PhD Thesis

Tesis Doctoral

Antioxidant and Phenolic Characterization, Bioavailability and Protective Effects under Oxidative Stress and Endothelial Dysfunction of a Red Wine Pomace Seasoning (RWPS)

Caracterización Fenólica y Antioxidante Biodisponibilidad y Efecto Protector frente al Estrés Oxidativo y la Disfunción Endotelial de un Sazonador obtenido a partir de Residuos de Vinificación.

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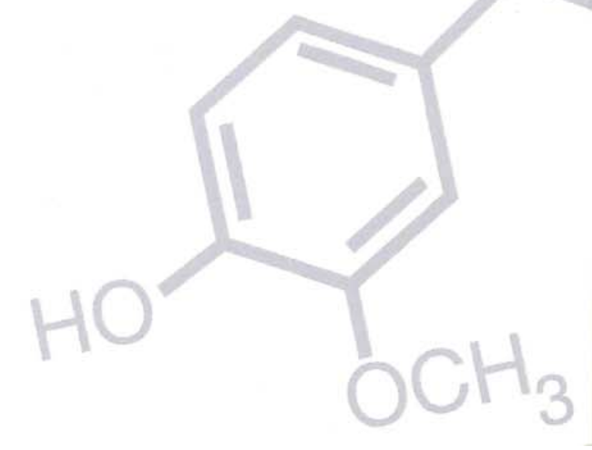

FACULTAD DE CIENCIAS
Dpto. de Biotecnología y Ciencia de los Alimentos
Area de Bioquímica y Biología Molecular

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TESIS DOCTORAL

Raquel del Pino García

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UNIVERSIDAD DE BURGOS
DEPARTAMENTO BIOTECNOLOGÍA Y CIENCIA DE LOS ALIMENTOS

Dña. Pilar Muñiz Rodríguez y Dña. María Dolores Rivero Pérez, profesoras titulares de la Universidad de Burgos y en su calidad de Directoras de la Tesis Doctoral,

CERTIFICAN

Que la memoria titulada "Antioxidant and Phenolic Characterization, Bioavailability and Protective Effects under Oxidative Stress and Endothelial Dysfunction of a Red Wine Pomace Seasoning (RWPS) " que presenta Dña. Raquel del Pino García, ha sido realizada bajo nuestra dirección y autorizamos su presentación para optar al Grado de Doctora.

Y para que así conste a los efectos oportunos, firmamos el presente certificado en Burgos, 5 de Abril de 2016.

Fdo. Dra. Pilar Muñiz Rodríguez

Fdo. Dra. Mª Dolores Rivero Pérez

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"El mundo necesita gente que ame lo que hace"

George H. Murdaskedano

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LIST OF ABBREVIATIONS AND NOMENCLATURE

ABTS, 2,2’-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid

ACE, angiotensin I-converting enzyme

ALA, α-lipoic acid

Ang, angiotensin

ANOVA, one-way analysis of variance

AP, associated protein

AREs, antioxidant responsive elements

AUC, area under the curve

BCF, potentially bioavailable fraction obtained after simulated colonic fermentation

BGID, potentially bioavailable fraction obtained after simulated gastrointestinal digestion

CAT, catalase

CF, colonic fermented

CFr, colonic fermented residue

CFs, colonic fermented supernatant

CGs, carbonyl groups

COX, cyclooxygenase

DAD, diode array detector

DM, diabetes mellitus

DMEM, Dulbecco's Modified Eagle Medium

DPPH, 2,2-diphenyl-1-picrylhydrazyl

ELISA, enzyme-linked immunosorbent assays

eNOS, endothelial nitric oxide synthase

F_2-IsoPs, F_2-isoprostanes

FC, Folin-Ciocalteu

FID, flame ionization detector

FRAP, ferric reducing/antioxidant power

FRSC, free radical scavenging capacity

GAE, gallic acid equivalents

GAR, global antioxidant response

GC-EI-MS, gas chromatography/electron ionization mass spectrometry

GC-MS, gas chromatography coupled to mass spectrometry

GC-NCI-MS, gas chromatography/electron capture-negative chemical ionization mass spectrometry

GID, gastrointestinal digested

GIDD, gastrointestinal digested+dialyzed

GPX, glutathione peroxidase

GR, glutathione reductase

GSH, glutathione reduced

GSSG, glutathione oxidised

GST, glutathione S-transferase

HDL-C, high density lipoprotein cholesterol

HO, heme oxygenase

HPLC, high performance liquid chromatography

HRSC, hydroxyl radical scavenging capacity

HRSC, hydroxyl radical scavenging capacity

HT-29, human colorectal adenocarcinoma

HUVECs, human umbilical vein endothelial cells

IC_{50}, concentration of each treatment that inhibited 50% of cell viability

iNOS, inducible nitric oxide synthase

LDH, lactate dehydrogenase

LDL-C, low density lipoprotein cholesterol

$LOO^{\cdot}$, lipid peroxyl

LPSC, lipid peroxyl scavenging capacity

LSD, Fisher's Least Significant Difference

MAPK, mitogen-activated protein-kinase

MDA, malondialdehyde

mDP, mean degree of polymerization

MTT, thiazolyl blue tetrazolium bromide

NAD(P)H, β-nicotinamide adenine dinucleotide phosphate

NF-κB, nuclear factor-kappa B

NO, nitric oxide

NO_2^-, nitrite

NO_3^-, nitrate

NOX, β-nicotinamide adenine dinucleotide phosphate oxidase

NQO, β-nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase

Nrf2, nuclear factor-erythroid 2-related factor 2

NT, non-treated seasonings

$^1O_2$, singlet oxygen

$O_2^{\cdot -}$, superoxide

$^{\cdot}OH$, hydroxyl

$OONO^-$, peroxynitrite

ORAC, oxygen radical absorbance capacity

PARP, poly(ADP-ribose) polymerase

PI3K, phosphatidylinositol 3-kinase

PKC, protein kinase C

qPCR, quantitative real-time polymerase chain reaction

QUENCHER, QUick, Easy, New, CHEap, and Reproducible assays

RAAS, renin-angiotensin aldosterone system

RNS, reactive nitrogen species

ROS, reactive oxygen species

RONS, reactive oxygen/nitrogen species

ROO$^{\cdot}$, peroxyl

RXNOs, nitros(yl)ated species

sBP, systolic blood pressure

SEM, standard error of the mean

RWPS, red wine pomace seasoning

SCFAs, short chain fatty acids

Sd-S, seasoning from wine pomace seeds

SHRs, spontaneously hypertensive rats

SIM, selected-ion monitoring

Sk-S, seasoning from wine seedless pomace

SOD, superoxide dismutase

SRSC, superoxide radical scavenging capacity

STZ, streptozotocin

TAC, total antioxidant capacity

TE, Trolox equivalents

Trx, thioredoxin

TrxR, thioredoxin reductase

UD, undigested

W-S, seasoning from whole wine pomace

WKY, Wistar-Kyoto

Introduction

1. Wine Pomace: Problems and Value-adding Opportunities

1.1. Grape and Wine Production Statistics

Vine cultivation and wine production represent two of the most extended agro-economic activities in the world, with an outstanding importance in Europe and, specifically, in Spain.

According to the latest statistics provided by the Food and Agriculture Organization of the United Nations (FAO), more than 77 million tons of grapes were produced globally in 2013. As regards regional classification, Europe led such statistics with around 38% of worldwide grape production, and regarding each country separately, Spain represented the fourth main contributor in the world producing almost 7.5 million tons of grapes (FAO 2016). However, concerning vineyard acreage, Spain is the leader country worldwide with around 2.34 million acres (corresponding to almost 0.95 million hectares) used for grape production in 2014, which represented 13% of world total, and an increasing trend has been observed during last years with a 1.7% change from 2011 to 2014 (Wine Institute 2015).

Of all grapes, cultivars of the *Vitis vinifera* L. species are the most cultivated and economically important throughout the world, and especially in Europe. Other important grape cultivars belong to *V. labrusca*, *V. rotundifolia*, *V. riparia* and *V. rupestris* species, as well as their hybrids with *V. vinifera* or with each other (Teissedre & Chervin 2011).

About 80% of grape crops are intended to be used by the wine industry for the winemaking process, with the worldwide wine production in 2013 being estimated in around 274 million hL. In this case, the leadership of Europe was even more remarkable, with 57% of production, and Spain was once more in the fourth position as top wine producer globally, with about 32 million hL (FAO 2016).

1.2. Wine Pomace: the Main Winemaking By-product

The winemaking process is based on ancestral procedures, being more an art than a science. As a general rule, many artisanal practices are strongly rooted in traditional wine production, and human resources or physical infrastructures during the winemaking operations are limited. Therefore, despite several technological advances and strategies have been addressed to minimize waste production (AWARENET 2004; Musee et al. 2007), their actual implementation and update in wineries is rather restrained, and extensive amounts of residues and potential by-products are generated by the wine industry.

As shown in **Figure 1**, such agro-industrial waste streams are generated along the different steps of winemaking, mainly during de-stemming, pressing, racking, and stabilization steps (Teixeira et al. 2014), and include:

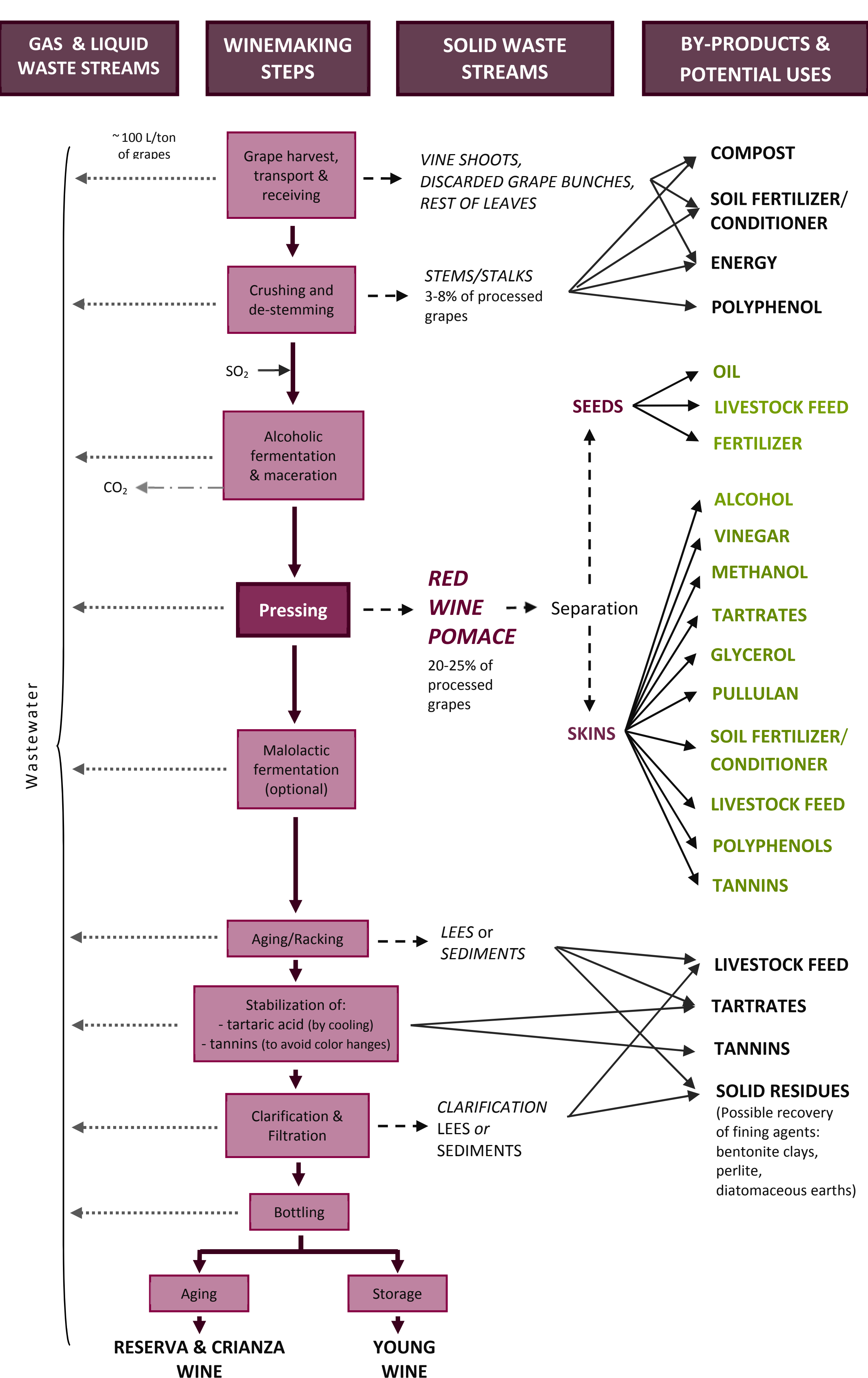


Figure 1. Red winemaking process, main waste streams, by-products and potential uses.

- Solid waste streams:
  - *Organic wastes*: mainly wine pomace and stems/stalks.
  - *Inorganic wastes*: such as bentonite clays, perlite, diatomaceous earths and other fining agents used for clarification processes.
- Liquid waste streams: considerable volumes of wastewater and lees (which also contain solid residual substances).
- Gas waste streams: emissions of greenhouse gases, including CO_2 and volatile organic compounds.

Wineries and wine-related businesses can choose between numerous ways to manage these waste streams, including either traditional or cutting-edge strategies for their value-adding, some of which have been also indicated in **Figure 1**.

Wine pomace is obtained after the pressing step of the winemaking process, and consists principally of the remaining grape skins, seeds, rest of pulp and some stems. It further comprises residual yeast and bacteria, which are the microbial agents in charge of carrying out alcoholic and malolactic fermentations. In the case of red wine pomace, the alcoholic fermentation takes place before pressing, so this solid by-product is previously in direct contact with wine. In contrast, since colour is undesirable to produce white wine, in this case the pomace is separated from the moisture before the alcoholic fermentative stage to avoid the extraction of colour compounds during this process (Dwyer et al. 2014)

It must be noted that wine pomace represents the main by-product of the wine industry. The amounts of wine pomace generated in each winery are dependent upon the species and varieties (cultivars) of grapes used as well as the equipment and pressing process applied, but many studies have estimated pomace to be approximately 20-25% (w/w) of the total amount of grapes processed (Yu & Ahmedna 2013; Dwyer et al. 2014). Convincing worldwide statistical data concerning amounts of wine pomace resulting from winemaking are not available as all producing regions are not included. As such, despite FAO data of 2013 indicated that around 3.44 million tons of wine pomace were generated, from which around 3.16 million tons were produced in Europe, and about 1.10 million tons in Spain (FAO 2016), factual values are expected to be much higher: around 16 million tons generated globally, about 6 million tons in Europe, and close to 1.5 million tonnes in Spain.

The maintenance of high volumes of wine production is not compatible with minimizing the huge amounts of wine pomace generated during winemaking because this is an inevitable intrinsic by-product of the process. Thus, appropriate management of this by-product is an important issue for the wine industry, as handling, storage and the final fate of wine pomace can be serious problems for wineries, but these tasks also offer interesting opportunities to improve the efficiency of the whole winemaking process.

1.3. Problems Associated to Traditional Wine Pomace Management

Wine pomace is produced in a seasonality way within a short period of time (from September to November) and the majority of this by-product is generally treated as a waste, being discarded in open areas next or close to the wineries and finally discarded in landfills.

