(3′,4′-dihydroxyphenyl)valeric acid 4-Oxo-5-(3′-hydroxyphenyl)valeric acid 1-(4-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol	Rat faecal microbiota	(Takagaki et al. 2013)
(−)-Epicatechin-3-*O*-gallate and (−)-epigallocatechin gallate	Gallic acid Pyrogallol (−)-5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (−)-5-(3',5'-Dihydroxyphenyl)-γ-valerolactone 5-(3′,4′-Dihydroxyphenyl)valeric acid 5-(3′-hydroxyphenyl)valeric acid 3-(3′,4-Dihydroxyphenyl)propionic acid 3-(3′-Hydroxyphenyl)propionic acid 3-(3′-Methoxyphenyl)valeric acid	Human feacal microbiota	(Meselhy et al. 1997)
Procyanidin dimers	5-(3′,4-Dihydroxyphenyl)-γ-valerolactone 3′,4′-Dihydroxyphenylacetic acid	Human faecal microbiota	(Appeldoorn et al. 2009a)
Proanthocyanidins	5-(3′-Hydroxyphenyl)valeric acid 3-(3′-Hydroxyphenyl)propionic acid 2-(4′-Hydroxyphenyl)acetic acid 3-(4′-Hydroxyphenyl)propionic acid 3-(3′-Hydroxyphenyl)propionic acid 3-(3′-Hydroxyphenyl)acetic acid 3-(Phenyl)propionic acid	Human feacal microbiota	(Déprez et al. 2000)

In another *in vitro* study (Meselhy et al. 1997), by incubating (–)-epicatechin, (–)-epigallocatechin and their 3-*O*-gallates with human faecal suspension, the authors detected pyrogallol, 5-(3′,4′-dihydroxyphenyl)valeric acid, 5-(3′-hydroxyphenyl)valeric acid, 3-(3′,4′-dihydroxyphenyl)propionic acid, 3-(3′-hydroxyphenyl)propionic acid, 3-(3′-methoxyphenyl)valeric acid and 2,3-dihydroxyphenoxyl 3-(3′,4′-dihydroxyphenyl)propionic acid (Table 2). This research also showed that gallates resist any degradation by a rat caecal suspension, suggesting species differences in metabolic activity of the two microbiota. A very recent investigation by Takagaki et al. (2013), investigated the *in vitro* catabolism of (+)-catechin and (–)-epicatechin by rat intestinal microbiota. Using LC-MS and NMR analyses, the authors have identified new metabolites: the 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid, 4-oxo-5-(3′,4′-dihydroxyphenyl)valeric acid, 4-oxo-5-(3′-hydorxyphenyl)valeric acid and 1-(4-hydroxyphenyl)-3-(2′,4′,6′-trihydroxyphenyl)propan-2-ol. A study in which volunteers drink green tea, (–)-epigallocatechin and (–)-epigallocatechin-3-*O*-gallate could generated 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(3′4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone both of which also appeared as sulphated and glucuronidated derivatives at high concentrations in urine and plasma (Table 3) (Lambert et al. 2007).

Table 3: Metabolites of dietary flavan-3-ols and proanthocyanidins from *in vivo* body fluid.

	Metabolites *in vivo*	Body Fluid	References
(+)-Catechin	3-(3′-Hydroxyphenyl)propionic acid 5-δ-(3',4'-Dihydroxyphenyl)-γ-valerolactone 5-δ-(3'-Hydroxyphenyl)-γ-valerolactone 3-(3′-Hydroxyphenyl)propionic acid 3′-Hydroxyhippuric acid	Human urine	(Das 1971)
(–)-Epigallocatechin gallate	(–)-5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone (M4) (–)-5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (M6) (–)-5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone(M6′)	Human urine	(Li et al. 2000; Meng et al. 2002; Lambert et al. 2007)
Proanthocyanidins	2-(3′,4′-Dihydroxyphenyl)acetic acid 3-(3′-Hydroxyphenyl)propionic acid 3′-Hydroxyphenylacetic acid Ferulic acid 3′-Methoxy-4′-hydroxyphenylacetic acid 3-Hydroxybenzoic acid	Human urine	(Rios et al. 2002)
	5-(3′,4′-Dihydroxyphenyl)valeric acid 5-(3′-Hydroxyphenyl)valeric acid 3-(3′,4′-Dihydroxyphenyl)propionic acid 3-(3′-Hydroxyphenyl)propionic acid 3,4-Dihydroxybenzoic acid 3-Methoxy-4-hydroxybenzoic acid 4-Hydroxybenzoic acid	Rat urine	(Gonthier et al. 2003)

II.2.2.b. Condensed tannins

In an early study, the used of simulated gastric fluid (pH 2.0, 37°C) suggested that proanthocyanidin oligomers (trimers to hexamers) were depolymerized into mixtures of (−)-epicatechin and dimers (Spencer et al. 2000). These authors also evidenced in an *ex vivo* perfusion of rat small intestines with procyanidin dimer B_2 or B_5 extracted from cocoa, that (−)-epicatechin was the major metabolites (Spencer et al. 2001). In addition, another report stipulated that once procyanidins were degraded, they yielded more readily absorbable flavan-3-ol monomers in an *in vitro* model of gastrointestinal condition (Kahle et al. 2011). However, numerous feeding studies with animals and humans using oligomeric and polymeric flavan-3-ols did not supported this conclusion (Donovan et al. 2002b; Rios et al. 2002; Tsang et al. 2005a). Most pass unaltered to the large intestine where they are catabolised by the colonic microflora yielding a diversity of phenolic acids (Selma et al. 2009) including 3-(3′-hydroxyphenyl)hydracrylic acid and 4-*O*-methyl-gallic acid (Déprez et al. 2000; Gonthier et al. 2003; Ward et al. 2004; Crozier et al. 2010) which are absorbed in the circulatory system and excreted in urine. A study in human demonstrated that depolymerization did not occur and proanthocyanidins were stable under gastric transit (Rios et al. 2002). A later investigation, confirmed that proanthocyanidin dimers and trimers were highly stable under gastric and duodenal digestion conditions (Serra et al. 2010). Ottaviani et al. (2012a), have recently compared the plasma concentration of (−)-epicatechin in human blood and urines after volunteers were given (−)-epicatechin, cocoa procyanidin monomers (predominantly (−)-epicatechin) through decamers, or cocoa procyanidins dimers through decamers. Results revealed that all the absorbed (−)-epicatechin in blood and urine were from ingested (−)-epicatechin. No (−)-epicatechin was derived from ingested oligomers and polymers.

Baba et al. (2002) detected procyanidins B_2 and (−)-epicatechin in rat plasma and urine after administration of pure procyanidins B_2. The authors suggested the hypothesis of degradation of a portion of dimer into (−)-epicatechin and the cleavage of interflavan bond was likely to occur in the large intestine microbiota. In two studies, where volunteers were given grape seed extract (Sano et al. 2003) and flavan-3-ol-rich cocoa (Holt et al. 2002), procyanidin dimers B_1 and B_2 were detected in human plasma. In the latter study, the T_{max} and pharmacokinetic profile of the B_2 dimer were similar to those of flavan-3-ol monomers, but the C_{max} was ca. 100-fold lower. Urpi-Sarda et al. (2009b) also detected and quantified procyanidins B_2 in human and rat urine after cocoa intake.

Using intestinal perfusion in rats, no glucuronidated or sulphated metabolites of dimers were detected in biological fluids (Appeldoorn et al. 2009b). Another investigation detected methylated B-type dimer and trimer, but not glucuronide forms, in rat plasma after ingestion of apple procyanidins. The amount of methylated metabolites was not determined but appeared to be low compared with the intact oligomers (Shoji et al. 2006). These results showed limited phase II metabolism on oligomers.

Recent studies using procyanidins B_2 and [^{14}C]procyanidin B_2 dimer have provided helpful information on their *in vitro* catabolism by the gut microbiota (Appeldoorn et al. 2009a; Stoupi et al. 2010a; Stoupi et al. 2010b) and rodent pharmacokinetics (Stoupi et al. 2010c). After ingestion of [^{14}C]procyanidin B_2 dimer, ca. 60% of the radioactivity was excreted in rat urine after 96 h with the vast majority in a form very different from the intact procyanidin dosed (Stoupi et al. 2010c). This observation is consistent with other *in vitro* studies that show extensive catabolism by the gut microbiota.

The role of gut microflora in the catabolism of proanthocyanidins was often explored using anaerobic incubation system in which proanthocyanidins were fermented with freshly collected human colonic faecal bacteria (Table 2) (Déprez et al. 2000). When proanthocyanidins enter the colon, they were transformed to phenolic acids. Condensed tannins were degraded by human colonic microbiota, producing several derivatives of phenylvaleric, phenylpropionic, phenylacetic, phenyllactic and benzoic acids with different patterns of hydroxylation, and the total yields decreased significantly according to the degree of polymerization. Other compounds such as phloroglucinol, 5-(3′-hydroxyphenyl)-γ-valerolactone, and 1-(3′-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol, were also produced (Groenewoud et al. 1986). In another study, condensed tannins also produced 4′-hydroxyphenylacetic acid, 3-(phenyl)propionic acid, 3′-hydroxyphenylacetic acid, 3-(4′-hydroxyphenyl)propionic acid, 5-(3′-hydroxyphenyl)valeric acid and 3-(3′-hydroxyphenyl)propionic acid whereas valerolactones were not detected (Déprez et al. 2000).

The degradation of B-type dimers was extensively studied because of their abundance in the human diet and the availability of commercial standards. The microbial catabolism pathway of procyanidin dimer B_2 is summarized in Figure 18. The scission of the interflavan bond to convert procyanidin B_2 into two (–)-epicatechin appeared to be slow and accounted for less than 10% of procyanidin B_2 (Appeldoorn et al. 2009a). The detection of (–)-epicatechin in an *in vitro* experiment incubating procyanidin B_2 with human faecal microbiota

has proven this minor pathway (Stoupi et al. 2010a). The dominant products were a series of phenolic acids, having one or two phenolic hydroxyls and between one and five aliphatic carbons in the side chain (Appeldoorn et al. 2009a; Stoupi et al. 2010a; Stoupi et al. 2010b). In addition, there were some C_6-C_5 catabolites with a side chain hydroxyl group and associated lactones, and several diaryl-propan-2-ols, most of which were produced from the flavan-3-ol monomers, whereas, 3′,4′-dihydroxyphenylacetic acid was derived from cleavage of the C-ring of the upper flavan-3-ol unit. However, Stoupi et al., (2010b) also indicated that a feature of flavan-3-ol catabolism is the conversion to C_6-C_5 valerolactones and the progressive β-oxydation to C_6-C_3 and C_6-C_1 products (Crozier et al. 2009; Del Rio et al. 2013).

Accordingly to the description above, monomeric metabolites formed by cleavage of interflavan bond or C-ring cleavage of procyanidins, were further degraded by gut microflora into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. This latter can be methylated by catechol-*O*-methyltransferase to form 5-(3′-methyoxy-4′-dihydroxyphenyl)-γ-valerolactone. Microflora can also removed a hydroxyl group from the 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and form 5-(3′-hydroxyphenyl)-γ-valerolactone. Actually, $C_{4'}$ and the aliphatic side chains were the favorably position for microbial dehydroxylation of metabolites. Phenylvalerolactones were slowly degraded into phenylvaleric acids after dehydroxylation of the side chain. Progressive shortening of the aliphatic chain by α- and β-oxidations further generated the phenylpropionic acids, phenylacetic acids and benzoic acid derivatives. An investigation by Appeldoorn et al. (2009a) showed in an *in vitro* study that procyanidin dimers, were degraded by human colonic microbiota to 5-(3′,4′-dihydoxyphenyl)-γ-valerolactone and 3′,4′-dihydroxyphenylacetic acid (Table 2).

Despite remarkable capabilities of the gut microflora, it should be noted that the ability of bacteria to catabolise proanthocyanidins and thus their bioavailability, decrease drastically with the increase of molecular size. The yield of phenolic acids in rat gut was 10% and 7% for monomers and dimers respectively. The capability decreased to 0.7% for trimers and 0.5% for polymers (Gonthier et al. 2003).

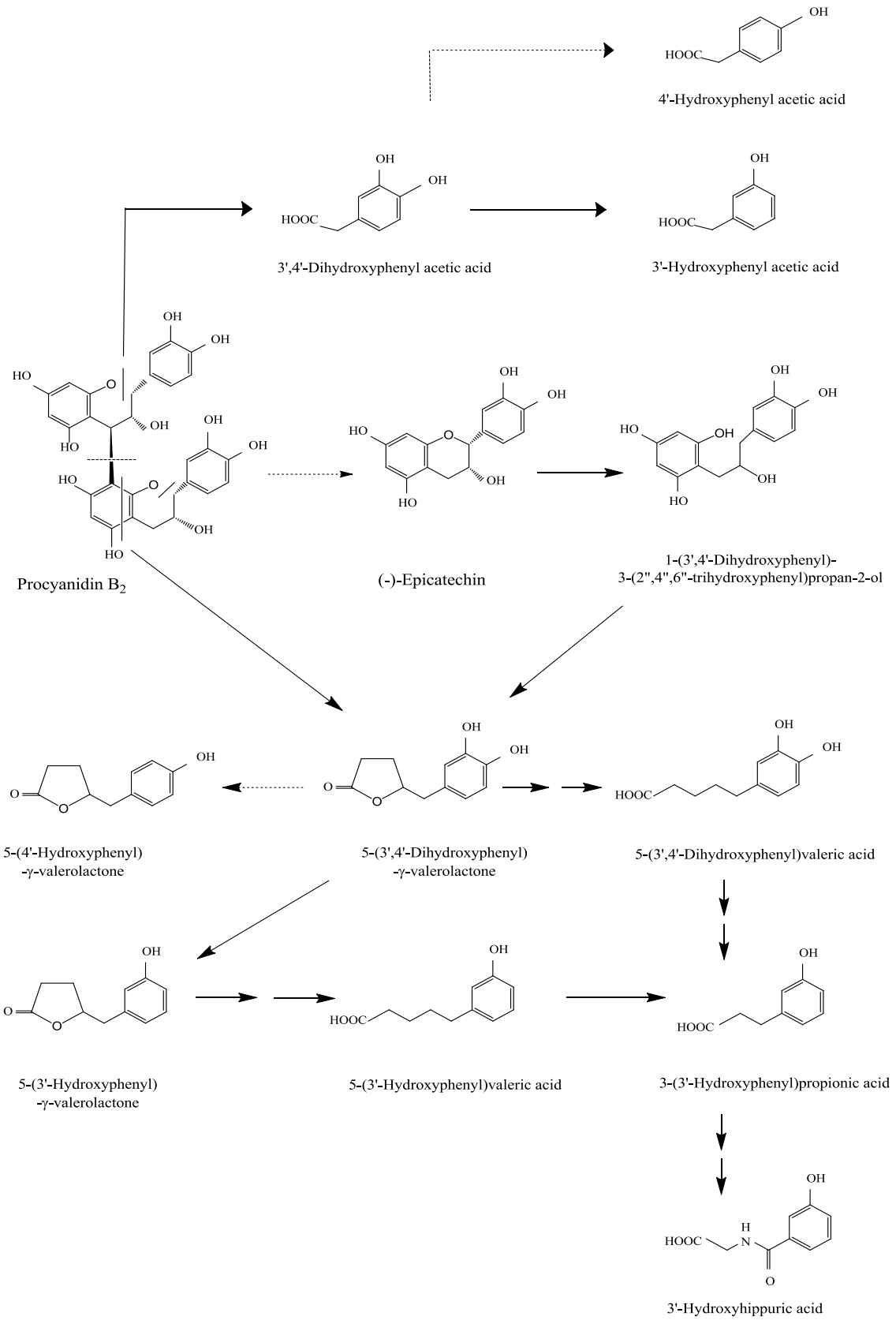


Figure 18: Proposed pathway for human microbial degradation of procyanidin B_2 dimer. Main routes are indicated with solid arrows and minor with dotted arrows. Adapted from Appeldoorn et al. (2009a), Stoupi et al. (2010a,b) and Del Rio et al. (2013).

II.2.2.c. Anthocyanins

Daily intake of anthocyanins has been estimated to be 12.5 mg/day per person in the United States but intakes could be easily over 200 mg/ day if a regular diet of berries and wine are consumed. Anthocyanins are among the few plant (poly)phenols that can be detected in the plasma in their native intact forms (i.e. glycosides). However, typically recoveries of anthocyanins in urine are ca. 0.1% of the ingested dose. The low bioavailability of anthocyanins can be influenced by the nature of the sugar moiety, the structure of the anthocyanin aglycones and by food matrices (Yang et al. 2011). In addition, low recoveries could be a consequence of anthocyanins undergoing structural rearrangements in response to pH. Anthocyanin actually occurs in equilibrium with essentially four molecular forms: the flavilium cation, the quinoidal base, carbinol pseudo-base form and chalcone. The relative amounts of these four forms vary with pH. The red flavilium cation predominates at pH 1-3 and transforms to other form as the pH increase to 4 and above. The colorless carbinol pseudobase becomes the major component along with smaller amounts of chalcone pseudobase and quinoidal base (Clifford 2000). Such changes are likely to occur *in vivo* thought the gastrointestinal tract, as anthocyanins pass from low pH in the stomach to the more-basic conditions of the small intestine.

Anthocyanins bioavailability in human and animal have been widely investigated. The wide array of anthocyanins in fruits and food supplements used in all the studies made the investigation of bioavailability more complex (Prior et al. 2006). Grapes contain more than 10 anthocyanins including glycosylated, acetylated, coumaroylated and caffeoyl-glucosylated anthocyanins. Other fruits such as raspberries also contain numerous different anthocyanins ranging from mono- to tri-saccharides and blueberries retain up to 14 anthocyanins with mainly 3-*O*-glucosides, galactosides and arabinosides of cyanidin, delphinidin, petunidin and malvidin. According to this observation, the evaluation of anthocyanin content of plasma and urine, the interpretation in terms of absorption, metabolism, excretion and potential phase I and phase II metabolism is very difficult, especially when 3′-*O*-methylation converts cyanidin to peonidin and delphinidin to petunidin, and 5′-*O*-methylation converts petunidin to malvidin (Crozier et al. 2009).

Numerous studies reported low urinary excretions, ranging from 0.004% to 0.1% of the intake, although Lapidot et al. (1998) and Felgines et al. (2003) measured high level of excretion up to 5% after red wine or strawberry consumption. In addition, it has been shown

that anthocyanins are absorbed and eliminated rapidly and once they are absorbed, their efficiency is poor (Prior et al. 2006). However, some studies revealed that the bioavailability of these compounds may be underestimated, because the metabolites and breakdown products of anthocyanins have not yet been identified (Manach et al. 2005).

Only a small part of anthocyanins are absorbed in their native forms as glycosides or as hydrolysis products in which the sugar moiety is removed. Using HPLC-MS, glucuronides and sulphates of anthocyanins have been identified (Wu et al. 2002; Felgines et al. 2003; Mullen et al. 2008). Lapidot el al. (1998) investigated the bioavailability of red wine anthocyanins. The consumption of 300 mL of red wine containing 218 mg of total anthocyanins has been shown to yield 1.5-5.1% of the amount ingested in urine within 12 hours. Two of the compounds found among the anthocyanins were unchanged in the urine but other anthocyanin compounds had undergone molecular modifications. In another study in which volunteers consumed 500 mL of red wine, dealcoholized red wine and red grape juice, containing 68, 58 and 117 mg of malvidin-3-*O*-glucoside, respectively, malvidin-3-*O*-glucoside was found in plasma and urine after ingestion of all the beverages studied whereas the aglycones, glucuronidated and sulphate conjugates were not detected either in urine or plasma (Bub et al. 2001). The authors suggested that malvidin-3-*O*-glucoside may be poorly absorbed after a single ingestion of red wine and seems to be differently metabolized compared to the other grape (poly)phenols.

Using HPLC-ESI-MS-MS, Felgines et al. (2003) studied the bioavailability of 200 g of strawberry (containing 179 µmol of pelargonidin-3-*O*-glucoside). Urinary excretion of ca. 1.8% of the ingested amount was obtained with more than 80% of this excretion related to monoglucuronide of pelargonidin. From the same authors, in a separate human feeding study, 200 g of blackberries containing 960 µmol of anthocyanins principally as cyanidin-3-*O*-glucoside were given to volunteers. In addition to cyanidin-3-*O*-glucoside, lower levels of methylated glycosides, glucuronides of anthocyanidins and anthocyanins, sulfoconjugate of cyanidin and anthocyanidins were identified. Total urinary excretion was estimated at 0.16 % of the intake (Felgines et al. 2005). These results indicate that pelargonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside are absorbed differently. Pelargonidin-3-*O*-glucoside showed to be better absorbed than cyanidin-3-*O*-glucoside. This is in keeping with the evidence obtained with other flavonoids that the presence of a substituent group at 3′- and 4′-positions can influence absorption.

Anthocyanins which are not absorbed in the small intestine may be transferred to the colon and transform by colonic microbiota. Feeding study with ileostomists who consumed raspberries has shown that 40 % of anthocyanin intake remained in the ileal fluid with a recovery of individual compounds ranging from 5.9% for cyanidin-3-*O*-glucoside to 93 % for cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside. The colonic metabolism includes deconjugation and ring-fission. Cyanidin is converted to protocatechuic acid (3,4-dihydroxybenzoic acid) (Vitaglione et al. 2007), malvidin to syringic acid (4-hydroxy-3,5-dimethoxybenzoic *acid),* peonidin to vanillic acid (4-hydroxy-3-methoxybenzoic *acid)* and pelargonidin to 4-hydroxybenzoic acid. González-Barrio et al. (2011), suggested in an *in vivo* and *in vitro* study that cyanidin-base anthocyanins are degraded by the colonic microbiota to a diversity of phenolic and aromatic catabolites. In a study by Keppler et al. (2005), various anthocyanin standards were metabolized to aldehydes and phenolic acids when incubated with pig cecum contents. Other metabolite produced *in vitro* by the pig gut microbiota include syringic acid, vanillic acid, phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde), gallic acid and 3-*O*-methylgallic acid (Forester et al. 2008).

II.2.2.d. Resveratrol

The interest of this compound begun when it was detected in wine (Siemann et al. 1992) and it was attributed some cardioprotective effects (Bertelli et al. 1995). After the publication in science by Jang et al. (1997) on resveratrol anticancer potential, the scientific community became really interested in resveratrol and the number of scientific reports on the effects and properties of this compound increased exponentially. Since resveratrol and other stilbenes are not very abundant in the diet, the vast majority of studies on the health effects have been carried out using the pure compound (either purified or synthetic) and often at a high concentration. *Trans*-resveratrol is widely credited with being responsible for the protective health effect of red wine. Found in a low concentration in wine (0.3-7 mg aglycones/L and 15 mg glycosides/L) (Vitrac et al. 2002), more than 60 liters would have to be consumed on a daily basis by human for intake to reach the amounts that are required to provide protective effects in animal model systems. *Trans*-resveratrol concentration reached by drinking within the safe limits is below the therapeutic range. Thus, any protective effect of this molecule is unlikely at normal nutritional intakes. No beneficial effects of *trans*-resveratrol should be attribute to wine.

The presence of *trans*-resveratrol in the diet is scarce and its bioavailability is considered as very low (ca. 1%). Walle et al. (2004) used ^{14}C-labelled resveratrol

administrated both orally and intravenously. While ca. 70% of resveratrol was absorbed following oral administration, only trace amount of <5 ng/mL of unmetabolised resveratrol could be detected in the systemic circulation following a 25 mg dose. Most of the oral dose was recovered in urine. HPLC-MS permitted to identify dihydroresveratrol glucuronides and sulphates. According to the authors, extremely rapid sulphate conjugation by the intestine/liver appears to be the rate-limiting step in resveratrol's bioavailability.

Absorption of resveratrol is thought to occur mainly by transepithelial diffusion. Extensive metabolism in the intestine and liver produced resveratrol-3-*O*-glucuronide, resveratrol-4′-*O*-glucuronide, resveratrol-3-*O*-sulphate, resveratrol-4′-*O*-sulphate and a resveratrol-*O*-disulphate. These metabolites appeared to be the main in human with the sulphates being predominant (Walle 2011). Resveratrol-3-*O*-glucuronide, resveratrol-4′-*O*-glucuronide, Resveratrol-3-*O*-sulphate, resveratrol-4′-*O*-sulphate, a resveratrol-*O*-disulphate and an *O*-glucuronide-*O*-sulphate were detected in plasma of both healthy humans and colorectal cancer patient ingesting 2.2 and 4.4 mmol of *trans*-resveratrol. In subjects who ingested 128 µmol of *trans*-piceid, two novel trans-resveratol *C/O*-conjugated diglucuronides were detected (Burkon et al. 2008). Concentrations of these metabolites are 3–8 fold higher than resveratrol postabsorption, and have longer half-lives, so that the body is more exposed (up to 23-fold) to the metabolites compared to the aglycones (Patel et al. 2011).

Aside from the extensive metabolism of *trans*-resveratrol which results in an oral bioavailability considerably less than 1%, a recent review focuses on the ABC transporter, specifically the multidrug resistance associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), involved on the efflux of trans-resveratrol glucuronides and sulphate to the intestinal lumen affecting not only the bioavailability but also its distribution in the different organs (Planas et al. 2012).

II.3. Bioavailability of wine-derived phenolic compounds

To date, data on bioavailability of the different wine-derived phenolic compounds in humans are limited. It has been hypothesized that phenolic compounds which are present as soluble forms in wine, should be more bioavailable than those polymeric, insoluble or tightly bound and compartmentalized forms in fruits and vegetables (Soleas et al. 1997).

De Vries et al. (2001) determined and compared the bioavailability of the flavonol quercetin, from red wine, tea and onions in humans. They concluded that although quercetin

and other flavonols are absorbed from the red wine, one 100 mL glass of red wine provides less available flavonols than one 125 mL cup of tea or one 15 g portion of onions. The absorption of flavonols from foods actually depends on the form in which the compound is present in food (Hollman et al. 1997a; Hollman et al. 1997b). For instance, glycosylation enhances absorption of quercetin. The bioavailability of the compound also depends on the sugar moiety. In fact, the bioavailability of quercetin glucoside is 20% greater than that of quercetin rutinoside, and this can be explain by the presence of different sites of absorption of the glucoside and rutinoside (Hollman et al. 1999).

Another study determined in humans that the hydroxycinnamate, caffeic acid, was rapidly absorbed from wine and reached a maximum concentration in the plasma at approximately 60 min post consumption (Simonetti et al. 2001). Other studies founded that the time taken to reach the maximum plasma concentration for the wine-derived phenolic compounds, whether free, conjugated or total, ranged from approximately 30 to 90 min (Donovan et al. 1999; Bell et al. 2000; Goldberg et al. 2003).

The bioavailability of catechin, quercetin and *trans*-resveratrol in white wine, grape juice and vegetable juice were determined and compared in humans (Goldberg et al. 2003). The authors observed that the three phenolic compounds were present in serum and urine primarily as glucuronide and sulfate conjugates. Actually, it had previously been observed that the phenolic compounds are primarily conjugated in serum and urine, which facilitates their elimination from the body (Paganga et al. 1997; Ader et al. 2000). Of the three compounds studied, *trans*-resveratrol was the most bioavailable phenolic compound followed by quercetin and catechin. However, the observed maximum serum concentration of 10 to 40 nmoL/L is 100-fold less than the 5 to 100 mmol/L required in *in vitro* and *ex vivo* studies to demonstrate antioxidant and other biological activities of the wine-derived phenolic compounds (Bhat et al. 2001; Tsang et al. 2005b; Kennedy et al. 2010). Goldberg et al. (2003) also concluded that absorption of the wine-derived phenolic compounds does not require the presence of ethanol. This result supports the observations of Donovan et al.(1999) and Bell et al. (2000) who observed that catechin was absorbed equally from dealcoholised red wine and red wine containing alcohol.

Although significant progress has been made, there are still some critical areas that need to be elucidated in order to truly understand the mechanisms linking (poly)phenol

consumption and health improvement. In view of the description about bioavailability and generated metabolites described above, research should be more concentrate on the health effect of circulating and excreted metabolites in their glucuronidated or sulphated forms and colonic ring-fission products rather than their native forms. Actually, it is not the (poly)phenolic compounds found in fruits, vegetables and beverages which are transported in the systemic circulation and reached the body tissues to elicit bioactive effects. Recently, methods to synthesize (poly)phenol metabolites and their colonic catabolites are emerging, making the evaluation of health effect easier and will help to understand their role in disease prevention by dietary (poly)phenols.

II.4. Biological effects of (poly)phenols

Phenolic compounds are currently receiving much attention because of their beneficial health effects related to their antioxidant, anti-microbial, anti-inflammatory, cardio-protective, cancer chemo-preventive and neuro-protective properties. The biological activity of phenolics has been extensively studied *in vitro* on pure enzymes, cultured cells or isolated tissues by using (poly)phenol aglycones or some glycosides that are present in food. Because of the lack of commercial standards, very little is known about the biological properties of the conjugated derivatives (i.e. glucuronides, sulphates and/or methylated). However, scientists start considering the importance of (poly)phenol metabolites and their colonic catabolites as these compounds are present in the systemic circulation and might elicit biological activity.

The assumption of beneficial biological effects of (poly)phenols originally came from *in vitro* studies showing the antioxidant properties of several (poly)phenols and their ability to modulate the activity of certain enzymes. More evidence of their protective role derives from numerous experiments on animal models, epidemiological studies and clinical trials. Most of the time, clinical studies used foods or beverages containing a mixture of different (poly)phenols and the exact nature of the most active compounds remains largely unknown. (poly)phenol families includes a wide range of diverse compounds with highly different bioavailability, thus, the results obtained for one type of (poly)phenols cannot be generalized to others.

In this chapter, cardiovascular protective effects of (poly)phenols will be highlight. Cardiovascular disease (CVD) is the leading cause of morbidity and mortality among adults in western countries. Although some of the major risk factors are not modifiable (e.g., genetic predisposition, sex, age), diet and lifestyle can be modulated. For instance, it is well

established that excessive dietary intake of saturated fats and cholesterol contribute to the development of CVDs via changes in low-density lipoprotein-cholesterol (LDL-c) (Murray et al. 1996). Several epidemiological studies suggest that regular consumption of foods and beverages rich in (poly)phenols is associated with a reduction in the relative risk to die for a range of pathological conditions, ranging from hypertension to coronary heart diseases, strokes and dementias. Some of the potential mechanisms of preventing CVD after consumption of (poly)phenols could be related to their antioxidant activity (Rice-Evans et al. 1996). In fact, (poly)phenols interfere with a large number of biochemical signaling pathways and, therefore, physiological and pathological processes. The protective effect is in part due to their ability to delay the development and progression of early atherosclerotic lesions to advances atherosclerotic plaques. Antioxidant flavonoids have been shown to reduce oxidation of LDL-c which is a key and early event of the atherogenic process (Frankel et al. 1993; Frankel et al. 1995; Frei 1995). Other potential mechanisms by which (poly)phenols may exert cardioprotective effects include reduction in oxidative stress (Bagchi et al. 2000; Rouanet et al. 2011), reduction of LDL-c oxidation (Stein et al. 1999; Sano et al. 2007), modulation of inflammatory cascade (Delmas et al. 2005; Castilla et al. 2006), improvement on vascular endothelial function and protection against atherothrombotic episode including myocardial ischemia and inhibition of platelet aggregation (Auger et al. 2002; Shanmuganayagam et al. 2002; Arts et al. 2005).

Moreover, (poly)phenols contribute to the prevention of high blood pressure and endothelial dysfunctions by preventing the NADPH oxidase vascular-dependent oxidative stress and the formation of vasoconstrictors. (poly)phenols have been shown, on the one hand to increase the formation of endothelium-derived relaxing factor such as nitric oxide (NO) (Fitzpatrick et al. 1993; Andriambeloson et al. 1997; Soares de Moura et al. 2002), the endothelium-derived hyperpolarizing factor (EDHF) (Kwan et al. 2004; Ndiaye et al. 2004) and prostacyclin (Mizugaki et al. 2000; Aldini et al. 2003) through the redox sensitive PI3-Kinase/Akt pathway and on the other hand, to inhibit the synthesis of the vasocontracting factors such as endothelin-1 (Corder et al. 2001).

II.4.1. (Poly)phenols as antioxidants

Some of the potential mechanisms of preventing CVD after consumption of (poly)phenols could be related to their antioxidant activity.

Oxidative property of oxygen plays both a vital role in various biological phenomena and can also aggravate the damages within the cells by excess oxidative events. Oxygen is used by the cell to generate energy and free radicals are form as a consequence of ATP (adenosine triphosphate) production by the mitochondria. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products resulting from the cellular redox process. Reactive oxygen species include different radicals described in Table 4.

Table 4: Example of Reactive Oxygen Species (ROS). Adapted from Mimić-Oka et al. (1999).

The radicals		
Superoxide	$O_2^{\bullet-}$	Oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems, by auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. Superoxide dismutase (SOD) accelerates the dismutation of $O2^{\bullet-}$.
Hydroxyl	$OH^{\bullet}$	A highly reactive oxygen-centered radical that attacks all molecules in the human body.
Peroxyl alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Typically, organic radicals often encountered as intermediates during the breakdown of peroxides of lipids in the free radical reaction of peroxidation.
Oxides of nitrogen	$NO^{\bullet}$, $NO_2^{\bullet}$	Nitric oxide is formed *in vivo* from the amino acid L-arginine. Nitrogen dioxide is formed when NO reacts with O_2 and is found in polluated air and smoke.
The nonradicals		
Hydrogen peroxide	H_2O_2	Formed *in vivo* when $O2^{\bullet-}$ dismutates and also by many oxidase enzymes. Higher levels of H_2O_2 can attack several cellular energy-producing systems. H_2O_2 also form $OH^{\bullet}$ in the presence of transition metal ion (Fe^{2+}) and $O2^{\bullet-}$ can facilitate this reaction.
Hypochlorous acid	HOCl	Powerful oxidant formed in the human neutrophils at sites of inflammation by action of the enzyme myeloperoxidase. May also react with $O2^{\bullet-}$ to generate $OH^{\bullet}$.
Ozone	O_3	This noxious gas has been shown to deplete plasma antioxidants vitamin D, vitamin E and uric acid.
Singlet oxygen	$^1O_2$	This molecule can react with unsaturated fatty acids producing a complex mixture of hydroperoxides.

The reactive species play both a beneficial and toxic role and the balance between them have to be maintained. To prevent an overload in free radicals and peroxides, aerobic organisms use a sophisticated defense system which operates both in intra- and extracellular aqueous phases and in membranes. Antioxidant defense strategies are committed to prevent

the oxidative attack in its early moment by the formation of priming radicals as well as during the initiation and chain propagation processes.

Actually, at low or moderate levels, reactive species exert beneficial effects on cellular redox signaling and immune function. For instance, free radicals take part in cascade events in the antimicrobial action of the phagocytic cells via NADPH-oxidase and act as regulator molecules. However, at high concentrations, they cause oxidative stress, a harmful process in which cell functions and structures can be damaged.

Due to the great diversity of (poly)phenols, the structure-activity relationship, bioavailability and therapeutic efficacy of the antioxidants differ extensively (Huang et al. 2005). Phenol itself is inactive as an antioxidant, but *ortho*- and *para*- diphenolics have antioxidant capacity which increases with the substitution of hydrogen atoms by ethyl or *n*-butyl groups. Flavonoids are among the most potent plant antioxidants because they possess one or more of the following structural elements involved on the antiradical activity: (1) an *o*-diphenolic group (in the ring B), (2) a 2-3 double bond conjugated with the 4-oxo function and (3) hydroxyl groups in positions 3 and 5 (Leopoldini et al. 2011). Quercetin is the flavonol that combines all of these characteristics and is one of the most potent natural antioxidant. Moreover, the antioxidant efficiency of flavonoids is directly correlated with their degree of hydroxylation and decreases with the presence of a sugar moiety (Cotelle 2001).

(Poly)phenols may act though various mechanisms leading to free radicals scavenging. These include inhibition or potentiation of the action of many enzymes and direct interaction with free radical (Mimić-Oka et al. 1999; Carillon et al. 2012). In addition, (poly)phenols can chelate metals such as iron which is involved in free radicals formation (Korkina et al. 1997). Indirectly, they can interfere with the cellular detoxification systems, such as superoxide dismutases (SOD), catalase or glutathione peroxidases (Krinsky 1992). Besides, (poly)phenols can also inhibit enzymes generating ROS such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Green tea flavonoids showed by *in vitro* investigations that they are strong antioxidants, being up to 5 times more effective than vitamin C and E (Dreosti 1996; Balentine et al. 1997). A study demonstrated that epigallocatechin-3-*O*-gallate from tea can modulate the oxidative stress by affecting the production of ROS, gluthatione and cytochrome P450 activity (Raza et al. 2005).

However, a gap between these antioxidant effects and the risk of CVDs exists. A direct scavenging of free radicals by (poly)phenols, as often suggested, may not be the key mechanism explaining their effects on oxidative stress biomarkers and CVD risk factors.

II.4.2. (Poly)phenols and vascular protection

II.4.2.a. Endothelial cells and regulation of vascular homeostatis

Endothelial cells of healthy blood vessels form a monolayer at the luminal surface and mediate chemically the vascular homeostasis. These cells prevent the contact of circulating blood with the underlying prothrombotic arterial wall. The endothelium plays a critical in the control of vascular tone by synthesizing and releasing various substances into the bloodstream which are classified as vasodilators and vasoconstrictors, based on their mechanism of action. Vasodilators are generally referred to as endothelium-derived relaxing factor (EDRF), represented mainly by nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2), and vasoconstrictors, known as endothelium-derived contracting factors (EDCFs), represented by endothelin-1.

The gas NO is generated from L-arginine by the enzyme endothelial NO synthase (eNOS) and diffuses toward the underlying vascular smooth muscle, where it stimulated guanylate cyclase in the sarcoplasm and increase the production of cyclic guanosine monophosphate (cGMP) (Vanhoutte 2009). NO is the most potent of all endogenous compounds causing vascular muscle relaxation, but also displays paracrine action by preventing platelet aggregation, adhesion and exerting an anti-proliferative effect on smooth muscle cells. In addition, NO also limits the oxidation of low-density lipoproteins (LDLs) and can lower the activity of reactive oxygen species (Frankel et al. 1995; Teissedre et al. 1996). The physiological effect of NO is very short (less than 20 sec) because of the inactivation of haemoglobin (Stankevicius et al. 2003; Stoclet et al. 2004).

PGI_2 can be synthesized through transcellular metabolism. Similarly to NO, PGI_2 dilates blood vessels, causing smooth muscle relaxation. This is mainly attributed to the stimulation of adenylate cyclase and thus to an increased content of intracellular adenosine monophosphate (cAMP) and the activation of potassium channels in smooth muscle cells (Kawashima et al. 2004; Fetalvero et al. 2007; Vanhoutte 2009).

Mechanical damages to endothelium or a loss of functional integrity disturbs the fragile multifactorial equilibrium ensured by endothelial cells and thus, causing the

development of pathological changes such as hypertension, atherosclerotic lesions, thrombi and disturbed tissues perfusion (Goch et al. 2009). Endothelial dysfunction is often associated with pronounced oxidative stress which is due, at least in part, to an increased expression of NADPH oxidase, an enzyme generating superoxide anions on the arterial wall (Griendling et al. 2000; Griendling et al. 2003). Superoxide anions react with NO to reduce its bioavailability and, hence, vascular protective effects. Furthermore, the dysfunction can activate the endothelium-dependent contractile responses involving the contractile actions of endothelin-1 and vasoconstrictor factors acting on tromboxane receptors. The latter stimulates platelet aggregation and vessel constriction.

II.4.2.b. Vascular protection by (poly)phenols

Pioneer study by Fitzpatrick et al. (1993), using rat aorta with various grape products including wines, grape juices and grape skin extracts, have showed that (poly)phenols increase the formation of NO by endothelial NO synthase action. Using another animal model, in porcine coronary artery ring, the endothelium-dependent relaxation induced by red wine (poly)phenols is observed at concentration of 3 µg/mL (Ndiaye et al. 2004). Several other aortic ring experiments using physiological concentrations of (poly)phenols have also shown that (poly)phenols induce endothelium-dependent relaxation (Fitzpatrick et al. 1993; Karim et al. 2000; Chin-Dusting et al. 2001; Woodman et al. 2004). This regulation of vascular nitric oxide is thought to involve the ability of (poly)phenols to interact with kinase signaling pathways such as the PI3-kinase/Akt pathway and intracellular Ca^{+2} on eNOS phosphorylation and subsequent NO production (Lorenz et al. 2004; Stoclet et al. 2004). However, *in vitro* experiment results are of limited value and (poly)phenol *in vivo* effects might not be effectively evaluated.

In humans, 30 min after the consumption of red wine, circulating NO concentration increased to 30-40 nM, respectively. In addition, a reduction of the blood pressure (11 mm Hg) and an increase of heart rate was observed (Matsuo et al. 2001). A study using olive oil, showed a reduction of blood pressure in hypertensive patients, possibly through enhanced NO levels stimulated by (poly)phenols. (Ferrara et al. 2000). Short-term and long-term effects of tea consumption were also investigated (Duffy et al. 2001). This study used water as a control beverage and results showed that both short-term and long-term consumption improved endothelial function. However, this study did not demonstrated any effect on plasma antioxidant capacity, on plasma concentration of F_2-isoprostanes, a marker of systemic lipid peroxidation or on 8-hydroxydeoxyguanosine, a marker of DNA oxidation.

These findings are consistent with several other well-conducted studies that failed to demonstrate a reduction in markers of oxidative stress after tea consumption (O'Reilly et al. 2001).

Several other studies demonstrated that flavonoid-containing beverages have beneficial effects on endothelial function. Stein et al. (1999) observed that consumption of grape juice for 14 days was associated with improved brachial artery flow-mediated dilation among 15 adults with angiographically proven coronary artery disease. A second study from the same group also indicated beneficial effects of purple grape juice on endothelial function (Chou et al. 2001). The effect of cocoa on flow-mediated dilation was also investigated. Among patients with at least one cardiovascular disease risk factor, impaired endothelial function was observed. Two hours after the patients consumed cocoa containing 176 mg/dL flavan-3-ols, the investigators observed a significant increase in flow-mediated dilation. They also observed increased in nitrosylated and nitrosated species in plasma, which suggested an increase in nitric oxide production (Heiss et al. 2003).

Endothelium- and NO-dependent relaxation has been reported for several isolated flavonoids, especially the anthocyanin delphinidin (Andriambeloson et al. 1998) and the flavone chrysin (Duarte et al. 2001). These effects are related to a pro-oxidant effect because it can be inhibited by superoxide dismutase and catalase and a subsequent increased in endothelial cystolic Ca^{2+} levels (Andriambeloson et al. 1998). Using ERα deficient mice, a recent study evidenced an activation of NO pathway leading to the induction of endothelial vasodilatation in aorta endothelial cells by the anthocyanin delphinidin (Chalopin et al. 2010). The authors also demonstrate the implication of the alpha isoform estrogen receptor (ERα) in the transduction of the vascular benefits of (poly)phenols. Actually, silencing the effects of ERα completely prevented the effects of delphinidin to activate NO pathway (Chalopin et al. 2010). Some group have also described that the effects of quercetin were partially endothelium-dependent and related to the release of endothelium-derived relaxing factors (Ajay et al. 2003; Khoo et al. 2010). A pro-oxidant mechanism involving the release of H_2O_2 has been proposed (Khoo et al. 2010).

A complex array of information has been reviewed by Stoclet et al. (2004), proposing a global view of vascular protection by dietary (poly)phenols. Briefly, plant (poly)phenols firstly act on endothelial cells by enhancing the production of vasodilating factors (i.e., NO, EDHF and prostacyclin) and inhibit the synthesis of vasoconstrictor endothelin-1. This

mechanism involves an increase of Ca^{2+} level and redox-sensitive activation of the phosphoinositide 3 (PI3)-kinase/Akt pathway (leading to rapid and sustained activation of nitric oxide synthase and formation of EDHF) and enhance expression of nitric oxide synthase. Secondly, (poly)phenols can operate in smooth muscle cells by inhibiting the expression of two major pro-angiogenic factors: vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2). The mechanism requires both redox-sensitive inhibition of the p38 mitogen-activated protein kinase (p38 MAPK) pathway activation (leading to inhibition of platelet-derived growth factor (PDGF)-induced VEGF gene expression) and redox-insensitive mechanisms (leading to inhibition of thrombin-induced MMP-2 formation). A more recent review by Schini-Kerth et al. (2011), describes the *in vitro* and *in vivo* vascular protection by natural product-derived (poly)phenols during the last 15 years. Grape-derived products (i.e. juices, extracts, wines and marc extracts), berries, tea and plants are able to improve the endothelial function in both *in vitro* and *in vivo* mostly by stimulating the endothelial formation of NO. The *in vitro* experiments are of limited value and cannot be generalized to *in vivo* effects.

II.4.3. (Poly)phenols and platelet function

II.4.3.a. Role of platelets in vascular hemostasis

Physiological hemostasis is a natural protection against excessive blood loss and is based on controlled thrombus formation at the site of damage of blood vessels. Platelets, the smallest (2–4 μ m) blood corpuscles, produced in the bone marrow from megakaryocytes at a rate of 40×10^3/ml/day, play the primary role in hemostasis. The role of platelets in hemostasis is not limited to the formation of platelets but also contribute to clotting reaction. When the endothelium is damaged, collagen and tissue factor (TF) present in the subendothelial matrix interact with the flowing blood, initiating the formation of a clot (Davì et al. 2007). The presence of collagen causes platelet pooling and activation while tissue factor initiates the formation of thrombin, which not only converts fibrinogen into fibrin, but also activates platelets (Furie et al. 2008). The interaction between platelets and the subendothelial layer is possible due to the presence of collagen receptors (including integrin $\alpha_2\beta_1$ and glycoprotein complex GPIb/IX/V) on the platelet surface (Jagroop et al. 2000). Association of these mechanisms lead to the platelet aggregation.

Increased activation of platelets, including their adhesion, secretion, and aggregation at the site of vessel damage or in vessels affected by atherosclerosis, plays an important role

in the pathogenesis of cardiovascular diseases (Jagroop et al. 2007). Chronic atherosclerotic lesions in coronary artery walls are associated with the formation of plaques which may narrow the vessel lumen. Biochemical markers of increase platelet activation include metabolites generated from arachidonic acid converted via cyclooxygenase (COX). Thromboxane A_2 (TXA_2) is the main platelet and a potent vasoconstrictor with proliferative and cell adhesion-stimulating properties. In addition, under conditions of increased platelet activation, two platelet surface molecules are also expressed: integrin $\alpha_{IIb}\beta_3$, a receptor for fibrinogen (GP IIb/IIIa) and P-selectin (Praticò et al. 2009). P-selectin is an adhesion molecule characteristic of platelets and is stored in α-granules of platelets and in endothelial cells. This molecule mediates the red blood flow along the endothelial surface and the activation of leukocyte binding processes to platelets, endothelial cells and other leukocytes at the tissue damage or inflammatory site. An increase of soluble P-selectin (sP-selectin), a procoagulant factor present in blood, is a predictive factor for cardiovascular events (Polek et al. 2009; Kaplan et al. 2011).

II.4.3.b. (Poly)phenols and anti-platelet action

The antioxidant action of (poly)phenols is important from the point of view of platelet activation, including platelet adhesion and aggregation. As mentioned above, the first stage of platelet activation is platelet adhesion to collagen. The interaction between platelet receptors and adhesion proteins, including fibrinogen, fibronectine, vitronectin and thrombospondin, mediates the adhesion of platelets, the triggering of the intracellular signaling pathway and as a consequence, platelet activation (Jennings 2009). The mechanisms responsible for the inhibitory effect of (poly)phenols on platelet adhesion have not been fully elucidated.

The platelet anti-aggregant effects of flavonols were initially described by Beretz et al (1982). Study reported the inhibition of platelet adhesion to collagen by extracts rich in (poly)phenolic compounds such as grape seed extracts. Resveratrol and its derivatives present in the extracts could reduced the adhesion of platelets stimulated by thrombin. Chokeberry fruit extract have also showed to display a similar effect. Integrin $\alpha_2\beta_1$ (GPIa/IIa) and glycoproteins GPIV and GPVI are responsible for the adhesion of platelets to collagen. An additional receptor for collagen may also be the $\alpha_{IIb}\beta_3$ receptor (Olas et al. 2008). (Poly)phenol effects on platelet adhesion is believed to be due to their capacity to regulate the expression of these receptors and the potential interaction with them.

In addition, (poly)phenols target molecule may be both cyclooxygenase and lipoxygenase (Vanhoutte 2009). Flavonols present in cocoa have been found to reduce TXA_2 synthesis. Moreover, they have also shown to inhibit the activity of phospholipase C, limiting the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which are secondary messengers in platelets (Nardini et al. 2007). Some flavonoid compounds, for example quercetin, were able to stimulate adenylate cyclase and guanylate cyclase and to inhibit phosphodiesterase, contributing to an elevation of cAMP and cGTP levels, limiting the activation of platelets. *In vitro* studies indicated that resveratrol was able to block calcium channels and inhibited Ca^{2+} ion flux on platelets activated by thrombin, while quercetin inhibited the intracellular mobilization of ca^{2+} from the endoplasmic reticulum. This is particular importance from the point of view of the anti-platelet properties of (poly)phenols, since many platelet enzymes are calcium dependent (Nardini et al. 2007).

It has been previously suggested that the inhibition of platelet reactivity by wine may partly explain some of its cardioprotective effects, for example, the French paradox as mentioned earlier (Renaud et al. 1992). Once red wine and grape juice, but not white wine were infused intravenously and intragastrically into anesthetized dogs, coronary artery blood flow parameters and inhibited platelet aggregation were improved (Demrow et al. 1995). In another study involving 10 subjects, the effects of drinking purple grape juice, grapefruit juice and orange juice for one week were examined (Keevil et al. 2000). Platelet aggregation responses to collagen were significantly reduced in the grape juice group only and appear to reflect the total phenolic contents of the test components. In fact, the total phenolics expressed as gallic acid equivalent were 2.26, 0.75 and 0.86 g/L for purple grape juice, orange juice and grape fruit, respectively. Another human study demonstrated a decrease in platelet aggregation, increase in platelet-derived NO release and a decrease in superoxide formation, both *in vitro* and after oral supplementation with purple grape juice, confirming the absorption and bioavailability of the bioactive compounds from purple grape juice (Freedman et al. 2001).

II.4.4. (Poly)phenols and anti-inflammatory properties

In cardiovascular diseases, important inflammatory processes take place and are associated with dysfunction of endothelial cells with accumulation of monocytes/ macrophages in the vascular intima, the formation of foam cells, and the proliferation of vascular smooth muscle (Wong et al. 2012). Many pro-inflammatory cytokines are release

such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, tumor necrosis factor (TNF-α), interferon-g (INF-γ), growth factors (e.g., granulocyte- macrophage colony-stimulating factor [GM-CSF], macrophage colony-stimulating factor [M-CSF], platelet- derived growth factor [PDGF]). In addition, other inflammation mediator factors are also release and can intensify the growth process and destabilization of the atherosclerotic plaque, such as the monocyte chemotactic protein-1 (MCP-1) and matrix metalloproteinases (MMPs) (Libby 2002).

Recent studies showed that catechins are able to inhibit the adhesion and migration of neutrophils through the monolayer of endothelial cells by inhibiting the production of chemokines at the site of inflammation and reducing the expression of VCAM-1 (Ludwig et al. 2004; Takano et al. 2004). Kawai and colleagues (2004) demonstrated that flavan-3-ols contributed to the reduced expression of CD11b molecules on monocytes and granulocytes, a circumstance which limited the recruitment of these cells to the inflammation site. In humans, Badia et al. (2004), observed that moderate red wine consumption was able to reduce the adhesion of monocytes to endothelial and associated this effect with the regulation of adhesion molecules located of the surface of the monocytes.

Nuclear factor kappa beta (NF-κB) can regulate the inflammatory process by modulating expression of pro-inflammatory genes. This protein is likely the major molecular target for the anti-inflammatory effects of (poly)phenols in the vasculature. Several mechanisms may be involved in the inhibition of NF-κB by (poly)phenols, and these compounds may inhibit ROS-mediated NF-κB activation. The efficient transcriptional activation of NF-κB depends on the phosphorylation of its active subunit P65, and (poly)phenols have showed to block P65 phosphorylation, rendering NF-κB transcriptionally inactive (Manna et al. 2000). Moreover, overexpression of SIRT1 mimic the effects of some (poly)phenols, including resveratrol (Kaeberlein et al. 2005). It has also been shown that resveratrol could promoted the deacylation of P65 by SIRT1 (Yeung et al. 2004). In addition, it was evidenced that curcumin was able to block cytokine-mediated NF-κB activation and pro-inflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity (Jobin et al. 1999).

II.4.5. (Poly)phenols and anti-atherogenic properties

One of the most frequently studied beneficial effects of (poly)phenols is their ability to improve lipid profile. The enzymes and free radicals released by immune system cells, platelets, and endothelial cells modify native LDLs by oxidation. Oxidized LDLs (oxLDLs)

are responsible for further development and destabilization of atherosclerotic plaque. Oxidative stress produces an increase in enzymes such as cyclooxygenase (COX) and lipooxygenase (LPO), which are implicated in the release of factors such as chemokines, pro-inflammatory substances, growth factors, free radicals, TF and proteolytic enzymes specialized in the digestion of connective tissue elements (MMPs) and other factors that have direct chemotactic properties for monocytes to adhere to the endothelium (Aviram et al. 1994). The accumulation of macrophage in this area eliminated the oxLDLs molecules but also provokes an inflammatory response, with requisite cell recruitment and proliferation accompanied by migration of smooth muscle cells. Oxidized LDL is preferentially taken up by macrophage cells via scavenger receptors, and they consequently become loaded with lipids and convert into "foam cell" (Aviram 1996). Extracellular matrix deposits increase around the inflamed area, and this permits the formation of so called atheroma plaque, which more or less blocks the vessel. Along with these processes, vasoconstriction episodes occur, caused by inhibition of NO formation and loss of arteries' natural relaxation capacity (Ross 1999).

The beneficial effects of (poly)phenols on atherosclerosis have been widely studied and it was proposed that these compounds are able to attenuate the onset and development of the disease thanks to their ability to limit LDL oxidation. Numerous studies investigated the protective effect of flavan-3-ols, both monomeric and oligomeric, against LDL oxidation (Fuhrman et al. 2001; Auger et al. 2004). The development of foam cells in the aorta is a good model and indicator of atherosclerotic lesions. Using a hamster model of atherosclerosis, Vinson et al. (2002) found that grape seed proanthocyanidins induced a pronounced reduction in plasma cholesterol (25%) and triglyceride levels (up to 34%). Accompanying these changes was a reduction in the percentage of aorta covered in foam cells. The latter was reduced by 50% and 63% after supplementation of the animals with 50 and 100 mg/kg grape seed proanthocyanidins, respectively. Furthermore, these beneficial effects were associated with a significant decrease in plasma lipid peroxidation levels.

In a randomized, double-blind, placebo-controlled study, grape seed proanthocyanidin extracts was given to 40 hypercholesterolemic patients for 8 weeks (Bagchi et al. 2003). There was a significant reduction in LDL-c levels and total cholesterol levels for the grape seed proanthocyanidins group, but only when this group was additionally supplemented with niacin-bound chromium. This phenomenon suggested that the mechanisms of action are complex and may require other "factors" for nutritional benefit.

Apolipoprotein $E^{-/-}$ ($ApoE^{-/-}$)-deficient mice represent a good model for atherosclerosis. They are characterized by accelerated development of atherosclerosis and are more susceptible to oxidative stress. Grape extract effects were extensively considered. Fuhrman et al. (2005) investigated atherosclerotic lesions using $ApoE^{-/-}$deficient mice following the dietary supplementation of freeze-dried extracts of fresh grapes. For $ApoE^{-/-}$-deficient mice that consumed 150 μg total (poly)phenolics per day for 10 weeks, a 41% reduction in the atherosclerotic lesion area was observed compared with control (no supplements) or placebo (glucose and fructose supplementation) groups. This effect was associated with a significant reduction in serum oxidative stress as indicated by an 8% reduction in plasma lipid peroxide concentration and an increase in antioxidant capacity (16%-20%) as well as a reduction in macrophage uptake of oxidized LDL (33%). Another study by Frederiksen et al. (2007) using Watanabe heritable hyperlipidemic rabbits demonstrated that consumption of a red grape skin extract was associated with a delay of the development of aortic atherosclerosis in male rabbits but not females, as determined by cholesterol content within the abdominal aorta.

Anti-atherogenic effects were also observed with other type of fruits and beverages. Administration of pomegranate juice, rich in anthocyanins, and ellagitannins such as punicalagin, to $apoE^{-/-}$ mice resulted in dramatic reductions in lipid peroxides and macrophage accumulations, without significantly affecting plasma cholesterol. After three months of pomegranate juice supplementation, atherosclerosis was reduced by 44% (Aviram et al. 2008). In another study, administration of pomegranate by-product to $apoE^{-/-}$ mice attenuated atherosclerosis development as a result of decreased macrophage oxidative stress and cellular uptake of oxidized LDL (Rosenblat et al. 2006).

It has been demonstrated that resveratrol, impeded LDL oxidation and lowered cytotoxicity caused by oxidized LDL in endothelial cells (Delmas et al. 2005). Using the same $apoE^{-/-}$ mouse model, four-month supplementation of a regular chow diet with resveratrol led to a reduction in total plasma cholesterol and LDL-c, and an increase in HDL cholesterol. The mechanism for the reduction in plasma cholesterol was through a reduction in hepatic cholesterol synthesis, which may have stimulated LDL receptor-mediated uptake of LDL from plasma (Do et al. 2008). Berrougui et al. (2009) showed in an *in vitro* experiment that resveratrol could prevented lipid peroxidation and increased cholesterol efflux from macrophages. Through these mechanisms, resveratrol significantly reduced atherosclerotic plaque development in the aortic arch of $apoE^{-/-}$ mice.

The reduction of vascular inflammation, the prevention of leukocyte adhesion, the inhibition of vascular smooth muscle proliferation and the stimulation of NO production may also contribute to the anti-atherosclerotic effects of (poly)phenols. Nie et al. (2006) showed that avenanthramides, polyphenols found in oats (*Avena sativa L.*), might contributed to the prevention of atherosclerosis through inhibition of smooth muscle proliferation and increasing NO production. A recent study by Choi et al. (2009), suggested that through its ability to inhibit type A scavenger receptors and CD36 (Cluster of Differentiation 36) on the macrophage surface, quercetin reduced oxLDLs uptake and absorption. Moreover, quercetin contributed to the production of pro-inflammatory and pro‑atherogenic vascular endothelial growth factor (VEGF) and inhibited the expression of MIP-2 (macrophage-inflammatory protein-2) and MCP-1. Quercetin has also been shown to reduce the activation of PPAR-γ (peroxisome proliferatoractivated receptor gamma) participating in the regulation of CD36 receptor expression on macrophages (Choi et al. 2009).

II.4.6. Conclusion on health effects

Although the beneficial effects, controversial results were found. Current limited evidence suggested that fruits containing relatively high concentrations of flavonols, anthocyanins and procyanidins, such as pomegranate, purple grapes and berries, were more effective at reducing CVD risk, particularly with respect to anti-hypertensive, inhibition of platelet aggregation and increasing endothelial-dependant vasodilatation than other fruits investigated. In fact, one of the reasons why it is difficult to draw a clear conclusion from the current evidence is the heterogeneity in study design. (Poly)phenols were mainly consumed in the form of fruit juices or fruits and a small number of studies provided (poly)phenols in the form of supplements (Conquer et al. 1998; Clifton 2004; Hubbard et al. 2004; Gorinstein et al. 2006). Between studies, duration of study period ranged from weeks to months and the dose of (poly)phenols investigated was not consistent between studies. Moreover, the dose administrated is often high and exceeds the dietary intake.

In addition, the types of subjects recruited differed, with some studies using healthy volunteers while others recruited subjects at risk of CVD or with CVD (Stein et al. 1999; Kurowska et al. 2000; Sumner et al. 2005; Gorinstein et al. 2006; Erlund et al. 2008; Wilson et al. 2008). It appears that observed effects were generally more marked in subjects with higher CVD risks. Moreover, subject compliance should also be considered because this could impact significantly the results. Difference in the degree of dietary compliance could

account for inconsistencies in the results obtained from various studies. Most of the time, compliance including diet diaries, log books, frequent interviews by the researchers and biomarkers, was not assessed.

Besides, analytical methodologies should be standardized to allow valid comparison between studies. This is particularly important in the quantification of specific (poly)phenols in fruits. Differences in the concentration of the bioactive flavonoid components within the fruits ingested in the various studies have been consistently highlighted as a possible factor influencing the variable effects of similar types of fruits/fruit juices. In some investigations, details of the (poly)phenols content of the fruits investigated were omitted and thus, generating inconsistent results.

All these factors may be the reasons for heterogeneous results and have to be considered for an acute investigation of (poly)phenol health effects. Long intervention periods and subject's compliance to treatments should be encouraged and evaluated in future studies. Improvements and standardization in the methodology for quantifying the (poly)phenolic content of fruits have to be taken into account.

III. Hypertension and treatments

III.1. Blood pressure

Blood pressure is defined as the force of blood exerts against the arteries wall. It is measured as millimeters of mercury (mm Hg) and recorded as two numbers: systolic pressure (i.e. rhythmic contraction of the heart) and diastolic pressure (i.e. relaxation and dilatation of the heart between two beats and refill with blood). An individual has a normal blood pressure when its systolic blood pressure (SBP) is less than 120 mm Hg and its diastolic blood pressure (DBP) less than 80 mm Hg. Cardiac output, peripheral resistance and blood volume are the three main factors influencing BP following this equation:

$$\text{Blood Pressure} = \text{cardiac output} \times \text{peripheral resistance}$$

Any change in cardiac output, peripheral resistance and blood volume will lead to a change in blood pressure.

III.2. Regulation of blood pressure

III.2.1. Short-term regulation

Generally, the regulatory system functions to correct deviations of arterial blood pressure from the setpoint value. Such a system requires three components:

• Sensors that respond to a pressure-related variable and convey an appropriately coded signal to an evaluator.

• Evaluators translate the incoming code from the sensors, compare the existing blood pressure with the setpoint and issue the appropriate commands for compensatory actions.

• Effector mechanisms bring changes in heart rate, cardiac performance and total peripheral vascular resistance to reduce the difference between existing blood pressure and setpoint.

The short term regulation occurs rapidly within second in response to posture, acute stresses and hemorrhage. Short term control of BP is mediated by the nervous system and need the contribution of the three important factors listed above (i.e; the sensors, the evaluators and the effectors).The dominant short-term regulator of arterial blood pressure is

the baroreceptor mechanism. The baroreceptors are unencapsulated nerve endings located in the wall of the carotid sinus and the aortic arch. They monitor the BP coming from the aorta and carotid artery (Ottesen et al. 2011). Stretch sensors in the cardiac atria and the stretch-sensitivity of a few cells in the juxtaglomerular apparatus of renal afferent arterioles also participate in this regulation. The evaluators are mostly situated in the pons/medulla region of the midbrain, particularly in the neurons of the nucleus tractus solitaries.

Arterial blood pressure is sensed by baroreceptors which translate wall stretch into action potentials that are conveyed to the midbrain region by fibres in the glossopharyngeal and vagus nerves. Neurons in the pons and medulla regions of the midbrain compare existing blood pressure against the setpoint, which represents the desirable blood pressure under the circumstances. If there is a deviation of arterial blood pressure from the setpoint then neurohumoral signals are generated and conveyed to the periphery in order to make appropriate corrective changes in heart rate, cardiac stroke volume and total peripheral vascular resistance (Convertino 1998). Figure 19 summarize this mechanism.

In order to ensure this regulation, the effectors have to complete these following mechanisms:

1/ Increase the production of angiotensin II (Ang II), a powerful vasoconstrictor via the renin-angiotensin cascade. Ang II is mediated by the activation of AT_1 receptors in the membranes of vascular smooth muscle cells. Activation elevates levels of intracellular calcium ions and leads to vasoconstriction. Angiotensin II also increases renal retention of sodium ions and water by acting on receptors in the proximal and late distal convoluted tubule of the nephron and by promoting the synthesis and release of the salt-retaining hormone aldosterone, from adrenal cortical zona glomerulosa cells .

2/ Synthesize vasopressin by the paraventricular and supraoptic nuclei of the hypothalamus which are transported along axons towards the posterior pituitary, and released by exocytosis into the circulation. Vasopressin result from the activation of V_1 receptors in vascular smooth muscle and such activation causes vasoconstriction. Vasopressin also acts on V_2 receptors in the cortical collecting duct of the nephron. Such action results mostly in the insertion of aquaporins into the luminal membrane and leads to increased reabsorption of water from the nephrons (Toba et al. 1999).

3/ Stimulate cardiac parasympathetic outflow from the neurons of the nucleus ambiguus and conveyed to the heart by the way of the vagus nerve. Efferent fibres of the vagus nerves innervate the cardiac pacemaker cells and, to some extent, the atrial myocytes and conductive tissue of the cardiac ventricles. Action potentials result in the release of acetylcholine from the terminal butons and subsequent activation of M_2 muscarinic receptors, which leads mainly to a decrease in heart rate.

4/ Stimulate sympathetic nervous outflow from the neurons of the rostral ventrolateral medulla. Potential action is translated into release of adrenaline from chromaffin cells in the adrenal medulla, renin release from renal juxtaglomerular cells, and noradrenaline release at peripheral synapses, which are found in cardiac myocytes and the vascular smooth muscle that surrounds blood vessels. Subsequent cardiovascular effects of adrenaline and noradrenaline are initiated when these catecholamines activate α and β adrenoreceptors located in cell membranes of the target tissues. The final effects of increased sympathetic nervous activity are the increase of heart rate and cardiac contractility by β1- activation and the increase of vascular resistance by α1-activation.

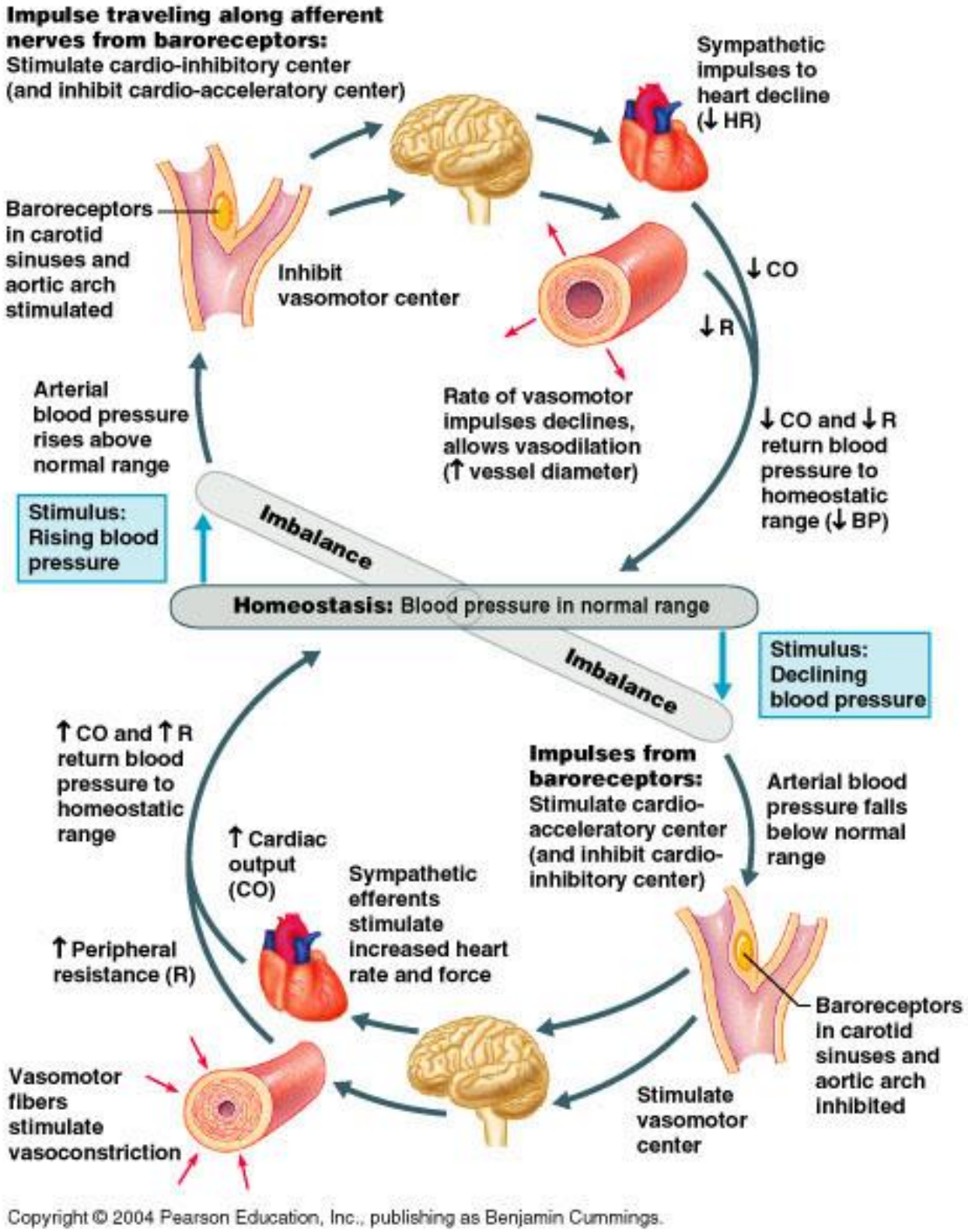


Figure 19: Baroreceptor mechanism of blood pressure control

III.2.2. Long term regulation

The long-term regulation system is mainly maintained by kidney and the renin-angiotensin system.

Early experiments suggested that hypertension develops when a small defect in kidney function causes renal retention of sodium ions and water, increases the volume of extracellular fluid and causes arterial blood pressure to rise to a level at which the pressure natriuresis mechanism can again maintain a balance between intake and output of sodium ions and water. It appears, therefore, that the long-term-controlled variable is not arterial blood pressure, but the balance between intake and output of fluid and electrolytes. While several organs excrete water and salt, only the kidney excretes them in a controlled manner. Kidneys have the capability to regulate the total body content of sodium ions and water. A key feature of the renal body fluid feedback control system is pressure natriuresis or the ability of the kidneys to respond to changes in arterial pressure by altering the renal excretion of salt and water. The nervous system induced changes in peripheral resistance and cardiac output, which are essential for rapid regulation of arterial pressure (Blaustein 1977; Hall 2003).

The sensitivity of the pressure natriuresis mechanism can be modified by a number of extra-renal neurohormonal regulatory systems. For instance, the renin-angiotensin system is one of the hormonal system which is particularly important to arterial pressure homeostasis (Hall et al. 1990). The renin-angiotensin system is a nonadapting hormonal mechanism that chronically alters the sensitivity of pressure natriuresis. As arterial pressure or sodium intake increases, the renin-angiotensin system is suppressed, which enhances the ability of the kidneys to excrete salt and water. Conversely, when either arterial pressure or sodium intake is reduced, high endogenous levels of Ang II decrease renal excretory function, which promotes sodium retention. In the absence of appropriate changes in the renin-angiotensin system, there is an abnormal shift in the pressure natriuresis relationship, resulting in sustained alterations in arterial pressure.

Angiotensin II (Ang II) plays an important role in the regulation. Ang II is produced in an enzymatic cascade which starts when the acid protease, renin, cleaves 4 amino acids from the terminal end of angiotensinogen, a 14-amino acid plasma globulin produced mainly in the liver. This step forms angiotensin I (Ang I), which has little biological activity. Angiotensin-converting enzyme (ACE), located on the plasma membrane of endothelial cells,

cleaves two amino acids from Ang I and produces Ang II. The major source of renin is the juxtaglomerular cells of the renal afferent arteriole, but a variety of tissues produce small quantities of renin that can have large local significance. Small quantities of renin may be significant if the local action involves a tissue (e.g., kidney, rostral ventrolateral medulla) and has the capacity to influence systemic function. The actions of angiotensin are initiated by its interaction with the membrane receptors AT_1 and AT_2. The most apparent actions of Ang II are due to activation of the AT_1 receptor (Oliverio et al. 1997; Zhuo et al. 2011).

III.3. Hypertension

Hypertension is the most important of cardiovascular risk factors worldwide. According to the World Health Organization 2012 (WHO 2012) data, hypertension accounted for approximately 9.4 million deaths a year, contributing to 45% of deaths due to heart disease and 51% of deaths due to stroke. The prevalence of hypertensive people under 25 years of age is low (5.5% in young men and 1.2% in young women) but steadily increases to ~40 % in 65 year olds and 90% at 85 years of age. It is estimated that by 2030, more than 23 million people will die annually from cardiovascular diseases (CVDs).

According the Mona Lisa epidemiological study established between 2005 and 2007 in France (Wagner et al. 2011), the age-adjusted prevalence of hypertension was 47% in men and 35% in women aged 35–74 years. These values were similar to the rates reached 44.8% and 30.6% in Italy, and 44.8% and 32.0% in Sweden. They were much lower than those reported in Germany (55.3%) or Finland (48.7%) but higher than that of North America (27.6%) (Wolf-Maier et al. 2004). Hypertension is one of the most prevalent modifiable risk factors for coronary heart disease, stroke, congestive heart failure and renal insufficiency, and clinical trials have demonstrated the efficacy of lowering blood pressure (BP) to decrease cardiovascular morbidity and mortality. The importance of oxidative stress, vascular inflammation and endothelial dysfunction has to be highlighted in the development of CVDs. The knowledge of the process has provided new perspectives to elaborate novel pharmaceutical or dietary strategies to control the development of vascular diseases.

The Sixth Report of the Joint National Committee on Prevention (1997), Detection, Evaluation, and Treatment of High Blood Pressure (JNC VI) defined and classified hypertension in adults, as shown in Table 5.

Table 5: Definition and classification of blood pressure levels according to the JNC VI

Category	Systolic, mm Hg		Diastolic, mm Hg
Optimal	< 120	and	< 80
Normal	< 130	and	< 85
High normal	130-139	or	85-89
Hypertension			
Stage 1 (mild)	140-159	or	90-99
Subgroup: borderline	140-149	or	90-94
Stage 2 (moderate)	160-179	or	100-109
Stage 3 (severe)	≥ 180	or	≥ 110
Isolated systolic hypertension	≥ 140	and	< 90
Subgroup: borderline	140-149	and	< 90

The diagnosis of hypertension is made when the average of 2 or more DBP measurements on at least 2 subsequent visits is ≥ 90 mm Hg or when the average of multiple SBP readings on 2 or more subsequent visits is consistently ≥ 140 mm Hg. Isolated systolic hypertension is defined as SBP ≥ 140mm Hg and DBP < 90 mm Hg. Individuals with high normal BP tend to maintain pressures that are above average for the general population and are at greater risk for development of definite hypertension and cardiovascular events than the general population. Hypertension prevalence changes according to different factors:

• **Race**: according to WHO, the prevalence of raised blood pressure was higher in the African region where it was 46% for both sexes combined and lower in the WHO region of the Americas, with 35% for both sexes.

• **Age**: in industrialized countries, SBP rises throughout life whereas DBP rises until age 55 to 60 years and thus the greater increase in prevalence of hypertension among the elderly is mainly due to systolic hypertension.

• **Geographical patterns**: hypertension is more prevalent in industrialized countries than rural ones.

• **Gender**: men had a slightly higher prevalence than women. In all WHO regions, men have slightly higher prevalence of raised blood pressure than women, but this difference was only statistically significant in the region of the Americas and the European region.

• **Socio-economic status**: the prevalence of raised blood pressure was consistently high, with low-, lower-middle- and upper-middle-income countries, all having rates of around 40% for both sexes. The prevalence in high-income countries was lower, at 35% for both sexes (WHO).

Two different type of hypertension exists: the essential or primary hypertension and the secondary hypertension. Essential hypertension is defined as high BP in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or mendelian forms (monogenic) are not present. Essential hypertension accounts for 85% of all cases of hypertension. Essential hypertension is a heterogeneous disorder, with different patients having different causal factors leading to high BP.

The evolution of gene sequencing techniques permit to provide information on genetic variations or genes that are overexpressed or underexpressed as well as the intermediary phenotypes that they regulate to cause high BP (Luft 1998). The elevation of blood pressure is multifactorial in origin, and probably represents a complex interaction of multiple genetic traits with lifestyle factors including obesity, insulin resistance, high alcohol intake, high salt intake (in salt-sensitive patients), aging, sedentary lifestyle, stress, low potassium intake and low calcium intake (Dyer et al. 1989; Sever et al. 1989). Furthermore, many of these factors are additive, such as obesity and alcohol intake.

The Seventh Report of the Joint National Commission on the Evaluation and Treatment of High Blood Pressure recommends considering secondary causes of hypertension if blood pressure cannot be controlled (<140/90 mm Hg) by a combination of three drugs (Chobanian et al. 2003). Secondary hypertension has been encountered with increasing frequency. The common causes of secondary hypertension include renal parenchymal disease, renal artery stenosis, primary aldosteronism, phaeochromocytoma, and Cushing's syndrome.

III.4. Mechanisms of hypertension

Many pathophysiologic factors have been implicated in the genesis of essential hypertension: increased sympathetic nervous system activity, perhaps related to heightened exposure or response to psychosocial stress; overproduction of sodium-retaining hormones and vasoconstrictors; long-term high sodium intake; inadequate dietary intake of potassium and calcium; increased or inappropriate renin secretion with resultant increased production of Ang II and aldosterone; deficiencies of vasodilators, such as prostacyclin, nitric oxide and the natriuretic peptides; diabetes mellitus; insulin resistance; obesity; increased activity of vascular growth factors; alterations in adrenergic receptors that influence heart rate, inotropic properties of the heart, and vascular tone; and altered cellular ion transport. Figure 20

facilitated the visualization of all these mechanisms. Recently, investigations also considered endothelial dysfunction, increased oxidative stress, vascular remodeling, and decreased compliance as a cause of hypertension and contribute to its pathogenesis.

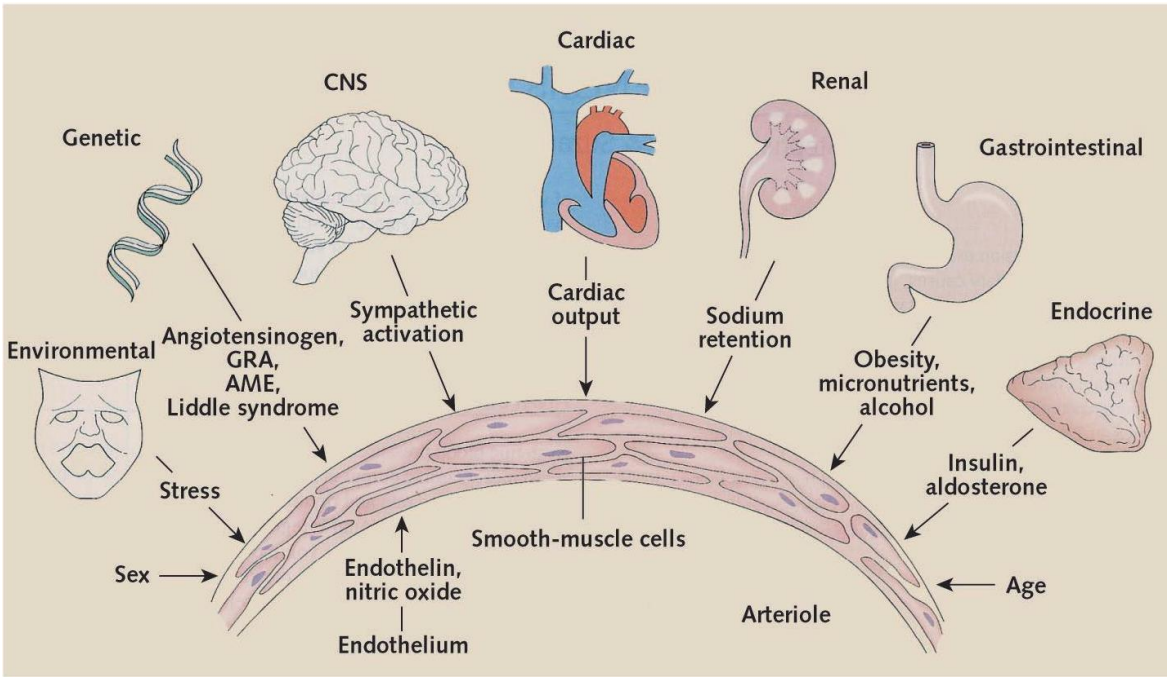


Figure 20: Pathophysiologic mechanisms of hypertension (Oparil et al. 2003)

In a review by Oparil et al. 2003, the authors provide a clear understanding of the pathogenesis of hypertension and underlined important factors contributing to its development.

III.4.1. Genetics

Oparil et al. 2003, firstly highlighted genetics evidence. Improvement of genetic analysis, especially genome-wide linkage analysis have enabled a search for genes that contribute to the development of primary hypertension in the population. Preliminary investigations evidenced that genetic might contribute to the development of hypertension. For instance, the most promising findings concern the genes of the renin–angiotensin–aldosterone system, such as the *M235T* variant in the angiotensinogen gene, which has been associated with increased circulating angiotensinogen levels and blood pressure in many distinct populations (Corvol et al. 1999; Staessen et al. 1999), and a common variant in the

angiotensin-converting enzyme (ACE) gene that has been associated in some studies with blood pressure variation in men (Fornage et al. 1998; O'Donnell et al. 1998).

III.4.2. Sympathetic nervous system

As previously described, sympathetic nervous system plays a key role in the regulation of a normal blood pressure. An important activity of the sympathetic nervous system activity increases blood pressure and contributes to the development and maintenance of hypertension through stimulation of the heart, peripheral vasculature, and kidneys, causing increased cardiac output, increased vascular resistance, and fluid retention (Mark 1996). Indeed, increased of the sympathetic nervous system activity in hypertension involve alterations in baroreflex and chemoreflex pathways at both peripheral and central level. In a normotensive individual, baroreflex function helps maintain reductions in arterial pressure by activating the sympathetic nervous system which has a direct action by increasing the heart rate and cardiac output and an indirect action via the stimulation of adrenalin and noradrenalin. In hypertensive patient, arterial baroreceptors are reset to a higher pressure, resulting in suppression of sympathetic inhibition after activation of aortic baroreceptor nerves (Guo et al. 1984b).

This baroreflex resetting seems to be mediated, at least partly, by a central action of Ang II (Guo et al. 1984a). Ang II also amplifies the response to sympathetic stimulation by a peripheral mechanism, that is, presynaptic facilitatory modulation of norepinephrine release. Additional small-molecule mediators that suppress baroreceptor activity and contribute to exaggerated sympathetic drive in hypertension include reactive oxygen species and endothelin (Chapleau et al. 1992; Li et al. 1996). In addition, chronic sympathetic stimulation induces vascular remodeling and left ventricular hypertrophy, by direct and indirect actions of norepinephrine on its own receptors, as well as on release of various trophic factors, including transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF-1), and fibroblast growth factors (FGF) (Brook et al. 2000).

III.4.3. Vascular remodeling

Peripheral vascular resistance is characteristically elevated in hypertension because of alterations in structure, mechanical properties, and function of small arteries. Remodeling of these vessels contributes to high blood pressure and its associated target organ damage (Folkow 1982; Mulvany et al. 1990). Peripheral resistance is determined at the level of the

precapillary vessels, including the arterioles (arteries containing a single layer of smooth-muscle cells) and the small arteries (lumen diameters < 300 µm). The elevated resistance in hypertensive patients is related to decrease in number of parallel-connected vessels and narrowing of the lumen of resistance vessels.

III.4.4. Nitric oxide (NO)

Nitric oxide has an important role in the vascular maintenance because of its potent vasodilator function, its inhibitor of platelet adhesion and aggregation role and its capacity to suppress migration and proliferation of vascular smooth-muscle cells. NO is produced during the metabolism of L-arginine by NO synthase (eNOS) (Govers et al. 2001). Reduced NO has often been reported in the presence of impaired endothelial function and may result from reduced activity of endothelial NO synthase and the decrease of NO bioavailability. ROS are known to quench NO with formation of peroxynitrite (Koppenol et al. 1992), which is a cytotoxic oxidant. Through nitration of proteins, peroxinitrite will affect protein function and therefore endothelial function. Peroxynitrite is an important mediator of oxidation of LDL and can causes degradation of the eNOS cofactor tetrahydrobiopterin (BH_4) (Milstien et al. 1999), leading to "uncoupling of eNOS". When this phenomenon occurs, formation of the active dimer of eNOS with oxygenase activity and production of NO is reduced. The reductase function of eNOS is activated and more ROS are formed. As a result, NO synthase goes from its oxygenase function producing NO to its reductase function producing ROS, with the consequent exaggeration of oxidant excess and deleterious effect on endothelial and vascular function.

III.4.5. Endothelin-1 and other factors

In addition to being the main determinant of basal vascular smooth muscle tone, NO opposes the actions of potent endothelium-derived contracting factors such as Ang II and endothelin-1 (ET-1). Nitric oxide inhibits platelet and leukocyte activation and maintains the vascular smooth muscle in a nonproliferative state. Beside its production of Ang II, prostaglandin endoperoxides, the endothelium is the source of the potent vasoconstrictor peptide ET-1 described as the most potent vasoconstrictor known and acts mainly in a paracrine manner by binding to two G-protein coupled receptors, ETA and ETB, which are located on endothelial cells (ETB), vascular smooth muscle cells, and fibroblasts (ETA and ETB). Endothelial ETB receptors can elicit endothelium-dependent relaxation by inducing NO release, whereas ETA and ETB receptors located on smooth muscle cells and fibroblasts

trigger vasoconstriction, cell proliferation, inflammation, and fibrosis. Importantly, ET receptor distribution has been shown to be modified in pathological conditions. ET-1 increases the vascular actions of others vasoactive peptides such as Ang II, norepinephrine, and serotonin; participates actively in leukocyte and platelet activation; and facilitates a prothrombotic and proatherogenic phenotype (Govers et al. 2001; Aird 2005).

Oxidative excess is also linked to a proinflammatory state of the vessel wall. ROS upregulate adhesion (VCAM-1 and ICAM-1) and chemotactic molecules (macrophage chemoattractant peptide-1 [MCP-1]) (Griendling et al. 2003). Reduced NO bioavailability, increased oxidant excess and expression of adhesion molecules contribute not only to the initiation but also to the progression of atherosclerotic plaque formation and triggering of cardiovascular events.

III.4.5. Endothelial dysfunction

The endothelium, the largest organ in the body, is strategically located between the wall of blood vessels and the blood stream. It senses mechanical stimuli, such as pressure and shear stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect hemostasis. Among the vasodilatory substances produced by the endothelium are nitric oxide (NO), prostacyclin, different endothelium-derived hyperpolarizing factors (EDHF), and C-type natriuretic peptide. Vasoconstrictors include endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A_2 (TXA_2), and reactive oxygen species (ROS) (Panza et al. 1990). The endothelium also contributes to mitogenesis, angiogenesis, vascular permeability, and fluid balance. Inflammatory modulators include NO, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM- 1), E-selectin, and NF-κB.

A chronic imbalance between endothelium-derived relaxing and contracting factors could lead to an abnormal vasodilating response and to high BP. Under physiological conditions, the vascular endothelium responds to mechanical and biochemical agonists by producing antiplatelet, anticlotting, fibrynolitic, vasodilating, and vasoconstricting factors (Deanfield et al. 2005). It basically involves either an increase/decrease in any of the endothelial cell-related chemical messenger and/or by alteration in any of the functional changes. Some examples of endothelial cell dysfunction include an increased permeation of macromolecules, increased or decreased production of vasoactive factors producing abnormal vasoconstriction/vasodilation, and increased prothrombotic and/or procoagulant activity (Vita

et al. 2002; Aird 2005; Förstermann et al. 2006). However, the most commonly accepted endothelial cell dysfunction alteration relates to abnormalities in the regulation of the lumen of vessels.

The best known endothelium-derived relaxing factors are nitric oxide, endothelium-derived hyperpolarizing factor, and the prostanoid prostaglandin I2 (prostacyclin, PGI2). Together with NO, EDHF contributes to relaxation of large conducting arteries, and appears to be a major determinant of vascular resistance in small arteries (Govers et al. 2001). Several studies have provided evidence that eNOS is important in the regulation of vascular functions. Oxidative stress in the vascular wall and vascular hypertrophy might contribute to decrease NO bioavailability and, thus, blunted endothelium-mediated vasodilation. When the homeostasis between endothelium-derived relaxing and contracting factors is disrupted, it predisposes the vasculature to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation, vascular inflammation, and atherosclerosis. Endothelial dysfunction has been described in many cardiovascular and metabolic disorders such as hypertension, coronary heart disease, dyslipidemia, and types 1 and 2 diabetes. Endothelial dysfunction appears to precede the clinical manifestations of many of these cardiovascular disorders (Govers et al. 2001; Endemann et al. 2004).

III.4.6. Dietary sodium and potassium

In today's society, Western industrialized society diets contain much more salt than truly required in order to ensure nutrient adequacy. The majority of daily salt intake (75–80%) comes from processed foods, while only 12% comes from natural sources, and approximately 10% comes from salt added during food preparation or consumption (Mattes et al. 1991). The most accurate measure of dietary salt intake is the amount of sodium excreted in a 24-h urine collection with over 90% of dietary sodium intake is excreted renally (Holbrook et al. 1984).