In addition, a number of traditional low-economic-value alternatives for wine pomace reutilization, taking into account the organic and nutrient contents of this by-product, have been developed (Rondeau et al. 2013; Fontana et al. 2013), such as its use for:

- Production of **alcohol** by distillation, and for recovery/production of **tartrates**, **glycerol**, and **methanol** in distilleries by applying further treatments to the remaining pomace.
- **Livestock feeding**.
- Direct dispersal on land as **soil amendment**, **conditioner** or **fertilizer**.
- **Composting**.

The first of these alternatives is the most profitable, but the residue remaining after distillation finally ends as any of the other options, which are ways of reusing wine pomace that still present several constraints. For example, certain polyphenols present in this winery by-product are known to be phytotoxic and display antimicrobial effects during composting, impairing their utilization for this purpose. When spreaded out on land (either directly or after composting) it should be considered that high concentrations of wine pomace per area of land may be detrimental to the environment, due to the phenolic compounds decreasing the pH of pomace and increasing its resistance to biological degradation. This fact should be also taking into account when wine pomace is discarded in landfills. Regarding its use as livestock feed, some animals show intolerance to certain components, such as condensed tannins, which negatively affects the digestibility of wine pomace as feedstuff (Arvanitoyannis et al. 2006; Dwyer et al. 2014).

These problems indicate that most of traditional wine pomace management practices can potentially lead to serious environmental pollution. Therefore, it is becoming a subject of increasing concern for wine processors and scientists to find a safe and profitable use for this winery by-product.

1.4. Current Value-Adding Proposals to Solve Wine Pomace Problems

The urgency of avoiding the environmental footprints of winemaking processes has recently encouraged a tough legal European framework (Regulation (EC) 479/2008) to ensure the efficiency of this agro-industrial activity, supporting measures to promote/improve the recovery and recycling processes. As a consequence, the utilization of wine pomace for alternative profitable uses has been a focus of intensive research and many applications have been proposed for the valorisation of this by-product (Teixeira et al. 2014; Yu & Ahmedna 2013), some of which have been also patented for their national/international industrial application and exploitation (Gollücke 2010; Gollücke & Ribeiro 2012).

In several cases, skins and seeds contained in wine pomace are separated and utilized for different proposes, seeking to isolate interesting molecules mostly concentrated in one of these wine pomace components.

Grape seeds are usually used as a valuable source of oil (around 10-17% v/w). Approximately 90% of such **grape seed oil** is unsaturated, with linoleic acid (C18:2) being the predominant fatty acid, followed by oleic acid (C18:1) and palmitic acid (C16) (Tangolar et al. 2009).

Grape skin pulp is considered the best substrate for **pullulan** production through its fermentation with the yeast *Aerobasidium pullulans*. Pullulan is a non-ionic exopolysaccharide that present several biomedical, pharmaceutical, cosmetic and food technological applications as it is water soluble, biodegradable, impermeable to oxygen, tasteless, odourless, and shows high film-forming capabilities, among other desirable properties for such industries (Rekha & Sharma 2007).

However, the use of whole wine pomace, as well as its separated constituents, as raw materials for production of **polyphenol-rich extracts** is currently considered the most profitable alternative for its value-adding (Zhu et al. 2015).

Firstly, anthocyanins were extracted to be utilized as natural pigments and food colorants, products that have a great interest as substitutes for synthetic colorants. The oldest anthocyanin extract used in the food industry is “**enocyanin**”, which was obtained from red grape pomace and marketed in Italy since 1879 (Mateus & de Freitas 2009).

More recently, polyphenol-rich extracts have been value-added due to their antioxidant potential and have attracted the attention of researchers and industries in order to be incorporated into different foods, nutraceuticals and cosmetics as **natural antioxidants** (Yu & Ahmedna 2013; Zhu et al. 2015). Extraction techniques have been widely investigated to obtain high recoveries of these bioactive phytochemicals from wine pomace. Depending on the application, solvents classified as GRAS (generally recognized as safe) or not are required for these extractions, obtaining satisfactory recoveries when extraction parameters have

been optimized (Louli et al. 2004). Most of traditional methods consist on solid-liquid extraction and Soxhlet extraction techniques, many of which are not considered “green” practices as they involved the use of organic solvents, which may cause significant land, air and water pollution. In addition, some conventional techniques require overheating of matrices for long time, which might alter the structure and bioactivity of polyphenols.

In the past few years, other alternatives to improve the sustainability and yields of the extractive processes have been developed in an attempt to gradually switch conventional methods by novel eco-friendly extraction techniques with shorter extraction times and lower or even non-consumption of organic solvents (Fontana et al. 2013; Teixeira et al. 2014; Zhu et al. 2015). Some of these emerging techniques include:

- Microwave-assisted extraction.
- Ultrasonic-assisted extraction.
- Enzyme-assisted extraction.
- Supercritical fluid extraction.
- Accelerated solvent extraction (also known as pressurized liquid extraction, pressurized fluid extraction or subcritical solvent extraction).
- High-voltage electric discharge.
- Pulsed ohmic heating.

Nevertheless, despite these novel techniques allow gaining operational efficiency, most of them have not even been tested at a pre-industrial scale, and they require specific equipment, qualified technicians in certain cases, and high energy expenditure. Consequently, total operational costs are once more increased, which might force producers to make an important investment and/or to raise final sale prizes of polyphenol-rich extracts obtained through these innovative extraction techniques.

Saura-calixto (1998) proposed another alternative that consisted on value-adding wine pomace by avoiding any extractions steps. This approach is desirable as it avoids most of the aforementioned drawbacks of obtaining extracts, which also entails incomplete recovery of polyphenols and losses of the bioactivity and potential synergisms between phytochemicals. In fact, when antioxidants are isolated and then used as dietary supplements or incorporated into foods, other compounds of wine pomace with potential biological properties are lost, which is unwanted from a nutritional point of view. Thus, Saura-Calixto and co-authors firstly suggested the use of **powdered products** (obtained through this non-extractive approach) as ingredients to increase the antioxidant capacity and fibre contents of foods (Goñi & Martin-Carrón 1998; Martin-Carrón et al. 1997; Saura-calixto 1998). Lately, the potential of these types of powders as antioxidant and hypocholesterolemic dietary complements has been also demonstrated (Saura-Calixto 2011; Pérez-Jiménez et al. 2008; Pérez-Jiménez et al. 2009).

1.5. Main Bioactive Compounds present in Wine Pomace

Several compounds contained in red wine pomace may display important biologically active properties, with a wide range of health effects and food technological applications (Yu & Ahmedna 2013; Fontana et al. 2013; Zhu et al. 2015). These bioactive compounds include:

- ### Antioxidants

Halliwell & Gutteridge (1989) defined the term 'antioxidant' for the first time as 'any substance which, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate'. Some years later, the same authors simplified and updated that definition to 'any substance that delays, prevents, or removes oxidative damage to a target molecule' (Halliwell & Gutteridge 2007).

The majority of antioxidants are synthesised by various microorganisms, fungi, even animals, and most often by plants. These compounds are all known as natural antioxidants (Pokorný 2007; Carocho & Ferreira 2013). Among plant-derived antioxidants, fruits represent one of the most importance sources in the Spanish population (Tresserra-Rimbau et al. 2013).

Antioxidant compounds present in wine pomace derive from the large amount of phytochemicals contained in grapes processed for winemaking. The most important grape antioxidants are numerous phenolic compounds, mainly including simple phenolic acids, stilbenes and flavonoids (Anastasiadi et al. 2010; Georgiev et al. 2014). In fact, grape is one of the richest sources of polyphenols among fruits, with an average concentration in the range of 0.5-5 mg/g of fresh matter (Bravo & Saura-Calixto 1998).

During winemaking, the grapes are crushed and pressed, which does not alter their chemical composition. Thus, in red wine processing, fermentation is the only remarkable process that occurs before the pomace is produced and may significantly modify its antioxidant contents. Despite large chemical changes are not induced, some phenolic compounds (mainly part of those present in cell vacuoles) are extracted from grapes into wine during fermentation. Such phytochemicals account for most of the sensory characteristics of the respective wines, including colour, aroma, browning, and astringency (Waterhouse 2002). Even so, approximately 70% of bioactive phenolic compounds are retained within wine pomace after pressing (Arvanitoyannis et al. 2006; Georgiev et al. 2014).

These natural bioactive compounds may exert health promoting effects when consumed as part of functional food and nutraceuticals, and can be also used as food antioxidants and antimicrobials (Yu & Ahmedna 2013; Fontana et al. 2013).

In addition to phenolic compounds, wine pomace contains other antioxidants with recognized beneficial health effects, such as ascorbic acid (vitamin C), α- and γ-tocopherols (two of the various form of vitamin E), and β-carotene (which is converted to vitamin A in the body) (Wojcik et al. 2010). However, all these non-phenolic antioxidants are present at much lower concentration than phenolic compounds. In this regard, Rho & Kim (2006) reported that total flavonoids contents in grape pomace were close to 6 mg/g of powder, whereas the concentration of other antioxidants corresponded to around 0.500 mg of ascorbic acid/g of powder, 0.115 mg of α-tocopherol/g of powder, 0.128 mg of γ-tocopherol/g of powder, and 0.013 mg of β-carotene/g of powder.

The average daily intake of natural antioxidants in the Western diet was estimated in about 1000 mg (about 50-200 mg phenolic compounds per meal), of which 290 mg may derive from fruits and juices, and around 420 mg from beverages such as wine, coffee, tea, cocoa, beer, etc. (Murkovic 2003). As regards only polyphenols, an intake of around 1000 mg/day has been reported to be achievable in people who eat several servings of fruit and vegetables per day (Scalbert & Williamson 2000; Manach et al. 2004). Accordingly, based in the plant food and beverage intakes listed in the Spanish National consumption survey of 2001, the total dietary antioxidant capacity (TDAC) of the Spanish Mediterranean diet was estimated showing that about 20% of TDAC came from fruits and vegetables and 68% from beverages, with a very low contribution from cereals (Saura-Calixto & Goñi 2006). Additionally, this study estimated the mean total phenolic intake in 1171 mg gallic acid equivalents/person/day. More recently, a large (7,200 participants), parallel-group, multicentre, randomized, controlled 5-year feeding trial, which aimed to assess the effects of the Mediterranean diet on the primary prevention of cardiovascular diseases in the Spanish population (the PREDIMED study), concluded that the mean total polyphenol intake was about 820 mg/day (around 443 mg/day of flavonoids and 304 mg/day of phenolic acids). Fruits were the main source of polyphenols, providing almost 44% of the total polyphenol intake (about 360 mg/day, corresponding to around 255 mg/day of flavonoids and 72 mg/day of phenolic acids) (Tresserra-Rimbau et al. 2013).

According to the WHO recommendations, increasing fruit and vegetable consumption over 400 g/day is a population nutritional goal. This is due to the cumulative epidemiological evidence indicating that diets rich in plant-derived foods and beverages can reduce the incidence of several non-communicable diseases, such as cardiovascular and neurodegenerative diseases, diabetes and cancer, health benefits that are partly attributed to the preventive/protective effects against oxidative stress provided by fruits and vegetables, mainly due to their high polyphenol contents (Scalbert et al. 2005; Pandey & Rizvi 2009; Crozier et al. 2009) and other above mentioned phytochemicals (Wojcik et al. 2010). Recent data obtained from a huge cohort study (451,151 participants from 10 European countries) performed from 2000 to 2010 (the EPIC study) have shown that the

median value of fruits and vegetable intake was 388 g/day, with a trend to increase according to a North-to-South gradient. In Spain, such intake was of around 520 mg/day, corresponding to about 271 mg/day of fruits and 221 mg/day of vegetables (Leenders et al. 2013). Therefore, the Spanish population surpassed the fruit and vegetable intake suggested by the WHO, but the overall European population is slightly below such recommendation.

o Dietary Fibre

Dietary fibre is defined in the CODEX Alimentarius (FAO 2010) as 'carbohydrate polymers with ten or more monomeric units that are not hydrolysed by the endogenous enzymes in the small intestine of humans'. However, the inclusion of oligomers with a mean degree of polymerization (mDP) of 3-9 is suggested to be adopted by all countries for international harmonization (Lupton 2010). Among the three categories described in the CODEX, wine pomace dietary fibre could belong to two of them:

- Edible carbohydrate polymers naturally occurring in the food as consumed.
- Carbohydrates polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

There is an increasing interest in dietary fibre by consumers and the food industry in the last decades in view of the essential role that dietary fibre plays in intestinal health, and the significant association between a high-fibre intake and a lower risk of developing coronary heart disease, hypertension, diabetes and obesity, which has been established in numerous epidemiological and clinical studies (IOM 1999; McBurney 2010). These effects are primarily due to (Goñi et al. 2005; Wong et al. 2006; Jones 2013):

- A reduction in intestinal transit time and increase in stool bulk, which might reduce macronutrients absorption in the small intestine.
- A decrease in the blood concentrations of total cholesterol (TC) and/or low density lipoprotein cholesterol (LDL-C).
- A reduction in postprandial blood glucose and/or insulin concentrations.
- An increase in fermentation by colonic microbiota, with the subsequent production of short chain fatty acids (SCFAs) which positively affect major regulatory systems, such as blood glucose and lipid levels, intestinal immune functions, and the colonic environment, thus mediating some of the beneficial effects of dietary fibre intake. Predominant SCFAs generated are acetic, propionic and butyric acids, which are mainly metabolized by the colonic epithelium (butyrate), muscle (acetate) and liver (propionate). The extent of fermentation is dependent on both substrate and host factors.

Physiological and physicochemical effects of dietary fibre depend on the relative amount of individual fibre components, especially as regards to the soluble and insoluble fractions. Soluble dietary fibre includes β-glucans, fructans, oligosaccharides, some hemicellulose, pectins and hydrocolloids, with fruit, vegetables and legumes being the best sources of these fibres (although their content in insoluble dietary fibre is always much higher). Insoluble dietary fibre includes cellulose, some hemicelluloses and lignin, and it is especially abundant in whole cereal grains (Zhou et al. 2013).

Despite the most extensively advertised and consumed products rich in dietary fibre are derived from cereals, fruit dietary fibre-rich materials have in general a better nutritional quality because of the presence of higher amounts of associated bioactive compounds (flavonoids, carotenoids, etc.) and their more balanced composition: higher dietary fibre content, soluble/insoluble dietary fibre ratio, and water and fat holding capacities; lower energy value, and phytic acid content (Saura-calixto 1998).

Wine pomace contains high levels of dietary fibre (around 50-60% of dry matter), with the insoluble fraction comprising more than 90% of total dietary fibre. The main constituents were Klason lignin (around 40% dry mater), pectins, cellulose, and hemicelluloses. However, it has been demonstrated that wine pomace dietary fibre fractions are not composed only of lignin and non-starch polysaccharides, but other indigestible compounds, namely condensed tannins and resistant proteins, are also present in high percentages. For example, in red grape pomace skins, condensed tannins and resistant proteins represent around 27% and 11% dry matter, respectively (Bravo & Saura-Calixto 1998).

The biological activities of wine pomace antioxidant dietary fibre and its protective effects against a range of illnesses have been recently reviewed (Zhu et al. 2015). These benefits take into account the combined effect of dietary fibre and the antioxidants retained in such fibre and are summarized in **Figure 2**.