Dietary sodium, mostly consumed as sodium chloride (NaCl), has been considered the main environmental determinant of hypertension pathogenesis (Jones 2004). It is currently well established that increased sodium intake is related to hypertension (Jones 2004). Kidneys play fundamental role for sodium handling and long-term blood pressure homeostasis (Guyton et al. 1972). The major pathophysiological mechanisms underlying the positive association between salt and blood pressure include intravascular fluid volume expansion and vascular dysfunction, both resulting from increased sodium intake (Meneton et

al. 2005; Safar et al. 2009). The salt-induced rise in blood pressure is followed by a phenomenon called pressure natriuresis, in which increased renal perfusion pressure leads to increased excretion of sodium and fluid. In essential hypertension, renal sodium excretion is functionally defective. It has been hypothesized that essential hypertension is mainly a genetic disorder, involving several individual genes that regulate renal sodium handling, which becomes clinically expressed in the context of an unhealthy dietary environment characterized particularly by excessive salt intake (O'Shaughnessy et al. 2004).

Experimental and clinical studies have revealed that, besides affecting blood pressure, high sodium intake exerts multiple detrimental effects independent of blood pressure elevation (Frohlich et al. 2004). In addition, strong correlations between sodium intake and left ventricular hypertrophy, myocardial fibrosis, arterial stiffness and arteriosclerotic thickening of vessel walls, abnormally increased platelet aggregation, loss of renal function, urolithiasis, and reduced bone density due to increased renal calcium excretion have been observed (Cirillo et al. 1994; Devine et al. 1995; du Cailar et al. 2002).

According to evidence-based medicine, the strongest evidence is provided by controlled randomized clinical trials, such as the DASH-Sodium Trial, the Trials of Hypertension Prevention (TOHP) I and II, and the Trial of Nonpharmacologic Interventions in the Elderly (TONE). The DASH-Sodium Trial revealed a direct and progressive blood pressure response to decreased sodium intake, independent of the diet followed (Sacks et al. 1995). Subgroup analyses of this trial have shown that reduced sodium intake could lower blood pressure in all participants studied, including men, women, blacks, nonblacks, hypertensive subjects, and nonhypertensive subjects, although the exact magnitude of this benefit may vary slightly (Vollmer et al. 2001; Bray et al. 2004). TOHP I and II confirmed the findings of the DASH-Sodium Collaborative Research Group in a large number of subjects with prehypertension, (TOHP 1997) while the TONE Trial has shown that even a moderate sodium reduction of only 40 mmol (0.9 g)/day was able to decrease by 30% the need for extra antihypertensive medications in elderly hypertensive patients (Whelton et al. 1998).

In contrast to sodium, which is the major extracellular cation of the human body, potassium is the major intracellular cation and plays an important physiological role in multiple body functions. There is also an association between dietary sodium and dietary potassium in terms of blood pressure regulation (Adrogué et al. 2007). More specifically,

increased potassium intake enhances natriuresis, and, conversely, increased sodium intake promotes kaliuresis. It has been demonstrated that the blood pressure-lowering effects of dietary potassium are more profound in the context of increased sodium intake, and vice versa.

Modern Western diets are sodium rich and potassium poor generate a sodium excess and a potassium deficit and promote increased peripheral vascular resistance and hypertension development (Adrogué et al. 2007). The deficit in intracellular potassium triggers cells, including renal epithelial cells, to preserve sodium in order to maintain their volume and osmolarity. Low potassium adversely affects blood pressure through several major pathophysiological mechanisms (Adrogué et al. 2007). First, hypokalemia stimulates both the sympathetic nervous system and the renin-angiotensin-aldosterone system, and this can lead to the acquired renal injury involved. Experimental data in rats fed a potassium-deficient diet have demonstrated that potassium depletion can induce intracellular acidosis, leading to significant alterations in the luminal transport systems of the rat renal cortex, such as an enhanced activity of sodium-hydrogen exchanger type 3, which promotes increased renal sodium reabsorption (Soleimani et al. 1990). Finally, long-term potassium depletion increases the activity of renal sodium pumps, leading again to abnormal sodium retention (Adrogué et al. 2007).

Although data from individual trials have been inconsistent, three meta-analyses of these trials have documented a significant inverse relationship between potassium intake and blood pressure in both normotensive and hypertensive individuals. (Cappuccio et al. 1991; Whelton et al. 1997; Geleijnse et al. 2003). Whelton et al. (1997) showed an average reductions of SBP/DBP associated with a net increase in urinary potassium excretion of 50 mmol/day were 4.4/2.5 mmHg in hypertensive individuals and 1.8/ 1.0 mmHg in normotensive individuals. In addition, INTERSALT researchers evidenced a decrease in urinary potassium excretion by 50 mmol/day was associated with an increase of SBP and DBP by 3.4 and 1.9 mmHg, respectively (Intersalt 1988). The urinary potassium:sodium ratio was a more significant inverse determinant of blood pressure than the urinary excretion of either electrolyte alone.

III.5. High blood pressure prevention

Treatments for all grades of hypertension are classified as either lifestyle or pharmacotherapy. The JNC 7 guidelines state that lifestyle modifications should be adopted

for the treatment of pre-hypertension, and in conjunction with drug therapy for stage 1 and stage 2 hypertension. Lifestyle modifications are generally beneficial in reducing a variety of CVD risk factors and promoting good health and should therefore be used in all hypertensive patients. In well-motivated patients with stage 1 or 2 hypertension, modifying lifestyle effectively lowers BP and may be more important than the initial choice of antihypertensive drug.

Although sustained modifications in diet and lifestyle are sometimes difficult to achieve, they may lower BP and obviate the need for drug treatment or reduce the dosages of antihypertensive drugs needed to control BP. A reasonable generalized approach for all patients includes (1) weight loss for the overweight patient; (2) regular physical activity; (3) moderation of alcohol consumption; (4) dietary modification to reduce sodium and fat and increase calcium, potassium, magnesium, vitamins, and fiber from food sources; (5) adoption of a DASH-type (Dietary Approaches to Stop Hypertension) dietary pattern. Each of the five modifiable lifestyle factors has substantial scientific evidence supporting its effectiveness, either alone or in combination, in promoting a significant reduction in BP. Bond Brill et al., (2011) reviewed a summary of well-established lifestyle strategies in addition to several novel interventions that have shown promise in the intervention and treatment of hypertension.

III.5.1. Weight reduction

Weight loss is closely correlated with reduction in BP and appears to be the most effective of all non-pharmacological measures used to treat hypertension. A body mass index (BMI) above 25 is a strong risk factor for hypertension (Forman et al. 2009). Numerous trials have shown that weight loss is an effective lifestyle intervention for lowering BP in overweight and obese individuals (Schotte et al. 1990; Davis et al. 1993; Neter et al. 2003) and according to JNC 7, a 10-kg weight loss promotes a 5- to 20-mm-Hg reduction in BP (Chobanian et al. 2003). Because sustained weight reduction is so difficult to achieve, more emphasis should be placed on prevention of weight gain, particularly in younger individuals with high normal BP and in families with a high prevalence of hypertension.

III.5.2. Increased physical activity

Considerable study demonstrates that physical activity lowers the risk of developing hypertension and is an effective BP-lowering treatment for diagnosed hypertension

(Pescatello et al. 2004). Epidemiological studies clearly show an inverse relationship between physical activity level and BP (Fagard 2005) with higher physical activity levels reducing risk of incident hypertension by approximately 15% (Cornelissen et al. 2005). According to the *Physical Activity Guidelines Advisory Committee Report* (2008) by the Department of Health and Human Services (DHHS), frequency, intensity, and mode of exercise yield important reductions in both SBP and DBP. Both aerobic exercise and resistance exercise reduce BP, but the evidence backing aerobic exercise is more substantial. Additional benefits of regular physical activity include weight loss, enhanced sense of well-being, improved functional health status, and reduced risk of CVD. Accordingly, regular aerobic physical activity is recommended for all hypertensive individuals, including those with target organ damage. Patients with advanced or unstable CVD may require a medical evaluation before initiation of exercise or a medically supervised exercise program. Isometric exercise such as heavy weight lifting can have a pressor effect and should be avoided.

III.5.3. The DASH-diet

Named simply from Dietary Approaches to Stop Hypertension, it was funded by the National Heart, Lung and Blood Institute (NHLBI) (Sacks et al. 1995). Published data in 1997 showed the main results of the DASH diet trial (Appel et al. 1997). For the time, it was evidenced that dietary intervention alone (holding body weight and sodium intake [3 g/d] constant) significantly lowered systolic and diastolic BP by an average of 11.4 and 5.5 mm Hg in hypertensive participants and by 3.5 and 2.1 mm Hg in normotensive participants, respectively. The magnitude of BP reduction is similar to that achieved by BP-lowering drug monotherapy for mild hypertension. The DASH diet is rich in fruits, vegetables, and whole grains, with the additional inclusion of legumes, nuts, lean poultry, fish, and low-fat or fat-free dairy products. The diet allows only a small amount of red meat, sweets, and added sugars and is exceptionally high in potassium, calcium, magnesium, and fiber.

III.5.4. Sodium restriction

According to a report of the Council on Science and Public Health regarding sodium and cardiovascular disease (Dickinson et al. 2007), the rise in BP with age and the prevalence of hypertension are directly related to sodium intake. This effect appears to be augmented by concomitant low potassium intake. Additional benefits of sodium reduction include reduced diuretic-induced hypokalemia and greater ease of BP control with diuretic therapy, protection from osteoporosis and fractures by reducing urinary calcium excretion, and favorable effects

on left ventricular hypertrophy. The recommended daily amount of salt by the US Departments of Agriculture and Health and Human Services is 3.7 g of salt (2300 mg of sodium). Oftenly, the consumption largely exceeds this recommendation. For instance, in the United States, 10.4 g of salt/d and 7.3 g of salt/d is consumed by man and woman respectively. Salt restriction can be achieved by avoiding obviously salty foods, not adding salt at the table, and eating more meals cooked directly from natural ingredients.

III.5.5. Potassium and calcium intake

Studies have shown that a high dietary intake of the minerals potassium, calcium, and magnesium has a BP-lowering effect.

Maintenance of adequate potassium intake (100 mmol/d), preferably from dietary sources, is recommended for hypertensive individuals and those with high normal BP. A diet rich in fruits and vegetables is better than pills or other supplements as a source of potassium because these foods contain other nutrients, for example, calcium, magnesium, and vitamins, which may also have beneficial effects on BP. Moreover, it is clear that potassium supplements can be harmful and should be avoided or used only with extreme caution in patients with renal insufficiency, diabetics, and those receiving potassium-sparing diuretics, ACE inhibitors, or angiotensin II receptor blockers. (Kassirer et al. 1985).

Inadequate calcium intake is particularly common in populations at high risk of developing hypertension. Calcium deficiency should be avoided. Maintaining the recommended daily allowance for calcium, preferably from food sources, is beneficial for a variety of reasons, such as preventing osteoporosis. The DASH trial showed that consuming calcium-rich low-fat or fat-free dairy foods in addition to a diet high in fruits and vegetables lowered SBP and DBP further than a diet high in fruits and vegetables alone (Appel et al. 1997).

III.5.6. Moderation of alcohol consumption

Alcohol consumption elevates BP both acutely and chronically. In cross-sectional and prospective studies involving all kinds of populations, the relationship between alcohol consumption, BP levels, and the prevalence of hypertension has been remarkably consistent (Briasoulis et al. 2012). The relationship is linear, but some studies show a threshold effect of 2 to 3 drinks a day. The effect increases with age, is independent of the type of alcoholic beverage, and is additive but independent of the effects of obesity, oral contraceptives, and

high salt intake (Arkwright et al. 1982). However, moderate alcohol consumption (3 standard drinks a day) reduces overall CVD risk in the general population (Puddey et al. 1997). Those having more than 2 standard alcohol drinks per day show an increase in both mortality rates and hypertension (Stranges et al. 2004). Excessive alcohol intake also appears to cause resistance to antihypertensive therapy. For unrelated health reasons, alcohol consumption is not recommended for nondrinkers and for drinkers, intake should be limited to 1 drink of alcohol per day (i.e., 12 oz beer, 5 oz of wine, 1.5 oz of spirit) in most men and half that amount in women. Actually, a standard drink is equivalent to 10 g of alcohol per drink.

III.5.7. Novel lifestyle interventions

Among the novel interventions, dark chocolate (Taubert et al. 2007; Desch et al. 2010), soy (Yang et al. 2005; Welty et al. 2007), relaxation therapy (Wang et al. 2013) were listed as having the ability to reduce BP. Although promising, the research on these novel therapies is preliminary and need more published trials before definitive conclusions can be drawn.

III.6. Hypertension treatments

Aside from preventing high blood pressure by changing lifestyle, reducing BP by pharmacological means clearly reduces CVD morbidity and mortality rates. Benefits include protection from stroke, coronary events, heart failure, progression of renal disease, progression to more severe hypertension, and, most importantly, mortality from all causes. Clinicians can choose from several classes of antihypertensive drugs which have proven their efficacy to lower BP: diuretics, anti-adrenergics, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs). Each of them possesses different mechanisms of action and different targets.

III.6.1 Diuretics

They are the oldest and least expensive class of drugs used to treat hypertension. Diuretics act by draining sodium and water from the body which decreases blood volume. Diuretics are used in patient with mild hypertension and take several weeks to produce vasodilating effects. In moderate to severe hypertension, the diuretics are combined with other antihypertensive drugs. However, one drawback of diuretics is the depletion of potassium and in order to counteract potassium depletion, clinicians prescribed another type of diuretic called potassium-sparing diuretics. The latter can also directly or indirectly block

aldosterone. As a result, potassium-sparing diuretics are sometimes also known as aldosterone-receptor blockers. Side effects of these drugs include frequent urination, lightheadedness, fatigue, diarrhea or constipation, and muscle cramps. Diuretics are contraindicated in patients with gout, a painful form of arthritis caused by the buildup of uric acid in the body.

III.6.2. Anti-adrenergics

Their anti-hypertensive action is lead to their ability to reduce the activity of sympatheric nervous system. Anti-adrenergics lower blood pressure by limiting the action of the hormones epinephrine and norepinephrine causing the relaxation of blood vessels. This class includes a variety of different agents acting differently: (1) peripheral adrenergic-receptor blockers, including β-adrenergics and α-adrenergics; (2) central acting agents; and (3) peripheral nerve acting agents.

Peripheral adrenergic-receptor blockers work by preventing the action of neurotransmitters and lead to a reduce activity of nerves to heart and blood vessels. β-blockers do not only block β-receptors which inhibited the action of epinephrine on heart leading to a decrease of cardiac output but also block the release of renin in kidneys. The cardioselective of β-blockers target primilary the β1 receptors whereas the non selective form target the β1 and β2 receptors in lungs, blood vessels and other tissues. Common side effects are fatigue, depression, erectile dysfunction, shortness of breath, insomnia, and reduced tolerance for exercise. α-blockers have similar action to β-blockers but work on α-receptors in the heart and vascular smooth cells, which normally interact with norepinephrine causing vessel constriction. Its action consists of a vasodilation and a decrease of peripheral resistance. In addition, α-adrenergic blockers have several advantages. It can improve insulin sensitivity in patients with glucose intolerance and hyperglycemia, lowers LDL and increases HDL. It is oftently prescribed to patient with benign prostatic hyperplasia, a noncancerous enlargement of the prostate gland, because of its ability to relax smooth muscles surrounding the prostate, relieving the constriction of the urethra and easing urine flow. Common side effects are orthostatic hypotension, heart palpitations, dizziness, nasal congestion, headaches, dry mouth and erectile dysfunction.

Central acting agents: these agents block the neurotransmitters implicate in the activation of the sympathetic nervous system to increase blood pressure. They are generally used in combination with other blood pressure medicines. Common side effects include

abnormally low blood pressure when standing up, dry mouth, depression, erectile dysfunction, and sedation.

Peripheral nerve acting agents: these anti-adrenergics deplete the autonomic nerves of norepinephrine. They are recently less used alone because of their frequent side effects but they are usually prescribed along with other antihypertensive drugs.

III.6.3. Calcium channel blockers

They interfere with the influx of calcium in cardiac and vascular smooth muscles resulting in slow depolarization in the atrioventricular and sinoatrial nodes of the heart, reduction of cardiac muscle contractility, relaxation of smooth muscles and vasodilation. Because, calcium-channel blockers have also the ability to slow nerve impulses in the heart, they are often prescribed for arrhythmias. Unkike diuretics, common side effects do not cause adverse metabolic effects but mild adverse effects like dizziness, fatigue etc. Calcuim channel blockers do not compromise haemodynamics, no renal and male sexual function impairment. They can be given to asthma and angina patients and because of its no adverse fetal effects, they can be given during pregnancy. In overall, calcuim channel blockers have minimal effect on quality of life.

III.6.4. Angiotensin-converting enzyme (ACE) inhibitors

These agents prevent the kidneys from retaining sodium and water by inhibiting the formation of Ang II. Angiotensin-converting enzyme which converts inactive angiotensin I to the active Ang II is desactivated. ACE inhibitors also decrease release of aldosterone and antidiuretic hormone. They reduce blood pressure in most patients and produce fewer side effects than many other antihypertensive drugs. The most common side effects of these medications are a reduced sense of taste, dry cough and potassium retention.

III.6.5. Angiotensin receptor blockers (ARBs)

ARBs block angiotensin receptor type 1 and 2 (AT_1 and AT_2) located the surface of the target cells. For instance, in smooth muscle cells, transducer mechanisms of AT_1 stimulate the phospholipase c-IP_3/DAG-intracellular leading to a Ca^+ release mechanism and thus a vascular and visceral smooth muscle contraction. By blocking these receptors, Ang II is prevented from constricting the blood. Because ARBs are highly effective and well tolerated by most of the people, ARBs have become quite popular.

Because of the substantial anti-hypertensive drugs and the numerous different side effects, the choice of the right medication for the right patient becomes very important. Drugs can be prescribed alone or in combination with other drugs. The patient's clinical state has to be examined meticulously. The key for a successful antihypertensive regime is to include highly effective medications that are well tolerated and affordable and keep a well-being lifestyle.

III.7. Hypertension and (poly)phenols

Hypertension causes modifications of the vascular walls that lead to hypertensive cardiomyopathy and heart failure. Changes in the mechanical properties of arteries affect vascular resistance by altering the pressure–lumen diameter relationship of small arteries. Part of the cardioprotective actions of (poly)phenols is due to their ability to lower blood pressure. Using pure compounds, an antihypertensive effect of resveratrol was reported in partially nephrectomised rats (Liu et al. 2005). In another study using double transgenic rats harbouring human renin and angiotensinogen genes, it was observed that resveratrol reduced blood pressure, ameliorated cardiac hypertrophy and prevented Ang II-induced mortality, probably by increasing mitochondrial biogenesis and SIRT1 activity (Biala et al. 2010). Resveratrol probably suppressed Ang II type 1 receptor expression through SIRT1 activation, suggesting that the inhibition of the renin–angiotensin system may contributed, at least in part, to the resveratrol-induced cardiprotective effects (Miyazaki et al. 2008). Other studies reported that resveratrol did not affected established hypertension in SHR (Rush et al. 2007), although it attenuated the compliance of arteries from SHR without changes in wall stiffness by reducing eutrophic remodelling (Behbahani et al. 2010).

Chronic treatment with quercetin (10 mg/kg) have shown to reduce systolic blood pressure and significantly reduced left ventricular and renal hypertrophy in SHR (Romero et al. 2010), in hypertension induced by the inhibition of NOS rats (Perez-Vizcaino et al. 2009), and in deoxycorticosterone acetate-salt hypertensive rats (Galisteo et al. 2004). It appeared that quercetin was effective in all animal models of hypertension studied, and acted independently of the status of renin–angiotensin system, oxidative stress, NO, etc (Perez-Vizcaino et al. 2009).

Short-term oral administration of (poly)phenols from red wine decreased blood pressure in normotensive rats. This haemodynamic effect was associated with an enhanced endothelium-dependent relaxation and an induction of gene expression within the arterial

wall, which together maintain unchanged agonist-induced contractility (Diebolt et al. 2001). It has been evidenced that (poly)phenols from red wine reduced blood pressure elevations caused by chronic inhibition of NOS, attenuated end-organ damage such as myocardial fibrosis and aortic thickening, and decreased protein synthesis in the heart and aorta (Bernátová et al. 2002). (Poly)phenols also prevented endothelium dysfunction by increasing eNOS activity, moderately enhancing eNOS expression and reducing oxidative stress in the left ventricle and aorta. Sarr et al. (2006) showed that endothelial dysfunction associated with excessive NADPH oxidase-dependent vascular formation of ROS in angiotensin II-induced hypertension was prevented by (poly)phenols. Thus, (poly)phenols from red wine could reduced hypertension by modulating the NO and ROS balance in the cardiovascular system.

When considering the effects of grape products on blood pressure, the ethanol content of red wine should be taken into account. It is well known that there is a linear relationship between alcohol intake and blood pressure (Briasoulis et al. 2012). Although several epidemiological studies have suggested that beer and spirits consumption may be associated with higher blood pressure, particularly SBP, than wine consumption (Klatsky et al. 1986; Fuchs et al. 2001), an *in vivo* trial (Zilkens et al. 2005) showed that daily consumption of 40 g alcohol as either red wine or beer for 4 weeks resulted in similar increases in SBP and heart rate. Therefore, studies on the effects of grape products on blood pressure should be focused more on grape products lacking ethanol.

Substantial studies on animals dealing with the effects of grape (poly)phenols on blood pressure showed an hypotensive effect of these compounds. Grape skin extracts, red wine (poly)phenols and red wine extracts significantly reduced blood pressure (in several cases, both SBP and DBP) in normotensive and hypertensive rats (Diebolt et al. 2001; Bernátová et al. 2002; Soares de Moura et al. 2002; Al-Awwadi et al. 2004; Ranaivo et al. 2004). In the case of human subjects, a study showed that the intake of a grape product rich in both dietary fibre and (poly)phenols by normotensive subjects for 16 weeks led to a non-significant decrease in SBP and DBP (Jiménez et al. 2008) whereas a significant reduction in SBP and DBP was observed in hypertensive or coronary artery disease subjects after the intake of grape juice (Park et al. 2004).

It has also been suggested that grape (poly)phenols could promoted the release by the vascular endothelium of NO, a compound with vasorelaxing and anti-aggregating effects and that in the long term induces the expression of protective genes for the cardiovascular system

(Chou et al. 2001; Diebolt et al. 2001). Wallerath et al. (2003) has proposed that wine may regulated the endothelial NO synthase gene in endothelial cells, through transcriptional and post-transcriptional factors. These hypotheses have only been partially proven. In an *ex-vivo* experiment performed by Andriambeloson et al. (1997), using aortic rings from rats incubated with red grape (poly)phenols, two-fold increase of Fe^{2+}-diethyldithiocarbamate electron paramagnetic resonance was observed, indicating the generation of NO. Similarly, after supplementing rats with red wine, an anti-thrombotic effect was observed indicating the involvement of NO in this process (Wollny et al. 1999).

In human subjects, although an acute intake of (poly)phenols did not significantly increase NO production (Matsuo et al. 2001), the supplementation to healthy volunteers with grape juice for 14 days led to a significant increase in platelet-derived NO production (Freedman et al. 2001). Another parameter that has been studied to investigate the effect of grape (poly)phenols on endothelial function has been the determination of effects on vasodilatation and, particularly, on flow-mediated dilatation of the brachial artery, which is considered to be an early marker of alterations in endothelial function. In animal studies, red wine (poly)phenols induced endothelium-dependent relaxation in rat aorta (Andriambeloson et al. 1997), red wine and dealcoholised red wine induced vasodilatation in isolated vessels from rats (Boban et al. 2006) and grape skin extracts had a vasodilator effect on the mesenteric cardiovascular bed of rats (Soares de Moura et al. 2002). In human subjects, it has been observed that an acute intake of red grape (poly)phenol extract, red wine or dealcoholised red wine cause an increase in flow-mediated dilatation, with a peak at 60 min (Hashimoto et al. 2001; Lekakis et al. 2005). Nevertheless, in another study by Boban et al. (2006), red wine, but not (poly)phenols from red wine, produced an enhancement of endothelial response, despite a similar catechin concentration in both products. Intake of grape juice or grape seed extract for 2–3 weeks also caused a significant increase in flow-mediated dilatation, compared with the control group (Stein et al. 1999; Clifton 2004). Interestingly, in the Clifton et al. study the addition of quercetin to the grape seed extract abolished this effect indicating probably a pro-oxidant effect due to an excessive amount of antioxidants. Bernátová et al. (2002) showed that grape (poly)phenols may also have a reducing effect in myocardiac fibrosis, a process occurring in cases of hypertension which is produced by an excessive accumulation of collagen and is associated with an increase in alterations of cardiac and vascular functions.

Chapter 2:

Materials and Methods

I. Grapes and grape pomaces characterisation

I.1. Experimental materials

I.1.1. Chemicals

Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA). HPLC grade acetonitrile (HPLC ≥ 99%), ethyl acetate (HPLC ≥ 99%), chloroform (HPLC ≥ 99%), methanol (HPLC ≥ 99%), ethanol and acetone purchased from Scharlau (Sentmenat, Barcelona, Spain). The following chemicals were obtained from Sigma Aldrich (Saint Louis, USA): (+)-catechin (≥ 98%), (–)-epicatechin (≥ 98%), B_1 [(–)-epicatechin-(4β-8)-(+)-catechin] (≥ 98%), procyanidin dimer B_2 [(–)-epicatechin-(4β-8)-(–)-epicatechin] (≥ 98%), cyanidin-3-*O*-glucoside chloride (≥ 98%), delphinidin-3-*O*-glucoside chloride (≥ 98%), malvidin-3-*O*-glucoside chloride (≥ 98%), peonidin-3-*O*-glucoside chloride (≥ 98%), gallic acid (≥ 98%), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (≥ 97%), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥ 98%), potassium persulfate (≥ 99%), fluorescein (≥ 98%), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) (≥ 97%), sodium dihydrogen phosphate dehydrate (≥ 98%), disodium hydrogen phosphate dodecahydrate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (≥ 98%), iron (III) chloride hexa-hydrate (≥ 98%), iron (II) sulfate hepta-hydrate (≥ 98%), Folin Ciocalteu's phenol (2N), sodium bisulfate (97 %), sodium carbonate (99 %), phloroglucinol (≥ 98%), L(+)-tartaric acid (≥ 99%), L-ascorbic acid (≥ 99%), hydrochloric acid (37%), sodium hydroxide (≥ 98%), acetic acid (≥ 99%) and formic acid (≥ 95%). The Laboratory of Organic Chemistry and Organometallic (Université Bordeaux 1) synthesized procyanidins dimers B_3 [(+)-catechin-(4α-8)-(+)-catechin], and B_4 [(+)-catechin-(4α-8)-(–)-epicatechin] and a trimer (T) [(+)-catechin-(4β-8)-(+)-catechin-(4β-8)-(–)-epicatechin] (Tarascou et al. 2006).

I.1.2. Plant materials

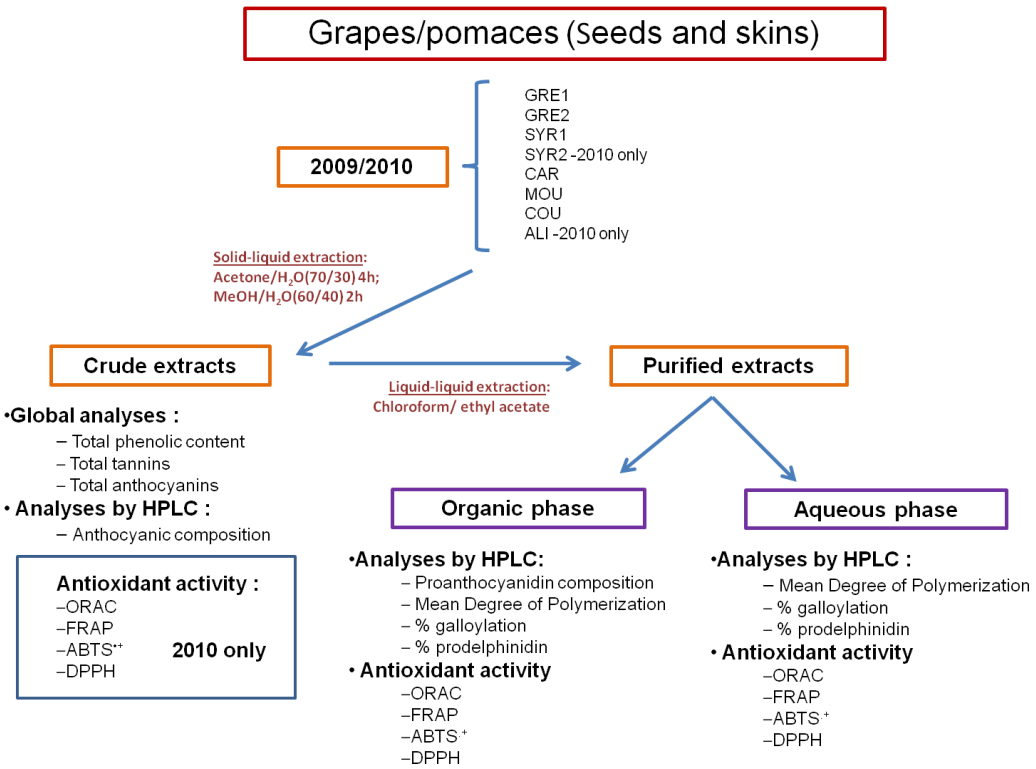
In the first instance, this study was conducted on crude materials, with 2009 and 2010 grapes at maturity and their respective grape pomaces from *V.vinifera* L. cv. Grenache (from two different locations [GRE1 and GRE2]), Syrah (from two different locations [SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI),

provided by the Château de Beaucastel located in the Vallée du Rhône, appellation of Châteauneuf-du-Pape. GRE2, COU and ALI pomaces were analysed only for the vintage 2010. Details are provided below (Table 6):

Table 6: Grape varieties provided by the Chateau de Beaucastel and abbreviations used

Varieties	Name used according to location	Abbreviation used
Grenache	Grenache long du Coudoulet	GRE1
Grenache	Grenache face aux Pins	GRE2
Syrah	Syrah Plantier	SYR1
Syrah	Syrah Haut de Julien	SYR2
Carignan	Carignan Pont des Voleurs	CAR
Mourvèdre	Mourvèdre Courrieux à beaucastel	MOU
Counoise	Counoise Quicaro à Beaucastel	COU
Alicante	Alicante au Grand Prébois	ALI

All the analysis performed on seeds and skins for the 2009 and 2010 vintage after skins and seeds separation was illustrated below.



Another type of extracts was obtained after extraction process in collaboration with 3INature Company and will be used for *in vivo* experiments. Grape pomace samples obtained after the vinification process (vintage 2010) from *V.vinifera* L. cv. Grenache (from two

different locations [GRE1 and GRE2]), Syrah (from two different locations [SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI) were collected by the Château de Beaucastel. Seeds were separated from skins and both were frozen (− 20°C) prior being sent for extraction by 3iNature company. Pomace samples were extracted using water and hydro-alcoholic 70 % solution, thus, giving two types of samples, aqueous samples (EAQ) and hydro-alcoholic 70% (EA70). Samples were shipped to the university Bordeaux Segalen, as summarize in the Table 7. All the analysis carried out on seeds and skins for the 2010 vintage after skins and seeds separation was illustrated below.

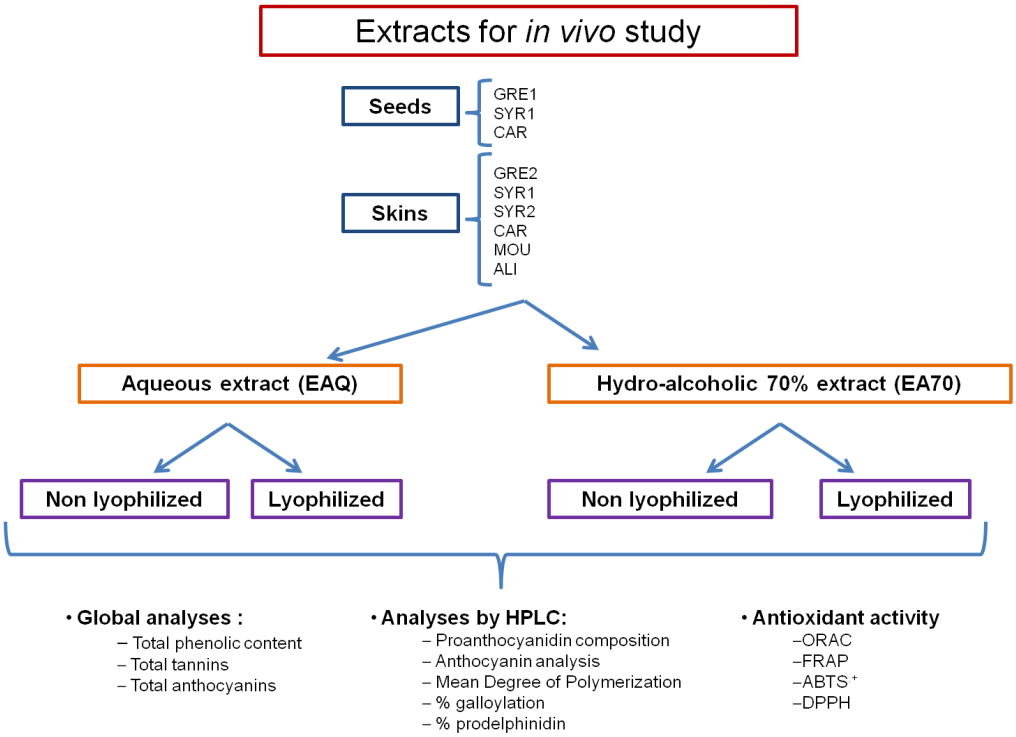


Table 7: Skin and seed extracts obtained from different varieties of grapes by EAQ and EA70 extraction

Samples	Skins		Seeds	
	EAQ	EA70	EAQ	EA70
Grenache long du Coudoulet (GRE1)			X	X
Grenache face aux Pins (GRE2)	X	X		
Syrah plantier (SYR1)	X	X	X	X
Syrah Haut de Julien (SYR2)	X	X		
Carignan Joseph (CAR)	X	X	X	X
Mourvèdre (MOU)	X	X		
Counoise (COU)				
Alicante (ALI)	X	X		

I.1.3. Samples preparation

Seeds and skins were carefully removed by hand from grapes and separated from pomaces, lyophilized and stored at −20°C prior to analysis. The dry seeds and skins were powdered in a ball grinder. Extracts of the powders were prepared in duplicate according to a previous study (Chira et al. 2009; Lorrain et al. 2011). A 6 g portion of powder was extracted using 55 mL of acetone/water (70:30, v/v) for 4 h followed by 55 mL of methanol/water (60:40, v/v) for 2.5 h. The centrifugal supernatants were combined and evaporated *in vacuo* at 30°C to remove organic solvents; the residue was dissolved in water and lyophilized to obtain a crude extract.

I.1.4. Grape and pomace tannins extraction

A small portion of the powders (eq. to 1 g of dried skin or seed powder from grape and pomace) was retained for further polyphenol analyses while the remaining (eq. to 3 g of dried skin powder and 900 mg of dried seed powder) was solubilized in 250 mL of water/ethanol (95:5, v/v) and partitioned three times with chloroform (250 mL) to remove lipophilic material. The aqueous phase was then extracted three times with ethyl acetate (250 mL) to obtain two distinctive fractions: a low molecular weight procyanidin fraction (monomeric/oligomeric tannins) in the organic phase and a high weight procyanidin fraction (polymeric tannins) in the aqueous phase. These two fractions were concentrated and lyophilized to produce dry extracts (Chira et al. 2009; Lorrain et al. 2011).

I.1.5. Grape and pomace anthocyanins extraction

Anthocyanins extraction was adapted from the method of Sriram et al. (1999). A 1 g portion of dried skin powder was extracted four times with 40 mL of acidified methanol (0.1% HCl 12N) successively for 4 h, 12 h, 4 h and 12 h. The centrifugal supernatants were combined and evaporated in vacuo at 30°C to remove methanol; the residue was dissolved in water and lyophilized to obtain an anthocyanin-rich powder.

I.2. Total phenolics, tannins and anthocyanins

Total polyphenol, tannin and anthocyanin contents of grapes and pomace skin and seed extracts were determined. Crude extracts were solubilized in water/ethanol (90:10, v/v; pH 3.5 adjusted with tartaric acid) at specific concentrations: for total phenol contents (TPC) and total anthocyanins determination, concentrations were of 2 g/L for the seed extracts and 6 g/L

for the skin extracts; for total tannin determination, they were of 0.25 g/L and 1 g/L for seed and skin extracts respectively.

TPC was determined by the Folin Ciocalteu assay (Singleton et al. 1965) and the data expressed as mg of gallic acid equivalents (GAE) per g dry weight. The total tannin contents was measured by acidic hydrolysis of proanthocyanidins resulting in carbocation formation with partial conversion into red cyanidin using the method of Ribereau-Gayon and Stonestreet (1966). The procedure required the preparation of two samples, each containing 4 mL of sample, 2 mL of water and 6 mL of HCl (12 N). One tube was heated at 100°C in a water bath for 30 min and 1 mL of pure ethanol was added. The other sample was not heated but received 1 mL of ethanol. The difference in absorbance was measured at 550 nm on a 10 mm optical path. The concentration was obtained in g/L by the calculation: 19.33/50 x ΔA. Anthocyanin content was determined by the SO_2 bleaching procedure (Ribéreau Gayon et al. 1965).

I.3. HPLC analysis of monomeric and oligomeric tannins

Monomeric/oligomeric tannin extracts were solubilized in a methanol/water solution (50:50, v/v) at concentrations of 1 g/L for seed extracts and 6 g/L for skin extracts. Analyses were carried out according to Silva et al. (2011). The equipment used for HPLC analysis consisted of a Thermo-Finnigan Surveyor HPLC system (Thermo-Fisher, San Jose, CA, USA) comprising a UV–Vis detector (Surveyor PDA Plus), a Thermo-Finnigan autosampler and a Thermo-Finnigan quaternary pump system (Surveyor LC pump Plus) controlled by an Xcalibur data treatment system. It is also coupled with a Thermo-Finnigan fluorescence detector (FL plus Detector) operated with ChromQuest 4.2 software. Separations were performed on a reversed-phase Lichrospher C18 (250 mm x 4 mm, 5 µm) column. A binary gradient system was employed using 1% (v/v) aqueous formic acid (solvent A) and 1% acid formic/acetonitrile (v/v) (solvent B) at a flow rate of 1 mL/min. Initial B were set at 8%. The mobile phase gradient was as following: 18% B at 21 min and 100% at 22 min. The column was then washed with 100% B for 3 min and re-equilibrated with 8% B for 5 min before the next injection. Eluting peaks were recorded at excitation and emission wavelengths of 280 nm and 320 nm respectively. Identification of mean peaks was carried out by co-chromatography with authentic standards [(+)-catechin, (–)-epicatechin, procyanidin dimers B_1, B_2, B_3, B_4, and trimer C_1] which were also used as reference compounds for quantitative analysis. The results were presented as amounts present per mg of dried skins or seeds.

I.4. HPLC analysis of proanthocyanidins

Aqueous and hydro-alcoholic 70% grape pomace extracts were solubilized in methanol/water (50:50, v/v) containing 1% formic acid. Analysis was carried out on a Surveyor HPLC system equipped with sampler cooler maintained at 4ºC, a PDA detector scanning from 200 to 600 nm and a fluorescence detector (FP-920m Jasco (U.K.) Ltd.) linked to a Finnigan LCQ Duo mass spectrometer set with a split volume at 0.2 mL/min and ESI operating in full-scan negative ionization mode scanning from *m/z* 200 to 1000. Fluorescence detection was set respectively at an excitation and emission wavelengths of 230 nm and 320 nm.

Separation was performed on a 250 × 4.6 mm i.d. 5 μm Develosil Diol 100Å column (Phenomenex, Cheshire, UK) maintained at 35 ºC in a column oven. The mobile phase pumped at 1 mL/min and consisted of acidic acetonitrile ((A), CH_3CN:HOAc, 98:2; v/v) and acidic aqueous methanol ((B), CH_3OH:H_2O:HOAc, 95:3:2; v/v/v). The gradient was set at 7% B for 3 min then increased to 37.6% over 57 min. The column was then washed with 100% B for 7 min prior to returning to 7% over 6 min. The column was re-equilibrating with 7% B during 10 min. The injection volume was 10 µL for seed extracts and 20 µL for skin extracts. Two different gain were used in order to quantify monomers and dimers on the one side and the procyanidins with Dpm higher than 2 on the other side.

Peak detection and quantification were mainly carried out by fluorescence because of its more selectivity and stronger signal than UV absorption detection for procyanidins. Identification was confirmed by mass spectrometry in full-scan negative ionization, data dependent MS. Procyanidins were quantified as (–)-epicatechin equivalents and expressed in mg per dry weight of seeds or skins ± SD (*n*=3).

I.5. Determination of mean degree of polymerization, %G and %P

The proanthocyanidin mean degree of polymerization (mDP), percentage of galloylation (%G) and percentage of prodelphinidins (%P) was determined for seeds and skins extracts both in monomeric/oligomeric and polymeric tannin fractions by the means of phloroglucinolysis (Drinkine et al. 2007). The oligomeric and polymeric products were depolymerized in the presence of a nucleophilic agent (phloroglucinol) in an acid medium. The reaction released the terminal unit as a flavan-3-ol monomer and ethylideneflavan-3-ol-

phloroglucinol adducts from extension units. Phloroglucinolysis reaction is summarized in Figure 21.

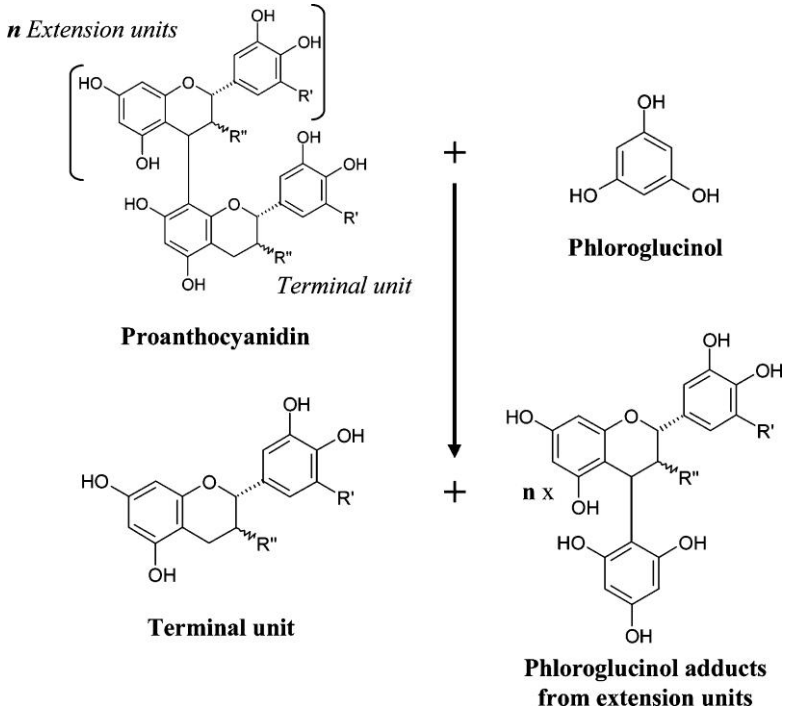
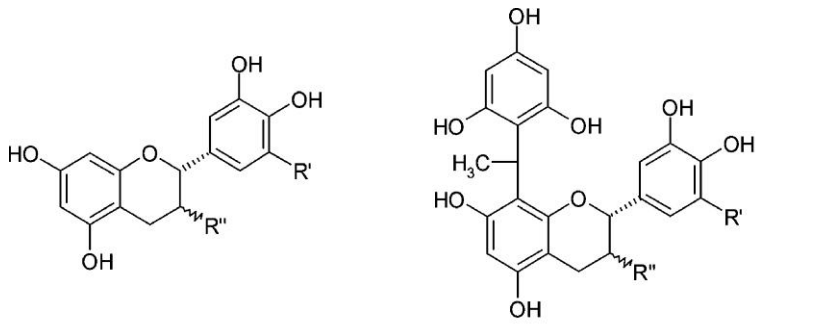


Figure 21: Reaction pathway of phloroglucinolysis. Adapted by Drinkine et al. (2007). Procyanidins, R' = H and R" = OH; prodelphinidins, R' = OH and R'' = OH.

For procyanidins, terminal units release were (+)-catechin (C), (–)-epicatechin (EC) and (–)-epicatechin gallate (ECG). Phloroglucinol adducts from extension units were the (+)-catechin–phloroglucinol (C-P), (–)-epicatechin phloroglucinol (EC-P) and (–)-epicatechin gallate phloroglucinol (ECG-P). Concerning prodelphinidins, in addition to the cited compounds, they also released the (–)-epigallocatechin (EGC) and his phloroglucinol adduct the (–)-epigallocatechin phloroglucinol (EGC-P) (Figure 22).



Flavan-3-ol monomer **Ethylidene-flavan-3-ol-phloroglucinol adduct**

R' = H,	**R'' = OH :**	**(+)-catechin, (-)-epicatechin**
R' = OH,	**R'' = OH :**	**(-)-epigallocatechin**
R' = H,	**R'' = O-gallate :**	**(-)-epicatechin gallate**

Figure 22: Proposed compounds released by the phloroglucinolysis of ethylidene-bridged flavan-3-ols. Adapted by Drinkine et al. (2007).

Monomeric/oligomeric tannin seed and skin extracts were prepared as followed: 100 µL of extracts solubilized in MeOH (5 g/L) and 100 µL of phloroglucinolyse reagent were placed in an amber glass vial and reacted at 50 °C for 20 min. The mixture was then combined with 1 mL of aqueous sodium acetate (40 mmol/L) to stop the reaction. Phloroglucinolyse reagent is a mix of phloroglucinol (50 g/L), ascorbic acid (10 g/L) dissolve in acidified methanol with 0.1N HCl. Phloroglucinolysis reaction were carried out in duplicate.

Reaction products were analyzed by HPLC-MS on a Hewlett-Packard 1100 HPLC (Agilent, Massy, France) system comprising a pump module and an absorbance detector coupled to a Micromass Platform II quadruple mass spectrometer (Micromass-Beckman, Roissy Charles de Gaulle, France) equipped with an electrospray ion source. The system was operated using Masslynx 3.4 software.

Separations were performed on a reversed-phase Waters XTerra RR C18 (100 mm x 4.6 mm, 3.5 µm) column at room temperature. A binary gradient system was employed using 1% (v/v) aqueous acetic acid (solvent A) and MeOH (solvent B) at a flow rate of 1 mL/min. The elution conditions were: 5% B at 0 min; 16% B at 1 min, 22% B at 7 min, 35% B at 8 min, 42% B at 15 min; the column was then washed with 100% B for 3 min and re-equilibrated with 5% B for 4 min before next injection. The mass spectrometer was operated in negative-ionisation mode. The source temperature was 120°C, the capillary voltage was set at 3.5 kV and the cone voltage was –30 eV. The absorbance was recorded at 280 nm and mass spectra were recorded in the range of 50 to 1500 amu.

Concentration of breakdown products was obtained thought molar extinction coefficient, specific for each product and expressed as mol/L using this formula:

$$C = \frac{\text{Area}}{\text{Molar extinction coefficient}}$$

Identification of reaction products was confirmed by mass spectrometry in full scan negative ionization, data MS dependant (Table 8).

Table 8: Reaction products and their MS characteristics

Compounds	[M-] (m/z)
(+)-Catechin (C)	289
(−)-Epicatechin (EC)	289
(−)-Epicatechin-*O*-gallate (ECG)	441
(−)-Epigallocatechin (EGC)	305
(+)-Catechin–phloroglucinol (C-P)	413
(−)-Epicatechin phloroglucinol (EC-P)	413
(−)-Epicatechin-*O*-gallate phloroglucinol (ECG-P)	565
(−)-Epigallocatechin phloroglucinol (EGC-P)	429

Reverse phase HPLC allowed the dosage of phloroglucinol breakdown products and thus, the determination of the mDP, %G and %P.

$$mDP = \frac{\sum \text{concentration terminal units (flavan-3-ols)} + \sum \text{concentration extension units}}{\sum \text{concentration terminal units liberated (flavan-3-ols)}}$$

$$\%P = \frac{\sum \text{concentration of terminal units (EGC)} + \sum \text{concentration of extension units (EGC-P)}}{\sum \text{concentration of terminal units (flavan-3-ols)} + \sum \text{concentration of extension units}}$$

$$\%G = \frac{\sum \text{concentration of terminal units (ECG)} + \sum \text{concentration of extension units (ECG-P)}}{\sum \text{concentration of terminal units (flavan-3-ols)} + \sum \text{concentration of extension units}}$$

I.6. HPLC analysis of anthocyanins

Powder skin extracts were dissolved in water/methanol solution (50:50, v/v) at a concentration of 10 mg/mL prior to UPLC-UV analyses using a Thermo-Accela HPLC-UV system (Thermo-Fisher, San Jose, CA, USA) composed of a PDA detector, an autosampler and a quaternary 600 series pump system controlled by an Xcalibur data system. Separation was performed on a C18 Kinetex column (100 mm x 2.1 mm, 1.7 µm). The injected volume

was 2 µL. The mobile phases were water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B) at a flow rate of 200 µL/min. Initial solvent B was set at 7%. The mobile phase gradient was as following: 26% B at 18 min, 100% B at 19 min. The column was then washed with 100% B for 3 min and re-equilibrated with 7% B for 5 min before next injection. Eluting peaks were monitored at 520 nm. Identification of mean peaks was performed by comparison to injected external standards. The data was expressed as malvidin-3-*O*-monoglucoside equivalent/g dry weight of skins.

Grape pomace extracts used for *in vivo* study were studied in another HPLC system. Grape pomace extracts were solubilized in methanol/water (50:50, v/v) containing 1% formic acid. Analysis were carried out using a Surveyor HPLC with sampler cooler maintained at 4ºC, a PDA detector scanning from 200 to 600 nm and a LCQ Advantage ion trap mass spectrometer with a split volume set at 0.2 mL/min and ESI operating in full-scan positive mode scanning from *m/z* 200 to 1000. Separation was performed on a 250 × 4.6 mm i.d. 4 *µ*m Synergi RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40 ºC in a column oven. The mobile phase pumped at 1 mL/min comprised a 65 min, 10-45% gradient of methanol in water with both solvents containing 1% formic acid. The injection volume was 10 µL. Peak detection and quantification were monitored at 520 nm and performed by comparison with available standards or confirmed by mass spectrometry in full-scan positive ionization, data dependent MS^2. Anthocyanins were quantified as malvidin-3-*O*-glucoside equivalents and expressed in mg per dry weight of seed or skin ± SE (*n*=3).

I.7 Antioxidant assays

I.7.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was applied according to the method of Ou et al. (2001), as modified by Dávalos et al. (2004). The ORAC assay was carried out on the BGM FLUOstar Omega plate reader with a fluorescence detector (BMG LABTECH GmbH, Ortenberg, Germany). The fluorescent filters were set at excitation and emission wavelengths of 485 nm and 530 nm respectively. The temperature of the incubator was set at 37°C. A black 96-well plate was loaded with phosphate buffer (blank, 30 µL), Trolox standard series (1 µM-100 µM, 30 µL) and samples (grape and pomace crude extract; solubilized in phosphate buffer at appropriate concentration, 30 µL) into the respective wells, fluorescein (117 nM, 180 µL) were then added to every well followed by AAPH (40 mM, 90 µL). Readings were taken every minute for 90 minutes. All samples were analyzed in triplicate. The area under the

curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC was calculated by subtracting the AUC of the blank. The final ORAC values were determined by linear regression equation of Trolox concentrations and are expressed as µM Trolox equivalents/g dry weights.

I.7.2. Ferric Reducing Antioxidant Potential assay (FRAP)

The ferric reducing power of crude extracts was measured based on the method of Benzie et al. (1996). The FRAP assay was carried out on the BGM FLUOstar Omega plate reader set at 593 nm absorbance readings. FRAP reagent were prepared daily by mixing 10 volumes of 300 mM sodium acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) and 1 volume 20 mM ferric chloride. A standard curve was prepared using various concentrations of $FeSO_4$ x 7 H_2O. 96-well plate was loaded with sodium acetate buffer (blank, 40 µL), $FeSO_4$ x 7 H_2O standard series (3 mM-0.50 mM, 40 µL) and samples (grape and pomace crude extract; solubilized in sodium acetate buffer at appropriate concentration, 40 µL) into the respective wells, FRAP reagent (300 µL) were then added to every well. Readings were taken after 4 minutes of reaction. All samples were analyzed in triplicate. Blank values were subtracted from samples and standards values then difference were used to calculate the FRAP value. In this assay, the reducing capacity of grape and pomace skin and seed extracts was calculated with the reference to the signal given by Fe^{2+} solution. FRAP values were expressed as µM $Fe^{2+/}$g of dry skin and seed weights.

I.7.3. ABTS assay

The free radical scavenging capacity of grape and pomace skin and seed crude extracts were also studied using the ABTS radical cation decolorization assay (Re et al. 1999). When combined with an oxidant (2.45 mM potassium persulfate), ABTS (7 mM in deionized water) reacts to create a stable dark blue-green $ABTS^{+\bullet}$ radical solution following a 12-16 h incubation in the dark at room temperature. Before use, the $ABTS^{+\bullet}$ solution was diluted with deionized water to an absorbance of 0.7 ± 0.02 at 734 nm using a Jenway-6305 UV-vis spectrophotometer (Jenway, Stone, Staffordshire, UK). This solution was prepared daily and all samples were analyzed in triplicate. One hundred µL of aqueous skin and seed crude extracts were added to 2 mL of $ABTS^{+\bullet}$ solution. The absorbance was taken 10 min after the initial mixing. An appropriate solvent blank reading was also taken. Blank values were subtracted from samples and standard values and a linear regression for the Trolox standards were constructed. Results were expressed as µM Trolox equivalents/g dry weights.

I.7.4. DPPH assay

This method was used according to Brand-Williams et al. (1995), modified by Miliauskas et al. (2004). $DPPH^{\bullet}$ has an intense violet color which turns colorless as unpaired electrons are scavenged by antioxidants. Reaction mixtures containing 100 µL of sample and 200 mL of $DPPH^{\bullet}$ solution (6×10^{-5} M, dissolve in methanol, prepared daily) were gently mixed and stored at room temperature during 20 min. The absorbance of the resulting solution was measured at 515 nm using a Jenway-6305 UV-vis spectrophotometer (Jenway, Stone, Staffordshire, United Kingdom). An appropriate solvent blank reading was also taken. Blank values were subtracted from samples and standard values. A linear regression for the Trolox standards was constructed. Results were expressed as µM Trolox equivalents/g dry weights.

I.8. Statistical analysis

All measurements were performed in triplicate. Results are expressed as means ± standard deviation (SD). One-way ANOVA was performed to test the effects of variation factors (different samples) on each variable (TPC, total tannin, anthocyanin, phenol concentrations, mDP, etc). If significant effects were found at a 95% confidence interval, ANOVA was followed by a Tukey's HSD and Duncan post hoc test to identify differences among groups. These analyses were performed using Statistica V.7 Software (Statsoft InC., Tulsa, O.K).

II. *In vivo* study

II.1. Chemicals

Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA). HPLC grade ethyl acetate, methanol and ethanol were purchased from Rathburn Chemicals (Walkerburn, Scotland). The following chemicals: (+)-catechin (≥ 98%), (−)-epicatechin (≥ 98%), (−)-Epigallocatechin (≥ 98%), (−)-epigalocatechin3-*O*-gallate (≥ 98%), ethyl gallate (≥ 96%), L-ascorbic acid (≥ 99%), derivatization reagent (pyridine (≥ 99.8%) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide [MSTFA] (≥ 98.5%)), *N*,*N*-dimethyl formamide (DMF) (≥ 99.8%) and all other chemicals including phenolic acid standards were purchased from Sigma Aldrich (Saint Louis, USA). Ferulic acid (≥ 99%), was obtained from AASC Chemicals (Southampton, U.K.). (±)-Verapamil hydrochloride ≥ 99%, EDTA and pentobarbital solution used in rats feeding studies were supplied by Sigma Aldrich. HCl (37%), acetic acid (≥ 99%), phosphoric acid (≥ 85%), formic acid (≥ 95%), were purchased from Fisher Scientific Ltd (Loughborough, Leicestershire, U.K.).

II.2. Plant materials

SHR rats used in this study were fed with aqueous (EAQ) and hydro-alcoholic 70% (EA70) grape pomace extracts provided by 3INature. Composition of these extracts is provided in the chapter III of the manuscript. Grape pomace samples were dissolved daily in 3% EtOH before administration.

II.3. Animals and experimental protocols

For all the experiments, spontaneously hypertensive rats (SHR) and normotensive control Wistar-Kyoto (WKY) rats were purchased from Laboratoire Janvier (Le Genest St. Isle, France). Animals were maintained at a temperature of 23°C with 12h light/dark cycles. Tap water and standard diet A 04 SAFE (Augy, France) composed of 83.9 % of cereals and cereal biproducts, 8.0 % of vegetable proteins (soya bean meal, yeast), 4.1 % of vitamin and mineral mixtures and 4 % of animal proteins (fish) were given *ad libitum*. Food intake and body weight of animals were recorded once a week. Prior to each experiment, adaptation period was allowed. Experiments were performed following European Community animal experiments ethical regulations.

II.3.1. 1st study: Grape pomace extracts screening

Male nine week-old spontaneously hypertensive rats (SHR) and normotensive control Wistar-Kyoto (WKY) rats were used to determine the *in vivo* effect of grape pomace extracts and their potential effect on hypertension. This experiment was held over 6 weeks including three weeks of treatment, one week of treatment resumption followed again by two weeks of treatment. Rats were treated orally by gavage with hydro-alcoholic 70% grape pomace samples at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. Extracts were solubilised with 3 % ethanol.

Rats were randomly assigned to different experimental group organized as follows:

- Control group (6 WKY rats)
- SHR control group (5 SHR treated with 3% EtOH)
- 6 groups of 4 SHR rats treated with hydro-alcoholic 70% grape pomace extracts dissolved in 3% EtOH:

*SHR1: **GRE1** (EA70) seed pomace extract
*SHR2: **SYR1** (EA70) seed pomace extract
*SHR3: **SYR2** (EA70) skin pomace extract
*SHR4: **CAR** (EA70) seed pomace extract
*SHR5: **MOU** (EA70) skin pomace extract
*SHR6: **ALI** (EA70) skin pomace extract

II.3.2. 2nd study: Effect of the verapamil (model validation)

Three male nine week-old spontaneously hypertensive rats (SHR) and three normotensive control Wistar-Kyoto (WKY) rats were used to study the effect of verapamil on SHR blood pressure. This experiment was held over twelve weeks. During the first two weeks, only blood pressure was followed. Then, SHR were treated with verapamil at 30, 20 and 40 mg/kg/day during 10 weeks. Throughout this time, blood pressure was monitored.

II.3.3. 3rd study: Effect of grape pomace extracts associated with verapamil

This study was carried out in three steps

1) "Grape pomace extract + verapamil model" development using extract GRE1 (EA70) seed pomace extract. Three experimental groups were established and experiment lasted during three weeks: group SHR control (3 SHR treated with 3% EtOH), group SHR

verapamil control (3 SHR treated with verapamil at 40 mg/k), group verapamil + extract GRE1 (EA70) seed pomace extract (3 SHR treated with 21 mg/kg of extract).

2) Comparison of grape pomace extracts efficiency in association with verapamil. Rats were randomly assigned to different experimental group organized as follows:

- Control group (3 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)
- SHR verapamil control group (3 SHR treated with verapamil at 40 mg/kg)
- 6 groups of 3 SHR rats treated with hydro-alcoholic 70% grape pomace extracts dissolved in 3% EtOH (21 mg/kg) associated with verapamil (40 mg/kg):

*SHR1+V: **GRE1** (EA70) seed pomace extract + verapamil
*SHR2+V: **SYR1** (EA70) seed pomace extract + verapamil
*SHR3+V: **SYR2** (EA70) skin pomace extract + verapamil
*SHR4+V: **CAR** (EA70) seed pomace extract + verapamil
*SHR5+V: **MOU** (EA70) skin pomace extract + verapamil
*SHR6+V: **ALI** (EA70) skin pomace extract + verapamil

Blood pressure was recorded all along the three weeks experiment.

3) **Results confirmation**. For this experiment, seven SHR rats were used in order to confirm previous results. Animals were fed with Alicante (EA70) skin pomace extract (extract n°6) at a concentration of 21 mg/kg associated with verapamil (40 mg/kg) and blood pressure was followed during three weeks.

II.3.4. Supplementary experiments of grape pomace extracts associated with verapamil screening

a/ Verapamil co-ingested with SYR1 seed (EAQ), SYR1 skin (EAQ), ALI skin (EAQ) and GRE2 skin (EA70) screening

Eighteen male nine week-old spontaneously hypertensive rats (SHR) and six male normotensive control Wistar-Kyoto (WKY) rats were used to determine the *in vivo* effect of additional grape pomace extracts associated with verapamil and their potential effect on hypertension. This experiment was held over 9 weeks including 7 weeks of treatment with gradual increasing dose of verapamil from 10 mg/kg/day to 40 mg/kg/day and 2 week of

grape pomace extracts in association with verapamil. Rats were treated orally by gavage with hydro-alcoholic 70% and aqueous extract of grape pomace samples at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. Extracts were solubilised with 3 % ethanol. Verapamil was administrated at a dose of 40 mg/kg/day.

Rats were randomly assigned to different experimental group organized as follows:

- Control group (6 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)
- SHR verapamil control group (3 SHR treated with verapamil at 40 mg/kg/day)
- 4 groups of 3 SHR rats treated with aqueous and hydro-alcoholic 70% grape pomace extracts dissolved in 3% EtOH in association with verapamil:

*SHR1+V: **SYR1** (EAQ) seed pomace extract + verapamil
*SHR2+V: **SYR1** (EAQ) skin pomace extract + verapamil
*SHR3+V: **ALI** (EAQ) skin pomace extract + verapamil
*SHR4+V: **GRE2** (EA70) skin pomace extract + verapamil

b/ Verapamil co-ingested with CAR seed (EAQ), GRE2 skin (EAQ), SYR2 skin (EAQ) and SYR1 skin (EA70) screening

Eighteen male twenty-three week-old spontaneously hypertensive rats (SHR) and six male normotensive control Wistar-Kyoto (WKY) rats were used to determine the *in vivo* effect of additional grape pomace extracts and their potential effect on hypertension. This experiment was held over 6 weeks including 15 days without treatment, 13 days of treatment with verapamil only, 7 days of grape pomace extracts in association with verapamil treatment followed again by 4 day of treatment with verapamil only. Rats were treated orally by gavage with hydro-alcoholic 70% and aqueous extract of grape pomace samples at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. Extracts were solubilised with 3 % ethanol. Verapamil was administrated at a dose of 40 mg/kg/day.

Rats were randomly assigned to different experimental group organized as follows:

- Control group (6 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)

- SHR verapamil control group (3 SHR treated with verapamil at 40mg/kg/day)

- 4 groups of 3 SHR rats treated with aqueous and hydro-alcoholic 70% grape pomace extracts dissolved in 3% EtOH in association with verapamil:

*SHR1+V: **CAR** (EAQ) seed pomace extract + verapamil

*SHR2+V: **GRE2** (EAQ) skin pomace extract + verapamil

*SHR3+V: **SYR2** (EAQ) skin pomace extract + verapamil

*SHR4+V: **SYR1** (EA70) skin pomace extract + verapamil

II.3.5. Feeding protocol and sample collection for the evaluation of grape pomace extracts bioavailability

Based on previous results, the extract GRE1 (EA70) seed pomace extract, MOU (EA70) skin pomace extract and ALI (EA70) skin pomace extract have been chosen to bioavailability study.

Male nine week-old SHR rats, weighting 207 ± 8 g, were randomly assigned to different experimental group organized as follows:

- SHR control group (6 SHR treated with 3% EtOH)

- SHR verapamil control group (6 SHR treated with verapamil at 40 mg/kg/day)

- 3 groups of 6 SHR rats treated with hydro-alcoholic 70 % grape pomace extracts at 21 mg/kg/day:

*E1: **GRE1** (EA70) seed pomace extract

*E5: **MOU** (EA70) skin pomace extract

*E6: **ALI** (EA70) skin pomace extract

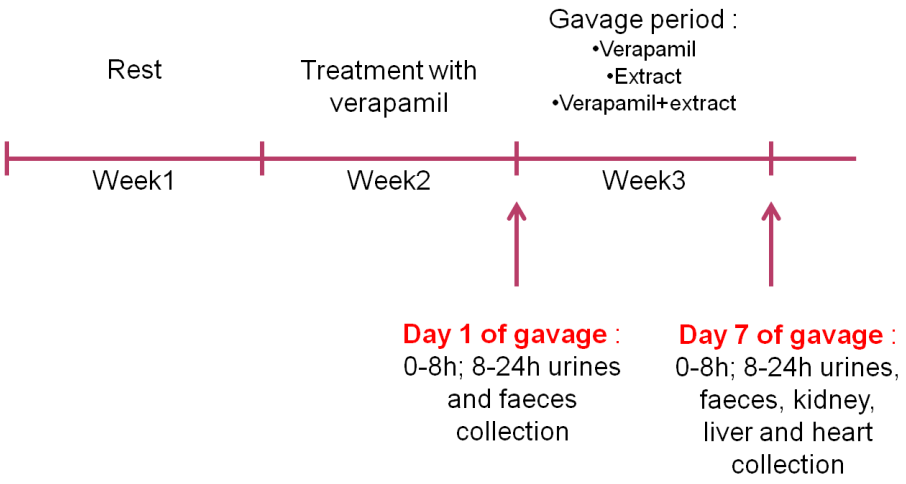
- 3 groups of 6 SHR rats treated with hydro-alcoholic 70% grape pomace extract in association with verapamil:

*VE1: **GRE1** (EA70) seed pomace extract + verapamil

*VE5: **MOU** (EA70) skin pomace extract + verapamil

*VE6: **ALI** (EA70) skin pomace extract + verapamil

Experiment lasted three weeks. After one week of adaption, only animal from SHR verapamil control, VE1, VE5 and VE6 group were treated with verapamil (40 mg/kg) by gavage at week two. At week three, all the animals were daily administrated as stated above during all the week. The experiment procedure is summarized below:



Urine and faeces were collected at two time point (0-8h and 8-24h) at day 1 and day 7 of gavage from metabolic cage in falcon tubes and immediately stored at -80 °C. At day 7, four hours after gavage, rats were anaeasthetized with lethal dose of pentobarbital (60 g/L pentobarbital, 60 mg/kg body weight) and sacrificied by decapitation. Blood was drawn in an EDTA-moistened tube. Tubes were centrifuged at 2300g for 10 min at 4 °C. Plasma was separated from erythrocytes before being stored at -80°C. Tissues including heart, liver and kidneys were collected, rinsed, blottled and stored at -80°C. Samples were shipped to the University of Glasgow on dry ice. Upon arrival, tissues and faeces were freeze dried (Thermo Savant SuperMosulyo), weighted, grounded to a powder and stored at -80°C along with plasmas and urines prior to analysis.

II.4. Blood pressure measurement

Blood pressure was followed and accessed by tail-cuff method with a LETICA LE 5002 Scientific Instrument electrosphygomanometer (Barcelona, Spain) under conscious condition, in a calmed and regulated between 29°C and 32°C darkened room. Rats were trained to the measurements, handled with care and covered with a fabric during the record in order to minimize stress. In this method, the reappearance of pulsation on a digital display of the blood pressure cuff is detected by a pressure transducer, amplified and recorded digitally

as the systolic blood pressure. The average of the three pressure readings was recorded if only the difference between two measurements was below 20 mmHg.

II.5. HPLC-PDA-ESI-MS^n analysis of urines, faeces, tissues and plasmas

II.5.1. Extraction of phenolics from faeces and tissues

Rat faeces and tissues from the same experimental group were pooled together in equal proportions. Faeces and tissues were extracted using a method optimized by Serra et al., (2011). To 100 mg of freeze-dried faeces and 60 mg to freeze-dried tissues were added 50 µL of ascorbic acid 1% and 100 µL of phosphoric acid 4%. Each sample was spiked with 1 µg of ethyl gallate as an internal standard. The samples were first extracted with 800 µL of water/methanol/phosphoric acid 4 % (94/4.5/1.5, v/v/v) using a sonicator during 30 secondes (Digital Sonifier® model S-150D ultrasonic cell disruptor, Branson, Teltow, Germany) and maintained in ice to avoid heat. Samples were then centrifuged for 15 min at 16 100 × g, 20 °C in a 0.2 µm Micro-Spin^{TM} Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, U.K.). The supernatant was decanted and the pellet re-extracted three more times with 500 µL of the same solvent as described above, after which it was centrifuged. The four supernatants were combined. A mixture of 1 mL of the assembled supernatant and 1 mL of phosphoric acid 4 % was loaded onto OASIS® HLB cartridges (3cc, 60 mg) previously conditioned with 1 mL of methanol and 1 mL of 0.2% acetic acid. Column was washed with 1 mL of phosphoric acid 4% and with 1 mL of 0.2% acetic acid. The retained compounds were eluted with 2 × 1 mL acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, v/v/v). The eluate was reduced to dryness using a Speedvac concentrator (SPS SpeedVac, Thermo Savant, Waltham, MA) and resuspended in 25 µL of acidified methanol (1% acid formic) to which was added 225 µL of 0.1% aqueous formic acid. Once resuspended, extracts were centrifuged at 16,100 × g for 10 min at 4°C in a 0.2 µm Micro-Spin^{TM} Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, U.K.) prior to analysis by HPLC-PDA-MS^n.

II.5.2. Extraction of phenolics from plasmas

Rat plasmas from the same group were pooled together in equal proportions before extraction based on Ottaviani et al., (2012c) method. Plasma samples were defrosted and spiked with 1 µg of ethyl gallate as an internal standard. Plasma was added drop wise while vortex to a 15 mL falcon tube containing 3.4 % phosphoric acid and kept on a freeze water

bath. Sample was then loaded onto OASIS® HLB cartridge (3 mL, 60 mg) previously conditioned with 1 mL of *N*,*N*-dimethyl formamide (DMF)/methanol (7/3, v/v) and 0.5% (v/v) acetic acid in water. Cartridge was washed with 3 mL of 0.5 % acetic acid in water (v/v) and 1 mL of water/methanol/acetic acid (80/20/0.5). For elution, cartridge was dried and eluted with 2 × 1 mL of DMF/methanol (7/3, v/v). The eluate was collected in a tube containing 200 µL of 0.5% (v/v) acetic acid in methanol and reduced to approximately 50 µL using a Speedvac concentrator (SPS SpeedVac, Thermo Savant, Waltham, MA). Sample was then resuspended in 25 µL of acidified methanol (1% acid formic) to which was added 225 µL of 0.1% aqueous formic acid. Extract was then centrifuged at 16,100 × g for 10 min at 4°C prior to analysis by HPLC-PDA-MS^n within 24 hours.

II.5.3. Urine analysis

Urine samples were defrosted, vortexed and centrifuged at 16,100 × g for 10 min at 4°C prior to the analysis by HPLC-PDA-MS^n.

II.5.4. HPLC-PDA-ESI-MS^n analysis of procyanidin metabolites

Quantification of metabolites in urines, faeces, plasmas and tissues was carried out using two Surveyor HPLC system both equipped with sampler cooler maintained at 4ºC, a PDA detector and a Finnigan LCQ Duo ion trap mass spectrometer for urine and faeces analysis or a LCQ Adavantage ion trap mass spectrometer for tissue and plasma analysis. Mass spectrometers were both fitted with an electrospay interface (ESI) (Thermo Fisher scientific, San Jose, CA). Separation was performed on a 250 × 4.6 mm i.d. 5 μm Kinetex phenyl-hexyl 100Å column (Phenomenex, Macclesfield, U.K.) maintained at 40 °C. The mobile phase was pumped at a flow rate of 1 mL/min with either a gradient over 20 min of 15-60 % methanol in 0.1% aqueous formic acid (analysis in faeces), a gradient over 30 min of 5-60% methanol in 0.1% aqueous formic acid (analysis of urines) or a gradient over 30 min of 5-65% in 0.1% aqueous formic acid (analysis of tissues and plasmas). The column eluate initially passed through the PDA detector and was then split, with 0.2 mL/min directed to the mass spectrometer fitted with an electrospay interface operating in negative ion mode. The tuning of the mass spectrometer was optimized by infusing a standard of (–)-epicatechin, dissolved in the initial HPLC mobile phase, into the source at a flow rate of 0.2 mL/min.

Procyanidin metabolites were firstly identified using full-scan data-dependent MS^2 scanning from *m/z* 100 to 700. Compound identities were confirmed by MS^3 consecutive

reaction monitoring with a collision energy set at 30%. Following HPLC separation and MS^3 identification, flavan-3-ols and their metabolites were quantified using selected ion monitoring (SIM mode) for faeces and selective reaction monitoring (SRM mode) for urine, tissues and plasma.

Quantification of 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide (*m/z*, 401/225) and 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate (*m/z*, 287/207) was by reference to the aglycone 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone while 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate (*m/z*, 305/225) levels were measured using a calibration curve obtained with 5-(3′,4′-dihydroxyphenyl)-4-hydroxyvaleric acid. Reference compounds were supplied by Daniel Del Rio (University of Parma, Italy).

The other metabolites were quantified as (−)-epicatechin equivalents and expressed as nmol ± SE (*n*=3).

II.6. GC-MS analysis of urines and faeces

II.6.1. Purification of phenolic acids in urines

Urine samples were prepared as described previously by Roowi et al. (2010), and further developed. After thawing, 1 mL of urine was added to 3 mL of 0.2 M HCl containing 60 µg of 3,4,5-trimethoxycinnamic acid (TMCA) as an internal standard. A styrene divinyl benzene solid phase extraction cartridge (Strata SDB-L 200 mg, Phenomenex, Macclesfield, U.K.) was used for sample purification. Acidified urine sample was loaded onto cartridge previously preconditioned with 3 mL of ethyl acetate, followed by 3 mL of methanol and 3 mL of 0.1 M HCl. Thereafter, cartridge was washed with 3 mL of 0.1 M HCl and dry with nitrogen gas for 20 min before being eluted with 3 mL of ethyl acetate. The eluate was transferred to an amber glass vial and reduced to dryness under nitrogen flow at 35 °C. The dried extract was silylated in sealed vial using 300 µL of a mix of pyridine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (1:4, v/v) heated at 80 °C for 20 min on a heating block. Samples were cooled in a closed dry container prior to analysis by GC-MS.

II.6.2. Purification of phenolic acid in faeces

Faecal extracts preparation was adapted from Grün et al. (2008). Briefly, 1 mL of distilled water was added to 50 mg of freeze-dried faecal sample, mixture was vortexed for 60 s and centrifuged at 16 100 × g at 8°C during 10 min in a 0.2 µm Micro-Spin™ Eppendorf

filter (Alltech Associates Applied Sciences, Lancashire, U.K.). The supernatant was collected, purified and silylated with the same procedure than those described for urines.

II.6.3. GC-MS analysis of derivatized urine and faeces

Derivatized phenolic acids in urine and faeces samples were analysed using an Agilent Technologies 6890 Chromatograph equipped with an autosampler and a mass selective detector 5973 (Agilent Technologies, Berkshire, U.K.). Helium was the carrier gas with a flow rate of 1.2 mL/min. One microliter of sample was injected in the split mode with a 25:1 ratio. The injector temperature as maintained at 220°C. The mass spectrometer was used in the positive ionization mode with the ion source and transfer line set at 230°C and 150°C, respectively. Separation was carried out using a ZB-5MS Zebron 30 m × 0.25 mm i.d. × 0.25 µm capillary column (Phenomenex, Macclesfield, U.K.). The initial temperature was set at 40 °C and raised to 160°C at 20°C/min, 200°C at 15°C/min, 250 °C at 10°C/min to a final temperature of 300°C at 40°C/min, held for 5 min. Data acquisition was performed in full scan mode (*m/z* 50-470) with ionization energy of 70 eV. Phenolic acids were identified according to the mass spectra and retention times obtained from authentic standards analysed under identical conditions. When standards were not available, identification was achieved through the integrated NIST mass spectral library with a confidence of 80 % or above. Quantification was based on calibration curves of the ratio between the target ion (*m/z*) of the standard of interest and the target ion of the internal standard (*m/z* 310). Data were expressed in µmol as mean values ± SE (n=3).

Analyses were carried out using MSD ChemStation Data Analysis (Agilent Technologies, Berkshire, U.K).

II.7. Statistical analysis

All measurements were performed in triplicate. Results are expressed as means ± standard deviation (SD). One-way ANOVA was performed to test the effects of variation factors (different samples) on each variable (concentration of different phenolic acids, procyanidin metabolites, etc). If significant effects were found at a 95% confidence interval following a Student's t-test, ANOVA was followed by a Tukey's HSD and Duncan post hoc test to identify differences among groups. These analyses were performed using Statistica V.7 Software (Statsoft InC., Tulsa, O.K).

Chapter 3:

Grapes and grape pomaces phenolic and antioxidant characterisation

I. State of the art: assessing the value of grape pomaces and their potential applications

Today's society is characterized by rising costs and often decreasing availability of raw materials together with much concern about environmental pollution. The demand for appropriate nutritional standards is increasing and thus, there is a considerable emphasis on the recovery, recycling and upgrading of wastes. The food processing industry permits waste effluents, residues and by-products to be recovered and often to be upgraded to higher value and useful products. Using appropriate technology, food industry by-products can be converted into commercial products either as raw materials for secondary processes or as ingredients of new products.

I.1. Grape pomace composition

Bustamante et al. (2008), analyzed different residues from the wine industry and found that winery and distillery waste has a low pH (mean values ranged from 3.8 to 6.8), electrical conductivity (1.62–6.15 dS m^{-1}) and high organic matter content (669–920 g/kg). Moreover winery residues have high concentrations of macronutrients, especially potassium (11.9–72.8 g/kg), as well as high concentrations of (poly)phenols (1.2–19.0 g/kg) and low concentrations of micronutrients and heavy metal contents (Bustamante et al. 2008).

Grape pomace represents an important under utilised residue of the winemaking process. The dry grape by-product consists of pressed skins, seeds and stems and accounts for ca. 20%-25% of the weight of the grapes used to make wine (Laufenberg et al. 2003; Llobera et al. 2007). During winemaking the extraction of grape (poly)phenols typically reaches only 30–40%, depending upon grape variety, vineyard location and the technological parameters used during the winemaking process including destemming, crushing, maceration and pressing (Kammerer et al. 2004; Ruberto et al. 2007). Kammerer et al. (2004), have screened pomaces after winemaking from red *Vitis vinifera* grape varieties (i.e. Cabernet Mitos, Lemberger, Spätburgunder, Trollinger) and white wine cultivars (Kerner, Müller-Thurgau, Weisser Riesling and Merzling) for their phenolic contents and results are presented in Table 9.

Table 9: Mean concentration of principal (poly)phenols in red grape seed and skin pomaces after winemaking after Kammerer et al. (2004).

Anthocyanins	Mean contents in skins (mg/kg DW)	Min[a]	Max[a]
Delphinidin 3-*O*-glucoside	1035	68	5552
Cyanidin 3-*O*-glucoside	403	37	1903
Petunidin 3-*O*-glucoside	1247	65	6680
Peonidin 3-*O*-glucoside	2639	515	12450
Malvidin 3-*O*-glucoside	12123	1117	20533
Delphinidin 3-*O*-(6″-acetyl-glucoside)	150	0	956
Petunidin 3-*O*-(6″-acetyl-glucoside)	213	27	1375
Peonidin 3-*O*-(6″-acetyl-glucoside)	329	27	1484
Malvidin 3-*O*-(6″-acetyl-glucoside)	1336	45	8688
Cyanidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	160	0	1071
Petunidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	381	0	2458
Peonidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	1077	68	6828
Malvidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	4934	271	31442

Flavanols	Skins (mg/kg DW)	Seeds (mg/kg DW)
Catechin	226.7 ± 24.6	790.2 ± 11.2
Epicatechin	134.6 ± 12.1	674.5 ± 24.9
Epicatechin gallate	35.5 ± 3.6	457.9 ± 35.8
Procyanidin B_1	191.5 ± 6.6	1053.7 ± 29.3
Procyanidin B_2	91.0 ± 2.5	506.2 ± 41.1
Quercetin	nd	nd
Quercetin-3-*O*-galactoside	156.7 ± 9.3	14.7 ± 0.9
Quercetin-3-*O*-glucoside	351.7 ± 23.3	32.6 ± 3.0
Quercetin -3-*O*-*g*lucuronide	509.9 ± 30.4	38.0 ± 0.8
Quercetin-3-*O*-rhamnoside	57.7 ± 3.5	14.4 ± 1.0
Kaempferol	nd	nd
Kaempferol-3-*O*-glucoside	247.6 ± 15.6	20.0 ± 1.4
Trans-resveratrol	86.4 ± 4.5	14.2 ± 1.8
Trans-polydatin	15.5 ± 0.1	4.7 ± 0.2
Isorhamnetin-3-*O*-glucoside	35.5 ± 0.1	nd

Phenolic acids	Skins (mg/kg DW)	Seeds (mg/kg DW)
Gallic acid	15.0 ± 0.2	106.5 ± 8.8
Protocatechuic acid	42.8 ± 0.5	102.8 ± 25.5
Caftaric acid	61.0 ± 2.8	9.3 ± 3.4
p-Hydroxybenzoic acid	31.1 ± 0.1	13.8 ± 0.9
Coutaric acid	54.5 ± 1.4	30.2 ± 16.3
Caffeic acid	1.7 ± 0.2	1.9 ± 0.6
Fertaric acid	17.3 ± 1.1	3.0 ± 0.1
Syringic acid	1.0 ± 1.2	1.1 ± 0.1
p-Coumaric acid	Nd	7.2 ± 0.7
Ferulic acid	2.6 ± 0.0	3.9 ± 0.4
Sinapic acid	Nd	1.0 ± 0.1

[a]Min, minimum concentration detected, Max, maximum concentration detected. Data are expressed as mg/kg.

Grape pomaces contain up to 75% of dietary fiber and over 60% of grape pomace dry matter that was indigestible *in vitro* (Bravo et al. 1998). The indigestible grape pomace dietary fibers include pectin, cellulose, lignin and (poly)phenols (Llobera et al. 2007; González-Centeno et al. 2010; Deng et al. 2011; González-Centeno et al. 2012) and the

composition depends on grape varieties and the part of pomace. White grape pomace had lower fiber concentrations (i.e. crude fibers, neutral detergent fibers and acid detergent fibers) than red wine pomace (Baumgärtel et al. 2007). Pomace and stems of Manto Negro red grape (*V.vinifera*) dietary fibers were studied by Llobera et al. (2007). A high percentage of soluble fibers (15%) in relation to the total dietary fibers was found in pomaces, while a high content of lignin, which had important amounts of condensed tannins and resistant proteins, was found in both by-products but especially in stems (31.6%). González-Centeno *et al. (2010)* investigated the dietary fiber compositions of pomace and stems from ten grape (*V.vinifera* L.) different varieties (six red and four white). Both presented considerable quantities of dietary fibers, ranging from 60 to 90% of total dry matter. Grape pomace and stem cell wall polysaccharides composition were very different with pectic substances being the main component of pomace cell walls (40–54% of total cell wall polysaccharides) and cellulose being predominant in stem cell wall polysaccharides (40–49% of total cell wall polysaccharides). Lignin accounted for around 20–25% of dietary fibers in both grape pomaces and stems. In addition, the pectin content and the degree of methyl-esterification of uronic acids of grape pomaces varied depending on the varieties of grapes. A study by Deng *et al.* (2011) using grape pomace from two white grape varieties and three red grape varieties showed that insoluble dietary fibers were composed of lignin (7.9–36.1% dry matter), neutral sugars (4.9–14.6% dry matter) and uronic acids (3.6–8.5% dry matter) and weighed more than 95.5% of total dietary fibers in all pomace samples. White grape pomaces were significantly lower in dietary fibers (17.3–28.0% dry matter) than those of red grape pomaces (51.1–56.3%).

Like other plant materials, grape pomace contain relatively higher amount of non-extractable (poly)phenols (NEP). Although the total (poly)phenol content in dry grape pomace are about 4.8–5.4% (Makris et al. 2007), only 2% of (poly)phenols is extractable under mild conditions (Bravo et al. 1998). The majority portion of grape pomace (poly)phenols has been reported to be highly polymerised condensed tannins, and some (poly)phenols form complexes with fibers and are non-extractable unless strong acidic treatments are applied (Arranz et al. 2010). Monomeric and oligomeric proanthocyanidins are certainly soluble in the organic solvents usually used for (poly)phenols extraction, but a major proportion of high-molecular-weight proanthocyanidins and (poly)phenols complexed with proteins or cell wall polysaccharides remain insoluble. The NEP quantification needs hydrolysis of grape pomace residual to release the bound phenolics from cell walls or

proteins after the extraction of soluble (poly)phenols (Ignat et al. 2011). The NEP content can be as high as 67 mg/g of dry matter in red grape pomace (var. Cencibel) and as low as 1.68 mg/g in white grape pomace (var. Thompson, seedless) (Pérez-Jiménez et al. 2009).

I.2. Grape pomace and their potential uses

Grape pomace is usually transformed in distilleries, after washing, to obtain alcohol and tartrates. It is often distilled in wineries to recover ethanol to produce spirits, although substantial amounts of distilled grape pomace are not used efficiently after winemaking process.

Grape pomace contain large amounts of hemicellulosic sugars, that can be hydrolyzed to produce solutions containing monomers of xylose and glucose, which in turn could be used as a substrate by *Lactobacillus pentosus* to produce lactic acid (Rivera et al. 2007). The same investigators found that the mineral and organic nitrogen present in grape pomace hydrolyzates enhance xylose consumption allowing *L. pentosus* to produced cell bound biosurfactants, which not only reduced the surface tension, but also have emulsifying properties that might facilitate the bioremediation of hydrocarbon contaminated sites (Rivera et al. 2009) using tomato plants. The effectiveness of this bioemulsifier was investigated by preparing kerosene/water emulsions in the presence and absence of emulsifiers. The volumes of kerosene/water emulsions stabilized by the bioemulsifiers produced by *L. pentosus* from hydrolyzed grape pomaces were approximately 74.5% after 72 hours of emulsion formation, with a stability capacity of emulsion about 97% (Portilla-Rivera et al. 2010). The authors reported that these results were better than those achieved using sodium dodecyl sulfate (SDS), a highly effective surfactant use in detergents, to stabilize kerosene/water emulsions. Another study found promising results by testing the biosurfactants obtained by *L. pentosus*-induced fermentation of hemicellulosic sugars from grape pomaces, on various hydrophobic plant substrates in order to reduce their water repellence (Paradelo et al. 2009b).

Most of the time, conditioning treatments such as composting, is necessary before by-products can be used for agricultural purposes. Actually, grape by-products contain large amounts of organic matter and macronutrients such as potassium, which are important factors in soil fertility, and also contain (poly)phenols, which may exert phytotoxic and antimicrobial effects. Studies on the co-composting of winery and distillery wastes with animal manures have indicated a reduction in phytotoxicity during the process (Bustamante et al. 2008). Efficiency of composts derived from winery wastes was evaluated by García-Martínez et al.

(2009). The authors compared the use of winery waste composts and perlite and did not find any differences in the tomato solid soluble content and titratable acidity which indicates that winery waste compost could be used as an alternative. Pardo et al (2007), used vineyard wastes to formulate a high quality, low cost substrate for the cultivation of the most widely consumed edible mushroom, *Agaricus bisporus* (Lange) Imbach. The study demonstrated that this bio-compost was found to be economically viable and environmentally advantageous. Grape pomace composts were also tested for their ability to remove color components from vinasse, the remaining residual liquid from the fermentation or distillation of alcoholic liquors. Results showed comparable results to those achieved with activated carbon (Paradelo et al. 2009a).

Grape pomace can also be used for other applications. Some authors proposed the use of grape marc as a metal adsorbent. For instance, Farinella et al. (2008), found that grape pomace can be used as a natural absorbent for cadmium and lead to the treatment of effluents. Other investigations proposed the use of grape by-products to obtain hydrolytic enzymes by solid state fermentation (Botella et al. 2005; Díaz et al. 2011). The possibility of using grape pomace as animal feeds was also studied (Brenes et al. 2008; Molina-Alcaide et al. 2008; Basalan et al. 2011). Basalan et al. (2011) investigated the nutrient contents and *in vitro* digestibility of twenty-eight fresh grape pomace samples from white and red wine grape varieties in Turkey. They found that *in vitro* the disappearance of dry matter and neutral detergent fiber at 48 h determined using ruminal fluid was similar for pomace from both white and red grapes. This study suggests that fresh grape pomace rich in skin and seed could be a suitable feed for ruminants and to non-ruminants with extensive cecal fermentation. Applications such as the use of grape pomace as a wood adhesive (Ping et al. 2011), bioethanol (Rodríguez et al. 2010) and (poly)phenol oil (Fiori 2010) have been proposed.