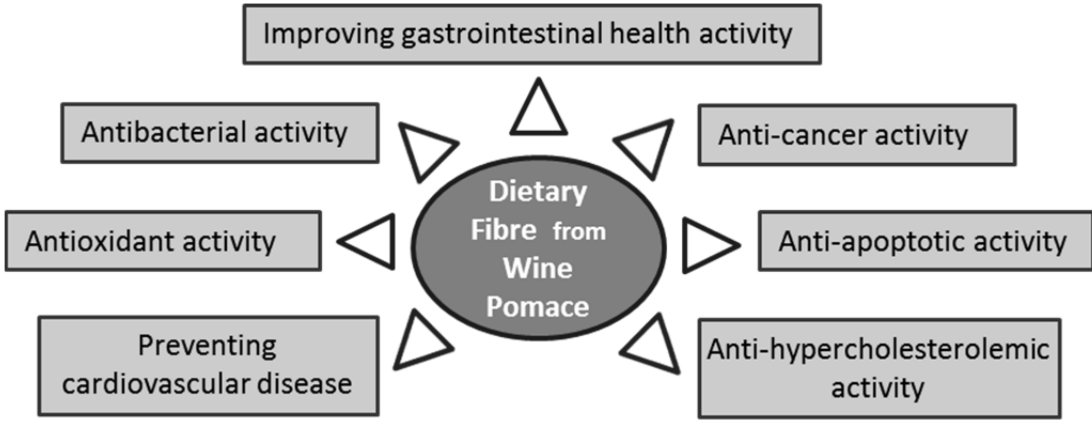


Figure 2. **The benefits of dietary fibre for human health.**
Adapted from Zhu et al. (2015).

In the past 20 years both the World Health Organization (WHO) and many national government bodies have written guidelines to increase the daily intake of dietary fibre-rich foods, as most countries do not reach the suggested dietary fibre intake. For instance, the European Food Safety Authority (EFSA) recommended a dietary reference intake of 25 g/day for adults and, despite European consumption of dietary fibre was estimated to be within this recommendation (ranging from 16-29 g/day) (EFSA 2010), the intake in several countries is actually below the suggested value. This is the case of Spain, where the average total dietary fibre consumption in the period 2004-2008 was estimated in 16.2 g/day (Ruiz-Roso Calvo de Mora & Pérez-Olleros Conde 2010). Therefore, the production and new formulation of high dietary fibre products to facilitate its increased intake is a challenge for the food industry.

- **Potassium**

Potassium is an essential nutrient needed for maintenance of total body fluid volume, acid and electrolyte balance, and normal osmotic pressure and function within cells. About 90% of ingested potassium is absorbed, irrespective of the amount consumed, and around 98% of the total body potassium is located in the intracellular fluid where the concentration can be 30 times higher than in the extracellular milieu. Potassium is also a cofactor for numerous enzymes and is required for insulin secretion by the pancreas, for carbohydrate metabolism and for protein synthesis, among other functions (Expert Group on Vitamins and Minerals 2003).

The function of potassium in the body is closely related to that of sodium, and an adequate balance between these minerals is needed for maintaining cell homeostasis and the membrane potential on which all contractibility and nerve functions depend.

Nowadays, the high consumption of dietary sodium, usually as sodium chloride (common salt), is an important nutritional and health problem. In populations on a salt intake around 3 g/day, the proportion of individuals with hypertension rises with age, and the phenomenon is more pronounced when levels of salt consumed are higher. In contrast, it has been demonstrated that a moderate salt restriction induces a significant fall in blood pressure (Meneton et al. 2005). In a multicentre, randomized, 30-day-trial including normotensive and hypertensive individuals (a total of 412 participants), the systolic blood pressure (sBP) was around 6.7 mmHg significantly lower in the subjects on the 8 g/day than on the 4 g/day salt diet. Greater reduction in sBP was observed in hypertensive than normotensive individuals, as well as when participants were given the 'Dietary Approach to Stop Hypertension' (DASH) than the control diet, with the former being rich in vegetables, fruits, and low-fat dairy products (Sacks et al. 2001). Based on this evidence, the WHO has recommended that sodium intake should not be higher than 2 g per day (equivalent to 5 g/day salt) (WHO 2012b).

On the other hand, there is evidence from randomized controlled trials that combining increased potassium and decreased sodium intake, which means a reduction in the Na/K ratio of the diet, is associated to a lower risk of hypertension (He & MacGregor 2001) and can be effective in reducing blood pressure and cardiovascular mortality (Geleijnse et al. 2003). In addition, scientific proof about the beneficial effect of potassium supplementation on hypertension is becoming increasingly important (Barden et al. 1988; Aburto et al. 2013). These blood pressure lowering effects of potassium involve several mechanisms, such as:

- Increased natriuresis.
- Reduced sympathetic nervous activity.
- Suppression of the renin-angiotensin-aldosterone system (RAAS).
- Decreased pressor response to noradrenaline and angiotensin II (Ang II).

Moreover, potassium may protect against cardiovascular diseases through mechanisms unrelated to blood pressure, as it has been also linked to antiatherosclerotic properties and to improvements of the glucose tolerance (Vaskonen 2003; Karppanen et al. 2005).

Potassium citrate and gluconate are the most common supplemented forms, followed by potassium chloride. In addition, this mineral is conventionally found at high concentrations in a variety of unrefined foods, such as fruit and vegetables. In these natural sources, potassium is mainly present as citrate or malate salts, and its content may reach more than 300 mg/serving (Bebel Stargrove et al. 2008).

Grapes, especially red grapes, are well-known as natural sources of this mineral element Among the various berry parts – skin, pulp and seeds – the skin has the highest concentration of potassium, followed by the seeds, but the total amount of potassium is greater in the pulp because the pulp accounts for most (90%) of the berry (Harbertson & Harwood 2009). The specific content depends greatly in the cultivars, although average values around 2 mg/g full weight (corresponding to about 10 mg/g of dry matter) have been reported in the literature (Mayer 1997; Sanchez-Castillo et al. 1998). During fermentation of grapes in the winemaking process, potassium has been suggested to be adsorbed by skins. Thus, the potassium content in skins of grape pomace was found to be around 20% higher than in skins of harvested grapes (Harbertson & Harwood 2009).

The WHO recommends that potassium intake is at least of 3.51 g/day (around 90 mmol/day), which could be supplied by a daily ingestion of 5 to 10 servings of fruit and vegetables. The population average potassium consumption in Spain has been estimated to reach the recommended levels, but data from around the world suggest that such consumption is below 70-80 mmol/day in many countries. Therefore, further steps to increase potassium intake are encouraged to be taken worldwide, with actions promoting a higher consumption of natural sources of this mineral being always much better than synthetic alternatives (WHO 2012a).

2. Oxidative Stress *vs.* Antioxidant Defences in Living Organisms

Oxidative stress can be defined as 'an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage' (Sies 1991).

The main oxidant molecules and antioxidant defences in living organisms are briefly described below.

2.1. Reactive Oxygen and Nitrogen Species (RONS)

The most important oxidants in biological systems are called reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are collectively named as reactive oxygen and nitrogen species (RONS). These reactive species are molecules of considerable variety, ranging from free radicals to non-radicals.

Free radicals are any species containing one or more unpaired electrons in atomic or molecular orbitals, which gives them a considerably degree of reactivity (Halliwell & Whiteman 2004). It must be noted that free radicals can be involved in chain reactions, which are a series of oxidation-reduction (redox) reactions leading to regeneration of new radicals that can begin a new cycle of reactions. These free radical chain reactions involve three distinct steps: initiation, propagation and termination (Sen et al. 2010).

Non-radicals are oxidizing agents and/or molecules easily converted into radicals, so they can also be involved in redox reactions (Halliwell & Gutteridge 1989).

ROS include free radicals such as superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$), peroxyl ($ROO^{\cdot}$), hydroperoxyl ($HOO^{\cdot}$), and lipid peroxyl ($LOO^{\cdot}$), as well as non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the singlet oxygen ($^1O_2$), among others (Halliwell & Whiteman 2004).

RNS include free radicals such as nitric oxide ($^{\cdot}NO$) and nitrogen dioxide ($^{\cdot}NO_2$), and non-radicals such as peroxynitrite ($OONO^-$), nitrous acid (HNO_2) and peroxynitrous acid (HNO_3), among others. $OONO^-$ and HNO_3 are usually also included in the ROS group (Halliwell & Whiteman 2004).

All these oxidant agents are produced in the body (endogenous RONS) and some of them can be also supplied in the diet or be present in the environment (exogenous RONS) (Lobo et al. 2010; Sen et al. 2010).

2.1.1. Endogenous Sources of RONS

The chemistry of endogenous ROS and RNS formation in living systems is a complex process, with some cellular pathways being intertwined (Fang et al. 2002; Valko et al. 2007; Carocho & Ferreira 2013), as seen in **Figure 3**.

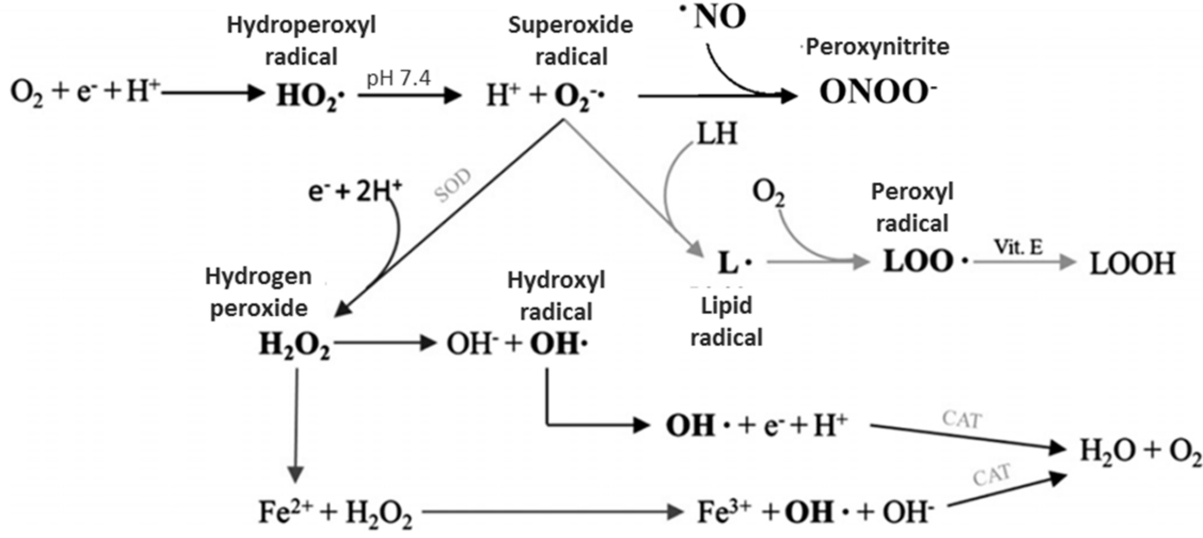


Figure 3. Overview of the reactions leading to the formation of RONS.
Adapted from Carocho & Ferreira (2013).

- **<u>Endogenous Production of ROS</u>**

ROS production generally begins with the one electron reduction of molecular oxygen (O_2), resulting in formation of $O_2^{\cdot -}$, which is considered the 'primary' ROS. The main enzyme systems responsible for $O_2^{\cdot -}$ generation include the isoforms of the nicotinamide adenine dinucleotide (phosphate) oxidase, which are generally known as NAD(P)H oxidases or NOXs, and the enzymes of the mitochondrial electron transport chain. Additionally, in the vessel wall, uncoupled endothelial nitric oxide synthase (eNOS) and xanthine oxidase isoforms, among other mechanisms, seem to play also an important role in $O_2^{\cdot -}$ production under detrimental conditions, such as endothelial dysfunction and ischemia/reperfusion (Pennathur & Heinecke 2007; Higashi et al. 2009).

Once generated, $O_2^{\cdot -}$ can further interact with other molecules to generate 'secondary' ROS, either directly or, prevalently, through enzyme- or metal-catalysed processes. Superoxide dismutase (SOD) isoforms catalyse the conversion of $O_2^{\cdot -}$ into H_2O_2, although this dismutation can also occur spontaneously in biological tissues, similarly to the non-enzymatically conversion of $O_2^{\cdot -}$ into $^1O_2$.

The highly reactive $^{\cdot}OH$ can be generated through processes known as the Fenton and Haber-Weiss reactions (Valko et al. 2007):

- In the Fenton reaction, H_2O_2 is converted into $^{\cdot}OH$ in the presence of reduced transition metals, such as Fe^{2+} or Cu^{+} ions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$$

- In the Haber-Weiss reaction, the Fenton reaction is combined with the ability of $O_2^{\cdot-}$ to reduce the Fe^{3+} contained in certain enzymes, yielding free Fe^{2+} and oxygen:

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^{-}$$

  This is an important mechanism of superoxide-mediated cytotoxicity, affecting some mitochondrial proteins containing iron-sulphur (Fe-S) centres, such as aconitase and fumarase (Faraci & Didion 2004).

A schematic representation of $O_2^{\cdot-}$ and other ROS production can be seen in **Figure 4**.

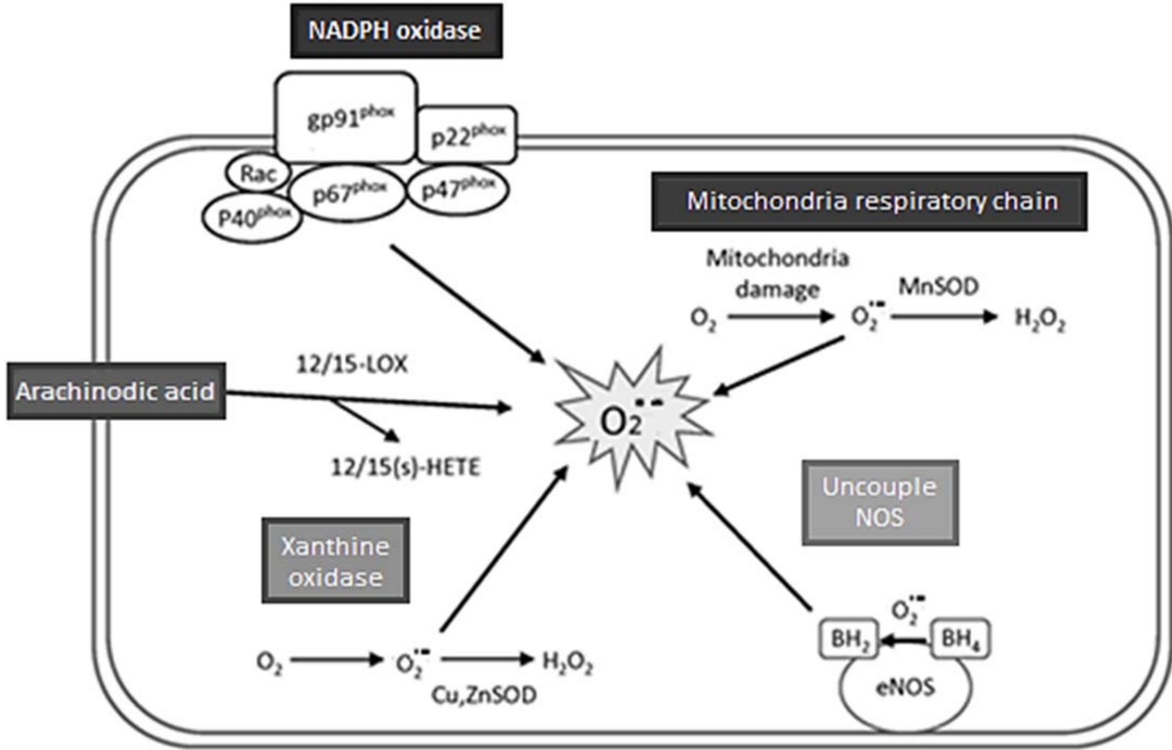


Figure 4. Main pathways leading to the generation of superoxide ($O_2^{\cdot-}$).
Adapted from Kayama et al. (2015).