In the health field, grape pomace can be of value as natural antioxidants replacing synthetic antioxidants, extracted with organic solvents and/or supercritical carbon dioxide (Vatai et al. 2009) and isothermal autohydrolysis (Conde et al. 2011). Some food processing methods such as extrusion increased extractable and bioavailable (poly)phenols and reduced the NEP by reducing the degree of polymerisation (Khanal et al. 2009). These studies suggest that thermal processing may increase the extractability and bioavailability of some (poly)phenols while destroying heat sensitive (poly)phenols in grape skins and seeds.

A large number of publications have shown the abundance quantity of (poly)phenols in grape seeds and skins, showing significant antioxidant capacity. It is therefore obvious that grape-derived products such as wines, grape juices and eventually pomace are natural sources of (poly)phenols. Significant effort has been made over the past decade to explore the potential of using grape pomace to produce functional food ingredients, such as natural antioxidants for nutrition fortification and food preservation. Sáyago-Ayerdi et al. (2009) showed by feeding chicken (3–6 weeks old) with different concentration of grape pomace (0, 30 and 60 mg/kg body weight) for 3 weeks that chicken's growth performance were not affected, but lipid oxidation of raw and cooked breast chicken patties were significantly inhibited compared with samples obtained from birds fed the control diet at 20 days and long-term frozen storage (6 months). These results indicate that dietary grape pomace (poly)phenols could be effective in inhibiting lipid oxidation of chilled and long-term frozen stored chicken patties.

Other alternative potential commercial uses of grape pomaces that have been advocated inclcude food coloring (Metivier et al. 1980; Ayed et al. 1999), dietary fibers (Saura-Calixto 1998; Llobera et al. 2007) and phytochemical products (González-Paramás et al. 2003).

Wines by-products have been recently assessed by several patents in the last few years. Galbreath et al. (2001)under the patent US 6190716, described a method for preparing a grape derived product with Muscadine grapes. Howard et al. (2001) had described a method of concentrating grape flavonols from wine, juice or de-alcoholized wine, grape skins and fermented grape skins. In this invention, several solvents were tested as eluents. Although the organic solvents are eliminated at the drying stage, the use of water would be preferable. Aware of the nutraceutical benefits of grape derived substances, recent patents have presented advanced extraction methods. Shrikhande et al. (2003) reviewed some patents concerning the extraction of polyphenols from whole grapes, grape seeds and grape pomace. The authors proposed a method using hot water extraction and an adsorb resin to obtain the concentration and purification of polyphenols.

Newly developed foods and beverages have incorporated grape derived extracts in order to obtain health appeal. Wild and Sass (2006) developed a concentrated food ingredient comprising the extracts of green tea, grape skin and grape seed. Shrikhande et al. (2007)

developed new grape extracts by maximizing the extraction of monomeric and oligomeric procyanidins and minimizing the content of polymeric procyanidins.

In 2008, a patent application by Perlman et al. (2008) presented an interesting use of the “rich” by-product of the grape juice industry. Based on the fact that grape pomace solids contain at least ten times greater amounts of polyphenols than one time pressed grape juice, the authors proposed the fortification of such juice with a pomace polyphenol extract. The beverage was tested at different extract concentrations for sensorial acceptance and antioxidant activity. Draijer et al. (2009) described the invention of a beverage containing 550 mg red wine polyphenols + 250 mg of red grape polyphenols added to 200 mL of a soy-based drink (3.4% protein and 2% fat contents).

Thus, grape pomace potentially constitutes a very abundant and relatively inexpensive source of a wide range of (poly)phenols including monomeric and oligomeric proanthocyanidins and a diversity of anthocyanins providing important economic advantages.

II. Grape and grape pomace extracts: phenolics and antioxidant activities characterisation

II.1 Grapes and grape pomaces composition of the different varieties in 2009 and 2010

Grapes at maturity and the pomace samples from six different *V.vinifera* L. varieties were analyzed for their phenolic contents and antioxidant activities in order to select extracts with the highest potential. Figure 23 summarized all the analysis performed on seeds and skins for the 2009 and 2010 vintage after skins and seeds separation. This study was conducted on crude materials, with 2009 and 2010 grapes at maturity and their respective grape pomace from *V.vinifera* L. cv. Grenache (from two different locations [GRE1 and GRE2]), Syrah (from two different locations [SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI), provided by the Château de Beaucastel located in the Vallée du Rhône, appellation of Châteauneuf-du-Pape. GRE2, COU and ALI pomaces were analysed only for the 2010 vintage. The ratio of initial phenolic compounds from grape to pomace was also determined.

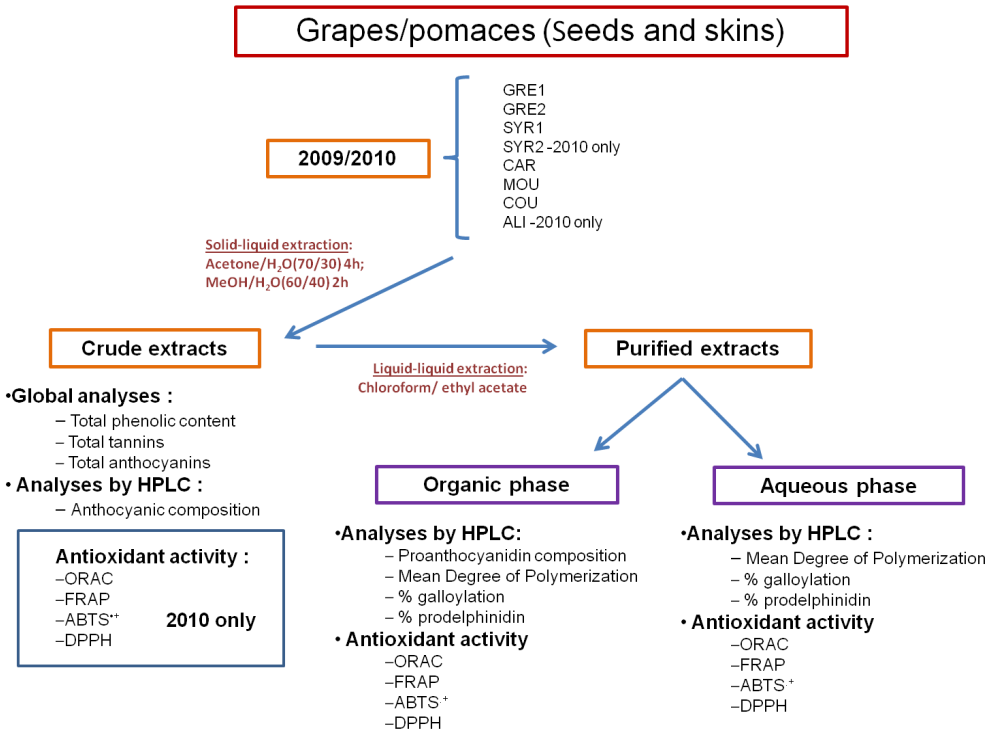


Figure 23: Analysis performed on grape and pomace seeds and skins

II.1.1 Grapes and grape seed pomace phenolic composition in 2009 and 2010

First, total phenolic contents (TPC) and total tannin contents were determined (Table 10 and 11). For the 2009 vintage five varieties were analyzed: Grenache from two different locations (GRE1, GRE2), Syrah (SYR1), Carignan (CAR), Mourvèdre (MOU) and Counoise (COU). The TPC of seed extracts varied slightly between varieties and ranged from 36.6 mg GAE/g DW in CAR to 49.7 mg GAE/g DW in MOU. Total tannins ranged from 62.3 mg/g DW in COU to 106.4 mg/g DW in GRE2. In grape pomaces, values ranged from 12.6 mg GAE/g DW in GRE2 to 35.3 mg GAE/g DW in GRE1 for TPC and from 39.1 mg/g DW to 105.8 mg/g DW for total tannins in GRE2 and GRE1, respectively (Table 10). The amount of extracted TCP was different according to grape varieties and locations (GRE1 *vs* GRE2). Indeed, in GRE2 and MOU, up to 70% of their initial TCP was extracted during fermentation whereas in GRE1, it was only 15%.

Table 10: Total phenol contents and total tannins in 2009 grape and pomace seed extracts

Grapes- 2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
TPC	41.2 ± 1.0	42.1 ± 1.7	47.4 ± 1.0	36.6 ± 0.8	49.7 ± 4.0	42.7 ± 1.7
Total tannins	89.8 ± 0.4	106.5 ± 4.4	89.1± 0.1	91.4 ± 0.8	101.0 ± 4.8	62.3 ± 1.9
Pomaces-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
TPC	35.3 ± 1.1	12.6 ± 1.0	30.2 ± 1.2	27.5 ± 1.0	19.0 ± 2.0	-
Total tannins	105.8 ± 1.0	39.1 ± 1.2	54.3 ± 0.9	59.8 ± 0.7	51.5 ± 0.9	-

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents.

In the case of the 2010 vintage, two more samples were added to the study: another Syrah sample (SYR2) and Alicante (ALI). A greater variability in the amount of (poly)phenols can be observed in grape seeds (Table 11). The highest levels of TPC were founded in GRE1 and COU (88.7 and 83.4 mg GAE/g DW respectively) while GRE2, MOU and CAR contained lowest amounts with an average of 59 mg GAE/g DW. Total tannin levels ranged from 115.6 mg/g DW in SYR2 to 167.8 mg/g DW in GRE1. After vinification the variability was smaller ranging from 33.0 to 44.5 mg GAE/g DW for TPC and 68.9 to 84.9 mg/g DW for total tannins (Table 11). With all the varieties, more than 45% of TCPs remained in the pomaces (Figure 24).

Table 11: Total phenol contents and total tannins in 2010 grape and seed pomace extracts

Grapes-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
TPC	88.7 ± 1.0	58.6 ± 0.2	72.8 ± 0.7	65.6 ± 0.2	58.6 ± 3.7	59.6 ± 1.5	83.4 ± 15.0	76.4 ± 8.0
TT	167.8 ± 0.9	136.8 ± 5.2	123.3 ± 1.4	115.6 ± 1.3	131.7 ± 1.4	153.0 ± 5.2	143.1 ± 21.9	148.4 ± 7.3
Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
TPC	40.5 ± 1.1	34.9 ± 0.2	35.6 ± 1.8	33.0 ± 1.4	38.8 ± 0.3	34.5 ± 0.1	40.8 ± 3.1	44.5 ± 0.4
TT	83.1 ± 0.0	74.9 ± 1.1	79.2 ± 1.4	68.9 ± 2.3	78.7 ± 0.2	69.4 ± 3.1	70.9 ± 4.4	84.9 ± 34.0

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents; TT, Total tannins.

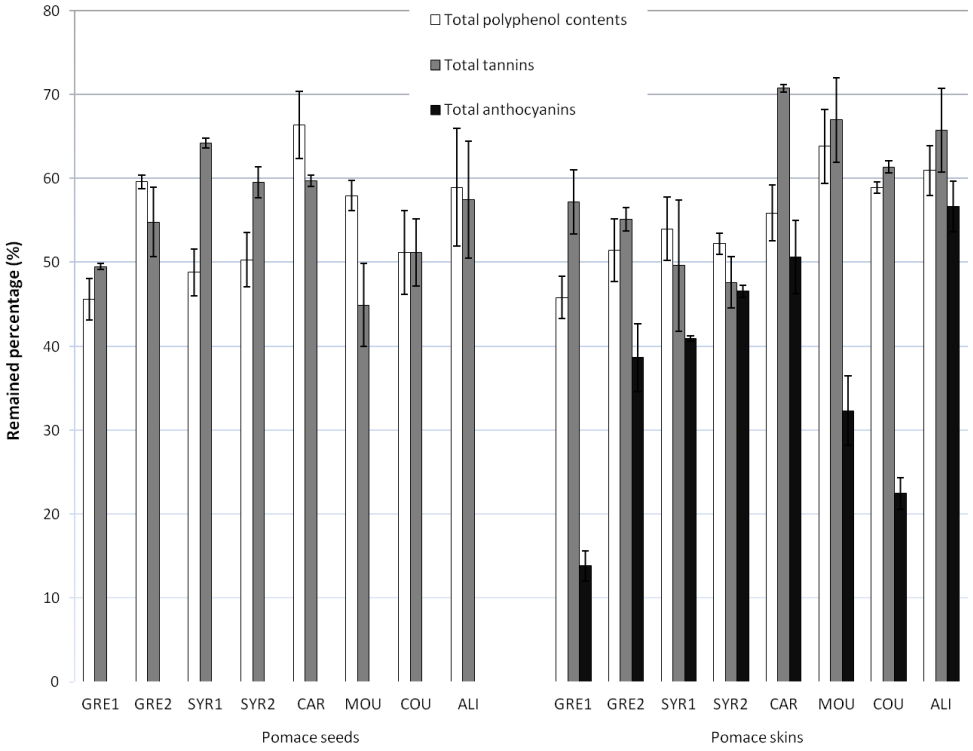


Figure 24: Residual phenolics (total polyphenol contents, total tannins and total anthocyanins) in 2010 grape seed and skin pomace extracts

Concerning proanthocyanidins characterisation, flavan-3-ol monomers [(+)-catechin, (−)-epicatechin] and oligomers (B_1, B_2, B_3, B_4) were identified and quantified (Table 12 and

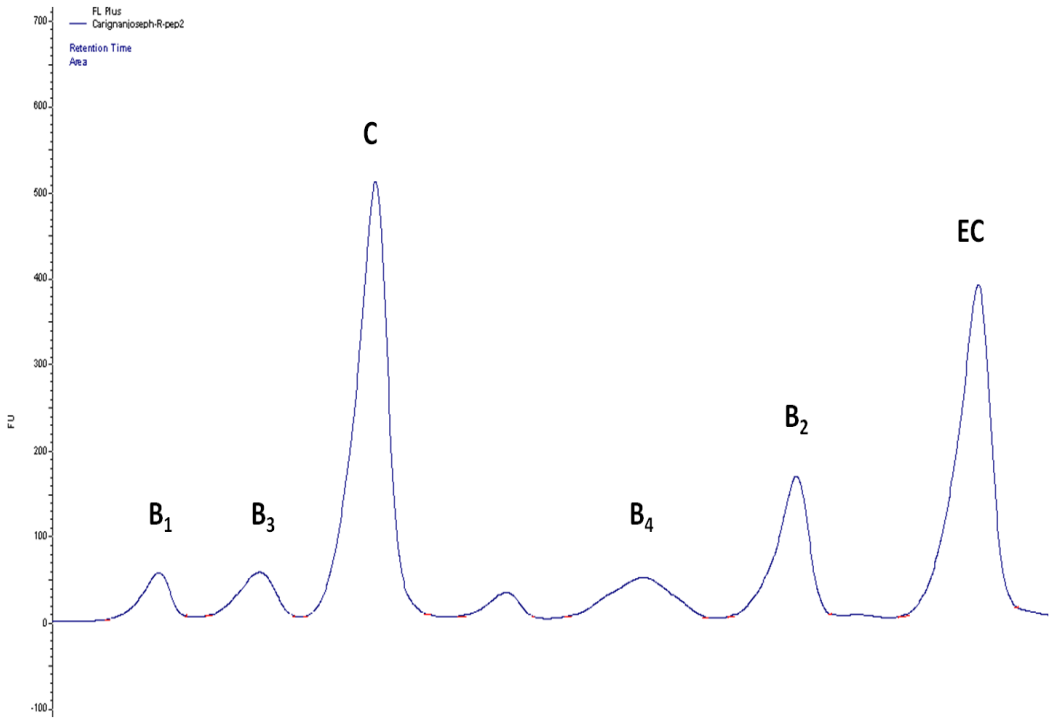


Figure 25: Typical HPLC-Fluo chromatogram of grape and grape pomace extracts with an excitation and emission wavelengths at 230 nm and 320 nm. In this chromatogram, CAR seed extract flavan-3-ol chromatogram is presented. C, (+)-Catechin; EC, (–)-Epicatechin; B1, B2; B3, B4, Procyanidin dimers B1, B2; B3, B4.

13). A typical HPLC-Fluo chromatogram of grape and grape pomace extracts with an excitation and emission wavelengths at 230 nm and 320 nm were showed in Figure 25.

In grapes, for both vintages, COU contained the highest amount of monomeric and oligomeric proanthocyanidins whereas CAR and MOU had the lowest. SYR1 contained a particular rich level of monomers represented by the sum of (+)-catechin and (–)-epicatechin (4.1 mg/g DW in 2009 and 7.8 mg/g DW in 2010) while ALI was a source of an appreciable quantity of proanthocyanidins (7.4 mg/g DW of monomers and 2.2 mg/g DW of dimers [Σ of B_1, B_2, B_3, B_4]). Regarding their respective grape pomaces, 2009 and 2010 SYR1 and ALI retained a high concentration of monomers with up to 6.5 mg/g DW remaining in ALI. The 2009 and 2010 GRE1, COU and ALI were still relatively rich in dimers (Table 12 and 13). Indeed, 90% of monomers and 55% of dimers remained in GRE1 seed pomace and the respective figures for ALI, were 88% and 62%.

Table 12: Proanthocyanidins characterisation of 2009 grape and pomace seed extracts from the vintage 2009

Grapes-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
C	2.33 ± 0.00	1.14 ± 0.13	1.81 ± 0.00	0.70 ± 0.01	0.43 ± 0.16	2.21 ± 0.04
EC	1.01 ± 0.00	0.91 ± 0.11	2.31 ± 0.00	0.62 ± 0.00	0.38 ± 0.14	2.52 ± 0.06
Σ Monomers	**3.34 ± 0.00**	**2.05 ± 0.23**	**4.12 ± 0.00**	**1.32 ± 0.01**	**0.81 ± 0.30**	**4.72 ± 0.09**
B_1	0.15 ± 0.00	0.13 ± 0.02	0.13 ± 0.00	0.10 ± 0.01	0.08 ± 0.03	0.14 ± 0.00
B_2	0.43 ± 0.00	0.43 ± 0.05	0.34 ± 0.00	0.37 ± 0.01	0.18 ± 0.07	0.57 ± 0.01
B_3	0.26 ± 0.00	0.18 ± 0.02	0.14 ± 0.00	0.13 ± 0.01	0.11 ± 0.04	0.14 ± 0.00
B_4	0.19 ± 0.00	0.17 ± 0.02	0.15 ± 0.00	0.15 ± 0.00	0.11 ± 0.04	0.19 ± 0.00
Σ Dimerss	**1.03 ± 0.00**	**0.91 ± 0.10**	**0.76 ± 0.00**	**0.76 ± 0.03**	**0.49 ± 0.18**	**1.04 ± 0.02**
Monomeric/oligomeric fraction						
mDP	2.70 ± 0.00	2.60 ± 0.10	2.24 ± 0.03	3.11 ± 0.05	4.56 ± 0.21	2.11 ± 0.00
%G	37.48 ± 0.00	34.90 ± 1.45	35.27 ± 1.50	36.05 ± 0.09	51.88 ± 0.71	28.70 ± 0.36
Polymeric fraction						
mDP	17.6 ± 0.62	16.70 ± 0.66	13.36 ± 0.08	15.16 ± 0.68	25.11 ± 0.14	11.78 ± 0.11
%G	58.16 ± 0.13	58.70 ± 0.34	54.38 ± 0.12	46.14 ± 0.16	58.90 ± 0.25	44.92 ± 10.76

Pomaces-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
C	0.75 ± 0.01	0.255 ± 0.020	0.63 ± 0.10	0.33 ± 0.00	0.19 ± 0.02	-
EC	0.46 ± 0.02	0.229 ± 0.020	0.75 ± 0.12	0.22 ± 0.00	0.13 ± 0.01	-
Σ Monomers	**1.20 ± 0.03**	**0.484 ± 0.04**	**1.38 ± 0.22**	**0.55 ± 0.01**	**0.32 ± 0.03**	-
B_1	0.09 ± 0.00	0.024 ± 0.002	0.08 ± 0.01	0.06 ± 0.00	0.03 ± 0.00	-
B_2	0.24 ± 0.01	0.073 ± 0.010	0.17 ± 0.03	0.15 ± 0.00	0.04 ± 0.00	-
B_3	0.11 ± 0.00	0.026 ± 0.003	0.07 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	-
B_4	0.11 ± 0.01	0.038 ± 0.010	0.08 ± 0.02	0.06 ± 0.00	0.03 ± 0.00	-
Σ Dimers	**0.56 ± 0.02**	**0.161 ± 0.019**	**0.40 ± 0.07**	**0.31 ± 0.00**	**0.12 ± 0.01**	-
Monomeric/oligomeric fraction						
mDP	3.97 ± 0.13	6.99 ± 0.37	2.95 ± 0.14	4.11 ± 0.00	9.20 ± 0.70	-
%G	45.34 ± 0.39	48.89 ± 2.45	41.86 ± 1.96	44.19 ± 0.00	54.10 ± 0.99	-
Polymeric fraction						
mDP	16.27 ± 0.63	25.81 ± 0.00	12.26 ± 0.49	19.21 ± 0.03	13.28 ± 0.00	-
%G	54.91 ± 0.50	34.30 ± 0.00	53.21 ± 0.51	50.12 ± 0.75	62.75 ± 0.00	-

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; mDP, mean degree of polymerization ; %G, percentage of galloylation ; %P, percentage of prodelphinidins.

Table 13: Proanthocyanidins characterisation of grape and pomace seed extracts from the vintage 2010

Grapes-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
C	1.85 ± 0.10	2.99 ± 0.15	4.53 ± 0.05	2.38 ± 0.06	3.82 ± 0.50	2.62 ± 0.03	2.71 ± 0.12	4.24 ± 0.01
EC	0.79 ± 0.00	1.39 ± 0.18	3.23 ± 0.24	2.93 ± 0.09	0.81 ± 0.13	0.87 ± 0.03	5.16 ± 0.77	3.12 ± 0.33
Σ Monomers	**2.63 ± 0.09**	**4.31 ± 0.16**	**7.76 ± 0.22**	**5.31 ± 0.12**	**4.63 ± 0.52**	**3.49 ± 0.03**	**7.87 ± 0.56**	**7.36 ± 0.29**
B_1	0.26 ± 0.03	0.38 ± 0.02	0.36 ± 0.01	0.27 ± 0.01	0.17 ± 0.04	0.17 ± 0.01	0.33 ± 0.03	0.46 ± 0.07
B_2	0.44 ± 0.01	0.53 ± 0.11	0.39 ± 0.01	0.40 ± 0.07	0.32 ± 0.06	0.20 ± 0.01	0.50 ± 0.01	0.64 ± 0.00
B_3	0.29 ± 0.04	0.49 ± 0.02	0.27 ± 0.04	0.20 ± 0.03	0.18 ± 0.03	0.10 ± 0.00	0.29 ± 0.01	0.71 ± 0.01
B_4	0.23 ± 0.01	0.26 ± 0.05	0.23 ± 0.03	0.22 ± 0.01	0.21 ± 0.05	0.13 ± 0.00	0.25 ± 0.03	0.41 ± 0.02
Σ Dimers	**1.22 ± 0.07**	**1.65 ± 0.06**	**1.25 ± 0.07**	**1.10 ± 0.09**	**0.88 ± 0.08**	**0.59 ± 0.01**	**1.37 ± 0.05**	**2.22 ± 0.06**
C1	0.23 ± 0.04	0.29 ± 0.04	0.29 ± 0.01	0.28 ± 0.01	0.19 ± 0.04	0.20 ± 0.00	0.34 ± 0.03	0.34 ± 0.03
Monomeric/oligomeric fraction								
mDP	1.98 ± 0.29	2.02 ± 0.24	2.02 ± 0.19	1.62 ± 0.32	3.04 ± 0.00	3.54 ± 0.49	1.77 ± 0.02	1.72 ± 0.02
%G	48.16 ± 2.27	47.13 ± 0.07	48.67 ± 0.12	33.35 ± 5.72	55.16 ± 0.00	68.05 ± 2.47	41.13 ± 2.23	43.26 ± 0.59
Polymeric fraction								
mDP	10.86 ± 0.41	11.44 ± 1.47	10.51 ± 1.9	9.93 ± 0.13	9.97 ± 0.46	13.23 ± 1.78	12.11 ± 1.01	10.82 ± 0.49
%G	80.35 ± 8.24	92.03 ± 4.95	91.28 ± 0.15	83.3 ± 0.15	90.73 ± 4.37	92.77 ± 0.3	89.49 ± 4.39	86.43 ± 1.57

Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
C	2.34 ± 0.01	2.33 ± 0.06	2.773 ± 0.162	1.86 ± 0.07	0.89 ± 0.09	0.43 ± 0.01	0.14 ± 0.02	4.11 ± 0.63
EC	0.03 ± 0.01	0.69 ± 0.02	2.61 ± 0.07	1.15 ± 0.02	0.39 ± 0.01	0.55 ± 0.02	2.70 ± 0.13	2.40 ± 0.43
Σ Monomers	**2.37 ± 0.01**	**3.02 ± 0.07**	**5.38 ± 0.14**	**3.01 ± 0.08**	**1.28 ± 0.08**	**0.99 ± 0.04**	**2.84 ± 0.09**	**6.51 ± 0.92**
B_1	0.12 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.17 ± 0.01	0.20 ± 0.01
B_2	0.21 ± 0.03	0.16 ± 0.02	0.09 ± 0.01	0.18 ± 0.03	0.15 ± 0.00	0.11 ± 0.01	0.22 ± 0.03	0.49 ± 0.02
B_3	0.15 ± 0.02	0.13 ± 0.02	0.03 ± 0.00	0.10 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.11 ± 0.01	0.33 ± 0.02
B_4	0.19 ± 0.02	0.16 ± 0.02	0.04 ± 0.00	0.17 ± 0.03	0.12 ± 0.01	0.11 ± 0.01	0.16 ± 0.01	0.33 ± 0.06
Σ Dimers	**0.67 ± 0.05**	**0.57 ± 0.06**	**0.26 ± 0.02**	**0.54 ± 0.08**	**0.46 ± 0.02**	**0.39 ± 0.02**	**0.65 ± 0.05**	**1.37 ± 0.08**
C1	0.13 ± 0.01	0.11 ± 0.02	0.05 ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.17 ± 0.01	0.32 ± 0.03
Monomeric/oligomeric fraction								
mDP	2.60 ± 0.12	2.69 ± 0.02	1.85 ± 0.21	2.26 ± 0.11	3.04 ± 0.03	4.23 ± 0.69	2.53 ± 0.30	1.90 ± 0.07
%G	62.67 ± 4.97	63.87 ± 4.11	49.63 ± 4.12	55.32 ± 0.34	69.67 ± 1.65	73.07 ± 3.94	57.14 ± 1.87	43.66 ± 2.98
Polymeric fraction								
mDP	14.55 ± 0.01	10.95 ± 1.07	7.99 ± 0.73	12.04 ± 1.80	10.43 ± 0.42	12.18 ± 1.13	11.02 ± 0.78	8.06 ± 0.05
%G	95.56 ± 0.01	92.37 ± 2.66	92.93 ± 2.75	92.42 ± 0.38	87.11 ± 1.95	91.67 ± 0.98	91.35 ± 2.53	88.51 ± 3.50

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; mDP, mean degree of polymerization ; %G, percentage of galloylation ; %P, percentage of prodelphinidins.

Concerning the 2009 monomeric/oligomeric fraction of the different grape pomace varities, the mDP ranged from 2.1 to 4.6 and %G from 28.7 to 51.9 while in 2010, it varied respectively from 1.6 to 3.5 (mDP) and %G from 33.3 to 68.1 (Table 12 and 13). Seeds from MOU were the most polymerized and galloylated followed by CAR seeds. The same trend was observed in their respective grape pomaces where MOU has a mDP of 9.2 and 4.2 and a %G of 54.1 and 73.1 in 2009 and 2010 respectively. Compared to grapes, mDP values were higher in grape pomaces especially in 2009 with an increase of 1.3-fold of %G being observed. Indeed, as the alcohol level increases during the winemaking process, tissues become more permeable and low molecular weight tannins are released from seeds into wines toward the mid-point of fermentation and as a consequence the remaining seed pomaces has a higher mDP.

For the polymeric fraction, higher values were generally observed for both vintages. MOU was still the most highly polymerized with grapes mDP values ranging from 11.78 to 25.11 in 2009 and from 9.93 to 13.23 in 2010 (Tables 12 and 13). These results are in agreement with those obtained in other studies with other V. *vinifera* varieties where mDP values of polymeric proanthocyanidins in grape seed extracts extended from 2.7 to 18.6 (Monagas et al. 2003; Vidal et al. 2003). In pomaces, mDP fluctuated from 12.3 to 25.8 in 2009 and from 7.9 to 14.5 in 2010. The %G of 2010 grapes and their pomaces on average were 1.7-fold more galloylated than in 2009. No significant changes in the mDP and %G in polymeric fractions between grapes and pomaces from the same variety and the same vintage were observed. Values were predominantly vintage dependant.

II.1.2. Grape and grape pomace skin phenolics composition in 2009 and 2010

The TPC, total tannin and total anthocyanin contents of grape skins were analysed (Tables 14 and 15). Samples were the same as for seed extracts and for the two vintages. As expected, grape skins contained a lower concentration of phenolic compounds than in seeds. The TPC in 2009, ranged from 20.2 mg GAE/g DW in COU to 35.5 mg GAE/g DW in SYR1 and in 2010 from 34.8 mg GAE/g DW in COU to 52.3 mg GAE/g DW in ALI (Tables 14 and 15). Varieties with the highest total tannin levels were 2009 GRE1 (72.5 mg/g DW) and 2010 ALI (85.8 mg/g DW). For total anthocyanins, SYR1, CAR, MOU and ALI possessed high amounts for both vintages. These results are in good agreement with an earlier report on the high anthocyanin contents of grapes from *Vitis vinifera* cultivars (Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon and Mourvèdre) (Jensen et al. 2008).

Table 14: Total phenol contents, total tannins and total anthocyanins in grape and pomace skin extracts from the 2009 vintage

Grapes-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
TPC	23.4 ± 0.7	21.2 ± 0.0	35.5 ± 1.5	21.7 ± 0.4	27.3 ± 0.7	20.2 ± 0.3
Total tannins	72.5 ± 3.0	57.9 ± 1.4	66.4 ± 6.9	44.9 ± 6.1	67.2 ± 6.1	49.4 ± 0.8
Total antho	4.3 ± 0.9	10.0 ± 0.6	12.9 ± 1.4	24.5 ± 0.3	17.8 ± 2.1	13.7 ± 0.5

Pomaces-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
TPC	18.7 ± 0.1	11.8 ± 0.7	15.5 ± 0.1	22.7 ± 0.1	12.1 ± 0.8	-
Total tannins	53.4 ± 1.2	31.5 ± 0.4	33.0± 0.1	56.1 ± 0.3	31.8 ± 4.9	-
Total antho	3.7 ± 0.0	3.3 ± 0.5	5.1 ± 0.0	7.1 ± 0.5	3.4 ± 0.7	-

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents; total antho, total anthocyanins.

Table 15: Total phenol contents, total tannins and total anthocyanins in grape and pomace skin extracts from the 2010 vintage

Grapes-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
TPC	37.4 ± 0.7	37.9 ± 0.1	45.2 ± 2.2	39.7 ± 0.3	45.0 ± 0.1	41.3 ± 1.6	34.8 ± 0.0	52.3 ± 3.9
TT	59.5 ± 5.2	63.8 ± 0.7	73.0 ± 6.0	66.3 ± 3.3	65.2 ± 0.7	70.8 ± 3.1	61.3 ± 1.1	85.8 ± 8.4
TA	11.2 ± 0.2	8.4 ± 0.7	12.1 ± 0.3	10.8 ± 0.1	15.2 ± 0.0	11.8 ± 0.3	8.7 ± 0.4	18.1 ± 2.5

Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
TPC	17.1 ± 0.4	19.5 ± 1.0	24.3 ± 0.0	20.8 ± 0.2	25.1 ± 1.1	26.3 ± 0.3	20.5 ± 0.2	31.6 ± 1.7
TT	33.9 ± 1.4	35.2 ± 0.2	35.9 ± 1.1	31.8 ± 0.1	46.1 ± 0.3	47.3 ± 1.3	37.6 ± 0.3	55.3 ± 5.7
TA	1.5 ± 0.1	3.2 ± 0.0	4.9 ± 0.1	5.0 ± 0.1	7.7 ± 0.5	3.8 ± 0.3	2.0 ± 0.0	10.0± 0.8

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents; TT, Total tannins; TA, total anthocyanins.

More than 45% of TPC and total tannins remained in the grape skin pomaces of all the varieties. A different trend was observed concerning total anthocyanins especially for MOU in 2009 and GRE1 in 2010 (Figure 24) where up to 80% of the initial amounts were extracted. Thus, anthocyanins appeared to be the most easily extractable phenolic compounds during vinification. Indeed, skins are more altered than seeds by the procedures such as pressing, crushing and maceration. During maceration, appreciable substantial quantities of anthocyanins are extracted into wine. As the level of alcohol increases during vinification, anthocyanins are solubilized and released in the acidic matrix (Ribéreau-Gayon et al. 2006).

Additional information was obtained when monomeric flavan-3-ols and oligomeric proanthocyanidins were analysed by HPLC. They showed substantial amounts of epicatechin, catechin, and procyanidin dimers B_2, B_3 and B_4 in 2009 GRE1 and SYR1 grape skins (Table 16). In their respective skin pomaces, GRE1 and MOU retained the highest concentration of flavan-3-ols (Table 16). SYR1 and CAR were the most extracted varieties and retaining less than 10% of monomers and dimers. In 2010, grape varieties with the highest amounts of monomers and dimers in skins were the CAR, MOU and especially ALI which contained 8.7 mg/g DW of monomers and 0.3 mg/g DW of dimers. The vinification process removed more than 65% of the monomers and especially affected catechin levels (Table 16). Pomaces from 2010 skins of COU and ALI were the richest in monomeric and oligomeric proanthocyanidins (Table 16 and Figure 24).

As already observed in previous studies (Prieur et al. 1994; Lorrain et al. 2011) the proanthocyanidins in skins differed from those in seeds primarily by the presence of prodelphinidins, higher mDP values and lower amounts of galloylated derivatives. In the case of the 2009 vintage, substantial difference in grape skins were observed between varieties especially concerning the %G which varied from 18.3 in MOU to 50.7 in COU and the %P which ranged 5.6 in COU to 52.1 in MOU in the monomeric/oligomeric fraction. mDP values varied from 1.4 in CAR to 5.7 in GRE1. In the polymeric fraction, mDP ranged from 14.1 to 23, %G from 23.2 to 46.1 and only the %P was found for GRE1, GRE2 and COU.

In 2010 grape skins, in the monomeric/oligomeric fractions, mDP varied from 4.9 to 11.1, %G from 30.5 to 59.1 and %P from 8.5 to 25.2 (Table 17). In the polymeric fraction, mDP fluctuated from 18.8 to 24.8. Non-significant differences were observed between varieties and values ranged from 6.9 to 9.5 for %G and from 22.2 to 30.5 for %P. These results are consistent with data concerning mDP values of polymeric proanthocyanidins which can vary from 10 to ~83, depending on the fractionation technique employed, the grape variety and the vintage (Souquet et al. 1996; Bordiga et al. 2011). Compared to other studies on Italian and Bordeaux grape varieties with vintages 2008, 2009 and 2010, our %G and %P are higher. Indeed, these results can be related to the varieties and vintage effects (Chira et al. 2009; Bordiga et al. 2011; Lorrain et al. 2011). Grape skin pomace analysis underlined that vinification affected the characteristics of proanthocyanidins in skins. Actually, for the two vintages, in grape skin pomace extracts, an increase in mDP and a decrease in %G in the monomeric/oligomeric fractions were observed.

Table 16: Proanthocyanidins characterisation of grape and pomace skin extracts from the vintage 2009

Grapes-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
C	0.14 ± 0.01	0.01 ± 0.00	0.28 ± 0.02	0.08 ± 0.01	0.04 ± 0.01	0.03 ± 0.00
EC	0.06 ± 0.00	0.001 ± 0.00	0.08 ± 0.06	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.00
Σ Monomers	**0.20 ± 0.01**	**0.01 ± 0.00**	**0.36 ± 0.07**	**0.10 ± 0.02**	**0.05 ± 0.01**	**0.05 ± 0.00**
B_1	0.05 ± 0.01	0.02 ± 0.00	0.92 ± 0.06	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
B_2	0.05 ± 0.01	0.0004 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
B_3	0.02 ± 0.00	0.003 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
B_4	0.02 ± 0.00	0.003 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Σ Dimers	**0.15 ± 0.02**	**0.02 ± 0.00**	**1.01 ± 0.07**	**0.09 ± 0.03**	**0.05 ± 0.02**	**0.04 ± 0.00**
Monomeric/oligomeric fraction						
mDP	5.68 ± 0.28	4.05 ± 0.00	1.67 ± 0.02	1.37 ± 0.04	5.34 ± 0.54	2.82 ± 0.02
%G	29.86 ± 8.46	28.3 ± 0.00	29.92 ± 0.21	18.85 ± 2.73	18.32 ± 1.13	50.67 ± 0.73
%P	47.41 ± 5.08	16.15 ± 0.00	11.27 ± 1.1	8.55 ± 0.38	52.12 ± 0.82	5.75 ± 0.24
Polymeric fraction						
mDP	17.28 ± 0.55	22.92 ± 0.40	18.54 ± 0.00	15.16 ± 0.68	14.14 ± 0.32	18.92 ± 0.4
%G	23.2 ± 3.95	27.37 ± 1.80	42.29 ± 2.13	46.14 ± 0.16	28.99 ± 0.24	26.61 ± 1.57
%P	16.77 ± 0.13	11.29 ± 2.18	nd	nd	nd	16.17 ± 0.02

Pomaces- 2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
C	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	-
EC	0.003 ± 0.00	0.003 ± 0.00	0.002 ± 0.00	0.002 ± 0.00	0.01 ± 0.00	-
Σ Monomers	**0.03 ± 0.00**	**0.011 ± 0.00**	**0.01 ± 0.00**	**0.008 ± 0.00**	**0.016 ± 0.00**	-
B_1	0.02 ± 0.00	0.003 ± 0.00	0.007 ± 0.00	0.008 ± 0.00	0.007 ± 0.00	-
B_2	0.002 ± 0.00	0.007 ± 0.00	0.001 ± 0.00	0.002 ± 0.00	0.007 ± 0.00	-
B_3	0.005 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.003 ± 0.00	-
B_4	0.004 ± 0.00	0.003 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.004 ± 0.00	-
Σ Dimers	**0.032 ± 0.00**	**0.013 ± 0.00**	**0.01 ± 0.00**	**0.012 ± 0.00**	**0.022 ± 0.01**	-
Monomeric/oligomeric fraction						
mDP	3.76 ± 0.03	3.80 ± 0.10	3.96 ± 0.25	2.23 ± 0.22	7.50 ± 0.37	-
%G	17.17 ± 0.46	19.81 ± 1.40	19.27 ± 0.40	10.00 ± 1.49	21.27 ± 2.13	-
%P	6.37 ± 0.89	28.19 ± 2.27	6.62 ± 0.40	14.22 ± 6.72	17.87 ± 4.98	-
Polymeric fraction						
mDP	13.69 ± 0.43	17.64 ± 0.00	13.00 ± 0.59	12.04 ± 0.24	11.09 ± 0.00	-
%G	33.26 ± 0.13	20.48 ± 0.00	38.02 ± 0.99	34.81 ± 0.71	4.70 ± 0.00	-
%P	7.11 ± 0.82	26.88 ± 0.00	ND	ND	58.37 ± 0.00	-

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; mDP, mean degree of polymerization ; %G, percentage of galloylation ; %P, percentage of prodelphinidins.

Table 17: Proanthocyanidins characterisation of grape and pomace skin extracts from the vintage 2010

Grapes-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
C	1.24 ± 0.06	2.81 ± 0.00	1.42 ± 0.02	1.81 ± 0.01	3.96 ± 0.05	2.86 ± 0.03	2.78 ± 0.00	7.59 ± 0.08
EC	0.33 ± 0.01	0.36 ± 0.03	0.40 ± 0.03	0.67 ± 0.13	0.34 ± 0.01	1.03 ± 0.02	0.78 ± 0.00	1.11 ± 0.01
Σ Monomères	**1.57 ± 0.01**	**3.19 ± 0.00**	**1.84 ± 0.00**	**2.48 ± 0.11**	**4.29 ± 0.02**	**3.89 ± 0.03**	**3.56 ± 0.00**	**8.69 ± 0.05**
B_1	0.04 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.00	0.17 ± 0.00
B_2	0.01 ± 0.00	0.004 ± 0.00	0.003 ± 0.00	0.003 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
B_3	0.02 ± 0.00	0.02 ± 0.00	0.013 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.08 ± 0.00
B_4	0.01 ± 0.00	0.004 ± 0.00	0.002 ± 0.00	0.002 ± 0.00	0.005 ± 0.00	0.02 ± 0.00	0.004 ± 0.00	0.01 ± 0.00
Σ Dimères	**0.07 ± 0.00**	**0.07 ± 0.00**	**0.04 ± 0.00**	**0.04 ± 0.00**	**0.11 ± 0.01**	**0.12 ± 0.00**	**0.10 ± 0.01**	**0.27 ± 0.00**
Monomeric/oligomeric fraction								
mDP	4.88 ± 0.00	5.19 ± 0.17	11.1 ± 2.04	7.35 ± 1.45	8.16 ± 0.55	6.45 ± 0.6	7.42 ± 1.11	5.35 ± 1.19
%G	37.55 ± 6.21	30.47 ± 6.62	44.18 ± 26.12	46.61 ± 7.28	59.12 ± 2.33	47.13 ± 7.48	46.91 ± 7.02	40.09 ± 9.59
%P	25.2 ± 6.93	19.42 ± 3.42	24.74 ± 15.65	17.56 ± 2.57	10.33 ± 1.50	14.57 ± 2.02	12.92 ± 0.84	8.49 ± 0.74
Polymeric fraction								
mDP	22.43 ± 1.23	19.42 ± 0.05	21.41 ± 2.28	18.76 ± 0.01	24.85 ± 0.73	21.36 ± 0.45	21.98 ± 0.57	22.10 ± 0.18
%G	7.65 ± 0.52	7.07 ± 0.46	6.89 ± 1.40	7.77 ± 0.01	9.00 ± 0.16	7.68 ± 0.44	9.54 ± 1.01	8.38 ± 0.45
%P	25.97 ± 2.80	28.52 ± 0.70	29.81 ± 3.28	26.46 ± 0.01	30.50 ± 0.35	23.51 ± 1	22.18 ± 1.10	25.85 ± 1.00

Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
C	0.50 ± 0.00	0.51 ± 0.05	0.40 ± 0.03	0.58 ± 0.12	0.43 ± 0.01	0.29 ± 0.02	0.65 ± 0.07	1.28 ± 0.07
EC	0.01 ± 0.00	0.26 ± 0.03	0.25 ± 0.03	0.23 ± 0.00	0.24 ± 0.01	0.23 ± 0.015	0.63 ± 0.08	1.14 ± 0.15
Σ Monomères	**0.51 ± 0.00**	**0.76 ± 0.07**	**0.65 ± 0.04**	**0.82 ± 0.10**	**0.67 ± 0.00**	**0.52 ± 0.03**	**1.29 ± 0.13**	**2.42 ± 0.19**
B_1	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
B_2	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03± 0.00	0.05 ± 0.00	0.03 ± 0.00
B_3	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
B_4	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
Σ Dimères	**0.07 ± 0.00**	**0.08 ± 0.00**	**0.15 ± 0.01**	**0.04 ± 0.00**	**0.08 ± 0.01**	**0.11 ± 0.00**	**0.16 ± 0.00**	**0.12 ± 0.01**
Monomeric/oligomeric fraction								
mDP	12.13 ± 0.89	7.76 ± 0.01	9.35 ± 0.02	10.22 ± 0.04	10.29 ± 0.13	9.20 ± 0.45	11.22 ± 0.45	9.94 ± 2.40
%G	9.70 ± 0.34	19.24 ± 0.01	20.86 ± 0.76	23.82 ± 0.14	17.27 ± 0.31	18.59 ± 0.09	25.27 ± 0.33	13.81 ± 0.81
%P	16.94 ± 2.13	24.88 ± 0.01	29.08 ± 0.16	26.27 ± 0.03	34.92 ± 0.64	26.06 ± 0.28	32.38 ± 0.55	31.05 ± 0.62
Polymeric fraction								
mDP	10.74 ± 0.21	10.97 ± 0.82	11.48 ± 0.75	12.20 ± 1.17	12.11 ± 0.57	12.20 ± 1.17	11.70 ± 0.32	10.85 ± 1.76
%G	10.04 ± 0.52	7.62 ± 0.47	7.16 ± 0.23	10.53 ± 0.18	6.07 ± 0.03	10.53 ± 0.18	10.20 ± 0.57	11.02 ± 0.90
%P	17.73 ± 0.30	19.23 ± 0.16	18.96 ± 0.43	18.12 ± 0.09	20.67 ± 0.36	18.12 ± 0.09	18.79 ± 0.02	16.72 ± 1.03

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; mDP, mean degree of polymerization ; %G, percentage of galloylation ; %P, percentage of prodelphinidins.

As for seeds, these values suggest that proanthocyanidins with low mDP were the most readily extracted into wines. In the polymeric fractions, the trend was opposite since mDP decreased in pomaces. This observation demonstrates that not only the small proanthocyanidins but also the more polymerized ones can be extracted from skins, probably during different periods of the vinification process. No conclusions can be drawn concerning %P in 2009 because of varietal differences. However, in 2010, %P tended to increase in monomeric/oligomeric fractions while the opposite was observed in polymeric fractions.

The anthocyanin contents of skin extracts was analysed by HPLC and the profiles obtained were in good agreement with those obtained in earlier studies with *V. vinifera* L. grapes (Romero-Cascales et al. 2005; Liang et al. 2008) and individual anthocyanin concentrations were well correlated with estimates of total anthocyanin contents. For both vintages and for all varieties, malvidin-3-*O*-monoglucoside was the major anthocyanin and accounted for 40% to 55% of total anthocyanins depending on the variety (Tables 18 and 19). In 2009 grapes, it is observed that SYR1, CAR and MOU contained more glycosylated, acetylated and *p*-coumaroylated anthocyanins than the other varieties. Values ranged from 1.4 mg/g DW to 10.6 mg/g DW for glycosylated anthocyanins, from 0.2 mg/g DW to 1.1 mg/g DW for acetyl-anthocyanins and from 0.2 mg/g DW to 2.7 mg/g DW for *p*-coumaroylated anthocyanins. In grape skin pomace samples, CAR and SYR1 still contained the highest amounts of glycosylated and *p*-coumaroylated anthocyanins, with 8.9 mg/g DW and 5.2 mg/g DW respectively. MOU was the most affected by vinification since more than 70 % of the initial anthocyanins were extracted into wines (Table 18).

Table 18: Anthocyanins characterisation of grape and pomace skin extracts from the vintage 2009

Grapes-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
Dp	0.38 ± 0.01	nd	nd	0.35 ± 0.01	0.51 ± 0.01	nd
Cy	0.42 ± 0.01	nd	nd	0.33 ± 0.00	0.98 ± 0.01	nd
Pt	0.49 ± 0.01	0.16 ± 0.00	0.31 ± 0.00	0.39 ± 0.01	0.89 ± 0.01	0.19 ± 0.00
Pn	0.91 ± 0.00	0.30 ± 0.00	1.17 ± 0.01	0.77 ± 0.00	2.24 ± 0.01	0.49 ± 0.00
Mv	3.90 ± 0.01	0.91 ± 0.01	5.81 ± 0.05	8.08 ± 0.05	5.96 ± 0.01	0.76 ± 0.00
Σ Glu	**6.10 ± 0.02**	**1.38 ± 0.01**	**7.28 ± 0.06**	**9.91 ± 0.05**	**10.59 ± 0.02**	**1.43 ± 0.00**
Σ Ace	**0.38 ± 0.01**	**0.17 ± 0.01**	**1.13 ± 0.01**	**0.48 ± 0.01**	**0.57 ± 0.01**	**0.22 ± 0.00**
Σ Coum	**0.87 ± 0.01**	**0.24 ± 0.01**	**2.72 ± 0.01**	**2.53 ± 0.01**	**1.58 ± 0.01**	**0.24 ± 0.00**
Pomaces-2009						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**
Dp	0.26 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.67 ± 0.01	0.22 ± 0.01	-
Cy	0.22 ± 0.00	0.21 ± 0.00	0.17 ± 0.00	0.21 ± 0.00	0.18 ± 0.01	-
Pt	0.32 ± 0.01	0.33 ± 0.00	0.31 ± 0.00	0.87 ± 0.01	0.32 ± 0.00	-
Pn	0.41 ± 0.00	0.45 ± 0.01	0.35 ± 0.00	0.42 ± 0.02	0.27 ± 0.00	-

Mv	1.73 ± 0.02	2.60 ± 0.00	3.09 ± 0.05	6.77 ± 0.01	1.96 ± 0.01	-
Σ Glu	**2.94 ± 0.01**	**3.83 ± 0.01**	**4.16 ± 0.06**	**8.96 ± 0.02**	**2.94 ± 0.02**	-
Σ Ace	**0.36 ± 0.00**	**0.45 ± 0.00**	**0.79 ± 0.01**	**0.43 ± 0.01**	**0.18 ± 0.01**	-
Σ Coum	**1.28 ± 0.00**	**1.43 ± 0.02**	**2.34 ± 0.00**	**5.23 ± 0.00**	**0.69 ± 0.00**	-

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. Dp, delphinidin-3-*O*-monoglucoside ; Cy, Cyanidin-3-*O*-monoglucoside ; Pt, Petunidin-3-*O*-monoglucoside ; Pn, Peonidin-3-*O*-monoglucoside ; Mv, Malvidin-3-*O*-monoglucoside ; Σ Glu, sum of monoglucoside anthocyanins; Σ Ace, sum of petunidin-3-*O*-acetylmonoglucoside, peonidin-3-*O*-acetylmonoglucoside and malvidin-3-*O*-acetylmonoglucoside; Σ Coum, sum of peonidin-3-(6-*O*-p-coumaroyl)monoglucoside and malvidin-3-(6-*O*-p-coumaroyl)monoglucoside; nd, not detected.

As previously noted, the anthocyanin contents of grape skins were higher in 2010 than 2009. Grape skin extracts from ALI contained the highest quantities of glycoside-, acetyl- and *p*-coumaroyl-anthocyanins, 17.40, 1.57 and 2.38 mg/g DW, respectively (Table 19). "Teinturier "cultivars had higher anthocyanin contents than "non-teinturier "grapes. It has been reported that Alicante skins contain principally malvidin-3-*O*-glucoside (39-48% of the total) as expected for the *V.vinifera* cultivars, but also contain unusually high amounts of peonidin-3-*O*-glucoside (19-31%) when compared to Cabernet Sauvignon and Tempranillo (Hermosin Gutierrez et al. 2004). Among "non-teinturier" varieties, SYR1 and CAR were particularly rich in glycosylated and *p*-coumaroylated anthocyanins, for both 2009 and 2010 vintages, while MOU was rich in acetylated anthocyanins, especially in 2010. Appreciable amounts of anthocyanins remained in grape skin pomaces of SYR1, CAR and ALI, with up to 14.3 mg/g DW, 0.22 mg/g DW and 5.3 mg/g DW of glycoside-, acetyl- and *p*-coumaroyl-anthocyanins, respectively, being retained (Table 18 and 19). In 2009 and 2010 skin pomaces of GRE1 and COU contained the lowest levels of anthocyanins whereas CAR (2009 and 2010), GRE2 (2010), SYR2 (2010) and MOU (2010) retained high quantities of glycoside-, acetyl- and *p*-coumaroyl-anthocyanins. Furthermore, for the two vintages, the data obtained with grape skins and pomace skins indicated that the winemaking process resulted in a relative increase *p*-coumaroyl derivatives and a decrease of the acetyl-anthocyanins. This phenomenon has also been observed in an earlier study which found that the relative content of *p*-coumaroyl derivatives of malvidin and peonidin was lower in wines than in fresh grape skins but higher in pomace. (García-Beneytez et al. 2002). Slow rates of extraction of the *p*-coumaroyl anthocyanins from skins during vinification have been reported, explaining the presence of similar amounts of these anthocyanins in fresh grape skins and pomace skins (Fournand et al. 2006).

Table 19: Anthocyanins characterisation of grape and pomace skin extracts from the vintage 2010

Grapes-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Dp	2.51 ± 0.31	0.74 ± 0.11	1.44 ± 0.02	1.51 ± 0.02	2.92 ± 0.10	1.16 ± 0.01	0.94 ± 0.00	1.45 ± 0.00
Cy	0.45 ± 0.05	0.11 ± 0.01	0.13 ± 0.00	0.15 ± 0.00	0.13 ± 0.02	0.50 ± 0.03	0.16 ± 0.01	0.14 ± 0.00
Pt	1.40 ± 0.03	0.58 ± 0.04	1.10 ± 0.00	1.03 ± 0.01	2.61 ± 0.66	1.24 ± 0.06	0.80 ± 0.00	1.40 ± 0.01
Pn	1.55 ± 0.09	0.86 ± 0.04	0.95 ± 0.00	0.89 ± 0.02	0.74 ± 0.16	1.43 ± 0.11	0.85 ± 0.01	2.73 ± 0.03
Mv	6.26 ± 0.47	4.10 ± 0.28	6.96 ± 0.03	4.47 ± 0.05	8.17 ± 0.33	2.45 ± 0.18	3.62 ± 0.01	11.53 ± 0.06
Σ Glu	**12.17 ± 0.00**	**6.39 ± 0.01**	**10.38 ± 0.20**	**8.15 ± 0.10**	**14.55 ± 0.01**	**6.78 ± 0.16**	**6.38 ± 0.01**	**17.40 ± 0.12**
Σ Ace	**0.61 ± 0.01**	**0.98 ± 0.30**	**0.70 ± 0.00**	**0.63 ± 0.00**	**0.74 ± 0.00**	**2.81 ± 0.03**	**0.73 ± 0.01**	**1.57 ± 0.00**
Σ Coum	**1.14 ± 0.00**	**0.74 ± 0.01**	**1.97 ± 0.00**	**1.12 ± 0.00**	**3.11 ± 0.05**	**1.62 ± 0.01**	**1.43 ± 0.00**	**2.38 ± 0.01**

Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Dp	0.34 ± 0.00	1.48 ± 0.31	1.15 ± 0.01	1.45 ± 0.00	4.23 ± 0.72	1.39 ± 0.21	0.56 ± 0.00	1.57 ± 0.09
Cy	0.06 ± 0.00	0.09 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.10 ± 0.01
Pt	0.27 ± 0.00	0.94 ± 0.17	0.92 ± 0.01	1.01 ± 0.00	1.88 ± 0.24	0.94 ± 0.13	0.39 ± 0.00	1.18 ± 0.06
Pn	0.29 ± 0.00	0.68 ± 0.13	0.64 ± 0.03	0.64 ± 0.01	0.46 ± 0.03	0.46 ± 0.06	0.45 ± 0.01	2.58 ± 0.11
Mv	1.63 ± 0.00	5.39 ± 0.75	7.77 ± 0.09	5.28 ± 0.04	7.09 ± 0.69	3.34 ± 0.33	1.75 ± 0.01	8.89 ± 0.11
Σ Glu	**2.58 ± 0.01**	**8.57 ± 0.01**	**10.54 ± 0.00**	**8.46 ± 0.00**	**13.74 ± 0.02**	**6.23 ± 0.00**	**3.28 ± 0.05**	**14.33 ± 0.00**
Σ Ace	**0.18 ± 0.00**	**0.19 ± 0.00**	**0.22 ± 0.00**	**0.18 ± 0.00**	**0.18 ± 0.00**	**0.17 ± 0.00**	**0.20 ± 0.00**	**0.20 ± 0.01**
Σ Coum	**0.37 ± 0.00**	**0.97 ± 0.00**	**5.29 ± 0.00**	**1.15 ± 0.10**	**3.05 ± 0.01**	**0.67 ± 0.01**	**0.60 ± 0.00**	**4.07 ± 0.00**

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, COU, Counoise ; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. Dp, delphinidin-3-*O*-monoglucoside ; Cy, Cyanidin-3-*O*-monoglucoside ; Pt, Petunidin-3-*O*-monoglucoside ; Pn, Peonidin-3-*O*-monoglucoside ; Mv, Malvidin-3-*O*-monoglucoside ; Σ Glu, sum of monoglucoside anthocyanins; Σ Ace, sum of petunidin-3-*O*-acetylmonoglucoside, peonidin-3-*O*-acetylmonoglucoside and malvidin-3-*O*-acetylmonoglucoside; Σ Coum, sum of peonidin-3-(6-*O*-p-coumaroyl)monoglucoside and malvidin-3-(6-*O*-p-coumaroyl)monoglucoside; nd, not detected.

Higher concentrations of (poly)phenols were found in 2010 vintage seeds and skins than in 2009. A previous study of vintage effects on Bordeaux grape phenolic compositions considered the recorded climatic conditions and weather indicators such as temperatures, sunlight exposure and vine water status, and hypothesized about the impact of these parameters (Lorrain et al. 2011). In the present investigation, cumulated precipitation 60 days before flowering in the Rhône Valley area was 127 mm in 2009 and 99 mm in 2010. According to Lorrain et al. (2011), the water deficit induced by low rain falls in 2010 could lead to an activation of the flavonoid pathway responsible for tannin and anthocyanin biosynthesis which occurs from the flowering stage and the beginning of berry growth (Gagné et al. 2009). This observation would explain the higher TPCs obtained with 2010 grapes. Other investigators have mentioned the impact of climatic conditions such as sunlight exposure and average temperatures as factors impacting (poly)phenols accumulation in grapes (Downey et al. 2004; Cortell et al. 2006; Chorti et al. 2010). In the Rhône valley region, sunlight exposure in 2009 and 2010 were 2958 and 2753 hours respectively while average temperatures from May to September were 22°C and 22.5 °C respectively. According to Chorti et al. (2010) sunlight exposure which is essential for grape berry ripening could be responsible for excessive sunburn, qualitative and quantitative vine damages especially on anthocyanins. The sunlight exposure in 2009 was higher than in 2010 which could also explain a lower phenolic content in 2009 grapes. This vintage impact had direct consequences on the phenolic contents of grape pomaces which followed the same pattern as their parent grapes, with higher concentrations being evident in the 2010 vintage material.

II.2. Antioxidant activities of grape pomace extracts

The antioxidant potential of each sample was determined in order to select the most active grape pomace seeds and skins among studied varieties. Antioxidant capacity of each extracts cannot be assessed by a single method. Indeed, antioxidant measurements can be related either to the capacity of extracts to directly transfer hydrogen to a radical (DPPH or ABTS) or to act as competitors for the peroxy radicals (ORAC test) (Roginsky et al. 2005). Hence, more than one type of antioxidant measurement needs to be performed to take into account the various mode of action of antioxidants (Huang et al. 2005). In that context, the free radical scavenging capacities of seed and skin extracts were evaluated by four types of analytical methods: the FRAP, $ABTS^{\bullet+}$ decolorization, DPPH and ORAC assays.

II.2.1 Antioxidant activities of grape pomace seed extracts

Concerning the vintage 2009, only purified extracts including the monomeric/oligomeric and polymeric fractions were tested in the antioxidant assays. Different classification of pomace seed varieties was obtained. Radical scavenging capacities of their grape seed and skins extracts for 2009 and 2010 vintages are presented in Table 23 and 24. With the monomeric/oligomeric fraction, GRE1 contained the most antioxidants (ORAC: 146.3 µM TE/g DW; FRAP: 114.6 µM Fe^{2+}/g DW, ABTS: 94.8 µM TE/g DW and DPPH: 56.4 TE/g DW) in all four assays (Table 20), followed by SYR1 seed extracts. These results are in accordance with tannin analysis since SYR1 and GRE1 contain appreciable amounts of TPC and tannins as well as high levels of monomeric and dimeric proanthocyanidins.

Table 20: Radical scavenging capacities of grape seed pomaces in 2009

	Pomaces-2009				
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**
Purified extracts:					
Monomeric/oligomeric fraction					
ORAC	146.3 ± 17.8	102.2 ± 16.0	84.4 ± 29.0	42.4 ± 10.8	41.8 ± 4.9
FRAP	114.6 ± 11.8	53.7 ± 2.4	106.8 ± 17.3	74.9 ± 8.0	60.4 ± 3.7
ABTS	94.8 ± 2.1	53.1 ± 3.6	83.3 ± 13.8	71.6 ± 6.0	62.6 ± 2.1
DPPH	56.4 ± 2.6	38.0 ± 5.7	49.7 ± 8.7	39.8 ± 2.2	38.7 ± 4.4
Polymeric fraction					
ORAC	94.1 ± 1.9	42.3 ± 8.9	66.5 ± 5.5	54.6 ± 2.7	51.9 ± 0.6
FRAP	185.2 ± 1.5	59.8 ± 6.3	118.4 ± 2.7	138.0 ± 15.1	93.2 ± 1.9
ABTS	421.4 ± 8.9	311.0 ± 48.7	262.9 ± 10.0	324.3 ± 26.6	234.0 ± 26.5
DPPH	300.9 ± 19.4	335.6 ± 23.0	208.4 ± 24.1	191.0 ± 11.8	212.7 ± 8.8

[a] GRE1 and GRE2, Grenache ; Syr1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as µmol Fe^{2+}/g DW.

Regression analyses (correlation coefficient R^2) were attempted in order to correlate results obtained with the different methods. The highest correlations were obtained with FRAP followed by DPPH and ABTS (R^2 = 0.94, R^2 = 0.87 and R^2 = 0.81 respectively) (Figure 26). A weaker correlation was obtained with the ORAC assay (R^2 = 0.41) confirming the variations in reactivity of the different assays (Figure 26). Positive correlations between TPC and antiradical activity using similar tests on grape seed samples and various plant samples have also been observed by other investigators (Bozan et al. 2008; Dudonné et al. 2009; Ma et al. 2011).

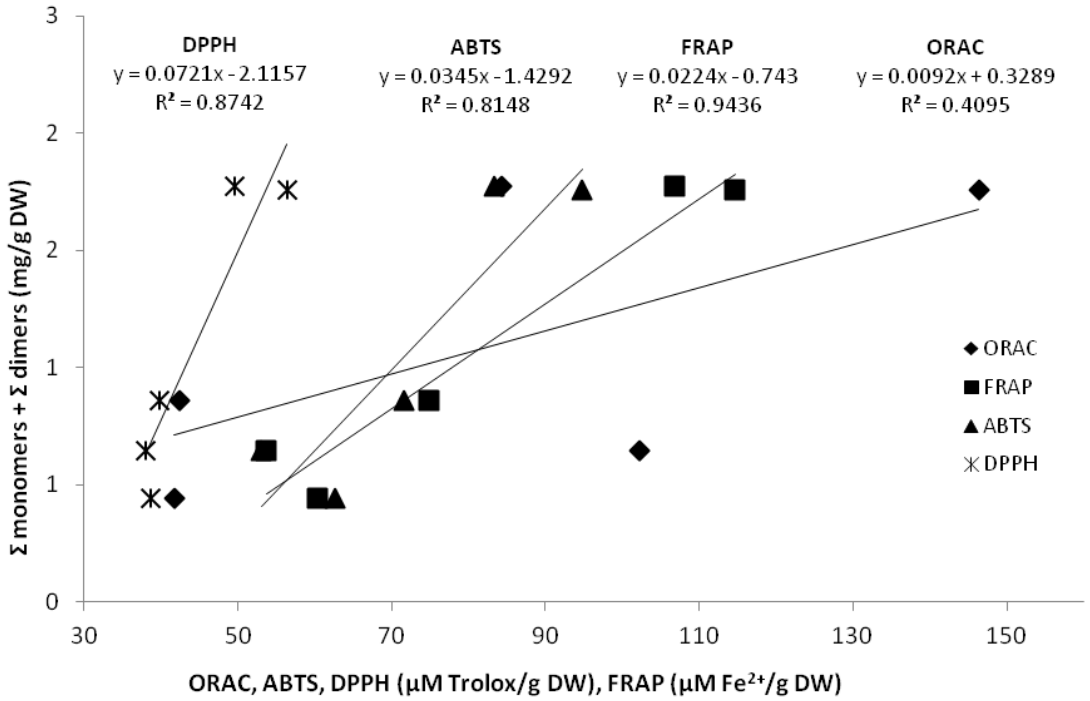


Figure 26: Correlations between radical scavenging capacity assays (ORAC, FRAP, ABTS and DDPH) and total proanthocyanidin contents (sum of monomers and dimers) in grape pomace seeds in 2009

In the current study, MOU was the most polymerized and galloylated sample but this feature did not seem to confer it particular higher antioxidant potential than the other samples in spite of the results of Plumb and co-workers who demonstrated that galloylated compounds have a higher antioxidant capacity in aqueous phase than their non galloylated homologues (Plumb et al. 1998). With the polymeric fraction, GRE1 again appeared as the most *in-vitro* active in all four assays. No particular correlation between mDP and %G in seed pomace and antioxidant activity were evident. The antioxidant capacity in the polymeric fraction was more important than in the monomeric/oligomeric fraction, especially for the $ABTS^{\bullet+}$, DPPH and FRAP assays while the ORAC values showed the opposite trend.

For 2010, antioxidant activities were evaluated on crude and purified extracts (Table 21). In agreement with previous analysis, the antioxidant potential of seed extracts was higher in 2010 than those from 2009. Considering crude extracts, the highest antioxidant activity were obtained in ALI (ORAC: 561.2 µM TE/g DW; FRAP: 267.5 µM Fe^{2+}/g DW, ABTS: 603.1 µM TE/g DW and DPPH: 450.1 TE/g DW) for all tests combined followed by GRE1.

Table 21: Radical scavenging capacities of grape seed pomaces in 2010

	Pomaces-2010							
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Crude extracts:								
ORAC	322.0 ± 20.5	303.1 ± 45.4	266.9 ± 10.9	267.9 ± 18.2	248.7 ± 14.8	201.8 ± 16.5	327.6 ± 34.2	561.2 ± 29.3
FRAP	212.2 ± 24.2	193.0 ± 19.9	232.7 ± 27.7	186.7 ± 9.9	176.4 ± 17.1	188.2 ± 29.1	205.4 ± 10.0	267.5 ± 25.9
ABTS	438.1 ± 54.2	445.7 ± 7.2	486.3 ± 13.0	425.8 ± 31.6	403.5 ± 23.0	526.4 ± 23.8	468.6 ± 51.9	603.1 ± 28.5
DPPH	450.7 ± 4.6	324.7 ± 6.0	322.0 ± 27.0	324.1 ± 48.3	318.5 ± 1.4	262.7 ± 15.9	536.2 ± 117.4	450.1 ± 18.3
Purified extracts:								
Monomeric/oligomeric fraction								
ORAC	189.0 ± 10.7	194.5 ± 16.5	72.1 ± 3.7	86.0 ± 15.1	241.0 ± 16.5	237.3 ± 18.6	291.6 ± 37.5	448.4 ± 35.4
FRAP	43.8 ± 4.7	43.1± 2.1	19.0 ± 1.9	41.3 ± 6.8	35.6 ± 0.8	40.2 ± 2.7	51.6 ± 2.7	88.9 ± 7.0
ABTS	81.5 ± 7.2	77.4 ± 7.3	28.4 ± 1.9	77.9 ± 6.2	64.4 ± 0.4	85.0 ± 4.6	96.5 ± 5.5	133.0 ± 9.6
DPPH	39.6 ± 4.8	41.4 ± 2.9	13.1 ± 1.0	35.5 ± 3.3	31.9 ± 1.2	42.1 ± 2.1	48.6 ± 0.5	73.0 ± 4.3
Polymeric fraction								
ORAC	281.1 ± 13.4	195.4 ± 6.4	234.6 ± 32.1	140.4 ± 1.9	180.8 ± 6.7	125.1 ± 9.8	242.4 ± 25.3	361.0 ± 6.1
FRAP	120.1 ± 0.7	115.8 ± 1.5	124.5 ± 21.1	112.0 ± 3.0	141.1 ± 4.8	149.2 ± 7.5	123.3 ± 12.5	208.3 ± 50.9
ABTS	355.9 ± 15.8	284.3 ± 8.1	384.4 ± 15.9	285.4 ± 0.7	329.6 ± 5.1	408.1 ± 16.0	322.3 ± 22.5	388.7 ± 10.6
DPPH	268.2 ± 7.9	236.1 ± 10.7	246.9 ± 42.7	212.7 ± 11.9	229.9 ± 3.1	301.9 ± 5.5	282.4 ± 28.9	338.6 ± 10.9

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as µmol Fe^{2+}/g DW.

In monomeric/oligomeric and polymeric purified extracts, ALI still retained the highest radical scavenging capacity. The lowest rate was observed in SYR1 monomeric /oligomerc fraction (ORAC: 72.1 µM TE/g DW; FRAP: 19 µM Fe^{2+}/g DW, ABTS: 28.4 µM TE/g DW and DPPH: 13.1 TE/g DW) and SYR2 in both type of extracts.

The antioxidant assay results were similar with the 2009 and 2010 vintages. The different tests conducted in different antioxidant activity classifications of grapes varieties. Regarding monomeric/oligomeric fraction, ALI and COU which contained high amounts of monomeric and dimeric proanthocyanidins possessed the highest antioxidant capacities for all types of tests (Table 21). The two Syrah samples exhibited the least *in vitro* activity. Furthermore, the most polymerized and galloylated (CAR and MOU seeds) did not exert a distinctive antiradical potential. In polymeric fractions, values from FRAP, ABTS and DPPH tests were higher than those obtained with monomeric/oligomeric fractions and established ALI as the most effective variety. Results obtained from ORAC analyses varied according to varieties. As already noticed, the 2009 and 2010 polymeric fraction exhibited a higher antioxidant than the monomeric and oligomeric fraction, in agreement with previous reports (Es-Safi et al. 2006; Spranger et al. 2008).

II.2.2 Antioxidant activities of grape pomace skin extracts

As anticipated, it was established that antioxidant activities in grape seed pomace extracts were higher than those in skins. GRE1 and CAR in 2009 and SYR1, CAR and ALI in 2010 contained the highest antioxidant activity (Table 22). These extracts consistently exhibited the highest values of TPC, proanthocyanidins and anthocyanins. Even though proanthocyanidin contents of grape pomace skins was low, a large amount of anthocyanins still remained and this could explain their antiradical activity.

Table 22: Radical scavenging capacities of grape skin pomace extracts in 2009 and 2010

	GRE1[a]	GRE2[a]	SYR1[a]	CAR[a]	MOU[a]			
Grapes-2009								
Purified extracts:								
Monomeric/oligomeric fraction								
ORAC	63.8 ± 3.2	63.8 ± 2.2	94.0 ± 1.4	37.4 ± 1.8	47.8 ± 14.7			
FRAP	57.3 ± 6.6	13.7 ± 2.4	53.1 ± 3.1	91.4 ± 2.6	15.2 ± 0.7			
ABTS	40.6 ± 4.2	25.3 ± 1.5	37.1 ± 1.5	62.0 ± 1.6	33.7 ± 0.9			
DPPH	20.5 ± 0.6	16.6 ± 0.6	18.8 ± 1.5	26.9 ± 1.4	19.3 ± 1.6			
Polymeric fraction								
ORAC	86.8 ± 0.3	55.4 ± 3.6	57.2 ± 1.5	66.9 ± 7.3	42.4 ± 4.0			
FRAP	120.9 ± 6.9	35.1 ± 4.4	78.7 ± 0.2	112.7 ± 14.1	78.0 ± 13.7			
ABTS	286.7 ± 19.9	77.9 ± 3.7	121.1 ± 7.1	197.0 ± 21.5	129.8 ± 8.8			
DPPH	203.1 ± 10.6	97.0 ± 11.0	76.1 ± 5.9	113.8 ± 13.4	110.7 ± 8.1			
Pomaces-2010								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Crude extracts:								
ORAC	200.6 ± 15.4	224.8 ± 17.0	355.8 ± 32.2	267.4 ± 18.0	326.2 ± 18.1	269.2 ± 21.5	212.3 ± 12.4	531.9 ± 30.2
FRAP	105.3 ± 4.9	137.2 ± 2.6	190.8 ± 6.4	157.4 ± 11.6	225.3 ± 5.9	202.7 ± 9.8	122.4 ± 8.8	266.8 ± 40.5
ABTS	263.4 ± 6.7	294.2 ± 22.5	299.3 ± 9.3	338.8 ± 11.3	390.8 ± 0.4	405.7 ± 2.0	255.6 ± 28.9	464.1 ± 44.5
DPPH	161.2 ± 3.7	134.6 ± 0.5	190.6 ± 1.5	151.0 ± 0.6	195.3 ± 7.4	161.3 ± 7.1	185.0 ± 12.6	290.7 ± 50.1
Purified extracts:								
Monomeric/oligomeric fraction								
ORAC	82.1 ± 3.4	83.9 ± 4.6	154.5 ± 11.1	87.1 ± 9.1	73.2 ± 7.1	72.2 ± 8.1	133.6 ± 11.3	204.9 ± 24.5
FRAP	15.4 ± 0.9	21.3 ± 1.9	31.9 ± 2.8	24.2 ± 2.1	27.0 ± 4.0	30.9 ± 1.9	24.9 ± 1.4	38.9 ± 2.1
ABTS	31.7 ± 4.4	42.0 ± 0.1	60.1 ± 9.1	36.9 ± 2.7	40.2 ± 3.0	68.2 ± 1.4	44.3 ± 2.0	64.6 ± 2.4
DPPH	14.5 ± 0.9	16.1 ± 0.4	35.3 ± 3.9	17.1 ± 0.3	19.3 ± 3.9	23.4 ± 0.9	16.0 ± 0.6	27.1 ± 1.6
Polymeric fraction								
ORAC	137.9 ± 6.8	132.5 ± 5.1	142.9 ± 15.6	117.2 ± 1.6	148.3 ± 3.2	125.9 ± 6.2	99.7 ± 2.6	266.8 ± 26.1
FRAP	85.3 ± 4.2	98.4 ± 7.1	135.9 ± 4.5	117.8 ± 1.3	162.8 ± 7.2	173.9 ± 10.4	91.7 ± 39.1	188.5 ± 14.8
ABTS	166.1 ± 20.4	175.2 ± 7.6	214.1 ± 7.5	176.7 ± 4.7	265.5 ± 17.6	295.9 ± 10.9	118.0 ± 10.1	307.6 ± 62.0
DPPH	107.8 ± 7.3	87.1 ± 12.7	113.9 ± 11.2	92.4 ± 13.3	121.8 ± 2.5	193.5 ± 15.4	133.0 ± 4.1	160.8 ± 24.4

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as µmol Fe^{2+}/g DW.

The highest correlations between total anthocyanins and antioxidant tests were obtained for the FRAP and ORAC tests (R^2 = 0.86 and R^2 = 0.84 respectively) (Figure 27) in 2010.

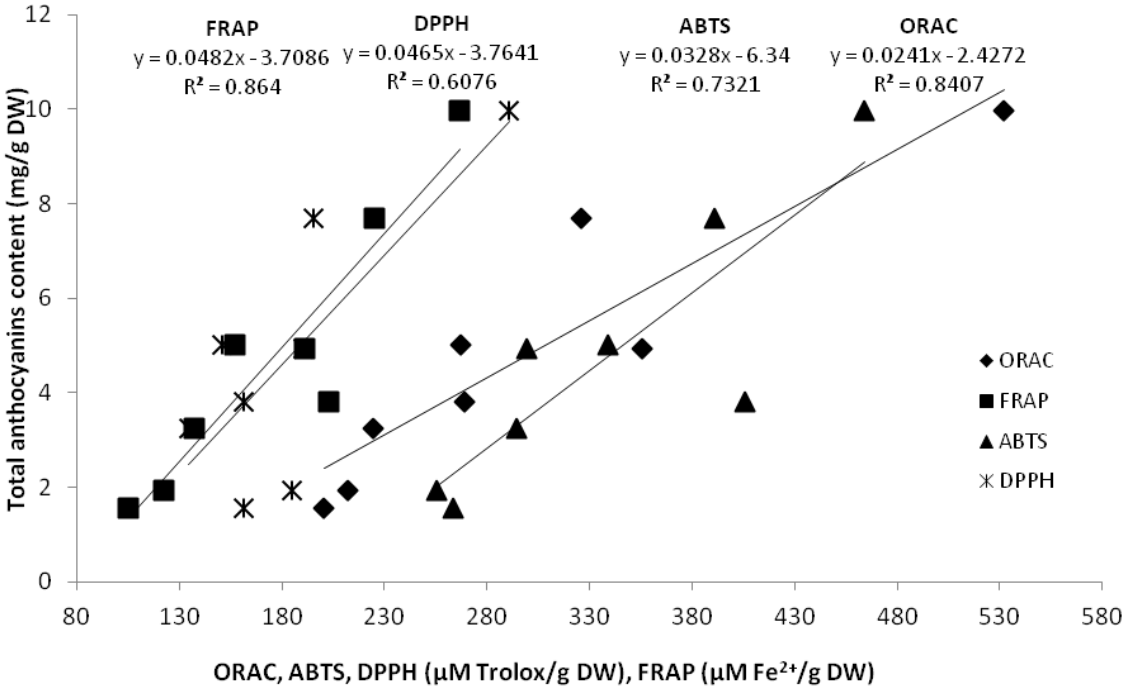


Figure 27: Correlations between radical scavenging capacity assays (ORAC, FRAP, ABTS and DPPH) and total anthocyanin contents in grape pomace skin extracts in 2010

Correlations between grape pomace contents and antioxidant levels were higher with total values than with the specific compound concentrations quantified by HPLC. As reported in a recent publication (Rockenbach et al. 2011), our result illustrate that antioxidant activity is more related to the total constituent levels than to the concentration of any individual compound despite the fact that some compounds may contribute more than the others.

Overall, grape seed pomaces exerted greater antioxidant capacities than grape skin pomaces. This observation can be explained by a greater concentration in total and individual phenolic contents in seeds. Furthermore, a lower amount of galloylated derivatives in skins may contributory factor as it has been demonstrated that galloylated compounds have a

higher antioxidant capacity in aqueous phase than their non-galloylated homologues (Plumb et al. 1998).

To conclude on this first characterisation, grape seed and skin pomace extracts still contained appreciable amounts of flavan-3-ols and anthocyanins, despite extraction during vinification. The quantitative and qualitative distribution of (poly)phenols in grape pomaces showed significant differences through varieties and vintages. Seeds from Grenache (GRE1), Syrah (SYR1) and Alicante and skins from Syrah (SYR1), Carignan and Alicante were evidenced as the most interesting fractions because of their richest (poly)phenol contents and highest antioxidant capacities. They, therefore represent useful by-products as a natural source of (poly)phenols and antioxidants. Varieties and part (seeds or skins) exhibiting the highest antioxidant capacity was pre-selected for further extraction using adequate extraction process for commercialization.

Table 23: Radical scavenging capacities of grape seeds and skins in 2009

Grapes-2009						
Seeds						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**GRE1[a]**
Purified extracts:						
Monomeric/oligomeric fraction						
ORAC[b]	289.48 ± 54.40	98.89 ± 16.55	328.73 ± 4.75	157.27 ± 4.82	95.22 ± 22.55	173.21 ± 10.48
FRAP[b]	153.06 ± 8.45	141.27 ± 37.42	247.26 ± 13.99	116.53 ± 1.14	86.11 ± 26.14	208.44 ± 8.47
ABTS[b]	145.25 ± 1.88	126.91 ± 1.26	174.05 ± 10.91	82.36 ± 3.59	126.12 ± 0.58	150.99 ± 4.37
DPPH[b]	74.58 ± 2.25	76.10 ± 1.82	108.32 ± 11.10	47.45 ± 1.62	62.12 ± 12.23	81.92 ± 4.79
Polymeric fraction						
ORAC[b]	87.23 ± 11.49	86.59 ± 10.80	97.96 ± 1.27	83.54 ± 2.82	91.01 ± 12.05	93.39 ± 7.58
FRAP[b]	157.40 ± 17.63	154.59 ± 10.04	159.68 ± 11.63	185.19 ± 9.29	286.73 ± 10.91	174.07 ± 10.76
ABTS[b]	437.68 ± 31.06	331.24 ± 17.24	370.67 ± 22.25	446.54 ± 19.28	573.25 ± 61.89	330.02 ± 61.02
DPPH[b]	282.03 ± 13.67	274.56 ± 5.09	305.02 ± 16.87	319.51 ± 8.52	447.67 ± 25.39	264.23 ± 12.96
Skins						
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**CAR[a]**	**MOU[a]**	**GRE1[a]**
Purified extracts:						
Monomeric/oligomeric fraction						
ORAC[b]	88.07 ± 9.92	85.69 ± 4.63	175.20 ± 17.32	133.57 ± 22.67	107.66 ± 35.69	43.02 ± 10.84
FRAP[b]	46.27 ± 2.29	32.75 ± 3.79	65.61 ± 2.29	45.35 ± 0.13	50.34 ± 16.25	36.17 ± 3.15
ABTS[b]	30.19 ± 0.90	18.11 ± 0.40	37.49 ± 2.32	29.39 ± 3.88	49.61 ± 1.15	24.51 ± 0.97
DPPH[b]	14.29 ± 0.78	-	23.37 ± 1.59	19.15 ± 1.14	20.72 ± 3.67	12.93 ± 1.29
Polymeric fraction						
ORAC[b]	108.44 ± 9.35	99.02 ± 2.46	125.66 ± 4.21	169.11 ± 8.80	142.91 ± 8.60	94.41 ± 1.84
FRAP[b]	25.62 ± 0.42	28.22 ± 0.81	43.02 ± 2.64	23.55 ± 1.80	21.30 ± 2.14	4.55 ± 0.38
ABTS[b]	218.71 ± 26.26	161.97 ± 5.89	272.50 ± 16.99	149.82 ± 14.34	184.58 ± 12.46	157.70 ± 13.63
DPPH[b]	228.93 ± 13.12	167.55 ± 10.89	312.24 ± 14.68	150.32 ± 4.78	205.30 ± 20.40	302.17 ± 26.82

[a] GRE1 and GRE2, Grenache ; Syr1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as µmol Fe^{2+}/g DW.

Table 24: Radical scavenging capacities of grape seeds and skins in 2010

Grapes-2010								
Seeds								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Crude extracts:								
ORAC[b]	1248.51 ± 192.02	1436.87 ± 61.95	1340.58 ± 69.22	1447.65 ± 100.79	860.34 ± 50.70	722.89 ± 52.82	1259.78 ± 101.81	1869.87 ± 100.83
FRAP[b]	356.95 ± 47.53	315.45 ± 36.85	335.90 ± 24.56	323.58 ± 28.40	303.12 ± 23.47	393.90 ± 34.30	340.89 ± 38.52	397.65 ± 42.29
ABTS[b]	1030.92 ± 74.24	815.61 ± 23.68	795.67 ± 49.32	734.51 ± 8.01	783.38 ± 59.40	1028.65 ± 33.02	996.57 ± 120.32	979.00 ± 103.03
DPPH[b]	781.15 ± 49.25	599.75 ± 31.24	514.11 ± 45.08	630.25 ± 12.50	623.66 ± 31.90	925.79 ± 113.11	632.98 ± 66.10	624.24 ± 36.06
Purified extracts:								
Monomeric/oligomeric fraction								
ORAC[b]	668.50 ± 59.85	804.15 ± 22.35	1014.42 ± 80.53	754.37 ± 101.85	428.45 ± 44.29	399.96 ± 77.17	852.29 ± 38.54	785.02 ± 57.45
FRAP[b]	99.81 ± 14.70	120.63 ± 12.36	142.86 ± 7.13	106.72 ± 22.59	57.83 ± 3.30	71.20 ± 2.22	138.35 ± 8.04	180.21 ± 15.59
ABTS[b]	148.96 ± 18.44	210.79 ± 12.73	254.97 ± 15.62	179.19 ± 40.71	103.98 ± 7.70	152.88 ± 10.88	255.47 ± 3.27	300.47 ± 24.10
DPPH[b]	70.73 ± 12.36	101.51 ± 5.68	121.46 ± 6.58	80.42 ± 19.47	54.29 ± 4.54	64.84 ± 7.64	92.66 ± 6.38	132.66 ± 6.86
Polymeric fraction								
ORAC[b]	715.44 ± 109.90	578.22 ± 41.35	555.65 ± 49.52	566.15 ± 97.30	525.55 ± 29.86	451.35 ± 27.78	540.56 ± 32.87	650.97 ± 17.42
FRAP[b]	322.19 ± 12.37	228.02 ± 15.46	232.31 ± 20.89	237.30 ± 17.25	234.20 ± 27.48	270.58 ± 15.01	256.24 ± 26.67	265.72 ± 9.40
ABTS[b]	876.83 ± 42.46	628.66 ± 21.01	540.72 ± 11.97	585.26 ± 38.01	617.25 ± 19.44	789.98 ± 4.41	646.66 ± 38.49	633.70 ± 24.78
DPPH[b]	737.86 ± 50.80	485.75 ± 2.09	455.56 ± 6.87	561.72 ± 32.97	456.18 ± 38.13	569.05 ± 24.43	464.87 ± 32.29	493.82 ± 17.95
Skins								
	GRE1[a]	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**COU[a]**	**ALI[a]**
Crude extracts:								
ORAC[b]	504.85 ± 33.60	663.67 ± 23.59	646.61 ± 24.63	476.18 ± 14.50	712.67 ± 54.94	763.83 ± 28.89	709.62 ± 58.98	866.78 ± 36.72
FRAP[b]	203.31 ± 10.61	181.74 ± 19.72	226.40 ± 20.64	192.68 ± 4.64	245.54 ± 17.89	238.59 ± 27.60	169.95 ± 13.50	284.88 ± 17.75
ABTS[b]	285.62 ± 16.68	283.25 ± 33.32	419.14 ± 82.81	363.59 ± 32.08	458.52 ± 8.42	430.95 ± 23.09	271.21 ± 4.37	576.71 ± 59.84
DPPH[b]	218.98 ± 17.99	162.79 ± 21.50	315.10 ± 26.89	209.38 ± 30.67	302.75 ± 24.30	92.42 ± 3.92	218.34 ± 2.16	337.07 ± 24.36
Purified extracts:								
Monomeric/oligomeric fraction								
ORAC[b]	142.02 ± 4.29	77.58 ± 10.23	269.96 ± 15.05	226.18 ± 23.10	231.41 ± 15.59	206.90 ± 14.68	187.70 ± 7.97	233.82 ± 31.16
FRAP[b]	70.45 ± 9.99	28.31 ± 1.90	45.13 ± 3.76	29.90 ± 1.43	37.90 ± 2.19	27.61 ± 1.59	26.99 ± 1.06	55.54 ± 4.14
ABTS[b]	53.55 ± 8.30	48.05 ± 3.05	68.03 ± 1.11	48.48 ± 6.09	70.63 ± 6.23	43.29 ± 14.40	43.67 ± 3.83	85.27 ± 13.15
DPPH[b]	15.45 ± 3.28	14.92 ± 0.61	25.39 ± 0.76	20.04 ± 1.65	25.23 ± 0.08	12.35 ± 2.67	12.78 ± 0.11	24.81 ± 2.33
Polymeric fraction								
ORAC[b]	477.21 ± 48.19	414.06 ± 42.91	500.03 ± 50.76	477.32 ± 17.80	481.80 ± 38.09	349.64 ± 4.07	320.64 ± 0.95	569.82 ± 15.61
FRAP[b]	193.75 ± 19.28	189.33 ± 18.60	282.55 ± 16.47	202.19 ± 17.50	277.53 ± 14.53	249.76 ± 12.72	204.37 ± 8.99	312.86 ± 27.83
ABTS[b]	153.66 ± 7.79	215.78 ± 24.59	277.17 ± 2.60	228.81 ± 7.68	294.81 ± 6.27	218.14 ± 20.03	254.52 ± 20.76	397.33 ± 19.04
DPPH[b]	104.86 ± 4.54	115.11 ± 5.32	429.63 ± 2.87	552.32 ± 41.09	151.55 ± 27.56	212.41 ± 4.06	318.84 ± 9.54	282.92 ± 4.83

[a] GRE1 and GRE2, Grenache ; Syr1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as µmol Fe^{2+}/g DW.

III. Grape pomace extracts for *in vivo* study phenolics and antioxidants characterisation

After vintage 2010 vinification, different pomaces from six varieties were collected. Seeds were separated from skins using a mechanic separator at the chateau de Beaucastel. Samples were frozen at -20°C and sent to the 3INature Company for extraction. The pre-selection of varieties and parts (seeds or skins) resulted from previous analysis on grape pomace extracts. Samples were extracted using aqueous solution and hydro-alcoholic 70% solution generating two types of extracts: an aqueous extract (EAQ) and a hydro-alcoholic extract (EA70). Figure 28 and Table 25 summarized varieties and part extracted by 3INature Company and analysis carried out on these grape pomace extracts. Some of these extracts will be chosen for *in vivo* study.

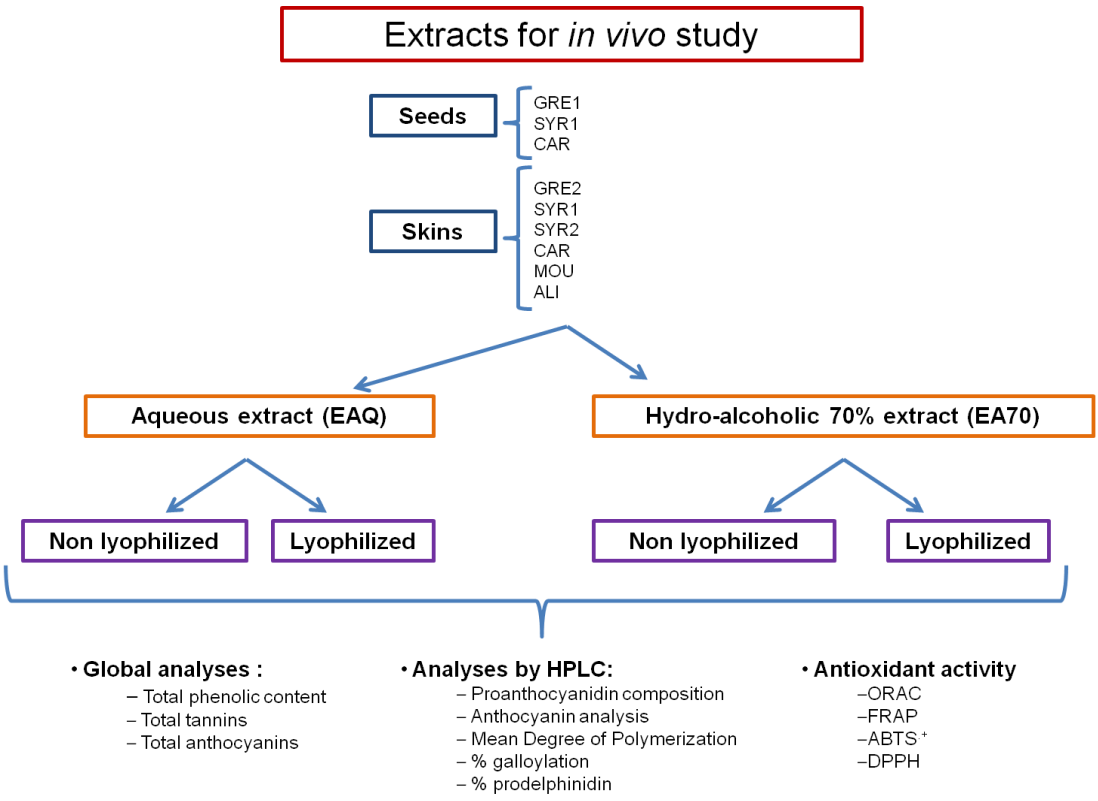


Figure 28: Analysis performed on EAQ and EA70 grape pomace seed and skin extracts

Table 25: EAQ and EA70 grape pomace skin and seed extracts

Samples	Skins		Seeds	
	EAQ	EA70	EAQ	EA70
GRE1			X	X
GRE2	X	X		
SYR1	X	X	X	X
SYR2	X	X		
CAR	X	X	X	X
MOU	X	X		
COU				
ALI	X	X		

III.1. Total phenol, tannin and anthocyanin analysis of grape pomace extracts

Aqueous and hydro-alcoholic 70% extracts were characterized for their overall composition by total phenol content, total tannin and total anthocyanin analysis *via* Folin Ciocalteu assay, acidic hydrolysis and SO_2 bleaching procedure, respectively, after being solubilized and diluted at appropriate concentration for each test. Results are presented in Table 26 and Table 27.

Table 26: Total phenol contents, total tannin and total anthocyanin contents in EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
TPC	128.22 ± 0.37	215.93 ± 1.17	186.08 ± 0.28
Total tannins	157.02 ± 0.56	266.87 ± 2.62	264.61 ± 2.39
Total anthocyanins	3.98 ± 0.16	10.55 ± 0.56	11.35 ± 0.51
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
TPC	195.66 ± 1.06	207.38 ± 2.15	215.84 ± 1.47
Total tannins	302.86 ± 4.85	455.42 ± 1.84	423.11 ± 15.13
Total anthocyanins	12.17 ± 0.51	38.67 ± 4.34	57.34 ± 1.86

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents.

In overall, results showed that an extraction with hydro-alcoholic 70% solution allowed a better extraction of phenolic compounds whether in seeds or in skins. Among seed extracts, seed from SYR1 and CAR were particularly rich in (poly)phenols, tannins and anthocyanins in EAQ and EA70 extracts (Table 26). SYR 1 (EA70) contained a higher

tannins concentration until 455.42 mg/g DW while CAR (EA70) has a higher quantity in total anthocyanins (57.34 mg/g DW). In both extracts, phenolic contents in GRE1 remained low compared in comparison with other varieties.

Table 27: Total phenol contents, total tannin and total anthocyanin contents in EAQ and EA70 grape pomace skin extracts

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
TPC	109.72 ± 0.19	146.50 ± 1.19	71.88 ± 0.08	120.83 ± 1.12	102.27 ± 0.38	196.71 ± 0.37
Total tannins	112.28 ± 2.67	156.63 ± 2.63	86.36 ± 1.86	161.61 ± 1.32	104.79 ± 20	221.40 ± 3.47
Total anthocyanins	8.70 ± 0.01	16.01 ± 0.01	1.76 ± 0.01	14.62 ± 0.75	5.65 ± 0.01	21.40 ± 0.20
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
TPC	195.15 ± 0.28	224.92 ± 0.18	173.58 ± 0.08	203.47 ± 0.83	219.88 ± 0.18	188.94 ± 0.69
Total tannins	256.07 ± 3.65	312.46 ± 10.77	250.17 ± 7.07	345.34 ± 4.18	268.6 ± 11.68	232.65 ± 3.14
Total anthocyanins	53.66 ± 0.83	86.68 ± 1.71	45.38 ± 0.20	88.44 ± 0.59	46.64 ± 0.39	54.41 ± 2.66

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents.

Concerning skins, results revealed in EAQ extracts that ALI contained the highest phenolic contents for the three test combined (TPC: 196.71 mg GAE/g DW, total tannins: 221.40 mg/g DW and total anthocyanins: 21.40 mg/g DW) while in EA70, SYR1 skins were predominantly high in phenolic contents (total phenol contents: 224.92 mg GAE /g DW, total tannins: 312.46 mg/g DW and total anthocyanins: 86.68 mg/g DW) (Table 27). The poorest extracts was SYR2 whether in EAQ or in EA70. As it was already observed in seeds extracts, EA70 were characterized by higher total phenol contents, total tannins and total anthocyanins. This result illustrated a better extraction by alcoholic 70 % solution. Ethanol could facilitate tissues dissolution and thus, liberate a greater amount of (poly)phenols. Actually, between EAQ and EA70, total (poly)phenols and total tannins rate were 1.5- to 2.5- and 1- to 3-fold respectively, higher in EA70. Anthocyanins extraction varied largely through varieties: by 2.5- to 25.8 -fold higher in ALI skin extracts (21.40 mg/g DW in EAQ and 54.41 mg/g DW in EA70) and SYR2 skins (1.76 mg/g DW in EAQ and 45.38 mg/g DW), respectively.

III.2. Flavan-3-ol and procyanidin analysis of grape pomace extracts

III.2.1. Flavan-3-ol analysis of grape pomace extracts

Flavan-3-ol monomers [(+)-catechin and (–)-epicatechin], dimers (B_1, B_2, B_3 and B_4) and trimer C_1 were identified and quantified in seed and skin aqueous and hydro-alcoholic 70% extracts by HPLC-UV-Fluo.

For both type of seed extracts, SYR1 was the richest whether in monomers (8.88 mg/g DW in EAQ and 13.84 mg/g DW in EA70), in dimers (6.87 mg/g DW in EAQ and 7.10 mg/g DW in EA70) and trimer C1 (2.00 mg/g DW in EAQ and 1.25 mg/g DW in EA70) as opposite to GRE1 (Table 28). This latter had already appeared to contain low amount of (poly)phenols in previous total analysis. However, despite this low content, GRE1 still possessed an exploitable potential especially when extracted with hydro-alcoholic 70 % solution.

Table 28: Flavan-3-ol monomers, dimers and trimers characterisation in EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
C	2.07 ± 0.09	5.12 ± 0.04	2.27 ± 0.00
EC	0.98 ± 0.04	3.76 ± 0.03	0.94 ± 0.00
Σ Monomers	**3.04 ± 0.09**	**8.88 ± 0.00**	**3.21 ± 0.00**
B_1	1.01 ± 0.08	2.94 ± 0.01	0.87 ± 0.01
B_2	0.70 ± 0.01	2.23 ± 0.02	0.68 ± 0.00
B_3	0.28 ± 0.02	0.86 ± 0.00	0.25 ± 0.00
B_4	0.51 ± 0.01	0.85 ± 0.04	0.11 ± 0.00
Σ Dimers	**2.50 ± 0.05**	**6.870 ± 0.03**	**1.90 ± 0.01**
C_1	0.48 ± 0.01	2.00 ± 0.06	0.53± 0.01
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
C	3.60 ± 0.02	8.60 ± 0.00	5.28 ± 0.03
EC	1.46 ± 0.00	5.24 ± 0.00	2.02 ± 0.06
Σ Monomers	**5.07 ± 0.01**	**13.84 ± 0.00**	**7.29 ± 0.02**
B_1	1.68 ± 0.01	3.53 ± 0.01	3.06 ± 0.00
B_2	0.84 ± 0.00	2.16 ± 0.02	1.29 ± 0.00
B_3	0.45 ± 0.02	0.87 ± 0.02	0.58 ± 0.01
B_4	Nd	0.53 ± 0.01	Nd
Σ Dimers	**2.97 ± 0.00**	**7.10 ± 0.03**	**4.92 ± 0.01**
C_1	0.54 ± 0.00	1.25 ± 0.03	0.83 ± 0.01

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. In units of mg/g DW seed or skin. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4; C1, procyanidin trimers C_1. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; C_1, trimer C_1; Nd, Not determined.

For grape pomace skin extracts (Table 29), values in EAQ ranged from 0.89 mg/g DW to 3.4 mg/g DW for the sum of monomers, from 0.91 mg/g DW to 2.34 mg/g DW for the sum of dimers and values from 1.88 mg/g W to 7.71 mg/g DW and 1.61 mg/g DW to 4.83 mg/g DW were found in EA70 extracts, for the sum of monomers and dimers, respectively. ALI and SYR1 showed a higher flavan-3-ol monomers and dimers in both type of extract. Among EAQ samples, SYR2 skin extracts was evidenced as having fewer amounts than other skin EAQ samples but in EA70 it was GRE2 and CAR.

Table 29: Flavan-3-ol monomers, dimers and trimers characterisation in EAQ and EA70 grape pomace skin extracts

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
C	0.76 ± 0.00	1.42 ± 0.01	0.52 ± 0.06	1.01 ± 0.00	0.66 ± 0.01	2.03 ± 0.13
EC	0.29 ± 0.00	1.04 ± 0.01	0.37 ± 0.03	0.35 ± 0.01	0.38 ± 0.00	1.39 ± 0.09
Σ Monomers	**1.05 ± 0.00**	**2.46 ± 0.02**	**0.89 ± 0.06**	**1.36 ± 0.01**	**1.03 ± 0.01**	**3.40 ± 0.15**
B_1	0.62 ± 0.00	0.92 ± 0.01	0.37 ± 0.00	0.74 ± 0.01	0.62 ± 0.00	0.91 ± 0.17
B_2	0.41 ± 0.00	0.66 ± 0.01	0.36 ± 0.04	0.43 ± 0.01	0.57 ± 0.00	0.77 ± 0.05
B_3	0.28 ± 0.00	0.39 ± 0.01	0.18 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	0.35 ± 0.12
B_4	ND	ND	ND	ND	ND	0.32 ± 0.09
Σ Dimères	**1.31 ± 0.00**	**1.96 ± 0.00**	**0.91 ± 0.03**	**1.45 ± 0.02**	**1.47 ± 0.01**	**2.34 ± 0.30**
C1	0.47 ± 0.00	0.82 ± 0.01	0.34 ± 0.08	0.54 ± 0.00	0.55 ± 0.13	0.67 ± 0.15
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
C	1.42 ± 0.00	2.29 ± 0.10	2.09 ± 0.05	1.44 ± 0.00	1.52 ± 0.02	5.08 ± 0.03
EC	0.44 ± 0.00	1.36 ± 0.01	1.10 ± 0.20	0.44 ± 0.00	0.69 ± 0.00	2.63 ± 0.01
Σ Monomers	**1.86 ± 0.00**	**3.65 ± 0.08**	**3.19 ± 0.11**	**1.88 ± 0.00**	**2.18 ± 0.02**	**7.71 ± 0.02**
B_1	0.92 ± 0.01	1.27 ± 0.01	1.19 ± 0.16	1.14 ± 0.01	1.15 ± 0.01	2.59 ± 0.00
B_2	0.40 ± 0.02	0.64 ± 0.01	0.60 ± 0.01	0.39 ± 0.02	0.57 ± 0.00	1.28 ± 0.00
B_3	0.30 ± 0.00	0.29 ± 0.00	0.36 ± 0.00	0.27 ± 0.00	0.31 ± 0.00	0.60 ± 0.01
B_4	Nd	Nd	Nd	Nd	Nd	0.35 ± 0.00
Σ Dimères	**1.61 ± 0.01**	**2.19 ± 0.00**	**2.15 ± 0.11**	**1.79 ± 0.03**	**2.02 ± 0.01**	**4.83 ± 0.01**
C1	0.34 ± 0.00	0.44 ± 0.00	0.66 ± 0.13	0.31± 0.00	0.37 ± 0.00	0.63 ± 0.00

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. In units of mg/g DW seed or skin. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4; C1, procyanidin trimers C_1. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B_1, B_2, B_3 and B_4; C_1, trimer C_1; Nd, Not determined.

III.2.2. Procyanidin analysis of grape pomace extracts

Detection of procyanidins was assessed by HPLC-Fluo-MS^n using a normal phase column where the stationary phase is made of polar packing material while the mobile phase is of non-polar solvents. Due to the complexity in their structural diversity, analytical methodology for procyanidins has been chromatographic separation based upon mDP as opposed to the individual compounds and specific isomers. This approach has permitted the measurement of the large structural diversity of these compounds in a categorical manner

(e.g. oligomeric size, %G and %P) but limited because no information about the molecular mass distribution can be obtained. Actually, reversed-phase HPLC is successful in separating monomers up to tetramers only and the increasing numbers of isomers with the degree of polymerization produce a very broad and unresolved UV-absorbing peak late in the chromatogram. By using Normal phase HPLC coupled with mass spectrometry and fluorescence detection, procyanidins can be separate and identify simultaneously on the basis of their molecular characteristics. Indeed, the resolution of these compounds has been improved recently (Núñez et al. 2006; Robbins et al. 2009; Hanlin et al. 2011). Normal phase HPLC technique proved to be a powerful tool for the analysis of oligomers > tetramers.

Peak detection and quantification were mainly carried out by fluorescence with an excitation and emission wavelengths at 230 nm and 320 nm because of its more selectivity and stronger signal than UV absorption detection for procyanidins. Identification was confirmed by mass spectrometry in full-scan negative ionization, data dependent MS (Robbins et al., 2009). In total, 17 compounds were founds and listed in Table 30. Figures 29 and 30 showed the typical HPLC-Fluo chromatogram of grape pomace extracts and the ESI (electrospray ionization) spectrum of grape pomace extracts of mass range from m/z 200 to 2000, respectively.

Table 30: Diol-HPLC and MS characteristics of procyanidins detected in grape pomace extracts

Peak	Rt (min)	Procyanidins		$[M^-]$ (m/z)
1	4.92	Monomers	(epi)C	289
2	6.98	(Epi)catechin-*O*-gallate	(epi)Cg	441
3	8.67	Dimers	(epi)C-(epi)C	577
4	13.26	Dimers-*O*-gallate	(epi)C-(epi)Cg	729
5	15.5	Trimers	(epi)C-(epi)C-(epi)C	865
6	17.25	(Epi)gallocatechin trimers	(epi)C-(epi)-(epi)GC	881
7	20.69	Trimers-*O*-gallate	(epi)C-(epi)C-(epi)Cg	1017
8	22.59	Tetramers	(epi)C-(epi)C-(epi)C-(epi)C	1153
9	23.52	(Epi)gallocatechin tetramers	(epi)C-(epi)C-(epi)-(epi)GC	1169
10	27.08	Tetramers-*O*-gallate	(epi)C-(epi)C-(epi)C-(epi)Cg	1305
11	29.28	Pentamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C	1441
12	30.23	(Epi)gallocatechin pentamers	(epi)C-(epi)C-(epi)C-(epi)-(epi) GC	1457
13	34.79	Hexamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C	1729
14	39.74	Heptamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)-(epi)C	2017
15	43.78	Octamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C	2305
16	47.16	Nonamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C	2593
17	50.21	Decamers	(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)C-(epi)-(epi)C	2881

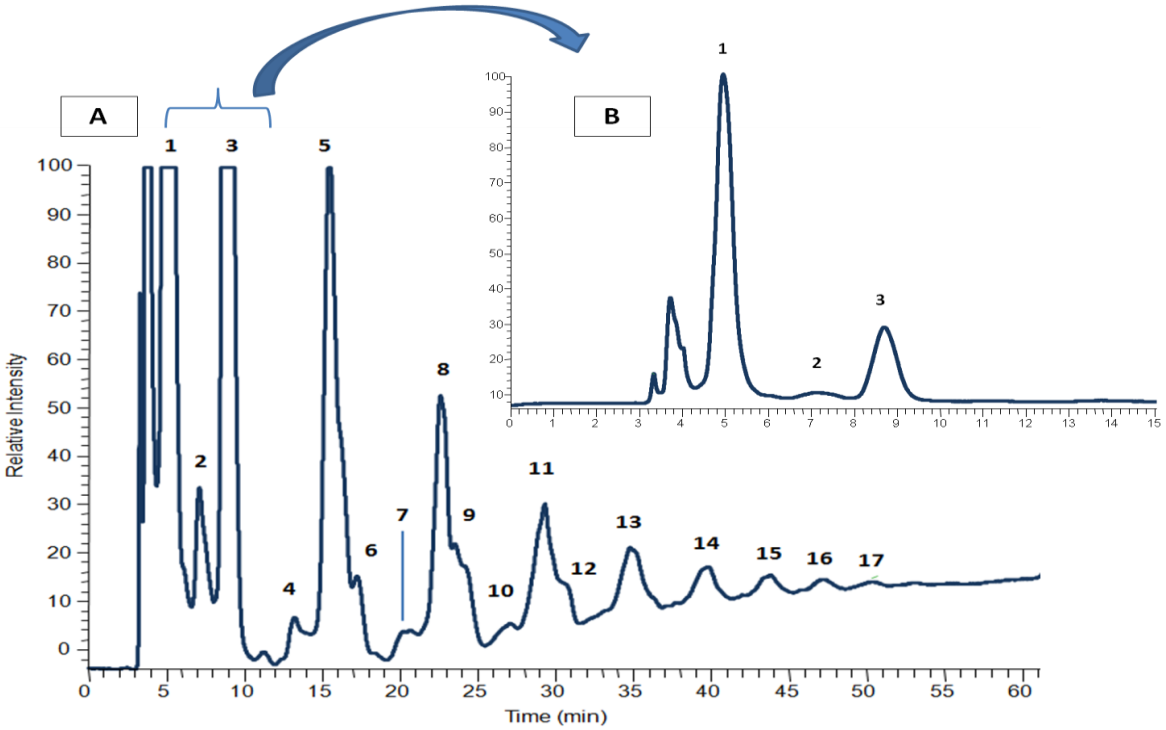


Figure 29: Typical HPLC-Fluo chromatogram of a grape pomace extract with an excitation and emission wavelengths at 230 nm and 320 nm using different sensitivity (A ten-fold higher than B)

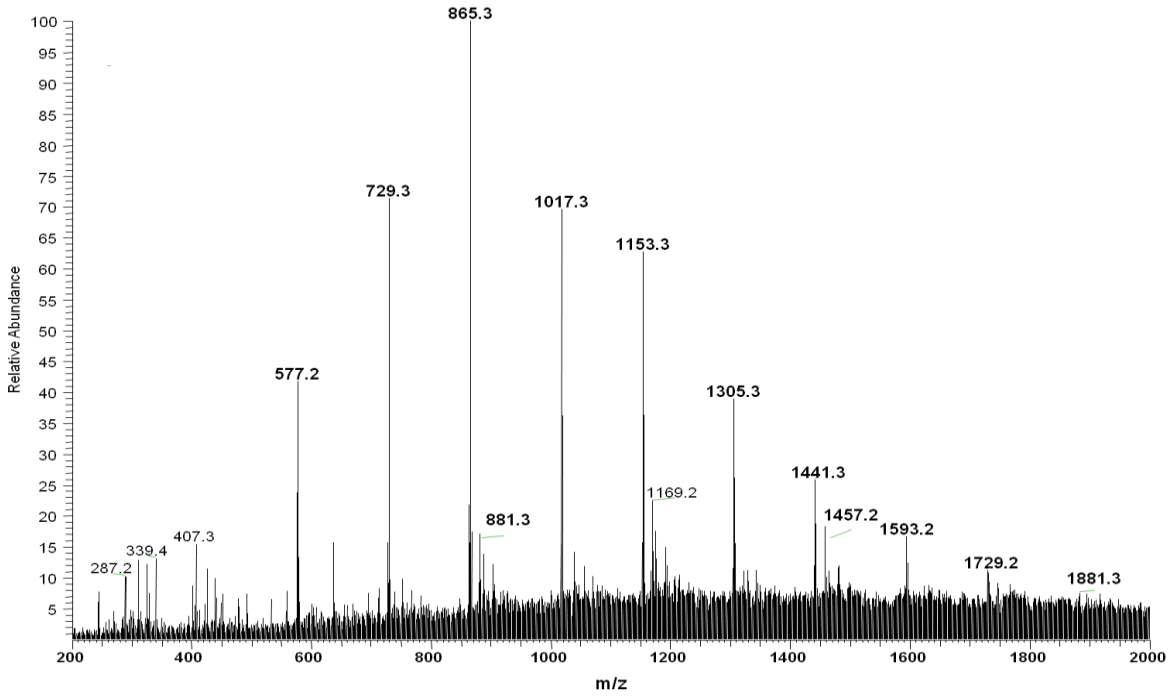


Figure 30: ESI Spectrum of a grape pomace extract of mass range from m/z 200 to 2000

In grape pomace seed extracts (Table 31), total procyanidin (Σ monomers-decamers) detected in EA70 extracts was more important than in EAQ, except for CAR where a total of 50.22 mg/g DW was found in EA70 and 73.34 mg/g DW in EAQ. SYR1 was the most interesting because of its particularly high amount of total procyanidins with up to 137.24 mg/g DW in EAQ and 157.12 mg/g DW in EA70. SYR1 also retained about 2- and 2.8-fold more procyanidins than GRE1 and CAR in EAQ and EA70, respectively. Hydro-alcoholic 70% solution permitted to extract more highly polymerized compounds.

Table 31: Procyanidins characterisation in EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
Procyanidins	**GRE1[a]**	**SYR1[a]**	**CAR[a]**
Monomers	37.74 ± 0.06	76.30 ± 0.39	45.42 ± 0.93
(Epi)catechin gallate	0.70 ± 0.00	0.77 ± 0.01	0.66 ± 0.01
Dimers	11.65 ± 0.13	21.52 ± 0.16	15.82 ± 0.22
Dimers gallate	0.22 ± 0.00	1.20 ± 0.01	0.40 ± 0.02
Trimers	3.81 ± 0.04	17.76 ± 0.21	4.94 ± 0.07
(Epi)gallocatechin trimers	0.25 ± 0.01	1.27 ± 0.03	0.21 ± 0.04
Trimers gallate	0.27 ± 0.02	0.68 ± 0.02	0.18 ± 0.01
Tetramers	1.56 ± 0.06	7.05 ± 0.05	1.89 ± 0.00
(Epi)gallocatechin tetramers	0.91 ± 0.04	2.66 ± 0.07	0.92 ± 0.01
Tetramers gallate	0.01 ± 0.00	0.15 ± 0.00	0.04 ± 0.00
Pentamers	0.79 ± 0.01	3.95 ± 0.06	1.29 ± 0.02
(Epi)gallocatechin pentamers	0.12 ± 0.01	0.61 ± 0.01	0.26 ± 0.00
Hexamers	0.55 ± 0.02	1.64 ± 0.06	0.67 ± 0.02
Heptamers	0.22 ± 0.01	1.01 ± 0.01	0.39 ± 0.02
Octamers	0.10 ± 0.00	0.43 ± 0.01	0.16 ± 0.01
Nonamers	0.02 ± 0.00	0.17 ± 0.01	0.07 ± 0.01
Decamers	nd	0.07 ± 0.00	0.02 ± 0.00
Seeds-EA70			
Procyanidins	**GRE1[a]**	**SYR1[a]**	**CAR[a]**
Monomers	32.59 ± 0.60	72.11 ± 0.93	29.72 ± 0.35
(Epi)catechin gallate	1.01 ± 0.02	1.34 ± 0.05	0.79 ± 0.02
Dimers	10.63 ± 0.17	28.15 ± 0.11	9.22 ± 0.15
Dimers gallate	0.33 ± 0.01	1.94 ± 0.00	0.34 ± 0.00
Trimers	4.16 ± 0.23	20.74 ± 0.03	3.49 ± 0.04
(Epi)gallocatechin trimers	0.63 ± 0.01	2.18 ± 0.02	0.58 ± 0.01
Trimers gallate	0.30 ± 0.00	1.42 ± 0.01	0.28 ± 0.00
Tetramers	1.98 ± 0.06	9.46 ± 0.19	1.69 ± 0.04
(Epi)gallocatechin tetramers	0.97 ± 0.13	4.50 ± 0.04	0.80 ± 0.01
Tetramers gallate	0.04 ± 0.01	0.82 ± 0.05	0.06 ± 0.00
Pentamers	1.30 ± 0.01	6.27 ± 0.15	1.25 ± 0.00
(Epi)gallocatechin pentamers	0.32 ± 0.01	1.37 ± 0.05	0.20 ± 0.00
Hexamers	0.92 ± 0.03	3.43 ± 0.05	0.83 ± 0.02
Heptamers	0.51 ± 0.00	1.82 ± 0.01	0.51 ± 0.00
Octamers	0.28 ± 0.00	0.90 ± 0.00	0.24 ± 0.01
Nonamers	0.13 ± 0.01	0.43 ± 0.00	0.15 ± 0.01
Decamers	0.09 ± 0.00	0.24 ± 0.01	0.07 ± 0.00

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. Data are expressed as the mean of triplicate ± standard deviation as mg (–)-epicatechin equivalents/g of dry weight.

Procyanidins composition in EAQ and EA70 grape pomace skin extracts were showed in Table 32. Interestingly, unlike seed extracts, a higher amount of procyanidins were evidenced in EAQ skin extracts, especially in SYR2, MOU and ALI in which total procyanidins were 2-fold more extracted by aqueous solution than hydro-alcoholic 70%. The most interesting extracts in term of procyanidins were the SYR 1 and ALI which contained up to 60 mg/g DW in EAQ, SYR1 and CAR in EA70 (51.65 mg/g DW and 39.08 mg/g DW respectively). MOU in both type of extract appeared to be the less interesting because of its low total procyanidin contents (27.35 mg/g DW in EAQ and 11.89 mg/g DW in EA70). A further extraction of highly polymerized procyanidins by hydro-alcoholic 70% solution was better illustrated in skin extracts. Actually, with the exception of ALI, nonamers were only detected in SYR1 and any decamers were evidenced in EAQ skin extracts.

Table 32: Procyanidins characterisation in EAQ and EA70 grape pomace skin extracts

Skins-EAQ						
Procyanidins	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Monomers	13.01 ± 0.34	26.30 ± 0.05	17.44 ± 0.00	14.20 ± 0.03	10.19 ± 0.15	12.20 ± 0.00
(Epi)catechin gallate	0.20 ± 0.01	0.37 ± 0.01	0.28 ± 0.02	0.31 ± 0.02	0.17 ± 0.00	0.24 ± 0.01
Dimers	4.17 ± 0.10	7.96 ± 0.06	5.32 ± 0.05	5.29 ± 0.02	3.92 ± 0.06	3.73 ± 0.07
Dimers gallate	0.37 ± 0.01	0.83 ± 0.03	0.35 ± 0.02	0.45 ± 0.01	0.37 ± 0.01	2.25 ± 0.01
Trimers	5.48 ± 0.00	11.24 ± 0.29	6.13 ± 0.15	6.68 ± 0.11	5.64 ± 0.09	19.44 ± 0.11
(Epi)gallocatechin trimers	0.82 ± 0.01	1.45 ± 0.01	0.90 ± 0.02	1.25 ± 0.04	0.48 ± 0.01	2.15 ± 0.01
Trimers gallate	0.23 ± 0.01	0.35 ± 0.07	0.17 ± 0.00	0.20 ± 0.03	0.16 ± 0.01	0.94 ± 0.00
Tetramers	1.94 ± 0.06	4.19 ± 0.00	2.14 ± 0.01	2.50 ± 0.04	2.33 ± 0.02	7.13 ± 0.01
(Epi)gallocatechin tetramers	0.89 ± 0.02	1.17 ± 0.00	0.70 ± 0.02	1.06 ± 0.04	0.73 ± 0.00	2.64 ± 0.09
Tetramers gallate	nd	nd	nd	nd	nd	nd
Pentamers	1.03 ± 0.00	2.41 ± 0.08	1.05 ± 0.02	1.45 ± 0.02	1.32 ± 0.01	3.42 ± 0.01
(Epi)gallocatechin pentamers	0.69 ± 0.00	0.66 ± 0.01	0.66 ± 0.03	1.04 ± 0.00	0.66 ± 0.02	1.26 ± 0.01
Hexamers	0.80 ± 0.00	1.68 ± 0.00	0.58 ± 0.02	0.70 ± 0.00	0.92 ± 0.01	1.90 ± 0.01
Heptamers	0.36 ± 0.01	0.86 ± 0.01	0.27 ± 0.01	0.34 ± 0.01	0.39 ± 0.03	0.92 ± 0.01
Octamers	0.08 ± 0.00	0.30 ± 0.02	nd	0.15 ± 0.01	0.07 ± 0.01	0.29 ± 0.03
nonamers	nd	0.10 ± 0.01	nd	nd	nd	0.10 ± 0.00
Decamers	nd	nd	nd	nd	nd	0.04 ± 0.00
Skins-EA70						
Procyanidins	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Monomers	9.89 ± 0.08	19.28 ± 0.28	9.62 ± 0.05	12.58 ± 0.05	3.80 ± 0.07	12.71 ± 0.03
(Epi)catechin gallate	0.28 ± 0.00	0.47 ± 0.01	0.64 ± 0.02	0.49 ± 0.03	0.02 ± 0.00	0.44 ± 0.01
Dimers	4.05 ± 0.03	6.76 ± 0.09	2.70 ± 0.02	4.65 ± 0.02	1.9 ± 0.05	4.51 ± 0.08
Dimers gallate	0.40 ± 0.01	0.89 ± 0.00	0.49 ± 0.00	0.61 ± 0.00	0.17 ± 0.00	0.44 ± 0.01
Trimers	6.31 ± 0.08	10.51 ± 0.01	4.35 ± 0.06	7.91 ± 0.14	2.34 ± 0.01	4.52 ± 0.06
(Epi)gallocatechin trimers	0.62 ± 0.00	0.78 ± 0.01	0.49 ± 0.04	0.35 ± 0.01	0.07 ± 0.00	0.08 ± 0.00
Trimers gallate	0.27 ± 0.00	0.47 ± 0.01	0.23 ± 0.01	0.38 ± 0.01	0.06 ± 0.00	0.31 ± 0.00
Tetramers	2.79 ± 0.01	4.79 ± 0.01	1.94 ± 0.01	3.58 ± 0.04	1.14 ± 0.00	1.97 ± 0.01
(Epi)gallocatechin	1.03 ± 0.00	1.16 ± 0.01	0.45 ± 0.01	1.30 ± 0.03	0.33 ± 0.02	0.67 ± 0.03

tetramers						
Tetramers gallate	nd	0.11 ± 0.01	0.09 ± 0.01	0.40 ± 0.01	nd	0.05 ± 0.00
Pentamers	1.87 ± 0.00	2.78 ± 0.00	1.10 ± 0.02	2.42 ± 0.01	0.84 ± 0.01	1.22 ± 0.00
(Epi)gallocatechin pentamers	0.56 ± 0.00	0.36 ± 0.00	0.37 ± 0.01	0.80 ± 0.04	0.18 ± 0.00	0.16 ± 0.00
Hexamers	1.16 ± 0.01	1.61 ± 0.00	1.02 ± 0.02	1.74 ± 0.01	0.47 ± 0.00	0.69 ± 0.01
Heptamers	0.87 ± 0.00	0.93 ± 0.01	0.32 ± 0.01	1.00 ± 0.07	0.30 ± 0.01	0.28 ± 0.00
Octamers	0.18 ± 0.00	0.44 ± 0.01	0.19 ± 0.00	0.59 ± 0.00	0.16 ± 0.00	0.17 ± 0.00
nonamers	0.16 ± 0.00	0.20 ± 0.01	nd	0.26 ± 0.01	0.07 ± 0.00	0.08 ± 0.00
Decamers	0.03 ± 0.00	0.11 ± 0.00	nd	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. In units of mg/g DW seed or skin. Data are expressed as the mean of triplicate ± standard deviation as mg (–)-epicatechin equivalents/g of dry weight. Nd, not determined.

III.3. mDP, %G, %P analysis of grape pomace extracts

For the analysis of mDP, %G (% galloylation) and %P (% prodelphinidins) and in order to facilitate the investigation, extracts were inevitably lyophilized because of their semi liquid viscous form, hard to manipulate and hard to dissolve with methanol. Results are presented in Table 33.

Table 33: mDP, %G and %P characterisation in EAQ and EA70 grape pomace seed and skin extracts

Seeds-EAQ						
	GRE1[a]	**SYR1[a]**	**CAR[a]**			
Dpm	4.36 ± 0.00	3.77 ± 0.09	4.13 ± 0.14			
% G	46.4 ± 0.35	64.06 ± 0.97	57.92 ± 1.12			
% P	26.93 ± 0.25	11.09 ± 0.47	17.82 ± 1.89			
Seeds-EA70						
	GRE1[a]	**SYR1[a]**	**CAR[a]**			
Dpm	5.21 ± 0.33	4.67 ± 0.10	4.05 ± 0.05			
% G	66.37 ± 0.46	71.75 ± 0.15	57.97 ± 0.71			
% P	16.63 ± 0.12	11.01 ± 0.00	17.07 ± 0.96			
Skins -EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Dpm	6.15 ± 0.64	4.44 ± 0.36	6.81 ± 0.15	4.92 ± 0.15	7.31 ± 0.30	3.94 ± 0.01
% G	39.44 ± 0.51	54.46 ± 1.90	20.33 ± 0.40	41.28 ± 2.91	31.8 ± 2.02	42.25 ± 1.68
% P	38.21 ± 1.43	22.23 ± 0.36	53.32 ± 0.56	33.17 ± 2.81	43.86 ± 1.24	28.58 ± 1.38
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Dpm	4.37 ± 0.16	5.34 ± 0.01	5.49 ± 0.04	3.35 ± 0.02	4.5 ± 0.09	3.4 ± 0.17
% G	47.16 ± 1.05	57.79 ± 0.17	44.3 ± 0.63	46.82 ± 0.18	51.85 ± 0.23	50.39 ± 3.08
% P	29.12 ± 0.58	23.65 ± 0.02	34.35 ± 0.44	23.38 ± 0.35	25.48 ± 0.70	22.01 ± 1.49

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. mDP, mean degree of polymerization ; %G, percentage of galloylation ; %P, percentage of prodelphinidins.

In seed grape pomace extracts, mDPs varied from 3.77 to 4.36 in EAQ and 4.05 to 5.21 in EA70 and were slightly higher in EA70 than EAQ. GRE1 and SYR1 presented a more important %G (66.4 and 71.8 respectively) in EA70 whereas in CAR, %G was similar in EAQ and EA70 (57.9 for both types of extracts). For %P, similar results were obtained for SYR1 and CAR in EAQ and EA70 but a greater %P was found in GRE1 aqueous extract (27 in EAQ *vs* 17 in EA70). GRE1 has the bigger mDP and %P values.

In skin pomace extracts, mDPs ranged in aqueous extracts from 3.9 in ALI to 7.3 in MOU and in hydro-alcoholic 70% extracts from 3.4 in ALI to 5.5 in SYR2. Higher mDPs were observed in EAQ skin extracts on the contrary to seed extracts. EA70 extracts were evidenced as being more galloylated than EAQ extracts and varied from 47.2 to 57.8. The contrary was evidenced concerning %P which was bigger in EAQ (from 22.2 in SYR1 to 53.3 in SYR2) than in EA70 (from 22 in ALI to 34.3 in SYR2).

Results were in accordance with previous studies evidencing seed extracts as more galloylated than skin extracts and %P as higher in skin extracts due to substantial quantities of galloylated compounds (Souquet et al. 1996; Chira et al. 2009). Previous procyanidins analysis evidenced that high polymerized procyanidins was better extracted with hydro-alcoholic 70% solution. In this current analysis, this observation was supported only by seed extracts but not in skin extracts where bigger procyanidins were evidenced in aqueous extracts. In overall, independently to mDP, aqueous extraction showed to increase the %P value while hydro-alcoholic 70% solution affected especially the %G.

III.4. Anthocyanin analysis of grape pomace extracts by HPLC-UV-MS^n

In total, 18 anthocyanins were detected by HPLC-PDA-MS. A typical chromatogram is illustrated in (Figure 31). Compounds were identified on the basis of absorbance spectra, retention time of commercially available standards, elution order, *m/z* of the positively charged molecular ion ($[M]^+$) and on the MS^2 fragmentation according to previous reports. Tables 34-36 summarize the anthocyanin contents of grape seed and skin pomace extracts in which total anthocyanins represented the sum of individual anthocyanins.

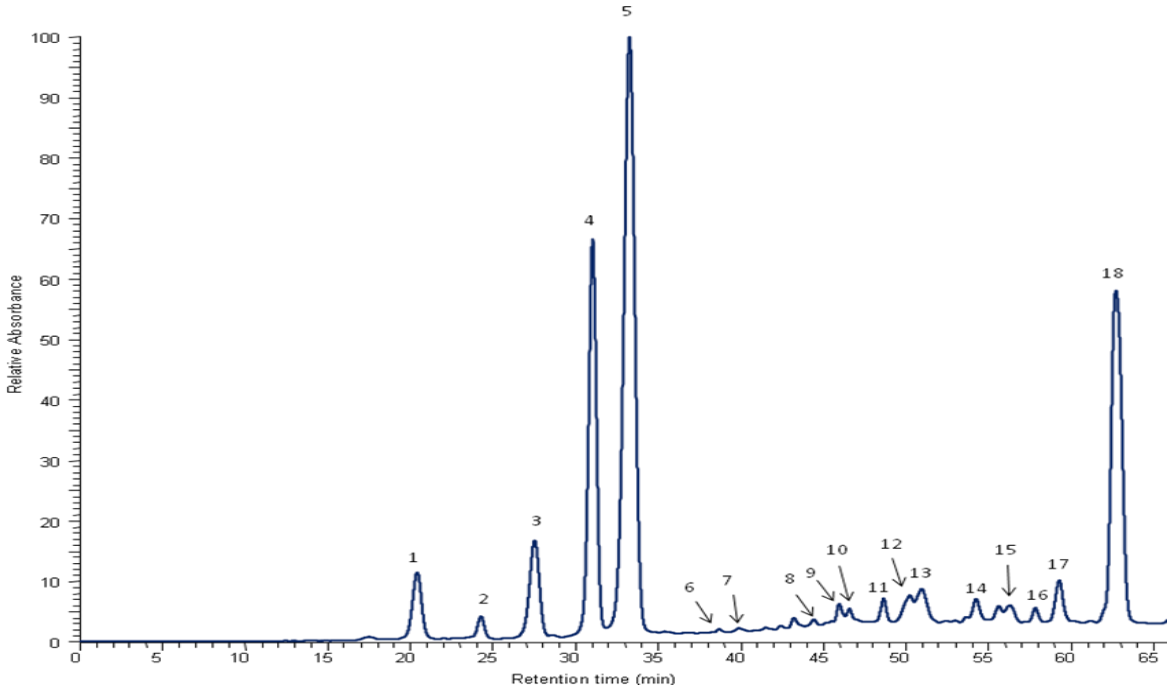


Figure 31: Typical HPLC chromatogram of grape pomace extract with detection wavelength at 520 nm

Table 34: Anthocyanins characterisation in EAQ and EA70 grape pomace seed extracts

Seeds-EAQ				
Peak	**Compounds**	**GRE1[a]**	**SYR1[a]**	**CAR[a]**
1	Delphinidin-3-*O*-glucoside	0.03 ± 0.00	0.08 ± 0.00	0.40 ± 0.00
2	Cyanidin-3-*O*-glucoside	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
3	Petunidin-3-*O*-glucoside	0.05 ± 0.00	0.14 ± 0.00	0.43 ± 0.00
4	Peonidin-3-*O*-glucoside	0.11 ± 0.00	0.12 ± 0.00	0.15 ± 0.01
5	Malvidin-3-*O*-glucoside	0.39 ± 0.00	0.93 ± 0.00	1.48 ± 0.02
6	Malvidin-3-*O*-glucoside-acetaldehyde (vitisin B)	Nd	0.02 ± 0.00	0.02 ± 0.00
7	Delphinidin-3-*O*-(6″-*O*-acetyl) glucoside	Nd	0.02 ± 0.00	Nd
8	Malvidin-catechin dimer	0.01 ± 0.00	0.03 ± 0.00	Nd
9	Malvidin-3-*O*-glucoside-pyruvate (vitisin A)	0.02 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
10	Malvidin-catechin dimer	0.002 ± 0.00	0.07 ± 0.00	0.05 ± 0.00
11	Malvidin-catechin dimer	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
12	Peonidin-3-*O*-(6″-*O*-acetyl) glucoside	0.01 ± 0.00	0.07 ± 0.00	Nd
13	Malvidin-3-*O*-(6″-*O*-acetyl) glucoside	0.02 ± 0.00	0.32 ± 0.00	0.09 ± 0.00
14	Delphinidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.01 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
15	Malvidin-3-*O*-(6″-*O*-caffeoyl) glucoside	0.01 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
16	Cyanidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
17	Petunidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.01 ± 0.00	0.11 ± 0.00	0.11 ± 0.00
18	Malvidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.06 ± 0.00	1.14 ± 0.01	0.65 ± 0.00
	Total anthocyanin glucosides	**0.60 ± 0.00**	**1.30 ± 0.00**	**2.52 ± 0.03**
	Total acetylated anthocyanins	**0.03 ± 0.00**	**0.41 ± 0.00**	**0.09 ± 0.00**
	Total coumaroylated anthocyanins	**0.08 ± 0.00**	**1.32 ± 0.01**	**0.88 ± 0.00**
	Total anthocyanins	**0.76 ± 0.00**	**3.26 ± 0.01**	**3.63 ± 0.03**

Seeds-EA70				
Peak	**Compounds**	**GRE1[a]**	**SYR1[a]**	**CAR[a]**
1	Delphinidin-3-*O*-glucoside	0.19 ± 0.00	0.31 ± 0.00	3.11 ± 0.02
2	Cyanidin-3-*O*-glucoside	0.09 ± 0.00	0.05 ± 0.00	0.23 ± 0.00
3	Petunidin-3-*O*-glucoside	0.33 ± 0.01	0.58 ± 0.02	3.18 ± 0.01
4	Peonidin-3-*O*-glucoside	0.56 ± 0.03	0.47 ± 0.02	1.11 ± 0.00
5	Malvidin-3-*O*-glucoside	2.36 ± 0.12	3.57 ± 0.08	10.52 ± 0.11
6	Malvidin-3-*O*-glucoside-acetaldehyde (vitisin B)	0.01 ± 0.00	0.05 ± 0.00	0.03 ± 0.00
7	Delphinidin-3-*O*-(6″-*O*-acetyl) glucoside	nd	0.09 ± 0.00	0.06 ± 0.00
8	Malvidin-catechin dimer	0.04 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
9	Malvidin-3-*O*-glucoside-pyruvate (vitisin A)	0.12 ± 0.00	0.07 ± 0.00	0.14 ± 0.00
10	Malvidin-catechin dimer	nd	0.13 ± 0.00	0.17 ± 0.00
11	Malvidin-catechin dimer	0.10 ± 0.00	0.13 ± 0.00	0.16 ± 0.00
12	Peonidin-3-*O*-(6″-*O*-acetyl) glucoside	0.07 ± 0.00	0.32 ± 0.01	0.12 ± 0.00
13	Malvidin-3-*O*-(6″-*O*-acetyl) glucoside	0.06 ± 0.00	0.90 ± 0.03	0.31 ± 0.00
14	Delphinidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.05 ± 0.00	0.18 ± 0.00	0.58 ± 0.01
15	Malvidin-3-*O*-(6″-*O*-caffeoyl) glucoside	0.08 ± 0.00	0.11 ± 0.00	0.33 ± 0.00
16	Cyanidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.07 ± 0.00	0.07 ± 0.00	0.11 ± 0.00
17	Petunidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.08 ± 0.00	0.35 ± 0.01	0.66 ± 0.00
18	Malvidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.80 ± 0.00	2.85 ± 0.00	4.51 ± 0.04
	Total anthocyanin glucosides	**3.53 ± 0.16**	**4.99 ± 0.12**	**18.15 ± 0.09**
	Total acetylated anthocyanins	**0.13 ± 0.00**	**1.30 ± 0.02**	**0.49 ± 0.00**
	Total coumaroylated anthocyanins	**1.00 ± 0.00**	**3.44 ± 0.01**	**5.86 ± 0.02**
	Total anthocyanins	**5.00 ± 0.17**	**10.28 ± 0.14**	**25.38 ± 0.11**

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. Data are expressed as the mean of triplicate ± standard deviation as mg malvidin-3-*O*-glucoside equivalents/g of dry weight. Nd, not determined.

Table 35: Anthocyanins characterisation in EAQ grape pomace skin extracts

		Skins-EAQ					
Peak	**Compounds**	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
1	Delphinidin-3-*O*-glucoside	0.24 ± 0.01	0.15 ± 0.00	0.01 ± 0.00	0.53 ± 0.00	0.20 ± 0.00	0.31 ± 0.00
2	Cyanidin-3-*O*-glucoside	0.09 ± 0.00	0.01 ± 0.00	0.004 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.11 ± 0.00
3	Petunidin-3-*O*-glucoside	0.33 ± 0.01	0.25 ± 0.01	0.03 ± 0.00	0.54 ± 0.00	0.30 ± 0.00	0.47 ± 0.02
4	Peonidin-3-*O*-glucoside	0.35 ± 0.01	0.20 ± 0.01	0.02 ± 0.00	0.19 ± 0.00	0.22 ± 0.00	1.49 ± 0.03
5	Malvidin-3-*O*-glucoside	1.78 ± 0.05	1.54 ± 0.05	0.11 ± 0.00	1.74 ± 0.02	1.01 ± 0.01	3.14 ± 0.03
6	Malvidin-3-*O*-glucoside-acetaldehyde (vitisin B)	0.01 ± 0.00	0.02 ± 0.00	nd	0.02 ± 0.00	0.01 ± 0.00	nd
7	Delphinidin-3-*O*-(6″-*O*-acetyl) glucoside	nd	0.05 ± 0.00	nd	0.02 ± 0.00	nd	nd
8	Malvidin-catechin dimer	0.01 ± 0.00	0.03 ± 0.00	nd	nd	0.01 ± 0.00	0.03 ± 0.00
9	Malvidin-3-*O*-glucoside-pyruvate (vitisin A)	0.05 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.10 ± 0.00
10	Malvidin-catechin dimer	nd	0.08 ± 0.00	0.002 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.05 ± 0.00
11	Malvidin-catechin dimer	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
12	Peonidin-3-*O*-(6″-*O*-acetyl) glucoside	0.02 ± 0.00	0.10 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.09 ± 0.00
13	Malvidin-3-*O*-(6″-*O*-acetyl) glucoside	nd	0.52 ± 0.00	0.02 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.19 ± 0.00
14	Delphinidin-3-*O*-(6″-*O*-coumaroyl) glucoside	nd	0.09 ± 0.00	nd	0.13 ± 0.00	0.02 ± 0.00	0.06 ± 0.00
15	Malvidin-3-*O*-(6″-*O*-caffeoyl) glucoside	0.02 ± 0.00	0.05 ± 0.00	nd	0.03 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
16	Cyanidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.01 ± 0.00	0.02 ± 0.00	nd	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
17	Petunidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.04 ± 0.00	0.16 ± 0.00	nd	0.12 ± 0.00	0.03 ± 0.00	0.07 ± 0.00
18	Malvidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.22 ± 0.00	1.59 ± 0.00	0.01 ± 0.00	0.68 ± 0.01	0.15 ± 0.00	1.11 ± 0.01
	Total anthocyanin glucosides	**2.80 ± 0.07**	**2.15 ± 0.06**	**0.17 ± 0.00**	**3.05 ± 0.02**	**1.80 ± 0.01**	**5.52 ± 0.08**
	Total acetylated anthocyanins	**0.02 ± 0.00**	**0.67 ± 0.00**	**0.03 ± 0.00**	**0.11 ± 0.00**	**0.05 ± 0.00**	**0.27 ± 0.00**
	Total coumaroylated anthocyanins	**0.27 ± 0.00**	**1.86 ± 0.00**	**0.01 ± 0.00**	**0.96 ± 0.01**	**0.24 ± 0.00**	**1.27 ± 0.01**
	Total anthocyanins	**3.19 ± 0.08**	**4.92 ± 0.06**	**0.24 ± 0.00**	**4.25 ± 0.03**	**2.20 ± 0.01**	**7.32 ± 0.08**

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± standard deviation as mg malvidin-3-*O*-glucoside equivalents/g of dry weight. Nd, not determined.

Table 36: Anthocyanins characterisation in EA70 grape pomace skin extracts

Skins-EA70							
Peak	**Compounds**	**GRE2[a]**	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
1	Delphinidin-3-*O*-glucoside	1.43 ± 0.00	0.78 ± 0.00	0.97 ± 0.00	5.35 ± 0.21	2.35 ± 0.00	1.06 ± 0.01
2	Cyanidin-3-*O*-glucoside	0.34 ± 0.02	0.05 ± 0.00	0.12 ± 0.00	0.39 ± 0.01	0.52 ± 0.00	0.24 ± 0.00
3	Petunidin-3-*O*-glucoside	2.05 ± 0.08	1.29 ± 0.00	1.53 ± 0.00	5.04 ± 0.21	3.38 ± 0.02	1.65 ± 0.03
4	Peonidin-3-*O*-glucoside	1.91 ± 0.02	0.87 ± 0.01	0.94 ± 0.02	1.71 ± 0.03	2.00 ± 0.01	5.32 ± 0.01
5	Malvidin-3-*O*-glucoside	10.96 ± 0.22	7.59 ± 0.04	6.76 ± 0.09	14.82 ± 0.42	10.55 ± 0.03	11.18 ± 0.05
6	Malvidin-3-*O*-glucoside-acetaldehyde (vitisin B)	0.03 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
7	Delphinidin-3-*O*-(6″-*O*-acetyl) glucoside	0.04 ± 0.00	0.16 ± 0.00	0.13 ± 0.00	0.09 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
8	Malvidin-catechin dimer	0.05 ± 0.00	0.07 ± 0.00	0.11 ± 0.00	nd	0.10 ± 0.00	0.06 ± 0.00
9	Malvidin-3-*O*-glucoside-pyruvate (vitisin A)	0.22 ± 0.01	0.17 ± 0.01	0.39 ± 0.00	0.18 ± 0.01	0.32 ± 0.00	0.20 ± 0.00
10	Malvidin-catechin dimer	0.12 ± 0.00	0.27 ± 0.01	0.21 ± 0.00	0.16 ± 0.00	0.07 ± 0.00	0.14 ± 0.00
11	Malvidin-catechin dimer	0.10 ± 0.00	0.11 ± 0.00	0.21 ± 0.01	0.10 ± 0.00	0.08 ± 0.00	0.23 ± 0.00
12	Peonidin-3-*O*-(6″-*O*-acetyl) glucoside	0.17 ± 0.00	0.52 ± 0.01	0.39 ± 0.01	0.10 ± 0.00	0.19 ± 0.00	0.52 ± 0.14
13	Malvidin-3-*O*-(6″-*O*-acetyl) glucoside	0.46 ± 0.01	2.11 ± 0.05	1.06 ± 0.00	0.41 ± 0.02	0.39 ± 0.00	0.61 ± 0.00
14	Delphinidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.25 ± 0.01	0.12 ± 0.00	0.35 ± 0.01	0.75 ± 0.00	0.30 ± 0.00	0.27 ± 0.00
15	Malvidin-3-*O*-(6″-*O*-caffeoyl) glucoside	0.34 ± 0.01	0.15 ± 0.00	0.56 ± 0.00	0.24 ± 0.00	0.32 ± 0.00	0.25 ± 0.00
16	Cyanidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.13 ± 0.00	0.05 ± 0.00	0.17 ± 0.01	0.13 ± 0.00	0.52 ± 0.00	0.14 ± 0.00
17	Petunidin-3-*O*-(6″-*O*-coumaroyl) glucoside	0.36 ± 0.00	0.20 ± 0.00	0.51 ± 0.01	0.82 ± 0.01	0.65 ± 0.00	0.55 ± 0.00
18	Malvidin-3-*O*-(6″-*O*-coumaroyl) glucoside	3.09 ± 0.02	1.53 ± 0.00	4.12 ± 0.00	3.80 ± 0.03	3.24 ± 0.00	6.25 ± 0.04
	Total anthocyanin glucosides	**16.68 ± 0.34**	**10.59 ± 0.05**	**10.33 ± 0.10**	**27.30 ± 0.88**	**18.79 ± 0.02**	**19.45 ± 0.08**
	Total acetylated anthocyanins	**0.67 ± 0.01**	**2.79 ± 0.04**	**1.59 ± 0.01**	**0.60 ± 0.02**	**0.62 ± 0.00**	**1.18 ± 0.14**
	Total coumaroylated anthocyanins	**3.82 ± 0.01**	**1.89 ± 0.00**	**5.14 ± 0.01**	**5.49 ± 0.04**	**4.71 ± 0.00**	**7.21 ± 0.04**
	Total anthocyanins	**22.03 ± 0.35**	**16.10 ± 0.10**	**18.60 ± 0.11**	**34.11 ± 0.95**	**25.06 ± 0.01**	**28.74 ± 0.01**

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± standard deviation as mg malvidin-3-*O*-glucoside equivalents/g of dry weight. Nd, not determined.

From all studied varieties, the main compounds consisted of 3-*O*-monoglucosides ([M – 162]$^{+}$) of delphinidin (*m/z* 303), cyanidin (*m/z* 287), petunidin (*m/z* 317), peonidin (*m/z* 301) and malvidin (*m/z* 331) which accounted for 63% in EAQ, 64% in EA70 to 72 % in EAQ, 70% in EA70 of the total anthocyanins content in seed and skin pomace extracts, respectively. Other compounds were largely represented by 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins ([M – 308] $^{+}$) followed by the 3-*O*-(6"-*O*-acetyl) glucoside one ([M – 204]$^{+}$) (Table 37 and 38). These results are in agreement with previously reported data which illustrated the monoglucoside character of *V.vinifera* species (Ribéreau-Gayon 1959; Mazza et al. 1995; Acevedo De la Cruz et al. 2012). Besides, malvidin-3-*O*-glucoside and its derivatives, mainly *p*-coumaroyl derivatives, petunidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were the major compounds. Malvidin-3-*O*-glucoside alone accounted for 30% in seeds to 40% in skins whereas the minor compound, the cyanidin-3-*O*-glucoside, represented no more than 2% of the total anthocyanins.

Table 37: Percentage of different forms of anthocyanins in seed pomace extracts of total anthocyanins. Data expressed in percentage (%).

Seeds-EAQ				
	GRE1[a]	**SYR1[a]**	**CAR[a]**	**Average (%)**
Cyanidin-3-*O*-glucoside	3	1	1	**2**
Malvidin-3-*O*-glucoside	52	29	41	**40**
Σ Anthocyanin glucosides	80	40	69	**63**
Σ Anthocyanins acetylated	4	13	2	**6**
Σ Anthocyanins coumaroylated	11	41	24	**25**
Seeds-EA70				
	GRE1[a]	**SYR1[a]**	**CAR[a]**	**Average (%)**
Cyanidin-3-*O*-glucoside	2	0.4	1	**1**
Malvidin-3-*O*-glucoside	47	35	41	**41**
Σ Anthocyanin glucosides	71	49	72	**64**
Σ Anthocyanins acetylated	3	13	2	**6**
Σ Anthocyanins coumaroylated	20	33	23	**26**

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan.

Table 38: Percentage of different forms of anthocyanins in skin pomace extracts of total anthocyanins. Data expressed in percentage (%).

Skins-EAQ							
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**	**Average (%)**
Cyanidin-3-*O*-glucoside	3	0.3	2	1	3	2	**2**
Malvidin-3-*O*-glucoside	56	31	44	41	46	43	**43**
Σ Anthocyanin glucosides	88	44	71	72	82	75	**72**
Σ Anthocyanins acetylated	1	14	11	3	2	4	**6**
Σ Anthocyanins coumaroylated	9	38	5	22	11	17	**17**
Skins-EA70							
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**	**Average (%)**
Cyanidin-3-*O*-glucoside	2	0.3	1	1	2	1	**1**
Malvidin-3-*O*-glucoside	50	47	36	43	42	39	**43**
Σ Anthocyanin glucoside	76	66	56	80	75	68	**70**
Σ Anthocyanins acetylated	3	17	9	2	2	4	**6**
Σ Anthocyanins coumaroylated	17	12	28	16	19	25	**19**

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante.

Furthermore, malvidin-3-*O*-glucoside-pyruvate (vitisin A) and malvidin-3-*O*-glucoside-acetaldehyde (vitisin B) have also been identified. These compounds form a group called pyranoanthocyanins which have been previously described in red wines (Bakker et al. 1997) and later isolated from grape pomaces after fermentation as stable anthocyanins even during SO_2 bleaching or when a change of pH occurred (Fulcrand et al. 1998; Amico et al. 2004; Boido et al. 2006). Indeed, vitisin A and vitisin B derived during the fermentation process from reactions of malvin-3-*O*-glucoside with pyruvic acid and acetaldehyde, respectively (Asenstorfer et al. 2003; Marquez et al. 2012).

III.4.1. Anthocyanin analysis of grape pomace seed extracts

In seed pomace extracts, an appreciable amount of anthocyanins still remained. This is due, in the first instance, to the contact between seeds and skins throughout the winemaking process, in particular during the pressing and maceration. Moreover, despite the separation of skins from seeds, the operation was not complete and some skins residues remained.

In the aqueous extracts, total anthocyanin contents ranged from 0.76 ± 0.001 mg/g DW to 3.63 ± 0.03 mg/g DW in GRE1 and CAR respectively. CAR samples possessed the highest level of 3-*O*-glucoside anthocyanins (2.52 ± 0.03 mg/g DW) while SYR1 contained more acetylated and coumaroylated anthocyanins (0.41 ± 0.001 mg/g DW and 1.32 ± 0.01 mg/g DW respectively). Extracts of these two varieties contained four times more anthocyanins than Grenache which was composed of only 0.76 ± 0.001 mg/g DW of total anthocyanins.

Regarding the hydro-alcoholic 70% extract, overall, the level of anthocyanins was higher: 6.6-, 3.2- and 7-fold more anthocyanins were extracted from GRE1, SYR1 and CAR respectively than with the aqueous extraction method (Figure 32).

Total anthocyanin levels ranged from 5.00 ± 0.17 mg/g DW in GRE1 to 25.38 ± 0.11 mg/g DW in CAR which also possessed the highest 3-*O*-glucosides and 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins (18.15 ± 0.09 mg/g DW and 5.86 ± 0.02 mg/g DW respectively). Besides, an important amount of 3-*O*-(6"-*O*-acetyl) glucoside (1.3 ± 0.02 mg/g DW) was founded in SYR1 grape seed pomaces.

Among the three studied varieties, CAR variety proved to be a promising source of anthocyanins especially in EA70 extract compared to EAQ and the two other varieties.

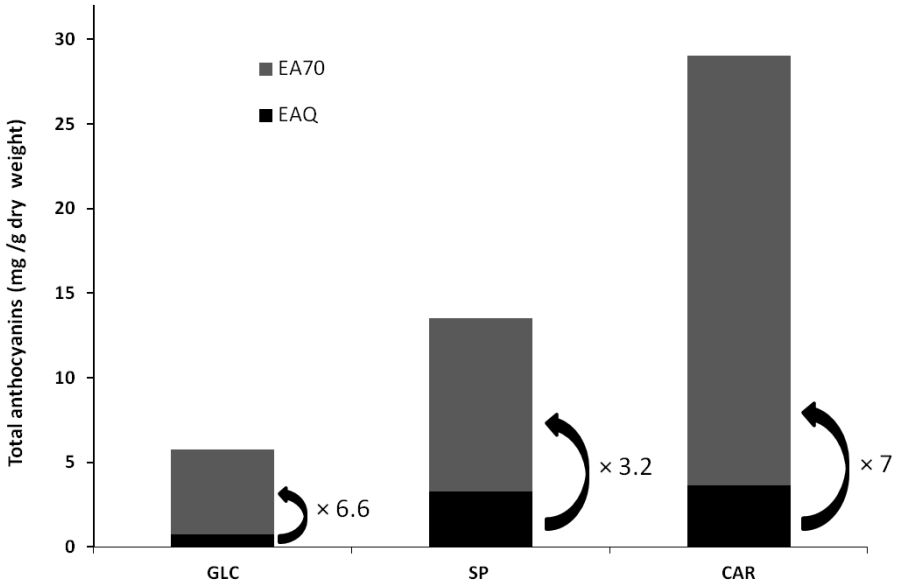


Figure 32: Comparison between aqueous and hydro-alcoholic 70% extraction of total anthocyanins in seed pomaces

III.4.2. Anthocyanin analysis of grape pomace skin extracts

As expected, anthocyanin levels in skin pomace extracts were higher than those in seeds and the predominant compound was the malvidin-3-*O*-glucoside mostly followed by the petudinin-3-*O*-glucoside and peonidin-3-*O*-glucoside. Indeed, despite the vinification process, a surprisingly large amount of anthocyanins still remained in grape skin pomaces.

With the aqueous extract, total anthocyanins value ranged from 0.24 ± 0.001 mg/g DW in SYR2 to 7.32 ± 0.08 mg/g DW in ALI. SYR1 possessed appreciable levels especially 3-*O*-(6"-*O*-acetyl) glucoside and 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins which reached up to 0.67 ± 0.001 mg/g DW and 1.86 ± 0.001 mg/g DW respectively. Concerning the hydro-alcoholic 70% extract, levels ranged from 16.1 ± 0.1 mg/g DW in SYR1 to 34.11 ± 0.95 mg/g DW in CAR. Moreover, CAR, ALI and MOU were the varieties which possessed the highest amounts of anthocyanin 3-*O*-glucosides (27.3 ± 0.88 mg/g DW, 19.45 ± 0.08, 18.79 ± 0.02 mg/g DW respectively). Regarding the 3-*O*-(6"-*O*-acetyl) glucoside anthocyanins, the two Syrah (SYR1 and SYR2) retained the highest amounts reaching 2.79 ± 0.04 mg/g DW and 1.59 ± 0.01 mg/g DW respectively. The 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins were predominant in ALI, CAR and SYR2.

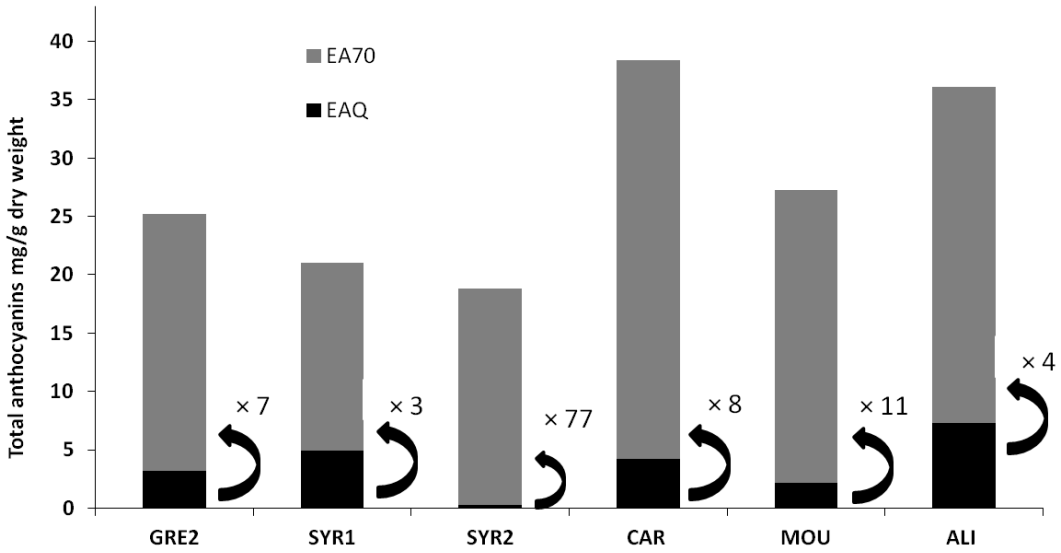


Figure 33: Comparison between aqueous extraction and hydro-alcoholic 70% extraction of total anthocyanins in skin pomaces

Considering the difference between the two extraction methods, extraction yield was superior in hydro-alcoholic 70% extracts with the amounts, depended on the variety, varying from 3- to 77-fold. Surprisingly, in SYR2, 77 times more of total anthocyanins were extracted. The level of 3-*O*-(6"-*O*-acetyl) glucoside and 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins reached up to 1.59 ± 0.01 mg/g DW and 5.14 ± 0.01 mg/g DW respectively (Figure 33).

Among the studied varieties, CAR and ALI were shown to be a rich source of anthocyanins especially with the hydro-alcoholic 70% extract. The SYR2 also contained substantial amounts of acetylated and coumaroylated anthocyanins in EA70.

In overall, extraction with 70% of ethanol was shown to improve the recovery of anthocyanins from the grape by-products. The grape pomaces were obtained after vinification and had been pressed to remove residual juice. During the maceration process, mainly in the aqueous phase, more soluble and less stable compounds are released. As the alcohol level rise up, the efficiency to degrade cell walls increase and rate of dissolved phenols are more important. Nevertheless, reaching a certain level of alcohol, a decrease is observed and this mechanism is mainly due to the adsorption on solids (yeast, pomace), modifications in their structure (formation of tannin-anthocyanin complexes) (Ribéreau Gayon et al. 1965). At this stage, extraction of anthocyanins is almost complete. By using a high alcohol level for the extraction of grape pomaces, less soluble and more stable compounds can be release while more soluble and less stable had already been extracted. Extraction yield were then optimized.

However, the two different methods of extraction did not strictly increase or decrease the ratio of 3-*O*-glucoside, 3-*O*-(6"-*O*-acetyl) glucoside and 3-*O*-(6"-*O*-coumaroyl) glucoside in grape skin and seed pomace extracts (Table 37 and 38). In some varieties, using hydro-alcoholic 70% extraction increased the relative amount of 3-*O*-(6"-*O*-coumaroyl) glucoside obtained but in other instances, it was decreased the ratio. For instance, GRE1 skin pomace extract comprised of 9% coumaroylated anthocyanins in EAQ which increased to 17% when using 70% alcohol whereas in SYR1, 38% were extracted in EAQ and the ratio decreased to 12% in EA70. Overall, the data showed that the two different extraction methods did not substantially affect the ratio of 3-*O*-glucoside, 3-*O*-(6"-*O*-acetyl) glucoside and 3-*O*-(6"-*O*-coumaroyl) glucoside anthocyanins.

III.5. Antioxidant activities evaluation of grape pomace extracts

Grape pomace seed and skin extracts radical scavenging potential were evaluated by three spectrophotometric tests: the FRAP, $ABTS^{\bullet+}$ and DPPH and a spectrofluorimetric test, the ORAC test.

Concerning seed extracts, the four antioxidant analytical techniques gave the same classification both in EAQ and EA70. The highest antioxidant activities were found in SYR1

for both types of extracts. Results were correlated with previous analysis which evidenced SYR 1 as having a substantial amount of flavan-3-ols, procyanidins and anthocyanins. GRE1 extract presented a low antioxidant activity as a consequence of its low phenolic contents (ORAC: 1466.4 µM TE/g DW; FRAP: 0.63 mM Fe^{2+}/g DW, ABTS: 1203.2 µM TE/g DW and DPPH: 410.8 TE/g DW in EAQ and ORAC: 1926.7 µM TE/g DW; FRAP: 1.28 mM Fe^{2+}/g DW, ABTS: 2813.1 µM TE/g DW and DPPH: 1277.6 TE/g DW in EA70). Antioxidant activities of EAQ and EA70 grape pomace seed extracts were showed in Table 39.

Table 39: Antioxidant activities characterisation in EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
ORAC[b]	1466.39 ± 29.58	2230.69 ± 101.74	2058.58 ± 85.11
FRAP[b]	0.63 ± 0.02	1.33 ± 0.08	1.06 ± 0.08
ABTS[b]	1203.20 ± 24.09	2432.62 ± 55.95	1948.75 ± 61.10
DPPH[b]	410.79 ± 43.30	1037.12 ± 64.04	1050.59 ± 30.11
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
ORAC[b]	1926.73 ± 108.55	2613.98 ± 150.86	2332.90 ± 91.94
FRAP[b]	1.28 ± 0.01	1.45 ± 0.16	1.20 ± 0.06
ABTS[b]	2813.15 ± 89.95	3601.20 ± 88.59	3495.58 ± 66.40
DPPH[b]	1277.59 ± 54.69	1685.87 ± 130.65	1536.77 ± 38.92

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as mmol Fe^{2+}/g DW.

In skins, results obtained by different antioxidant analysis were more disparate especially in EA70 extracts (Table 40). In aqueous extracts, the highest antioxidant activity was found in SYR1 and ALI. This observation was observed by every test and well correlated with previous results evidencing these extracts as containing high phenolic content. In EA70, different antioxidant tests did not give the same extract classification. Despite this fact, SYR1 skin extract was classified as being the first or second extract showing the highest antioxidant capacity between the four tests (ORAC: 1912.6 µM TE/g DW; FRAP: 1.52 mM Fe^{2+}/g DW, ABTS: 2614.5 µM TE/g DW and DPPH: 1391.7 TE/g DW).

Table 40: Antioxidant activities characterisation in EAQ and EA70 grape pomace skin extracts

	GRE2[a]	SYR1[a]	SYR2[a]	CAR[a]	MOU[a]	ALI[a]
Skins EAQ						
ORAC[b]	1190.70 ± 183.58	1345.94 ± 19.15	1065.98 ± 84.21	1077.76 ± 60.16	1033.76 ± 77.61	1714.62 ± 14.77
FRAP[b]	0.56 ± 0.01	0.88 ± 0.01	0.14 ± 0.02	0.67 ± 0.02	0.32 ± 0.01	1.13 ± 0.00
ABTS[b]	934.12 ± 11.90	1427.98 ± 54.80	668.30 ± 29.99	1048.83 ± 101.57	965.59 ± 16.63	1760.08 ± 91.03
DPPH[b]	99.45 ± 10.82	690.29 ± 147.01	263.85 ± 71.54	591.01 ± 85.59	279.43 ± 61.65	1057.12 ± 45.22
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
ORAC[b]	1828.26 ± 40.37	1912.56 ± 6.09	1701.83 ± 88.34	1238.38 ± 11.09	2070.03 ± 60.64	1628.45 ± 82.58
FRAP[b]	1.32 ± 0.03	1.52 ± 0.05	0.94 ± 0.03	1.34 ± 0.03	1.03 ± 0.02	1.13 ± 0.01
ABTS[b]	2612.08 ± 130.93	2614.50 ± 10.42	2010.64 ± 146.96	2555.92 ± 146.04	2674.84 ± 187.30	1923.37 ± 87.01
DPPH[b]	876.96 ± 74.32	1391.69 ± 37.24	1164.91 ± 55.55	1075.39 ± 46.16	833.28 ± 26.37	1749.31 ± 112.65

[a] GRE1 and GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC, ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as mmol Fe^{2+}/g DW.

III.6. Complementary phenolic analysis on grape pomace lyophilized extracts

As already mention above in the mDP, %G and %P analysis of grape pomace extracts paragraph, extracts were lyophilized in other to facilitate analysis. Further investigations such as total analysis, flavan-3-ol and anthocyanin analysis and evaluation of antioxidant activity were achieved to evaluate the potential of completely dry samples. Results are presented in Tables 41-46 for total contents, flavan-3-ols, anthocyanins and antioxidant activity analysis respectively.

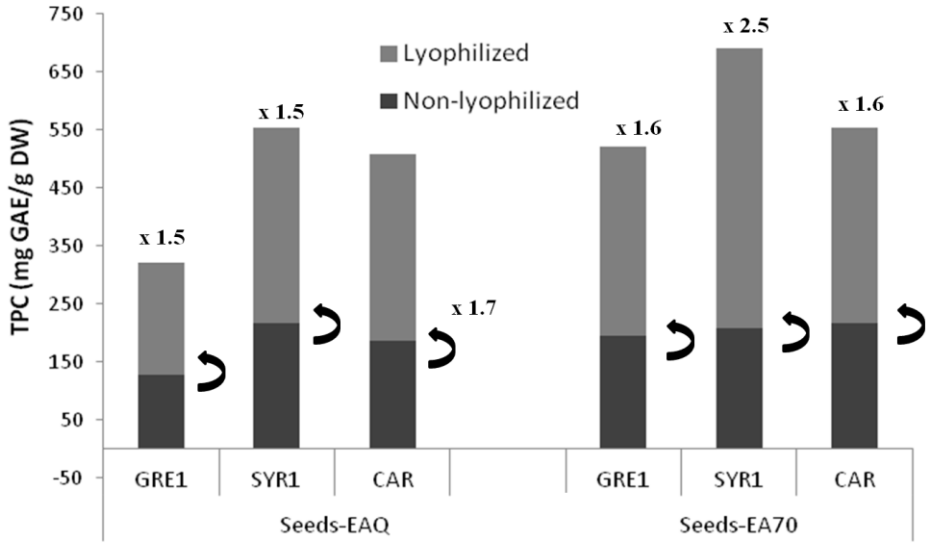


Figure 34: Comparison between non-lyophilized and lyophilized EAQ and EA70 seeds pomace extracts

In overall, lyophilization increased the potential of seed and skin grape pomace extracts especially in EA70 by concentrating compounds. For instance, all the analysis in both seed and skin extracts showed that lyophilized extracts contained 1- to 2.5-fold more (poly)phenolic compounds than non-lyophilized ones. Figures 34 and 35 compared the seed and skin TPC values in non-lyophilized and lyophilized extracts in EAQ and EA70.

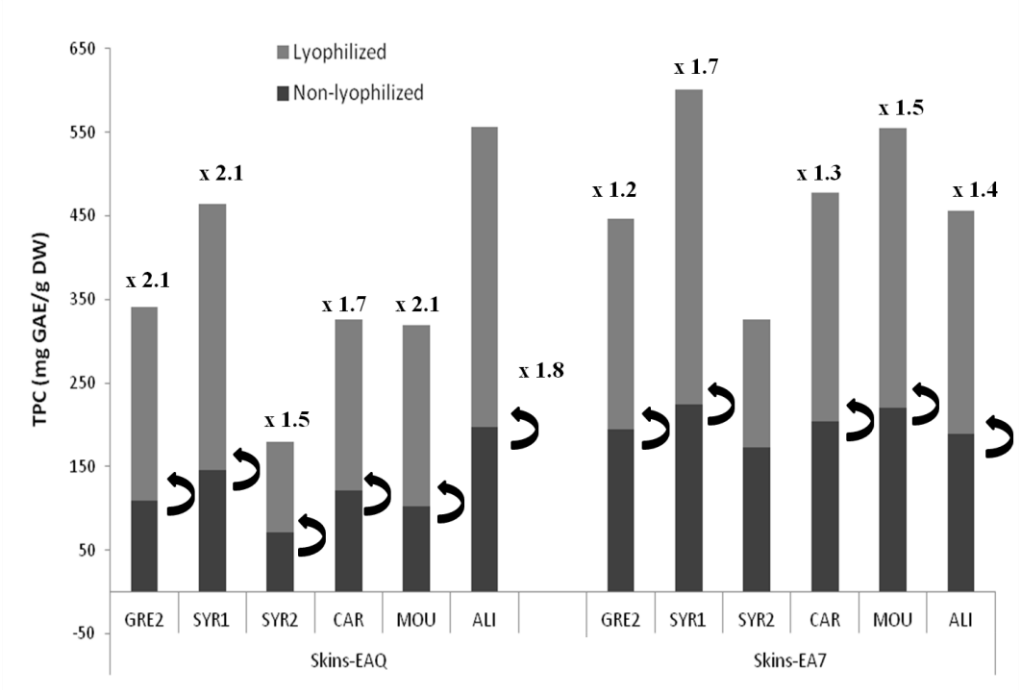


Figure 35: Comparison between non-lyophilized and lyophilized EAQ and EA70 skins pomace extracts

Actually, the techniques employed by 3INature Company, suitable for commercialization, didn't allow a complete drying of the samples and the use of lyophilization will increase the cost of production. This additional step in the samples elaboration can be beneficial and have to be further discussed. Lyophilization can be taken into account if it doesn't intensively increase the cost of production. However, even if the analysis showed a better potential of lyophilized extracts, their *in vivo* capacities have to be evaluated and might not have the same effects as the non-lyophilized extracts.

Table 41: Total phenol contents, total tannin and total anthocyanin contents in lyophilized EAQ and EA70 grape pomace seed and skin extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
TPC	193.18 ± 2.97	337.50 ± 5.35	323.27 ± 10.19
Total tannins	254.19 ± 0.89	439.16 ± 16.61	475.87 ± 18.75
Total antho	3.47 ± 0.23	11.94 ± 0.00	26.53 ± 0.66
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
TPC	324.98 ± 5.01	483.58 ± 3.33	338.10 ± 11.36
Total tannins	550.22 ± 14.99	738.50 ± 12.11	724.42 ± 4.42
Total antho	19.51 ± 0.96	47.43 ± 0.96	95.29 ± 4.06

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
TPC	231.55 ± 24.71	318.26 ± 10.17	108.53 ± 3.68	205.74 ± 9.17	216.68 ± 2.68	359.5 ± 1.28
Total tannins	250.86 ± 16.39	402.53 ± 1.44	154.61 ± 4.69	278.58 ± 7.81	281.29 ± 5.88	469.33 ± 1.23
Total anthocyanins	15.01 ± 0.56	37.93 ± 0.77	1.001 ± 0.00	28.73 ± 0.00	23.31 ± 2.00	49.14 ± 0.35
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
TPC	250.97 ± 34.48	375.6 ± 15.64	153.08 ± 6.67	273.75 ± 24.59	335.51 ± 10.81	267.52 ± 17.78
Total tannins	476.24 ± 19.79	731.04 ± 57.01	306.68 ± 10.38	536.31 ± 18.57	555.56 ± 8.13	488.63 ± 13.11
Total anthocyanins	83.95 ± 3.14	117.25 ± 0.90	58.467 ± 0.96	146.48 ± 0.96	103.12 ± 1.92	104.34 ± 0.00

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre, ALI, Alicante. In units of mg GAE/g DW seeds or skins for TPC and mg/g DW seeds or skins for total tannins and total anthocyanins. Data are expressed as the mean of triplicate ± standard deviation. TPC, total phenol contents.

Table 42: Flavan-3-ol monomers, dimers and trimers characterisation in lyophilized EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
C	3.96 ± 0.00	10.26 ± 0.01	4.15 ± 0.01
EC	1.80 ± 0.06	7.25 ± 0.06	1.66 ± 0.02
Σ Monomers	**5.77 ± 0.04**	**17.51 ± 0.03**	**5.81 ± 0.02**
B_1	1.76 ± 0.03	6.46 ± 0.17	1.91 ± 0.05
B_2	1.41 ± 0.01	4.36 ± 0.01	1.20 ± 0.02
B_3	0.96 ± 0.02	2.18 ± 0.09	0.85 ± 0.03
B_4	Nd	1.28 ± 0.03	Nd
Σ Dimers	**4.13 ± 0.06**	**14.28 ± 0.21**	**3.95 ± 0.07**
C_1	1.34 ± 0.11	3.67 ± 0.08	1.14 ± 0.02
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
C	6.13 ± 0.19	11.22 ± 0.19	8.45 ± 0.15
EC	2.44 ± 0.15	7.15 ± 0.15	3.45 ± 0.00
Σ Monomers	**8.57 ± 0.24**	**18.37 ± 0.24**	**11.90 ± 0.10**
B_1	2.88 ± 0.29	4.88 ± 0.43	5.20 ± 0.21
B_2	1.56 ± 0.27	2.99 ± 0.17	2.38 ± 0.12
B_3	1.00 ± 0.20	1.31 ± 0.01	1.28 ± 0.06
B_4	Nd	0.80 ± 0.12	Nd
Σ Dimers	**5.44 ± 0.54**	**9.97 ± 0.50**	**8.85 ± 0.28**
C_1	1.00 ± 0.04	2.28 ± 0.23	1.72 ± 0.04

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4; C_1, procyanidin trimers C_1. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B1, B2, B3 and B4; C_1, trimer C_1. Nd, Not determined.

Table 43: Flavan-3-ol monomers, dimers and trimers characterisation in lyophilized EAQ and EA70 grape pomace skin extracts

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
C	1.34 ± 0.01	2.40 ± 0.18	0.50 ± 0.00	1.61 ± 0.01	1.11 ± 0.04	3.38 ± 0.02
EC	0.48 ± 0.06	1.76 ± 0.15	0.36 ± 0.00	0.48 ± 0.01	0.63 ± 0.08	2.17 ± 0.01
Σ Monomers	**1.82 ± 0.03**	**4.16 ± 0.02**	**0.86 ± 0.00**	**2.09 ± 0.00**	**1.73 ± 0.08**	**5.55 ± 0.00**
B_1	1.16 ± 0.01	1.64 ± 0.05	0.46 ± 0.01	1.19 ± 0.00	1.03 ± 0.05	1.82 ± 0.01
B_2	0.80 ± 0.03	1.20 ± 0.01	0.56 ± 0.02	0.71 ± 0.01	0.97 ± 0.10	1.32 ± 0.01
B_3	0.62 ± 0.02	0.77 ± 0.01	0.39 ± 0.01	0.59 ± 0.00	0.54 ± 0.04	0.93 ± 0.01
B_4	Nd	Nd	Nd	Nd	Nd	0.55 ± 0.01
Σ Dimers	**2.57 ± 0.00**	**3.60 ± 0.05**	**1.40 ± 0.00**	**2.48 ± 0.01**	**2.54 ± 0.14**	**4.62 ± 0.01**
C_1	0.78 ± 0.06	1.05 ± 0.21	0.41 ± 0.00	0.68 ± 0.00	0.78 ± 0.05	0.98 ± 0.02
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
C	2.57 ± 0.05	6.34 ± 0.13	2.86 ± 0.02	4.03 ± 0.07	2.99 ± 0.02	12.00 ± 0.18
EC	0.76 ± 0.00	3.76 ± 0.02	1.76 ± 0.03	1.32 ± 0.05	1.51 ± 0.01	6.56 ± 0.06
Σ Monomers	**3.33 ± 0.04**	**10.10 ± 0.11**	**4.62 ± 0.04**	**5.35 ± 0.09**	**4.50 ± 0.00**	**18.56 ± 0.08**
B_1	1.61 ± 0.00	3.58 ± 0.03	1.92 ± 0.08	3.15 ± 0.05	2.56 ± 0.03	6.44 ± 0.21
B_2	0.71 ± 0.01	1.82 ± 0.00	1.08 ± 0.07	1.09 ± 0.07	1.27 ± 0.03	3.34 ± 0.08
B_3	0.56 ± 0.00	0.94 ± 0.03	0.57 ± 0.04	0.72 ± 0.04	0.74 ± 0.01	1.85 ± 0.09
B_4	Nd	Nd	Nd	Nd	Nd	0.88 ± 0.07
Σ Dimers	**2.87 ± 0.01**	**6.35 ± 0.04**	**3.57 ± 0.13**	**4.96 ± 0.11**	**4.58 ± 0.05**	**12.51 ± 0.33**
C_1	0.60 ± 0.00	1.25 ± 0.00	0.73 ± 0.03	0.83 ± 0.08	0.91 ± 0.02	1.87 ± 0.05

[a] GRE2, Grenache ; SYR1 and SYR2, Syrah ; CAR, Carignan ; MOU, Mourvèdre; ALI, Alicante. In units of mg/g DW seeds or skins. Data are expressed as the mean of triplicate ± standard deviation. C, (+)-Catechin; EC, (–)-Epicatechin; B_1, B_2; B_3, B_4, Procyanidin dimers B_1, B_2; B_3, B_4; C_1, procyanidin trimers C_1. Σ Monomers, sum of catechin and epicatechin; Σ Dimers, sum of B1, B2, B3 and B_4. Nd, Not determined.

Table 44: Anthocyanins characterisation in lyophilized EAQ and EA70 grape pomace seed extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
Delphinidin-3-*O*-glucoside	0.60 ± 0.00	0.26 ± 0.02	1.47 ± 0.02
Cyanidin-3-*O*-glucoside	0.48 ± 0.00	0.09 ± 0.00	0.14 ± 0.00
Petunidin-3-*O*-glucoside	1.05 ± 0.00	0.24 ± 0.00	0.79 ± 0.03
Peonidin-3-*O*-glucoside	0.92 ± 0.00	0.19 ± 0.01	0.30 ± 0.02
Malvidin-3-*O*-glucoside	1.40 ± 0.00	1.12 ± 0.01	2.45 ± 0.21
Σ Anthocyanin glucosides	**4.44 ± 0.01**	**1.89 ± 0.01**	**5.16 ± 0.27**
Petunidin-3-*O*-(6"-*O*-acetyl) glucoside	0.99 ± 0.00	0.13 ± 0.00	0.19 ± 0.00
Peonidin-3-*O*-(6"-*O*-acetyl) glucoside	0.99 ± 0.00	0.18 ± 0.00	0.20 ± 0.00
Malvidin-3-*O*-(6"-O-acetyl) glucoside	1.00 ± 0.00	0.49 ± 0.01	0.29 ± 0.00
Σ Acetylated anthocyanins	**2.99 ± 0.00**	**0.79 ± 0.01**	**0.68 ± 0.00**
Peonidin-3-*O*-(6"-*O*-coumaroyl) glucoside	1.00 ± 0.00	0.22 ± 0.01	0.21 ± 0.02
Malvidin-3-*O*-(6"-*O*-coumaroyl) glucoside	1.03 ± 0.00	0.95 ± 0.02	1.05 ± 0.04
Σ Coumaroylated anthocyanins	**2.03 ± 0.00**	**1.17 ± 0.01**	**1.27 ± 0.02**

Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
Delphinidin-3-*O*-glucoside	0.48 ± 0.01	0.81 ± 0.00	5.83 ± 0.14
Cyanidin-3-*O*-glucoside	0.13 ± 0.00	0.13 ± 0.00	0.17 ± 0.00
Petunidin-3-*O*-glucoside	0.48 ± 0.01	0.85 ± 0.02	3.4 ± 0.16
Peonidin-3-*O*-glucoside	0.53 ± 0.02	0.57 ± 0.01	0.94 ± 0.07
Malvidin-3-*O*-glucoside	2.75 ± 0.06	4.79 ± 0.00	12.49 ± 0.56
Σ Anthocyanin glucosides	**4.37 ± 0.10**	**7.14 ± 0.02**	**22.83 ± 0.94**
Petunidin-3-*O*-(6"-*O*-acetyl) glucoside	0.22 ± 0.00	0.25 ± 0.00	0.25 ± 0.01
Peonidin-3-*O*-(6"-*O*-acetyl) glucoside	0.19 ± 0.00	0.44 ± 0.01	0.24 ± 0.01
Malvidin-3-*O*-(6"-O-acetyl) glucoside	0.19 ± 0.00	1.73 ± 0.05	0.60 ± 0.02
Σ Acetylated anthocyanins	**0.6 ± 0.00**	**2.42 ± 0.05**	**1.09 ± 0.04**
Peonidin-3-*O*-(6"-*O*-coumaroyl) glucoside	0.34 ± 0.01	0.65 ± 0.01	0.55 ± 0.06
Malvidin-3-*O*-(6"-*O*-coumaroyl) glucoside	0.66 ± 0.00	2.85 ± 0.08	5.07 ± 0.24
Σ Coumaroylated anthocyanins	**0.99 ± 0.01**	**3.49 ± 0.10**	**5.62 ± 0.30**

[a] GRE1, Grenache ; SYR1, Syrah ; CAR, Carignan. Data are expressed as the mean of triplicate ± standard deviation as mg malvidin-3-*O*-glucoside equivalents/g of dry weight. Nd, not determined.