Other enzymes such as cyclooxygenases (COXs), lipoxygenases and myeloperoxidases are also important sources of RONS generation (Bhattacharyya et al. 2014).

Of the cited mechanisms of ROS production, the NOX family of enzymes deserves further description. Although NOX was originally considered to be present only in phagocytic cells, where the NOX2 (catalytic subunit: gp91phox) isoform is the main representative, it is now evident that there are several homologues of this isoform (with different catalytic and regulatory subunits) which are functionally active in other cells.

In mammalians, the NOX family comprises seven members (Bedard & Krause 2007; Brandes et al. 2010): from NOX1 to NOX5, Duox1 and Duox2 (**Figure 5**). The expression of NOXs isoforms is dependent upon the type of tissue and cells. For example, in the colon epithelial cells NOX1 seems to be the predominant isoform (Bedard & Krause 2007). As regards endothelial cells, they have at least four NOX isoforms: NOX1, NOX2, NOX4 and NOX5, with the expression of NOX4 being particularly important (Montezano & Touyz 2011; Ray & Shah 2005).

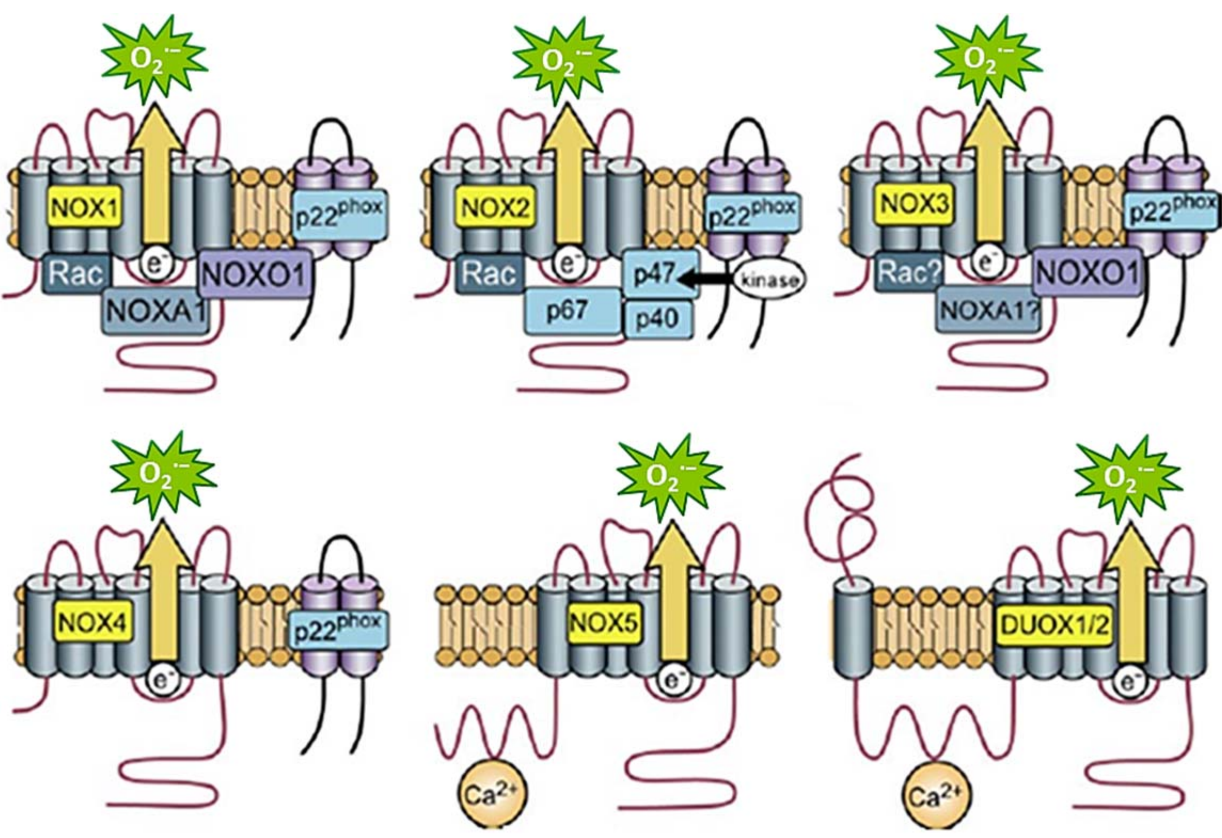


Figure 5. **NADPH oxidase (NOX) isoforms.**
Adapted from Bedard & Krause (2007).

- **Endogenous Production of RNS**

Nitric oxide radical ($^{\cdot}NO$) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine, thus yielding citrulline and $^{\cdot}NO$. This process is catalysed by the isoforms of NOS in the presence of the co-factor tetrahydrobiopterin (BH_4) and plays key constitutive roles in the vasculature (endothelial NOS – eNOS) and the nervous system (neuronal NOS), as well as under inflammatory conditions in a number of body tissues (inducible NOS – iNOS). However, as aforementioned, certain conditions, such as the deficiency of its co-factor BH_4 and other forms of oxidative damage to the enzyme structure, may result in NOS uncoupling (**Figure 6**) which contributes to a reduced $^{\cdot}NO$ formation and an increased ROS production (Montezano & Touyz 2011; Kietadisorn et al. 2012).

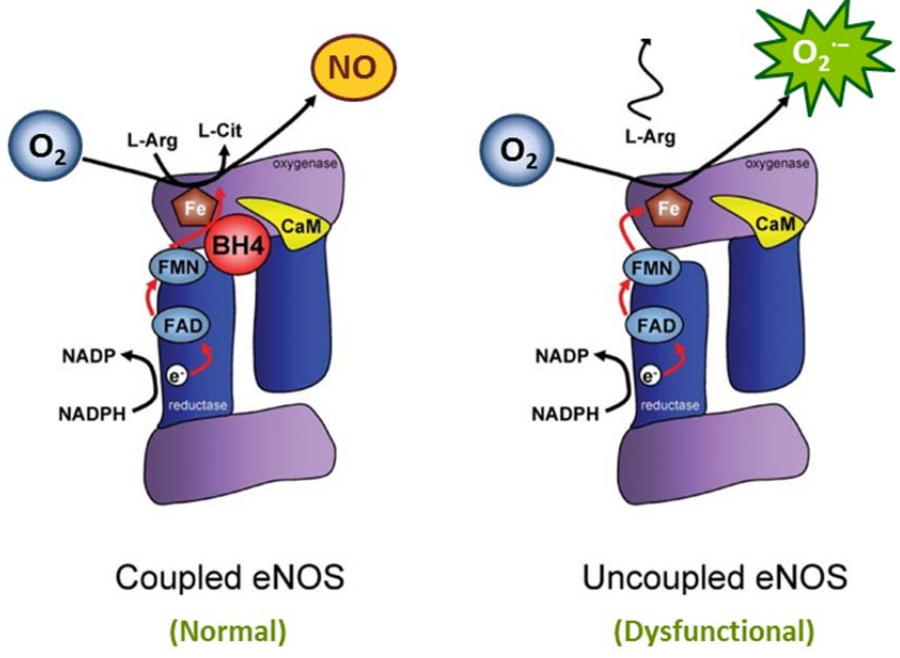


Figure 6. Formation of ·NO by endothelial nitric oxide synthase (eNOS) under normal circumstances and in the 'uncoupled' state.
Adapted from Schmidt & Alp (2007).

Nitric oxide radical ($^{\cdot}NO$) has a half-life of only a few seconds in an aqueous environment, especially in the presence of high oxygen concentrations. However, since $^{\cdot}NO$ is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes, having effects mainly in vascular function and neuronal transmission.

Once generated, $^{\cdot}NO$ can be converted to various other RNS (**Figure 7**), being $ONOO^{-}$ one of the most potent oxidant, which is generated following reaction of $^{\cdot}NO$ with $O_2^{\cdot-}$. This process is regulated by the rate of diffusion of both radicals and results in decreased $^{\cdot}NO$ bioavailability (Dröge 2002).

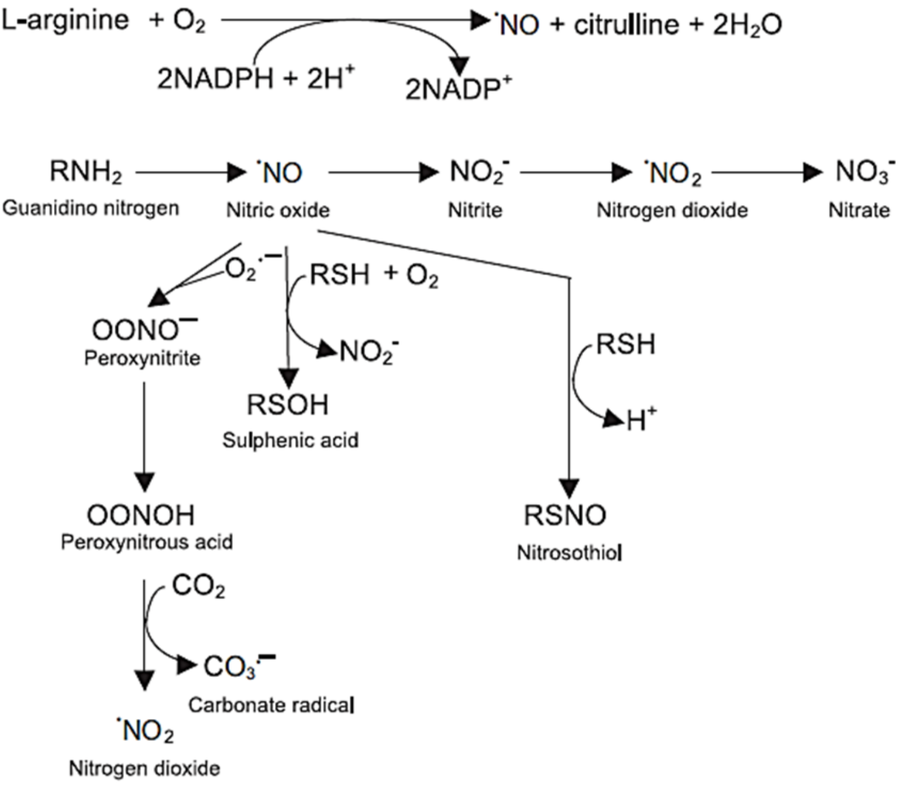


Figure 7. Reactions leading to the formation of RNS.
Adapted from Bhattacharyya et al. (2014).

In the extracellular milieu, $^{\cdot}NO$ reacts also with molecular oxygen and H_2O to form nitrate (NO_3^-) and nitrite (NO_2^-) anions. These $^{\cdot}NO$ metabolites are rather stable, so they are not included among RNS. On the other hand, as they circulate in blood and are excreted in urine, they are commonly used as biomarkers of $^{\cdot}NO$ production in the organisms (Tsikas et al. 2006). Moreover, other nitroso species can be found in plasma derived from the rapid $^{\cdot}NO$ breakdown, which are named nitros(yl)ated compounds (RXNOs) and mainly include: S-nitrosothiols, N-nitrosamines, and nitrosylhemes (Feelisch et al. 2002). Both plasma NO_2 and RXNOs have been also proposed as useful markers of $^{\cdot}NO$ bioavailability, with a depletion of these NO metabolites being a surrogate index of endothelial dysfunction and correlating with cardiovascular disease risk factors (Heiss et al. 2006; Kleinbongard et al. 2006).

- **<u>Beneficial and Detrimental effects of Endogenous RONS</u>**

Endogenous RONS are well recognized for playing a dual role as both beneficial and deleterious species in living systems (Valko et al. 2007):

- Beneficial effects of certain RONS (such as $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}NO$) occur at low/moderate concentrations. A small oxidative burst is elicited in most cell types when they are stimulated by several cytokines, growth factors and hormones, such as certain interleukins, tumour necrosis factor-α, Ang II, etc. These molecules and the generated

RONS can interact with several kinases and phosphatases which activate transcription factors that finally induce the expression of specific genes. Therefore, RONS are involved as secondary messengers in several cellular responses under normal physiological conditions, such as the defence against infectious agents, the induction of numerous cellular signalling pathways and gene expression, and the regulation of vascular tone, among many others.

- In contrast, overproduction of RONS (especially those highly reactive, such as $^{\cdot}OH$, and $OONO^{-}$) may lead to increased oxidative and nitrosative burdens, which are referred to as oxidative and nitrosative stress, respectively, although the term ‘oxidative stress’ is frequently used to encompass both detrimental processes. Such excessive levels of RONS may be generated either by excessive stimulation of otherwise tightly regulated enzymes (such as NOXs and NOSs from neutrophils, macrophages and vascular cells under inflammatory responses and other stressor conditions) and/or by other situations that produce ROS ‘accidentally’ in a non-regulated way (such as overproduction by the mitochondrial electron transfer chain as a consequence of altered energetic metabolism). The resultant oxidative stress leads to oxidative damage to biomolecules and impairs cell normal functions.

The delicate equilibrium between beneficial and harmful effects of RONS is a very important aspect of living organisms. It is achieved by mechanisms called ‘redox regulation’, which are responsible for the modulation of the defence systems in response to the fluctuating levels of oxidative stress. As such, these mechanisms maintain and re-establish ’redox homeostasis’ under physiological conditions. However, defence mechanisms may be overwhelmed by an excessive RONS production or a deficiency of enzymatic and non-enzymatic antioxidants, resulting in oxidative stress (Dröge 2002).

2.1.2. Exogenous Sources of RONS

Exogenous RONS usually provoke oxidative damage in living systems. These generally detrimental molecules can derive from several sources (Bouayed & Bohn 2010; Sen et al. 2010), such as:

- Radiations: X-rays, γ-rays, UV radiations, microwave radiation, etc.
- Environmental pollutants: industrial and urban effluents (solid, liquid and gaseous), automobile exhausts fumes, cigarette smoke, polycyclic aromatic hydrocarbons, etc.
- Burning of organic matter during forest fires, volcanic activities, and thermal treatments such as cooking.
- Other xenobiotics: allergens, microbes, pesticides, antibiotics, chemotherapeutics, alcohol, diets excessive in fat and carbohydrates, etc.

2.2. Antioxidant Defences

A series of defence mechanisms have been developed in all aerobic organisms as the production of RONS is inevitable and, within certain concentrations, essential. Hence, there is a continuous requirement for their inactivation once generated. In addition, our defence system has to be efficient against the various stressful conditions and situations leading to RONS overproduction (Halliwell & Gutteridge 2015).

As previously indicated, an 'antioxidant' is defined as 'any substance that delays, prevents, or removes oxidative damage to a target molecule' (Halliwell & Gutteridge 2007). This simple but complete definition tries to encompass the various complications to define clearly and comprehensively the term 'antioxidant', which can have specific connotations depending on the context and, consequently, different scientific groups have their own view on what is a 'good' antioxidant (Finley et al. 2011; Halliwell & Gutteridge 2015). Despite there is no universal 'best' antioxidant (Valko et al. 2006) described the desired characteristics, since a biologically point of view, of such a 'good' antioxidant, which should:

- Specifically quench free radicals.
- Chelate redox metals.
- Have a positive effect on gene expression.
- Be readily absorbed.
- Have a concentration in tissues and biofluids at a physiologically relevant level.
- Work in both the aqueous and/or membrane domains.
- Interact with other antioxidants within 'antioxidant networks' where the reduced forms of oxidised antioxidants are regenerated in a highly coordinated and controlled process.