Table 45: Anthocyanins characterisation in lyophilized EAQ and EA70 grape pomace skin extracts

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Delphinidin-3-*O*-glucoside	0.66 ± 0.03	0.81 ± 0.01	0.23 ± 0.02	1.86 ± 0.00	1.19 ± 0.01	4.34 ± 3.57
Cyanidin-3-*O*-glucoside	0.15 ± 0.00	0.15 ± 0.00	0.10 ± 0.00	0.15 ± 0.00	0.18 ± 0.00	0.15 ± 0.00
Petunidin-3-*O*-glucoside	0.52 ± 0.00	0.66 ± 0.01	0.17 ± 0.00	0.94 ± 0.01	0.89 ± 0.00	0.81 ± 0.01
Peonidin-3-*O*-glucoside	0.47 ± 0.01	0.48 ± 0.01	0.14 ± 0.00	0.37 ± 0.01	0.59 ± 0.00	1.69 ± 0.04
Malvidin-3-*O*-glucoside	2.48 ± 0.01	3.83 ± 0.10	0.32 ± 0.02	3.10 ± 0.00	2.74 ± 0.00	4.82 ± 0.04
Σ Anthocyanin glucosides	**4.29 ± 0.05**	**5.93 ± 0.09**	**0.95 ± 0.05**	**6.43 ± 0.01**	**5.60 ± 0.01**	**11.81 ± 3.57**
Petunidin-3-*O*-(6"-*O*-acetyl) glucoside	0.19 ± 0.00	0.21 ± 0.00	ND	0.19 ± 0.00	0.19 ± 0.00	0.18 ± 0.00
Peonidin-3-*O*-(6"-*O*-acetyl) glucoside	0.21 ± 0.00	0.40 ± 0.01	ND	0.20 ± 0.00	0.21 ± 0.01	0.27 ± 0.01
Malvidin-3-*O*-(6"-O-acetyl) glucoside	0.31 ± 0.00	1.32 ± 0.02	0.16 ± 0.01	0.31 ± 0.00	0.23 ± 0.02	0.45 ± 0.01
Σ Acetylated anthocyanins	**0.72 ± 0.01**	**1.92 ± 0.01**	**0.16 ± 0.01**	**0.70 ± 0.00**	**0.63 ± 0.03**	**0.89 ± 0.02**
Peonidin-3-*O*-(6"-*O*-coumaroyl) glucoside	0.23 ± 0.00	0.51 ± 0.02	ND	0.25 ± 0.01	0.23 ± 0.02	0.51 ± 0.00
Malvidin-3-*O*-(6"-*O*-coumaroyl) glucoside	0.38 ± 0.00	2.51 ± 0.06	0.15 ± 0.01	1.17 ± 0.00	0.39 ± 0.05	1.32 ± 0.03
Σ Coumaroylated anthocyanins	**0.61 ± 0.00**	**3.02 ± 0.09**	**0.15 ± 0.01**	**1.42 ± 0.01**	**0.62 ± 0.07**	**1.82 ± 0.02**
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
Delphinidin-3-*O*-glucoside	3.66 ± 0.19	2.38 ± 0.03	1.60 ± 0.02	11.42 ± 0.29	4.64 ± 0.00	1.87 ± 0.02
Cyanidin-3-*O*-glucoside	0.27 ± 0.00	0.76 ± 0.00	0.50 ± 0.00	0.28 ± 0.01	0.85 ± 0.00	0.16 ± 0.00
Petunidin-3-*O*-glucoside	2.88 ± 0.18	2.94 ± 0.00	1.94 ± 0.00	6.17 ± 0.37	4.52 ± 0.02	1.61 ± 0.01
Peonidin-3-*O*-glucoside	2.16 ± 0.03	1.98 ± 0.02	1.24 ± 0.00	1.62 ± 0.15	2.53 ± 0.01	3.90 ± 0.06
Malvidin-3-*O*-glucoside	16.82 ± 1.27	10.37 ± 0.00	5.55 ± 0.00	19.85 ± 1.19	12.16 ± 0.03	11.58 ± 0.10
Σ Anthocyanin glucosides	**25.78 ± 1.67**	**18.40 ± 0.03**	**10.83 ± 0.02**	**39.35 ± 2.02**	**24.71 ± 0.00**	**19.12 ± 0.13**
Petunidin-3-*O*-(6"-*O*-acetyl) glucoside	0.26 ± 0.01	ND	1.19 ± 0.03	0.28 ± 0.01	1.62 ± 0.01	0.23 ± 0.00
Peonidin-3-*O*-(6"-*O*-acetyl) glucoside	0.37 ± 0.01	2.07 ± 0.05	1.26 ± 0.058	0.33 ± 0.01	1.81 ± 0.02	0.41 ± 0.00
Malvidin-3-*O*-(6"-O-acetyl) glucoside	0.92 ± 0.03	4.71 ± 0.01	1.90 ± 0.08	0.98 ± 0.06	1.89 ± 0.01	0.54 ± 0.03
Σ Acetylated anthocyanins	**1.55 ± 0.05**	**6.78 ± 0.04**	**4.35 ± 0.12**	**1.59 ± 0.08**	**5.32 ± 0.00**	**1.18 ± 0.04**
Peonidin-3-*O*-(6"-*O*-coumaroyl) glucoside	0.91 ± 0.04	1.84 ± 0.02	1.65 ± 0.00	0.57 ± 0.04	2.29 ± 0.010	1.99 ± 0.01
Malvidin-3-*O*-(6"-*O*-coumaroyl) glucoside	3.69 ± 0.24	2.70 ± 0.00	3.34 ± 0.04	4.53 ± 0.38	4.09 ± 0.05	4.36 ± 0.08
Σ Coumaroylated anthocyanins	**4.59 ± 0.29**	**4.54 ± 0.02**	**4.99 ± 0.02**	**5.10 ± 0.42**	**6.38 ± 0.00**	**6.36 ± 0.09**

[a] GRE2. Grenache ; SYR1 and SYR2. Syrah ; CAR. Carignan ; MOU. Mourvèdre; ALI. Alicante. Data are expressed as the mean of triplicate ± standard deviation as mg malvidin-3-*O*-glucoside equivalents/g of dry weight. Nd. not determined.

Table 46: Antioxidant activities characterisation in lyophilized EAQ and EA70 grape pomace skin extracts

Seeds-EAQ			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
ORAC	3094.2 ± 209.0	4070.8 ± 199.0	6059.2 ± 597.1
FRAP	1.3 ± 0.2	3.2 ± 0.0	2.5 ± 0.0
ABTS	2110.9 ± 381.3	3964.9 ± 250.9	4062.0 ± 417.9
DPPH	961.7 ± 42.4	1248.3 ± 37.4	1351.5 ± 290.2
Seeds-EA70			
	GRE1[a]	**SYR1[a]**	**CAR[a]**
ORAC	4374.4 ± 290.6	6252.5 ± 430.7	5125.1 ± 43.2
FRAP	2.9 ± 0.1	4.0 ± 0.2	2.4 ± 0.1
ABTS	4666.1 ± 668.0	7662.6 ± 261.7	4605.9 ± 46.1
DPPH	3322.5 ± 26.7	4076.8 ± 79.7	2552.9 ± 150.7

Skins-EAQ						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
ORAC	3209.3 ± 109.8	4528.0 ± 65.8	2783.6 ± 115.1	3834.5 ± 451.2	3651.8 ± 166.9	5666.7 ± 216.5
FRAP	1.7 ± 0.2	2.9 ± 0.0	1.3 ± 0.0	2.0 ± 0.1	1.8 ± 0.0	2.9 ± 0.1
ABTS	2232.0 ± 305.0	4217.9 ± 597.3	1335.5 ± 97.9	2722.2 ± 207.5	2539.2 ± 131.2	2624.2 ± 340.0
DPPH	484.3 ± 93.8	606.9 ± 125.7	750.3 ± 0.0	575.0 ± 98.2	685.7 ± 41.1	1571.4 ± 94.9
Skins-EA70						
	GRE2[a]	**SYR1[a]**	**SYR2[a]**	**CAR[a]**	**MOU[a]**	**ALI[a]**
ORAC	4293.7 ± 102.0	4829.2 ± 32.0	2769.1 ± 14.9	4964.7 ± 102.3	3913.8 ± 467.1	4969.3 ± 32.2
FRAP	2.4 ± 0.1	2.9 ± 0.1	1.3 ± 0.0	3.2 ± 0.1	1.9 ± 0.1	1.8 ± 0.2
ABTS	3880.7 ± 648.1	4539.9 ± 253.5	1713.9 ± 29.9	3695.6 ± 333.1	4494.5 ± 336.4	3580.4 ± 128.5
DPPH	2340.3 ± 0.0	1886.9 ± 53.5	947.1 ± 69.5	1912.9 ± 170.3	2285.1 ± 31.4	1681.9 ± 67.9

[a] GRE1 and GRE2. Grenache ; Syr1 and SYR2. Syrah ; CAR. Carignan ; MOU. Mourvèdre; ALI. Alicante. Data are expressed as the mean of triplicate ± SD. [b]ORAC. ABTS and DPPH are expressed as µmol Trolox/g DW and FRAP as mmol Fe^{2+}/g DW.

IV. Conclusions

The first part of our work screened the phenolic compositions of by-products obtained after vinification of different Mediterranean grape varieties, in order to assess their potential for nutraceutical applications against hypertension. The comparison of several wine industry by-products with their respective grapes provided evidence that pomaces are very rich sources of antioxidants, despite extraction during vinification. Grape seed and skin pomace extracts still contained appreciable amounts of flavan-3-ols and anthocyanins. The quantitative and qualitative distribution of (poly)phenols in grape pomaces showed significant differences through varieties and vintages. In addition, seeds were shown to contain particularly high amounts of phenolics and thus an important antioxidant potential compared to skins.

In the first study of grape and grape pomace characterisations, seeds from Grenache (GRE1), Syrah (SYR1) and skins from Syrah (SYR1), Carignan and Alicante were evidenced as the most interesting fractions because of their higher (poly)phenol contents in term of flavan-3-ols (i.e. monomers, dimers and trimer) and anthocyanins (i.e. glycosides, acetylated and coumaroylated). These extracts also exerted the highest antioxidant capacities thought four different tests. As a result, these varieties and their parts (seeds and skins) were chosen for further extraction with aqueous and hydro-alcoholic 70% solution suitable for consumption and commercialization.

The investigation of aqueous and hydro-alcoholic 70% evidenced seeds from Carignan and Syrah (SYR1) and skins from Carignan and Alicante as containing high phenolic contents and antioxidant activity. Hydro-alcoholic 70% solution showed to be more effective because of a higher flavan-3-ol, procyanidin and anthocyanin contents in EA70 extracts. However, EAQ extracts displayed also interesting amount of (poly)phenolic and radical scavenging capacity and should not be ignored. Lyophilization step permitted to obtain extracts with more concentrated (poly)phenolic compounds. Nevertheless, this step can intensively increases the cost of production and has to be further discussed.

Although selected extracts presented high content of (poly)phenolic compounds, the *in vivo* effects might not be that effective because bioavailability greatly differs between (poly)phenols. Several extracts, even those which contain low phenolic amounts, have to be

tested. The next chapter will highlight the *in vivo* biological effects on hypertension using a SHR rat model (spontaneously hypertensive rats), with our selected seed and skin grape pomace extracts.

Résumé chapitre 3

Actuellement, les polyphénols sont bien connus pour leurs effets bénéfiques sur la santé et sont actuellement au centre de nouvelles collaborations entre scientifiques et industries. De nombreuses études épidémiologiques ont démontré qu'une alimentation riche en polyphénols est associée à une réduction de la mortalité globale avec une diminution du nombre de décès dus aux maladies cardiovasculaires et aux cancers (Mennen et al. 2004; He et al. 2007; Chong et al. 2010). Les composés phénoliques sont dotés de propriétés antioxydantes qui protègent l'organisme contre la production excessive de radicaux libres. Ces derniers possèdent une forte réactivité pouvant mener à un désordre dans les structures moléculaires par oxydation des lipides membranaires, des protéines cellulaires et des acides nucléiques et provoquer ainsi la mort cellulaire (Halliwell 1994; Dröge 2002). Outre ces particularités, les polyphénols peuvent aussi contribuer à réduire le stress oxydatif (Bagchi et al. 2000), à moduler la cascade inflammatoire (Delmas et al. 2005; Castilla et al. 2006), à réduire l'oxydation des LDL-c responsable des évènements athérogéniques (Stein et al. 1999; Sano et al. 2007) ainsi qu'à la protection contre les épisodes athérothrombotiques incluant l'ischémie cardiaque et l'inhibition des agrégations plaquettaires (Keevil et al. 2000; Shanmuganayagam et al. 2002).

De plus, les composées phénoliques peuvent prévenir l'hypertension artérielle et le dysfonctionnement endothélial induit par l'angiotensine II en partie en prévenant le stress oxydant vasculaire-dépendant de la NADPH oxydase et la formation de métabolites vasoconstricteurs. D'une part, les polyphénols accroissent la production des facteurs vasodilatatrices tel que le monoxyde d'azote (NO) (Fitzpatrick et al. 1993; Andriambeloson et al. 1997; Soares de Moura et al. 2002), l'EDHF (endotheluim-derived hyperpolarizing factor) (Kwan et al. 2004; Ndiaye et al. 2004) et la prostacycline (Mizugaki et al. 2000; Aldini et al. 2003) via la stimulation de la voie PI3-Kinase/Akt redox-sensible et d'autre part, ils inhibent la synthèse de vasoconstricteur, l'endotheline-1 dans les cellules endothéliales (Corder et al. 2001).

La protection contre les maladies cardiovasculaires par les polyphénols citée précédemment résulte d'association de différents effets produits par différents mécanismes et dans certains cas, par différents composés. En effet, les composés phénoliques sont présents sous des formes diverses (plus de 8000 composés phénoliques identifiés dans les végétaux en quantité variable) mais présentent toutefois une structure commune, constituée d'un ou

plusieurs cycles benzéniques portant une ou plusieurs fonctions hydroxyles. Parmi ces composés, les flavonoïdes sont les constituants majeurs avec plus de 4000 composés et sont sous divisés en plusieurs familles: les flavan-3-ols (catéchine, épicatéchine), les flavonols (quercétine, myricétine, kaempférol..), les anthocyanidines (malvidine, cyanidine, delphinidine..), les flavones (apigénine, lutéoline…), et les flavanonols. Du fait de leurs structures, leurs modes d'action au niveau l'organisme ainsi que leurs biodisponibilités sont différents (Kris-Etherton et al. 2002; Crozier et al. 2010; Del Rio et al. 2010). Il est pour cela intéressant de connaitre la composition des différents extraits utilisés lors des expériences *in vivo*.

A l'heure actuelle, les polyphénols, notamment ceux issus des raisins et du vin ont prouvé leur efficacité à réduire l'HTA sur les modèles animaux (Al-Awwadi et al. 2004). La plupart des études concernant les effets bénéfiques des ces composés sur la santé ont été effectué avec les extraits de pépins et/ou de pellicules de raisins et des vins. Ces derniers renferment une quantité non négligeable de polyphénols. Une revue récente de (Garrido et al. 2011) en illustre la composition. La vinification permet l'extraction de polyphénols contenus dans les raisins que l'on peut retrouver dans les vins. Leurs concentrations dépendent des cépages, de la localisation du vignoble et des différents paramètres techniques appliqués lors de la vinification (Kammerer et al. 2004; Ruberto et al. 2007). Cependant l'extraction n'est pas complète (environ 30 à 40 % seulement), une certaine quantité subsiste dans les marcs et sont essentiellement composés de pellicules et de pépins pressés qui comptent pour environ 20 % du poids originale des raisins utilisé pour l'élaboration du vin (Laufenberg et al. 2003; Llobera et al. 2007).

Ce chapitre étudie la composition phénolique des sous-produits de la vigne obtenus après la vinification de différents cépages caractéristiques de la Vallée du Rhône dans le but de procéder au développement d'extraits phénoliques de marcs en tant que complément alimentaire préventif de l'hypertension artérielle. Les analyses globales telles que les polyphénols totaux, les tannins totaux et les anthocyanes totales ont été effectuées. Une analyse poussée par HPLC-UV-Fluo a permis d'obtenir la composition en tannins et anthocyanes individuels. Le pouvoir antioxydant des extraits a été évalué à l'aide de quatre tests antioxydants différents : l'ORAC, le FRAP, l'ABTS et le DPPH. La comparaison des extraits de raisins et de leurs marcs respectifs montre que les marcs représentent une source importante d'antioxydants phénoliques malgré le processus de vinification. Les pépins et pellicules de marcs renferment des quantités appréciables de flavan-3-ols et anthocyanines.

La distribution qualitative et quantitative des polyphenols dans les marcs de raisins présentent des différences significatives au travers des variétés et millésimes allant de 15% à 70% de polyphénols extraits. Par ailleurs, les pépins possèdent une quantité importante en polyphénols et par conséquent un pouvoir antioxydant plus élevé comparé aux pellicules.

Dans une première étude de caractérisation de raisins et de marcs après séparation pépins-pellicules, il a été démontré que les pépins de Grenache (GRE1), Syrah (SYR1) et les pellicules de Syrah (SYR1), Carignan et Alicante sont les fractions les plus intéressantes dues a la présence d'importantes quantités de flavan-3-ols (monomères, dimères et trimère) jusqu'à 8.7 mg/g MS et d'anthocyanes (glycosylées, acétylées et coumaroylées jusqu'à 17.40 mg/g MS, 1.57 mg/g MS et 2.38 mg/g MS respectivement). De même, ces extraits présentent les pouvoirs antioxydants les plus élevés pour tous les tests confondus. Par conséquent, ces variétés et leurs parties (pépins/pellicules) ont été sélectionnées pour des extractions supplémentaires avec d'autres types de solvants tels que l'eau et une solution hydro-alcoolique 70 % compatible à la consommation et la commercialisation. Deux différentes extractions ont été effectuées afin d'obtenir deux types d'extraits pour chaque échantillons : un extrait aqueux (EAQ) et un extrait hydro-alcoolique 70% (EA70).

L'analyse des extraits aqueux (EAQ) et hydro-alcoolique 70% (EA70) indique que les pépins de Carignan et Syrah (SYR1) et les pellicules de Carignan et d'Alicante contiennent les plus fort taux en composés phénoliques et activités antioxydantes. La solution hydro-alcoolique 70% permet une meilleure extraction de flavan-3-ols, procyanidins et anthocyanes dans les extraits EA70. Cependant, les extraits aqueux (EAQ) affichent tout de même des quantités de polyphénols et de pouvoir antioxydants intéressants et ne devrait pas être ignoré. La lyophilisation permet une meilleure concentration des composés phénoliques dans les extraits. Toutefois, cette étape augmentera considérablement le coût de production des extraits. Ce point devrait être discuté d'avantage lors de l'élaboration des extraits finaux.

Bien que les extraits sélectionnés présentent une quantité importante en composés phénoliques et de forts pouvoirs antioxydants *in vitro,* ceci ne permet pas d'évaluer leur efficacité biologique *in vivo* dûe à une différence de biodisponibilité de chaque composé phénolique dans le milieu gastro-intestinale. Certains extraits, même ceux contentant de faible quantité en polyphénols ont été choisis afin d'évaluer leur efficacité *in vivo*.

Chapter 4:

In vivo study of grape pomace extracts

I. State of knowledge: SHR rats

For centuries, animals have been used by humans to understand their own biology. In cardiovascular research, animal models have allowed the study of cardiovascular disease in the early stages, as well as the investigation of the mechanisms of the pathogenesis of cardiovascular disease, pathophysiology, complications and the effects of drug intervention.

An ideal animal model for any cardiovascular disease in humans should fulfill the following criteria:

1/ mimic the human disease,

2/ allow studies in chronic, stable disease,

3/ produce symptoms which are predictable and controllable,

4/ satisfy economical, technical and animal welfare considerations,

5/ allow measurement of relevant cardiac, biochemical and haemodynamic parameters.

Over the past 50 years various animal models of hypertension have been developed, predominantly in the rat. The spontaneously hypertensive rat (SHR) is the most widely used rat model and reflects the primary stages hypertension of human hypertension. Many other aspects of the etiology of hypertension are found in other rat models such as the Dahl/Salt sensitive (Dhal/SS) which develop hypertension in response to a high NaCl diet (Dahl et al. 1962b), deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Katholi et al. 1980), fructose-fed rats which develop insulin resistance and hypertension (Hwang et al. 1987), two-kidney one-clip and one kidney one clip hypertensive rats or even the transgenic Ren-2 TGR(mRen2)27 hypertensive rat (Langheinrich et al. 1996). However, these models are less frequently employed.

The most commonly used model of cardiovascular disease is the spontaneously hypertensive rat (SHR) with the Wistar Kyoto rat (WKY) as the normotensive control. SHR represents the genetic strain of hypertensive rats and is the animal of choice for screening antihypertensive agents.

SHR rats introduced by Okamoto and Aoki in 1963 represented a new model of experimental hypertension that required no physiological, pharmacological or surgical intervention. The SHR model was developed by meticulous genetic inbreeding that uniformly resulted in 100% of the progeny having naturally occurring hypertension. Indeed, SHRs are descendants of an outbred Wistar male with spontaneous hypertension mating with a female with an elevated blood pressure, and then brother × sister mating continued with selection for spontaneous hypertension, defined as a SBP of over 150 mm Hg persisting for more than one month. From 1968, this inbred strain of SHRs was further developed in the USA (Kurtz et al. 1987). The various colonies of SHR are pre-hypertensive for the first 6-8 weeks of their lives with systolic blood pressure around 100-120 mm Hg, and then hypertension develops over the next 12-15 weeks (Adams et al. 1989). In SHRs male, mean arterial pressure is approximately 190-200 mm Hg as compared to 115-130 mm Hg in normal rats.

The WKY normotensive controls were established in 1971 by the National Institutes of Health (USA) as an inbreed of the Wistar Kyoto colony via brother × sister mating (Kurtz et al. 1987). The degree of genetic difference between the SHR and WKY strains and within different colonies of each strain is substantial and comparable to the maximum divergence possible between unrelated humans. WKY may share some of the genes responsible for hypertension with the SHRs because they were derived from the WKY and hypertension may develop spontaneously in the WKY (Johnson et al. 1992; St Lezin et al. 1992).

The male SHR is commonly used as a model of established human hypertension and to test new antihypertensive medication. Because substantial individual variation in polygenetic disposition and excitatory environmental factors exist, many variations in the direct and indirect effects on the cardiovascular system are difficult to differentiate and complicate the study of human hypertension. Researchers in hypertension have commonly resorted to the use of SHRs which have, within each colony, uniform polygenetic disposition and excitatory factors which produce uniform changes in the indirect and direct effects on the cardiovascular system. This lack of inter-individual variation is one of the major advantages of the SHR but it means that the SHR can only model one of many possible causes of human hypertension (Lindpaintner et al. 1992).

Another advantage of the SHRs is that it follows the same progression of hypertension as human hypertension with pre-hypertensive, developing and sustained hypertensive phases with each phase lasting at least several weeks (Folkow 1993). However the SHRs differ from

human hypertension in that SHRs reproducibly develop hypertension in young adulthood rather than in middle age as in humans. Because SHRs have a pre-hypertensive state, they have the important potential to be used in studies of the cause and development of hypertension. The SHR model is also suitable for gender studies in hypertension but only a few recent studies have considered gender differences in SHR responses (Kauser et al. 1995).

Spontaneous hypertension has been observed in a number of different strains of common laboratory animals. The New Zealand strain of Smirk seems to be most similar to the Japanese SHR, though this has not been studied as widely (Smirk et al. 1958). In contrast, the Milan strain developed by Bianchi *et al*, (1975) seems to be different involving primarily alterations in renal sodium and water metabolism; therefore it may not be analogous to essential hypertension. Another developed by Dahl and coworkers shows a high sensitivity ("S" strain) to sodium intake in comparison to its normotensive control ("R" strain), which is sodium resistant (Dahl et al. 1962a). Furthermore, other strain such as Milan (Bianchi et al. 1975), Prague (Heller et al. 1993), Lyon (Sassard et al. 1997) and San Juan (Crespo et al. 1996) hypertensive rats were developed.

Among these strains, SHRs develop not only moderate to severe hypertension but also typical complications of hypertension. Stroke prone SHRs (SHRSP), which are selectively bred from among SHRs, are extreme examples that develop cerebrovascular lesions spontaneously in over 80% of rats (Ogata et al. 1982). Because of high mortality rate of stroke in man, the similarity of stroke in SHRSP is important for further application of this model to studies of stroke in man. Other variants derived from SHRs is the arteriolipidosis-prone (Yamori et al. 1981).

Extreme environmental conditions such as stress and excess salt accelerate the development of hypertension and aggravate the hypertensive complications (Pinto et al. 1998). Complications such as cerebral hemorrhage, thrombosis, nephrosclerosis, myocardial lesions in SHRs and especially cerebral lesions in SHRSPs, are pathogenically, pathologically and epidemiologically similar to those observed in essential hypertension in man. Therefore, these models can be used to study not only the pathogenesis and therapy but also prophylaxis in essential hypertension and its complications. Because of apparent similarities of the SHRs to essential hypertension, SHR models are highly recommended for screening potential drug candidates for hypertension (Kacew et al. 1996). Experimental models of genetic hypertension have also been developed in animals other than the rat.

Though it has been produced in one strain of rabbit and dog they have not been studied extensively for practical, financial and other reasons. Rats are mainly used because of their small size, short lifespan and low cost.

Multiple quantitative trait loci associated with haemodynamic and metabolic parameters have been mapped in the SHR. Recently, it has become possible to identify some of the specific quantitative trait gene (QTG) variants that underline quantitative trait loci linked to complex cardiovascular and metabolic traits in SHR related strains. As in humans, spontaneous hypertension and associated metabolic disturbances in the SHRs are governed by complex polygenic traits. Identification of responsible genes in the SHRs could be used to study the role of human orthologous genes in complex cardiovascular and metabolic diseases and to identify new pathways and targets for pharmaceutical interventions (Pravenec et al. 2007). Linkage analyses of crosses between the SHRs and various control strains have revealed multiple quantitative trait loci (QTL) associated with blood pressure variation and with parameters of lipids and glucose metabolism on practically all rat chromosomes. However, only a few of the responsible quantitative trait genes (QTGs) underlying these QTL for blood pressure and metabolic phenotypes has been confirmed in SHR-related models. The first one is the deletion variant of Cd36 (fatty acid translocase) and has been confirmed as a genetic determinant of multiple features of the metabolic syndrome including dyslipidemia, insulin resistance, and hypertension (Pravenec et al. 2008a). The second one consisted of the mutated form of Ogn (osteoglycin) which has been validated as a gene predisposing to cardiac hypertrophy (Petretto et al. 2008), and the last one is a variant in Srebf1 (sterol regulatory element binding factor 1c isoform), which proved to exert effects on hepatic lipid levels (Pravenec et al. 2008b). This illustrates the inherent difficulty of identifying causative QTGs even in animal models in which the effects of genetic background and environmental variables can be strictly controlled.

Origin of hypertension is multiple. As in humans, hypertension develops more rapidly and becomes more severe in male than female SHR. *In vivo* studies have shown that, in the early stages of hypertension, SHRs have an increased cardiac output with normal total peripheral resistance. As the SHRs progress into the established hypertension state, the cardiac output returns to normal and the hypertrophied blood vessels produce an increase in the total peripheral resistance (Smith et al. 1979). In addition, According to Dietz et al (1984), SHRs presented an abnormality of intracellular electrolyte balance with increased intracellular sodium and calcium concentration. Calcification of vasculature which sometimes

occurs in humans has also been demonstrated by Kanemaru et al. (2008) in the SHRs smooth muscle cells that Osterix (a transcription factor for osteogenesis), and alkaline phosphatise (ALP) (a marker of vascular calcification) were significantly increased in aortic smooth muscle cells from SHRs compared to similar cells from WKYs. Reduction in active transport of calcium by the intestine (Shibata et al. 1990) and excess loss through urine (Hsu et al. 1987) may lead to hypocalcemia.

It has been showed that SHR have fewer glomeruli than WKY rats, but still with similar size, resulting in a reduced glomerular volume. This is consistent with the hypothesis that the kidney plays an important role in hypertension (Skov et al. 1994).

Compared to normotensive rats, the abnormal development of the intra renal renin-angiotensin system (RAS) is thought to contribute to adult-onset hypertension in the SHRs (Tikellis et al. 2006). Studies have shown the expression and angiotensin-converting enzyme 2 (ACE2) activities are significantly increased in the SHRs kidney at birth. With the onset of hypertension, the tubular expression of ACE2 falls in SHRs compared to WKYs and remains reduced in the adult SHR kidney. The overall developmental pattern of ACE2 expression in the SHRs kidney is also modified, with declining expression over the course of renal development (Tikellis et al. 2006). The developmental pattern of ACE2 expression in the SHRs kidney is altered before the onset of hypertension, consistent with the key role of the RAS in the pathogenesis of adult-onset hypertension (Obata et al. 2000).

It is well established that the RAS has a central role in the regulation of blood pressure and angiotensin-converting enzyme 2 (ACE2), is involved in the regulation of the RAS (Crackower et al. 2002). ACE2 is a carboxypeptidase converting Angiotensin I (Ang I) to the nonapeptide angiotensin(1–9) (Ang 1–9) and Angiotensin II (Ang II) to the angiotensin(1–7) (Ang 1–7) by removing a single amino-acid residue from the carboxy-terminus of the substrate (Donoghue et al. 2000). This action is distinct and complementary to the dipeptidyl carboxypeptidase activity of ACE that generates Ang II from Ang I. ACE2 is thought to represent the major renal source of Ang 1–7, a peptide with anti-angiogenic and vasodilatory actions that antagonize the effects of Ang II (Ferrario et al. 2004), thus, a deficit of ACE2 induces an increase of Ang II which lead to a vasoconstriction.

Dysfunction and vascular hypertrophy in SHRs is also a part due to an important oxidative stress. Oxidative stress is actually caused, on the one hand, by an excess of superoxide anion ($\bullet O_2^-$) production by nicotinamide adenine dinucleotide/nicotinamide

adenine dinucleotide phosphate (NADH/NADPH) oxidase (Grunfeld et al. 1995; Zalba et al. 2000) and on the other hand, by a low activity of the endothelial NO synthase (eNOS) which catalyses the production of nitric oxide (NO), a potent vasodilatator agent generated from L-arginine (Palmer et al. 1987). Actually, $\bullet O_2^-$ and NO are known to rapidly react to form the stable peroxynitrite anion (ONOO-), a powerful oxidant, which will prevent the vasodilatating action of NO (Beckman et al. 1990; Pacher et al. 2007; Vera et al. 2007).

Thus, SHRs have been used extensively and successfully to test drugs for their effectiveness in lowering blood pressure and to study the mechanisms of established hypertension. The SHR is a chronic stable model producing symptoms which are predictable and controllable and avoiding difficult or life-threatening technical interventions. Similarity of SHRs to essential hypertension and its complication made the SHR the main animal model of hypertension.

II. Evaluation of grape pomace extracts anti-hypertensive effects on SHR rats

In this part, *in vivo* anti-hypertensive effects of grape pomace extracts on SHR were investigated. Different experimental groups were made and blood pressure (BP) was followed. Rats were fed with aqueous (EAQ) and hydro-alcoholic 70% (EA70) grape pomace extracts at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. Grape pomace extracts were dissolved in 3% EtOH solution in order to facilitate the complete dissolution before administration. Moreover, the effect of verapamil administrated alone or in association with grape pomace extracts were evaluated.

II.1. 1st study: Grape pomace extracts screening

In this experiment, six EA70 grape pomace extracts were screened for their anti-hypertensive effect. The study was conducted for a period of six weeks: three weeks of treatment, a one week break followed again by two weeks of treatment on male nine-week-old SHR and WKY. Blood pressure was recorded during all along the experiment. Rats were randomly assigned to different experimental group as follows:

- Control group (6 WKY rats)
- SHR control group (5 SHR treated with 3% EtOH)
- 6 groups of 4 SHR rats treated with hydro-alcoholic 70% grape pomace extracts (21 mg/kg/day) dissolved in 3% EtOH:

*SHR1: **GRE1** (EA70) seed pomace extract
*SHR2: **SYR1** (EA70) seed pomace extract
*SHR3: **SYR2** (EA70) skin pomace extract
*SHR4: **CAR** (EA70) seed pomace extract
*SHR5: **MOU** (EA70) skin pomace extract
*SHR6: **ALI** (EA70) skin pomace extract

As expected, the growth of WKYs (3.3 ± 0.1 g/day) was higher than that of SHR rats (2.5 ± 0.2 g/day) without a significant influence of grape pomace extracts on weight gain. The mean systolic blood pressure (SBP) of SHR rats was ranged from 150 mm Hg at the beginning of the experiment to 190 mm Hg after five weeks (Figure 36). (Poly)phenolic extracts given to SHR rats had little effect on SBP which increased gradually (except for the SHR1 after 2 weeks of treatment) (Figure 37).

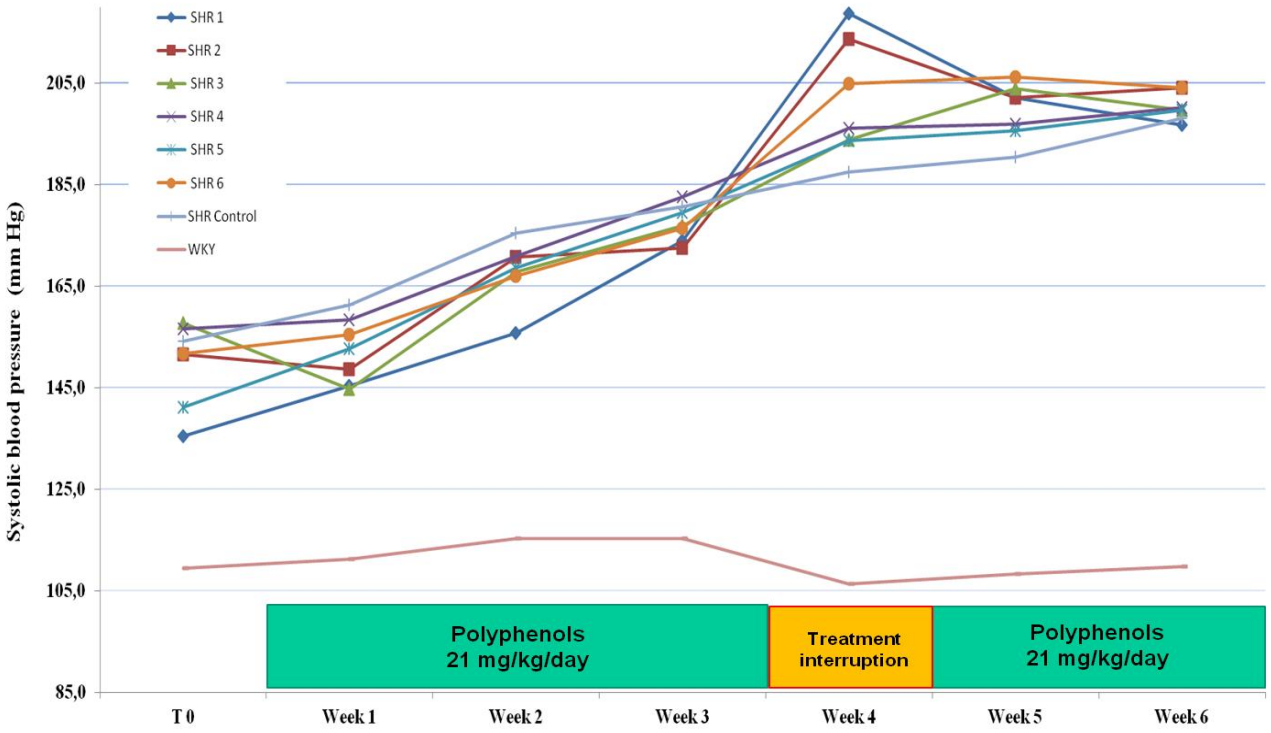


Figure 36: Effect of (poly)phenolic extracts on the mean systolic blood pressure (mm Hg) during the 6-weeks study. Results correspond to the mean of triplicate measurements ± standard deviation.

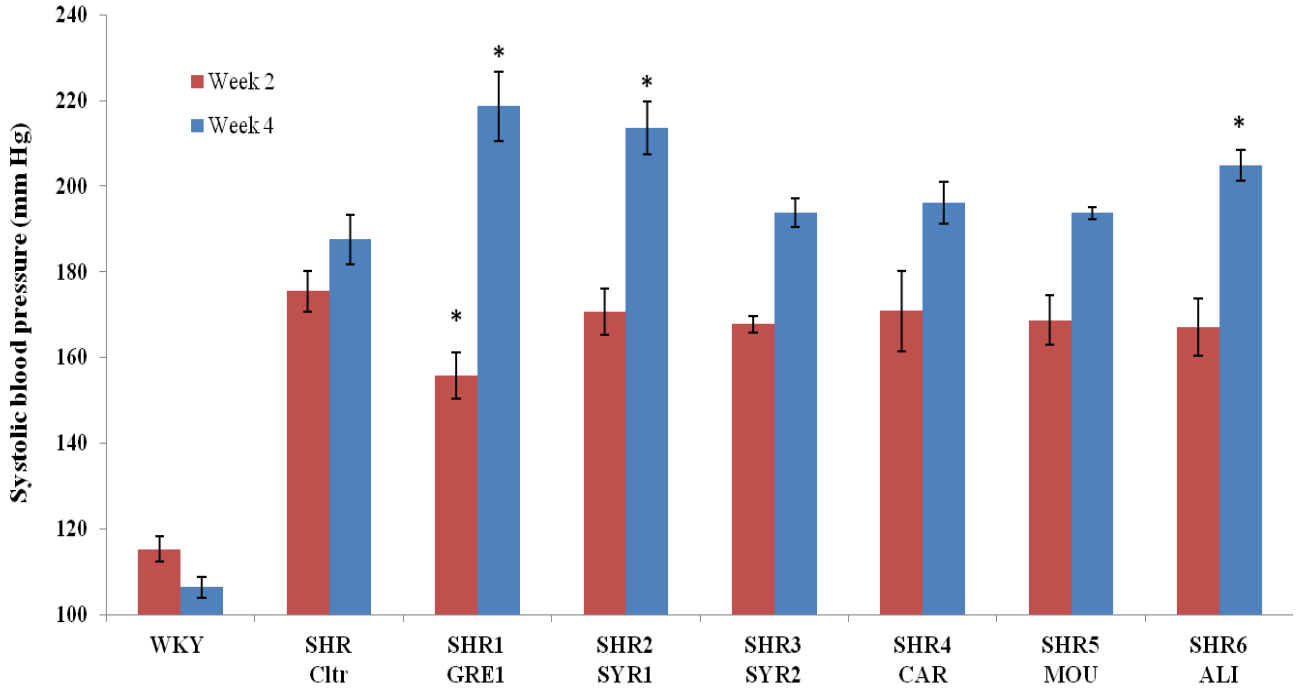


Figure 37: Mean systolic blood pressure (mm Hg) measured at week 2 (after 14 days of treatment with pomace extracts) and at week 4 (after 7 days of a therapeutic break). Results correspond to the mean of triplicate measurements ± Std. *p<0.05).

However, after three weeks, gavage intolerance was observed and caused difficulties to the extracts administration of the extracts, with the necessity to interrupt the treatment for one week (Figure 36). This interruption was followed by an increase of the SBP in SHR1 (Grenache [GRE1] seed pomace extract), SHR2 (Syrah [SYR1] seed pomace extract) and SHR6 (Alicante [ALI] skin pomace extract) group compared to the SHR control group. It is well know that some anti-hypertensive agents that are used to lower arterial pressure may acutely increase arterial pressure and a rebound increase in pressure may be encountered after discontinuation of certain antihypertensive agents (Grossman et al. 1995). Thus, this phenomenon can be interpreted as a "rebound effect" and may reveal an anti-hypertensive effect of grape pomace extracts (Figure 38).

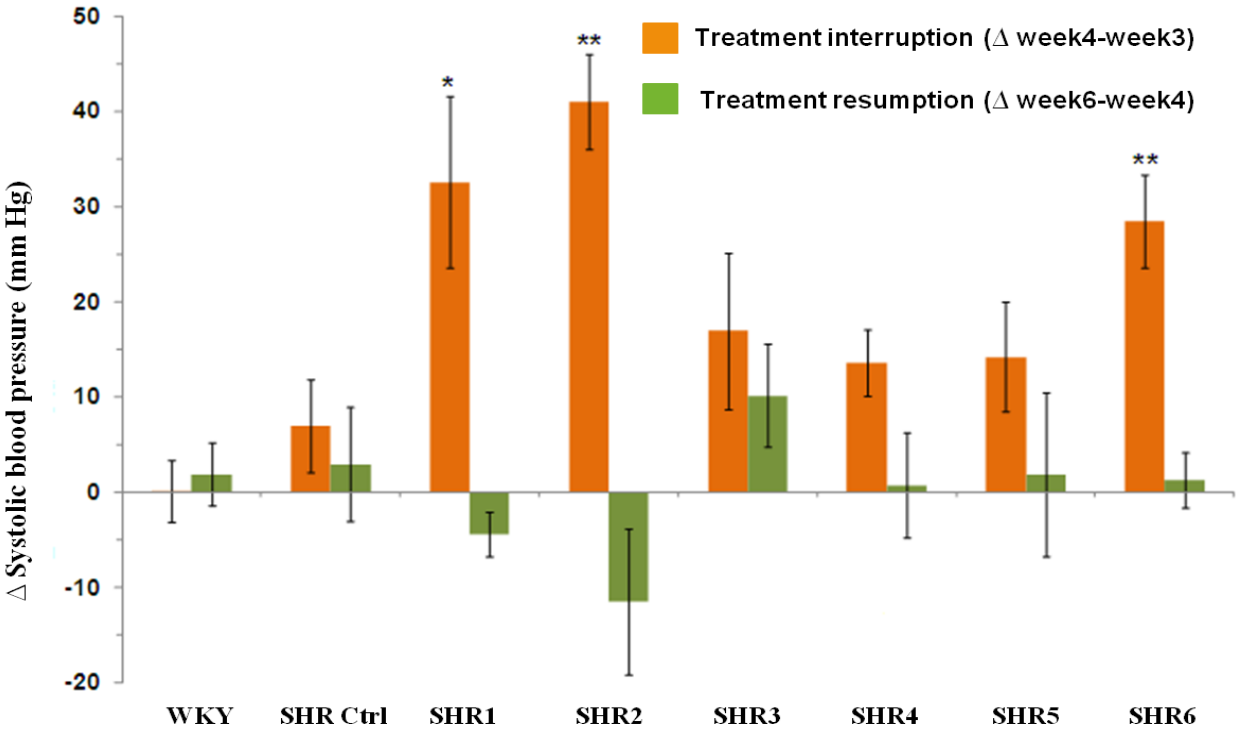


Figure 38: Variations of the mean systolic blood (mm Hg) pressure during interruption and resumption period. Results correspond to the mean of triplicate measurements ± Std. (*p<0.05, **p<0.01).

However, treatment resumption at weeks 5 and 6 was not followed by a decrease of the SBP nor was another "rebound effect" observed during the re-interruption of the treatment during 48 h at week 8 (Figure 39).

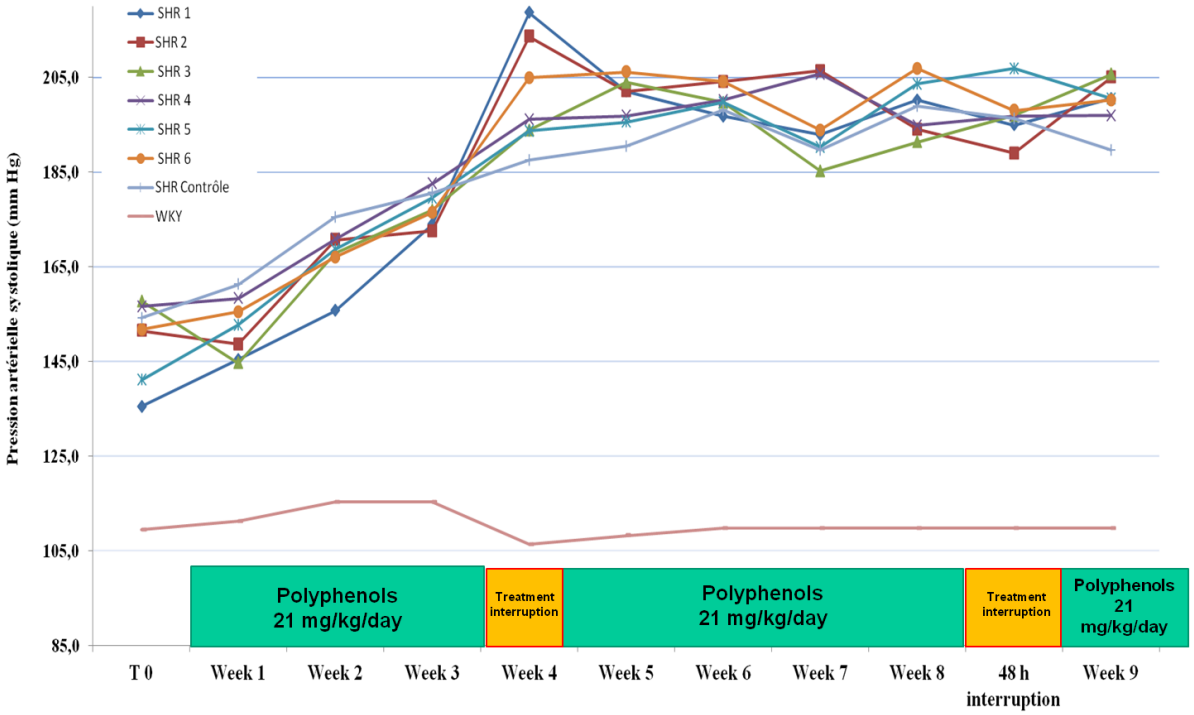


Figure 39: Effect of (poly)phenolic extracts on the mean systolic blood pressure (mm Hg) during the 9-weeks study. Results correspond to the mean of triplicate measurements ± Std.

This first experiment evidenced the ability of Grenache seeds (GRE1), Syrah seed (SYR1) and Alicante skin (ALI) hydro-alcoholic 70% grape pomace extracts to regulate blood pressure which was illustrated by a rebound effect.

II.2. 2nd study: Effect of the verapamil (model validation)

Verapamil or the 5-[*N*-(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (Figure 40) is a calcium channel antagonist with anti-arrhythmic (PR-interval prolongation), angina, and antihypertensive effects (Harder et al. 1993). Verapamil can be found as the (R)-, (S)-verapamil and racemate which contains equal amounts of (R)-verapamil and (S)-verapamil. The enantiomers have different pharmacodynamic properties. (S)-Verapamil is 20-fold more potent than (R)-verapamil for prolongation of the PR interval in healthy volunteers. In contrast, (R)-verapamil causes a decrease in mean blood pressure (Busse et al. 2006). Verapamil is lipophilic and is easily absorbed from the gut. The drug is, however, the subject of extensive first-pass metabolism in the gut wall and the liver and the total oral bioavailability is only 10% to 30% (Echizen et al.

1986). After multiple dosing of controlled-release verapamil, the oral bioavailabilty was 27% for (S)-verapamil and 55% for (R)-verapamil (Busse et al. 2001).

Verapamil is the most extensively characterized and a reference compound of P-gp inhibitor and multi-drug resistance (MDR) reversal agent (Potschka et al. 2001; Potschka et al. 2002). P-glycoprotein (P-gp) was first studied in the context of cancer research where its overexpression in tumor cells has been associated with the multidrug MDR phenotype (Kartner et al. 1983; Gottesman et al. 1993). In cancer cells, P-gp acts as an eflux pump that extrudes chemotherapeutic agents out of the cells and decreasing their intracellular concentration. P-gp is also expressed in normal tissues. High levels of P-gp expression have been observed in the endothelial cells of brain capillaries, in kidney and in adrenal glands while moderate expression has been detected in lung, liver and intestines (Fojo et al. 1987; Cordon-Cardo et al. 1990; Barrand et al. 1995). P-gp is involved in organism detoxification by excreting toxic compounds into the bile, urine and gastrointestinal tract (Schinkel et al. 1994; Trambas et al. 1997). Moreover, it seems to play an important role in brain protection at the blood-brain barrier by preventing the passage of many drugs into the brain (Schinkel 1999; Potschka et al. 2001).

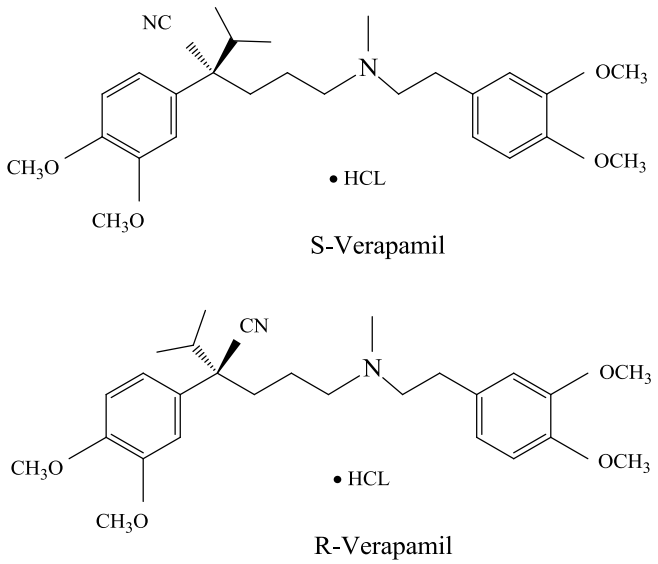


Figure 40: S-verapamil and R-verapamil structures

The effect of verapamil on SHR blood pressure was evaluated using three male nine week-old SHRs and three WKY rats. This experiment was held over twelve weeks. During the first four weeks, only blood pressure was followed. Then, SHR were treated with verapamil at 30, 20 and 40 mg/kg/day during 8 weeks. Throughout this time, blood pressure was monitored.

WKY blood pressure remained stable between 100 mm Hg and 110 mm Hg throughout the experiment. At the beginning of the experiment, without any drug administration, SHR blood pressure gradually increased from 158 mm Hg to 194 mm Hg. At day 28, treatment with 30 mg/kg/day of verapamil caused a sudden drop of SBP to 120 mm Hg followed by a progressive blood pressure rise to 183 mm Hg within two weeks and reached 217 mm Hg after five weeks of treatment with 20 mg/kg/day of verapamil. An increase dose of verapamil at 40 mg/kg/day caused a drop of blood pressure to 180 mm Hg followed again by an increase to 200 mm Hg in 10 days (Figure 41).

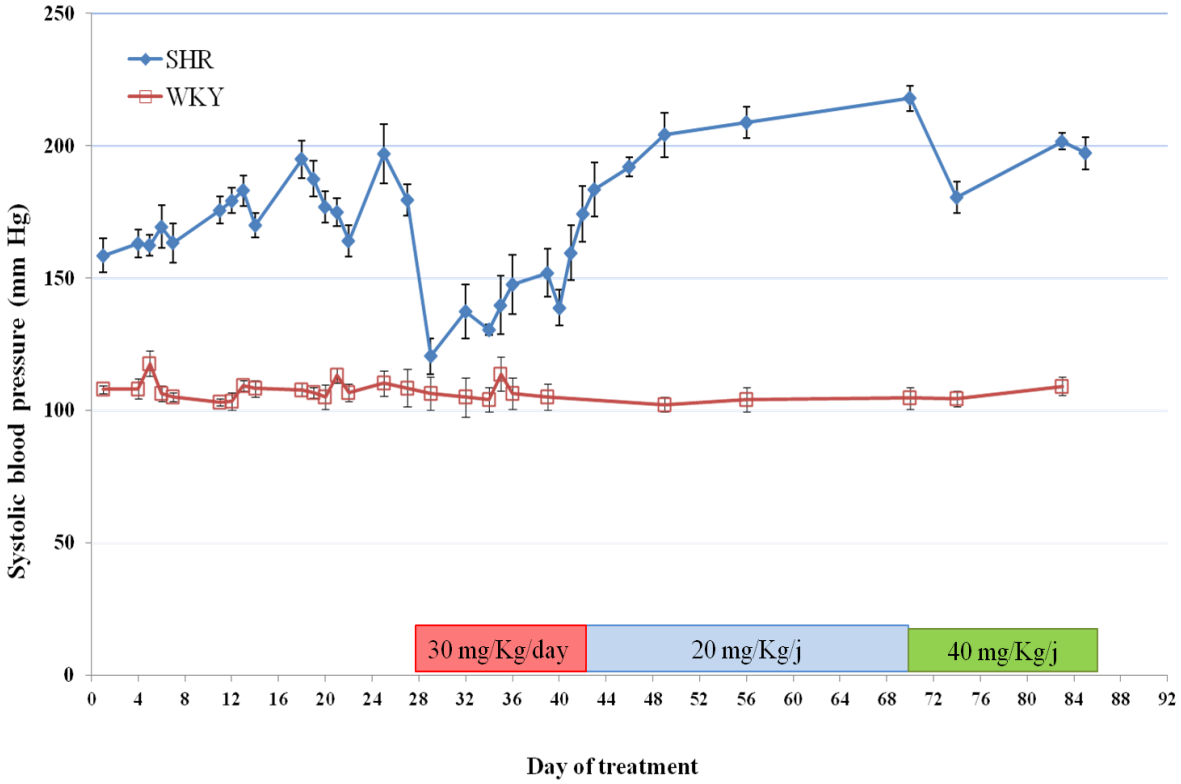


Figure 41: Effect of different concentration of verapamil on SHR and WKY on the mean systolic blood pressure (mm Hg). Symbols correspond to the mean of triplicate ± standard deviation.

Despite the administration of a higher dose of verapamil at 40 mg/kg/day, SBP remained high and fluctuated around 200 mm Hg. These results indicate that over time, SHR rats develop resistance to anti-hypertensive drugs including the calcium channel blocker drug, verapamil.

II.3. 3rd study: Effect of grape pomace extracts associated with verapamil

It is well known that anti-hypertensive drugs including diuretics, anti-adrenergics, calcium channel blockers or even those affecting the Renin–angiotensin-aldosterone system (RAAS) are not effective in every patients and a combination of drugs is often needed to treat high blood pressure. The previous study demonstrated that SHR rats develop a resistance to verapamil over time and considering the anti-hypertensive potential of grape pomace extracts, the combined intake of grape pomace extracts rich in (poly)phenols with verapamil might have beneficial effect on blood pressure regulation by amplifying (poly)phenols or verapamil action. In order to evaluate the potential of grape pomace extracts in association with verapamil, study was carried out in three steps.

1/ « Grape pomace extract + verapamil » model development

The first step consisted of the development of "grape pomace extract + verapamil" model using extract GRE1 (EA70) seed pomace extract. Three experimental groups were established and experiment lasted during three weeks:

- Group SHR control (3 SHR treated with 3% EtOH),
- Group SHR verapamil control (3 SHR treated with verapamil at 40 mg/kg/day),
- Group verapamil + extract GRE1 (EA70) seed pomace extract (3 SHR treated with 21 mg/kg/day of extract + 40mg/kg/day of verapamil).

Hydro-alcoholic 70% extract of GRE1 seed grape pomace was chosen because of its highest ability to regulate blood pressure evidenced in the 1st study. Following the treatment, a gradual decrease of blood pressure was observed and the minimum SBP was reached after 3 weeks compared to SHR control and WKY group (Figure 42 and Table 47). The association of GRE1 (EA70) seed pomace extract + verapamil showed its efficacy to lower blood pressure, thus, the model is validated.

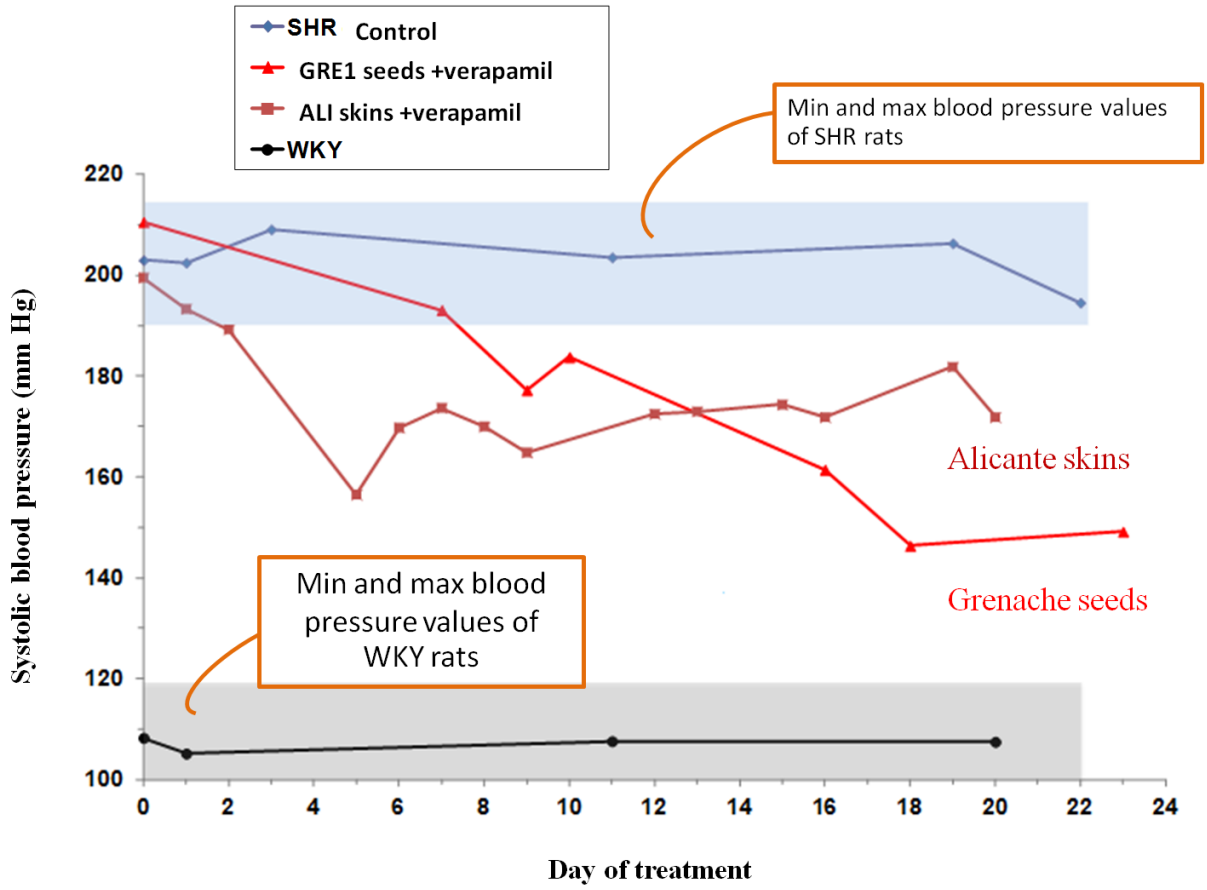


Figure 42: Effect of GRE1 (EA70) seed pomace extract and ALI (EA70) skin pomace extract (21 mg/kg/day) in association with verapamil (40 mg/kg/day) on blood pressure in SHR rats.

The shades area correspond to maximum deviation between maximum and minimum values of SHR (blue) and WKY (grey) individual SBP measurements along the experiment.

2/ Comparison of grape pomace extracts efficiency in association with verapamil.

Following the "grape pomace extract + verapamil" model validation, the other grape pomace extracts in association with verapamil were also tested. Rats were randomly assigned to different experimental group organized as follows and blood pressure was recorded all along the three weeks experiment:

- Control group (3 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)
- SHR verapamil control group (3 SHR treated with verapamil at 40 mg/kg/day)
- 6 groups of 3 SHR rats treated with hydro-alcoholic 70% grape pomace extracts dissolved in 3% EtOH (21 mg/kg/day) associated with verapamil (40 mg/kg/day):

*SHR1+V: **GRE1** (EA70) seed pomace extract + verapamil
*SHR2+V: **SYR1** (EA70) seed pomace extract + verapamil
*SHR3+V: **SYR2** (EA70) skin pomace extract + verapamil
*SHR4+V: **CAR** (EA70) seed pomace extract + verapamil
*SHR5+V: **MOU** (EA70) skin pomace extract + verapamil
*SHR6+V: **ALI** (EA70) skin pomace extract + verapamil

Results are presented in Table 47 and evidenced an anti-hypertensive effect of ALI (EA70) skin extract without any effect from the other extracts. According to this comparative study, ALI (EA70) skin pomace extract, which already showed its anti-hypertensive ability in the 1st screening study, was chosen to confirm the preliminary results obtained with GRE1 (EA70) seed extracts (Table 47).

Table 47: The impact over time of "grape pomace extracts + verapamil" on mean systolic blood pressure

Groups	6 days	13 days	20-23 days
WKY	108,5 ± 1,2 (102-121)	108, 5 ± 1,2 (102-121)	108,5 ± 1,2 (102-121)
SHR	200,3 ± 1,8 (190-214)	200,3 ± 1,8 (190-214)	200,3 ± 1,8 (190-214)
SHR+ V[a]	198,0 ± 1,5 (195-202)	198,0 ± 1,5 (195-202)	198,0 ± 1,5 (195-202)
SHR1+V[a]	193,1 ± 3,2 **(183, 189, 197)**	161,4 ± 10,5 **(144, 157, 183)**	146,3 ± 5,9 **(138, 143, 158)**
SHR2+V[a]	191,7 ± 3,7 **(188, 195)**	192,5 ± 0,8 **(192, 193)**	
SHR3+V[a]	188,7 ± 2,7 **(186, 191)**	192 ± 1 ,0 **(191, 193)**	
SHR4+V[a]	187,2 ± 5,8 **(181, 193)**	189,5 ± 5,5 **(184, 195)**	
SHR5+V[a]	186,3 ± 0,0 **(186,2, 186,4**	185,3 ± 5,7 **(183, 194)**	
SHR6+V[a]	168,8 ± 10,6 **(144,182 124,191, 206, 167, 173)**	173,0 ± 24,7 **(148, 197)**	171,8 ± 18,8 **(153, 190)**
SHR6[a]	200,9 ± 2,8 (195-204)		

[a]V, Verapamil; SHR1 + V to SHR6 + V, experimental group fed with GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract, SYR2 (EA70) skin pomace extract, CAR (EA70) seed pomace extract, MOU (EA70) skin pomace extract, ALI (EA70) skin pomace extract in association with verapamil respectively; SHR6, experimental group fed with ALI (EA70) skin. Number in parentheses indicated individual SBP values measured during the "grape pomace extract + verapamil" model validation (in green), during the comparative study (in red) and during the result confirmation (in blue). Number in parentheses without any colour corresponded to the minimum and maximum SBP values along the experiment.

3/ Results confirmation

The extract ALI (EA70) was chosen to confirm results obtained with GRE1 (EA70) seed pomace extracts. The number of SHR rats fed with ALI (EA70) skin pomace extract (21 mg/kg) in association with verapamil (40 mg/kg) was increased to seven rats. The results obtained showed a gradual decrease of blood pressure after 3 weeks of treatment (Table 47 and Figure 42). It should to be noted that, as in human species (Hanes et al. 1996), individual responses of SHR rats is variable from one animal to another with the existence of highly receptive and non-receptive rats. In this experiment, one of the SHR rats had normal blood pressure which is exceptional for the SHR rat model. The experiment confirmed the efficiency of ALI (EA70) skin pomace extract to lower blood pressure in agreement with the first screening experiment in which ALI (EA70) skin pomace extract was administrated alone (Figure 7). Thus, GRE1 (EA70) seed and ALI (EA70) skin grape pomace extracts in association with verapamil proved to be efficient in lowering blood pressure.

II.4. Supplementary experiments of grape pomace extracts associated with verapamil

II.4.1. Verapamil co-ingested with SYR1 seed (EAQ), SYR1 skin (EAQ), ALI skin (EAQ) and GRE2 skin (EA70) screening

As previously demonstrated, certain grape pomace extracts in association with verapamil have an ability to lower blood pressure. Supplemental extracts associated with verapamil were then tested for their anti-hypertensive ability. In the first experiment, 18 male nine week-old spontaneously hypertensive rats (SHR) and 6 male normotensive control Wistar-Kyoto (WKY) rats were used. This experiment was held over 9 weeks including 7 weeks of treatment with a gradual increase of verapamil dose from 10 mg/kg/day to 40 mg/kg/day and 2 week treatment with grape pomace extracts in association with verapamil. Rats were randomly assigned to different experimental group organized as follows:

- Control group (6 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)
-SHR verapamil control group (3 SHR treated with verapamil at 40mg/kg/day)
- 4 groups of 3 SHR rats treated with aqueous and hydro-alcoholic 70% grape pomace extracts (21 mg/kg/day) dissolved in 3% EtOH in association with verapamil (40 mg/kg/day):

*SHR1+V: **SYR1** (EAQ) seed pomace extract + verapamil
*SHR2+V: **SYR1** (EAQ) skin pomace extract + verapamil

*SHR3+V: **ALI** (EAQ) skin pomace extract + verapamil
*SHR4+V: **GRE2** (EA70) skin pomace extract + verapamil

WKY blood pressure remained stable between 100 and 110 mm Hg. No significant differences were founded between SHR control group and SHR administrated with verapamil even with a dose of 40 mg/kg. Administration of a gradual dose of verapamil from 10 mg/kg/day to 40 mg/kg/day during 7 weeks led to SHR resistance and SBP was stabilized around 170 mm Hg (Figure 43).

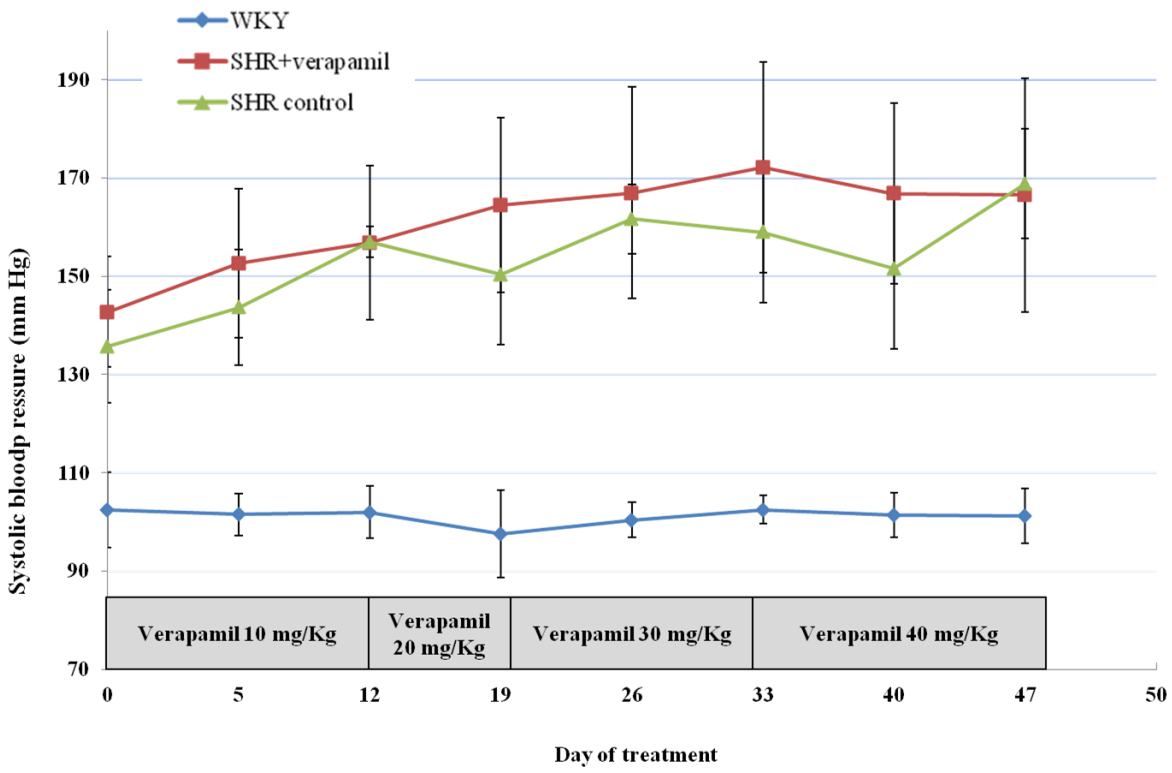


Figure 43: Mean systolic blood pressure evolution in SHR and WKY and effect of verapamil. Results correspond to the mean of triplicate measurements ± standard deviation.

After seven weeks of administration of verapamil, different grape pomace extracts associated to verapamil were administrated to SHRs. After 8 days of administration, lower SBP was observed (Figure 44). The percentage of SBP variation after 8 days of grape pomace associated with verapamil treatment reached and varied from 4% in SHRs fed with ALI (EAQ) skin grape pomace extract to 14% in SHRs fed with SYR1 (EAQ) seed grape pomace extract (Figure 45). However, only SYR1 (EAQ) seed grape pomace extract showed a significant anti-hypertensive effect (Figures 44 and 45).

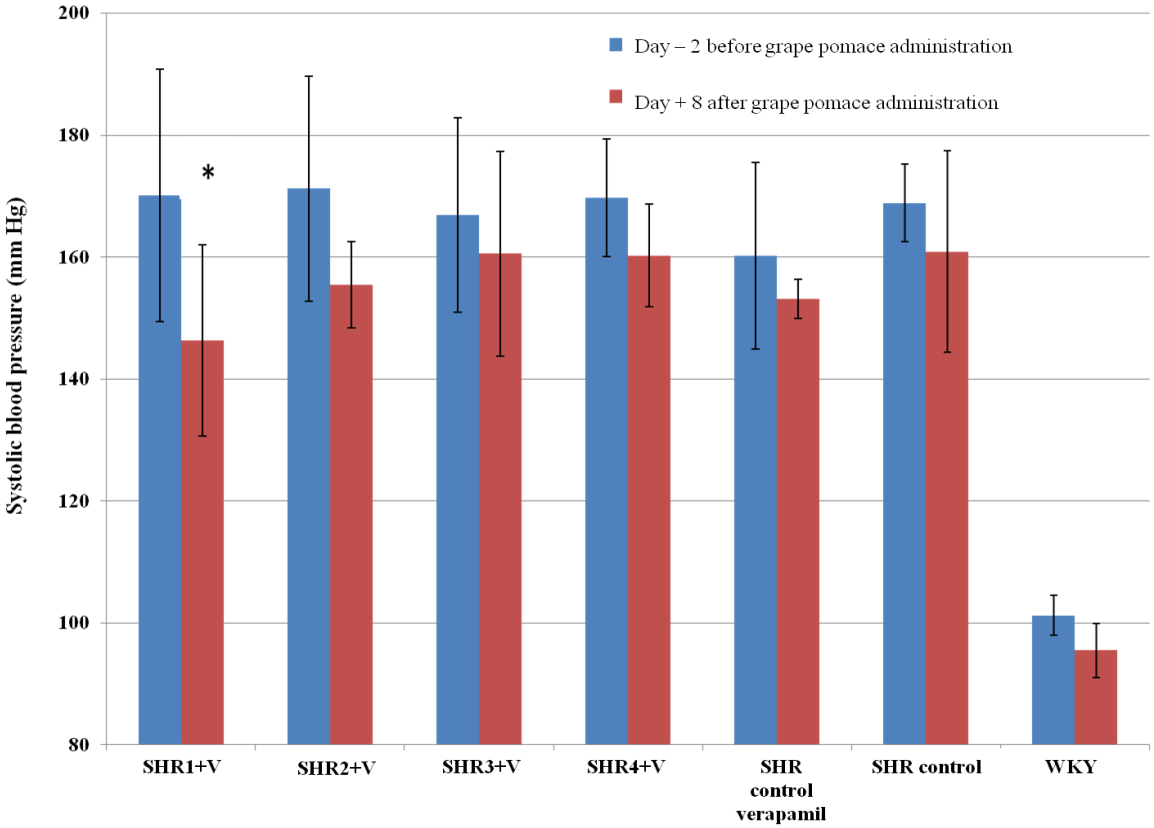


Figure 44: Mean systolic blood pressure (mm Hg) measured 2 days before and 8 days after grape pomace ex tract associated with verapamil administration.* Paired Student's t-test, (*p = 0.06)*.

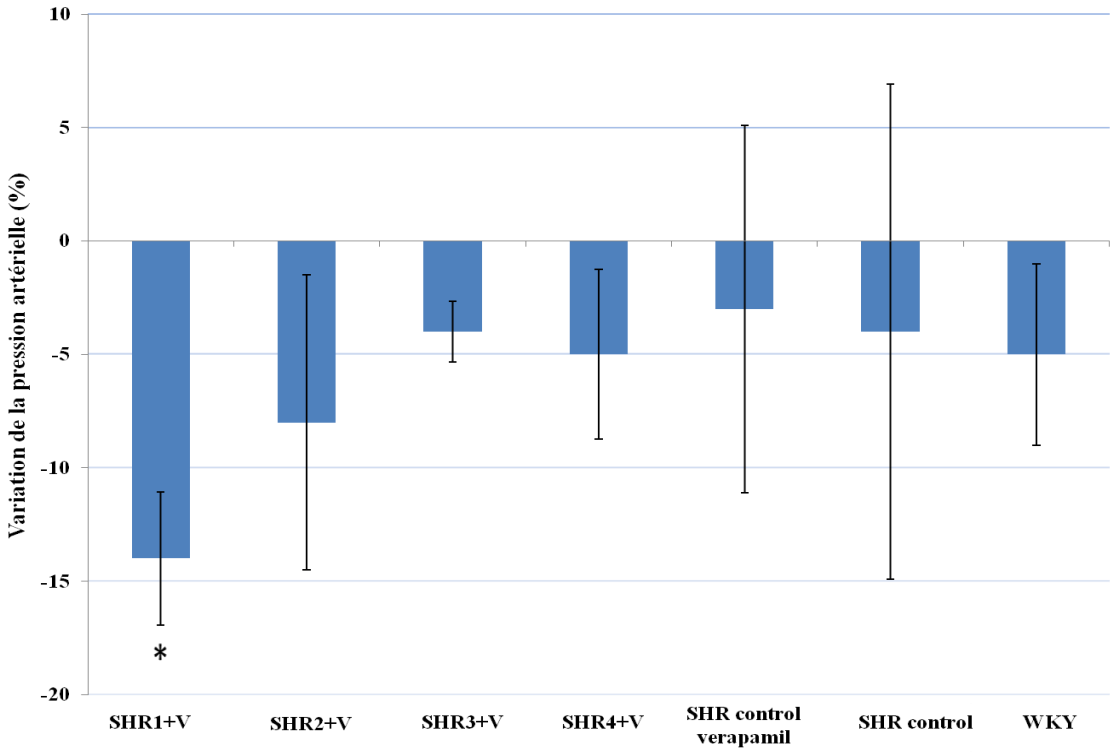


Figure 45: Mean systolic blood pressure variation (%) after 8 days of grape pomace extract associated with verapamil administration. * Significant decrease compared to blood pressure measurement before grape pomace extract treatment (*p < 0.05*).

This experiment again indicated that verapamil was not effective in treating high blood pressure in SHR rats. Among the extract tests, only SYR1 (EAQ) seed pomace extract was shown to have an anti-hypertensive capacity. In addition, previous screening study has demonstrated by a rebound effect that SYR1 (EA70) seed pomace extract administrated alone also exerted an anti-hypertensive effect.

II.4.2. Verapamil co-ingested with CAR seed (EAQ), GRE2 skin (EAQ), SYR2 skin (EAQ) and SYR1 skin (EA70) screening

For this complementary experiment, 18 male twenty-three week-old SHR and six male WKY rats were used to determine the *in vivo* effect of additional grape pomace extracts and their potential effect on hypertension. This experiment was held over 6 weeks including 15 days without treatment, 13 days of treatment with verapamil only, 7 days of grape pomace extracts in association with verapamil treatment followed again by 4 days of treatment with verapamil only. Rats were randomly assigned to different experimental group organized as follows:

- Control group (6 WKY rats)
- SHR control group (3 SHR treated with 3% EtOH)
- SHR verapamil control group (3 SHR treated with verapamil at 40 mg/kg/day)
- 4 groups of 3 SHR rats treated with aqueous and hydro-alcoholic 70% grape pomace extracts (21 mg/kg/day) dissolved in 3% EtOH in association with verapamil (40 mg/kg/day):

*SHR1+V: **CAR** (EAQ) seed pomace extract + verapamil
*SHR2+V: **GRE2** (EAQ) skin pomace extract + verapamil
*SHR3+V: **SYR2** (EAQ) skin pomace extract + verapamil
*SHR4+V: **SYR1** (EA70) skin pomace extract + verapamil

SBP of normotensive WKY rats fluctuated between 125 and 140 mm Hg all along the experiment. During the first 15 days in which any rats received any treatments, SHR rats SBP was around 200 mm Hg. At day 15, verapamil at 40 g/kg/day was administrated to SHR (SHR1+V-SHR4+V) rats and SHR verapamil control. A rapid and significant decrease of SBP down to 175 mm Hg was observed after 3 days. However, this fall was transient as blood pressure increased and reached a similar SBP to that of Sthe HR control (Figure 46).

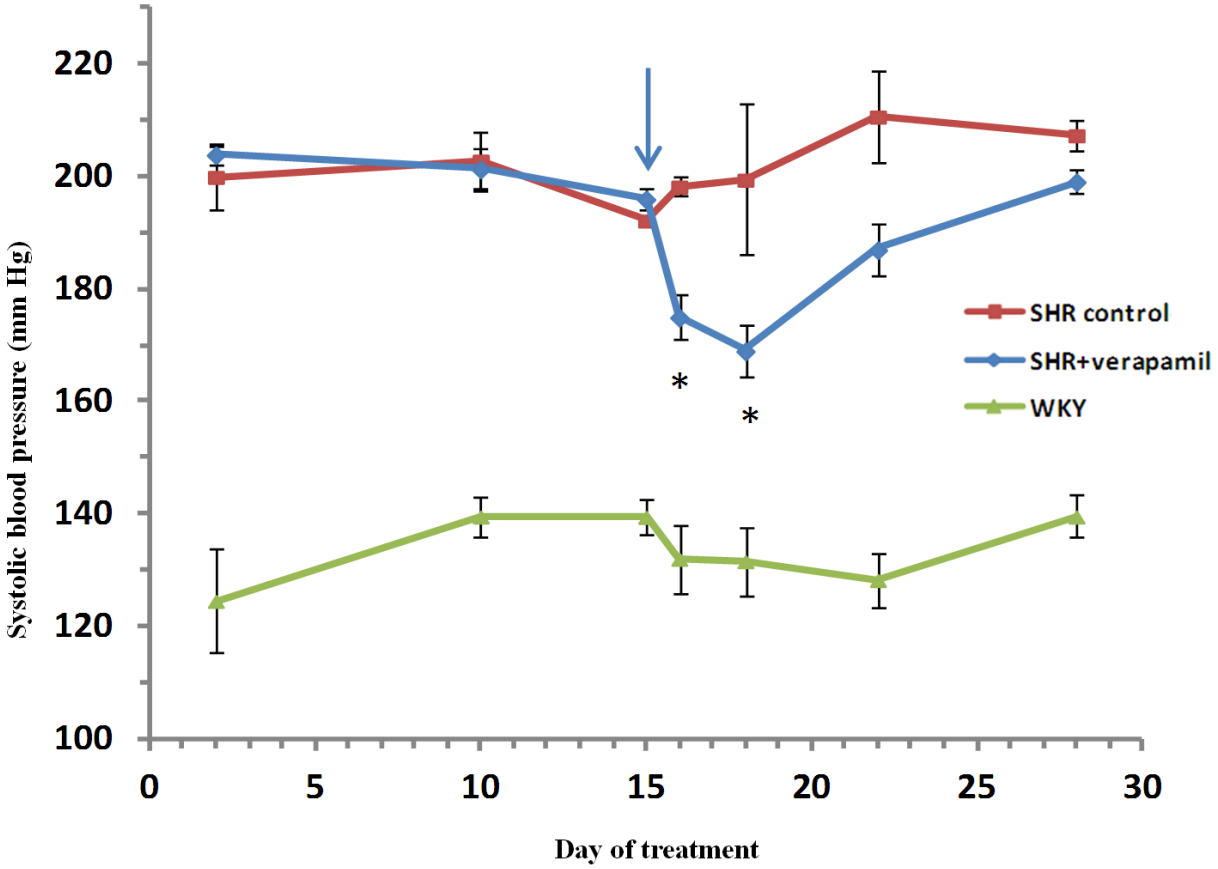


Figure 46: Mean systolic blood pressure (mmHg) evolution in SHR and WKY and effect of verapamil. The arrow indicated the beginning of verapamil treatment. Results correspond to the mean of triplicate measurements ± standard deviation. * Significant decrease compared to the SHR control group ($p < 0.05$).

The administration of different grape pomace extracts began once the SBP of SHR rats which were treated with verapamil reached the same values as those in SHR control (i.e. 200 mm Hg). Figure 47 showed the mean SBP at day 28 (the first day of treatment with grape pomace extracts) and at day 35 (7 days after). A decrease of SBP on the borderline of statistical significance was observed in SHR rats fed with SYR2 (EAQ) skin pomace extract associated with verapamil with a $p = 0.056$ (difference between SBP measured at day 28 and 35 using Paired Student's t-test). Expressing results as percentage of SBP variation, the mean variation (%) for SYR2 (EAQ) skin pomace extract associated with verapamil was significantly different from 0 indicating a significant decrease of SBP ($p < 0.05$) (Figure 48). However, SYR1 (EA70) skin pomace extract induced a significant elevation of SBP once associated with verapamil (Figures 47 and 48).

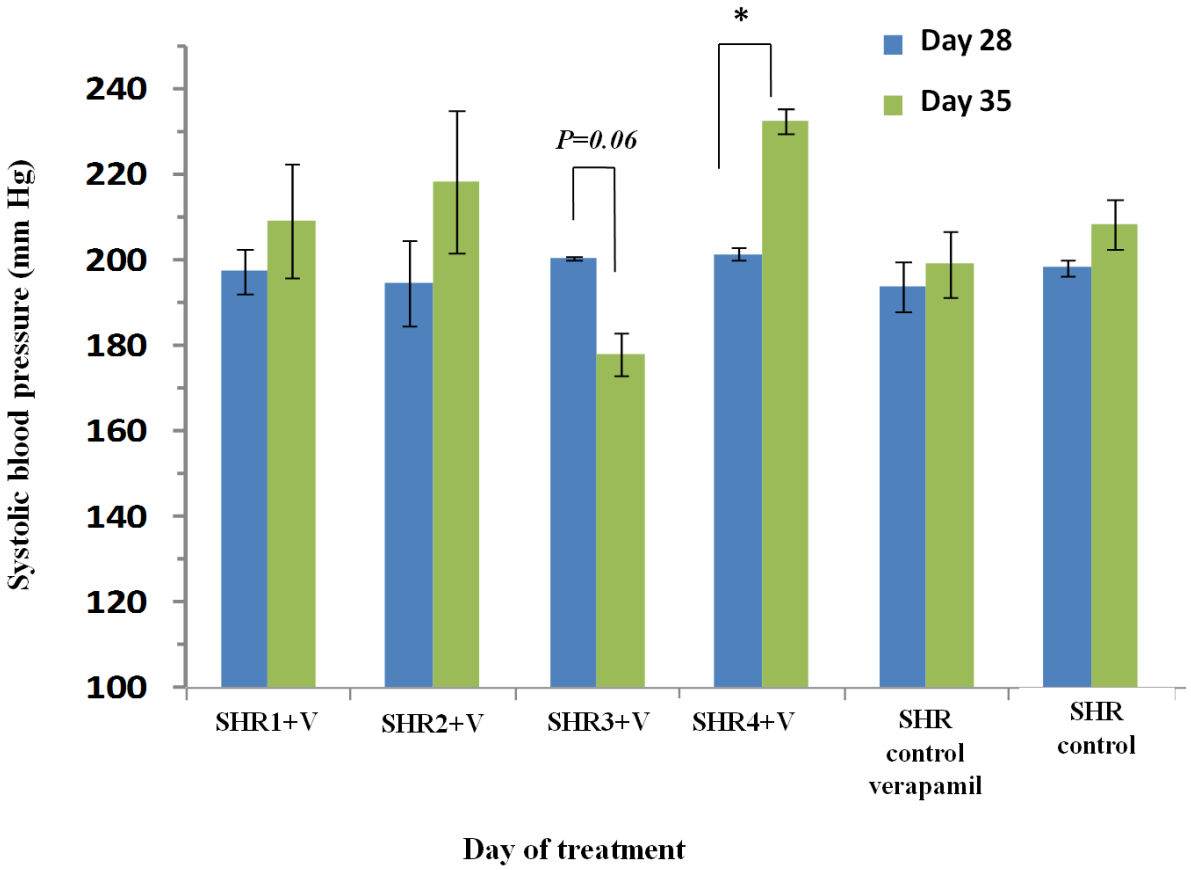


Figure 47: Mean systolic blood pressure before (day 28) and after (day 35) grape pomace extracts associated with verapamil treatment. Results correspond to the mean of triplicate measurements ± standard deviation. * $p < 0.05$.

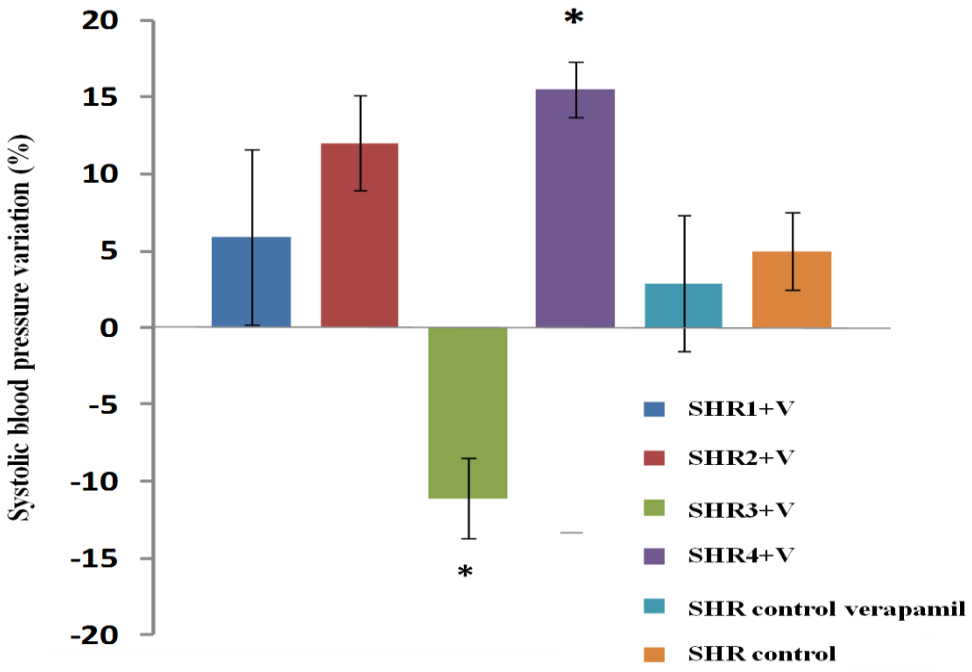


Figure 48: Variation of mean systolic blood pressure (%) between day 35 and day 28 of grape of grape pomace extracts associated with verapamil. * Significant decrease or increase of blood pressure after 7 days of administration ($p < 0.05$).

After being treated for 7 days with grape pomace extract in association with verapamil, at day 35, SHR1+V to SHR4+V did not receive any grape pomace extract but were still administrated with verapamil at 40 mg/kg/day. SBP was measured four days later. In the SHR1+V, SHR2+V and SHR4+V group, SBP did not vary and remained stable at around 200 mm Hg. Nevertheless, in SHR3+V group which was observed previously as having anti-hypertensive ability when SYR2 (EAQ) skin pomace extract was associated with verapamil (SBP = 178 ± 5 mm Hg at day 35), SBP increased significantly after the treatment with grape pomace extract was interrupted (SBP = 202 ± 11 mm Hg at day 39) (Figure 49).

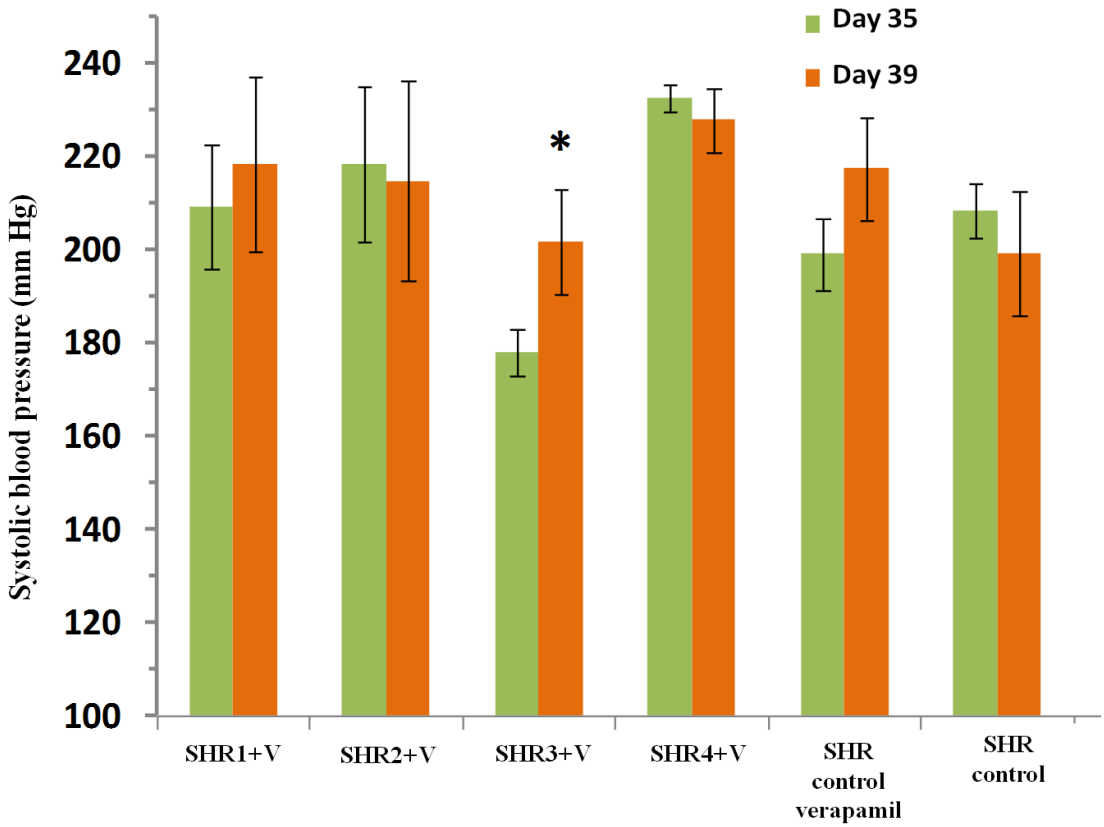


Figure 49: Mean systolic blood pressure at day 39, after grape pomace extracts administration interruption. * Significant increase of blood pressure between day 35 and day 39 ($p < 0.05$).

This experiment confirmed the resistance of SHR rat to verapamil treatment. However, once verapamil was associated with SYR2 (EAQ) skin pomace extract, an anti-hypertensive effect was apparent. This observation was confirmed by an increase of blood pressure after the interruption of SYR2 (EAQ) skin grape pomace extract while SHR rats were still under verapamil treatment. Nevertheless, SYR1 (EA70) skin pomace extract

induced an elevation of SBP. These results demonstrated the possibility using grape pomace polyphenols to modulate drug activity on blood pressure.

II.5. Conclusions

The experiments demonstrated that some grape pomace extracts administrated alone or in association with verapamil possessed an anti-hypertensive potential. This ability was evidenced with GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract, ALI (EA70) skin pomace extract administrated alone; and with GRE1 (EA70) seed pomace extract, SYR1 (EAQ) seed pomace extract, ALI (EA70) skin pomace extract and SYR2 (EAQ) skin pomace extract administrated in association with verapamil. In the last *in vivo* experiment, SYR1 (EA70) skin pomace extract with verapamil showed to exert an adverse effect by elevating SBP. Table 48 summarized the anti-hypertensive efficiency of different tested grape pomace extracts.

Table 48: Anti-hypertensive efficiency of different grape pomace extracts

Grape pomace extracts tested	Treatment time	Effect on hypertension	Efficiency
GRE1 seed (EA70)	3 weeks	+	Indirect effect, rebound effect, **+ 19%**
SYR1 seed (EA70)	3 weeks	+	Indirect effect, rebound effect, **+24 %**
SYR 2 skin (EA70)	3 weeks	~	-
CAR seed (EA70)	3 weeks	~	-
MOU skin (EA70)	3 weeks	~	-
ALI skin (EA70)	3 weeks	+	Indirect effect, rebound effect, **+16 %**
GRE1 seed (EA70) + verapamil	3 weeks	+	Direct effect, **-26 %**
SYR1 seed (EA70) +verapamil	3 weeks	~	-
SYR 2 skin (EA70) + verapamil	3 weeks	~	-
CAR seed (EA70) + verapamil	3 weeks	~	-
MOU skin (EA70) +verapamil	3 weeks	~	-
ALI skin (EA70) + verapamil	3 weeks	+	Direct effect, **-13 %**
SYR1 seed (EAQ) + verapamil	2 weeks	+	Direct effect, **-14 %**
SYR1 skin (EAQ) + verapamil	2 weeks	~	-
ALI skin (EAQ) + verapamil	2 weeks	~	-
GRE2 Skin (EA70) + verapamil	2 weeks	~	-
CAR seed (EAQ) + verapamil	1 week	~	-
GRE2 skin (EAQ) + verapamil	1 week	~	-
SYR2 skin (EAQ) + verapamil	1 week	+	Direct effect, **-11 %**
SYR1 skin (EA70) + verapamil	1 week	−	Direct effect, **+15 %**

Grape pomace extracts were administrated at a dose of of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine and verapamil at a dose of 40 mg/kg/day. **+**, positive effect on hypertension regulation; − negative effect on hypertension regulation; **~** no effect. Extract efficiency fed to SHR rats was illustrated by the percentage of variation (%) of blood pressure before and after the ingestion.

Grape pomace extracts ingested without verapamil were shown to exert anti-hypertensive effects indirectly, by increasing blood pressure after a rebound effect. An increase of +19 %, +24 % and +16 % were observed, respectively, for GRE1 seed (EA70), SYR1 seed (EA70) and ALI skin (EA70) after three weeks of treatment. Grape pomace extracts co-ingested with verapamil, lowering blood pressure by -26 % with GRE1 seed (EA70) + verapamil and -13% using ALI skin (EA70) + verapamil after 3 weeks of treatment and by -14 % after 2 weeks of treatment. Moreover, experiments showed that one week of treatment was sufficient to induce anti-hypertensive effect in rats fed with SYR2 skin (EAQ) + verapamil in which an 11 % reduction in blood pressure while a pro-hypertensive effect was observed in rats fed with SYR1 skin (EA70) + verapamil (+ 15 %).

According to the extract used, it is actually possible to modulate anti-hypertensive drug effects. The composition of the grape pomace extracts used in this study might have a crucial role and should be taken into account. Both EAQ and EA70 proved to be effective even though the overall grape pomace characterisation study showed that EA70 extracts were richer in (poly)phenolic compounds than EAQ.

Among seed extracts, CAR seed pomace extract in both EAQ and EA70 contained higher amounts of (poly)phenols compare to GRE1 seed pomace extract, but did not exert any anti-hypertensive effects whereas GRE1 seed pomace extract did. Seed extracts exerting anti-hypertensive effects (i.e. GRE1 [EA70] seed pomace extract, SYR1 [EA70] seed pomace extract and SYR1 [EAQ] seed pomace extract) contained the highest amount of total procyanidins ranging from 56 to 157 mg/g of DW, especially in the Syrah varieties where 137 and 157 mg/g of DW of total procyanidins was found in EAQ and EA70, respectively. In addition, these extracts also contained high amount of gallo- and gallate derivatives and were highly galloylated compared to CAR seed EAQ and EA70 extracts. In addition, grape pomace characterisation also showed seed from Syrah contained the highest amount of monomer and dimer flavan-3-ols in both types of extracts. In fact, Syrah was the only variety which exhibited anti-hypertensive in both EAQ and EA70 extracts. In term of anthocyanins, the highest concentration was found in CAR seed extracts. Actually, GRE1 and SYR1 possessed only 5 mg/g DW and 10.3 mg/g DW in EA70 compared to 25.4 mg/g DW in CAR (EA70). Evaluation of antioxidant activity indicated the highest radical scavenging activity of SYR1 in both types of extracts. *In vivo* anti-hypertensive ability of GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract and SYR1 (EAQ) seed pomace extract is

most likely conferred by the large amount of flavan-3-ols and procyanidins rather than anthocyanins.

Among the nine skin extracts tested, only two exhibited anti-hypertensive effect, the ALI (EA70) skin pomace extract and SYR2 (EAQ) skin pomace extract. In pomace characterisation analysis, ALI skin in both EAQ and EA70 was shown to be one of the extracts which contains high phenolic compounds and high antioxidant capacity. Even if ALI (EA70) has higher amounts of flavan-3-ol monomers and dimers but only 28 mg/g DW of total procyanidins was found compared to its EAQ extract which has 59 mg/g DW but did not show any ability to lower SBP. The extract anti-hypertensive effect is more likely linked to its anthocyanins content. Actually, ALI (EA70) skin contained up to 19.5 mg/g DW, 1.2 mg/g and 7.2 mg/g DW of anthocyanins total glucosides, acetylated and coumaroylated anthocyanins, respectively. According to these observations, it is not surprising that ALI (EA70) skin was not classified as the most antioxidant by every test. However, the high content of (poly)phenol in an extract did not always lead to an *in vivo* effect. Paradoxically, SYR2 (EAQ) skin pomace extract which contained the lowest levels of phenolics for every analysis combined, was able to lower SBP once associated with verapamil while other skin pomace extracts, much richer in (poly)phenolic compounds, did not showed any anti-hypertensive effects and could also increase SBP (i.e. SYR1 (EA70) skin pomace extract). Unlike seed extracts, anti-hypertensive ability of skin extracts is more difficult to predict. Other compounds in these skin extracts such as polysaccharides or fibers might prevent the anti-hypertensive effect by affecting (poly)phenol absorption.

It is well established that the renin–angiotensin system (RAS) has a central role in the regulation of blood pressure and angiotensin-converting enzyme 2 (ACE2), is involved in the regulation of the RAS (Crackower et al. 2002). Compared to normotensive rats, SHR presented an abnormal development on the intra renal RAS (Obata et al. 2000; Tikellis et al. 2006). Studies have shown that the expression and ACE2 activities are significantly increased in the SHR kidney at birth. With the onset of hypertension, the tubular expression of ACE2 falls in SHR compared to WKY and remains reduced in the adult SHR kidney (Tikellis et al. 2006). As previously explain in the “chapter 4-I. State of knowledge: SHR rats”, a deficient in ACE2 induces an increase of Ang II which reduces the production of NO and lead to vasoconstriction phenomenon and vascular dysfunction. Oxidative stress contributes to vascular dysfunction and it is actually caused, on the one hand, by an excess of superoxide anion ($\bullet O_2^-$) production by NADH/NADPH oxidase (Grunfeld et al. 1995; Zalba et al. 2000)

and on the other hand, by a low activity of eNOS which catalyses the production of nitric oxide (Palmer et al. 1987). Actually, $\bullet O_2^-$ and NO are known to rapidly react to form the stable peroxynitrite anion (ONOO-), a powerful oxidant, which will prevent the vasodilatating action of NO (Beckman et al. 1990; Pacher et al. 2007; Vera et al. 2007).

As oxidative stress is involved in the pathogenesis of hypertension, scientists have focused their interest on anti-hypertensive effects of (poly)phenolic compounds which have been confirmed in several studies (Galisteo et al. 2004; Sarr et al. 2006; Perez-Vizcaino et al. 2009) and might be linked to the capacity of (poly)phenols to regulate the eNOS and NADPH oxidase activity. A study by Al Awwadi et al.(2004) showed that a red wine (poly)phenolic extract was able to prevent cardiac hypertrophy and inhibit production of reactive oxygen species in the insulin resistant fructose-fed rat. More precisely, another paper from the same group (Al-Awwadi et al. 2005), showed that anthocyanins and catechin oligomers prevented hypertension, cardiac hypertrophy and production, whereas procyanidins had only minor effects on hypertension. This result illustrated that antioxidant activity is not the only mode of action of (poly)phenolic compounds. These observations are in accordance with our results, in which highest amounts of monomer and dimer flavan-3-ols were found in seed extracts exerting anti-hypertensive effects.

Grape pomace extracts on their own might be used alone but their impact may be enhanced by co-ingestion with verapamil. It is now widely recognized that P-gp influences drug transport across various biological membranes. P-gp transporter is occurs prominently in the luminal surface of enterocytes, biliary canicule surfaces of hepatocytes, apical surface of proximal tubular cells of kidney, endothelial cells of blood brain barrier, etc. and consequently can affect absorption, distribution, metabolism and excretion of xenobiotics. In fact, more than fifty percent of existing anti-cancer drugs undergo P-gp-mediated efflux. Consequently, there is an increasing trend to optimize pharmacokinetics, enhance antitumour activity and reduce systemic toxicity of existing anti-cancer drugs by inhibiting P-gp mediated transport. Although a wide variety of P-gp inhibitors have been discovered, flavonoids form the third generation of non-pharmaceutical category of P-gp inhibitors (Bansal et al. 2009). The effects produced by some of these components are found to be comparable to those of well-known P-gp inhibitors verapamil and cyclosporine.

As verapamil is the reference compound of P-gp inhibitors and multi-drug resistance (MDR) reversal agents, several researchers have demonstrated that (poly)phenols presented

inhibitor properties on P-gp transport affecting the bioavailability and uptake of certain drugs (Jodoin et al. 2002; Kitagawa 2006; Eichhorn et al. 2012). The association of grape pomace extracts rich in (poly)phenols and verapamil might have beneficial effect on blood pressure regulation by modifying the absorption of (poly)phenols and/or verapamil.

III. Absorption, metabolism and excretion of grape pomace extracts: metabolite analysis

Based on previous results, two of the extracts exerting anti-hypertensive effects, the GRE1 (EA70) seed pomace extract and ALI (EA70) skin pomace extract, and one which didn't showed any anti-hypertensive potential, the MOU (EA70) skin pomace extract, were chosen for bioavailability studies. Feeding protocol was detailed in the chapter II: materials and methods. Briefly, male nine week-old SHR rats were used for this experiment and were randomly assigned to different experimental group organized as follows:

- SHR control group (6 SHR treated with 3% EtOH)
- SHR verapamil control group (6 SHR treated with verapamil at 40 mg/kg)
- 3 groups of 6 SHR rats treated with EA70 grape pomace extracts at 21 mg/kg:

  *E1: **GRE1** (EA70) seed pomace extract
  *E5: **MOU** (EA70) skin pomace extract
  *E6: **ALI** (EA70) skin pomace extract

- 3 groups of 6 SHR rats treated with EA70 grape pomace extracts in association with verapamil:

  *VE1: **GRE1** (EA70) seed pomace extract + verapamil
  *VE5: **MOU** (EA70) skin pomace extract + verapamil
  *VE6: **ALI** (EA70) skin pomace extract + verapamil

The experiment procedure and different collection is summarized in Figure 50:

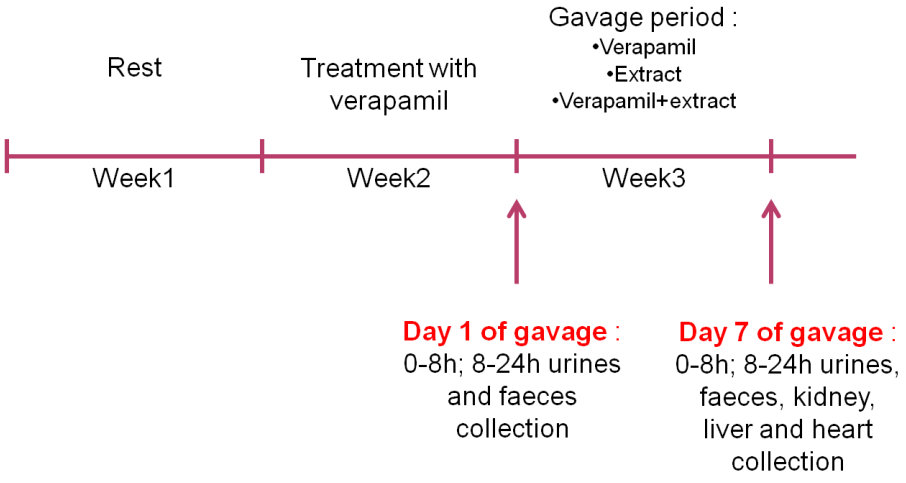


Figure 50: Experimental protocol and different collection periods

Urines and faeces were collected at 0-8 h and 8-24 h at day 1 and day 7 after grape pomace extracts ingestion. Tissues (i.e. heart, liver and kidneys) and plasma were collected 4 h after of gavage at day 7. Grape pomace extracts analysis (chapter III) allowed the evaluation of polyphenols ingested by SHR rats in µmol. A total of 5.20 µmol, 2.4 µmol and 4.1 µmol of polyphenols were ingested by SHR rats fed with GRE1 (EA70) seed pomace extract (E1/VE1), MOU (EA70) skin pomace extract (E5/VE5) and ALI (EA70) skin pomace extract (E6/VE6), respectively (Table 49).

Table 49: Amounts of polyphenols in µmol ingested by SHR rats fed with GRE1 (EA70) seed pomace extract (E1/VE1), MOU (EA70) skin pomace extract (E5/VE5), ALI (EA70) skin pomace extract (E6/VE6). Data are expressed as nmol ± standard deviation.

	Ingested amount (nmol)					
	E1/VE1		E5/VE5		E6/VE6	
	Mean	SD	Mean	SD	Mean	SD
Anthocyanins						
Delphinidin-3-*O*-glucoside	12.7	0.1	141.5	2.9	75.5	2.3
Cyanidin-3-*O*-glucoside	6.4	0.1	32.2	0.7	17.8	0.5
Petunidin-3-*O*-glucoside	21.9	0.2	197.2	4.1	114.2	3.5
Peonidin-3-*O*-glucoside	38.3	0.4	120.5	2.5	380.6	11.5
Malvidin-3-*O*-glucoside	151.5	1.5	597.8	12.3	751.1	22.8
Malvidin-3-*O*-glucoside-acetaldehyde (vitisin B)	0.5	0.0	2.2	0.0	1.8	0.1
Delphinidin-3-*O*-(6″-*O*-acetyl) glucoside	0.0	0.0	2.5	0.1	3.2	0.1
Dimer malvidin-catechin	1.5	0.0	3.3	0.1	2.3	0.1
Malvidin-3-*O*-glucoside-pyruvate (vitisin A)	6.7	0.1	16.0	0.3	11.7	0.4
Dimer malvidin-catechin	0.0	0.0	2.6	0.1	5.5	0.2
Dimer malvidin-catechin	3.8	0.0	2.7	0.1	9.5	0.3
Peonidin-3-*O*-(6″-*O*-acetyl) glucoside	4.6	0.0	10.4	0.2	33.8	1.0
Malvidin-3-*O*-(6″-*O*-acetyl) glucoside	3.3	0.0	20.4	0.4	38.0	1.2
Delphinidin-3-*O*-(6″-*O*-coumaroyl) glucoside	2.5	0.0	13.6	0.3	14.6	0.4
Malvidin-3-*O*-(6″-*O*-caffeoyl) glucoside	3.9	0.0	13.5	0.3	12.7	0.4
Cyanidin-3-*O*-(6″-*O*-coumaroyl) glucoside	4.3	0.0	24.5	0.5	8.0	0.2
Petunidin-3-*O*-(6″-*O*-coumaroyl) glucoside	3.7	0.0	28.9	0.6	28.9	0.9
Malvidin-3-*O*-(6″-*O*-coumaroyl) glucoside	39.8	0.4	141.8	2.9	323.7	9.8
Total anthocyanin glucosides	230.9	2.4	1089.2	22.4	1339.2	40.6
Total anthocyanins acetylated	7.9	0.1	33.3	0.7	75.0	2.3
Total anthocyanins coumaroylated	50.2	0.5	208.9	4.3	375.3	11.4
Total anthocyanins	**305.3**	**3.1**	**1371.7**	**28.2**	**1833.0**	**55.6**
Procyanidins						
Monomers	3553.6	36.2	365.0	7.5	1448.6	43.9
(Epi)catechin-*O*-gallate	72.1	0.7	1.5	0.0	33.2	1.0
Dimers	581.7	5.9	91.6	1.9	258.0	7.8
Dimers gallate	14.3	0.1	6.5	0.1	19.8	0.6
Trimers	151.9	1.5	75.3	1.5	172.7	5.2
(Epi)gallocatechin trimers	22.6	0.2	2.3	0.0	3.1	0.1
Trimers gallate	9.3	0.1	1.6	0.0	10.2	0.3
Tetramers	54.2	0.6	27.5	0.6	56.3	1.7
(Epi)gallocatechin tetramers	26.3	0.3	8.0	0.2	18.9	0.6
Tetramers gallate	0.0	0.0	0.0	0.0	1.3	0.0
Pentamers	28.5	0.3	16.3	0.3	28.0	0.8
(Epi)gallocatechin pentamers	7.0	0.1	3.5	0.1	3.7	0.1
Hexamers	16.8	0.2	7.6	0.2	13.1	0.4
Heptamers	8.0	0.1	4.1	0.1	4.6	0.1
Octamers	3.8	0.0	1.9	0.0	2.4	0.1
nonamers	1.6	0.0	0.7	0.0	1.0	0.0
Decamers	1.0	0.0	0.3	0.0	0.4	0.0
Total procyanidins	**4552.8**	**46.4**	**613.7**	**12.6**	**2075.3**	**62.9**
Quercetin-3-*O*-glucuronide	62.6	0.6	102.1	2.1	49.7	1.5
Resveratrol	23.7	0.2	18.9	0.4	25.8	0.8
Quercetin	244.7	2.5	251.0	5.2	111.1	3.4
Total	**5189.1**	**52.9**	**2357.3**	**48.5**	**4094.8**	**124.1**

III.1. HPLC-ESI-MS^n analysis of urines, tissues, plasma and faeces

In this study, reversed-phase HPLC with multistage mass detection (i.e. MS^n) without any pre-treatment of samples with enzymatic preparations was used to identified and quantified metabolites in body fluid, tissues and faeces. The use of glucuronidase/sulfatase preparations for urine/plasma analysis converts metabolites to the native aglycones and provides indirect information together with varying and unmeasured error factors. In the first instance, urine was screened for metabolites using full-scan MS. Metabolites which were present in higher concentrations in most of the samples were then selected for quantification. The same procedure was used for tissues and plasma. Concerning faeces, only compounds found in grape pomace extracts were targeted, especially procyanidins and flavan-3-ol dimers and monomers.

III.1.1 HPLC-ESI-MS^n analysis of SHR rat urines

III.1.1.a. Identification of phenolic compounds in SHR rat urines

SHR rat urines were firstly screened for anthocyanins and flavan-3-ol procyanidin metabolites. Because of generally low levels in urine, anthocyanin metabolites were undetectable. Purification and concentration steps were undertaken but the levels detected in SHR rat urine were still very low. Previous studies have shown that unlike other flavonoids that are absorbed and excreted, anthocyanins do not appear to undergo extensive metabolism to glucuronide and sulfate derivatives. Typical recoveries in human urine are < 0.1% of intake (Crozier et al. 2009). These low recoveries could be a consequence of anthocyanins undergoing structural rearrangements in response to pH. The red flavilium cation predominates at pH 1-3, but as the pH increases to 4 and above, the colorless carbinol pseudobase becomes the major component along with smaller amounts of a colorless chalcone pseudobase and a blue quinoidal base (Clifford 2000). Such changes are likely to occur *in vivo* as anthocyanins pass from low pH in the stomach to the more-basic conditions of the small intestine. As a consequence, the study focused on procyanidins and flavan-3-ol oligomers and monomers.

SHR rat urine from different experimental groups and different time points (i.e. 0-8 h and 8-24 h) were screened for procyanidin and flavan-3-ol metabolites using HPLC with the MS scanning from *m/z* 100 to 700 in negative ionization mode. Identification of HPLC peaks was based on data dependent MS^2 and on previous studies (Touriño et al. 2009; Urpi-Sarda et al. 2009a; van der Hooft et al. 2012). For this experiment, urine was defrosted, centrifuged at

16,100 × g for 10 min at 4 °C prior to the analysis. Separation was performed on a 250 × 4.6 mm i.d. 5 μm Luna phenyl-hexyl 100Å column (Phenomenex, Macclesfield, U.K.) using a gradient over 120 min of 5-35% methanol in 0.1% aqueous formic acid. Identification results are presented in Table 50.

Table 50: Identification of metabolites after ingestion of grape pomace extracts by SHR rats: HPLC retention times and MS fragmentation patterns

Peak	Rt (min)	Metabolites identified	$[M-H]^-$ $(m/z)^a$	MS^2 fragment $(m/z)^b$
		Flavan-3-ol conjugates		
M1	18.0	(Epi)catechin-*O*-glucuronide 1	465	289
M2	24.7	(Epi)catechin-*O*-glucuronide 2	465	289
M3	33.2	(Epi)catechin-*O*-glucuronide 3	465	289
M4	48.0	*O*-Methyl-(epi)catechin-*O*-glucuronide 1	479	303
M5	57.1	*O*-Methyl-(epi)catechin-*O*-glucuronide 2	479	303
M6	80.6	*O*-Methyl-(epi)catechin-*O*-sulfate	383	303
M7	95.7	(Epi)catechin-*O*-sulfate 1	369	289
M8	97.8	(Epi)catechin-*O*-sulfate 2	369	289
		Hydroxyphenylvalerolactone conjugates		
M9	9.8	5-(*O*-Methyl-phenyl)-γ-valerolactone-*O*-glucuronide	381	205
M10	11.2	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	223	179
M11	15.9	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 1	383	207, 163
M12	27.0	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 2	383	207, 163
M13	46.1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 3	383	207, 163
M14	76.8	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate 1	287	207
M15	78.0	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate 2	287	207
M16	78.8	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone-*O*-glucuronide	399	223
M17	89.6	5-(Phenyl)-γ-valerolactone-*O*-sulfate	271	191
M18	113.3	5-(3′-Methoxy-4′-phenyl)-γ-valerolactone-*O*-sulfate	301	221
		Hydroxyphenylvaleric acid conjugates		
M19	13.7	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide	401	225
M20	46.1	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	305	225, 207
M21	50.2	5-(Phenyl)-4-hydroxyvaleric acid-*O*-glucuronide	385	209
M22	57.5	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate 1	289	209
M23	84.5	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate 2	289	209
		Hydroxyphenylacetic acid conjugates		
M24	13.0	Hydroxy-methoxyphenylacetic acid-*O*-glucuronide / 3-(3′,4′-dihydroxyphenyl)propionic acid-*O*-glucuronide*	357	181
M25	16.9	Hydroxy-methoxyphenylacetic acid-*O*-glucuronide / 3-(3′,4′-dihydroxyphenyl)propionic acid-*O*-glucuronide*	357	181
M26	21.0	Dihydroxyphenylacetic acid-*O*-glucuronide / *O*-methyl-dihydroxybenzoic acid-*O*-glucuronide*	343	167
M27	25.0	Hydroxyphenyl-acetic acid-*O*-sulfate / *O*-methyl-hydroxybenzoic acid-*O*-sulfate*	231	151
M28	27.6	Dihydroxyphenylacetic acid-*O*-sulfate / *O*-methyl-dihydroxybenzoic acid-*O*-sulfate*	247	167

M29	28.7	Hydroxy-*O*-methylphenylacetic acid-*O*-sulfate / dihydroxyphenylpropionic acid-*O*-sulfate*	261	181
M30	30.2	Hydroxyphenylacetic acid-*O*-sulfate / *O*-methyl-hydroxybenzoic acid-*O*-sulfate*	231	151
M31	33.6	Hydroxyphenylacetic acid-*O*-glucuronide	327	151
M32	36.0	Hydroxyphenylacetic acid-*O*-sulfate / *O*-methyl-hydroxybenzoic acid-*O*-sulfate*	231	151
M33	46.3	Dihydroxyphenylacetic acid-*O*-sulfate / *O*-methyl-dihydroxybenzoic acid-*O*-sulfate*	247	167
		Hydroxybenzoic acid conjugates		
M34	15.0	Hydroxybenzoic acid-*O*-glucuronide	313	137
M35	15.5	Dihydroxybenzoic acid-*O*-sulfate	233	153
M36	21.0	*O*-Methyl-dihydroxybenzoic acid-*O*-glucuronide / dihydroxyphenylacetic acid-*O*-glucuronide*	343	167
M37	25.0	Hydroxyphenylacetic acid-*O*-sulfate / *O*-methyl-hydroxybenzoic acid-*O*-sulfate*	231	151
M38	27.6	*O*-Methyl-dihydroxybenzoic acid-*O*-sulfate / dihydroxyphenylacetic acid-*O*-sulfate*	247	167
M39	30.2	*O*-Methyl-hydroxybenzoic acid-*O*-sulfate / hydroxyphenylacetic acid-*O*-sulfate*	231	151
M40	22.2	Hydroxybenzoic acid-*O*-sulfate	217	137
M41	27.8	Dihydroxybenzoic acid-*O*-sulfate	233	153
M42	27.3	Hydroxybenzoic acid glucuronide-*O*-sulfate	393	217, 137
M43	33.2	Hydroxybenzoic acid-*O*-sulfate	217	137
M44	36.0	*O*-Methyl-hydroxybenzoic acid-*O*-sulfate / Hydroxyphenylacetic acid-*O*-sulfate*	231	151
M45	46.3	*O*-Methyl-dihydroxybenzoic acid-*O*-sulfate / Dihydroxyphenylacetic acid-*O*-sulfate*	247	167
		Hydroxyphenylpropionic acid conjugates		
M46	13.0	3-(Dihydroxyphenyl)propionic acid-*O*-glucuronide / *O*-methyl-hydroxyphenylacetic acid-*O*-glucuronide*	357	181
M47	16.9	3-(Dihydroxyphenyl)propionic acid-*O*-glucuronide / *O*-methyl-hydroxyphenylacetic acid-*O*-glucuronide*	357	181
M48	28.7	3-(Dihydroxyphenyl)propionic acid-*O*-sulfate /Hydroxy-methoxy-phenylacetic acid-*O*-sulfate*	261	181
M49	37.7	3-(*O*-Methyl-hydroxyphenyl)propionic acid-*O*-sulfate/ caffeic acid-*O*-sulfate*	259	179
M50	44.5	3-(*O*-Methyl-dihydroxypheny)propionic acid-*O*-sulfate	275	195
M51	53.6	3-(-*O*-Methyl-hydroxyphenyl)propionic acid-*O*-sulfate/ caffeic acid-*O*-sulfate*	259	179
M52	56.1	3-(Hydroxyphenyl)propionic acid-*O*-sulfate	245	165
M53	60.7	3-(*O*-Methyl-dihydroxyphenyl)propionic acid-*O*-sulfate	275	195
M54	67.8	3-(Hydroxyphenyl)propionic acid-*O*-sulfate	245	165
		Hydroxycinnamic acid conjugates		
M55	37.7	Caffeic acid-*O*-sulfate / 3-(*O*-methyl-3′-hydroxyphenyl)propionic acid-*O*-sulfate*	259	179
M56	33.7	Ferulic acid-*O*-glucuronide*	369	193
M57	53.6	Caffeic acid-*O*-sulfate / 3-(*O*-methyl-3′-hydroxyphenyl)propionic acid-*O*-sulfate*	259	179
M58	60.0	Isoferulic acid-*O*-sulfate 1	273	193
M59	62.2	Isoferulic acid-*O*-sulfate 2	273	193
M60	70.4	Coumaric acid	163	119
		Hydroxyhippuric acid conjugates		
M61	17.3	3′-Hydroxyhippuric acid	194	150

[a] Tentative identification based on mass spectra, [b]molecular ion *(m/z)* identification in negative ionization mode. * MS^3 required for identification; Rt, retention time; $[M-H]^-$, negatively charged molecular ion.

Following ingestion of 21 mg/kg of grape pomace extracts, a total of 61 metabolites and polyphenolic derivatives were identified or tentatively identified in urine samples collected from SHR rats. Some of them need to be subjected to MS^3 analysis in order to be further fragment and provide information necessary to distinguish between different compounds with the same parent ion mass.

Metabolites were classified into different groups of compounds, namely flavan-3-ol conjugates, 5-(hydroxyphenyl)-γ-valerolactone conjugates, 5-(hydroxyphenyl)valeric acid conjugates, hydroxyphenylacetic acid conjugates, hydroxybenzoic acid conjugates, 3-(hydroxyphenyl)propionic acid conjugates, hydroxycinnamic acid conjugates and a hydroxyhippuric acid.

Eight flavan-3-ol metabolites were detected in urine. Peak M1-M8 were identified as a complex array of glucuronide, sulfate, and methyl metabolites of (epi)catechin which have been detected in previous studies following the ingestion of grape seed extracts (Tsang et al. 2005a; Serra et al. 2009). Epicatechin and catechin metabolites are referred as (epi)catechin as without reference compounds which can be separated by reversed phase HPLC, mass spectrometry is unable to distinguish between epicatechin and catechin metabolites.

Peak M9-M18 were identified as 5-(phenyl)-γ-valerolactone derivatives and were presented as substituted phenyl structures in the form of glucuronide and sulfate conjugates, or as a combination of methoxy-sulfate derivatives. These compounds could undergo in-source fragmentation resulting in signals corresponding to the loss of the glucuronide (176 *amu*) or sulfate (80 *amu*) moieties. The MS/MS spectra of 5-(phenyl)-γ-valerolactone conjugates were characterized by a product ion at *m*/*z* 163 resulting from the decarboxylation (44 *amu*) of the the γ-valerolactone ring, as previously described (Urpi-Sarda et al. 2009a; Sang et al. 2008). The *m*/*z* 207 and *m*/*z* 221 fragments correspond to the loss of different conjugate groups from hydroxy- and methoxy-phenylvalerolactones. These 5-(phenyl)-γ-valerolactone conjugates are derived the interaction of valerolactones, produced from the ring-fission of (epi)catechin/procyanidins by the colonic microbiota (Gonthier et al. 2003; Appeldoorn et al. 2009; Déprez et al. 2009), with UDP-glucuronosyl-transferases, sulfotransferases and catechol-*O*-methyltransferases.

Peak M19-M61 corresponded to five metabolites of 5-(hydroxyphenyl)valeric acid (peak M19-M23), ten metabolites of hydroxyphenylacetic acid (peak M24-M33), twelve

metabolites of hydroxybenzoic acid (peak M34-M45), nine metabolites of 3-(hydroxyphenyl)propionic acid (peak 46-54), six metabolites of hydroxycinnamic acids (peak M55-M60) and one hydroxyhippuric acid (peak M61) were identified in urine. They were presented as non- and mono-, and di-substituted phenyl structures in the form of glucuronide and sulfate conjugates, or as a combination of methyl-glucuronide, methyl-sulfated and methyl-sulfate derivatives. Their identification was obtained by comparing metabolite fragmentation patterns to those found in previous studies (Touriño et al. 2009; Urpi-Sarda et al. 2009a; van der Hooft et al. 2012). The aglycones of these metabolites and 5-(hydroxyphenyl)propionic acid conjugates (sulfates) were found by Touriño et al. 2009 in rat urine after ingestion grape dietary fiber containing a large proportion of polyphenolic compounds, mainly oligomeric and polymeric procyanidins, and by Gonthier et al. (2003) who fed wine powder to rats.

Phenylvalerolactones are derived from microbial degradation of monomeric flavan-3-ols and procyanidins (Gonthier et al. 2003; Sang et al. 2008; Appeldoorn et al. 2009a). Mediated by the action of UDP-glucuronosyl-transferases, sulfotransferases and catechol-*O*-methyltransferases on the aglycones, glucuronidated, sulfated, and methylated metabolites are formed in the liver and kidney before being excreted in the urine. Microbial degradation involves firstly a reductive cleavage of the (–)-epicatechin heterocyclic C-ring resulting in the formation of diphenylpropan-2-ols followed by a lactonization of diphenylpropan-2-ols into hydroxyphenylvalerolactones. Fission of the valerolactone ring leads to hydroxyphenylvaleric acids (Meselhy et al. 1997; Gonthier et al. 2003). Oxidation of hydroxyphenylvaleric acid side chains results in the formation of hydroxyphenylpropionic and hydroxybenzoic acids (Meselhy et al. 1997). Hydroxyphenylpropionic acids can undergo α- and β-oxidation and form phenylacetic acids which can be subjected to 3′- and 4′-dehydroxylation reactions (Gonthier et al. 2003). This colonic catabolism pathway of flavan-3-ol monomer is outlined in Figure 51.

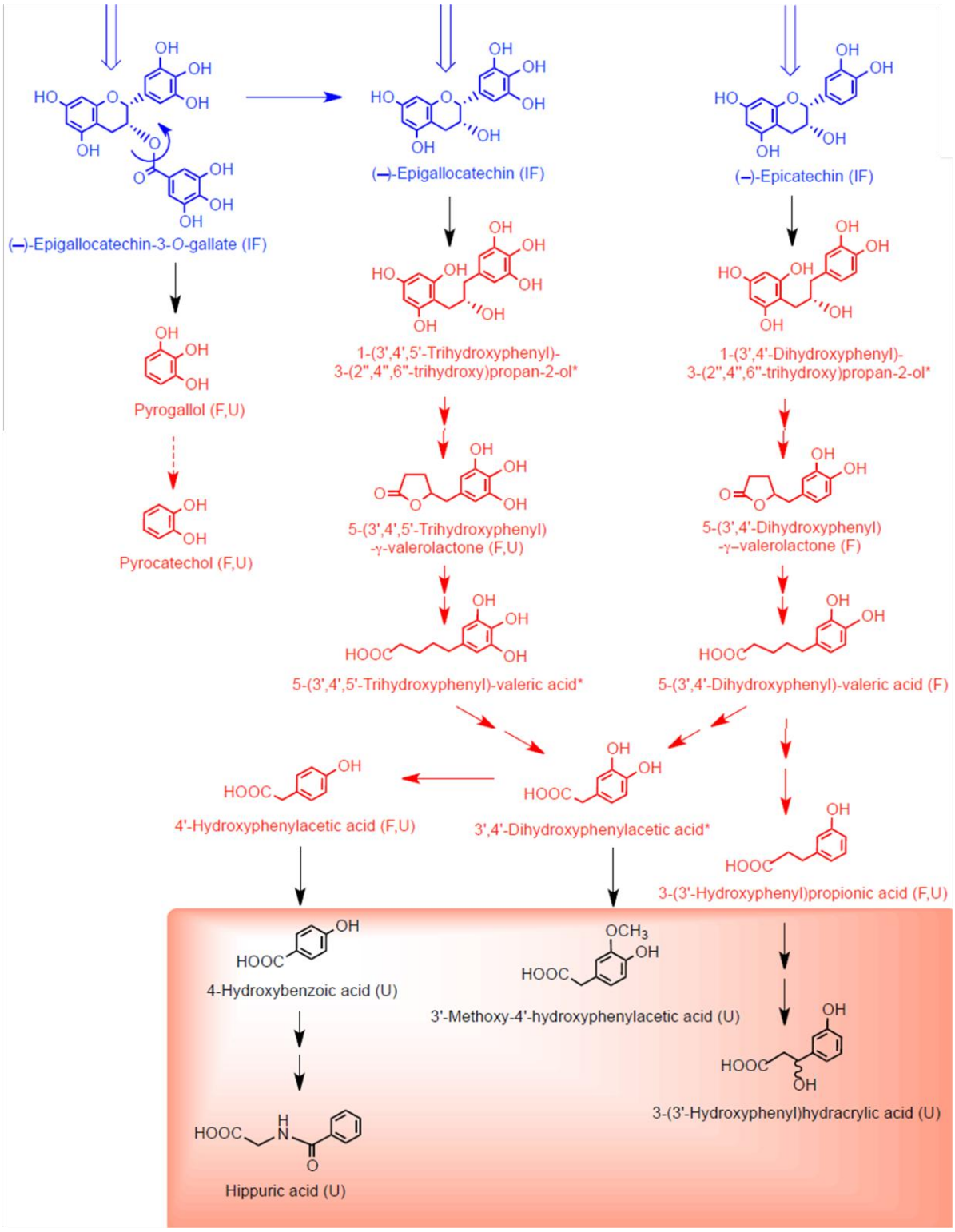


Figure 51: Proposed pathways involved in the colonic catabolism and urinary excretion of green tea flavan-3-ols.

Following consumption of green tea more than 50% of the ingested (–)-epicatechin, (–)-epigallocatechin and (–)-epigallocatechin-3-*O*-gallate (blue structures) pass into the large intestine. When incubated with faecal slurries these compounds are catabolised by the colonic microflora probably via the pathways illustrated with red structures. Analysis of urine after green tea consumption indicates that some of the colonic catabolites enter the circulation and undergo further metabolism before being excreted in urine. Boxed black structures indicate catabolites detected in urine but not produced by faecal fermentation of (–)-epicatechin, (–)-epigallocatechin or (–)-epigallocatechin-3-*O*-gallate. The dotted arrow between pyrogallol and pyrocatechol indicate this is a minor conversion. Double arrows indicate conversions where the intermediate(s) did not accumulate and are unknown. Compounds detected in ileal fluid after green tea consumption (IF); catabolites detected in fecal slurries (F) and in urine (U); potential intermediates that did not accumulate in detectable quantities in fecal slurries (*). After Roowi et al. (2010).

III.1.1.b. Quantification of polyphenolic compounds in SHR rat urines

After the initial qualitative analysis, a targeted analysis of urine samples was carried out by HPLC with MS in the SRM (selected reaction monitoring) mode. As described previously, a considerable number of metabolites were found in urine but only metabolites presented in high amounts and in all urine samples screened were chosen for quantification.

In addition, because of the lack of glucuronidated or sulfated reference compounds, some of the metabolites were not quantified. Indeed, the quantification based on a calibration curve made of aglycone reference compounds for the quantification of glucuronidated and sulfated metabolites might introduce a potential source of error in the quantitative estimates caused by different slopes of the glucoside and glucuronide SRM dose-response curves. In fact, different aglycones of phenolic acids were tested in order to determine their ionization characteristics and their limit of detection. The 3-hydroxybenzoic acid *(m/z 137)*, 4-hydroxybenzoic acid *(m/z 137)*, 3′-hydroxyphenylacetic acid *(m/z 151)*, 4′-hydroxyphenylacetic acid *(m/z 151)*, 3-(3′-hydroxyphenyl)propionic acid *(m/z 165)* and 4′-hydroxyhippuric acid *(m/z 194)* were identified and quantified using SRM mode. Results revealed a limit of detection of 150 ng for the 3-hydroxybenzoic acid, 67 ng for the 4-hydroxybenzoic acid, 170 ng for the 3-(3′-hydroxyphenyl)propionic acid and 16 ng for 4′-hydroxyhippuric acid. 3′-Hydroxyphenylacetic acid, 4′-hydroxyphenylacetic acid underwent limited fragmentation and as a consequence the limit of detection was 2 µg. Therefore, 3-(hydroxyphenyl)propionic acid, hydroxyphenylacetic acid, hydroxybenzoic acid and hydroxycinnamic derivatives were identified but not quantified by HPLC-ESI-MS^n. Phenolic acid excretion derived from colonic microbiota in urine were analysed by GC-MS as will be outlined later.

Compounds chosen were listed by elution order in Table 51 and their chromatograms are illustrated in Figure 52.

Table 51: Identification of urinary metabolites selected for quantification: HPLC retention times and MS fragmentation patterns obtained by SRM

Peak	Rt (min)	Compounds	$[M-H]^-$ *(m/z)*[a]	MS^2 fragment *(m/z)*[b]
1	**6.13**	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	383	207, 163
2	**7.28**	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide	401	225, 113
3	**7.76**	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	383	207, 163
4	**9.39**	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	383	207, 163
5	**9.48**	(Epi)catechin-*O*-glucuronide	465	289, 245
6	**10.05**	(Epi)catechin-*O*-glucuronide	465	289, 245
7	**11.31**	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	305	225, 207
8	**13.14**	(Epi)catechin-*O*-glucuronide	465	289, 245
9	**13.83**	*O*-Methyl-(epi)catechin-*O*-glucuronide	479	303, 223
10	**14.23**	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	305	225, 207
11	**15.22**	*O*-Methyl-(epi)catechin-*O*-glucuronide	479	303, 223
12	**17.03**	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	289	209
13	**17.08**	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	305	225, 207
14	**18.63**	Isoferulic acid-4-*O*-sulfate	273	193
15	**19.33**	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	289	209
16	**20.11**	5-(Hydroxyphenyl)-γ-valerolactone sulfate	287	207, 163
17	**22.55**	5-(Hydroxyphenyl)-γ-valerolactone sulfate	287	207, 163
18	**24.45**	Isoferulic acid-4-*O*-sulfate	273	193

[a]Tentative identification based on mass spectra, [b]molecular ion *(m/z)* identification in negative ionization mode. Rt, retention time; $[M-H]^-$, negatively charged molecular ion.

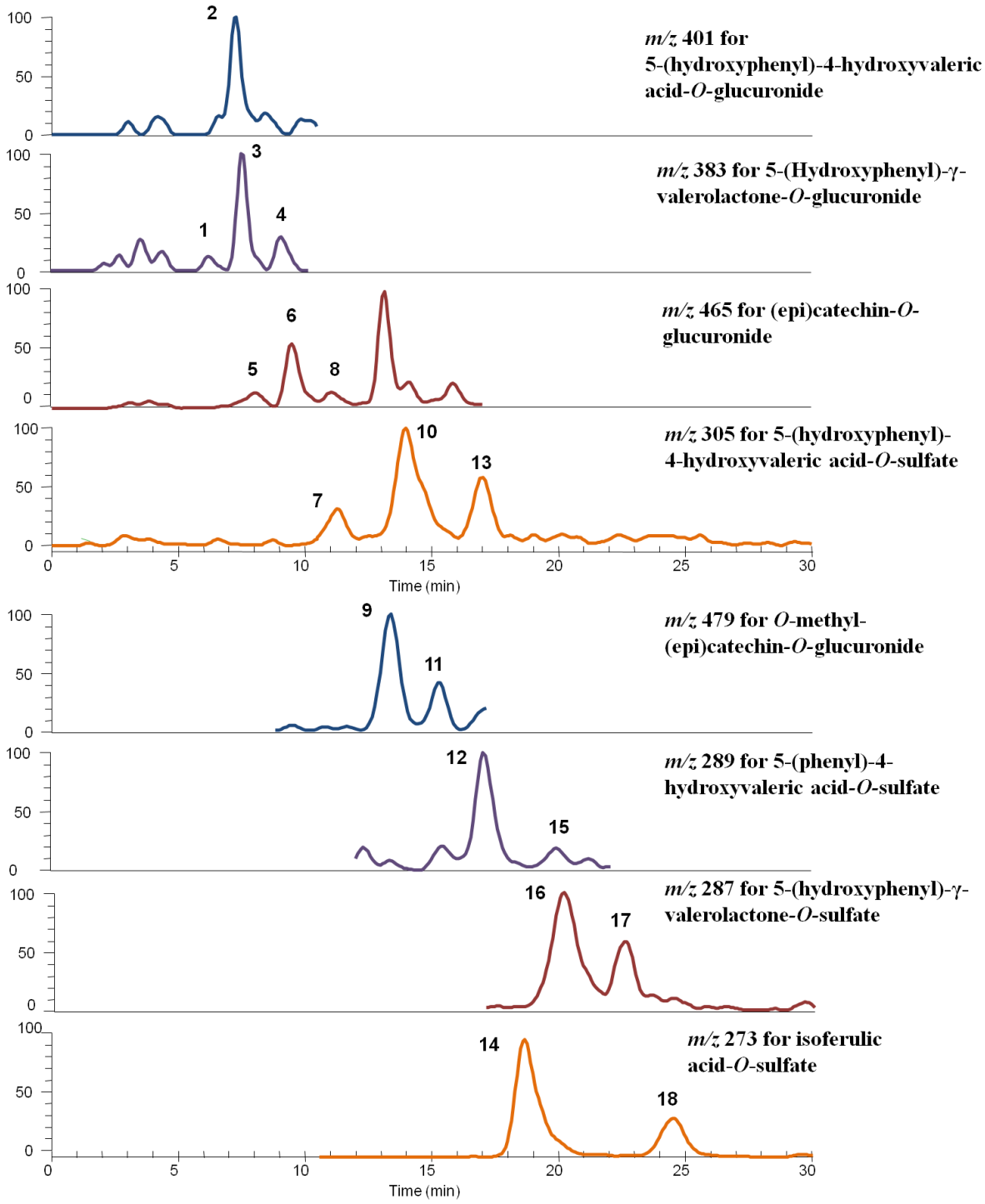


Figure 52: HPLC-SRM quantification of flavan-3-ol and procyanidin metabolites in SHR rat urines collected after ingestion of 21 mg/kg/day

Chromatograms represent gradient RP-HPLC analysis with detection of flavan-3-ol and procyanidin metabolites by using SRM at *m/z* 401, *m/z* 383, *m/z* 465, *m/z* 305, *m/z* 479, *m/z* 289, *m/z* 287 and *m/z* 273. Peak number in chromatograms corresponded to compounds having the same peak number provided in Table 51.

Urine samples were collected at 0-8 h and 8-24 h on day 1 and day 7 after the ingestion of different grape pomace extracts. The urine did not contain any of the original grape pomace extract flavan-3-ols and procyanidins but glucuronides and methyl-glucuronides of (epi)catechin, as well as valerolactone and valeric acid phase II glucuronide and sulfate metabolites were detected. Compared to other studies in which rats were fed grape derived products (Tsang et al. 2005a; Touriño et al. 2009; Serra et al. 2011), sulfate derivatives were found as one of the main metabolites, whereas in this work, sulfates were present in a very low amounts. The reason for these varying metabolite profiles, especially the dominance of glucuronides in some studies and sulfates in order could be due to losses of (epi)catechin-*O*-sulfates during sample processing before analysis (Day et al. 2003). The difference in the metabolic profile of (epi)catechins may also be attributed to the different flavan-3-ols composition of the matrix (e.g.,. grape seed/skin extracts, tea, cocoa).

Without reference compounds which can be separated by reversed phase HPLC, mass spectrometry is unable to distinguish between epicatechin and catechin metabolites. Therefore, epicatechin and catechin metabolites are referred as (epi)catechin. Moreover, it was not possible to determine the position on the flavan-3-ol skeleton of the methyl and glucuronic acid substituents. In this study, three (epi)catechin-*O*-glucuronides (*m/z* 465) and two *O*-methyl-(epi)catechin-*O*-glucuronides (*m/z* 479/303, 223) were detected. According to Baba et al. (2000), who studied the bioavailability of (–)-epicatechin, the authors suggested that (–)-epicatechin bioavailability was higher than that of (+)-catechin in rats and that chemical features such as solubility and lipophilicity of each substances might be involve in these differences. Other investigations also found that (–)-epicatechin was highly bioavailable (Manach et al. 2005; Auger et al. 2008). Considering these observations, at least two of the metabolites found could be partially identified as (–)-epicatechin-*O*-glucuronide and *O*-methyl-(–)-epicatechin-*O*-glucuronide. They could be (–)-epicatechin-7-*O*-glucuronide and *O*-methyl-(–)-epicatechin-7-*O*-glucuronide as demonstrated by Natsume et al. (2003) who identified these metabolites in rat urine after (–)-epicatechin ingestion. Unlike human subjects, which produce 4′-*O*- and 3′-*O*-methylated derivatives (Ottaviani et al. 2012b), rats accumulated predominantly 3′-*O*-methylated metabolites of flavan-3-ols (Nakamura et al. 2003; Natsume et al. 2003). Therefore, it is probable that one of the metabolites found was the 3′-*O*-methyl-(–)-epicatechin-7-*O*-glucuronide. However, these are tentative identifications and require further investigation.

Detection of valerolactone and valeric acid conjugates in this study is in agreement with previous reports. Their presence in urine as aglycones has been established in human and rats feeding studies with flavan-3-ol- and procyanidin-rich products (i.e. grape derived products, cocoa, green tea) (Gonthier et al. 2003; Serra et al. 2009; Urpi-Sarda et al. 2009a; Del Rio et al. 2010). 5-(Hydroxyphenyl)-γ-valerolactones are considered to be the main metabolites deriving from the first stages of the colonic microbial metabolism of flavan-3-ols, after the opening of the C-ring to form hydroxyphenylpropan-2-ols (Meselhy et al. 1997) (see Figure 51). The presence of methylated, glucuronide and sulfate metabolites results from the action of UDP-glucuronosyl-transferases, sulfotransferases and catechol-*O*-methyltransferases in the liver and kidneys and arguably the wall of the colon. Several studies have detected the presence of glucuronide and sulfate metabolites of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone after green tea consumption and procyanidin-rich products such as almond skins (Lee et al. 1995; Meng et al. 2002; Sang et al. 2008; Urpi-Sarda et al. 2009a; Del Rio et al. 2010). Subsequent fission of the valerolactone ring leads to the formation of 5-(hydroxyphenyl)valeric acids which were also detected and quantified in this study.

Quantification of 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide (*m/z*, 401/225) and 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate (*m/z*, 287/207) was by reference to the aglycone 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone while 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate (*m/z*, 305/225) levels were measured using a calibration curve obtained with 5-(3′,4′-dihydroxyphenyl)-4-hydroxyvaleric acid. Data are expressed as nmol ± SE (n=3). Details are provided in chapter II: material and methods. The other metabolites were quantified as (–)-epicatechin equivalents and expressed as nmol ± SE (n=3).

Quantification of metabolites in urine collected of 0-8 h and 8-24 h after pomace extract ingestion at day 1 and day 7 are presented in Tables 52 to55. Compounds present in significantly higher amounts in urine from SHRs fed with grape pomace extracts than the control urine are mark by an asterisk (*). A paired student's t-test was performed at a threshold of $p < 0.05$.

Table 52: Urinary excretion at day 1, 0-8 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SE (*n*=3).

		E1		VE1		E5		VE5		E6		VE6	
Peak	**Metabolites**	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**1.0***	**0.1**	**1.3***	**0.2**	1.2	0.4	0.7	0.1	**1.1***	**0.2**	0.8	0.2
2	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-glucuronide	**0.8***	**0.1**	0.8	0.2	0.9	0.3	0.6	0.1	0.6	0.1	0.8	0.1
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.0	Nd
5	(Epi)catechin-*O*-glucuronide	0.1	0.1	Nd	Nd	**1.3***	**0.0**	Nd	Nd	**1.3***	**0.1**	0.2	Nd
6	(Epi)catechin-*O*-glucuronide	**1.0***	**0.3**	0.5	0.2	**0.7***	**0.2**	**0.2***	**0.1**	**0.7***	**0.2**	0.3	0.2
7	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	**0.6***	**0.2**	**0.5***	**0.2**	**0.7***	**0.3**	0.3	0.0	0.3	0.1	**0.5***	**0.1**
8	(Epi)catechin-*O*-glucuronide	0.3	0.0	**0.5***	**0.0**	**0.6***	**0.0**	0.5	Nd	0.5	Nd	Nd	Nd
9	*O*-Methyl-(epi)catechin-*O*-glucuronide	**6.7***	**1.2**	**10.5***	**3.5**	3.2	0.4	3.4	0.8	**8.7***	**2.1**	6.8	1.6
10	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	**1.4***	**0.3**	**1.0***	**0.2**	**1.3***	**0.3**	1.1	0.1	**1.3***	**0.3**	**2.8***	**0.9**
11	*O*-Methyl-(epi)catechin-*O*-glucuronide	**1.8***	**0.5**	**3.0***	**1.3**	0.5	0.2	**0.6***	**0.2**	**1.8***	**0.6**	**2.2***	**0.7**
12	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	6.9	1.3	**9.4***	**0.6**	7.6	1.6	4.1	1.1	**6.1***	**3.7**	9.0	3.4
13	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	**0.5***	**0.1**	**0.6***	**0.2**	1.7	1.2	**0.5***	**0.1**	0.3	0.1	**1.1***	**0.4**
14	Isoferulic acid-4-*O*-sulphate	98.3	22.2	**88.5***	**6.0**	**75.0***	**15.6**	69.1	8.3	47.1	12.4	85.7	10.6
15	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
16	5-(Hydroxyphenyl)-γ-valerolactone sulfate	1.4	0.4	**1.6***	**0.5**	4.2	2.9	**1.6***	**0.4**	0.8	0.1	**3.2***	**1.2**
17	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.3	0.2	0.7	0.4	0.7	0.0	0.4	0.2	0.7	0.4	1.1	0.9
18	Isoferulic acid-4-*O*-sulphate	**58.7***	**13.2**	39.0	5.2	Nd	Nd	22.9	4.7	35.8	7.5	30.6	6.5

*Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. Nd, not determined; SE, standard error.

Table 53: Urinary excretion at day 1, 8-24 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SE (*n*=3).

		E1		VE1		E5		VE5		E6		VE6	
Peak	**Metabolites**	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.2	0.0	0.3	0.0	0.6	0.1	0.2	0.1	**0.4***	**0.1**	**0.4***	**0.0**
2	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-glucuronide	0.1	0.0	**0.2***	**0.1**	0.3	0.1	0.2	0.1	0.2	0.1	**0.2***	**0.0**
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	Nd	Nd	Nd	Nd	Nd	Nd	Nd		Nd	Nd	Nd	
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	Nd	Nd	Nd	Nd	Nd	Nd	Nd		Nd	Nd	0.0	
5	(Epi)catechin-*O*-glucuronide	0.2	0.1	0.3	Nd	0.2	Nd	Nd		Nd	Nd	0.5	0.0
6	(Epi)catechin-*O*-glucuronide	1.0	0.2	1.1	0.3	1.7	0.6	0.8	0.1	1.4	0.3	**1.6***	**0.2**
7	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	0.1	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.3	0.1	**0.2***	**0.0**
8	(Epi)catechin-*O*-glucuronide	Nd	Nd	Nd	Nd	Nd	Nd	Nd		Nd	Nd	Nd	
9	*O*-Methyl-(epi)catechin-*O*-glucuronide	3.8	0.7	4.0	0.7	7.6	1.5	3.4	0.7	**7.1***	**1.3**	5.5	0.9
10	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	0.6	0.1	1.5	0.6	2.0	0.8	**1.4***	**0.4**	**1.4***	**0.4**	**2.1***	**0.6**
11	*O*-Methyl-(epi)catechin-*O*-glucuronide	Nd	Nd	Nd	Nd	**1.7***	**0.0**	Nd		Nd	Nd	0.0	
12	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	11.7	0.8	12.8	2.5	13.2	3.6	13.4	4.0	13.9*	0.8	12.1*	1.4
13	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate	0.3	0.0	0.4	0.1	0.5	0.2	**0.7***	**0.2**	**0.6***	**0.1**	**0.8***	**0.3**
14	Isoferulic acid-4-*O*-sulphate	**39.2***	**4.3**	**56.4***	**7.0**	**45.0***	**0.0**	31.2	8.1	55.2	14.1	83.0	7.6
15	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	8.9	0.8	10.9	2.2	16.8	3.2	14.6	1.7	17.6	1.2	16.2	4.4
16	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.9	0.1	**1.3***	**0.3**	1.3	0.4	1.7	0.6	1.1	0.2	1.8	0.5
17	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.4	0.1	**0.6***	**0.2**	0.6	0.2	**0.7***	**0.2**	0.5	0.1	**0.8***	**0.2**
18	Isoferulic acid-4-*O*-sulphate	41.2	9.3	43.6	11.0	54.7	16.1	57.1	8.9	**56.4***	**11.5**	**69.4***	**5.4**

*Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. Nd, not determined; SE, standard error.

Table 54: Urinary excretion at day 7, 0-8 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SE (*n*=3).

		E1		VE1		E5		VE5		E6		VE6	
Peak	**Metabolites**	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**0.2***	**0.0**	**0.2***	**0.0**	0.1	0.0	0.1	0.0	0.1	0.0	0.2	0.0
2	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide	0.1	0.0	**0.2***	**0.1**	**0.3***	**0.1**	0.1	0.0	0.2	0.1	0.1	0.0
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.3	0.2	**0.3***	**0.0**	0.3	0.1	**0.5***	**0.0**	0.3	0.1	0.1	0.0
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	Nd	Nd	Nd	Nd	0.1	Nd	Nd	Nd	Nd	Nd	**0.1***	**0.0***
5	(Epi)catechin-*O*-glucuronide	0.0	0.0	0.3	Nd	Nd	Nd	**0.2***	**0.0**	**0.1***	**0.0**	0.0	0.0
6	(Epi)catechin-*O*-glucuronide	1.0	0.5	0.7	0.3	1.6	0.9	0.6	0.2	**2.1***	**0.9**	1.7	1.0
7	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	**0.2***	**0.1**	0.1	0.0	0.2	0.1	0.2	0.1	**0.3***	**0.1**	0.3	0.1
8	(Epi)catechin-*O*-glucuronide	1.1	1.0	0.3	0.0	3.1	1.7	Nd	Nd	**0.9***	**0.3**	**1.5***	**0.6***
9	*O*-Methyl-(epi)catechin-*O*-glucuronide	**4.6***	**1.7**	**2.1***	**0.4**	4.2	1.5	**4.3***	**1.3**	**5.9***	**2.3**	5.2	2.0
10	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.2	0.0	0.5	0.2	0.4	0.1	**0.6***	**0.1**	0.3	0.2	**0.6***	**0.0***
11	*O*-Methyl-(epi)catechin-*O*-glucuronide	1.2	0.6	**1.8***	**0.5**	3.0	0.9	0.8	0.3	1.9	0.1	1.8	0.8
12	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	5.9	1.2	2.9	0.6	7.6	2.3	7.4	2.2	7.2	0.6	2.6	0.0
13	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.1	0.1	**0.2***	**0.1**	**0.1***	**0.0**	0.2	0.1	0.7	0.6	0.2	0.1
14	Isoferulic acid-4-*O*-sulphate	**73.0***	**11.3**	**65.3***	**11.8**	**76.8***	**14.8**	**73.4***	**14.0**	**62.5***	**16.8**	38.3	9.5
15	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	4.4	0.8	**7.8***	**2.9**	6.3	1.9	6.6	1.5	5.3	1.9	6.4	1.4
16	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.2	0.0	**0.5***	**0.2**	**0.4***	**0.2**	**0.6***	**0.2**	**0.6***	**0.3**	**0.5***	**0.1***
17	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.1	Nd	0.1	0.0	0.0	0.0	0.4	0.2	0.0	0.0	0.2	0.0
18	Isoferulic acid-4-*O*-sulphate	58.3	14.6	94.3	23.1	72.4	19.1	60.9	23.7	74.9	29.1	**94.8***	**8.6***

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p< 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. Nd, not determined; SE, standard error.

Table 55: Urinary excretion at day 7, 8-24 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SE (*n*=3).

		E1		VE1		E5		VE5		E6		VE6	
Peak	**Metabolites**	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.4	0.1	0.3	0.0	0.5	0.1	0.2	0.1	**0.5***	**0.0**	0.2	0.1
2	5-Hydroxyphenyl-4-hydroxyvaleric acid-*O*-glucuronide	0.7	0.3	0.2	0.0	0.3	0.1	0.1	0.0	0.2	0.0	0.2	0.0
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**0.5***	**0.0**	0.2	0.0	0.8	0.2	0.5	0.1	**0.7***	**0.2**	0.4	0.1
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.1	0.0	Nd	Nd	0.0	Nd	**0.1***	**0.0**	Nd		0.1	0.1
5	(Epi)catechin-*O*-glucuronide	0.7	0.5	1.1	0.0	1.1	0.4	0.9	0.0	1.3		1.6	Nd
6	(Epi)catechin-*O*-glucuronide	1.1	0.1	**0.9***	**0.1**	1.1	0.2	0.7	0.1	2.0	0.5	1.1	0.5
7	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.6	0.4	0.3	0.1	0.4	0.1	0.5	0.3	0.4	0.1	1.0	0.5
8	(Epi)catechin-*O*-glucuronide	Nd	Nd	0.8	Nd	0.7	Nd	Nd	Nd	0.3		0.5	Nd
9	*O*-Methyl-(epi)catechin-*O*-glucuronide	8.1	1.8	**3.2***	**0.6**	5.7	1.4	2.5	0.5	5.2	0.8	**3.6***	**0.8**
10	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.7	0.2	0.8	0.3	1.5	0.7	0.5	0.2	0.9	0.3	0.7	0.2
11	*O*-Methyl-(epi)catechin-*O*-glucuronide	Nd	Nd	Nd	Nd	1.3	0.9	Nd	Nd	0.0	0.0	0.5	0.3
12	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	14.2	2.6	11.2	3.5	16.0	2.7	8.9	1.6	16.0	3.7	8.0	1.8
13	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.2	0.0	0.2	0.1	0.2	0.1	0.2	0.1	0.3	0.1	0.3	0.1
14	Isoferulic acid-4-*O*-sulphate	152.2	26.0	87.2	20.2	152.8	41.0	61.8	17.1	129.8	30.5	85.1	20.3
15	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	11.2	1.2	**10.8***	**2.8**	6.2	0.0	1.2	0.6	3.4	0.0	12.4	2.7
16	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.6	0.1	0.7	0.1	1.0	0.3	0.6	0.2	0.7	0.1	1.0	0.2
17	5-(Hydroxyphenyl)-γ-valerolactone sulfate	0.3	0.1	0.6	0.2	**0.9***	**0.2**	0.2	0.1	**1.2***	**0.1**	0.5	0.2
18	Isoferulic acid-4-*O*-sulphate	**57.3***	**12.6**	**36.2***	**4.9**	**65.4***	**19.6**	27.8	7.6	**41.7***	**12.5**	**31.4***	**9.9**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p< 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. Nd, not determined; SE, standard error.

In order to have an overall picture of metabolite excretion, the sum of urinary (epi)catechin-*O*-glucuronide, *O*-methyl-(epi)catechin-*O*-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate, 5-hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulfate, 5-(phenyl)-4-hydroxyvaleric acid-*O*-sulfate, isoferulic acid-4-*O*-sulphate was calculated. The results are presented in Tables 56 and 57.

At day 1 of gavage, SHR urines from the experimental group E1 exhibited significantly higher excretion of metabolites than other SHR group at the 0-8 h collection period whereas rats from the E6 and VE6 groups (i.e. fed with GRE1 [EA70] seed pomace extract and ALI [EA70] skin pomace extract + verapamil, respectively), excreted high amounts at over the later 8-24 h period. Urinary excretion of VE5 group rats (i.e. fed with MOU [EA70] skin pomace extract + verapamil) was not different from that detected in verapamil control group urine. At day 7, urinary excretion from VE1 group (i.e. fed with GRE1 [EA70] seed pomace extract + verapamil) at 0-8 h contained substantial amounts of metabolites. In contrast to day 1, VE5 rat urine at day 7, in the 0-8 h sample, showed sizable metabolite excretion. Overall, higher amounts of metabolites were observed in SHR urine of rats fed grape pomace extracts alone or in association with verapamil, especially for (epi)catechin-*O*-glucuronide, *O*-methyl-(epi)catechin-*O*-glucuronide and isoferulic acid-4-*O*-sulfate at day 1 and day 7.

Table 56: Sum of structurally related urinary metabolites at day 1, 0-8 h and 8-24 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SE.

Peak	Σ of structurally related metabolites (Day 1, 0-8 h)	E1		VE1		E5		VE5		E6		VE6		Control		Verapamil control	
		Mean	SD	Mean	SD	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	(Epi)catechin-*O*-glucuronide	**1.0***	**0.3**	**0.6***	**0.3**	**2.0***	**0.2**	0.2	0.1	**1.2***	**0.5**	0.3	0.1	0.1	0.0	0.1	0.0
2	*O*-Methyl-(epi)catechin-*O*-glucuronide	**8.5***	**1.5**	10.8	4.3	3.5	0.4	3.9	0.9	6.1	2.7	9.0	2.2	3.5	1.0	4.2	1.6
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**1.0***	**0.1**	0.8	0.3	1.2	0.4	0.7	0.1	0.5	0.3	0.8	0.2	0.4	0.2	0.5	0.2
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	1.5	0.3	1.6	0.5	4.4	2.9	1.9	0.3	1.2	0.4	**3.7***	**1.1**	1.1	0.3	1.0	0.2
5	5-(Hydroxyphenyl-)4-hydroxyvaleric acid-*O*-sulfate	**2.5***	**0.6**	1.8	0.4	3.5	1.3	2.0	0.2	**2.0***	**0.5**	**4.3***	**1.3**	0.9	0.3	1.2	0.3
6	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	**6.9***	**1.3**	6.3	2.0	7.6	1.6	4.1	1.1	6.1	3.7	**9.0***	**3.4**	3.1	0.7	4.8	0.8
7	Isoferulic acid-4-*O*-sulfate	**157.0***	**35.5**	**127.5***	**11.2**	**75.0***	**15.6**	92.0	13.1	82.8	19.9	116.3	17.1	82.2	19.9	75.6	18.4

Peak	Σ of structurally related metabolites (Day 1, 8-24 h)	E1		VE1		E5		VE5		E6		VE6		Control		Verapamil control	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	(Epi)catechin-*O*-glucuronide	1.1	0.2	1.1	0.2	1.8	0.6	0.8	0.1	1.4	0.3	**1.8***	**0.2**	1.0	0.3	0.6	0.2
2	*O*-Methyl-(epi)catechin-*O*-glucuronide	3.8	0.7	4.0	0.7	**6.9***	**2.1**	3.4	0.7	**7.1***	**1.3**	5.5	0.9	2.7	1.0	3.3	1.2
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.2	0.0	0.3	0.0	0.4	0.1	0.2	0.1	**0.4***	**0.1**	**0.4***	**0.0**	0.2	0.0	0.2	0.0
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	1.3	0.2	**1.9***	**0.5**	1.8	0.6	**2.4***	**0.8**	1.6	0.2	**2.7***	**0.7**	1.3	0.3	0.8	0.2
5	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	1.0	0.1	2.0	0.7	**2.6***	**0.9**	**2.3***	**0.4**	**2.2***	**0.4**	**3.1***	**0.9**	0.7	0.1	0.7	0.1
6	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	12.7	1.8	12.2	1.7	14.4	2.5	13.8	2.6	**15.8***	**1.1**	**13.5***	**1.7**	8.7	0.9	9.9	2.4
7	Isoferulic acid-4-*O*-sulfate	80.4	13.6	100.0	18.0	99.7	16.1	88.2	17.0	**111.5***	**25.7**	**152.4***	**13.0**	74.2	4.3	83.6	12.6

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SE, standard error.

Table 57: Sum of structurally related urinary metabolites at day 7, 0-8 h and 8-24 h after ingestion of grape pomace extracts.
Data are expressed as nmol ± SE

Peak	Σ of structurally related metabolites (Day 7, 0-8 h)	E1		VE1		E5		VE5		E6		VE6		Control		Verapamil control	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	(Epi)catechin-*O*-glucuronide	1.6	1.0	**0.7***	**0.3**	3.1	1.9	0.6	0.2	**2.9***	**1.0**	1.9	1.0	0.5	0.3	0.1	0.0
2	*O*-Methyl-(epi)catechin-*O*-glucuronide	5.7	2.2	**2.9***	**0.4**	5.7	2.3	**4.0***	**1.3**	**5.9***	**2.1**	**6.1***	**2.6**	1.7	0.4	1.5	0.4
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.2	0.1	0.2	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.2	0.0	0.2	0.1	0.1	0.0
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	0.2	0.0	**0.5***	**0.2**	0.4	0.2	**0.7***	**0.3**	0.5	0.2	**0.6***	**0.1**	0.2	0.1	0.2	0.1
5	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	0.4	0.1	**0.6***	**0.2**	**0.6***	**0.1**	**0.9***	**0.2**	0.9	0.4	**0.9***	**0.2**	0.4	0.2	0.3	0.1
6	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	6.6	1.6	7.5	2.5	6.7	2.6	**10.3***	**2.7**	8.4	2.3	7.3	1.1	7.7	2.0	3.5	1.2
7	Isoferulic acid-4-*O*-sulfate	**131.2***	**25.9**	**159.6***	**34.9**	**149.2***	**34.0**	134.3	37.7	137.4	45.8	**133.1***	**18.1**	71.8	29.5	82.5	7.5

Peak	Σ of structurally related metabolites (Day 7, 8-24 h)	E1		VE1		E5		VE5		E6		VE6		Control		Verapamil control	
		Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
1	(Epi)catechin-*O*-glucuronide	1.6	0.4	**1.4***	**0.2**	1.6	0.1	1.0	0.3	**2.2***	**0.4**	1.4	0.5	1.1	0.3	0.6	0.2
2	*O*-Methyl-(epi)catechin-*O*-glucuronide	8.1	1.8	**3.2***	**0.6**	6.2	1.3	2.5	0.5	**5.2***	**0.8**	**3.8***	**0.8**	3.7	0.8	2.1	0.7
3	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.5	0.1	0.3	0.0	0.7	0.3	0.4	0.1	**0.7***	**0.1**	0.3	0.1	0.4	0.1	0.3	0.1
4	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	0.8	0.1	1.1	0.3	1.4	0.5	0.8	0.2	1.6	0.3	**1.1***	**0.2**	1.1	0.3	0.4	0.1
5	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate	1.2	0.4	1.1	0.3	1.8	0.6	1.1	0.3	1.4	0.3	**1.7***	**0.5**	1.1	0.3	0.4	0.1
6	5-(Phenyl)-4-hydroxyvaleric acid-*O*-sulfate	13.2	4.2	**12.9***	**2.6**	15.4	3.6	8.0	1.7	14.5	4.0	10.8	2.8	12.4	2.8	5.5	1.7
7	Isoferulic acid-4-*O*-sulfate	**209.4***	**38.6**	123.3	25.1	**218.2***	**60.6**	89.6	24.7	171.5	43.0	116.5	30.2	121.2	20.8	96.4	27.6

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SE, standard error.

Higher levels of *O*-methyl-(epi)catechin-*O*-glucuronide relative to (epi)catechin-*O*-glucuronide were found in every urine sample. Sulfate derivatives of 5-(hydroxyphenyl)-γ-valerolactone were excreted in greater amounts than glucuronides. Glucuronidated 5-(hydroxyphenyl)-4-hydroxyvaleric acid and 5-(phenyl)-4-hydroxyvaleric acid were identified (Table 51) but because of their low concentration, they were not quantified unlike their sulfated counterparts. A decrease of 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate from 0-8 h to 8-24 h at day 1 was observed while an increase of 5-(phenyl)-4-hydroxyvaleric acid-*O*-sulfate occurred at 8-24 h compared to 0-8 h in all rat urines at day 1 and day 7. A previous report by Llorach et al. (2010) on a human almond skin polyphenol bioavailability suggested that partial dehydroxylation reactions occur gradually as colonic metabolism progresses. These authors observed a change in the hydroxylation pattern of the phenyl ring from di- to mono- and unhydroxylated forms. Dihydroxylated derivatives were found 6-10 h after the intake, monohydroxylated forms were observed at 6-24 h, and unhydroxylated derivatives were found 10-24 h after the intake.

In order to compare efficiency of grape pomace extracts, the sum of metabolites that are excreted in significantly higher amounts compared to their respective control ($p < 0.05$) are presented in Figure 53 for day 1 of extract ingestion and Figure 54 for day 7 of extract intake.

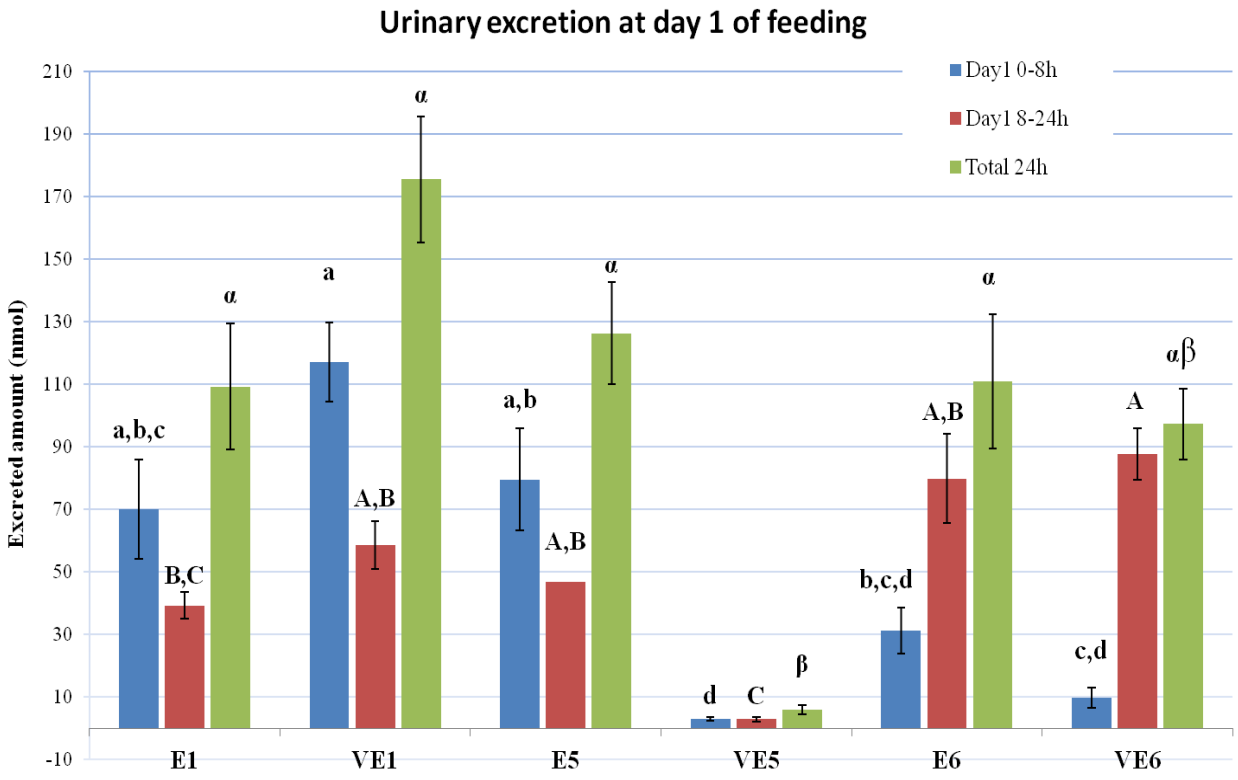


Figure 53: Total urinary metabolites excreted at day 1, 0-8 h, 8-24 h and 0-24h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α; Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 1. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in nmol ± standard error.

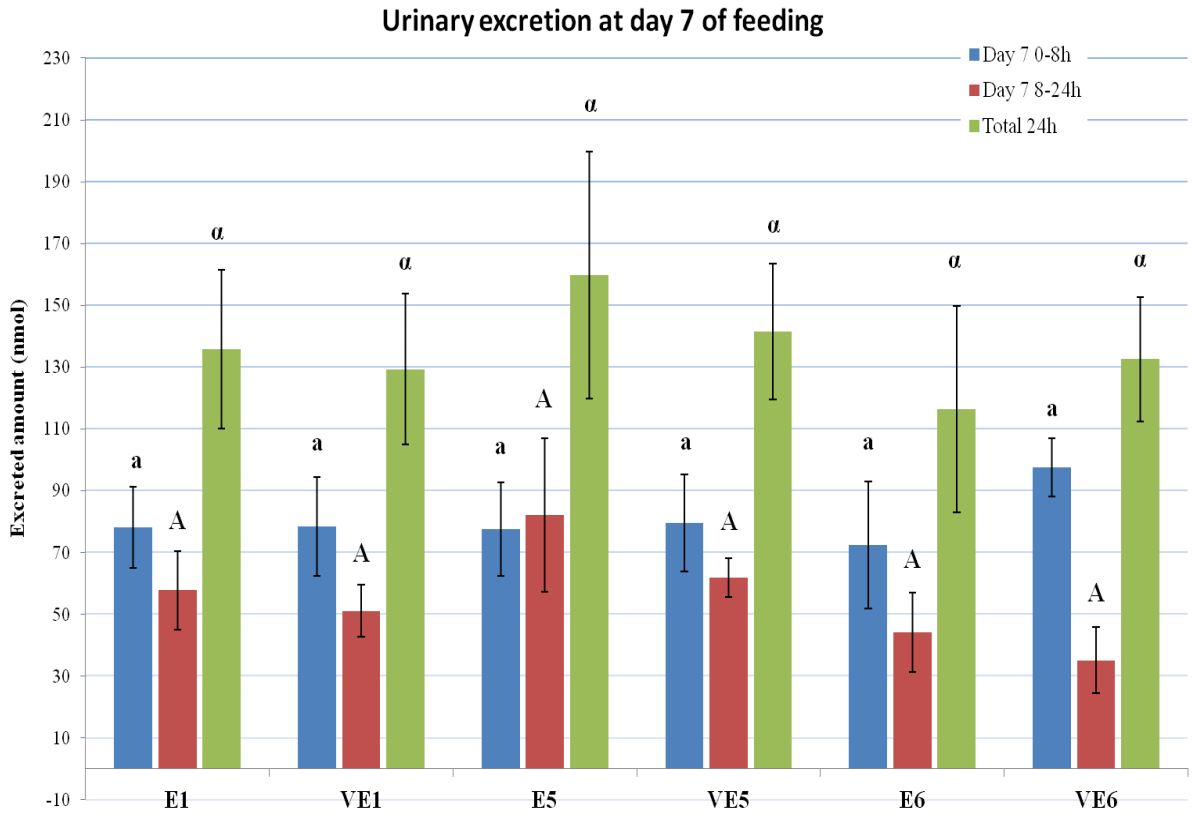


Figure 54: Total urinary metabolites excreted at day 7, 0-8 h, 8-24 h and 0-24h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α; Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 7. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in nmol ± standard error.

At day 1 of SHR rat gavage, substantial 0-8 h urinary excretion of metabolites was observed in the E1, VE1 and E5 experimental groups unlike E6 and VE6 in which highest excretion occurred in 8-24 h urine. Considering the 0-8 h samples, high urinary excretion was found in VE1 (117 ± 12 nmol) followed by E5 (80 ± 16 nmol) and E1 (70 ± 16 nmol). The lowest amount was in VE5 with only 2.9 ± 0.7 nmol of metabolites excreted. During 8-24 h collection period, excretion of metabolites was more pronounced with VE6 and E6 (88 ± 3 nmol and 80 ± 7 nmol respectively). VE1 showed the highest 0-24 h excretion metabolites with 175 ± 20 nmol. However, the excretion rate was not significantly

different from those obtained in E1 E5 E6 and VE6. Only VE5 presented a significant lower rate of excretion (6 ± 1.4 nmol).

Unlike day 1 in which high variability of excretion pattern was observed, at day 7, all SHR groups excreted metabolites in the same way with the highest amount at 0-8 h (from 98 ± 9 nmol in VE6 to 72 ± 21 nmol in E6) rather than 8-24 h time point (from 35 ± 11 nmol in VE6 to 82 ± 24 nmol in E5). No significant difference was observed between different experimental groups in both 0-8 h, 8-24 h and even over 0-24h. This phenomenon can be due to a high absorption and excretion reducing variability between subjects of the same group. Overall, total 24 h urinary excretion at day 7 ranged from 117 ± 33 nmol in E6 to 160 ± 40 nmol in E5 and was higher than that obtained at day 1 which varied from 6 ± 1.4 to 175 ± 20 nmol. The percentage of intake of grape pomace extracts administrated alone or in association with verapamil to SHR rats was calculated for each time point (Table 58).

Table 58: Percentage of intake of different grape pomace extracts excreted in urine at day 1 and day 7, 0-8 h, 8-24 h and 0-24 h after intake

Percentage of intake day 1 (%)

	E1 [a]	VE1 [a]	E5 [a]	VE5 [a]	E6 [a]	VE6 [a]
Day1 0-8 h	0.83	0.99	0.83	0.08	0.51	0.19
Day1 8-24 h	0.19	0.30	0.74	0.08	0.51	0.83
Total 24 h	1.02	1.29	1.57	0.16	1.02	1.02

Percentage of intake day 7 (%)

	E1 [a]	VE1 [a]	E5 [a]	VE5 [a]	E6 [a]	VE6 [a]
Day7 0-8 h	0.94	0.64	2.10	1.63	1.03	1.32
Day7 8-24 h	0.58	0.43	2.31	2.62	0.39	0.32
Total 24 h	1.52	1.08	4.41	4.25	1.43	1.64

[a] SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

At day 1, excretion as a percentage of intake varied from 0.16 % to 1.57 %. Highest recoveries were observed in the VE1 and E5 SHR groups (1.29 % and 1.57 % respectively, over 0-24 h). The lowest recovery was obtained in SHR group fed with Mourvèdre [MOU EA70] skin pomace extract + verapamil (0.16%). At day 7, an increase of intake was observed for the two collection periods and, as a consequence, over 0-24 h. The recoveries ranged from 1.08 % in VE1 to 4.25 % in VE5. A large increase in metabolite excretion

was observed in VE5 SHR group with 4.25% of intake at day 7 compared to 0.16 % at day 1. These observations suggested that over time, SHR rats may be able to ingest higher doses of polyphenols especially those contained in Mourvèdre [MOU EA70] skin pomace extract.

The recoveries obtained in this study were low compared to other studies. For instance, Tsang et al. (2005), found urinary excretion corresponding to 33% of intake 0-24 h after ingestion of a grape seed extract containing a wide array of monomeric to polymeric flavan-3-ols ingestion. In humans, urinary recovery of flavan-3-ols accounted 0.5-6 % of intake for some tea catechins (Lee et al. 1995; Yang et al. 2000), 2-10% for red wine catechins (Donovan et al. 2002a), and up to 30 % for cocoa epicatechin (Baba et al. 2000). However, higher amounts of flavan-3-ol monomers and polymers were ingested by humans than rats. Auger et al. (2008) showed in a green tea flavan-3-ol feeding study with ileostomists that (epi)gallocatechins are subjected to strictly limited absorption whereas (epi)catechins can be readily absorbed even with an increasing dose. Thus, the low amount of polyphenols fed in this study compared to other studies could be the cause of these recovery differences.

III.1.2. HPLC-ESI-MS^n analysis of SHR rat plasma

SHR rats plasma were screened for flavan-3-ol and procyanidin metabolites using, in the first instance, the full-scan MS mode from *m/z* 100 to 700 in negative ionization mode. Peak identifications was based on data dependent MS^2 and on previous studies (Tsang et al. 2005a; Touriño et al. 2009; Urpi-Sarda et al. 2009a; van der Hooft et al. 2012). Metabolites detected were listed in Table 59. Their levels, expressed as nmol ± SD, were determined using SRM, calculated by the same procedure to that used with urine.

Table 59: Identification of metabolites detected in SHR rat plasma at day 7, 4 h after ingestion of grape pomace extracts: HPLC and MS characteristics obtained in SRM mode

Peak	Rt (min)	Compounds	$[M-H]^-$ $(m/z)^a$	MS^2 fragment $(m/z)^b$
1	9.34	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 1	383	207, 163
2	9.4	(Epi)catechin-*O*-glucuronide 1	465	289, 245
3	11.03	(Epi)catechin-*O*-glucuronide 2	465	289, 245
4	13.3	*O*-Methyl-(epi)catechin-*O*-glucuronide 1	479	303, 223
5	15.05	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 2	383	207, 163
6	15.16	Methyl-(epi)catechin-*O*-glucuronide 2	479	303, 223
7	16.2	Methyl-(epi)catechin-*O*-glucuronide 3	479	303, 223
8	16.87	Di-methyl-(epi)catechin-*O*-glucuronide 1	493	493, 289
9	18.43	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate 2	305	225, 207
10	19.49	Di-methyl-(epi)catechin-*O*-glucuronide 2	493	493, 289
11	25.67	5-(Hydroxyphenyl)-γ-valerolactone sulfate 1	287	207, 163

[a]Tentative identification based on mass spectra, [b]molecular ion *(m/z)* identification in negative ionization mode. Rt, retention time; $[M-H]^-$, negatively charged molecular ion.

A total of 11 metabolites were found in SHR rat plasma collected 4 h after grape pomace extract ingestion. Plasma did not contain any of the original grape pomace extract flavan-3-ols and procyanidins but glucuronide, methyl-glucuronide and di-methyl-glucuronide of (epi)catechin and glucuronide, sulfate and mono-substituted valerolactone and valeric acid were present. These metabolites were also detected in urine with the exception of the di-methyl-(epi)catechin-*O*-glucuronide. Metabolites were occurred mainly as glucuronides rather than sulfates. These observations were in accordance with a previous study by Tsang et al. (2005) in which rats were fed grape seed extracts. These investigators detected only glucuronidated forms of (epi)catechins in plasma while sulfates predominated in urine.

Quantitative data are presented in Table 60. Generally, lower amounts of metabolites was observed in plasma than in urine showing that flavan-3-ols are rapidly turned over the circulatory system, and, rather than accumulating, are excreted via the kidneys. Figure 55, summarizes plasma metabolite amounts in different experimental group at day 7, 4 hours after grape pomace extracts ingestion *($p < 0.05$)* and their respective recoveries (%).

Table 60: Mean plasma metabolite levels at day 7, 4 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SD.