Living organisms possess an extensive, sophisticate and cooperative range of antioxidant defences, which can be classified according to several of their features, such as their nature, origin, hydrophobicity, or functional role. Several of the most important natural antioxidants in mammals are shown in **Table 1**, where they have been divided according to their enzymatic or non-enzymatic nature. Secondarily, the latter are separated, depending upon their origin, in endogenous or exogenous antioxidants.

Table 1. Classification of the antioxidant defence system in mammals.

Antioxidant Defence System	
Enzymatic Antioxidants	**Non-enzymatic antioxidants**
- Superoxide dismutases (SODs) SOD1 (Cytosolic Cu, Zn-SOD) SOD2 (Mitochondrial Mn-SOD) SOD3 (Extracellular SOD) - Catalase (CAT) - Glutathione peroxidases (GPXs) GPX1 – GPX8 - Glutathione S-transferases (GSTs) GSTA (alpha) GSTM (mu) GSTP (pi) GSTS (sigma) GSTO (omega) GSTZ (zeta) GSTT (theta) Etc. - Glutathione reductase (GR) - Thioredoxin reductases (TrxRs) TrxR1,TrxR2 - Peroxiredoxins (PRXs) PRX1 – PRX6 - Other peroxidases - Heme oxygenase (HO) system HO-1 – HO-3 - NAD(P)H:quinone oxidoreductases (NQOs) - Repair systems - Etc.	***Endogenous antioxidants:*** - Glutathione (GSH) - Thioredoxins (Trxs) Trx1, Trx2 - α-Lipoic acid (ALA) - Uric acid - Melatonin - Bilirubin - Ferritin - Coenzyme Q10 - Etc. ***Exogenous antioxidants:*** - Vitamins Vitamin C (ascorbic acid) Vitamin E (tocopherols and tocotrienols) Etc. - Phenolic compounds Phenolic acids Stilbenes Flavonoids Tannins Etc. - Carotenoids β-carotene Lycopene Zeaxanthin Lutein Etc. - Trace elements Selenium (Se) Zinc (Zn) Manganese (Mn) Etc.

2.2.1. Enzymatic Antioxidants

Several antioxidant enzyme systems catalyse reactions to neutralize free radicals and reduce RONS concentration in living organisms. Other enzymes may be implicated in repair or adaptation mechanisms under conditions of oxidative stress and damage (Sies 1993). The contents of most of these antioxidant enzymes may increase readily in response to the induction of certain transcription factors, which are at the same time tightly regulated by changes in the cell redox state. This gene regulation is also responsible for the increased expression of certain enzymes and isoforms in particular tissues (Matés et al. 1999; Valko et al. 2006; Valko et al. 2007).

Some of the most efficient representatives among enzymatic antioxidants include:

- **Superoxide dismutases (SODs)**

  SODs are considered the first line of antioxidant enzyme defence systems against ROS (Zelko et al. 2002). SOD isoforms efficiently detoxify $O_2^{\cdot-}$, with the subsequent generation of H_2O_2 and molecular oxygen:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

  SOD isoforms differ in the nature of the active metal centre and amino acid composition, their number of subunits, cofactors, and other features (Miao & St. Clair 2009). In mammals there are three SOD isoforms (**Figure 8**):

  - *Cytosolic Cu, Zn-SOD (SOD1)*: this enzyme has two identical subunits and is localised in the cytosol and the intermembrane space of mitochondria.
  - *Mitochondrial Mn-SOD (SOD2)*: this is a homotetramer enzyme localised in the mitochondrial matrix.
  - *Extracellular SOD (EC-SOD or SOD3)*: this is a secretory, homotetrameric, Cu and Zn containing glycoprotein, localised in the interstitial spaces of tissues (e.g. between endothelium and vascular muscle) and also in the extracellular fluids such as plasma.

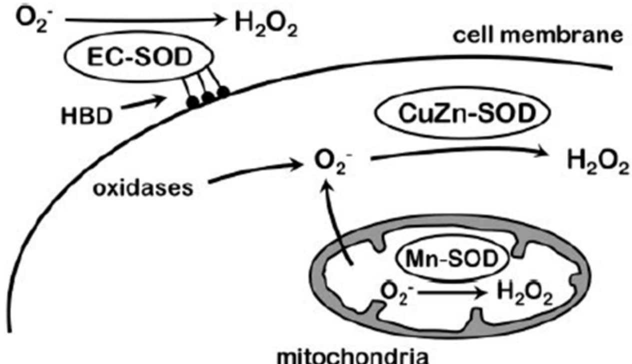


Figure 8. Superoxide dismutase (SOD) isoforms and their subcellular localization.
Adapted from Faraci & Didion (2004).

Transcriptional regulation of all three SOD isoforms is highly controlled based on extra- and intracellular conditions, with SOD1 and SOD2 being expressed in most tissues, whereas SOD3 expression appears to be restricted to only a few cell types (Zelko et al. 2002).

In the gastrointestinal tract, most investigations are focussed in SOD1 and SOD2. The pattern of gene expression, protein levels and activities of these SODs may change in subsequent stages of clinical advancement and grades of differentiation of colorectal cancer, which seems to be an adaptive response to the oxidative stress emerging during progression of this cancer (Skrzycki et al. 2009). In this regard, SOD2 seems to be particularly responsive to such oxidative stress (Dhar & St. Clair 2012).

In aortic tissue, the pattern of SOD expression may range between 50-80% for SOD1, 2-12% for SOD2, and the remaining % for SOD3. Much higher SOD2 expression has been detected in endothelial cells, but it appears to be masked by other cell types when evaluated in intact vessels (Faraci & Didion 2004). Transcriptional regulation of SOD 1 and SOD2 is dependent on the cell redox state, whereas modulation of SOD3 may occur primarily in a manner coordinated by cytokines, rather than as a cell response to oxidants (Matés et al. 1999). In particular, SOD2 expression is highly responsive to vascular oxidative stress and may change in a temporal pattern along disease states such as diabetes, hypertension and hypercholesterolemia (MacMillan-Crow & Cruthirds 2001; Faraci & Didion 2004).

- **Catalase (CAT)**

This enzyme reacts very efficiently with H_2O_2 to form H_2O and molecular oxygen; and, secondarily, with H donors (methanol, ethanol, or phenols), with peroxidase activity (Matés et al. 1999):

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

CAT is a homotetrameric enzyme containing a single ferriprotoporphyrin (heme) group per subunit. It is primarily present in a cell organelle called peroxisome, being ubiquitously expressed in all eukaryotic cells, and it has been also described in the nucleus, the sarcoplasm, and the mitochondria of certain higher organism tissues (Zhou & Kang 2000).

- **Glutathione peroxidases (GPXs)**

At least eight isoforms of GPX (GPX1 – GPX8) have been so far identified in humans (Brigelius-Flohé & Maiorino 2013). All of them have arisen from a cysteine-containing ancestor and they act in conjunction with reduced glutathione (GSH). GPX1 – GPX3 and 5 and 6 are homotetramers. Mammalian GPX1 – GPX4 contain a single Se-cysteine residue in each of their catalytic centres and are the most studied members of this antioxidant enzyme family.

It should be noted that not all GPXs isoforms are able to catalyse the same reactions (Brigelius-Flohé & Maiorino 2013):

- GPX1 – GPX4 are known to decompose soluble low-molecular-mass lipid hydroperoxides (LOOH) and synthetic hydroperoxides (such as *tert*-butylhydroperoxide, t-BOOH) to the corresponding alcohols while oxidizing GSH simultaneously (Brigelius-Flohé, 2013). Respective global reactions are:

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

- GPX4 (phospholipid-hydroperoxide GPX or PHGPX) can additionally reduce complex LOOH such as phospholipid (PLOOH), cholesterol, and cholesterol-ester hydroperoxides, even when they are inserted into biomembranes or lipoproteins. The global reaction in this case is:

$$2GSH + PLOOH \rightarrow GSSG + PLOH + H_2O$$

- GPX1, GPX2, GPX4, GPX7 and GPX8 can catalyse the conversion of H_2O_2 to H_2O, but at a minor rate than CAT, and requiring GSH for their peroxidase activity:

$$2GSH + H_2O_2 \rightarrow GSSG + H_2O$$

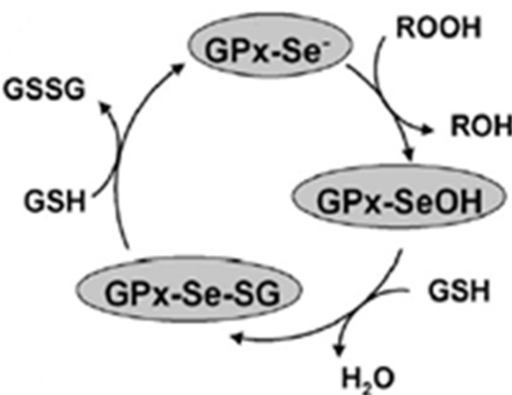


Figure 9. Catalytic cycle of Se-dependent glutathione peroxidases (GPXs).
Adapted from Brigelius-Flohé & Maiorino (2013).

It should be noted that, during catalysis by GPX Se-dependent enzymes, peroxidation reactions occur indeed in three phases (**Figure 9**), as the selenol ($GPX\text{-}Se^-$) reacts first with any hydroperoxide (ROOH) to give a selenenic acid (GPX-SeOH), and then two GSH molecules bind subsequently (Halliwell & Gutteridge 2015):

$$ROOH + GPX\text{-}Se^- + H^+ \rightarrow ROH + GPX\text{-}SeOH$$

$$GPX\text{-}SeOH + GSH \rightarrow GPX\text{-}Se\text{-}SG + H_2O$$

$$GPX\text{-}Se\text{-}SG + GSH \rightarrow GPX\text{-}Se\text{-}SG\text{-}GSH \rightarrow GPX\text{-}Se^- + H^+ + GSSG$$

With regard to specific features of the most studied GPXs, such as their cell localization and specific tissue expression (Matés et al. 1999; Brigelius-Flohé & Kipp 2009; Brigelius-Flohé & Maiorino 2013):

- *Glutathione peroxidase-1 (GPX1)*: this is a homotetramer selenoprotein present in the cytosol and mitochondria, thus working in the water phase. It has a primary antioxidant function *in vivo* and cannot be replaced by any other selenoprotein in protecting from generalized oxidative stress.
- *Glutathione peroxidase-2 (GPX2)*: this isoform is closely related to GPX1, but presents a higher specificity for GSH.
- *Glutathione peroxidase-3 (GPX3)*: the peroxidase activity of this isoform is as efficient as GPX1 but GPX3 is mainly an extracellular enzyme. Thus, it is released into the plasma or present in extracellular fluids, where it is found as a glycosylated protein.
- *Glutathione peroxidase-4 (GPX4)*: this is a monomeric selenoprotein located in both the cytosol and the membrane fraction as its main role is protecting phospholipid membranes from oxidative challenges.

The levels of each isoform of these Se-dependent enzymes vary depending on the type of tissue. GPX1 and GPX 4 are present ubiquitously, whereas GPX2 is mainly expressed in the intestinal epithelium (where it is suggested to act as a barrier against absorption of food-born ROOH), and GPX3 is present primarily in the kidneys and plasma (Brigelius-Flohé & Kipp 2009; Matés et al. 1999).

The ranking of the biological importance of GPXs, which is determined by the stability of their mRNA and their resistance to decline upon selenium deficiency (hierarchy of selenoproteins), have been described as follows (Brigelius-Flohé & Kipp 2013):

$$GPX2 \geq GPX4 > GPX1 = GPX3$$

- **Glutathione S-transferases (GSTs)**

This complex group of transferases exhibits various activities and participates in several different types of reaction, some of which give rise to detoxification of xenobiotics (phase II drug metabolism) and antioxidant effects. Most of GSTs can catalyse the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic centre (usually a functional group introduced during phase I drug metabolism by members of the cytochrome P450 sugergene family), such as: epoxide-containing compounds, alkyl- and aryl-halides, isothiocyanates, α,β-unsaturated carbonyls, and quinones, among others (Sherratt & Hayes 2002).

The numerous reactions catalysed by GSTs can be divided in 4 main types (Kulinsky & Kolesnichenko 2009a):

- Nucleophilic addition of the whole GSH molecules to substrate (R):

$$R + GSH \rightarrow HRSG$$

- Nucleophilic substitution of a leaving group (X^-) by GS^-:

$$RX + GSH \rightarrow RSG + HX$$

- Reduction of organic hydro- and endoperoxides containing compounds such as LOOH, PLOOH, etc. to alcohols in a reaction that is not dependent on Se but does require GSH. The global reaction is:

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

- Isomerization though mechanisms involving intermediate GSH addition.

There are at least 18 different GST isoforms identified in humans, most of them with several polymorphic variants (Board & Menon 2013), classified in 7 distinct classes of catalytically active enzymes termed Alpha (GSTA), Mu (GSTM), Pi (GSTP), Sigma (GSTS), Omega (GSTO), Zeta (GSTZ) and Theta (GSTT).

GSTs are present in several cell compartments (nucleus, cytoplasm, mitochondrion, and endoplasmic reticulum). They are expressed in an organ-specific fashion, although several tissues (including intestinal and vascular tissues) contain most of the isoforms (Sherratt & Hayes 2002).

- **Glutathione reductase (GR)**

This enzyme is a member of the flavoprotein disulphide reductase family that is in charge of maintaining high reduced glutathione (GSH) and low oxidised glutathione (GSSG) levels within cells by the regeneration of GSSG to GSH in an irreversible reaction:

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

This enzyme is mainly localized in the cytosol, although can be also detected in mitochondrions and the nucleus. In mammals, GR is located in all tissues, with a high activity being found in the small intestine, liver, kidneys, etc. (Kulinsky & Kolesnichenko 2009b).

- **Thioredoxin reductases (TxrRs)**

TrxR isoforms are also members of the flavoprotein disulphide reductase family and catalyses the regeneration of oxidised Trx (Trx-S_2) to the reduced form (Trx-$(SH)_2$), at the expense of NADPH (Mustacich & Powis 2000):

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Trx-(SH)}_2 + \text{NADP}^+$$

Mammalian cells contain three TrxR isoforms: TrxR1, a cytosolic protein; TrxR2, a mitochondrial protein; and, TGR, the testis specific thioredoxin glutathione reductase (Karlenius & Tonissen 2010). TrxR1 and TrxR2 are expressed in most tissues. In addition to their role in maintaining Trxs in their reduced forms, TrxRs can reduce a number of small-molecule substrates, such as: dehydroascorbic acid, LOOH, α-lipoic acid, etc. (Mustacich & Powis 2000).

- **Peroxiredoxins (PRXs)**

Mammalian cells express six PRX isoforms (PRX1 – PRX6). They are usually present as homodimers and can be divided into three subgroups, distinguished by the number and location of catalytic cysteines (Cys): typical 2-Cys PRXs (PRX1 – PRX4); atypical 2-Cys PRX (PRX5); and, 1-Cys PRX (PRX6).