		E1		VE1		E5		VE5		E6		VE6	
Peak	**Metabolites**	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 1	**0.62***	**0.06**	0.31	0.02	0.71	0.02	**0.63***	**0.01**	0.46	0.01	0.40	0.07
2	(Epi)catechin-*O*-glucuronide 1	Nd	Nd	0.05	0.05	Nd	Nd	Nd	Nd	**0.25***	**0.02**	Nd	Nd
3	(Epi)catechin-*O*-glucuronide 2	**0.34***	**0.01**	0.34	0.04	0.32	0.03	**0.29***	**0.00**	**0.39***	**0.03**	0.25	0.02
4	*O*-Methyl-(epi)catechin-*O*-glucuronide 1	**0.24***	**0.02**	0.16	0.01	**0.17***	**0.02**	**0.33***	**0.01**	**0.23***	**0.01**	**0.13***	**0.00**
5	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 2	**0.26***	**0.01**	0.16	0.02	**0.31***	**0.00**	0.23	0.02	**0.22***	**0.01**	0.18	0.01
6	Methyl-(epi)catechin-*O*-glucuronide 2	**0.62***	**0.05**	**0.62***	**0.01**	0.41	0.00	0.41	0.04	**0.71***	**0.04**	**0.60***	**0.04**
7	Methyl-(epi)catechin-*O*-glucuronide 3	Nd	Nd	**0.26***	**0.01**	Nd	Nd	Nd	Nd	**0.37***	**0.01**	**0.33***	**0.01**
8	Di-methyl-(epi)catechin-*O*-glucuronide 1	**0.54***	**0.06**	0.00	0.00	**0.86***	**0.03**	**1.99***	**0.03**	**0.50***	**0.02**	Nd	Nd
9	5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulfate 2	Nd	Nd	**0.20***	**0.01**	Nd	Nd	Nd	Nd	Nd	Nd	**0.35***	**0.02**
10	Di-methyl-(epi)catechin-*O*-glucuronide 2	**0.42***	**0.04**	Nd	Nd	**0.35***	**0.04**	**0.83***	**0.00**	**0.19***	**0.01**	Nd	Nd
11	5-(Hydroxyphenyl)-γ-valerolactone sulfate 1	**0.42***	**0.04**	**0.80***	**0.05**	**0.31***	**0.05**	**0.49***	**0.01**	**0.42***	**0.06**	**1.08***	**0.04**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

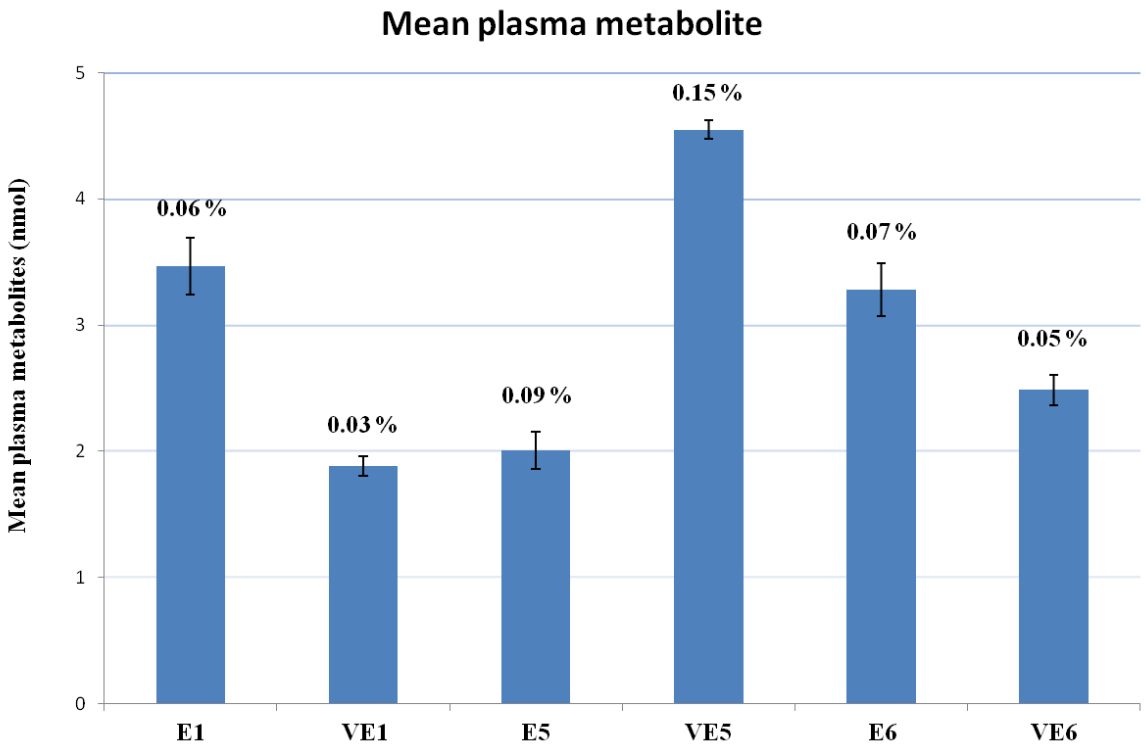


Figure 55: Mean plasma metabolite levels in different experimental group at day 7, 4 h after ingestion of grape pomace extracts *(p< 0.05)* and their respective recoveries (%). Data expressed as nmol ± standard deviation. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

The highest metabolite amount was evidenced in plasma from VE5 experimental group in which SHR rats were fed MOU (EA70) skin pomace extracts + verapamil. Up to 4.6 ± 0.07 nmol and a recovery of 0.15 % were observed compared to other plasma in which only 0.03-0.09 % recoveries were found. These results are in accordance with those obtained with urine, showing a high intake of grape pomace polyphenols in VE5 experimental group (4.25 %). E5 urinary excretion indicated substantial absorption of polyphenols (4.41 % of intake) but a recovery of 0.09 % in plasma implies a rapid turnover of polyphenols in the circulatory system. Grape pomace polyphenols were absorbed to a lesser extent by rats fed with other extracts (i.e. E1, VE1, E6 and VE6), as illustrated by low recoveries of metabolites in urine and plasma.

III.1.3. HPLC-ESI-MS^n analysis of SHR rat tissues (heart, liver and kidneys)

Metabolites in SHR rat tissues were detected using the same procedure as described for urine and plasma. No (epi)catechin conjugates were found but a 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide (*m/z* 383/207, 163) and a 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate (*m/z* 287/207, 163) were detected and quantified by SRM. Their distribution in tissues is presented in Table 61. Data are expressed as nmol per organ ± standard deviation. Both glucuronide and sulfate metabolites of the 5-(hydroxyphenyl)-γ-valerolactone were detected but the glucuronide predominated.

Heart tissues contained more glucuronidated metabolites than sulfated, ranging from 1.32 ± 0.02 nmol in VE6 to 2.64 ± 0.03 nmol in E5. Significantly higher amounts were observed in the E1, VE1, E5, VE5 and E6 experimental groups. 5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate, was detected only in E1 and E5 experimental groups at a level of 0.11 ± 0.00 nmol.

Kidneys contained more 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate than the liver and the amount was significantly higher in all the SHR experimental group which received grape pomace extracts compared to control groups. Rats from VE5 experimental group contained up to 9.19 ± 0.85 nmol of 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide followed by VE6 group which retained 7.73 ± 0.65 nmol.

Liver contained the highest level of metabolites with most present as glucuronides. A 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate was only detected in SHR rats fed with ALI (EA70) skin pomace extract + verapamil at 7.24 ± 0.6 nmol. Only SHR from E5, VE5 and VE6 experimental groups contained significantly higher amounts (210 ± 12 nmol, 198 ± 3 nmol and 194 ± 7 nmol, respectively) of 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide compared to control groups.

Table 61: Mean tissue metabolite levels at day 7, after 4 hours of grape pomace extracts ingestion. Data are expressed as nmol per organ ± standard deviation.

Peak	Heart	E1		VE1		E5		VE5		E6		VE6	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**2.50***	**0.25**	**2.04***	**0.23**	**2.64***	**0.03**	**1.83***	**0.07**	**2.27***	**0.08**	1.32	0.02
2	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	**0.11***	**0.00**	Nd	Nd	**0.11***	**0.00**	Nd	Nd	Nd	Nd	Nd	Nd

Peak	Kidneys	E1		VE1		E5		VE5		E6		VE6	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	5.55	0.11	6.27	0.07	6.62	0.08	**9.19***	**0.85**	5.23	0.34	**7.73***	**0.65**
2	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	**0.38***	**0.00**	**1.55***	**0.07**	**0.37***	**0.02**	**0.55***	**0.04**	**0.45***	**0.03**	**1.34***	**0.05**

Peak	Liver	E1		VE1		E5		VE5		E6		VE6	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	167.19	8.77	180.88	3.44	**209.98***	**12.20**	**197.73***	**2.87**	179.26	0.25	**193.64***	**6.53**
2	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulfate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	**7.24***	**0.58**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p< 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

Table 62: Metabolites in heart, kidneys and liver as a % of polyphenol intake

Heart	**E1[a]**	**VE1[a]**	**E5[a]**	**VE5[a]**	**E6[a]**	**VE6[a]**
% intake	0.024	0.011	0.059	0.016	0.022	0.000
Kidneys	**E1[a]**	**VE1[a]**	**E5[a]**	**VE5[a]**	**E6[a]**	**VE6[a]**
% intake	0.005	0.025	0.010	0.137	0.008	0.063
Liver	**E1[a]**	**VE1[a]**	**E5[a]**	**VE5[a]**	**E6[a]**	**VE6[a]**
% intake	Nd	Nd	1.76	1.64	Nd	1.02

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

It has to be noted that rats fed with MOU (EA70) skin pomace extract + verapamil (VE5 SHR group) contained high amounts of these metabolites and with higher accumulation after ingestion of MOU (EA70) skin pomace extract + verapamil than other extracts, especially in the kidneys and liver (0.14 % and 1.64 %, respectively) (Table 62). A higher accumulation of metabolites in rats fed with grape pomace extracts + verapamil was observed in kidneys. Accumulation of metabolites was 5-fold higher in VE1 than E1, 14-fold higher in VE5 than E5 and 8-fold higher in VE6 than E6.In contrast, the heart presented 2-fold and 4-fold higher accumulation of polyphenol metabolites in rats fed with grape pomace extracts without co-ingestion with verapamil in E1 and E5, respectively. No significant accumulation was found in VE6 heart tissues compared to E6 which contained metabolites corresponding to 0.022 % of the ingested polyphenols.

The absorption, and arguably metabolism, of flavan-3-ols and procyanidins initially takes place during transfer through the wall of the small intestine after which further phase II metabolism occurs in the liver. As liver appear to be the most important organs involved in flavonoid metabolism, it was not surprising to find such a high level of metabolites. Some of the conjugated metabolites can be actively effluxed back into the lumen of the small intestinal and/or may be transported to other organs through the bloodstream as shown by their presence in the heart. In addition, metabolites were also detected in kidneys no doubt as a consequence of renal excretion. It should be noted that the time of tissue sampling may be of importance, and metabolites detection depend on the kinetics of their accumulation and

elimination in the tissues. In this study, tissues which were shown to contain metabolites were collected 4 h after the ingestion of grape pomace extracts. Tsang et al. (2005) found flavan-3-ol metabolites in the liver of rats 1 h and 4 h after ingestion of a grape seed extract but none were detected 6, 12 and 24 h after intake. This indicates a rapid elimination of flavan-3-ol metabolites in keeping with them being treated as xenobiotics by the body.

III.1.4. HPLC-ESI-MS^n analysis of SHR rat faeces

In the first instance, faecal extracts were subjected to normal phase HPLC with mass spectrometry and fluorescence detection for the analysis of procyanidins polymers. Faeces were then screened for the original grape pomace flavan-3-ols and procyanidins using HPLC-MS^n in SIM mode (selected ion monitoring). Seven compounds were partially identified and quantified, including (epi)catechin (*m/z* 289/245, 205), (epi)catechin-3-*O*-gallate (*m/z* 441/289,169), two dimers (*m/z* 577/425, 407, 289), one trimer (*m/z* 865/577, 289), two (epi)gallocatechin trimers (*m/z* 881, 729, 577, 407) and one tetramer (*m/z* 153/865, 577). Quantitative data are presented in Table 63.

Table 63: Faecal excretion of polyphenolics at day 1 and day 7 after ingestion of grape pomace extracts. Data are expressed in nmol ± SD.

J1 0-8h	**E1**		**VE1**		**E5**		**VE5**		**E6**		**VE6**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(Epi)catechin	**3.6***	**0.0**	**3.9***	**0.2**	**4.2***	**0.3**	2.1	0.1	**3.2***	**0.0**	**4.5**	**0.2**
(Epi)catechin-*O*-gallate	Nd	Nd	Nd	Nd	Nd			Nd	Nd			
Dimer	1.8	0.0	**2.1***	**0.1**	2.1	0.2	0.6	0.0	2.1	0.2	**2.5**	**0.1**
Trimer	Nd	Nd	Nd	Nd	Nd			Nd	Nd			
(Epi)gallocatechin trimer 1	**1.4***	**0.0**	**1.4***	**0.1**	**1.3***	**0.0**		Nd	**1.2***	**0.0**	**1.4**	**0.0**
(Epi)gallocatechin trimer 2	1.7	0.0	Nd	Nd	1.5	0.1		Nd	1.8	0.0	1.3	0.0
Tetramer	**5.5***	**0.2**	2.0	0.1	**3.7***	**0.1**	1.8	0.1	**3.9***	**0.2**	**9.3**	**0.5**
J1 8-24 h	**E1**		**VE1**		**E5**		**VE5**		**E6**		**VE6**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(Epi)catechin	**32.9***	**1.2**	**33.7***	**1.6**	**34.3***	**1.7**	27.2	1.6	27.5	1.1	28.5	2.5
(Epi)catechin-*O*-gallate	Nd	Nd	**37.1***	**1.4**	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Dimer	28.7	0.6	27.1	1.1	27.3	2.5	**31.1***	**1.2**	31.4	1.6	**28.3***	**0.6**
Trimer	9.3	0.4	9.5	0.9	8.9	0.6	10.8	0.7	**11.5***	**0.9**	10.3	1.0
(Epi)gallocatechin trimer 1	**17.3***	**0.1**	17.4	0.9	**19.4***	**0.8**	**21.9***	**1.5**	**20.7***	**0.9**	15.4	0.5
(Epi)gallocatechin trimer 2	7.3	0.3	11.8	0.2	13.5	1.1	**16.8***	**0.4**	**11.1***	**0.7**	**11.0***	**0.1**
Tetramer	**52.3***	**0.6**	20.7	1.1	**51.6***	**3.4**	23.3	1.8	**27.1***	**0.7**	28.5	2.2
J7 0-8 h	**E1**		**VE1**		**E5**		**VE5**		**E6**		**VE6**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(Epi)catechin	4.7	0.1	**6.5***	**0.2**	5.0	0.3	5.0	0.2	4.1	0.1	**7.6***	**0.3**
(Epi)catechin-*O*-gallate	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Dimer	2.2	0.2	3.0	0.1	2.7	0.1	2.9	0.1	4.3	0.4	3.4	0.1
Trimer	Nd	Nd	Nd	Nd	**2.2***	**0.1**	Nd	Nd	Nd	Nd	2.3	0.2
(Epi)gallocatechin trimer 1	2.7	0.1	3.0	0.1	3.9	0.4	**3.4***	**0.0**	**4.7***	**0.2**	**4.7***	**0.3**

(Epi)gallocatechin trimer 2	**2.5***	**0.1**	2.0	0.0	**2.6***	**0.2**	1.9	0.1	**3.0***	**0.1**	2.5	0.2
Tetramer	**11.0***	**0.4**	4.1	0.1	4.7	0.2	1.8	0.1	**6.5***	**0.1**	7.8	0.6

J7 8-24 h	**E1**		**VE1**		**E5**		**VE5**		**E6**		**VE6**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(Epi)catechin	**31.0***	**1.0**	**35.7***	**2.4**	**34.1***	**1.3**	**44.6***	**2.8**	**32.2***	**1.0**	**41.8***	**3.3**
(Epi)catechin-*O*-gallate	Nd		**29.1***	**0.2**	Nd	Nd	**42.5***	**2.1**	Nd	Nd	**29.2***	**1.6**
Dimer	17.4	0.2	**22.9***	**1.4**	17.6	0.2	**25.8***	**0.1**	**27.3***	**0.3**	**17.2***	**1.3**
Trimer	4.4	0.1	**7.2***	**0.2**	7.1	0.1	**10.0***	**0.9**	7.7	0.1	**9.3***	**0.3**
(Epi)gallocatechin trimer 1	12.6	1.0	**15.9***	**1.2**	12.6	0.9	**29.6***	**1.6**	**19.7***	**0.6**	**19.3***	**1.3**
(Epi)gallocatechin trimer 2	**13.3***	**1.4**	**15.1***	**0.2**	**12.5***	**0.9**	**13.1***	**0.5**	**19.4***	**0.2**	**15.3***	**0.7**
Tetramer	**68.1***	**3.5**	25.9	0.8	**33.5***	**1.6**	21.8	0.1	**27.8***	**0.4**	27.5	1.8

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

In order to have a clear picture, Figures 56 and 57 illustrate the total excreted compounds in faeces at day 1 and day 7, 0-8 h and 8-24 h and 0-24 h after pomace extract intake.

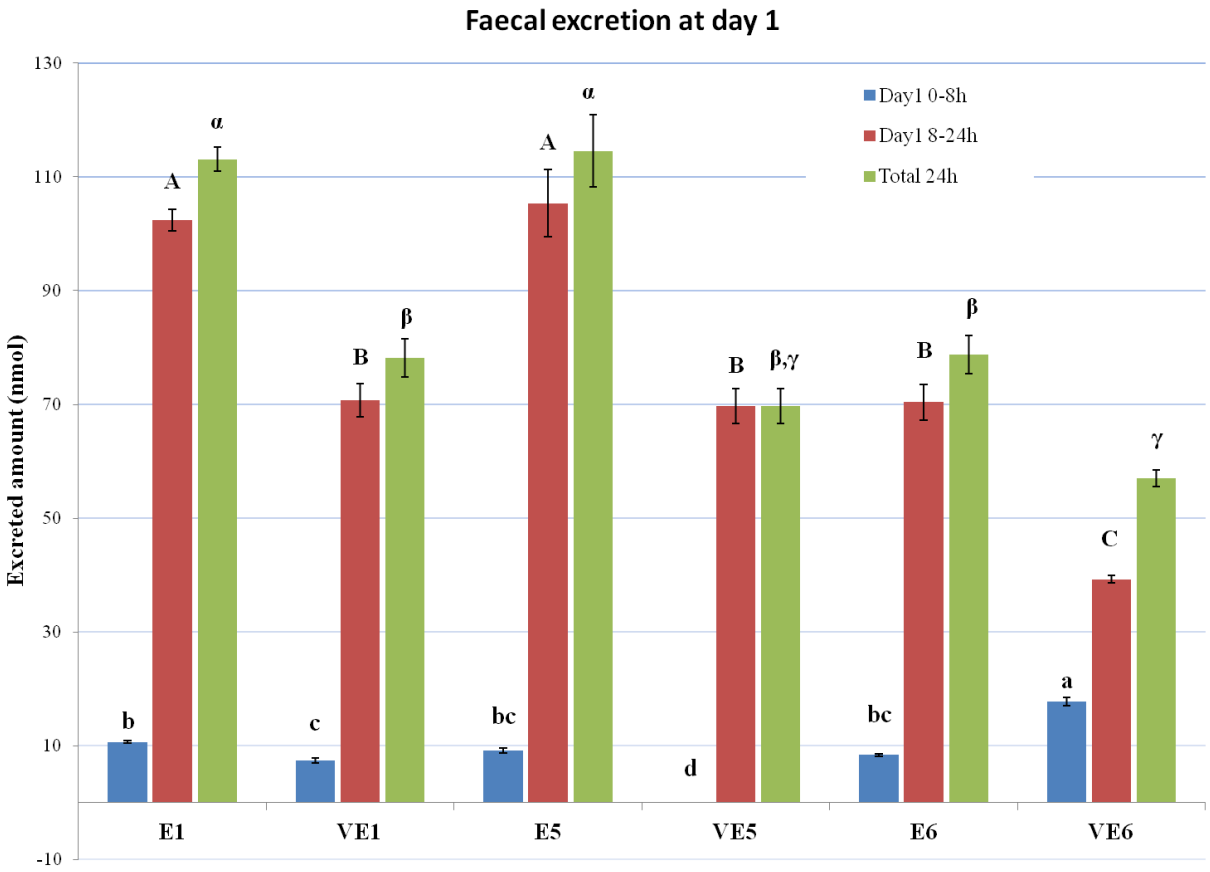


Figure 56: Total faecal excretion of phenolic metabolites in nmol at day, 0-8h, 8-24h and 0-24h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract + verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α; Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 1. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in nmol ± Standard deviation.

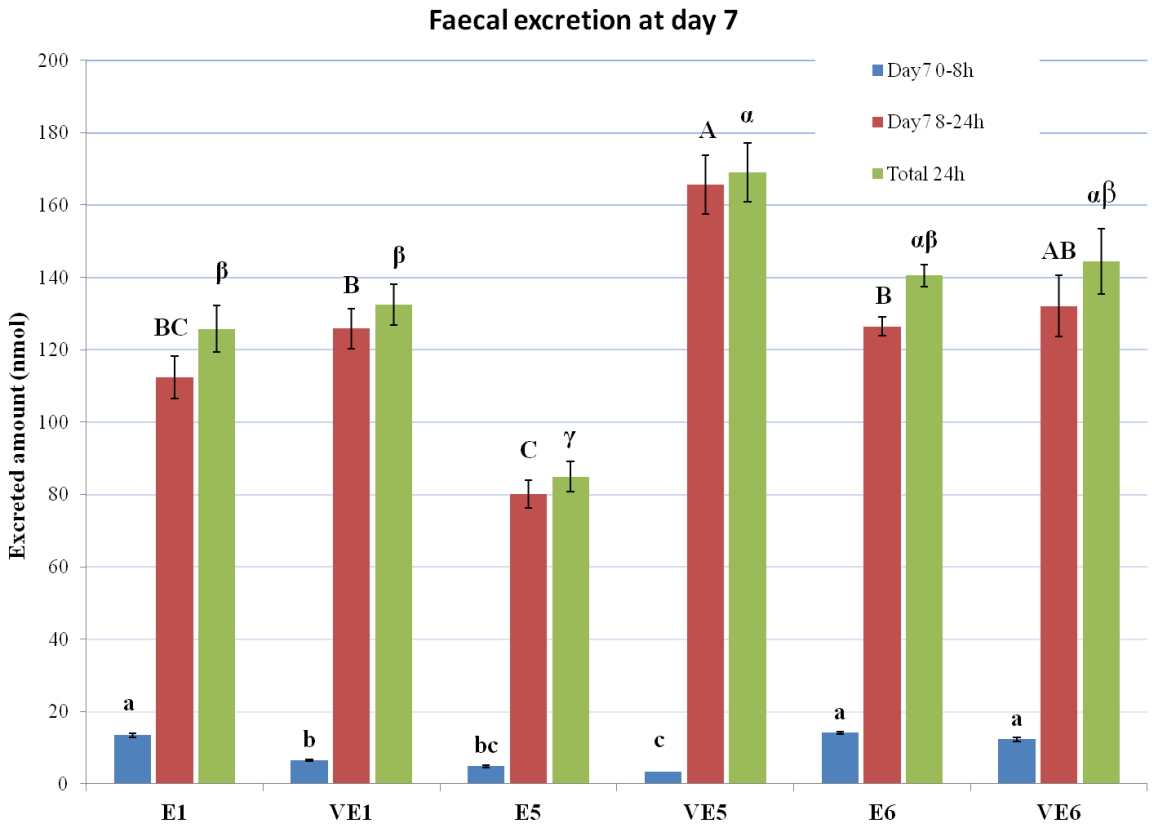


Figure 57: Total faecal excretion of phenolic metabolites in nmol at day 7, 0-8 h, 8-24 h and 0-24 h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract + verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α; Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 7. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in nmol ± standard deviation.

Faecal phenolic excretion occurred predominantly at 8-24 h after pomace intake. At day 1, SHR rats fed with GRE1 (EA70) seed pomace extract and MOU (EA70) skin pomace extract excreted the highest amount of flavan-3-ol monomers and oligomers over the 0-24 h collection period (113 ± 0.1 nmol and 115 ± 0.1 nmol, respectively) corresponding to 1.05 % and 2.37 % of polyphenolic intake (Table 64). The lowest excretion was observed in VE6 SHR group (57 ± 0.2 nmol over 24 h, 0.3 % of polyphenolic intake). At day 7, unlike day 1, higher level of flavan-3-ol monomers and oligomers was observed in faeces from VE5 SHR group (169 ± 0.01 nmol) and corresponding to up to 3.93 % of the intake of grape pomace polyphenols.

Table 64: Total faecal excretion of phenolic compounds as % of intake 0-8 h, 8-24 h and 0-24 h after the ingestion of different grape pomace extracts at day 1 and day 7

Percentage of intake day 1

	E1[a]	**VE1[a]**	**E5[a]**	**VE5[a]**	**E6[a]**	**VE6[a]**
Day1 0-8 h	0.08	0.03	0.12	0.00	0.05	0.17
Day1 8-24 h	0.97	0.30	2.26	0.89	0.84	0.13
Total 0-24 h	1.05	0.34	2.37	0.89	0.88	0.30

Percentage of intake day 7

	E1[a]	**VE1[a]**	**E5[a]**	**VE5[a]**	**E6[a]**	**VE6[a]**
Day7 0-8 h	0.18	0.02	0.20	0.01	0.15	0.09
Day7 8-24 h	1.31	1.02	1.51	3.92	1.27	1.44
Total 0-24h	1.49	1.03	1.72	3.93	1.42	1.53

[a]SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract + verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

III.2. GC-MS analysis of urines and faeces

III.2.1 GC-MS analysis of urines

Urine samples at day 1 and day 7 were collected at 0-8 h and 8-24 h after feeding the different grape pomace extracts by gavage. The phenolic acids in urine and faecal samples were analysed quantitatively by GC-MS. Catabolites detected represented urinary excretion of phenolic acids in their unconjugated form.

A total of 21 phenolic acids and aromatic compounds were identified in urines of SHR rats. In SHR group fed with grape pomace extracts, the production of some compounds increased significantly. Thus, only catabolites that are excreted in significantly higher amounts than the control were taken into account in order to exclude the background noise. Information of the 21 phenolic acid GC-MS properties identified in urine is presented in Table 65.

Table 65: GC-MS identification of phenolic acids detected in urines following ingestion of seed or skin grape pomace extracts

Rt (min)	Compounds	Target ion *(m/z)*[a]	Qualifier ion *(m/z)*[a]
6.19	Benzoic acid	179	105, 135, 77, 147
6.72	Pyrocatechol (1,2-dihydroxybenzene)	73	254, 239
9.73	3-Hydroxybenzoic acid	267	73, 147, 193, 223, 282, 253
10.03	Phenylacetic acid	193	147 73, 220, 267, 295
10.49	3′-Hydroxyphenylacetic acid	73	164, 147, 296, 281
10.91	4-Hydroxybenzoic acid	267	223, 193, 73, 282
11.14	4′-Hydroxyphenylacetic acid	73	179, 164. 252, 281, 296
12.98	3′,4′-Dimethoxyphenylacetic acid	209	73, 268, 151, 253
13.28	3-(3′-Hydroxyphenyl)propionic acid	205	192, 310, 177,73
14.16	3-(3′,4′-Dihydroxyphenyl)propionic acid	179	73, 192, 174, 310
14.28	3-Methoxy-4-hydroxybenzoic acid	297	267, 312, 73, 223, 253, 282, 126, 193
14.46	4′-Hydroxy-3′-methoxyphenylacetic acid	73	326, 209, 311, 179, 267, 147
14.76	4′-Hydroxmandelic acid	267	73, 147, 341
15.55	Hippuric acid	105	206, 73
18.65	3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	209	340, 73, 192, 79, 310, 325
20.41	*p*-Coumaric acid	219	293, 308, 73, 249
21.91	Gallic acid	281	398, 293, 73
27.11	Ferulic acid	338	309, 323, 247, 73, 293
29.47	Caffeic acid	396	219, 291, 73, 307, 381
29.70	3′-Hydroxyhippuric acid	294	73, 193, 147
33.37	Sinapic acid	193	294, 73, 147

[a,] based on the retention time and mass spectra of commercially available standards and from previous published works (Roowi et al. 2010; Stalmach et al. 2013).

Some catabolites detected in this study have been previously showed to derived from procyanidins and flavan-3-ol monomers (Aura et al. 2008; Touriño et al. 2009; Roowi et al. 2010). An investigation by Ward et al. (2004) reported significant increase in 3-(3′-hydroxyphenyl)propionic and 3′-hydroxyphenylacetic acids in 24-h urine samples after regular consumption of grape seed polyphenols in humans compared with a placebo group. The 3-(3′-hydroxyphenyl)propionic acid was evidenced as an important metabolite of procyanidins in humans. Another study by Cueva et al. (2013), showed by *in vitro* fermentation of grape seed momomeric and oligomeric flavan-3-ol fractions with human faecal microbiota that fraction can influence subsequent stages of catabolism. Larger concentration of 3-(3′,4′-dihydroxyphenyl)propionic acid was produced rapidly once incubated with oligomeric fraction than monomeric one and was progressively transformed to 3-(4′-hydroxyphenyl)propionic acid and then to phenylpropionic acid. In addition, the detection of 3-(3′-hydroxyphenyl)propionic acid and 3-(3′,4′-dihydroxyphenyl)propionic acid indicated the capacity of microflora to carry out side chain hydrogenation and dehydroxylation of the phenyl ring of hydroxycinnamates (Chesson et al. 1999; Poquet et al. 2008). This phenomenon was already observed in urines analysed by HPLC-ESI-MS^n and has been discussed previously in paragraph III.1.1.b. Actually, Appeldoorn et al. (2009a), proposed that the colonic catabolism of procyanidin dimers involved the production of 3′,4′-dihydroxyphenylacetic acid, yielding 3′-hydroxyphenylacetic acid from the degradation of the upper unit, whereas the lower unit resulted in the formation of 5-(3′-4′-dihydroxyphenyl)-γ-valerolactone and 3-(3′-hydroxyphenyl)propionic acid.

Unlike previous study in which hydroxyphenyl-γ-valerolactone acids were found after grape seed extracts ingestion and products rich in flavan-3-ol monomers and polymers (Touriño et al. 2009; Roowi et al. 2010; Cueva et al. 2013), no unconjugated of hydroxyphenyl-γ-valerolactone acids was detected by GC-MS. However, their conjugated forms were found in urine and quantified by HPLC-ESI-MS^n. In addition, HPLC-ESI-MS^n analysis of SHR rat urines evidenced the presence of 3-(3′-hydroxyphenyl)propionic acid, 3-(3′,4′-dihydroxyphenyl)propionic acid, 3′,4′-dihydroxyphenylacetic acid and 3′-methoxy-4′-hydroxyphenylacetic acid in glucuronidated and sulfated form suggesting glucuronidation and sulfatation of these catabolites either in the wall of the colon or post-absorption in the liver and kidneys. Quantification results of catabolites are presented in Tables 66-69.

Table 66: Quantification of phenolic acids detected in rat urine 0-8 h 8-24 h and 0-24 h after ingestion of seed and skin grape pomace extracts. Data are expressed as µmol ± SD.

	E1			E5			E6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	**0.10 ± 0.00***	**0.12 ± 0.00***	**0.22 ± 0.00***	**0.06 ± 0.00***	**0.17 ± 0.00***	**0.23 ± 0.00***	0.05 ± 0.00	**0.15 ± 0.00***	**0.21 ± 0.01***
Pyrocatechol	0.18 ± 0.01	0.30 ± 0.01	0.48 ± 0.02	0.14 ± 0.00	**0.63 ± 0.02***	**0.77 ± 0.02***	0.14 ± 0.01	0.49 ± 0.03	0.63 ± 0.03
3-Hydroxybenzoic acid	**0.03 ± 0.00***	0.04 ± 0.00	0.08 ± 0.00	0.03 ± 0.00	**0.11 ± 0.00***	**0.13 ± 0.00***	0.02 ± 0.00	**0.06 ± 0.00***	0.09 ± 0.00
Phenylacetic acid	**0.06 ± 0.00***	0.07 ± 0.00	0.13 ± 0.01	0.05 ± 0.00	0.10 ± 0.00	0.15 ± 0.01	0.04 ± 0.00	0.08 ± 0.00	0.12 ± 0.00
3′-Hydroxyphenylacetic acid	**0.18 ± 0.00***	0.23 ± 0.01	0.41 ± 0.01	0.13 ± 0.01	**0.49 ± 0.02***	**0.62 ± 0.03***	0.12 ± 0.00	0.37 ± 0.02	0.49 ± 0.02
4-Hydroxybenzoic acid	**0.04 ± 0.00***	0.05 ± 0.00	0.09 ± 0.00	0.03 ± 0.00	**0.11 ± 0.01***	**0.15 ± 0.01***	0.03 ± 0.00	**0.08 ± 0.00***	**0.12 ± 0.00***
4′-Hydroxyphenylacetic acid	**0.55 ± 0.00***	0.96 ± 0.04	1.51 ± 0.04	0.48 ± 0.03	**1.93 ± 0.10***	**2.42 ± 0.12***	**0.54 ± 0.01***	1.48 ± 0.07	2.02 ± 0.08
3′,4′-Dimethoxyphenylacetic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd
3-(3′-Hydroxyphenyl)propionic acid	**0.36 ± 0.02***	0.93 ± 0.01	1.29 ± 0.03	0.29 ± 0.02	1.14 ± 0.07	1.43 ± 0.08	0.27 ± 0.01	**1.40 ± 0.07***	**1.67 ± 0.08***
3-(3′,4′-Dihydroxyphenyl)propionic acid	0.07 ± 0.00	0.11 ± 0.00	0.18 ± 0.00	0.07 ± 0.00	0.15 ± 0.01	0.22 ± 0.01	0.07 ± 0.00	**0.21 ± 0.01***	**0.28 ± 0.01***
3-Methoxy-4-hydroxybenzoic acid	**0.04 ± 0.00***	0.02 ± 0.00	0.06 ± 0.00	**0.03 ± 0.00***	**0.08 ± 0.00***	**0.11 ± 0.00***	0.02 ± 0.00	**0.04 ± 0.00***	**0.07 ± 0.00***
4′-Hydroxy-3′-methoxyphenylacetic acid	0.08 ± 0.00	0.14 ± 0.00	0.22 ± 0.00	0.07 ± 0.00	**0.19 ± 0.01***	0.27 ± 0.01*	0.06 ± 0.00	**0.18 ± 0.01***	0.24 ± 0.01
4′-Hydroxymandelic acid	**0.11 ± 0.00***	**0.04 ± 0.00***	**0.15 ± 0.00***	**0.10 ± 0.00***	**0.09 ± 0.00***	**0.19 ± 0.01***	**0.09 ± 0.00***	**0.10 ± 0.00***	**0.18 ± 0.01***
Hippuric acid	20.52 ± 0.73	33.83 ± 0.49	54.34 ± 1.22	16.22 ± 1.08	**47.39 ± 2.86***	63.61 ± 3.95	14.56 ± 1.54	42.24 ± 0.74	56.80 ± 2.28
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	**0.06 ± 0.00***	0.08 ± 0.00	0.14 ± 0.01	0.03 ± 0.00	0.08 ± 0.00	0.11 ± 0.00	0.03 ± 0.00	**0.13 ± 0.00***	**0.16 ± 0.00***
p-Coumaric acid	**0.03 ± 0.00***	0.07 ± 0.00	0.10 ± 0.00	**0.03 ± 0.00***	0.12 ± 0.01	**0.15 ± 0.01***	**0.05 ± 0.00***	**0.15 ± 0.01***	**0.20 ± 0.01***
Gallic acid	1.07 ± 0.03	1.26 ± 0.04	2.33 ± 0.07	0.95 ± 0.04	**5.43 ± 0.30***	**6.38 ± 0.34***	0.86 ± 0.03	2.79 ± 0.01	3.65 ± 0.04
Ferulic acid	0.13 ± 0.01	0.23 ± 0.01	0.35 ± 0.01	0.08 ± 0.00	**0.69 ± 0.00***	**0.76 ± 0.00***	0.06 ± 0.00	0.51 ± 0.03	0.56 ± 0.03
Caffeic acid	**0.01 ± 0.00***	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	**0.04 ± 0.00***	**0.04 ± 0.00***	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
3′-Hydroxyhippuric acid	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.02 ± 0.00	**0.09 ± 0.00***	**0.12 ± 0.00***	0.02 ± 0.00	**0.04 ± 0.00***	0.06 ± 0.00
Sinapic acid	0.04 ± 0.00	0.05 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	**0.10 ± 0.01***	**0.14 ± 0.01***	0.03 ± 0.00	**0.07 ± 0.00***	**0.10 ± 0.00***
Total	**23.66 ± 0.71**	**38.56 ± 0.63**	**62.22 ± 1.09**	**18.85 ± 1.19**	**59.14 ± 3.41***	**77.99 ± 4.60***	**17.05 ± 1.49**	**50.61 ± 1.02***	**67.65 ± 2.51**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *(p< 0.05)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; E5, Mourvèdre (MOU) EA70 skin pomace extract and E6, Alicante (ALI) EA70 skin pomace extract. SD, standard deviation.

Table 67: Quantification of phenolic acids detected in rat urine 0-8 h, 8-24 and 0-24 h after ingestion of seed and skin grape pomace extracts in association with verapamil. Data are expressed as µmol ± SD.

	VE1			VE5			VE6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	0.05 ± 0.00	0.15 ± 0.00	0.21 ± 0.00	0.05 ± 0.00	0.10 ± 0.00	0.15 ± 0.00	0.06 ± 0.00	0.11 ± 0.00	0.17 ± 0.01
Pyrocatechol	0.17 ± 0.01	0.40 ± 0.01	0.57 ± 0.02	0.18 ± 0.01	0.35 ± 0.01	0.53 ± 0.02	0.19 ± 0.01	**0.52 ± 0.00***	**0.72 ± 0.01***
3-Hydroxybenzoic acid	0.03 ± 0.00	0.05 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	**0.08 ± 0.00***	**0.11 ± 0.00***
3-Phenylacetic acid	**0.06 ± 0.00***	0.10 ± 0.00	**0.16 ± 0.00***	0.04 ± 0.00	0.09 ± 0.00	0.13 ± 0.01	0.05 ± 0.00	0.11 ± 0.00	0.16 ± 0.00
3′-Hydroxyphenylacetic acid	**0.15 ± 0.01***	0.36 ± 0.01	**0.52 ± 0.02***	0.10 ± 0.00	0.35 ± 0.02	0.45 ± 0.02	0.12 ± 0.00	**0.42 ± 0.00***	**0.54 ± 0.01***
4-Hydroxybenzoic acid	**0.04 ± 0.00***	**0.08 ± 0.00***	**0.12 ± 0.00***	0.03 ± 0.00	0.06 ± 0.00	0.10 ± 0.00	**0.04 ± 0.00***	**0.09 ± 0.00***	**0.13 ± 0.01***
4′-Hydroxyphenylacetic acid	0.56 ± 0.03	1.60 ± 0.02	**2.15 ± 0.05***	0.37 ± 0.01	1.53 ± 0.14	1.90 ± 0.14	0.54 ± 0.02	1.40 ± 0.01	1.95 ± 0.03
3′,4′-Dimethoxyphenylacetic acid	2.68 ± 0.05	1.28 ± 0.01	3.96 ± 0.06	2.19 ± 0.01	1.48 ± 0.05	3.67 ± 0.06	2.82 ± 0.06	1.79 ± 0.03	4.61 ± 0.08
3-(3′-Hydroxyphenyl)propionic acid	**0.31 ± 0.01***	**1.08 ± 0.01***	**1.39 ± 0.02***	0.22 ± 0.01	0.88 ± 0.06	1.10 ± 0.07	0.23 ± 0.01	**1.28 ± 0.04***	**1.51 ± 0.05***
3-(3′,4′-Dihydroxyphenyl)propionic acid	**0.05 ± 0.00***	0.13 ± 0.00	0.18 ± 0.01	0.04 ± 0.00	0.14 ± 0.01	0.18 ± 0.01	**0.05 ± 0.00***	0.17 ± 0.01	**0.22 ± 0.01***
3-Methoxy-4-hydroxybenzoic acid	**0.03 ± 0.00***	**0.03 ± 0.00***	**0.06 ± 0.00***	**0.03 ± 0.00***	0.02 ± 0.00	0.06 ± 0.00	**0.03 ± 0.00***	**0.05 ± 0.00***	**0.09 ± 0.00***
4′-Hydroxy-3′-methoxyphenylacetic acid	0.11 ± 0.01	0.22 ± 0.01	0.33 ± 0.01	0.10 ± 0.00	0.21 ± 0.00	0.31 ± 0.01	0.11 ± 0.00	**0.25 ± 0.00***	0.35 ± 0.01
4′-Hydroxymandelic acid	0.08 ± 0.00	nd	0.08 ± 0.00	0.09 ± 0.00	nd	0.09 ± 0.00	**0.13 ± 0.01***	nd	**0.13 ± 0.01***
Hippuric acid	15.16 ± 0.07	39.93 ± 0.65	55.10 ± 0.72	13.26 ± 0.40	34.39 ± 5.34	47.65 ± 5.74	**16.88 ± 0.67***	45.94 ± 1.02	**62.82 ± 1.69***
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	0.04 ± 0.00	0.08 ± 0.00	0.12 ± 0.00	0.03 ± 0.00	0.09 ± 0.00	0.11 ± 0.00	0.04 ± 0.00	**0.10 ± 0.01***	**0.14 ± 0.01***
p-Coumaric acid	**0.03 ± 0.00***	**0.11 ± 0.00***	**0.13 ± 0.00***	**0.03 ± 0.00***	0.10 ± 0.00	**0.13 ± 0.00***	**0.06 ± 0.00***	**0.18 ± 0.01***	**0.24 ± 0.01***
Gallic acid	0.99 ± 0.05	**2.79 ± 0.13***	**3.78 ± 0.18***	1.02 ± 0.05	1.70 ± 0.15	2.72 ± 0.20	**1.29 ± 0.04***	**4.16 ± 0.04***	**5.45 ± 0.08***
Ferulic acid	0.10 ± 0.00	**0.41 ± 0.02***	**0.50 ± 0.02***	0.07 ± 0.00	0.26 ± 0.01	0.33 ± 0.02	0.08 ± 0.00	**0.62 ± 0.00***	**0.70 ± 0.00***
Caffeic acid	0.00 ± 0.00	**0.01 ± 0.00***	**0.01 ± 0.00***	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	**0.01 ± 0.00***	**0.02 ± 0.00***	**0.03 ± 0.00***
3′-Hydroxyhippuric acid	**0.02 ± 0.00***	**0.05 ± 0.00***	**0.07 ± 0.00***	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	**0.02 ± 0.00***	**0.07 ± 0.00***	**0.09 ± 0.00***
Sinapic acid	0.03 ± 0.00	**0.06 ± 0.00***	**0.08 ± 0.00***	0.03 ± 0.00	0.04 ± 0.00	0.07 ± 0.00	**0.04 ± 0.00***	**0.08 ± 0.00***	**0.11 ± 0.00***
Total	**20.70 ± 0.23**	**48.91 ± 0.49**	**69.61 ± 0.72**	**17.93 ± 0.50**	**41.86 ± 5.80**	**59.80 ± 6.30**	**22.81 ± 0.83**	**57.44 ± 1.03***	**80.25 ± 1.86***

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

Table 68: Quantification of phenolic acids detected in rat urine 0-8 h 8-24 h and 0-24 h after ingestion of seed and skin grape pomace extracts. Data are expressed as µmol ± SD.

	E1			E5			E6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	**0.73 ± 0.02***	**2.75 ± 0.11***	**3.48 ± 0.12***	0.09 ± 0.00	0.29 ± 0.01	0.38 ± 0.01	0.08 ± 0.00	0.25 ± 0.00	0.33 ± 0.01
Pyrocatechol	0.20 ± 0.01	1.20 ± 0.04	1.41 ± 0.05	0.26 ± 0.01	1.10 ± 0.02	1.36 ± 0.03	0.22 ± 0.01	1.37 ± 0.04	1.59 ± 0.05
3-Hydroxybenzoic acid	**0.04 ± 0.00***	0.21 ± 0.00	0.25 ± 0.00	**0.05 ± 0.00***	0.19 ± 0.01	0.24 ± 0.01	**0.05 ± 0.00***	0.18 ± 0.010	0.24 ± 0.01
3-Phenylacetic acid	0.04 ± 0.00	0.15 ± 0.00	0.20 ± 0.01	0.04 ± 0.00	0.12 ± 0.00	0.16 ± 0.00	0.04 ± 0.00	0.11 ± 0.00	0.15 ± 0.00
3′-Hydroxyphenylacetic acid	**0.18 ± 0.01***	0.70 ± 0.03	0.88 ± 0.03	**0.17 ± 0.00***	0.74 ± 0.03	0.91 ± 0.03	0.16 ± 0.00	0.61 ± 0.03	0.77 ± 0.03
4-Hydroxybenzoic acid	**0.04 ± 0.00***	**0.23 ± 0.00***	**0.27 ± 0.00***	**0.05 ± 0.00***	0.18 ± 0.00	0.24 ± 0.00	**0.05 ± 0.00***	0.18 ± 0.01	0.23 ± 0.01
4′-Hydroxyphenylacetic acid	**0.60 ± 0.01***	2.35 ± 0.10	2.95 ± 0.11	**0.64 ± 0.01***	2.29 ± 0.10	2.93 ± 0.11	**0.77 ± 0.03***	2.42 ± 0.07	3.19 ± 0.09
3′,4′-Dimethoxyphenylacetic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd
3-(3′-Hydroxyphenyl)propionic acid	**0.39 ± 0.00***	1.26 ± 0.04	1.65 ± 0.04	**0.41 ± 0.01***	1.24 ± 0.08	1.64 ± 0.09	**0.65 ± 0.01***	1.46 ± 0.08	**2.11 ± 0.09***
3-(3′,4′-Dihydroxyphenyl)propionic acid	**0.06 ± 0.00***	0.21 ± 0.00	**0.27 ± 0.00***	0.05 ± 0.00	0.17 ± 0.01	0.22 ± 0.01	**0.07 ± 0.00***	0.19 ± 0.00	0.26 ± 0.00
3-Methoxy-4-hydroxybenzoic acid	0.03 ± 0.00	0.15 ± 0.00	0.19 ± 0.00	**0.05 ± 0.00***	0.10 ± 0.00	0.15 ± 0.01	**0.05 ± 0.00***	0.13 ± 0.00	0.18 ± 0.00
4′-Hydroxy-3′-methoxyphenylacetic acid	**0.09 ± 0.00***	**0.29 ± 0.01***	**0.38 ± 0.01***	**0.10 ± 0.00***	0.24 ± 0.01	0.34 ± 0.01	**0.14 ± 0.00***	0.22 ± 0.00	**0.36 ± 0.00***
4′-Hydroxymandelic acid	0.08 ± 0.00	**0.37 ± 0.02***	**0.45 ± 0.02***	**0.13 ± 0.01***	**0.39 ± 0.01***	**0.52 ± 0.01***	0.06 ± 0.00	**0.36 ± 0.02***	**0.42 ± 0.02***
Hippuric acid	16.70 ± 1.34	48.80 ± 2.79	65.50 ± 4.14	**23.97 ± 0.37***	46.55 ± 6.22	70.52 ± 6.60	**26.93 ± 1.71***	49.23 ± 3.41	**76.16 ± 5.12***
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	**0.07 ± 0.00***	0.16 ± 0.00	**0.24 ± 0.00***	**0.06 ± 0.00***	**0.18 ± 0.00***	**0.24 ± 0.01***	0.05 ± 0.00	0.13 ± 0.00	0.18 ± 0.00
p-Coumaric acid	0.04 ± 0.00	0.18 ± 0.00	0.22 ± 0.01	**0.07 ± 0.00***	0.18 ± 0.00	0.25 ± 0.01	**0.11 ± 0.00***	0.17 ± 0.00	**0.27 ± 0.00***
Gallic acid	1.78 ± 0.11	8.03 ± 0.50	9.81 ± 0.62	**2.66 ± 0.10***	5.51 ± 0.07	8.17 ± 0.18	**2.73 ± 0.06***	5.97 ± 0.30	8.70 ± 0.37
Ferulic acid	**0.30 ± 0.01***	0.61 ± 0.02	0.91 ± 0.03	**0.37 ± 0.00***	0.64 ± 0.02	1.01 ± 0.03	**0.35 ± 0.02***	0.48 ± 0.02	0.84 ± 0.03
Caffeic acid	**0.01 ± 0.00***	0.05 ± 0.00	0.06 ± 0.00	**0.02 ± 0.00***	0.03 ± 0.00	0.05 ± 0.00	**0.01 ± 0.00***	0.03 ± 0.00	0.04 ± 0.00
3′-Hydroxyhippuric acid	0.02 ± 0.00	0.11 ± 0.00	0.13 ± 0.00	**0.03 ± 0.00***	0.11 ± 0.00	0.14 ± 0.00	**0.04 ± 0.00***	0.11 ± 0.00	0.15 ± 0.01
Sinapic acid	0.04 ± 0.00	0.16 ± 0.01	0.19 ± 0.01	**0.05 ± 0.00***	0.11 ± 0.00	0.16 ± 0.01	**0.04 ± 0.00***	0.12 ± 0.00	0.16 ± 0.00
Total	**21.46 ± 1.42**	**67.98 ± 1.94**	**89.44 ± 3.36**	**29.26 ± 0.50***	**60.35 ± 6.55**	**89.61 ± 7.05**	**32.60 ± 1.82***	**63.73 ± 3.99**	**96.33 ± 5.81**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *(p< 0.05)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; E5, Mourvèdre (MOU) EA70 skin pomace extract and E6, Alicante (ALI) EA70 skin pomace. SD, standard deviation.

Table 69: Quantification of phenolic acids detected in rat urine 0-8 h, 8-24 and 0-24 h after ingestion of seed and skin grape pomace extracts in association with verapamil. Data are expressed as µmol ± SD.

	VE1			VE5			VE6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	0.07 ± 0.00	0.15 ± 0.01	0.22 ± 0.01	0.06 ± 0.00	**0.20 ± 0.01***	**0.27 ± 0.01***	0.06 ± 0.00	0.17 ± 0.00	**0.23 ± 0.00***
Pyrocatechol	0.24 ± 0.00	**1.06 ± 0.01***	**1.30 ± 0.02***	**0.29 ± 0.02***	0.74 ± 0.01	1.03 ± 0.03	0.23 ± 0.00	0.83 ± 0.00	1.05 ± 0.01
3-Hydroxybenzoic acid	**0.04 ± 0.00***	**0.16 ± 0.00***	**0.20 ± 0.00***	**0.04 ± 0.00***	0.12 ± 0.00	**0.16 ± 0.00***	**0.03 ± 0.00***	**0.16 ± 0.01***	**0.20 ± 0.01***
3-Phenylacetic acid	**0.05 ± 0.00***	**0.11 ± 0.00***	**0.17 ± 0.00***	**0.05 ± 0.00***	**0.13 ± 0.00***	**0.18 ± 0.00***	0.04 ± 0.00	**0.12 ± 0.01***	**0.16 ± 0.01***
3′-Hydroxyphenylacetic acid	0.13 ± 0.00	**0.58 ± 0.03***	**0.71 ± 0.03***	0.14 ± 0.00	0.54 ± 0.01	0.68 ± 0.01	0.12 ± 0.00	0.53 ± 0.03	0.65 ± 0.03
4-Hydroxybenzoic acid	**0.04 ± 0.00***	**0.16 ± 0.00***	**0.20 ± 0.00***	**0.04 ± 0.00***	0.14 ± 0.01	**0.18 ± 0.01***	**0.04 ± 0.00***	0.14 ± 0.00	**0.18 ± 0.01***
4′-Hydroxyphenylacetic acid	**0.60 ± 0.00***	1.85 ± 0.07	2.45 ± 0.07	**0.62 ± 0.00***	2.11 ± 0.08	**2.73 ± 0.08***	0.48 ± 0.00	1.51 ± 0.07	1.99 ± 0.07
3′,4′-Dimethoxyphenylacetic acid	**2.27 ± 0.02***	1.09 ± 0.08	**3.36 ± 0.10***	**2.58 ± 0.01***	**1.34 ± 0.05***	**3.92 ± 0.06***	**2.14 ± 0.02***	**1.40 ± 0.02***	**3.54 ± 0.03***
3-(3′-Hydroxyphenyl)propionic acid	**0.33 ± 0.01***	**0.97 ± 0.00***	**1.30 ± 0.02***	**0.36 ± 0.00***	**1.04 ± 0.03***	**1.40 ± 0.03***	**0.31 ± 0.01***	**1.24 ± 0.03***	**1.54 ± 0.04***
3-(3′,4′-Dihydroxyphenyl)propionic acid	0.05 ± 0.00	0.14 ± 0.01	0.19 ± 0.01	0.05 ± 0.00	0.13 ± 0.00	0.18 ± 0.01	0.04 ± 0.00	**0.16 ± 0.00***	**0.20 ± 0.00***
3-Methoxy-4-hydroxybenzoic acid	**0.04 ± 0.00***	0.12 ± 0.01	**0.15 ± 0.01***	**0.04 ± 0.00***	0.09 ± 0.00	0.13 ± 0.00	**0.04 ± 0.00***	0.10 ± 0.01	0.14 ± 0.01
4′-Hydroxy-3′-methoxyphenylacetic acid	**0.12 ± 0.00***	**0.23 ± 0.00***	**0.35 ± 0.00***	**0.14 ± 0.00***	**0.23 ± 0.01***	**0.37 ± 0.01***	**0.10 ± 0.00***	**0.25 ± 0.00***	**0.35 ± 0.00***
4′-Hydroxymandelic acid	**0.08 ± 0.00***	0.19 ± 0.01	0.27 ± 0.02	**0.05 ± 0.00***	0.22 ± 0.02	0.27 ± 0.02	**0.07 ± 0.00***	0.24 ± 0.01	0.31 ± 0.01
Hippuric acid	**22.77 ± 0.10***	**41.99 ± 1.23***	**64.76 ± 1.33***	**20.5 ± 0.31***	**44.02 ± 0.36***	**64.52 ± 0.66***	16.15 ± 0.35	**46.99 ± 1.42***	**63.14 ± 1.77***
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	0.04 ± 0.00	0.11 ± 0.01	0.15 ± 0.01	0.04 ± 0.00	0.12 ± 0.01	0.16 ± 0.01	0.04 ± 0.00	0.11 ± 0.00	0.15 ± 0.01
p-Coumaric acid	**0.05 ± 0.00***	**0.17 ± 0.00***	**0.22 ± 0.00***	**0.07 ± 0.00***	**0.17 ± 0.01***	**0.24 ± 0.01***	**0.09 ± 0.00***	**0.18 ± 0.01***	**0.27 ± 0.01***
Gallic acid	**2.05 ± 0.13***	6.21 ± 0.23	**8.25 ± 0.36***	**1.83 ± 0.05***	4.34 ± 0.21	6.16 ± 0.26	**1.69 ± 0.08***	5.64 ± 0.20	7.33 ± 0.28
Ferulic acid	**0.27 ± 0.01***	**0.53 ± 0.02***	**0.80 ± 0.02***	**0.25 ± 0.01***	0.48 ± 0.03	0.73 ± 0.04	**0.23 ± 0.01***	**0.59 ± 0.00***	**0.83 ± 0.01***
Caffeic acid	**0.01 ± 0.00***	0.04 ± 0.00	0.06 ± 0.00	0.01 ± 0.00*	0.03 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
3′-Hydroxyhippuric acid	**0.02 ± 0.00***	**0.14 ± 0.00***	**0.17 ± 0.01***	**0.03 ± 0.00***	**0.1 ± 0.00***	**0.13 ± 0.00***	**0.02 ± 0.00***	**0.15 ± 0.00***	**0.17 ± 0.00***
Sinapic acid	**0.03 ± 0.00***	**0.15 ± 0.00***	**0.18 ± 0.00***	**0.03 ± 0.00***	0.1 ± 0.00	0.13 ± 0.00	**0.03 ± 0.00***	**0.14 ± 0.00***	**0.17 ± 0.00***
Total	**29.32 ± 0.02***	**56.15 ± 1.23***	**85.47 ± 1.25***	**27.21 ± 0.38***	**56.41 ± 0.63***	**83.62 ± 1.01***	**21.97 ± 0.47***	**60.67 ± 1.28***	**82.64 ± 1.74***

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

Total significant urinary catabolites excreted are illustrated in Figures 58 and 59.

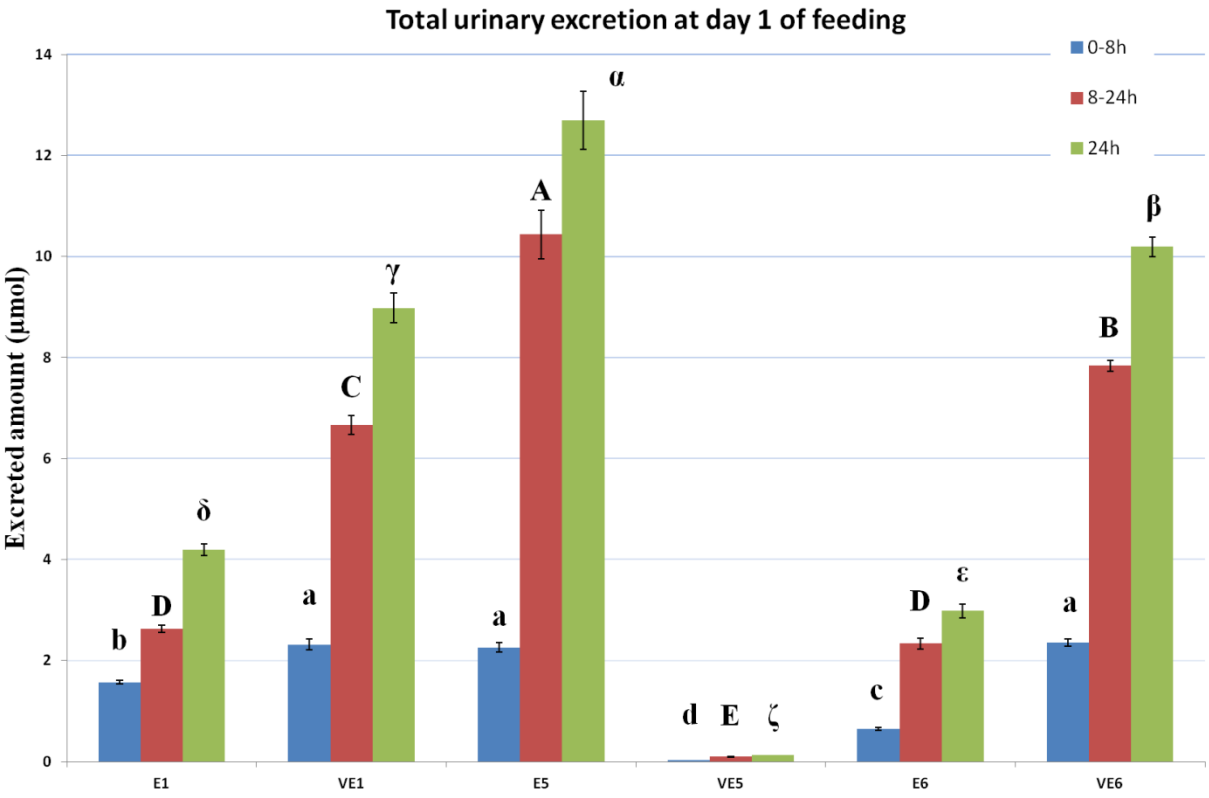


Figure 58: Total urinary catabolites excreted at day 1 during 0-8h, 8-24h and over 24h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α: Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 1. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in µmol ± Std.

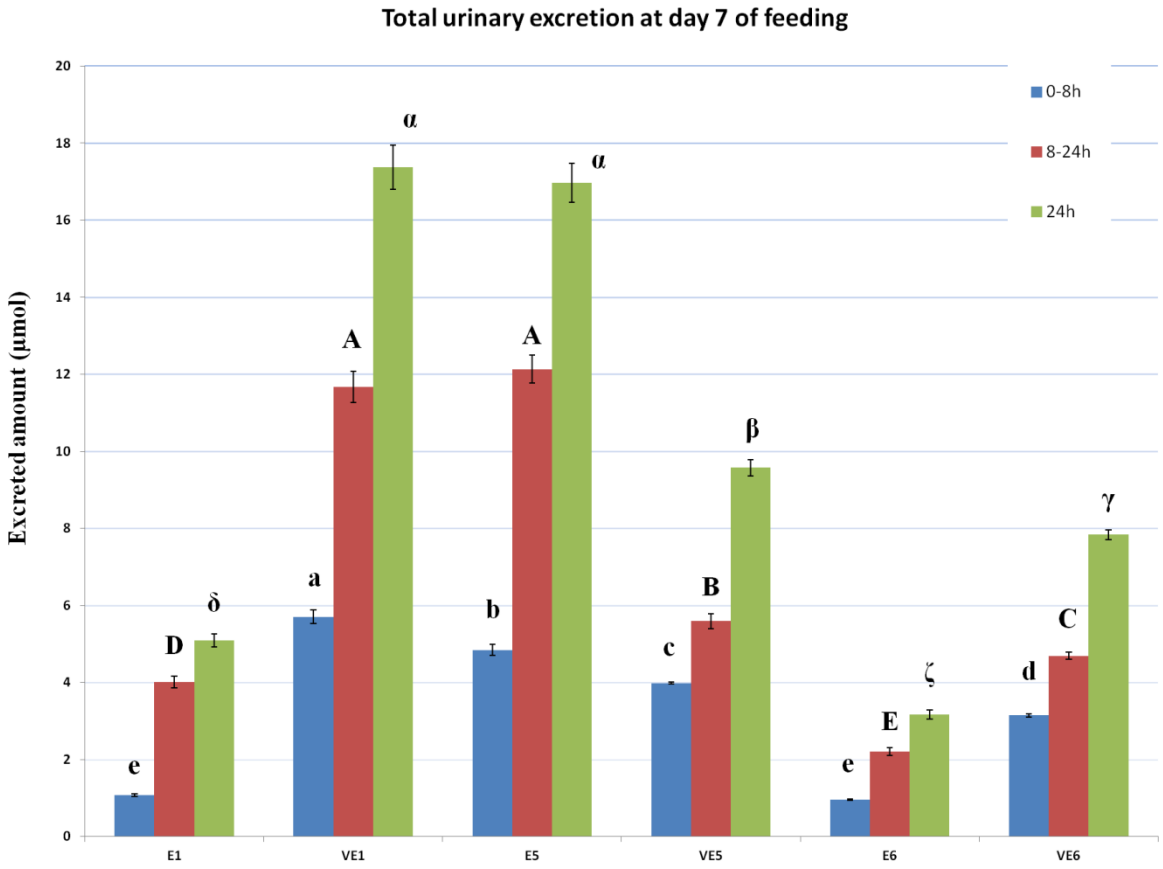


Figure 59: Total urinary catabolites excreted at day 7 during 0-8h, 8-24h and over 24h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α: Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 7. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in µmol ± Std.

The great majority of the phenolic acids were excreted in 8-24 h urine after ingestion of grape pomace extracts. Total significant catabolites excreted values over 24 h ranged from 0.16 µmol in VE5 to 12.7 µmol in E5 at day 1 and from 3.17 µmol in E6 to 17.4 µmol in VE1 at day 7. The maximum excretion rates were observed in rats fed with E5 followed by VE1 whether at day 1 or day 7.

At day 1, rats from E5 and VE6 experimental group absorbed higher amount of grape pomace polyphenols (182 % and 106% of intake, respectively) (Table 70) unlike rats fed with MOU (EA 70) skin pomace extract (i.e.VE5 group) which excreted catabolites at a level equivalent to only 1.2 % of ingested polyphenols. At day 7, an increase of absorption was evidenced in VE5 group (up to 102 % of intake) followed by E5 group (76%). Results are in accordance with those obtained by HPLC-ESI-MS^n which showed that solution fed to VE5 group (i.e. MOU (EA 70) skin pomace extract + verapamil) presented a low bioavailability at day 1 of gavage but increase at day 7 and metabolites were observed in high quantities in plasma and body tissues compared to other extracts. Using GC-MS, a difference of absorption between E1/VE1 and E6/VE6 was evidenced. At day 1, rats fed with GRE1 (EA70) seed pomace extract + verapamil absorbed 12-fold more grape pomace polyphenols than those fed with GRE1 (EA70) seed pomace extract alone and rats fed with ALI (EA70) skin pomace extract + verapamil absorbed 5-times more than those fed with extract without verapamil. At day 7, the same observation can be seen but to a lower extent (same absorption rate for E1/VE1 and 4-fold higher for E6/VE6).

Table 70: Percentage of intake of each type of extracts in urines analysed by GC-MS (in %)

	Day 1					
	E1	**VE1**	**E5**	**VE5**	**E6**	**VE6**
0-8h	2.0	6.1	0.0	0.7	1.9	11.9
8-24h	1.5	35.8	181.7	0.4	20.0	93.6
24h	3.5	41.9	181.7	1.2	22.0	105.5
	Day 7					
	E1	**VE1**	**E5**	**VE5**	**E6**	**VE6**
0-8h	14.2	31.7	69.7	53.5	12.6	17.3
8-24h	52.2	34.1	6.2	48.7	1.1	36.1
24h	66.4	65.8	75.9	102.2	13.7	53.4

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

Considering the percentage of intake, HPLC-ESI-MS^n results did not show clear differences in absorption between E1/VE1, E5/VE5 and E6/VE6 group in urine except for the fact that polyphenols from MOU (EA70) skin pomace extract had a 27-fold higher excretion at day 7 of gavage than at day 1 especially when associated with verapamil (VE5 group). However, a higher accumulation of metabolites in rats fed with grape pomace extracts + verapamil was observed in kidneys. Accumulation of metabolites was 5-fold higher in VE1 than E1, 14-fold in VE5 than E5 and 8-fold in VE6 than E6.

High percentage of intake evidenced by GC-MS might be explained by extensive activity of colonic microbiota which degraded phenolic compounds especially flavan-3-ol polymers to numerous smaller compounds such as phenolic acids. As grape pomace extracts contain monomeric, oligomeric and polymeric flavan-3-ols, different catabolism pathway might be involved as proposed by previous studies (Tzounis et al. 2008a; Appeldoorn et al. 2009a; Serra et al. 2009).

III.2.1 GC-MS analysis of faeces

Rat faeces were collected at 0-8 h and 8-24 h time points after grape pomace extracts ingestion at day 1 and day 7 of gavage. A total of 10 phenolic acids were detected in SHR faeces (Table 71).

Table 71: GC-MS identification of phenolic acids detected in faeces following ingestion of seed or skin grape pomace extracts

Rt (min)	Compounds	Target ion *(m/z)*[a]	Qualifier ion *(m/z)*[a]
6.22	Benzoic acid	179	105, 77, 135, 147, 194
6.60	Phenylacetic acid	73	147, 164, 193, 91
7.80	3-(Phenyl)propionic acid	104	75, 147, 207, 179, 222, 91
10.09	3-(Phenyl)lactic acid	193	73, 147, 220, 295
10.56	3′-Hydroxyphenylacetic acid	73	147, 164, 281, 296
10.88	4-Hydroxybenzoic acid	267	147, 223, 193, 282
11.21	4′-Hydroxyphenylacetic acid	73	179, 164, 296, 281, 252, 147
13.38	3-(3′-Hydroxyphenyl)propionic acid	205	192, 310, 177, 73
18.60	3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	340	209, 192, 73, 310. 179, 222
27.28	Ferulic acid	338	249, 323, 308, 293, 73, 147

[a] based on the retention time and mass spectra of commercially available standards and from previous published works (Roowi et al. 2010; Stalmach et al. 2013).

These catabolites were produced from ingested polyphenols degradation by colonic microbiota and represented catabolites which have not been absorbed in the colon. Tables 72-75 showed catabolite quantifications in faeces at day 1 and day 7 in SHR rats which received grape pomace extracts.

Table 72: Quantification of phenolic acids detected in rat faeces 0-8 h 8-24 h and 0-24 h after ingestion of seed and skin grape pomace extracts. Data are expressed as µmol ± SD.

	E1			E5			E6		
Compounds	**0-8h**	**8-24h**	**0-24h**	**0-8h**	**8-24h**	**0-24h**	**0-8h**	**8-24h**	**0-24h**
Benzoic acid	**0.35 ± 0.02***	0.67 ± 0.01	1.01 ± 0.02	**0.33 ± 0.02***	0.73 ± 0.00	**1.06 ± 0.02***	0.18 ± 0.03	0.50 ± 0.02	0.67 ± 0.05
Phenylacetic acid	**0.73 ± 0.05***	0.75 ± 0.02	1.48 ± 0.07	0.50 ± 0.01	0.78 ± 0.03	1.29 ± 0.05	0.43 ± 0.15	0.70 ± 0.02	1.13 ± 0.17
3'-Phenylpropionic acid	**1.61 ± 0.18***	0.78 ± 0.07	**2.40 ± 0.25***	1.08 ± 0.12	0.69 ± 0.07	1.77 ± 0.19	0.91 ± 0.37	0.49 ± 0.04	1.40 ± 0.41
3-(Phenyl)-lactic acid	**0.01 ± 0.00***	0.07 ± 0.00	0.08 ± 0.00	**0.01 ± 0.00***	0.06 ± 0.00	0.07 ± 0.00	**0.01 ± 0.00***	**0.08 ± 0.00***	**0.09 ± 0.00***
3'-Hydroxyphenylacetic acid	0.16 ± 0.01	**0.17 ± 0.00***	0.34 ± 0.01	0.18 ± 0.00	**0.22 ± 0.00***	**0.40 ± 0.00***	0.14 ± 0.05	0.10 ± 0.00	0.24 ± 0.05
4-Hydroxybenzoic acid	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	**0.03 ± 0.00***	**0.03 ± 0.00***	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
4'-Hydroxyphenylacetic acid	0.36 ± 0.00	**0.52 ± 0.01***	**0.88 ± 0.02***	**0.41 ± 0.00***	**0.43 ± 0.01***	**0.84 ± 0.01***	0.36 ± 0.09	0.23 ± 0.01	0.59 ± 0.10
3-(3'-Hydroxyphenyl)propionic acid	0.03 ± 0.00	**0.94 ± 0.02***	**0.97 ± 0.02***	0.09 ± 0.01	**0.77 ± 0.01***	**0.86 ± 0.02***	0.09 ± 0.01	0.59 ± 0.00	0.68 ± 0.01
3-(3'-Methoxy-4'-hydroxy)phenylpropionic acid	**0.01 ± 0.00***	**0.06 ± 0.00***	**0.07 ± 0.00***	**0.01 ± 0.00***	0.04 ± 0.00	**0.05 ± 0.01***	**0.01 ± 0.00***	**0.09 ± 0.01***	**0.10 ± 0.01***
Ferulic acid	0.03 ± 0.00	0.16 ± 0.00	0.19 ± 0.00	0.04 ± 0.00	0.10 ± 0.00	0.13 ± 0.00	0.03 ± 0.00	0.12 ± 0.00	0.15 ± 0.01
Total	**3.28 ± 0.27***	**4.15 ± 0.13**	**7.43 ± 0.40***	**2.66 ± 0.17**	**3.83 ± 0.13**	**6.49 ± 0.31**	**2.16 ± 0.71**	**2.92 ± 0.10**	**5.08 ± 0.81**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *(p< 0.05)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; E5, Mourvèdre (MOU) EA70 skin pomace extract and E6, Alicante (ALI) EA70 skin pomace extract. SD, standard deviation.

Table 73: Quantification of phenolic acids detected in rat faeces 0-8 h, 8-24 and 0-24 h after ingestion of seed and skin grape pomace extracts in association with verapamil. Data are expressed as µmol ± SD.

	VE1			VE5			VE6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	0.16 ± 0.01	**0.46 ± 0.03***	0.62 ± 0.03	0.18 ± 0.02	**0.50 ± 0.01***	0.68 ± 0.03	0.10 ± 0.00	0.38 ± 0.01	0.48 ± 0.02
Phenylacetic acid	0.24 ± 0.00	**0.86 ± 0.00***	1.11 ± 0.01	0.23 ± 0.03	**0.85 ± 0.02***	1.08 ± 0.05	0.19 ± 0.00	0.56 ± 0.01	0.75 ± 0.01
3'-Phenylpropionic acid	0.65 ± 0.01	**0.79 ± 0.01***	1.44 ± 0.02	0.55 ± 0.07	**0.98 ± 0.03***	1.53 ± 0.10	0.34 ± 0.01	**0.63 ± 0.01***	0.96 ± 0.03
3-(Phenyl)-lactic acid	0.01 ± 0.00	**0.10 ± 0.00***	**0.11 ± 0.00***	0.00 ± 0.00	**0.10 ± 0.00***	**0.10 ± 0.00***	0.01 ± 0.00	**0.06 ± 0.00***	**0.06 ± 0.00***
3'-Hydroxyphenylacetic acid	0.10 ± 0.00	**0.25 ± 0.00***	**0.35 ± 0.01***	0.07 ± 0.01	**0.22 ± 0.00***	**0.29 ± 0.01***	0.08 ± 0.00	0.10 ± 0.01	0.17 ± 0.01
4-Hydroxybenzoic acid	0.00 ± 0.00	**0.03 ± 0.00***	**0.03 ± 0.00***	0.00 ± 0.00	**0.03 ± 0.01***	0.03 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
4'-Hydroxyphenylacetic acid	0.21 ± 0.00	**0.63 ± 0.01***	**0.84 ± 0.01***	0.24 ± 0.02	**0.60 ± 0.00***	**0.84 ± 0.02***	0.13 ± 0.02	0.10 ± 0.00	0.23 ± 0.02
3-(3'-Hydroxyphenyl)propionic acid	0.12 ± 0.00	**0.99 ± 0.00***	**1.11 ± 0.01***	0.05 ± 0.00	**0.80 ± 0.01***	**0.85 ± 0.01***	**0.21 ± 0.01***	**0.50 ± 0.01***	**0.71 ± 0.02***
3-(3'-Methoxy-4'-hydroxy)phenylpropionic acid	nd	nd	nd	0.01 ± 0.00	**0.04 ± 0.00***	**0.04 ± 0.00***	nd	nd	nd
Ferulic acid	0.03 ± 0.00	**0.32 ± 0.00***	**0.34 ± 0.00***	0.03 ± 0.00	**0.21 ± 0.01***	**0.25 ± 0.01***	0.06 ± 0.00	**0.18 ± 0.00***	**0.23 ± 0.00***
Total	**1.52 ± 0.03**	**4.44 ± 0.06***	**5.96 ± 0.09***	**1.37 ± 0.15**	**4.33 ± 0.08***	**5.70 ± 0.24***	**1.11 ± 0.05**	**2.51 ± 0.06***	**3.62 ± 0.11**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

Table 74: Quantification of phenolic acids detected in rat faeces 0-8 h, 8- 24 h and 0-24 h after daily ingestion of seed and skin grape pomace extracts for a period of seven days. Data are expressed as µmol ± SD.

	E1			E5			E6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	**0.20 ± 0.01***	1.07 ± 0.03	1.27 ± 0.05	0.25 ± 0.01	0.82 ± 0.05	1.06 ± 0.06	0.15 ± 0.02	0.81 ± 0.05	0.96 ± 0.08
Phenylacetic acid	0.53 ± 0.03	**2.14 ± 0.00***	**2.67 ± 0.03***	0.48 ± 0.01	1.60 ± 0.00	2.08 ± 0.01	0.38 ± 0.12	**1.82 ± 0.01***	2.20 ± 0.13
3'-Phenylpropionic acid	1.10 ± 0.08	1.79 ± 0.00	2.89 ± 0.08	1.03 ± 0.11	1.83 ± 0.10	2.85 ± 0.20	0.80 ± 0.29	1.86 ± 0.05	2.66 ± 0.34
3-(Phenyl)-lactic acid	**0.01 ± 0.00***	0.05 ± 0.00	0.06 ± 0.00	**0.01 ± 0.00***	0.08 ± 0.00	0.09 ± 0.00	0.01 ± 0.00	0.10 ± 0.01	**0.11 ± 0.01***
3'-Hydroxyphenylacetic acid	0.11 ± 0.00	0.24 ± 0.01	0.35 ± 0.02	0.14 ± 0.00	**0.29 ± 0.00***	**0.43 ± 0.00***	0.11 ± 0.03	0.19 ± 0.00	0.30 ± 0.03
4-Hydroxybenzoic acid	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
4'-Hydroxyphenylacetic acid	0.21 ± 0.00	0.66 ± 0.00	0.87 ± 0.00	0.29 ± 0.00	**0.68 ± 0.00***	0.98 ± 0.00	0.24 ± 0.05	**0.92 ± 0.01***	**1.16 ± 0.06***
3-(3'-Hydroxyphenyl)propionic acid	0.02 ± 0.00	0.75 ± 0.05	0.77 ± 0.05	0.06 ± 0.01	0.53 ± 0.01	0.60 ± 0.02	0.07 ± 0.02	0.77 ± 0.02	0.83 ± 0.03
3-(3'-Methoxy-4'-hydroxy)phenylpropionic acid	**0.00 ± 0.00***	**nd**	0.00 ± 0.00	**0.01 ± 0.00***	**0.00 ± 0.00**	**0.01 ± 0.00***	**0.01 ± 0.00***	**0.06 ± 0.00***	**0.07 ± 0.00***
Ferulic acid	0.02 ± 0.00	0.24 ± 0.00	0.26 ± 0.00	0.03 ± 0.00	0.22 ± 0.00	0.25 ± 0.01	0.02 ± 0.01	**0.39 ± 0.02***	**0.41 ± 0.03***
Total	**2.21 ± 0.12**	**6.95 ± 0.11**	**9.16 ± 0.23**	**2.30 ± 0.14**	**6.07 ± 0.17**	**8.38 ± 0.31**	**1.79 ± 0.54**	**6.95 ± 0.18**	**8.74 ± 0.71**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *($p < 0.05$)*. SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; E5, Mourvèdre (MOU) EA70 skin pomace extract and E6, Alicante (ALI) EA70 skin pomace extract. SD, standard deviation.

Table 75: Quantification of phenolic acids detected in rat faeces 0-8 h, 8-24 h and 0-24 h after daily ingestion of seed and skin grape pomace extracts in association with verapamil for a period of seven days. Data are expressed as µmol ± SD.

	VE1			VE5			VE6		
Compounds	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**	**0-8h**	**8-24h**	**24h**
Benzoic acid	0.16 ± 0.01	**0.87 ± 0.00***	1.03 ± 0.01	0.24 ± 0.00	**1.05 ± 0.01***	**1.29 ± 0.01***	0.10 ± 0.01	0.71 ± 0.04	0.81 ± 0.05
Phenylacetic acid	0.32 ± 0.00	**1.56 ± 0.04***	1.88 ± 0.04	0.46 ± 0.02	**1.67 ± 0.08***	**2.13 ± 0.10***	0.21 ± 0.01	1.28 ± 0.06	1.49 ± 0.07
3'-Phenylpropionic acid	0.82 ± 0.02	**1.84 ± 0.03***	2.66 ± 0.05	0.92 ± 0.05	**2.09 ± 0.02***	**3.02 ± 0.07***	0.37 ± 0.02	1.57 ± 0.09	1.95 ± 0.11
3-(Phenyl)-lactic acid	0.01 ± 0.00	**0.11 ± 0.03***	0.12 ± 0.03	0.00 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.01 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
3'-Hydroxyphenylacetic acid	0.11 ± 0.00	**0.20 ± 0.01***	**0.31 ± 0.02***	0.12 ± 0.01	**0.28 ± 0.00***	**0.40 ± 0.01***	0.07 ± 0.00	0.15 ± 0.00	0.22 ± 0.00
4-Hydroxybenzoic acid	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
4'-Hydroxyphenylacetic acid	0.20 ± 0.01	0.42 ± 0.00	0.62 ± 0.01	0.35 ± 0.02	**0.73 ± 0.03***	**1.08 ± 0.05***	0.11 ± 0.01	0.17 ± 0.00	0.28 ± 0.01
3-(3'-Hydroxyphenyl)propionic acid	**0.13 ± 0.00***	**0.38 ± 0.04***	**0.50 ± 0.04***	0.07 ± 0.00	**0.59 ± 0.01***	**0.66 ± 0.01***	**0.18 ± 0.00***	**0.40 ± 0.00***	**0.59 ± 0.00***
3-(3'-Methoxy-4'-hydroxy)phenylpropionic acid	**nd**	**0.03 ± 0.00***	**0.03 ± 0.00***	0.01 ± 0.00	**0.06 ± 0.00***	**0.07 ± 0.01***	**nd**	**nd**	nd
Ferulic acid	0.03 ± 0.00	**0.26 ± 0.04***	0.30 ± 0.04	0.04 ± 0.00	**0.35 ± 0.01***	**0.39 ± 0.01***	0.05 ± 0.00	0.21 ± 0.01	0.26 ± 0.01
Total	**1.78 ± 0.05**	**5.71 ± 0.19***	**7.49 ± 0.24***	**2.22 ± 0.11**	**6.93 ± 0.17***	**9.15 ± 0.28***	**1.10 ± 0.05**	**4.57 ± 0.20***	**5.67 ± 0.25**

* Metabolites that were excreted in significantly higher amounts compared to their respective control *(p< 0.05)*. SHR rats fed with: VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. SD, standard deviation.

Significant catabolites excreted from different SHR experimental groups compared to their controls were presented in Figure 60 for day1 and Figure 61for day 7.

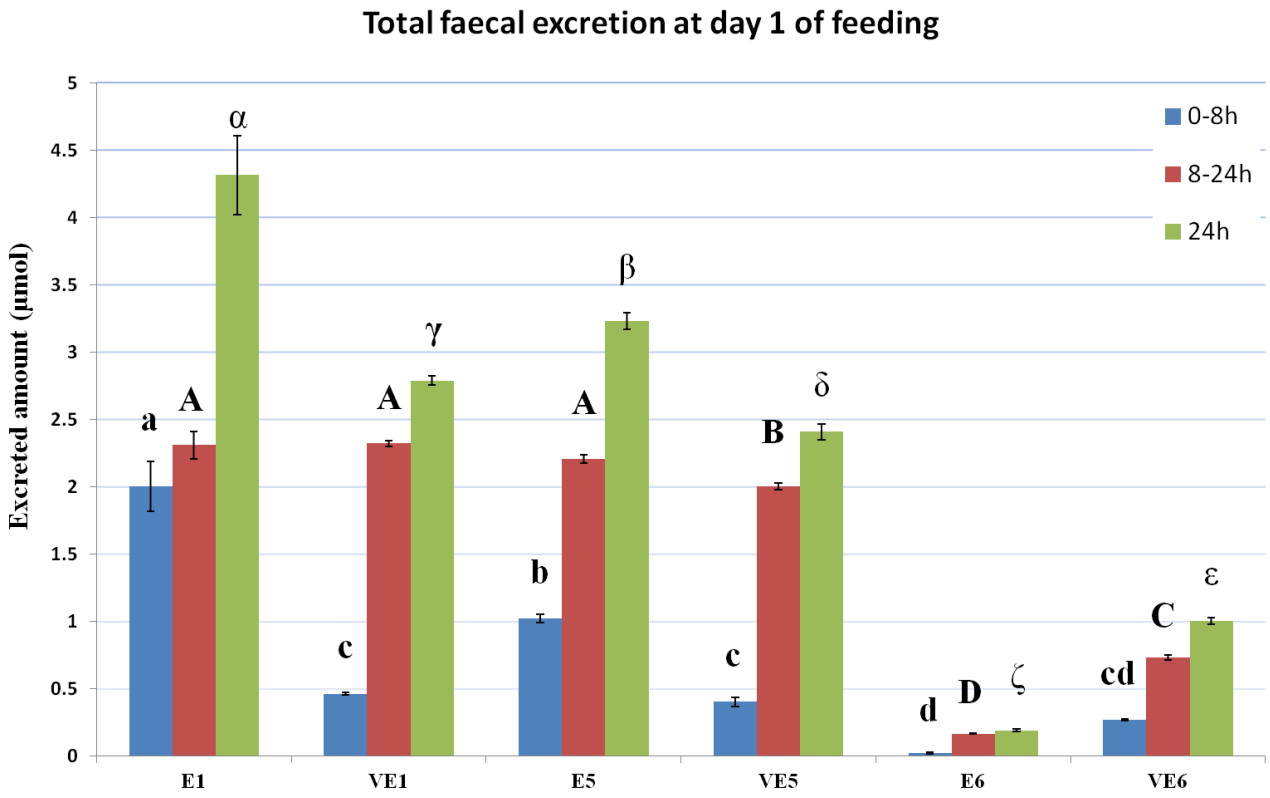


Figure 60: Total faecal catabolites excreted 0-8 h, 8-24h and 0-24 h after ingestion of grape pomace extracts

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α: Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 1. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in µmol ± Std.

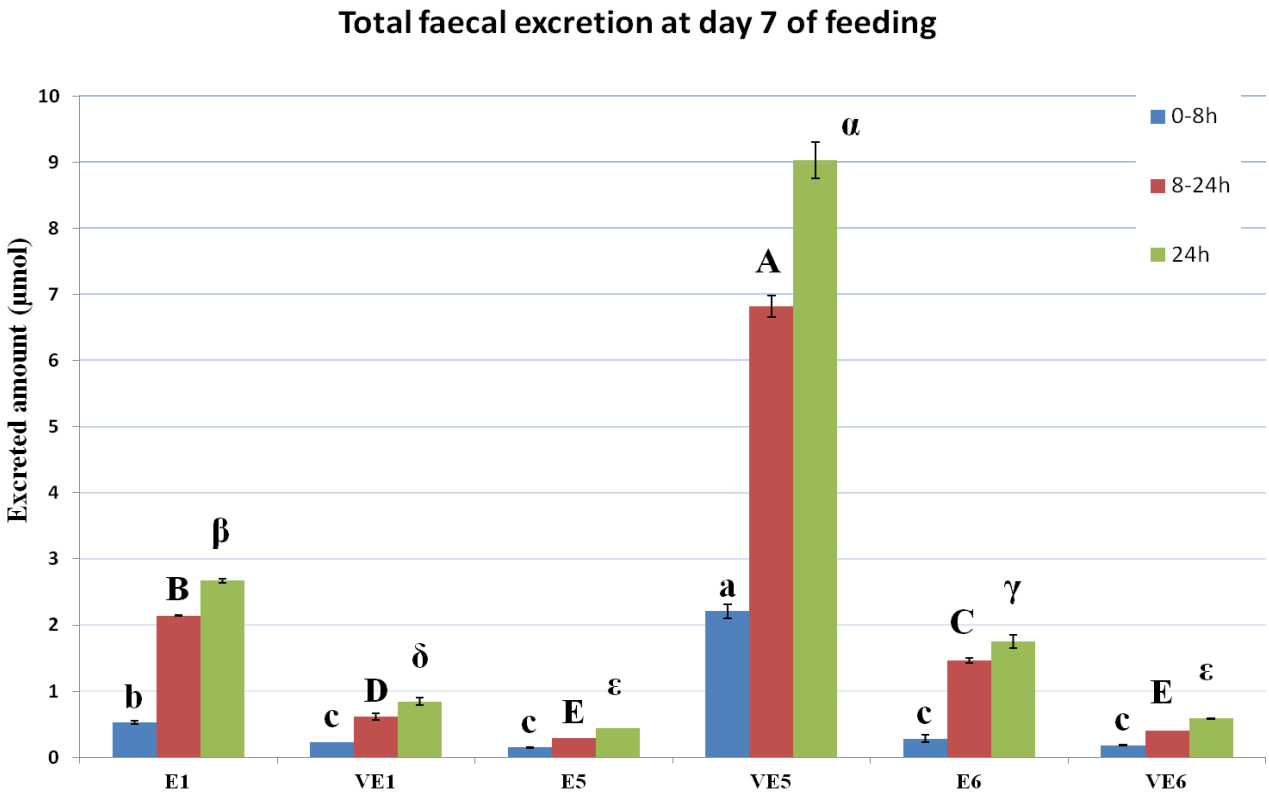


Figure 61: Total faecal catabolites excreted 0-8 h, 8-24 h and 0-24 h after ingestion of grape pomace extracts for a period of seven days

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil. a,A,α: Anova was made to compare values between different experimental groups during 0-8h, 8-24 and 24h at day 7. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in µmol ± Std.

The highest faecal excretion was observed in rats from E1 group with a maximum excretion of 4.3 µmol followed by E5 group with 3.23 µmol excreted at day 1. At day 7, the maximum excretion was observed in VE5 experimental group (9.03 µmol) compared to E5 group which only excreted 0.44 µmol.

Table 76 showed the corresponding percentage of intake of polyphenols excreted from different SHR experimental group. Higher catabolites were excreted at day 1 than at day 7, showing once again a better absorption of polyphenols by SHR rats over time. The highest excretion rate was evidenced in rat fed with MOU (EA70) skin pomace extract and excretions corresponded to 54 % at day 1 and 108 % at day 7 of polyphenols ingested, suggesting extensive catabolism of polyphenols contain in this extract by colonic microbiota.

Table 76: Percentage of intake of each type of extracts in faeces analysed by GC-MS

Day 1

	E1	VE1	E5	VE5	E6	VE6
0-8h	23	0	21	0	0	2
8-24h	7	31	6	54	2	7
0-24h	29	31	28	54	2	9

Day 7

	E1	VE1	E5	VE5	E6	VE6
0-8h	0	0	1	0	0	2
8-24h	9	6	2	108	10	5
0-24h	9	6	4	108	11	7

SHR rats fed with: E1, Grenache (GRE1) EA70 seed pomace extract; VE1, Grenache (GRE1) EA70 seed pomace extract + verapamil; E5, Mourvèdre (MOU) EA70 skin pomace extract; VE5, Mourvèdre (MOU) EA70 skin pomace extract +verapamil; E6, Alicante (ALI) EA70 skin pomace extract and VE6, Alicante (ALI) EA70 skin pomace extract + verapamil.

III.3. Conclusions

This chapter described the bioavailability of polyphenolic compounds of three different grape pomace extracts, in which two of them exerted anti-hypertensive activity with or without co-ingestion with verapamil. HPLC-ESI-MS^n permitted the detection of a wide array of metabolites including glucuronide, sulfate and methylated derivatives of flavan-3-ol conjugates, non-, mono- and di-substituted phenyl structures in the form of glucuronide and sulfate conjugates, or as a combination of methoxy-sulfate derivatives of hydroxyphenylvalerolactone conjugates, hydroxyphenylvaleric acid conjugates, hydroxyphenylacetic acid conjugates, hydroxybenzoic acid conjugates, hydroxyphenylpropionic acid conjugates, hydroxycinnamic acid conjugates and hydroxyhippuric acid conjugates. A total of 61 metabolites were detected but only 18 in amounts that facilitated quantification. It should be noted that in the absence of reference compounds quantification using aglycone standards might introduce varying and unmeasured error factors due to the different aglycone/glucuronide and aglycone/sulfate SIM/SRM dose response curves.