Peroxiredoxins (PRXs) are a ubiquitous family of thiol specific antioxidant enzymes, which exert their protective antioxidant role through their peroxidase activity, whereby a wide range of ROOH, as well as H_2O_2, are reduced and detoxified (Wood et al. 2003). The global reactions are:

$$\text{ROOH} + 2e^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O}$$
$$\text{H}_2\text{O}_2 + 2e^- + 2\text{H}^+ \rightarrow 2\ \text{H}_2\text{O}$$

Similarly as occurs in GPXs, these reactions take place in three phases (Halliwell & Gutteridge, 2015). In all cases, the ROOH oxidizes a Cys–SH group on the PRX to cysteine sulphenic acid (Cys-SOH). Then, in PRX1-5, the Cys-SOH reacts with another Cys–SH on the enzyme to give H_2O and a disulphide (PRX-Cys_2-S_2) that is then reduced by thioredoxin (Trx-$(SH)_2$). The second Cys–SH group is either on the other subunit (typical 2-Cys PRXs) or on the same subunit (atypical 2-Cys PRX):

$$ROOH + PRX\text{-}(Cys\text{-}SH)_2 \rightarrow ROH + PRX\text{-}Cys\text{-}SH/Cys\text{-}SOH$$

$$PRX\text{-}Cys\text{-}SH/Cys\text{-}SOH \rightarrow PRX\text{-}Cys_2\text{-}S_2 + H_2O$$

$$PRX\text{-}Cys_2\text{-}S_2 + Trx\text{-}(SH)_2 \rightarrow PRX\text{-}(Cys\text{-}SH)_2 + Trx\text{-}S_2$$

In PRX6, reduced glutathione (GSH) seems to be the electron donor that regenerates the Cys–SH group, catalysed by certain GST enzymes:

$$ROOH + PRX\text{-}Cys\text{-}SH \rightarrow ROH + PRX\text{-}Cys\text{-}SOH$$
$$PRX\text{-}Cys\text{-}SOH + 2\ GSH \rightarrow PRX\text{-}Cys\text{-}SH + GSSG + H_2O$$

Prx isoforms are expressed ubiquitously but distributed differentially within the cell: PRX1 and PRX2 are localised in the cytosol, PRX3 in mitochondria, PRX4 in the extracellular space, PRX5 in mitochondria and peroxisomes, and PRX6 in the cytosol (Rhee et al. 2005).

- **Heme oxygenase (HO) system**

The HO system is a highly conserved, inducible, cytoprotective and homeostatic stress-responsive heat-shock protein system. HO is the rate-limiting enzyme that catalyses the NADPH-cytochrome P450 reductase-dependent oxidative degradation of heme to yield carbon monoxide, bilirubin and active iron (Fe^{++}) which is rapidly sequestered by the anti-oxidant ferritin (in charge of Fe storage and transport) (**Figure 10**).

In addition to its primary role in heme catabolism, HO-1 exhibits anti-oxidative and anti-inflammatory functions via the actions of biliverdin and CO, respectively (Otterbein & Choi, 2000).

Three isoform of HO (HO-1 – HO-3) have been identified in humans, all of which are cytosolic proteins that anchor to the endoplasmic reticulum at the C-terminus (Wilks 2002).

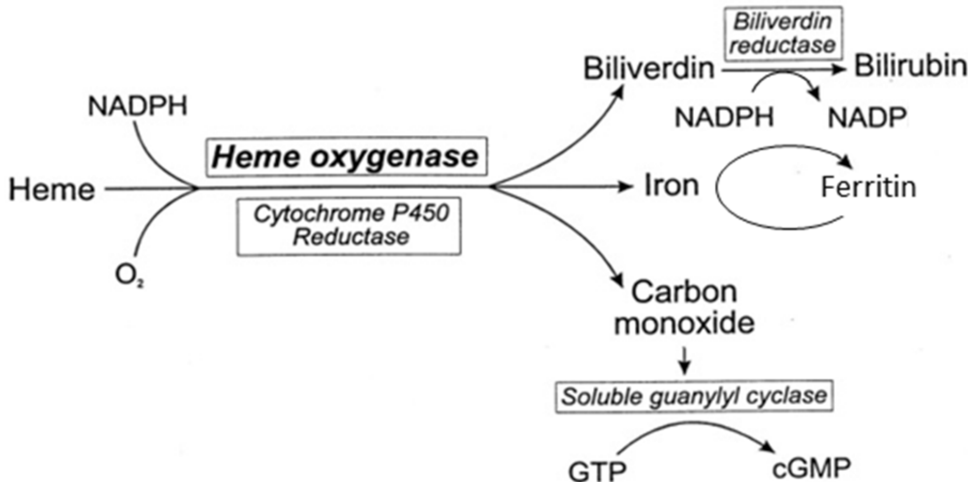


Figure 10. Heme degradation catalysed by heme oxygenase (HO).
Adapted from Otterbein & Choi (2000).

HO-1 is the only inducible isoform, which is expressed ubiquitously in almost every tissue and is regulated by a broad spectrum of different stimuli, including oxidative stress and dietary antioxidants (Otterbein & Choi 2000; Ogborne et al. 2004), thus playing an important role as an endogenous defence mechanism under several pathological conditions, such as carcinogenesis and tumour progression (Chau 2015) and several cardiovascular diseases (Calay & Mason 2014), including diabetes (Mishra & Ndisang 2014) and hypertension (Tiwari & Ndisang 2014).

- **Repair mechanisms**

As prevention and interception mechanisms of antioxidant defence are not totally effective, damage products are continuously formed and may accumulate. Therefore, repair of damage once it has occurred is essential for protecting cells from the detrimental effects of oxidants (Sies 1993). There are multiple enzyme systems involved in DNA repair (Evans et al. 2004; D'Errico et al. 2008). There are also several lipolytic and proteolytic enzymes that recognize and degrade/remove/replenish the respective oxidised biomolecules, thus avoiding their accumulation (Pacifici & Davies 1991; Grune et al. 1997). However, complete repair of damage is not always possible, as observed in several pathological conditions and diseases (Dalle-Donne et al. 2006; Valko et al. 2007; Khansari et al. 2009).

2.2.2. Non-Enzymatic Antioxidants

Non-enzymatic antioxidants involved in the defence system can be divided in endogenous and exogenous antioxidants. This classification depends on the living organism considered, as the formers are those that can be synthesised *de novo* by their cells, whereas the latters must be incorporated from the environment. In addition, it is remarkable that the hydrophobicity of antioxidant defences is a key factor which determines their target molecules and preferential site of location/reaction within cells. Thus, some antioxidants act in a hydrophilic environment, others in a hydrophobic environment, and some in both of them (Valko et al. 2006).

The main non-enzymatic natural antioxidants for mammals have been indicated in **Table 1**, and comprise:

- *Endogenous Non-Enzymatic Antioxidants*

Main endogenous non-enzymatic antioxidants include thiol antioxidants, such as GSH, thioredoxin and lipoic acid (Fang et al. 2002; Valko et al. 2006; Lobo et al. 2010; Ndhlala et al. 2010; Carocho & Ferreira 2013):

- **Glutathione (GSH)**

The tripeptide L-γ-glutamyl-L-cystinyl-glycine, or GSH, is the major thiol compound in animals and plants. GSH can be partly absorbed from the diet in the small intestine, but its synthesis *de novo* within cells is more important, thus being usually classified as an endogenous antioxidant. This molecule is synthesised from its amino acid constituents (L-glutamate, L-cysteine, and glycine) in two consecutive steps, catalysed by glutamyl-cysteine ligase (GCL) and glutathione synthase (GS) (**Figure 11**). The reaction catalysed by GCL is the rate-limiting step in GSH biosynthesis, which takes place mainly in the cytosol (Lu 2013).

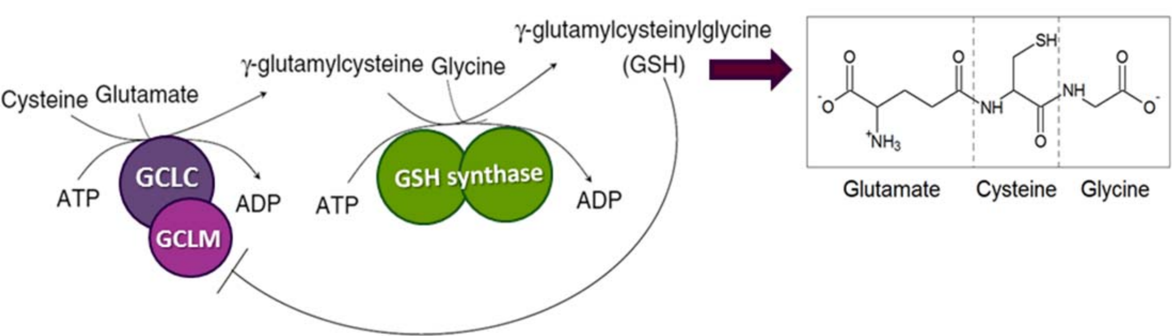


Figure 11. Glutathione (GSH) synthesis.
Adapted from Lu (2013).

GSH plays a key role in the defence against RONS, protecting against oxidative damage to biomolecules (Sáez et al. 1993), and in the maintenance of cell homeostasis by acting as a thiol 'redox buffer' to preserve a given thiol/disulphide redox potential (Viña et al. 1986; Sies 1999). This multifunctional non-enzymatic antioxidant exists either in a reduced (GSH) or an oxidised (GSSG) form, also referred to as glutathione disulphide, and participates in numerous cellular redox reactions by the reversible oxidation of its active thiol group.

As seen in **Figure 12**, GSH may play a direct role in free RONS interception (e.g. as a scavenger of $^{\bullet}OH$, $ROO^{\bullet}$, $ONOO^{-}$, etc.) and regeneration of other antioxidants (e.g. vitamins C and E). GSH can also act as a substrate of reactions catalysed by GSTs (leading to the formation of several thioethers, called GS-conjugates, which are usually committed to detoxification and elimination), as well as GPXs and glutaredoxins (e.g. reduction of ROOH and H_2O_2, regeneration of other antioxidants, etc.) (Masella et al. 2005; Valko et al. 2006). In addition, the thiol redox state is involved in practically all major biological processes, such as signal transduction, gene expression, protein folding, cell proliferation, apoptosis, etc. (Sies 1999; Groitl & Jakob 2014).

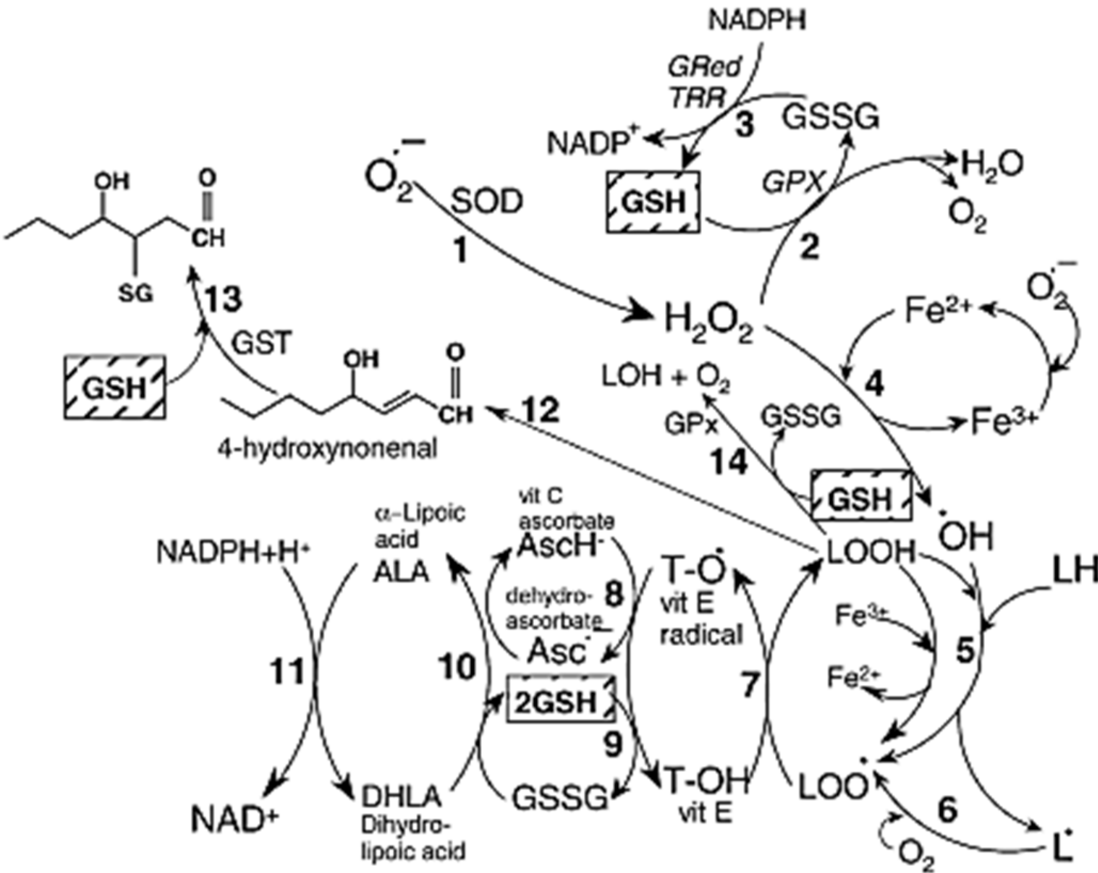


Figure 12. The various pathways of glutathione (GSH) in the management of oxidative stress.
Adapted from Valko et al. (2006).

GSH can be found in most intracellular compartments, including cytosol, endoplasmic reticulum, mitochondria, and nucleus. On average, the GSH concentration in the cytosol is 1-11 mM but, depending on several cell signals and processes, GSH can be redistributed to other cell compartments according to their redox requirements and changes in the GSH/GSSG ratio (Schafer & Buettner 2001).

When referring to 'total glutathione', all free or bound glutathione moieties would be included. However, only GSH plus twice the GSSG levels (free forms) are usually denoted as total glutathione in most analyses. Of note, this definition does not normally include an important pool of bound glutathione (e.g. the glutathionylated proteins generated by reaction of GSSG with protein –SH groups), but it is generally accepted (Sies 1999). In fact, GSSG is the main oxidised form of glutathione, and evaluating the GSH/GSSG ratio can serve as an important indicator of overall cell redox environment and responses to oxidative insults (Moskaug et al. 2005).

GSH is ubiquitously present in all cell types at mM concentrations. The overall cell GSH/GSSG ratio is typically greater than 30:1, but it may greatly differ from one cell type to another (Schafer & Buettner 2001; Masella et al. 2005).

- **Thioredoxin (Trx)**

Thioredoxin (Trx) is a small, multifunctional, and ubiquitously expressed protein which contains a conserved active site (Cys-Gly-Pro-Cys) with two adjacent –SH groups. Reduced Trx (Trx-$(SH)_2$) catalyses the reduction of oxidised cysteines of many proteins, both intracellularly and extracellularly, while Trx itself becomes oxidised (Trx-S_2) during this process. Trx can then be reduced back to the dithiol form by the action of TrxR, at the expense of NADPH (**Figure 13**) (Karlenius & Tonissen 2010; Valko et al. 2006).