Urine analysis by HPLC-ESI-MS^n revealed a higher urinary excretion of metabolites in SHR rats fed with grape pomace extracts and verapamil. However, considering the percentage of intake corresponding to the excreted amount in urine, the data did not show clear differences between E1/VE1 (1.02 *vs* 1.29 % at day 1 and 1.52 % *vs* 1.08 % at day 7) and E6/VE6 (1.02% *vs* 1.02% at day1 and 1.42% *vs* 1.64% at day 7) except for the fact that polyphenols from MOU (EA70) skin pomace extract were 27-fold better absorb at day 7 of gavage than at day 1, especially when co-ingested with verapamil (0.16 % at day 1 to 4.25 % at day 7). This result suggested that polyphenols were better absorbed or accumulated over time in SHR rats.

A total of 11 metabolites were found in SHR rat plasma collected 4 h after grape pomace extracts ingestion. Plasma did not contain any of the original grape pomace extract flavan-3-ols and procyanidins but contained glucuronide, methyl-glucuronide and di-methyl-glucuronide of (epi)catechin and glucuronide, sulfate, non- and mono-substitutes valerolactones and valeric acid. These metabolites were also detected in urine with the exception of the di-methyl-(epi)catechin-*O*-glucuronide. Metabolites were mainly present as glucuronides rather than sulfates. Generally, lower concentrations of metabolites were observed in plasma than in urine. The highest metabolites concentration was observed in

plasma from VE5 experimental group in which SHR rats were fed MOU (EA70) skin pomace extracts + verapamil. In fact, an amount up to 4.6 ± 0.07 nmol and a recovery of 0.15 % were observed compared to other plasma in which only a 0.03 % to 0.09 % recovery was found. These results are in accordance with those obtained in urine, showing a high intake of grape pomace polyphenols in VE5 experimental group (4.25 %). The E5 group also showed substantial urinary excretion (poly)phenols (4.41 % of intake) while no more than 0.09 % of intake accumulated in plasma. This indicates a rapid excretion of metabolites from the circulatory system. Grape pomace polyphenols were less well absorbed by rats fed with other extracts (i.e. E1, VE1, E6 and VE6), as illustrated by low levels in urine and plasma.

In SHR rat tissues, no (epi)catechin conjugates were found but only the 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide (*m/z* 383/207, 163) and the 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulfate (*m/z* 287/207, 163) was detected and quantified by SRM mode. Liver, heart and kidneys all contained these metabolites. It was observed that rats fed with MOU (EA70) skin pomace extract + verapamil (VE5 SHR group) there was a higher accumulation of these metabolites. Likewise there were higher accumulations of metabolites after ingestion of MOU (EA70) skin pomace extract + verapamil than other extracts, especially in the kidneys and liver (0.14 % and 1.64 %, respectively). In addition, a higher accumulation of metabolites in rats fed with grape pomace extracts + verapamil was also observed in kidneys. Accumulation of metabolites was 5-fold higher in VE1 than E1, 14-fold in VE5 than E5 and 8-fold in VE6 than E6.

Faeces were screened for the original grape pomace extract flavan-3-ols and procyanidins using HPLC-MS^n in SIM mode for the detection of flavan-3-ol oligomers and monomers. Seven compounds were identified and quantified, including (epi)catechin (*m/z* 289/245, 205), (epi)catechin gallate (*m/z* 441/289,169), two dimers (*m/z* 577/425, 407, 289), trimers (*m/z* 865/577, 289), two gallo(epi)catechin trimers (*m/z* 881, 729, 577, 407) and tetramers (*m/z* 153/865, 577). Faecal phenolic excretion occurred predominantly at 8-24 h time point. Recoveries ranged from 0.3 % in VE6 to 2.31 % in E5 at day 1 and 1.03 % in VE1 to 1.72 % in E5 after seven days of grape pomace extracts ingestion.

Urine and faeces were further analysed for catabolites using GC-MS techniques. A total of 21 phenolic acids and aromatic compounds were identified in urines of SHR rats. The great majority of the phenolic acids were excreted in 8-24 h urine after grape pomace extract ingestion. Total 24 h excretion of catabolites over 24 h ranged from 0.16 µmol in VE5 to 12.7

µmol in E5 at day 1 and from 3.17 µmol in E6 to 17.4 µmol in VE1 at day 7. Results obtained by GC-MS confirmed those obtained by HPLC-ESI-MS^n and showed an increase of bioavailability of MOU (EA 70) skin pomace extract + verapamil which was low at day 1 but increased up to 85-fold at day 7 (1.2 % of intake at day 1 and 102 % of intake at day 7). Using GC-MS, the difference of absorption between E1/VE1 and E6/VE6 was better evidenced. At day 1, rats fed with GRE1 (EA70) seed pomace extract + verapamil absorbed 12-fold more grape pomace polyphenols than those fed with GRE1 (EA70) seed pomace extract alone and rats fed with ALI (EA70) skin pomace extract + verapamil absorbed 5-times more than those fed with an extract without verapamil. At day 7, the same observation can be evidenced but with a lower extent (same absorption rate for E1/VE1 and 4-fold higher for E6/VE6).

Analysis of faeces by GC-MS reflected phenolic acids which had not been absorbed in the colon. Higher catabolites were excreted at day 1 than at day 7, showing once again a better absorption of polyphenols by SHR rats over time. The highest excretion rate was evidenced in rat fed with MOU (EA70) skin pomace extract and excretions corresponded to 54 % and 108 % of polyphenols ingested, suggesting extensive catabolism of polyphenols contain in this extract by colonic microbiota.

This study investigated the bioavailability of different grape pomace extracts in SHR rats, including both phase II and colonic microbial metabolism. Grape pomace extracts and grape pomace extracts associated to verapamil were absorb as phase II metabolites mainly including glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin which arise from the metabolism of monomeric flavan-3-ols. The detection of microbial- derived metabolites of flavan-3-ols, hydroxyphenyl-γ-valerolactones in their glucuronide and sulfate forms confirmed the absorption of metabolites derived from both monomeric and polymeric flavan-3-ols from grape pomace extracts and subsequent conjugation in the liver. Numerous metabolites derived from further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavan-3-ols, indicating the important role of intestinal bacteria in the metabolism of highly polymerized compounds. All these metabolites may have exerted biological effects during the period in which they circulated in the bloodstream.

The association of grape pomace extracts with verapamil showed to have beneficial effects according to results obtained from *in vivo* study on SHR rats. GRE1 (EA70) seed pomace extract (fed to rats belonging to EI/VE1experimental group) and ALI (EA70) skin pomace extract (fed to rats belonging to E6/VE6 experimental group) administrated alone or in association to verapamil were able lower blood pressure of SHR rats whereas MOU (EA70) skin pomace extract (fed to rats belonging to E5/VE5 experimental group) administrated alone or in association with verapamil did not exerted any anti-hypertensive effects. Bioavailability studies indicated that rats fed with grape pomace extracts in association with verapamil excreted higher amount of metabolites and catabolites than those administrated with only grape pomace extracts. However, the intake of polyphenols in E1 and E6 group appeared to be slightly higher to those fed with verapamil (VE1 and VE6). Unlike E1/VE1 and E6/VE6, E5/VE SHR rats absorbed higher amounts of polyphenols. Analysis by HPLC-ESI-MS^n showed at day 1 of feeding that E5 group exhibited the highest polyphenols excretion over 24 h (1.57 %) compared to other groups (1.02 % - 1.29%). The lowest excretion occurred with the VE5 group at 0.16 % of intake. The same observation was obtained by GC-MS in which E5 group exhibited the highest 0-24 h excretion of catabolites (181% of polyphenol intake) compared to other groups (3.5% - 105.5%). Once again the VE5 group showed the lowest excretion level (1.2 %). After 7 days of ingestion, E5 group still appeared to exhibit the 0-24 h excretion, and hence absorption, of the ingested polyphenols (4.41 % of intake estimated by HPLC-ESI-MS^n and 76 % by GC-MS). Unlike day 1, excretion by VE5 rats was than other groups (4.25 % of intake was detected by HPLC-ESI-MS^n and 102 % by GC-MS). Substantial accumulation of metabolites was evidenced in VE5 rat tissues and plasma.

Anti-hypertensive effects of polyphenols have been confirmed in several studies (Al-Awwadi et al. 2004; Galisteo et al. 2004; Sarr et al. 2006; Perez-Vizcaino et al. 2009) and might be linked to the capacity of polyphenols to regulate the eNOS and NADPH oxidase activity. Verapamil is a P-gp inhibitor and multi-drug resistance (MDR) reversal agent. Several studies have demonstrated that polyphenols inhibit P-gp transport affecting the bioavailability and uptake of certain drugs (Jodoin et al. 2002; Kitagawa 2006; Eichhorn et al. 2012). Therefore, considering these observations and those obtained in this study, anti-hypertensive effects could be attributed to the action of polyphenols contained in grape pomace extracts (in the case rats from E1 and E6 group) and/or by the action of verapamil. In E1, E5 and E6, polyphenols, especially flavan-3-ol monomers and procyanidins, contained in

each grape pomace extracts were responsible for the non- or anti-hypertensive effect. In fact, GRE1 (EA70) seed pomace extract and ALI (EA70) skin pomace extract were showed to contain higher levels of polyphenols than MOU (EA70) skin pomace extract. In VE1 and VE6 group, verapamil might allow a higher absorption of polyphenols or vice versa. The anti-hypertensive might be due to the action of polyphenols and/or verapamil. In the case of E5/VE5 group, polyphenols was showed to be highly absorbed in E5 group and when associated to verapamil, the polyphenol metabolite/catabolite urinary excretion, and hence absorption, increased. Thus, verapamil might allow better absorption of polyphenols, but, because of low concentration or the composition of MOU (EA70) skin pomace extract no anti-hypertensive effects were observed.

Résumé chapitre 4

Dans un premier temps, différents groupes expérimentaux ont été mis en place et les extraits de pépins et pellicules de raisins et de marcs ont été administrés seuls ou en association avec le vérapamil aux SHR. La pression artérielle a été suivie et l'effet des extraits sur l'hypertension a été apprécié. Trois extraits ont été choisis afin d'étudier leur biodisponibilité. Les urines, fèces, plasmas et organes (foie, reins et cœur) ont été récoltés. Les métabolites ont été identifiés et quantifiés par HPLC-UV-MS^n et GC-MS.

Les expériences *in vivo* ont été réalisées sur un modèle animal de rat génétiquement hypertendus communément appelé SHR pour Spontaneaously Hypertensive Rats. Parmi les modèles de rats hypertendus, le SHR constitue le modèle de choix pour le screening des agents anti-hypertenseurs (Wada et al. 1996; Bravo et al. 1998; Maghrani et al. 2005; Quiñones et al. 2010, Mukai et al. 2011; Yang et al. 2012) et il est actuellement le plus utilisé pour étudier l'HTA. Leurs contrôles normotendus sont les rats Wistar-Kyoto (WKY). La souche de rat SHR a été sélectionnée par Okamota et al. (1963) dans les années 60, à partir de deux rats Wistar de leur élevage à Kyoto présentant une pression artérielle relativement élevée et persistante pendante plus d'un mois. Par croissement intra-génération successif, ils obtiennent à partir de la génération F3 approximativement 100 % de rats spontanément hypertendus. La naissance d'une telle souche hypertendue uniquement par reproduction démontre l'extrême importance du facteur génétique dans la transmission de cette pathologie.

Les résultats des expériences *in vivo* démontrent que certains extraits administrés seuls ou en association avec le vérapamil possèdent un effet anti-hypertenseur. Cette capacité a été mise en évidence une fois que les extraits de pépins de marcs de GRE1 (EA70) et SYR1 (EA70) et de pellicules de marcs d'ALI (EA70) ont été administrés seuls ou lorsque les extraits de pépins de marcs GRE1 (EA70) et SYR1 (EAQ) et les pellicules de marcs d'ALI (EA70) et SYR2 (EAQ) ont été administrés en association avec le vérapamil. Dans la dernière expérience *in vivo*, les pellicules de marcs de SYR1 (EA70) en association avec le vérapamil révèle un effet inverse en élevant la pression systolique. Le tableau ci-dessous résume l'efficacité *in vivo* des extraits de pépins et de pellicules de marcs testés seuls ou en association avec le vérapamil sur la pression artérielle (PA).

Table 1: Effet *in vivo* des extraits de pépins et pellicules de marcs sur la pression artérielle.

Extraits de marcs testés	Temps de traitement	Effet sur la PA	Efficacité
GRE1 pep (EA70)	3 semaines	+	Effet indirect, effet rebond, **+ 19%**
SYR1 pep (EA70)	3 semaines	+	Effet indirect, effet rebond, **+24 %**
SYR2 pell (EA70)	3 semaines	~	-
CAR pep (EA70)	3 semaines	~	-
MOU pell (EA70)	3 semaines	~	-
ALI pell (EA70)	3 semaines	+	Effet indirect, effet rebond, **+16 %**
GRE1 pep (EA70) + vérapamil	3 semaines	+	Effet direct, **-26 %**
SYR1 pep (EA70) +vérapamil	3 semaines	~	-
SYR2 pell (EA70) + vérapamil	3 semaines	~	-
CAR pep (EA70) + vérapamil	3 semaines	~	-
MOU pell (EA70) +vérapamil	3 semaines	~	-
ALI pell (EA70) + vérapamil	3 semaines	+	Effet direct, **-13 %**
SYR1 pep (EAQ) + vérapamil	2 semaines	+	Effet direct, **-14 %**
SYR1 pell (EAQ) + vérapamil	2 semaines	~	-
ALI pell (EAQ) + vérapamil	2 semaines	~	-
GRE2 Pell (EA70) + vérapamil	2 semaines	~	-
CAR pep (EAQ) + vérapamil	1 semaine	~	-
GRE2 pell (EAQ) + vérapamil	1 semaine	~	-
SYR2 pell (EAQ) + vérapamil	1 semaine	+	Effet direct, **-11 %**
SYR1 pell (EA70) + vérapamil	1 semaine	−	Effet direct, **+15 %**

Les extraits ont été administrés à une dose de 21mg/kg/jour de polyphénols par jour ce qui équivaut à une consommation de polyphénols contenus dans 500 mL de vin rouge par un adulte de 70kg et le vérapamil à une dose de 40 mg/kg/jour. Pell, pellicules; pép, pépins; +, effet positif sur la régulation de l'hypertension; −, effet négatif sur la régulation de l'hypertension ; ~, pas d'effet. L'efficacité des extraits administrés aux rats SHR a été illustrée par le pourcentage de variation (%) de la pression artérielle avant et après l'ingestion des extraits.

Les extraits de marcs administrés seuls régulent la pression artérielle et a été démontré par l'effet rebond bien connu dans ce type de traitement. Une augmentation de + 19%, +24% et +16% a été observée pour les extraits de pépins GRE1 (EA70) et SYR1 (EA70) et de pellicules ALI (EA70) après 3 semaines de traitement. Ingéré en association avec le vérapamil, les pépins de GRE1 (EA70) diminuent la pression artérielle de -26% et les pellicules d'ALI (EA70) de -13% après 3 semaines de traitement et de -14% après 2 semaines de traitement. De plus, les expériences montrent qu'une semaine suffise pour induire des effets antihypertenseurs dans le groupe de rats SHR ayant reçu l'extrait de pellicules de marcs SYR2 (EAQ) + vérapamil dans lequel une réduction de 11 % a été observé et des effets pro-hypertenseurs dans le groupe de rat ayant reçu l'extrait de pellicules SYR1 (EA70) + vérapamil (+15%).

Selon le type d'extraits administrés aux rats SHR, il est possible de moduler les effets antihypertenseurs du médicament. La composition des extraits de marcs en composés phénoliques joue un rôle crucial et devrait être pris en compte. Malgré une quantité plus importante en composés phénoliques dans les extraits EA70, les extraits EAQ ont tout de même prouvé leurs efficacités dans la régulation de la pression artérielle.

Afin d'approfondir les connaissances sur le niveau d'absorption des polyphénols et leurs contributions aux effets antihypertenseurs, une étude de biodisponibilité des trois extraits de marcs administrés seuls ou associés au vérapamil, dont deux ayant montré une capacité anti-hypertenseur, a été réalisée par la suite. L'HPLC-ESI-MS^n a permit la détection de grandes variétés de métabolites y compris les dérivés méthylés, glucuronidés et sulfatés de flavan-3-ols, des structures non-, mono- et di- phényles glucuronides et sulfates ou même des methoxy-sulfates d'acide hydroxyphenylvalérolactone, d'acide hydroxyphenylvalerique, d'acide hydroxyphenylacétique, d'acide hydroxybenzoique, d'acide hydroxyphenylpropionique, d'acide hydroxycinnamique et d'acide hydroxyhippurique. Un totale de 61 métabolites a été détecté mais seulement 18 ont été quantifiés. En l'absence de standards glucuronidés et sulfatés, une quantification par le biais d'aglycones pourrait introduire des erreurs de quantification due a des réponses SIM/SRM différentes entre aglycones/glucuronides et aglycones/sulfates. L'analyse des urines par HPLC-ESI-MS^n révèle une plus grande excrétion de métabolites chez les SHR ayant reçu des extraits de marcs associés au vérapamil.

Compte tenu du pourcentage de l'apport correspondant à la quantité excrétée dans l'urine, les données n'ont pas montré de différences significatives entre E1/VE1 (1,02% vs 1,29% au 1^{er} jour de gavage et de 1,52% vs 1,08% au $7^{ème}$ jour de gavage) et E6/VE6 (1,02 % vs 1,02% au 1^{er} jour de gavage et 1,42% vs 1,64% au $7^{ème}$ jour de gavage), excepté pour les polyphénols contenus dans les extraits de pellicules de MOU (EA70) qui sont 27 fois mieux absorber au $7^{ème}$ jour de gavage qu'au 1^{er} jour de gavage, en particulier lorsqu'il est associé au vérapamil (0,16% 1^{er} jour de gavage à 4,25% au $7^{ème}$ jour de gavage). Ce résultat suggère qu'au fil du temps, les polyphénols sont mieux absorbés ou accumulés chez les rats SHR.

Concernant les plasmas, un total de 11 métabolites composé de dérivés glucuronidés, méthyl-glucuronidés et di-méthylglucuronidés d'épicatéchine ainsi que des dérivés glucuronidés, sulfatés, non- et mono-substitués de valerolactone et d'acide valérique ont été retrouvés 4 heures suivant l'ingestion des extraits de marcs. Aucun composé détecté dans les

extraits de pépins et pellicules de marcs tels que les flavan-3-ols et procyanidins n'a été décelé dans les plasmas. Ces métabolites ont aussi été retrouvés dans les urines à l'exception du di-méthyl-(épi)catéchin-*O*-glucuronide et sont principalement présents sous forme de glucuronides que sulfates. Généralement, de plus faibles concentrations ont été retrouvées dans les plasmas que dans les urines. La plus forte concentration a été décelée dans les plasmas des rats SHR appartenant au groupe expérimental VE5, ayant reçu l'extrait de pellicules MOU (EA70) + verapamil. En effet, jusqu'à 4,6 ± 0,07 nmol et 0.15 % d'absorption a été observé par rapport aux autres plasmas dans lequel seulement un taux de 0.03% à 0.09% a été retrouvée. Ces résultats sont en accords avec ceux observés dans les urines montrant une importante absorption des polyphenols dans le groupe expérimental VE5 (4.25%). Le groupe E5 excrète une grande quantité de polyphénols dans l'urine (4.41% de polyphénols ingérés) alors qu'une petite quantité a été détectée dans les plasmas. Ce résultat indique une rapide excrétion des métabolites du système circulatoire. Une faible absorption illustrée par une légère excrétion de métabolites dans les urines et plasmas a été mise en évidence dans les groupes de rat (E1, VE1, E6 et VE6).

Dans les tissus des rats SHR (foie, reins et cœur), aucuns dérivés de l'épicatéchine n'ont été retrouvés mis à part la 5-(hydroxyphényl)-γ-valérolactone-*O*-glucuronide (m/z 383/207, 163) et la 5-(hydroxyphényl)-γ-valérolactone-*O*-sulfate (m/z 287/207, 163) qui ont été détectées et quantifiées en mode SRM. Il a été observé que les rats ayant reçu les extraits de pellicules de MOU (EA70) + vérapamil (groupe VE5) concentre une plus forte concentration de métabolites dans leurs tissus par rapport aux autres groupes de rats SHR, particulièrement dans les reins et le foie (0.14% et 1.64%, respectivement). De plus, une plus importante accumulation de métabolites dans les rats administrés avec des extraits + vérapamil a été décelée dans les reins avec 5 fois plus dans le groupe VE1 que dans le groupe E1, 14 fois plus dans VE5 que dans E5 et 8 fois plus dans VE6 que dans E6.

Les fèces ont été dépistés pour les composés contenus dans les extraits de marcs par HPLC-MS^n en mode SIM pour la détection d'oligomères et de monomères flavan-3-ols. Sept composés incluant l'épicatéchine (m/z 289/245, 205), l' (épi)catéchine gallate (m / z 441/289, 169), un dimère (m/z 577/425, 407, 289), un trimère (m/z 865/577, 289), deux gallo (épi)catéchine trimères (m/z 881, 729, 577, 407) et un tétramère (m/z 153/865, 577) ont été détectés.

L'excrétion fécale de polyphénols survient principalement entre 8-24 h. Le pourcentage de polyphénols ingérés et excrétés dans les fèces varie de 0.3% dans VE6 à 2.31% dans E5 au 1er jour de gavage et de 1.03% dans VE1 à 1.72% dans E5 après sept jours de gavage avec les extraits de marcs.

Les urines et les fèces ont été analysés pour les catabolites utilisant les techniques de GC-MS. Un total de 21 acides phénoliques a été identifié dans les urines des rats SHR. La grande majorité des acides phénoliques ont été excrétée dans l'urine après 8-24 h d'ingestion d'extraits de pépins et pellicules de marcs. L'excrétion de catabolites au bout de 24 h varie de 0,16 µmol dans VE5 à 12,7 µmol dans E5 au 1er jour de gavage et de 3,17 µmol dans E6 à 17,4 µmol dans VE1 au 7ème jour de gavage. Les résultats obtenus par GC-MS confirment ceux obtenus par HPLC-ESI-MS^{n} et ont montré une augmentation de la biodisponibilité de l'extrait de pellicule MOU (EA 70) associé au vérapamil. En effet, au 7ème jour de gavage, 85 fois plus de polyphénols a été absorbé par rapport au 1er jour de gavage (1.2 % de polyphénols ingérés détecté dans les urines au 1er jour et 102% au 7ème jour). Au 1er jour de gavage, les rats ayant reçu des les extraits de pépins de GRE1 (EA70) + vérapamil ont absorbé 12 fois plus de polyphénols que ceux ayant reçu GRE1 (EA70) seul et les rats ayant reçu l'extrait de pellicules de marcs ALI (EA70) + vérapamil ont absorbés 5 fois plus de polyphénols que ceux ayant eux l'extrait seul. Au 7ème jour de gavage, la même observation a été constatée mais avec une plus faible ampleur (même taux d'absorption pour E1/VE1 et 4 fois plus pour E6/VE6).

L'analyse des fèces par GC-MS reflète les acides phénoliques qui n'ont pas été absorbés au niveau du colon. Les résultats montrent une plus importante quantité de métabolites excrétés au 1er jour de gavage par rapport au 7ème jour illustrant encore une fois de plus une meilleure absorption des polyphénols au cours du temps. Le plus fort taux d'excrétion a été détecté dans les fèces des rats administré par l'extrait MOU (EA70) et correspond à 54 % de polyphénols ingérés au 1er jour de gavage et à 108% de polyphénols ingérés au 7ème jour de gavage. Cette observation suggère un catabolisme extensif des polyphénols contenus dans cet extrait par la microflore intestinale.

Cette étude met en évidence la biodisponibilité des extraits de pépins et de pellicules de marcs des rats SHR incluant à la fois le métabolisme de phase II et de la microflore intestinale. Les extraits de marcs administrés seuls et en association au vérapamil sont absorbés en tant que métabolites de phase II présent sous forme de dérivé *O*-

methylglucuronidé, sulfaté, *O*-methylsulfaté d'(épi)catéchine dérivant du métabolisme des monomères de flavan-3-ols. La détection de métabolites microbiens dérivés de flavan-3-ols, d'hydroxyphényl-γ-valérolactones sous leurs formes glucuronidés et sulfatés confirme l'absorption des métabolites dérivés des flavan-3-ols, la fois monomères et polymères des extraits de marcs et des conjugaisons supplémentaires dans le foie. De nombreux métabolites issus de la dégradation microbienne des hydroxyvalerolactones ont également été détectés. L'excrétion urinaire de ces métabolites représente une plus grande proportion de polyphénols ingérés comparé à ceux issus de métabolisme de phase II des monomères de flavan-3-ols, indiquant un rôle important des bactéries intestinales dans le métabolisme des molécules hautement polymérisées. Ces métabolites peuvent avoir exercé leurs effets biologiques lors de leur passage dans la circulation sanguine.

Suite aux résultats obtenus lors des tests *in vivo*, l'association des extraits de pépins et de pellicules de marcs avec le vérapamil a prouvé son efficacité chez les rats SHR. L'extrait de pépins GRE1 (EA70) (administré au groupe E1 et VE1) et l'extrait de pellicules d'ALI (EA70) (administré aux groupe E6 et VE6) avec ou en association avec le vérapamil sont capables de faire diminuer la pression artérielle des rats SHR alors que dans les groupe de rats E5 et VE5 ayant reçu l'extrait de pellicules de MOU (EA70) seul ou en association avec le vérapamil, aucun effet sur la pression artérielle n'a été observé. Les études de biodisponibilité ont indiqué que les rats administrés avec les extraits de marcs associés au vérapamil génèrent une quantité plus importante de métabolites et catabolites que ceux ayant reçu l'extrait seul. Cependant, l'absorption de polyphénols dans les groupes E1 et E6 semble tout de même être légèrement supérieure à ceux ayant reçu du vérapamil (VE1 et VE6). Contrairement aux E1/VE1 et E6/VE6, les SHR des groupes E5/VE5 absorbent de plus grandes quantités de polyphénols. L'analyse par HPLC-ESI-MS^n a montré qu'au 1^{er} jour de gavage, les SHR du groupe E5 excrètent la plus grande quantité de polyphénols sur 24 h (1,57 %) comparé aux autres groupes (1,02%-1,29%). La plus faible excrétion a été observé chez les VE5 avec une excrétion de seulement 0.16 % des polyphénols ingérés. La même observation a été obtenue par GC- MS où il a été démontré que les SHR du groupe E5 excrètent une importante quantité de catabolites (181 % de l'apport en polyphénols) par rapport aux autres groupes (3,5%-105,5 %) même après 7 jours d'administration (excrétion de 4.41 % de polyphénols ingérés estimés par HPLC-ESI-MS^n et 76 % par GC-MS). Une fois de plus, les SHR du groupe VE5 excrètent la plus faible quantité de polyphénols (1.2% des polyphénols ingérés). Contrairement au 1^{er} jour de gavage, l'excrétion des SHR du groupe

VE5 est plus élevé par rapport aux autres groupes avec 4.25% d'absorption détecté par HPLC-ESI-MS^n et 102% par GC-MS). En effet, une importante accumulation de métabolites a été démontrée dans les tissus et plasmas des rats appartenant au groupe VE5.

Il a été établi que les SHR développent spontanément une hypertension artérielle qui augmente avec l'âge de vie. L'HTA se manifeste à partir de la sixième semaine d'âge et se stabilise autour de la 20ème semaine d'âge avec une pression artérielle systolique atteignant 200 à 220 mmHg. L'origine de cette hypertension est multiple. Par rapport aux rats normotendus, les rats SHR présentent un déficit de l'enzyme de conversion 2 (ACE2), une mono-carboxypeptidase qui catalyse à la fois l'angiotensine I (Ang I) en angiotensine $_{(1-9)}$ (Ang 1-9) et l'angiotensine II (Ang II) en angiotensine $_{(1-7)}$ (Ang 1-7) (Donoghue et al. 2000). L'ACE2 est impliquée dans la régulation du système rénine-angiotensine (RAS) (Tikellis et ak. 2006) et est reconnue pour son rôle majeur dans le contrôle de la pression artérielle. En effet, l'heptapeptide Ang 1-7 est un vasodilatateur et natriurétique dont les effets s'opposent à ceux de son précurseur. Le déficit d'ACE2 induit alors une augmentation de la concentration d'Ang II et conduit à une vasoconstriction. De plus, les rats SHR possèdent une relaxation endothéluim-dépendante réduite due à un dysfonctionnement endothéliale souvent associé à un fort stress oxydant. Ceci est causé d'un côté par un excès d'anion superoxyde ($O_2^{\bullet-}$) produit par l'enzyme NADPH oxydase (Grunfeld et al. 1995; Zalba et al. 2000) et d'un autre côté par une faible activité de la eNOS (endothelial NO synthase) qui catalyse la production du monoxyde d'azote ou NO, un puissant agent vasodilatateur produit par l'endothéluim à partir de la L-Arginine (Palmer et al. 1987). En effet, en présence du NO, l'ion superoxyde $O_2^{\bullet-}$ forme le peroxynitrite ($ONOO^-$) empêchant ainsi le NO d'exercer sa fonction vasodilatatrice (Beckman et al. 1990; McIntyre et al. 1999; Pacher et al. 2007). Physiquement, l'étude des tissus du rat SHR montre qu'au cours de son âge, l'oxygénation tissulaire diminue et entraine progressivement ainsi une hypertrophie cardiaque (Engelmann et al. 1987) ainsi qu'une augmentation de la résistance périphérique (Smith et al. 1979; Folkow et al. 1992).

Du fait de l'implication du stress oxydant dans la pathogenèse de l'hypertension, les chercheurs se sont intéressés aux effets antihypertenseurs des polyphénols retrouvés dans l'alimentation. Leur effet hypotenseur a été confirmé dans de nombreuses études (Galisteo et al. 2004 ; Sarr et al 2006 ; Perez-Vizcaino et al. 2009) et serait lié a la capacité des polyphénols de réguler l'activité de la eNOS et de la NADPH oxydase. Une étude par Al-

Awwadi et al. (2004) a montré qu'un extrait de polyphénols issus du vin rouge permettait de diminuer l'hypertension chez des rats ayant développé un syndrome métabolique suite à un régime riche en fructose. Plus précisément, des extraits polyphénoliques enrichis en anthocyanes et en catéchines préviennent l'hypertension chez le même modèle animal ce qui n'est pas le cas des procyanidines (Al-Awwadi et al. 2005). L'effet antioxydant n'est donc pas le seul mode d'action de ces molécules. Ces observations sont en adéquation avec nos résultats dans lequel de fortes concentrations en monomères et dimères de flavan-3-ols ont été détectés dans les extraits de pépins de marcs exerçant des effets antihypertenseurs.

Les extraits de marcs peuvent être utilisés seuls mais leurs effets peuvent être amplifiés une fois co-ingéré avec le vérapamil. Il est largement reconnu que la glycoprotéine P souvent nommé « P-gp » influence le transport des médicaments au travers des membranes. La P-pg se retrouve principalement au niveau des cellules du tubule contourné proximal du rein, des cellules de l'intestin, de l'endothélium de la barrière hémato-encéphalique etc. Par conséquent, elle peut influencer l'absorption, la distribution, le métabolisme et l'excrétion des xénobiotiques. La P-pg provoque par efflux, un phénomène de multirésitances à plus de 50% des agents anti-cancéreux. Par conséquent, les chercheurs tentent d'optimiser la pharmacocinétique, d'améliorer les activités anti-tumorales et de réduire la toxicité systémique des médicaments anti-cancéreux en inhibant les efflux de la P-glycoprotéine. Bien qu'une grande variété d'inhibiteur de la P-gp ont été découverts, les flavonoïdes constituent la troisième génération de la catégorie non-pharmaceutique d'inhibiteurs de la P-gp (Bansal et al. 2009). Il a été démontré que les effets produits par certains de ces composants sont comparables à ceux des inhibiteurs connus de la P-pg tels que le vérapamil et la cyclosporine.

Comme le vérapamil, un inhibiteur de référence de la P-pg et des phénomènes de multi-résistance aux médicaments (MDR), les polyphénols ont été démontrés comme ayant des propriétés inhibitrices sur le transport de la P-pg affectant la biodisponibilité et l'absorption de certains médicaments (Jodoin et al. 2002; Kitagawa 2006; Eichhorn et al. 2012). De ce fait, l'association d'extraits de pépins et de pellicules de marcs riches en polyphénols au vérapamil peut avoir un effet bénéfique sur la régulation de la pression artérielle en modifiant l'absorption du (poly)phénols et/ou du vérapamil.

En tenant compte de ces observations et des résultats obtenus lors de l'étude *in vivo,* les effets antihypertenseurs des polyphénols peuvent être attribués à l'action des polyphénols

contenus dans les extraits de marcs (dans le cas des rats des groupes E1 et E6) et/ou par l'action du vérapamil. Dans les groupes E1, E5 et E6, les polyphénols, en particulier les monomères et procyanidins de flavan-3-ols, peuvent contribuer aux effets antihypertenseurs des extraits. En effet, les extraits de pépins de GRE1 (EA70) et de pellicules d'ALI (EA70) renferment une importante quantité de polyphénols que l'extrait de pellicules de MOU (EA70). Dans le groupe VE1 et VE6, le vérapamil pourrait permettre une meilleure absorption de polyphenols ou vice versa et l'effet antihypertenseur pourrait être du à l'action des polyphénols et/ou le vérapamil. Dans les cas des groupes E5 et VE5, il a été démontré que les polyphénols sont absorbés de manière importante dans le groupe E5 et une fois associée au vérapamil, l'absorption est encore plus élevée. De ce fait, le vérapamil devrait permettre une meilleure absorption des polyphénols mais dû à une faible teneur ou à la composition en polyphénols des pellicules de MOU (EA70), cet extrait ne possède aucun effet antihypertenseur.

Conclusions and perspectives

This study investigated the beneficial effects of grape pomaces obtained after winemaking of different Mediterranean grape varieties from crude materials to its *in vivo* effectiveness. Grapes and their respective grape pomaces from six different *V. vinifera* L. cv. Grenache (from two different locations [GRE1 and GRE2]), Syrah (from two different locations [SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI) grape varieties from Rhône Valley region were studied.

Grapes and their respective grape pomaces, including seeds and skins were screened for their (poly)phenolic contents (total phenols, anthocyanins [glucosides, acetylated glucosides and coumarylic glucosides], tannins and monomeric and oligomeric proanthocyanidins. The *in vitro* antioxidant capacities of all the extracts were evaluated by four different analytical methods: $ABTS^{\bullet+}$, DPPH, FRAP and ORAC.

The comparison of several wine industry by-products with their respective grapes provided evidence that pomaces are very rich sources of antioxidants, despite extraction during winemaking. The quantitative and qualitative distribution of polyphenols in grape pomaces showed significant differences through varieties and vintages. In addition, seed pomace was shown to contain particularly high amounts of phenolics and thus had a more substantial antioxidant potential than skin pomace. Grape seed and skin pomace extracts contained appreciable amounts of flavan-3-ols and anthocyanins. Seeds from Grenache (GRE1), Syrah (SYR1) and skins from Syrah (SYR1), Carignan and Alicante were of particular interest because of their higher polyphenol contents in terms of flavan-3-ols (monomers, dimers and trimers) and anthocyanins (glycosides, acetylated and coumaroylated derivatives). These extracts also exerted the highest antioxidant activity in four different assay systems. As a result, these varieties and their parts (seeds and skins) were chosen for further extraction with aqueous and hydro-alcoholic 70% solution suitable for consumption and commercialization.

The investigation of aqueous and hydro-alcoholic 70% extracts of seeds from Carignan and Syrah (SYR1) and skins from Carignan and Alicante was carried out as they contained high levels of total phenols and antioxidant activity. Hydro-alcoholic 70% solution was shown to be more effective because of higher flavan-3-ol, procyanidin and anthocyanin contents in EA70 extracts. However, EAQ extracts also displayed interesting amounts of polyphenolics and radical scavenging capacity and should not be ignored. Lyophilization enabled extracts to contain more concentrated polyphenolic compounds. Nevertheless, this step can intensively increases the cost of production and has to be further discussed.

Although selected extracts had a high content of polyphenolic compounds, the *in vivo* effects might not be that effective because bioavailability greatly differs between polyphenols. Therefore, several extracts, even those which contain low phenolic amounts were tested in order to evaluate the *in vivo* biological effects on hypertension using a spontaneously hypertensive rat (SHR) model. Grape pomace extracts were administrated at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. Different experimental groups were made, blood pressure was followed and the effect of grape pomace on hypertension determined. In the first instance, different grape pomace extracts were screened for their anti-hypertensive effects. Among extracts screened, Grenache GRE1 (EA70) seed pomace extract, Syrah SYR1 (EA70) seed pomace extract and Alicante ALI (EA70) skin pomace extract were able to reduce blood pressure which was followed by a rebound effect once extract administration was interrupted.

SHR were also fed verapamil, a calcium-channel blocker anti-hypertensive drug commonly use to treat hypertension in humans. Results showed that throughout the experiment, SHR systolic blood pressure fluctuated depending on the administered verapamil dose (30 mg/kg/day – 20 mg/kg/day – 40 mg/kg/day), but finally end up with a constantly high SBP around 200 mm Hg. These results indicate that over time, SHR rats develop resistance to the verapamil. A model of "grape pomace extract + verapamil" was validated and a series of different grape pomace extracts associated with verapamil were tested in order to evaluate the anti-hypertensive ability. Results showed that the co-ingestion of verapamil with grape pomace extracts rich in polyphenols had beneficial effects on blood pressure regulation by amplifying the effects of polyphenols and/or verapamil.

All *in vivo* experiments demonstrated that some grape pomace extracts administrated with or without co-ingestion with verapamil possessed an anti-hypertensive potential and was

evidenced with GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract, ALI (EA70) skin pomace extract once administrated alone and with GRE1 (EA70) seed pomace extract, SYR1 (EAQ) seed pomace extract, ALI (EA70) skin pomace extract and SYR2 (EAQ) skin pomace extract once administrated in association with verapamil. However, SYR1 (EA70) skin pomace extract with verapamil was shown to exert an adverse effect by elevating systolic blood pressure. Table 77 summarized the anti-hypertensive efficiency of different tested grape pomace extracts.

Table 77: Anti-hypertensive efficiency of different grape pomace extracts.

Grape pomace extracts tested	Treatment time	Effect on hypertension	Efficiency to regulate blood pressure
GRE1 seed (EA70)	3 weeks	+	Indirect effect, rebound effect, **+ 19%**
SYR1 seed (EA70)	3 weeks	+	Indirect effect, rebound effect, **+24 %**
SYR 2 skin (EA70)	3 weeks	~	-
CAR seed (EA70)	3 weeks	~	-
MOU skin (EA70)	3 weeks	~	-
ALI skin (EA70)	3 weeks	+	Indirect effect, rebound effect, **+16 %**
GRE1 seed (EA70) + verapamil	3 weeks	+	Direct effect, **-26 %**
SYR1 seed (EA70) +verapamil	3 weeks	~	-
SYR 2 skin (EA70) + verapamil	3 weeks	~	-
CAR seed (EA70) + verapamil	3 weeks	~	-
MOU skin (EA70) +verapamil	3 weeks	~	-
ALI skin (EA70) + verapamil	3 weeks	+	Direct effect, **-13 %**
SYR1 seed (EAQ) + verapamil	2 weeks	+	Direct effect, **-14 %**
SYR1 skin (EAQ) + verapamil	2 weeks	~	-
ALI skin (EAQ) + verapamil	2 weeks	~	-
GRE2 Skin (EA70) + verapamil	2 weeks	~	-
CAR seed (EAQ) + verapamil	1 week	~	-
GRE2 skin (EAQ) + verapamil	1 week	~	-
SYR2 skin (EAQ) + verapamil	1 week	+	Direct effect, **-11 %**
SYR1 skin (EA70) + verapamil	1 week	−	Direct effect, **+15 %**

Grape pomace extracts were administrated at a dose of of 21 mg/kg/day equivalent to a daily 70 kg human consumption of phenolic contained in 0.5 L of wine and verapamil at a dose of 40 mg/kg/day. +, positive effect on hypertension regulation; − negative effect on hypertension regulation; ~ no effect. Extract efficiency fed to SHR rats was illustrated by the percentage of variation (%) of blood pressure before and after the ingestion.

Grape pomace extracts ingested without verapamil were shown to exert anti-hypertensive effects indirectly, by increasing blood pressure after a rebound effect. An increase of +19 %, +24 % and +16 % were observed, respectively, for GRE1 seed (EA70), SYR1 seed (EA70) and ALI skin (EA70) after three weeks of treatment. Grape pomace extracts co-ingested with verapamil, lowering blood pressure by -26 % with GRE1 seed

(EA70) + verapamil and -13% using ALI skin (EA70) + verapamil after 3 weeks of treatment and by -14 % after 2 weeks of treatment. Moreover, experiments showed that one week of treatment was sufficient to induce anti-hypertensive effect in rats fed with SYR2 skin (EAQ) + verapamil in which an 11 % reduction in blood pressure while a pro-hypertensive effect was observed in rats fed with SYR1 skin (EA70) + verapamil (+ 15 %).

According to the extract used and its composition, it is actually possible to modulate anti-hypertensive drug effects. *In vivo* anti-hypertensive ability of GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract and SYR1 (EAQ) seed pomace extract is most likely conferred by the large amount of flavan-3-ols and procyanidins rather than anthocyanins. Skin pomace extracts contained large amount of anthocyanins and flavan-3-ols but unlike seed extracts, anti-hypertensive ability of skin extracts is more difficult to predict. Other compounds in these skin extracts such as polysaccharides or fibers might prevent the anti-hypertensive effect by affecting polyphenol absorption.

The bioavailability of polyphenolic compounds in GRE1 (EA70) seed pomace extracts (E1/VE1 group), MOU (EA70) skin pomace extracts (E5/VE5 group) and ALI (EA70) skin pomace extracts (E6/VE6 group), with or without co-ingestion with verapamil was studied. Urine, faeces, tissues (heart, liver and kidneys) and plasma were collected from SHR rats and screened in order to determine the accumulation of polyphenol metabolites. A total of 61 metabolites were detected in urine but only 18 in amounts that facilitated quantification. Urine analysis by HPLC-ESI-MS^n revealed a higher urinary excretion of metabolites in SHR rats fed with grape pomace extracts and verapamil. However, considering the percentage of intake corresponding to the excreted amount in urine, the data did not show clear differences between E1/VE1 (1.02 *vs* 1.29 % at day 1 and 1.52 % *vs* 1.08 % at day 7) and E6/VE6 (1.02% *vs* 1.02% at day1 and 1.42% *vs* 1.64% at day 7) except for the fact that polyphenols from MOU (EA70) skin pomace extracts were 27-fold better absorb at day 7 of gavage than at day 1, especially when co-ingested with verapamil (0.16 % at day 1 to 4.25 % at day 7). This result suggested that polyphenols were better absorbed or accumulated over time in SHR rats.

A total of 11 metabolites were found in SHR rat plasma collected 4 h after grape pomace extracts ingestion. Generally, lower concentrations of metabolites were observed in plasma than in urine. The highest metabolite accumulation was observed in plasma from VE5 experimental group in which SHR rats were fed MOU (EA70) skin pomace extracts +

verapamil. In fact, an amount up to 4.6 ± 0.07 nmol and a recovery of 0.15 % were observed compared to other plasma in which only a 0.03 % to 0.09 % recovery was found. These results are in accordance with those obtained with urine, showing a high intake of grape pomace polyphenols in VE5 experimental group (4.25 %). The E5 group also showed substantial urinary excretion polyphenols (4.41 % of intake) while no more than 0.09 % of intake accumulated in plasma. This indicates a rapid excretion of metabolites from the circulatory system. Grape pomace polyphenols were less well absorbed by rats fed with other extracts (i.e. E1, VE1, E6 and VE6), as illustrated by low levels in urine and plasma.

In SHR rat tissues, it was observed that rats fed with MOU (EA70) skin pomace extracts + verapamil (VE5 SHR group) accumulated high amounts of metabolites. Likewise there were higher accumulations of metabolites after ingestion of MOU (EA70) skin pomace extracts + verapamil than other extracts, especially in the kidneys and liver (0.14 % and 1.64 %, respectively). Faecal phenolic excretion in SHR rats occurred predominantly at 8-24 h time point. Recoveries ranged from 0.3 % in VE6 to 2.31 % in E5 at day 1 and 1.03 % in VE1 to 1.72 % in E5 after seven days of grape pomace extracts ingestion.

A total of 21 phenolic acids and aromatic compounds were identified in urine of SHR rats by GC-MS. Results obtained by GC-MS confirmed those obtained by HPLC-ESI-MS^n and showed an increase of bioavailability of MOU (EA 70) skin pomace extracts + verapamil which was low at day 1 but increased up to 85-fold at day 7 (1.2 % of intake at day 1 and 102 % of intake at day 7). Using GC-MS, the difference of absorption between E1/VE1 and E6/VE6 was more evident. At day 1, rats fed with GRE1 (EA70) seed pomace extracts + verapamil absorbed 12-fold more grape pomace polyphenols than those fed with GRE1 (EA70) seed pomace extracts alone and rats fed with ALI (EA70) skin pomace extracts + verapamil absorbed 5-times more than those fed with an extract without verapamil. At day 7, the same profiles were apparent but to a lesser extent (same absorption rate for E1/VE1 and 4-fold higher for E6/VE6). Analysis of faeces revealed higher excretion of catabolites at day 1 than at day 7, showing once again a better absorption of polyphenols by SHR rats over time. The highest excretion rate was by rats fed MOU (EA70) skin pomace extract with excretions corresponding to 54 % and 108 % of polyphenols ingested, suggesting extensive catabolism of polyphenols contain in this extract by colonic microbiota.

Grape pomace extracts with or without co-ingestion with verapamil were absorb as phase II metabolites mainly including glucuronide, *O*-methyl glucuronide, sulfate, and *O*-

methyl sulfate derivatives of (epi)catechin which arise from the metabolism of monomeric flavan-3-ols. The detection of microbial-derived metabolites of flavan-3-ols, hydroxyphenyl-γ-valerolactones in their glucuronide and sulfate forms confirmed the absorption of metabolites derived from both monomeric and polymeric flavan-3-ols from grape pomace extracts and subsequent conjugation in the liver. Numerous metabolites derived from further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavan-3-ols, indicating the important role of intestinal bacteria in the metabolism of highly polymerized compounds. All these metabolites may have exerted biological effects during the period in which they circulated in the bloodstream.

Therefore, considering these observations, anti-hypertensive effects could be attributed to the action of polyphenols contained in grape pomace extracts (in the case rats from E1 and E6 group) and/or by the action of verapamil. In E1, E5 and E6, polyphenols, especially flavan-3-ol monomers and procyanidins, contained in each grape pomace extracts were responsible for the non- or anti-hypertensive effect. In fact, GRE1 (EA70) seed pomace extracts and ALI (EA70) skin pomace extracts were showed to contain higher levels of polyphenols than MOU (EA70) skin pomace extracts. In VE1 and VE6 group, verapamil might allow a higher absorption of polyphenols or vice versa. The anti-hypertensive might be due to the action of polyphenols and/or verapamil. In the case of E5/VE5 group, polyphenols was showed to be highly absorbed in E5 group and when associated to verapamil, the polyphenol metabolite/catabolite urinary excretion, and hence absorption, increased. Thus, verapamil might allow better absorption of polyphenols, but, because of low concentration or the composition of MOU (EA70) skin pomace extracts no anti-hypertensive effects were observed.

This study constitutes the first step of assessing grape pomace as an enhancer of the verapamil, an anti-hypertensive drug. Substantial levels of polyphenols after the winemaking process, especially flavan-3-ols, procyanidins and anthocyanins remain in pomace in quantities sufficient to exert anti-hypertensive effects. In addition, according to the extract used and its composition, it is feasible to modulate anti-hypertensive effects by amplifying or decreasing polyphenols and/or verapamil absorption. Therefore, it will be interesting to elucidate the exact mechanisms and compounds involved in this phenomenon in order to have a better control on blood pressure regulation and facilitate the choice of effective grape pomace extracts for further experiments. Moreover, it will be useful to investigate the effect

of each flavan-3-ol fractions (i.e. oligomeric, monomeric) and anthocyanin fractions (i.e. glucosides, acetylated glucosides and coumarylic glucosides) in order to identify whether anti-hypertensive effects are linked to a particular compound or to the extracts as a whole.

Additional bioavailability studies could be useful to confirm results obtain with the E5/VE5 group showing that verapamil might allow better absorption of polyphenols, but, because of low concentration or the composition of MOU (EA70) skin pomace extracts no anti-hypertensive effects were observed. In addition, the quantification of verapamil metabolites might be of great interest to evaluate verapamil absorption levels compared to those of polyphenols.

As SHR represents a good model to investigate hypertension, study could be extended to human clinical trials. For clinical tests, different parameters have to be taken into account such as the subjects (i.e. pre-hypertensive or hypertensive subjects), the dose used, the diet, biological fluid collections, and biological markers to be quantified. In addition, different processes will have to be considered such as the choice grape pomace varieties and their parts (seeds/skins), the extraction processes which will be used, the dosage and the galenic formulation used in order to provide great stabilisation of the active substances.

Cette étude examine les effets bénéfiques des marcs de raisins obtenus après vinification de différents cépages caractéristiques de la Vallée du Rhône, de leurs compositions phénoliques à leurs effets *in vivo*. Les raisins et leurs marcs respectifs des cépages Grenache (provenant de deux endroits différents [GRE1] et [GRE2]), Syrah (provenant de deux endroits différents [SYR1] et [SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) et Alicante (ALI) ont été étudiés. Le contenu en polyphénols des extraits a été dosé de façon globale (composés phénoliques totaux, tanins totaux et anthocyanes totales) et individuelles par HPLC-UV-Fluo-MS (tanins monomériques et condensés, degrés de polymérisation, anthocyanes glycosylées, acétylées et coumaroylées). Par la suite, l'activité antioxydante a été appréciée en utilisant quatre tests différents d'activités antioxydantes : $ABTS^{\bullet+}$, DPPH, FRAP et ORAC. Les ratios entre la quantité de polyphénols issus des raisins et la quantité finale retrouvée dans les marcs ont été estimés et la corrélation entre le contenu en composés phénoliques et l'activité antioxydante a été établie.

La comparaison des extraits de raisins et de leurs marcs respectifs montre que les marcs représentent une source importante d'antioxydants phénoliques malgré le processus de vinification. Les pépins et pellicules de marcs renferment des quantités appréciables de flavan-3-ols et anthocyanes. La distribution qualitative et quantitative des polyphenols dans les marcs de raisin présentent des différences significatives au travers des variétés et millésimes allant de 15% à 70% de polyphénols extraits.

Dans une première étude de caractérisation de raisins et de marcs après séparation pépins-pellicules, il a été démontré que les pépins de Grenache (GRE1), Syrah (SYR1) et les pellicules de Syrah (SYR1), Carignan et Alicante sont les fractions les plus intéressantes dues a la présence d'importantes quantités de flavan-3-ols (monomères, dimères et trimère) jusqu'à 8.7 mg/g MS et d'anthocyanes (glycosylées, acétylées et coumaroylées jusqu'à 17.40 mg/g MS, 1.57 mg/g MS et 2.38 mg/g MS respectivement). De même, ces extraits présentent les pouvoirs antioxydants les plus élevés pour tous les tests confondus. Par conséquent, ces

variétés et leurs parties (pépins/pellicules) ont été sélectionnées pour des extractions supplémentaires avec d'autres types de solvants tels que l'eau et une solution hydro-alcoolique 70 % compatible à la consommation et la commercialisation. Deux différentes extractions ont été effectuées afin d'obtenir deux types d'extrait pour chaque échantillons : un extrait aqueux (EAQ) et un extrait hydro-alcoolique 70% (EA70).

L'analyse des extraits aqueux (EAQ) et hydro-alcoolique 70% (EA70) indique que les pépins de Carignan et Syrah (SYR1) et les pellicules de Carignan et d'Alicante contiennent les plus fort taux en composés phénoliques et activités antioxydantes. La solution hydro-alcoolique 70% permet une meilleure extraction de flavan-3-ols, procyanidins et anthocyanes dans les extraits EA70. Cependant, les extraits aqueux (EAQ) affichent tout de même des quantités de polyphénols et de pouvoir antioxydants intéressants et ne devrait pas être ignoré. La lyophilisation permet une meilleure concentration des composés phénoliques dans les extraits. Toutefois, cette étape augmentera considérablement le coût de production des extraits. Ce point devrait être discuté d'avantage lors de l'élaboration des extraits finaux.

Bien que les extraits sélectionnés présentent une quantité importante en composés phénoliques et de forts pouvoirs antioxydants *in vitro*, ceci ne permet pas d'évaluer leur efficacité biologique *in vivo* dûe à une différence de biodisponibilité de chaque composé phénolique dans le milieu gastro-intestinale. Certains extraits, même ceux contentant de faible quantité en polyphénols ont été choisis afin d'évaluer leur efficacité *in vivo*.

Dans un premier temps, différents groupes expérimentaux ont été mis en place et les extraits de pépins et pellicules de raisins et de marcs ont été administrés seuls ou en association avec le vérapamil aux SHR. La pression artérielle a été suivie et l'effet des extraits sur l'hypertension a été apprécié. Trois extraits ont été choisis afin d'étudier leur biodisponibilité. Les urines, fèces, plasmas et organes (foie, reins et cœur) ont été récoltés. Les métabolites ont été identifiés et quantifiés par HPLC-UV-MS^n et GC-MS.

Les résultats des expériences *in vivo* démontrent que certains extraits administrés seuls ou en association avec le vérapamil possèdent un effet anti-hypertenseur. Cette capacité a été mise en évidence une fois que les extraits de pépins de marcs de GRE1 (EA70) et SYR1 (EA70) et de pellicules de marcs d'ALI (EA70) ont été administrés seuls ou lorsque les extraits de pépins de marcs GRE1 (EA70) et SYR1 (EAQ) et les pellicules de marcs d'ALI (EA70) et SYR2 (EAQ) ont été administrés en association avec le vérapamil. Dans la dernière expérience *in vivo*, les pellicules de marcs de SYR1 (EA70) en association avec le

vérapamil révèle un effet inverse en élevant la pression systolique. Le tableau ci-dessous résume l'efficacité *in vivo* des extraits de pépins et de pellicules de marcs testés seuls ou en association avec le vérapamil sur la pression artérielle (PA).

Table 1: Effet *in vivo* des extraits de pépins et pellicules de marcs sur la pression artérielle.

Extraits de marcs testés	Temps de traitement	Effet sur la PA	Efficacité
GRE1 pep (EA70)	3 semaines	+	Effet indirect, effet rebond, **+ 19%**
SYR1 pep (EA70)	3 semaines	+	Effet indirect, effet rebond, **+24 %**
SYR2 pell (EA70)	3 semaines	~	-
CAR pep (EA70)	3 semaines	~	-
MOU pell (EA70)	3 semaines	~	-
ALI pell (EA70)	3 semaines	+	Effet indirect, effet rebond, **+16 %**
GRE1 pep (EA70) + vérapamil	3 semaines	+	Effet direct, **-26 %**
SYR1 pep (EA70) +vérapamil	3 semaines	~	-
SYR2 pell (EA70) + vérapamil	3 semaines	~	-
CAR pep (EA70) + vérapamil	3 semaines	~	-
MOU pell (EA70) +vérapamil	3 semaines	~	-
ALI pell (EA70) + vérapamil	3 semaines	+	Effet direct, **-13 %**
SYR1 pep (EAQ) + vérapamil	2 semaines	+	Effet direct, **-14 %**
SYR1 pell (EAQ) + vérapamil	2 semaines	~	-
ALI pell (EAQ) + vérapamil	2 semaines	~	-
GRE2 Pell (EA70) + vérapamil	2 semaines	~	-
CAR pep (EAQ) + vérapamil	1 semaine	~	-
GRE2 pell (EAQ) + vérapamil	1 semaine	~	-
SYR2 pell (EAQ) + vérapamil	1 semaine	+	Effet direct, **-11 %**
SYR1 pell (EA70) + vérapamil	1 semaine	−	Effet direct, **+15 %**

Les extraits ont été administrés à une dose de 21mg/kg/jour de polyphénols par jour ce qui équivaut à une consommation de polyphénols contenus dans 500 mL de vin rouge par un adulte de 70kg et le vérapamil à une dose de 40 mg/kg/jour. Pell, pellicules; pép, pépins; +, effet positif sur la régulation de l'hypertension; −, effet negatif sur la régulation de l'hypertension ; ~, pas d'effet. L'efficacité des extraits administrés aux rats SHR a été illustrée par le pourcentage de variation (%) de la pression artérielle avant et après l'ingestion des extraits.

Les extraits de marcs administrés seuls régulent la pression artérielle et a été démontré par l'effet rebond bien connu dans ce type de traitement. Une augmentation de + 19%, +24% et +16% a été observée pour les extraits de pépins GRE1 (EA70) et SYR1 (EA70) et de pellicules ALI (EA70) après 3 semaines de traitement. Ingéré en association avec le vérapamil, les pépins de GRE1 (EA70) diminuent la pression artérielle de -26% et les pellicules d'ALI (EA70) de -13% après 3 semaines de traitement et de -14% après 2 semaines de traitement. De plus, les expériences montrent qu'une semaine suffise pour induire des effets antihypertenseurs dans le groupe de rats SHR ayant reçu l'extrait de pellicules de marcs SYR2 (EAQ) + vérapamil dans lequel une réduction de 11 % a été observé et des effets pro-

hypertenseurs dans le groupe de rat ayant reçu l'extrait de pellicules SYR1 (EA70) + vérapamil (+15%).

Selon le type d'extraits administrés aux rats SHR, il est possible de moduler les effets antihypertenseurs du médicament. La composition des extraits de marcs en composés phénoliques joue un rôle crucial et devrait être pris en compte. Malgré une quantité plus importante en composés phénoliques dans les extraits EA70, les extraits EAQ ont tout de même prouvé leurs efficacités dans la régulation de la pression artérielle.

Parmi les extraits de pépins de marcs, les extraits aqueux et hydro-alcoolique 70% de CAR qui contiennent une grande quantité de polyphenols comparé aux extraits de pépins GRE1, ne révèle aucun effet anti-hypertensive contrairement à GRE1. Les extraits de pépins exerçant un effet anti-hypertensif (GRE1 [EA70], SYR1 [EA70] et SYR1 [EAQ]) contiennent les plus grandes quantités en procyanidines (56 à 157 mg/g MS), en particulier dans la Syrah où 137 mg/g et 157 mg/g MS de procyanidins ont été détectés dans les EAQ et EA70, respectivement. De plus, ces extraits contiennent d'importantes quantités de dérivés gallates et sont fortement galloylés comparés aux extraits EAQ et EA70 de pépins de CAR. Les études de caractérisation ont aussi montré que les pépins de Syrah contiennent de grande quantité de monomères et dimères de flavan-3-ols dans les deux types d'extraits. En effet, parmi les extraits administrés aux SHR, la Syrah est la seule variété possédant des effets antihypertenseurs dans les deux types d'extraits. En terme d'anthocyanes, la plus grande concentration a été détectée dans les pépins de CAR. En effet, GRE1 et SYR1 possèdent seulement 5 mg/g MS et 10.3 mg/g MS dans les EA70 comparé à 25.5 mg/g MS dans CAR (EA70). L'évaluation des activités antioxydantes met en évidence la SYR1 EAQ et EA70 comme ayant le plus fort potentiel. L'efficacité *in vivo* à moduler la pression artérielle des extraits de pépins de GRE1 (EA70), SYR1 (EA70) et SYR1 (EAQ) est serait plutôt liée à la grande quantité de flavan-3-ols et procyanidins que par les anthocyanes.

Concernant les extraits des pellicules de marcs, parmi les neuf extraits testés, seulement deux révèlent des effets antihypertenseur : ALI (EA70) et SYR2 (EAQ). La caractérisation phénoliques des extraits a mis en évidence les extraits aqueux et hydro-alcooliques de l'ALI comme étant la plus riche en composés phénoliques et ayant le plus fort pouvoir antioxydant. L'ALI (EA70) présente en effet une grande quantité en monomères et dimères de flavan-3-ols et 28 mg/g MS de procyanidines. Malgré une quantité de 59 mg/g MS de procyanidines, l'extrait aqueux d'ALI ne possède aucune capacité à moduler la

pression artérielle. La capacité antihypertensive des extraits serait probablement dûe au contenu en anthocyanes. En effet, les pellicules de marcs d'ALI (EA70) possèdent jusqu'à 19.5 mg/g MS, 1.2 mg/g MS et 7.2 mg/g MS d'anthocyanes glycosylées, acétylées et coumaroylées respectivement. Toutefois, une grande composition en composés phénoliques ne se traduit pas toujours par un effet *in vivo*. SYR2 (EAQ) qui a été évalué comme possédant les plus faibles quantités en polyphénols pour toutes les analyses confondues, démontre tout de même une capacité à diminuer la pression systolique une fois administré en association avec le vérapamil aux SHR, alors que les autres extraits possédant une plus grande concentration en polyphénols ne possèdent aucuns effets antihypertenseurs et pourrait même augmenter la pression systolique comme dans le cas des extraits de SYR1 (EA70). Contrairement aux extraits de pépins de marcs, la capacité antihypertenseur des extraits de pellicules de marcs est plus difficile à prédire. D'autres composés contenus dans les pellicules tels que les polysaccharides ou les fibres pourraient empêcher les effets antihypertenseurs des extraits en affectant l'absorption des polyphénols.

Afin d'approfondir les connaissances sur le niveau d'absorption des polyphénols et leurs contributions aux effets antihypertenseurs, une étude de biodisponibilité des trois extraits de marcs administrés seuls ou associés au vérapamil, dont deux ayant montré une capacité anti-hypertenseur, a été réalisée par la suite. L'HPLC-ESI-MS^n a permit la détection de grandes variétés de métabolites y compris les dérivés méthylés, glucuronidés et sulfatés de flavan-3-ols, des structures non-, mono- et di- phényles glucuronides et sulfates ou même des methoxy-sulfates d'acide hydroxyphenylvalérolactone, d'acide hydroxyphenylvalerique, d'acide hydroxyphenylacétique, d'acide hydroxybenzoique, d'acide hydroxyphenylpropionique, d'acide hydroxycinnamique et d'acide hydroxyhippurique. Un totale de 61 métabolites a été détecté mais seulement 18 ont été quantifiés. En l'absence de standards glucuronidés et sulfatés, une quantification par le biais d'aglycones pourrait introduire des erreurs de quantification due a des réponses SIM/SRM différentes entre aglycones/glucuronides et aglycones/sulfates. L'analyse des urines par HPLC-ESI-MS^n révèle une plus grande excrétion de métabolites chez les SHR ayant reçu des extraits de marcs associés au vérapamil.

Compte tenu du pourcentage de l'apport correspondant à la quantité excrétée dans l'urine, les données n'ont pas montré de différences significatives entre E1/VE1 (1,02% vs 1,29% au 1^{er} jour de gavage et de 1,52% vs 1,08% au $7^{ème}$ jour de gavage) et E6/VE6 (1,02 % vs 1,02% au 1^{er} jour de gavage et 1,42% vs 1,64% au $7^{ème}$ jour de gavage), excepté pour les

polyphénols contenus dans les extraits de pellicules de MOU (EA70) qui sont 27 fois mieux absorber au 7ème jour de gavage qu'au 1er jour de gavage, en particulier lorsqu'il est associé au vérapamil (0,16% 1er jour de gavage à 4,25% au 7ème jour de gavage). Ce résultat suggère qu'au fil du temps, les polyphénols sont mieux absorbés ou accumulés chez les rats SHR.

Concernant les plasmas, un total de 11 métabolites composé de dérivés glucuronidés, méthyl-glucuronidés et di-méthylglucuronidés d'épicatéchine ainsi que des dérivés glucuronidés, sulfatés, non- et mono-substitués de valerolactone et d'acide valérique ont été retrouvés 4 heures suivant l'ingestion des extraits de marcs. Aucun composé détecté dans les extraits de pépins et pellicules de marcs tels que les flavan-3-ols et procyanidins n'a été décelé dans les plasmas. Ces métabolites ont aussi été retrouvés dans les urines à l'exception du di-méthyl-(épi)catéchin-*O*-glucuronide et sont principalement présents sous forme de glucuronides que sulfates. Généralement, de plus faibles concentrations ont été retrouvées dans les plasmas que dans les urines. La plus forte concentration a été décelée dans les plasmas des rats SHR appartenant au groupe expérimental VE5, ayant reçu l'extrait de pellicules MOU (EA70) + verapamil. En effet, jusqu'à 4,6 ± 0,07 nmol et 0.15 % d'absorption a été observé par rapport aux autres plasmas dans lequel seulement un taux de 0.03% à 0.09% a été retrouvée. Ces résultats sont en accords avec ceux observés dans les urines montrant une importante absorption des polyphenols dans le groupe expérimental VE5 (4.25%). Le groupe E5 excrète une grande quantité de polyphénols dans l'urine (4.41% de polyphénols ingérés) alors qu'une petite quantité a été détectée dans les plasmas. Ce résultat indique une rapide excrétion des métabolites du système circulatoire. Une faible absorption illustrée par une légère excrétion de métabolites dans les urines et plasmas a été mise en évidence dans les groupes de rat (E1, VE1, E6 et VE6).

Dans les tissus des rats SHR (foie, reins et cœur), aucuns dérivés de l'épicatéchine n'ont été retrouvés mis à part la 5-(hydroxyphényl)-γ-valérolactone-*O*-glucuronide (m/z 383/207, 163) et la 5-(hydroxyphényl)-γ-valérolactone-*O*-sulfate (m/z 287/207, 163) qui ont été détectées et quantifiées en mode SRM. Il a été observé que les rats ayant reçu les extraits de pellicules de MOU (EA70) + vérapamil (groupe VE5) concentre une plus forte concentration de métabolites dans leurs tissus par rapport aux autres groupes de rats SHR, particulièrement dans les reins et le foie (0.14% et 1.64%, respectivement). De plus, une plus importante accumulation de métabolites dans les rats administrés avec des extraits + vérapamil a été décelée dans les reins avec 5 fois plus dans le groupe VE1 que dans le groupe E1, 14 fois plus dans VE5 que dans E5 et 8 fois plus dans VE6 que dans E6.

Les fèces ont été dépistés pour les composés contenus dans les extraits de marcs par HPLC-MS^n en mode SIM pour la détection d'oligomères et de monomères flavan-3-ols. Sept composés incluant l'épicatéchine (m/z 289/245, 205), l' (épi)catéchine gallate (m / z 441/289, 169), un dimère (m/z 577/425, 407, 289), un trimère (m/z 865/577, 289), deux gallo (épi)catéchine trimères (m/z 881, 729, 577, 407) et un tétramère (m/z 153/865, 577) ont été détectés.

L'excrétion fécale de polyphénols survient principalement entre 8-24 h. Le pourcentage de polyphénols ingérés et excrétés dans les fèces varie de 0.3% dans VE6 à 2.31% dans E5 au 1^{er} jour de gavage et de 1.03% dans VE1 à 1.72% dans E5 après sept jours de gavage avec les extraits de marcs.

Les urines et les fèces ont été analysés pour les catabolites utilisant les techniques de GC-MS. Un total de 21 acides phénoliques a été identifié dans les urines des rats SHR. La grande majorité des acides phénoliques ont été excrétée dans l'urine après 8-24 h d'ingestion d'extraits de pépins et pellicules de marcs. L'excrétion de catabolites au bout de 24 h varie de 0,16 µmol dans VE5 à 12,7 µmol dans E5 au 1^{er} jour de gavage et de 3,17 µmol dans E6 à 17,4 µmol dans VE1 au $7^{ème}$ jour de gavage. Les résultats obtenus par GC-MS confirment ceux obtenus par HPLC-ESI-MS^n et ont montré une augmentation de la biodisponibilité de l'extrait de pellicule MOU (EA 70) associé au vérapamil. En effet, au $7^{ème}$ jour de gavage, 85 fois plus de polyphénols a été absorbé par rapport au 1^{er} jour de gavage (1.2 % de polyphénols ingérés détecté dans les urines au 1^{er} jour et 102% au $7^{ème}$ jour). Au 1^{er} jour de gavage, les rats ayant reçu des les extraits de pépins de GRE1 (EA70) + vérapamil ont absorbé 12 fois plus de polyphénols que ceux ayant reçu GRE1 (EA70) seul et les rats ayant reçu l'extrait de pellicules de marcs ALI (EA70) + vérapamil ont absorbés 5 fois plus de polyphénols que ceux ayant eux l'extrait seul. Au $7^{ème}$ jour de gavage, la même observation a été constatée mais avec une plus faible ampleur (même taux d'absorption pour E1/VE1 et 4 fois plus pour E6/VE6).

L'analyse des fèces par GC-MS reflète les acides phénoliques qui n'ont pas été absorbés au niveau du colon. Les résultats montrent une plus importante quantité de métabolites excrétés au 1^{er} jour de gavage par rapport au $7^{ème}$ jour illustrant encore une fois de plus une meilleure absorption des polyphénols au cours du temps. Le plus fort taux d'excrétion a été détecté dans les fèces des rats administré par l'extrait MOU (EA70) et correspond à 54 % de polyphénols ingérés au 1^{er} jour de gavage et à 108% de polyphénols

ingérés au 7ème jour de gavage. Cette observation suggère un catabolisme extensif des polyphénols contenus dans cet extrait par la microflore intestinale.

Cette étude met en évidence la biodisponibilité des extraits de pépins et de pellicules de marcs des rats SHR incluant à la fois le métabolisme de phase II et de la microflore intestinale. Les extraits de marcs administrés seuls et en association au vérapamil sont absorbés en tant que métabolites de phase II présent sous forme de dérivé *O*-methylglucuronidé, sulfaté, *O*-methylsulfaté d'(épi)catéchine dérivant du métabolisme des monomères de flavan-3-ols. La détection de métabolites microbiens dérivés de flavan-3-ols, d'hydroxyphényl-γ-valérolactones sous leurs formes glucuronidés et sulfatés confirme l'absorption des métabolites dérivés des flavan-3-ols, la fois monomères et polymères des extraits de marcs et des conjugaisons supplémentaires dans le foie. De nombreux métabolites issus de la dégradation microbienne des hydroxyvalerolactones ont également été détectés. L'excrétion urinaire de ces métabolites représente une plus grande proportion de polyphénols ingérés comparé à ceux issus de métabolisme de phase II des monomères de flavan-3-ols, indiquant un rôle important des bactéries intestinales dans le métabolisme des molécules hautement polymérisées. Ces métabolites peuvent avoir exercé leurs effets biologiques lors de leur passage dans la circulation sanguine.

Suite aux résultats obtenus lors des tests *in vivo*, l'association des extraits de pépins et de pellicules de marcs avec le vérapamil a prouvé son efficacité chez les rats SHR. L'extrait de pépins GRE1 (EA70) (administré au groupe E1 et VE1) et l'extrait de pellicules d'ALI (EA70) (administré aux groupe E6 et VE6) avec ou en association avec le vérapamil sont capables de faire diminuer la pression artérielle des rats SHR alors que dans les groupe de rats E5 et VE5 ayant reçu l'extrait de pellicules de MOU (EA70) seul ou en association avec le vérapamil, aucun effet sur la pression artérielle n'a été observé. Les études de biodisponibilité ont indiqué que les rats administrés avec les extraits de marcs associés au vérapamil génèrent une quantité plus importante de métabolites et catabolites que ceux ayant reçu l'extrait seul. Cependant, l'absorption de polyphénols dans les groupes E1 et E6 semble tout de même être légèrement supérieure à ceux ayant reçu du vérapamil (VE1 et VE6). Contrairement aux E1/VE1 et E6/VE6, les SHR des groupes E5/VE5 absorbent de plus grandes quantités de polyphénols. L'analyse par HPLC-ESI-MS^n a montré qu'au 1er jour de gavage, les SHR du groupe E5 excrètent la plus grande quantité de polyphénols sur 24 h (1,57 %) comparé aux autres groupes (1,02%-1,29%). La plus faible excrétion a été observé chez les VE5 avec une excrétion de seulement 0.16 % des polyphénols ingérés. La même

observation a été obtenue par GC- MS où il a été démontré que les SHR du groupe E5 excrètent une importante quantité de catabolites (181 % de l'apport en polyphénols) par rapport aux autres groupes (3,5%-105,5 %) même après 7 jours d'administration (excrétion de 4.41 % de polyphénols ingérés estimés par HPLC-ESI-MS^n et 76 % par GC-MS). Une fois de plus, les SHR du groupe VE5 excrètent la plus faible quantité de polyphénols (1.2% des polyphénols ingérés). Contrairement au 1^{er} jour de gavage, l'excrétion des SHR du groupe VE5 est plus élevé par rapport aux autres groupes avec 4.25% d'absorption détecté par HPLC-ESI-MS^n et 102% par GC-MS). En effet, une importante accumulation de métabolites a été démontrée dans les tissus et plasmas des rats appartenant au groupe VE5.

Il a été établi que les SHR développent spontanément une hypertension artérielle qui augmente avec l'âge de vie. L'HTA se manifeste à partir de la sixième semaine d'âge et se stabilise autour de la 20ème semaine d'âge avec une pression artérielle systolique atteignant 200 à 220 mmHg. L'origine de cette hypertension est multiple. Par rapport aux rats normotendus, les rats SHR présentent un déficit de l'enzyme de conversion 2 (ACE2), une mono-carboxypeptidase qui catalyse à la fois l'angiotensine I (Ang I) en angiotensine $_{(1-9)}$ (Ang 1-9) et l'angiotensine II (Ang II) en angiotensine $_{(1-7)}$ (Ang 1-7) (Donoghue et al. 2000). L'ACE2 est impliquée dans la régulation du système rénine-angiotensine (RAS) (Tikellis et al. 2006) et est reconnue pour son rôle majeur dans le contrôle de la pression artérielle. En effet, l'heptapeptide Ang 1-7 est un vasodilatateur et natriurétique dont les effets s'opposent à ceux de son précurseur. Le déficit d'ACE2 induit alors une augmentation de la concentration d'Ang II et conduit à une vasoconstriction. De plus, les rats SHR possèdent une relaxation endothéluim-dépendante réduite due à un dysfonctionnement endothéliale souvent associé à un fort stress oxydant. Ceci est causé d'un côté par un excès d'anion superoxyde ($O_2^{\bullet-}$) produit par l'enzyme NADPH oxydase (Grunfeld et al. 1995; Zalba et al. 2000) et d'un autre côté par une faible activité de la eNOS (endothelial NO synthase) qui catalyse la production du monoxyde d'azote ou NO, un puissant agent vasodilatateur produit par l'endothéluim à partir de la L-Arginine (Palmer et al. 1987). En effet, en présence du NO, l'ion superoxyde $O_2^{\bullet-}$ forme le peroxynitrite ($ONOO^-$) empêchant ainsi le NO d'exercer sa fonction vasodilatatrice (Beckman et al. 1990; McIntyre et al. 1999; Pacher et al. 2007). Physiquement, l'étude des tissus du rat SHR montre qu'au cours de son âge, l'oxygénation tissulaire diminue et entraine progressivement ainsi une hypertrophie cardiaque (Engelmann et al. 1987) ainsi qu'une augmentation de la résistance périphérique (Smith et al. 1979; Folkow et al. 1992).

Du fait de l'implication du stress oxydant dans la pathogenèse de l'hypertension, les chercheurs se sont intéressés aux effets antihypertenseurs des polyphénols retrouvés dans l'alimentation. Leur effet hypotenseur a été confirmé dans de nombreuses études (Galisteo et al. 2004; Sarr et al. 2006; Perez-Vizcaino et al. 2009) et serait lié a la capacité des polyphénols de réguler l'activité de la eNOS et de la NADPH oxydase. Une étude par Al-Awwadi et al. (2004) a montré qu'un extrait de polyphénols issus du vin rouge permettait de diminuer l'hypertension chez des rats ayant développé un syndrome métabolique suite à un régime riche en fructose. Plus précisément, des extraits polyphénoliques enrichis en anthocyanes et en catéchines préviennent l'hypertension chez le même modèle animal ce qui n'est pas le cas des procyanidines (Al-Awwadi et al. 2005). L'effet antioxydant n'est donc pas le seul mode d'action de ces molécules. Ces observations sont en adéquation avec nos résultats dans lequel de fortes concentrations en monomères et dimères de flavan-3-ols ont été détectés dans les extraits de pépins de marcs exerçant des effets antihypertenseurs.

Les extraits de marcs peuvent être utilisés seuls mais leurs effets peuvent être amplifiés une fois co-ingéré avec le vérapamil. Il est largement reconnu que la glycoprotéine P souvent nommé « P-gp » influence le transport des médicaments au travers des membranes. La P-pg se retrouve principalement au niveau des cellules du tubule contourné proximal du rein, des cellules de l'intestin, de l'endothélium de la barrière hémato-encéphalique etc. Par conséquent, elle peut influencer l'absorption, la distribution, le métabolisme et l'excrétion des xénobiotiques. La P-pg provoque par efflux, un phénomène de multirésitances à plus de 50% des agents anti-cancéreux. Par conséquent, les chercheurs tentent d'optimiser la pharmacocinétique, d'améliorer les activités anti-tumorales et de réduire la toxicité systémique des médicaments anti-cancéreux en inhibant les efflux de la P-glycoprotéine. Bien qu'une grande variété d'inhibiteur de la P-gp ont été découverts, les flavonoïdes constituent la troisième génération de la catégorie non-pharmaceutique d'inhibiteurs de la P-gp (Bansal et al. 2009). Il a été démontré que les effets produits par certains de ces composants sont comparables à ceux des inhibiteurs connus de la P-pg tels que le vérapamil et la cyclosporine.

Comme le vérapamil, un inhibiteur de référence de la P-pg et des phénomènes de multi-résistance aux médicaments (MDR), les polyphénols ont été démontrés comme ayant des propriétés inhibitrices sur le transport de la P-pg affectant la biodisponibilité et l'absorption de certains médicaments (Jodoin et al. 2002; Kitagawa 2006; Eichhorn et al. 2012). De ce fait, l'association d'extraits de pépins et de pellicules de marcs riches en

polyphénols au vérapamil peut avoir un effet bénéfique sur la régulation de la pression artérielle en modifiant l'absorption du (poly)phénols et/ou du vérapamil.

En tenant compte de ces observations et des résultats obtenus lors de l'étude *in vivo,* les effets antihypertenseurs des polyphénols peuvent être attribués à l'action des polyphénols contenus dans les extraits de marcs (dans le cas des rats des groupes E1 et E6) et/ou par l'action du vérapamil. Dans les groupes E1, E5 et E6, les polyphénols, en particulier les monomères et procyanidins de flavan-3-ols, peuvent contribuer aux effets antihypertenseurs des extraits. En effet, les extraits de pépins de GRE1 (EA70) et de pellicules d'ALI (EA70) renferment une importante quantité de polyphénols que l'extrait de pellicules de MOU (EA70). Dans le groupe VE1 et VE6, le vérapamil pourrait permettre une meilleure absorption de polyphenols ou vice versa et l'effet antihypertenseur pourrait être du à l'action des polyphénols et/ou le vérapamil. Dans les cas des groupes E5 et VE5, il a été démontré que les polyphénols sont absorbés de manière importante dans le groupe E5 et une fois associée au vérapamil, l'absorption est encore plus élevée. De ce fait, le vérapamil devrait permettre une meilleure absorption des polyphénols mais dû à une faible teneur ou à la composition en polyphénols des pellicules de MOU (EA70), cet extrait ne possède aucun effet antihypertenseur.

Cette étude constitue une première étape de valorisation de marcs de raisins après vinification, en tant qu'activateur de vérapamil, un médicament couramment utilisé contre l'hypertension. Des quantités suffisantes de composés phénoliques subsistent dans les marcs, en particulier en terme de flavan-3-ols et d'anthocyanes, pour exercer des effets antihypertenseurs. En effet, selon le type et la composition des extraits, il est possible de moduler les effets antihypertenseurs en amplifiant ou en diminuant l'absorption des polyphénols et/ou du vérapamil. Par conséquent, il serait intéressant d'élucider les mécanismes exacts et des composés impliqués dans ce phénomène afin de mieux contrôler la régulation de la pression artérielle et de faciliter le choix des extraits de pépins et de pellicules de marcs. En outre, il serait utile d'étudier l'effet de chaque flavan-3-ols (monomères individuels, oligomères…) et des fractions d'anthocyanes (glycosylées, acétylées et coumaroylées) afin de déterminer si les effets antihypertenseurs sont liés à un composé particulier ou à l'extrait dans son ensemble.

Des études complémentaires de biodisponibilité pourraient être utiles afin de confirmer les résultats obtenus avec le groupe E5/VE5 montrant que le vérapamil permet une

meilleure absorption des polyphénols mais en raison de la faible concentration ou de la composition en polyphénols de l'extrait de pellicules de marcs MOU (EA70), aucuns effets antihypertenseurs n'ont été observés. De plus, la quantification de métabolites de vérapamil pourrait être d'un grand intérêt pour évaluer les niveaux d'absorption du vérapamil par rapport à ceux de polyphénols.

Ayant montré leurs efficacités dans un le modèle de rat SHR, les extraits de pépins et de pellicules de marcs seuls ou en association avec le vérapamil pourrait faire l'étude d'essais cliniques humains. Différents paramètres tels que les sujets (sujets pré-hypertendus ou hypertendus), la dose à administrer, l'alimentation, les différentes collections (plasmas, urines) et les marqueurs biologiques à quantifier devrait être pris en compte. De plus, le choix des variétés des extraits de pépins et de pellicules, les procédés d'extraction à employer, le dosage, la forme galénique à utiliser afin de stabiliser les principes actifs devraient être pris en compte.

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Résumé: CONFIDENTIEL

Mot-clefs: polyphénols, hypertension, rats SHR, verapamil, biodisponibilité, métabolismes phénoliques, HPLC-UV-Fluo-MS^n, GC-MS

Cette étude examine les effets bénéfiques des marcs de raisins obtenus après vinification de différents cépages caractéristiques de la Vallée du Rhône, de leurs compositions phénoliques à leurs effets *in vivo*. Les raisins et leurs marcs respectifs des cépages Grenache (provenant de deux endroits différents [GRE1] et [GRE2]), Syrah (provenant de deux endroits différents [SYR1] et [SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) et Alicante (ALI) ont été étudiés.

La comparaison des extraits de raisins et de leurs marcs respectifs montre que les marcs représentent une source importante d'antioxydants phénoliques malgré le processus de vinification. Les pépins et pellicules de marcs renferment des quantités appréciables de flavan-3-ols et anthocyanes. La distribution qualitative et quantitative des polyphenols dans les marcs de raisin présentent des différences significatives au travers des variétés et millésimes allant de 15% à 70% de polyphénols extraits. Les pépins de Grenache (GRE1), Syrah (SYR1) et les pellicules de Syrah (SYR1), Carignan et Alicante sont les fractions les plus intéressantes dues a la présence d'importantes quantités de flavan-3-ols (monomères, dimères et trimère) jusqu'à 8.7 mg/g MS et d'anthocyanes (glycosylées, acétylées et coumaroylées jusqu'à 17.40 mg/g MS, 1.57 mg/g MS et 2.38 mg/g MS respectivement). L'analyse des extraits aqueux (EAQ) et hydro-alcoolique 70% (EA70) indique que les pépins de Carignan et Syrah (SYR1) et les pellicules de Carignan et d'Alicante contiennent les plus fort taux en composés phénoliques et activités antioxydantes.

Certains extraits on été afin d'évaluer leur efficacité *in vivo* sur l'hypertension utilisant un modèle de rat SHR. Les extraits de marcs ont administrés à une dose de 21mg/kg/jour de polyphénols par jour ce qui équivaut à une consommation de polyphénols contenus dans 500 mL de vin rouge par un adulte de 70kg et le vérapamil à une dose de 40 mg/kg/jour. Les résultats des expériences *in vivo* démontrent que certains extraits administrés seuls ou en association avec le vérapamil possèdent un effet anti-hypertenseur. Cette capacité a été mise en évidence une fois que les extraits de pépins de marcs de GRE1 (EA70) et SYR1 (EA70) et de pellicules de marcs d'ALI (EA70) ont été administrés seuls ou lorsque les extraits de pépins de marcs GRE1 (EA70) et SYR1 (EAQ) et les pellicules de marcs d'ALI (EA70) et SYR2 (EAQ) ont été administrés en association avec le vérapamil.

Cette étude met en évidence la biodisponibilité des extraits de pépins et de pellicules de marcs des rats SHR incluant à la fois le métabolisme de phase II et de la microflore intestinale. Les extraits de marcs administrés seuls et en association au vérapamil sont absorbés en tant que métabolites de phase II présent sous forme de dérivé *O*-methylglucuronidé, sulfaté, *O*-methylsulfaté d'(épi)catéchine dérivant du métabolisme des monomères de flavan-3-ols. La détection de métabolites microbiens dérivés de flavan-3-ols, d'hydroxyphényl-γ-valérolactones sous leurs formes glucuronidés et sulfatés confirme l'absorption des métabolites dérivés des flavan-3-ols, la fois monomères et polymères des extraits de marcs et des conjugaisons supplémentaires dans le foie. De nombreux métabolites issus de la dégradation microbienne des hydroxyvalerolactones ont également été détectés. L'excrétion urinaire de ces métabolites représente une plus grande proportion de polyphénols ingérés comparé à ceux issus de métabolisme de phase II des monomères de flavan-3-ols, indiquant un rôle important des bactéries intestinales dans le métabolisme des molécules hautement polymérisées. Ces métabolites peuvent avoir exercé leurs effets biologiques lors de leur passage dans la circulation sanguine.

Cette étude constitue une première étape de valorisation de marcs de raisins après vinification, en tant qu'activateur de vérapamil, un médicament couramment utilisé contre l'hypertension. Des quantités suffisantes de composés phénoliques subsistent dans les marcs, en particulier en terme de flavan-3-ols et d'anthocyanes, pour exercer des effets antihypertenseurs. En effet, selon le type et la composition des extraits, il est possible de moduler les effets antihypertenseurs en amplifiant ou en diminuant l'absorption des polyphénols et/ou du vérapamil.

Summary: CONFIDENTIAL

Keywords: polyphenols, hypertension, SHR rats, verapamil, bioavailability, phenolics metabolism, HPLC-UV-Fluo-MS^n, GC-MS

This study investigated the beneficial potential effects of grape pomaces obtained after winemaking of different Mediterranean grape varieties from crude materials to their *in vivo* effectiveness. Grapes and their respective grape pomaces from six different *V. vinifera* L. cultivar were studied namely Grenache (from two different locations [GRE1 and GRE2]), Syrah (from two different locations [SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI) grape varieties from the Rhône Valley.

The comparison of several wine industry by-products with their respective grapes provided evidence that pomace remaining at the end of the winemaking process can be very rich sources of antioxidants. The quantitative and qualitative distribution of polyphenols by HPLC-PDA-Fluo-MS in grape pomaces showed significant differences through varieties and vintages varying from 15% to 70% of polyphenols extracted. Seeds from Grenache (GRE1), Syrah (SYR1) and skins from Syrah (SYR1), Carignan and Alicante were of particular interest because of their higher polyphenol contents in terms of flavan-3-ols (monomers, dimers and trimers) up to 8.7 mg/g DW and anthocyanins (glycosides, acetylated and coumaroylated derivatives up to 17.40, 1.57 and 2.38 mg/g DW, respectively). The investigation of aqueous and hydro-alcoholic 70% extracts of seeds from Carignan and Syrah (SYR1) and skins from Carignan and Alicante was carried out as they contained high levels of total phenols and antioxidant activity.

Several extracts, were tested in order to evaluate their *in vivo* biological effects on hypertension using a spontaneously hypertensive rat (SHR) model. Grape pomace extracts were administrated at a dose of 21 mg/kg/day which in terms of phenolic compounds is equivalent to a 70 kg human consuming of ~0.5 L of red wine. A series of different grape pomace extracts were tested in association with verapamil. All *in vivo* experiments demonstrated that some grape pomace extracts administrated with or without co-ingestion with verapamil possessed an anti-hypertensive activity. This was evident with GRE1 (EA70) seed pomace extract, SYR1 (EA70) seed pomace extract, ALI (EA70) skin pomace extract administrated alone and with GRE1 (EA70) seed pomace extract, SYR1 (EAQ) seed pomace extract, ALI (EA70) skin pomace extract and SYR2 (EAQ) skin pomace extract administrated in association with verapamil.

Grape pomace extracts with or without co-ingestion with verapamil were absorb as phase II metabolites mainly including glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin which arise from the metabolism of monomeric flavan-3-ols. The detection by HPLC-PDA-Fluo-MS^n and GC-MS of microbial-derived metabolites of flavan-3-ols, hydroxyphenyl-γ-valerolactones in their glucuronide and sulfate forms confirmed the absorption of metabolites derived from both monomeric and polymeric flavan-3-ols from grape pomace extracts and subsequent post-absorption conjugation. Numerous metabolites derived from further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavan-3-ols, indicating the important role of intestinal bacteria in the metabolism of polymerized procyanidins. All these metabolites may have exerted biological effects during the period in which they circulated in the bloodstream.

This study constitutes the first step of assessing grape pomace as an enhancer of the verapamil, an anti-hypertensive drug. Substantial levels of polyphenols, especially flavan-3-ols, procyanidins and anthocyanins, remain in pomace after the winemaking process in quantities sufficient to exert anti-hypertensive effects. In addition, according to the extract used and its composition, it is feasible to modulate anti-hypertensive effects by amplifying or decreasing polyphenols and/or verapamil absorption.