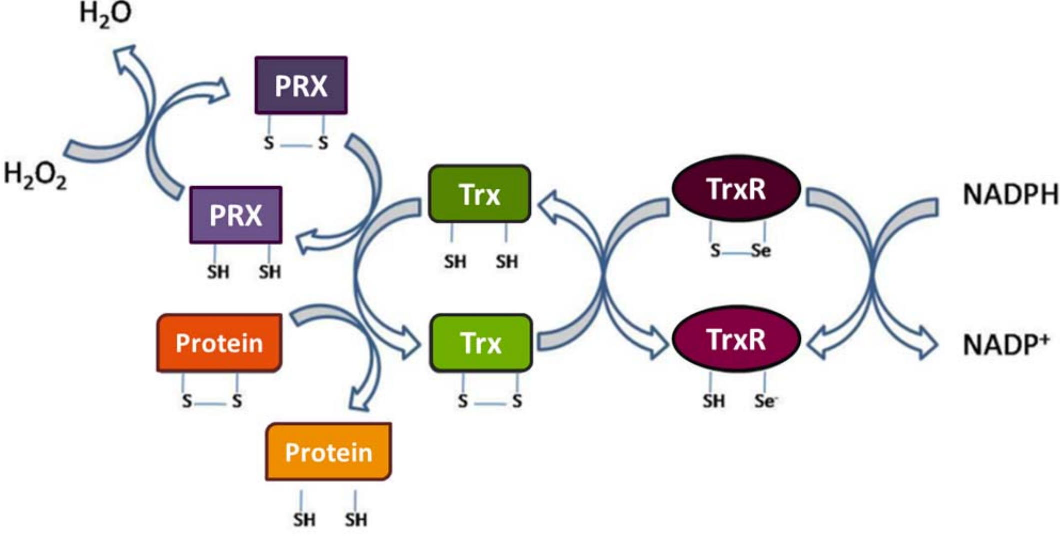


Figure 13. Mechanisms of action of the thioredoxin (Trx) redox system.
Adapted from Karlenius & Tonissen (2010).

Three isoforms of Trx have been identified in mammalian cells. They are: Trx1, mainly a cytoplasmic protein; Trx2, a mitochondrial protein; and SpTrx, mainly expressed in spermatozoa (Karlenius & Tonissen 2010).

Trx can thus exist in the extracellular environment, cytoplasm and nucleus, being expressed in most of body tissues but showing distinct roles in each of the different environments. Extracellularly, Trx exhibits chemokine like activity. In the cytoplasm, Trx regulates the cell redox balance and the activity of a number of proteins. Trx can exert its antioxidant function either directly through quenching $^{1}O_2$ and scavenging $^{\cdot}OH$ or indirectly by reducing oxidised proteins. As stated above, Trx performs most of its antioxidant functions through peroxiredoxins (PRXs) (Karlenius & Tonissen 2010). Trx may also interact with many transcription factors in the nucleus and thereby regulate gene expression. Hence, Trx participates in many important cellular processes that are dependent on thiol-redox states (Gromer et al. 2004; Kondo et al. 2006). In fact, Trx and GSH may have overlapping functions, although the levels of Trx are usually 100- to 1000-fold less than those of GSH (Karlenius & Tonissen 2010; Valko et al. 2006).

- **α-Lipoic acid (ALA)**

This is the common name for 1,2-dithiolane-3-pentanoic acid, which is a thiol antioxidant both water and fat-soluble. Therefore, it is widely distributed in both the cell membranes and the cytosol. Once absorbed from the diet, ALA is converted in several tissues to its reduced dithiol form, dihydrolipoic acid (DHLA). Both ALA and DHLA are powerful antioxidants, being able to: quench RONS, regenerate GSH and vitamins C and E, chelate transition metals such as Fe^{++} and Cu^{++}, etc. (Valko et al. 2006).

o *Exogenous Non-Enzymatic Antioxidants*

Main exogenous non-enzymatic antioxidants include vitamin C, vitamin E, phenolic compounds, carotenoids, and minerals (Fang, 2002; Valko et al., 2006; Lobo et al., 2010; Ndhlala et al., 2010, Carocho et al., 2013):

- **Vitamins C (ascorbic acid)**

Vitamin C is a water-soluble antioxidant that can be found at high concentrations in several vegetables and fruits, including peppers, dark leafy greens, broccoli, tomatoes, berries, citrus fruits, etc.

Vitamin C is also known as ascorbic acid, which has two ionisable hydroxyl groups, being thus a di-acid ($AscH_2$) with really effective antioxidant capacity. As seen in **Figure 14**, at physiological pH almost all vitamin C is present as $AscH^-$ (99.9%), and only very small proportions as $AscH_2$ (0.05%) and as Asc^{2-} (0.004%). $AscH^-$ is a donor antioxidant that reacts with radicals to produce the resonance stabilised tricarbonyl ascorbate free radical ($AscH^{\cdot}$) which under physiological pH is present in the non-protonated form ($Asc^{\cdot-}$), a poorly reactive terminal radical. Then $Asc^{\cdot-}$ can be regenerated by other antioxidants, such as GSH, Trx, and ALA (Valko et al. 2006).

Ascorbic acid acts in the aqueous environments of cells and extracellular fluids by directly quenching a variety of reactive species (e.g. $O_2^{\cdot-}$, $LOO^{\cdot\cdot}$, H_2O_2, $^1O_2$, $^{\cdot}NO$, etc.) and chelating transition metals such as Fe^{++} and Cu^{++} (Sies et al., 1992; Carocho et al., 2013).

Despite the majority of *in vivo* studies showed a reduction in markers of oxidative damage to biomolecules after supplementation with vitamin C, under certain circumstances, vitamin C may function also as a pro-oxidant, which is attributed to the release of metal ions from damaged cells (Valko et al., 2006).

Figure 14. Various forms of vitamin C and its reaction with radicals ($R^{\cdot}$).
(Valko et al. 2006)

- **Vitamin E (tocopherols and tocotrienols)**

High contents of this fat-soluble antioxidant are present in certain nuts and seeds, most oils (including fish oil), dark leafy greens, broccoli, avocados, seafood, etc.

Vitamin E is composed of eight isoforms, four tocopherols (α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol) and four tocotrienols (α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol), with α-tocopherol being the most potent and abundant isoform in biological systems (Carocho & Ferreira 2013). Vitamin E functions in cell membranes, lipoproteins and lipid droplets as one of the most effective lipid peroxidation chain-breaking antioxidants. In the case of α-tocopherol, it donates its phenolic hydrogen to $L^{\cdot}$ or $LOO^{\cdot}$, thus forming the α-tocopheroxyl radical which, despite also being a radical, is unreactive and unable to continue the oxidative chain reaction (Sies et al. 1992) (**Figure 15**). This mechanism of action can be translated to the rest of vitamin E forms. Then, the vitamin E oxidised forms can be regenerated, mainly by ascorbic acid, but also by GSH and ALA (Valko et al. 2006).

Several beneficial health effects have been described for vitamin E (Wojcik et al. 2010).

Figure 15. Reaction of vitamin E with lipoperoxides ($LOO^{\cdot}$).

- **Phenolic compounds**

Phenolic compounds are the most abundant dietary antioxidants. They are present in large quantities in numerous plant-derived foods and beverages: spices, dried herbs, darkly coloured berries (including grapes), cocoa, coffee, tea, wine, olive, artichokes, nuts, some seeds, etc. (Pérez-Jiménez et al. 2010).

These compounds are broadly covered by the term ‘polyphenols’. More than 8,000 different structures have been described, ranging from simple phenolic molecules to highly polymerized compounds with molecular weights of greater than 30,000 Da. Regarding the chemical structure of their basic skeleton, phenolic compounds can be divided into at least 10 different classes (Bravo 1998; Heim et al. 2002; Andersen & Markham 2005; Serrano et al. 2009) (**Table 2**).

The water solubility of these compounds is dependent on several factors related to their chemical structure: degree of polymerization, number of hydroxyl groups, conjugation with other molecules (glycosylation, galloylation, reactions during phase II metabolism, etc.) (Hemingway 1989; Gökmen et al. 2009).

There is increasing evidence that polyphenols, acting both through direct or indirect mechanisms, may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress through several health promoting activities: antioxidant, anti-inflammatory, anticarcinogenic, hypotensive, anti-atherosclerotic, anti-thrombogenic, anti-diabetic, antiosteoporotic, etc. (Nijveldt et al. 2001; Scalbert et al. 2005; Crozier et al. 2009; Pandey & Rizvi 2009; Del Rio et al. 2013).

Table 2. Main classes of phenolic compounds.

Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	OH
Benzoquinones	C_6	O= =O
Phenolic acids	C_6- C_1	COOH
Acetophenones	C_6- C_2	$COCH_3$
Phenylacetic acids	C_6- C_2	CH_2-COOH
Hydroxycinnamic acids	C_6- C_3	CH=CH-COOH
Phenylpropenes	C_6- C_3	CH_2-CH=CH_2
Coumarins, isocoumarins	C_6- C_3	
Chromones	C_6- C_3	
Naftoquinones	C_6- C_4	
Xanthones	C_6- C_1-C_6	
Stilbenes	C_6- C_2-C_6	
Anthraquinones	C_6- C_2-C_6	
Flavonoids	C_6- C_3-C_6	
Lignans, neolignans	(C_6- C_3)$_2$	
Lignins	(C_6- C_3)$_n$	

- **Carotenoids**

These phytochemicals are a group of more than 600 natural pigments that are mainly found in several red, orange and yellow fruits and vegetables, such as tomatoes, carrots, dark leafy greens, broccoli, etc.

They can be classified in two major groups: carotenoid hydrocarbons, such as β-carotene and lycopene; and, oxygenated carotenoids, which are also known as xanthophylls, like zeaxanthin and lutein (Krinsky 1989). β-carotene (**Figure 16**) is the most important natural precursor for vitamin A in mammals. In fact, all naturally occurring vitamin A derives by enzymatic oxidative cleavage from carotenoids with provitamin A activity, leading to at least dozen forms of vitamin A, such as retinol (Carocho & Ferreira 2013), which can hence be absorbed by mammals also when eating animal food sources rich in this carotenoid, such as milk, eggs, tuna, etc. The rest of carotenoids do not possess provitamin A activity, but their antioxidant capacities are similar to, or even greater than, the actions of β-carotene (Fang et al. 2002).

Figure 16. Chemical structure of β-carotene.

Carotenoids are polyenoic terpenoids having conjugated *trans*- double bonds. Therefore, they are hydrophobic molecules and their main antioxidant properties take place in the lipid phases by, mainly, $^1O_2$ quenching, as well as $ROO^{\cdot}$, $^{\cdot}OH$ and $O_2^{\cdot -}$ scavenging. These reactions result in excited carotenoids which dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This can occur while the carotenoids have conjugated double bonds within (Sies et al. 1992).

- **Minerals**

Minerals are a small proportion of dietary antioxidants, which are present mainly in plants (nuts, seeds, beans, dark leafy greens, etc.), seafood and fish, whereas they are found only in trace quantities in mammals. Even though, these molecules are essential for various pathways in metabolism. Regarding antioxidant activity, the most important minerals are Se, Zn, Cu and Mn. They do not attack directly RONS but, as an indispensable part of most antioxidant enzymes (Se: metalloenzymes, GPX1-GPX4, TrxR; Zn and Cu: SOD1; Mn: SOD2), they are important in the prevention of RONS formation. Some minerals have also inhibitory activities of RONS generating enzymes, such as Zn, which is an inhibitor of NOXs (Carocho & Ferreira 2013).

In addition to the consumption of antioxidants naturally contained in plant foods (mainly fruits, vegetables, cereals, and derived products, as aforesaid), further sources of exogenous dietary antioxidants for mammals are food fortification and dietary supplementation with antioxidants from natural and/or synthetic origin (Bouayed & Bohn 2010).

Physiological doses of exogenous antioxidants are required to maintain or re-establish redox homeostasis. However, high doses of exogenous antioxidants given as part of supplements may disrupt the delicate redox balance. As a consequence, controversial results are often observed using antioxidant supplements in epidemiological trials, reporting beneficial, detrimental and non-effects (Ndhlala et al. 2010). Therefore, the anti-oxidant and pro-oxidant properties of natural antioxidants over different concentration ranges should be always considered (Rietjens et al. 2002), being critical the investigation of doses that exert beneficial instead of detrimental effects in the organism.

With regard to supplementation with dietary antioxidant phytochemicals, such desired beneficial effects have been seen predominantly when they are consumed within their natural food matrices. In fact, non-extracted antioxidants have been suggested to be safer and healthier than isolated ones (usually supplied at high concentrations), which can be explained by two main factors: the general lower contents of antioxidants within natural food matrices, and the synergistic or additive actions of complex mixtures of phytochemicals and nutrients in these supplements based on non-extracted natural antioxidants (Saura-calixto 1998; Bouayed & Bohn 2010).

o *Interactions between Non-enzymatic Antioxidants*

Both endogenous and exogenous non-enzymatic antioxidants act interactively to maintain or re-establish redox homeostasis (Jacob 1995; Choe & Min 2009). These interactions can be synergistic, antagonistic, or merely additive:

- Synergism is a phenomenon in which a net interactive antioxidant effect is higher than the sum of the individual effects. Several mechanism may be involved, such as:
    - A combination of two or more different free radical scavengers in which a more effective antioxidant (primary antioxidant) is regenerated by other less effective antioxidants (co-antioxidants), which occurs when the primary free radical scavenger has a higher reduction potential than the other.
    - A sacrificial oxidation of a less effective antioxidant to protect another more effective antioxidant.
    - A combination of two or more antioxidants whose antioxidant mechanisms are different, such as the combination of metal chelators and free radical scavengers.

- Antagonism is a phenomenon in which a net interactive antioxidant effect is lower than the sum of the individual antioxidant effects. This effect can arise by:
  - Regeneration of the less effective antioxidant by the more effective one.
  - Alteration of microenvironment of one antioxidant by another antioxidant.
- Additive interaction means that a net interactive antioxidant effect is the same as the sum of individual effects.

In this regard, the 'antioxidant network' constituted by vitamin E, vitamin C and thiol redox cycles (**Figure 17**), which plays a key role in lipid peroxidation prevention, is one of the best examples of synergistic interactions between antioxidants (Valko et al. 2007).

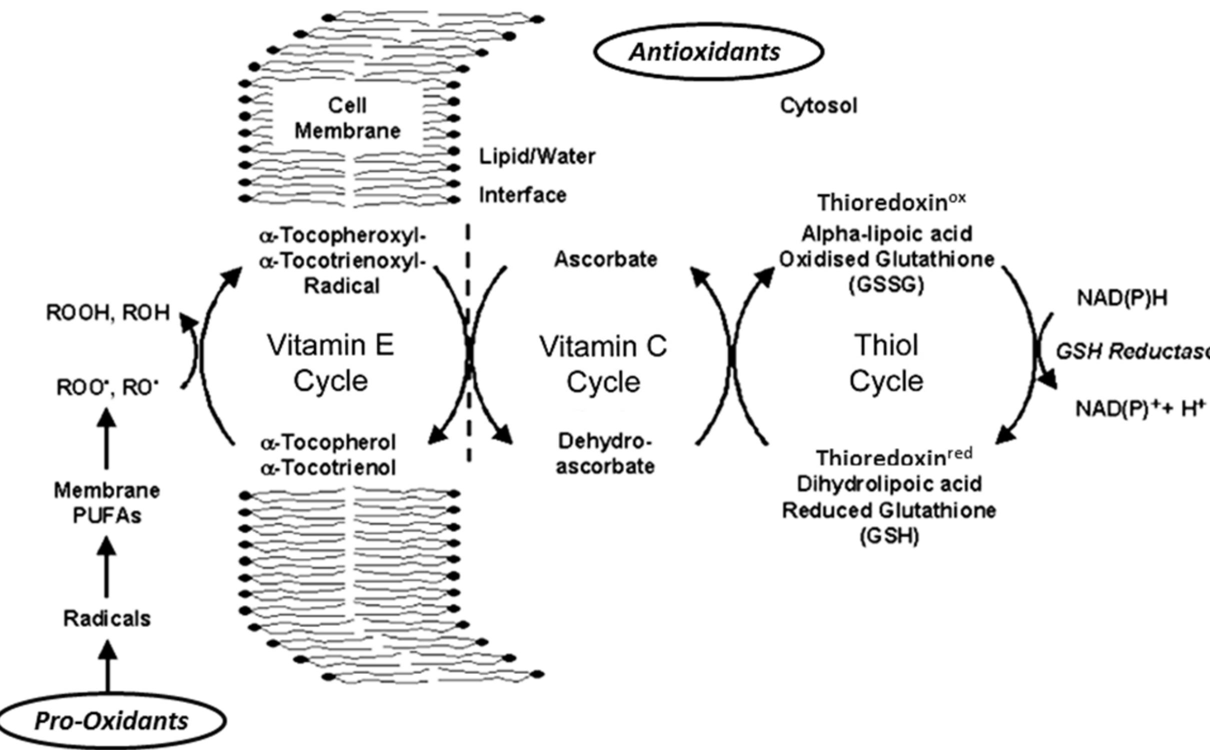


Figure 17. The antioxidant network showing interactions between vitamin E, vitamin C and thiol redox cycles.
Adapted from Packer et al. (2001).

Furthermore, all type of interactions between the above mentioned antioxidants and others, such as polyphenols and carotenoids, have been described in literature, which mainly depends on their respective reduction potentials (Choe & Min 2009).

2.2.3. Adaptive Antioxidant Response

The adaptation of cells to oxidative conditions by regulating the expression of protective stress genes/proteins, as well as by modulating the activity of enzymes implicated in RONS formation/elimination, is crucial for survival of aerobic organisms and has been a topic of active research. Several signal transduction pathways, which enable information to be transmitted from the extracellular milieu to various functional elements inside the cell, together with some exclusively intracellular signalling pathways, are involved in the response of cells to their redox state (redox signalling).

Extracellular signals that trigger signal transduction include cytokines, hormones, growth factors, etc. Then, several cell receptors (cytokine receptors, interleukin receptors, growth factor receptor tyrosine kinases, etc.) and non-receptors (serine/threonine kinases, protein tyrosine phosphatases, non-receptor tyrosine kinases, etc.) transmit the information from outside the cell into the cytoplasm and the nucleus (Valko et al. 2007). In this context, a number of antioxidants and RONS can act as signalling molecules on different levels in such signalling cascades (Bellezza et al. 2010; Finley et al. 2011; Marinho et al. 2014).

Signals sent to the transcription machinery are normally transmitted to the cell nucleus by a class of proteins called transcription factors, which finally modulate gene expression.

Two of the major transcription factors implicated in redox signalling pathways are:

- **Nuclear factor-kappaB (NF-κB)**

  This is an inducible and ubiquitously expressed transcription factor involved in cell survival, differentiation, inflammation, and growth. The configuration of the binding protein that interacts with target genes is a dimer of two members of the NF-κB/Rel/Dorsal family of proteins. This family is constituted by at least 5 members: RelA (also called p65), cRel, RelB, p50 (also called NF-κB1) and p52 (also called NF-κB2). The classical dimer is composed of p50 and p65, with the latter containing the transactivation domain (Valko et al. 2006).

  In non-stimulated cells, NF-κB is generally sequestered in the cytoplasm due to its interaction with IκBα, a member of the inhibitory (IκB) family. Activation of NF-κB occurs in response to a wide variety of extracellular stimuli, including cytokines, DNA damage, RONS, etc. RONS are also implicated as second messengers in the signalling cascades that promote the dissociation of IκBα, which unmasks the nuclear localization sequence (Bellezza et al. 2010; Brigelius-Flohé & Flohé 2011). At least three pathways have been thus described for the activation of NF-κB as the dimer p50/p65, and there is also an alternative pathway that leads in the formation of the dimer p52/RelB (**Figure 18**).

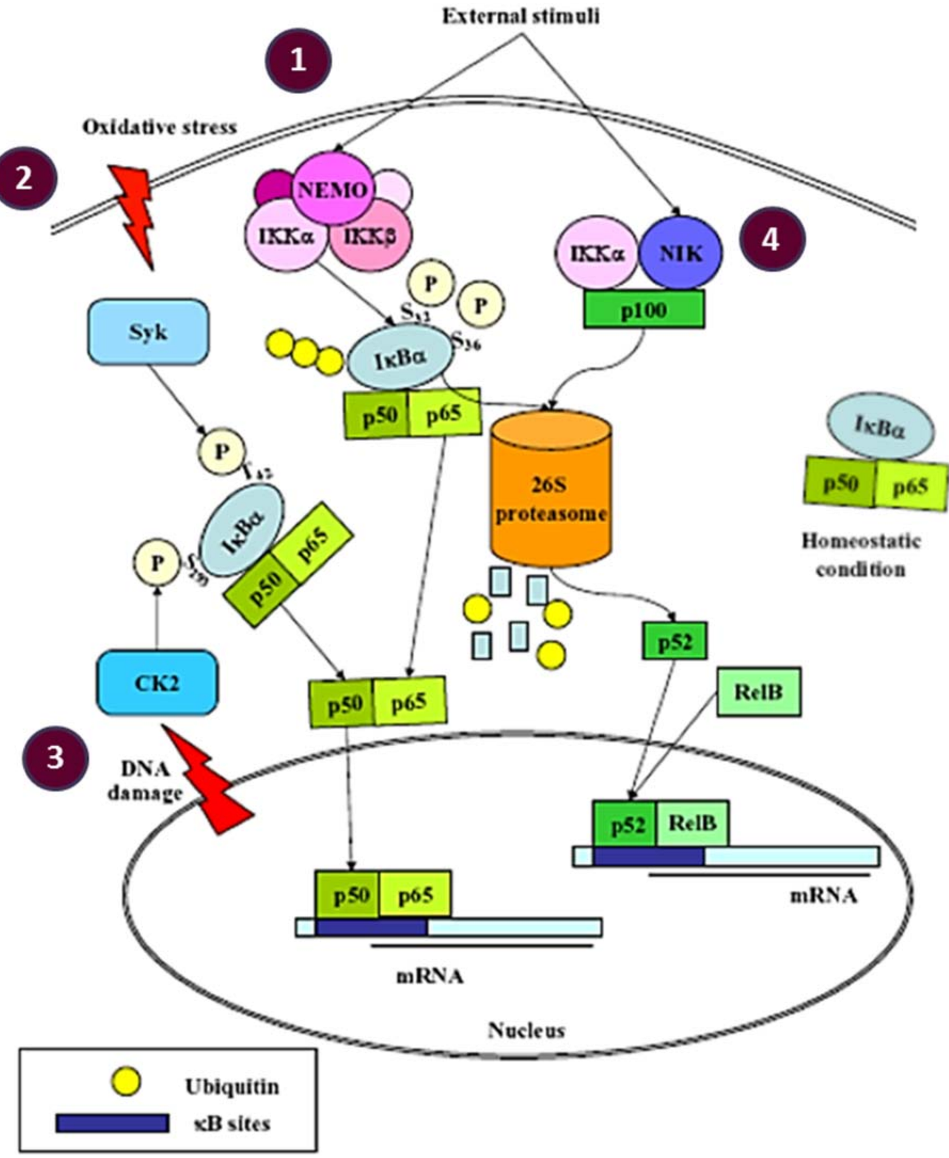


Figure 18. Proposed mechanisms for NF-κB activation.
In homeostatic conditions NF-κB is sequestered in the cytoplasm. Upon stimulation, different pathways can be followed: **1)** Classical pathway; **2)** Oxidative stress-induced pathway; **3)** Atypical pathway; **4)** Alternative pathway.
Adapted from Bellezza et al. (2010).

The consequence in all cases is the entry of NF-κB into the nucleus, its binding to κB regulatory elements in the promoter/enhancer regions of target genes, and the activation of their transcription. On the other hand, under excessive oxidative stress, DNA binding of NF-κB might be inhibited due to the sensitivity to oxidative modification of one of the residues in p50 (Bellezza et al. 2010). Termination of the NF-κB response involves also the resynthesis of IκB proteins induced by activated NF-κB.

Target genes of NF-κB implicated in the inflammatory/oxidative stress response include cytokines, chemokines, growth factors, Ang II, and enzymes such as COX-2, iNOS, SOD2, HO-1, NQO1, and the catalytic subunit of GCL (GCLC) (Pahl 1999; Lavrovsky et al. 1994).

- **<u>Nuclear factor-erythroid 2-related factor 2 (Nrf2)</u>**

The redox-sensitive transcription factor Nrf2, via the antioxidant responsive elements (AREs) contained in the promoter region of specific genes, mediates the coordinated up-regulation of antioxidant and detoxifying proteins in response to endogenous/exogenous oxidants and antioxidants.

Under normal homeostatic conditions, Nrf2 is maintained at a low level because it is targeted constitutively for proteosomal degradation by ubiquitylation. This transcription factor is activated by alteration of cysteine residues of its inhibitor, the Kelch-like ECH-associated protein 1 (Keap1), and/or by phosphorylation at specific serine and threonine residues by kinases such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC). As a result, Nrf2 is released from Keap1 and, subsequently, this transcription factor translocates into the nucleus and forms a heterodimer with small MAF protein. This heterodimer binds to the AREs and up-regulates transcription of many genes encoding antioxidant and detoxifying enzymes (Bellezza et al. 2010; Finley et al. 2011) (**Figure 19**).

The magnitude and duration of Nrf2 activation is also controlled by other mechanisms (Hayes & Dinkova-Kostova 2014), including the enhanced expression of the Nrf2 gene which is up-regulated by itself (as the Nrf2 promoter also contains ARE-like sequences) and other transcription factors, thus expanding the types of stressors that induce Nrf2-target genes.

The Nrf2/ARE pathway induces the expression of more than 200 genes. They give rise to several detoxification, antioxidant, and NAD(P)H regeneration/metabolism enzymes and proteins, which include numerous GSTs, some GPXs, GR-1, SODs, CAT, NQO1 and GCLC, among many others (Acharya et al. 2010; Hybertson et al. 2011; Hayes & Dinkova-Kostova 2014). Moreover, this pathway is intimately involved in the regulation of Fe homeostasis, as ferritin, HO-1 and ferroportin genes also contain ARE sequences in their promoters/enhancers (Finley et al. 2011).

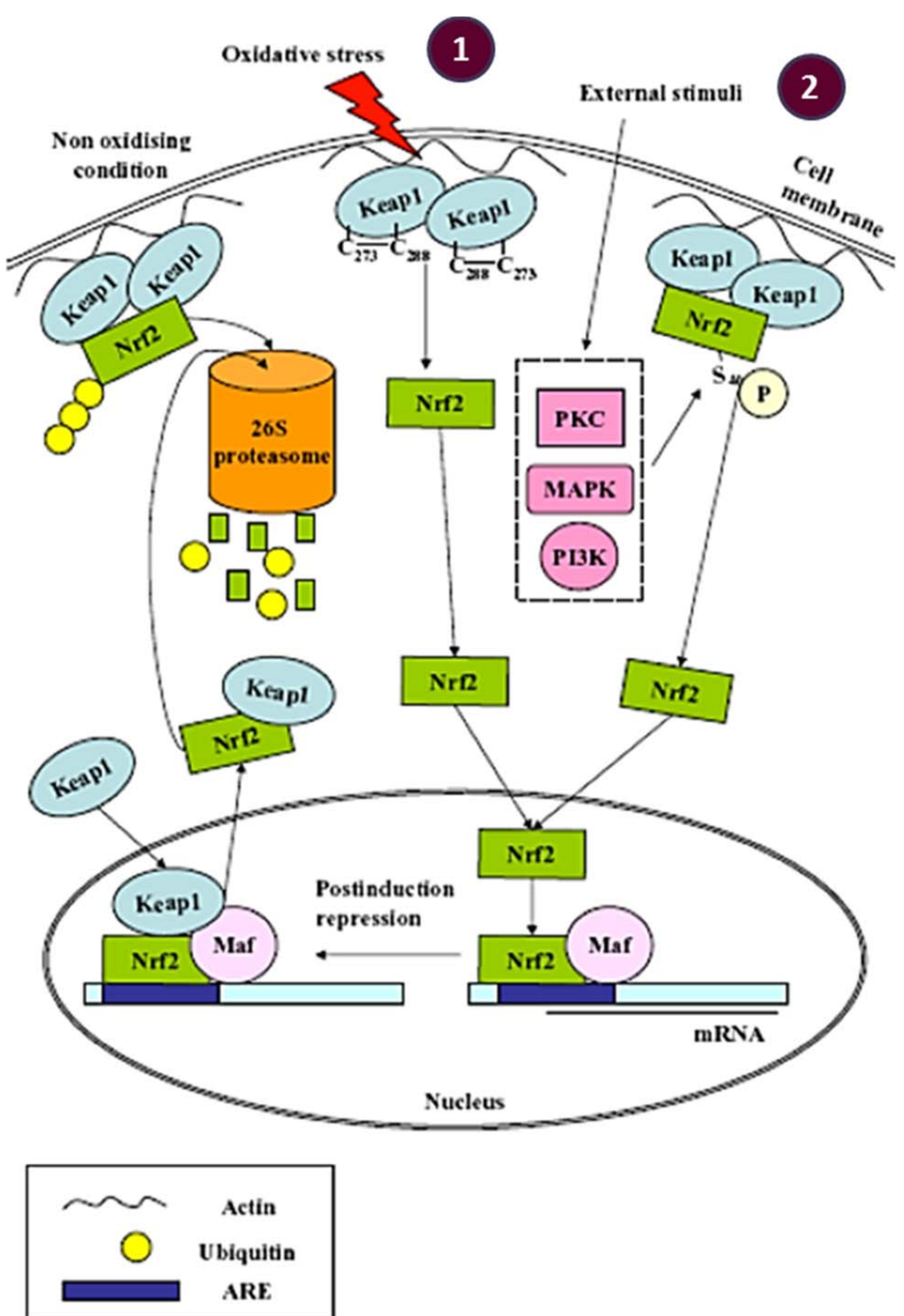


Figure 19. Proposed mechanisms for Nrf2 activation.
In non-oxidising conditions Nrf2 is retained in the cytoplasm. Upon stimulation, two main pathways can be followed:
1) Oxidative stress-induced pathway; **2)** External stimuli-induced pathway.
Adapted from Bellezza et al. (2010).

Activation of Nrf2/ARE pathway and suppression of NF-κB signalling are considered antioxidant adaptive responses to oxidative stress and, interestingly, there seem to be a crosstalk between them, with several anti-inflammatory and antioxidant phytochemicals being known to act simultaneously in this way (Bellezza et al. 2010; Buelna-Chontal & Zazueta 2013).

Activation of the Nrf2 pathway may hinder NF-κB signalling, mainly due to the inhibition of IκB kinase (IKK) activity and consequently interruption of IκB inhibitory proteins degradation. On the other hand, within the nucleus, NF-κB appears to compete with Nrf2 for the co-activator CREB-binding protein (CBP), and it also may recruit the histone deacetylase-3 (HDAC3) causing a local hypoacetylation of ARE regions that hampers trans-activation of the Nrf2 downstream genes. The MAPK family has been suggested to contribute to the concerted modulation of both transcription factors (Bellezza et al. 2010; Buelna-Chontal & Zazueta 2013) (**Figure 20**).

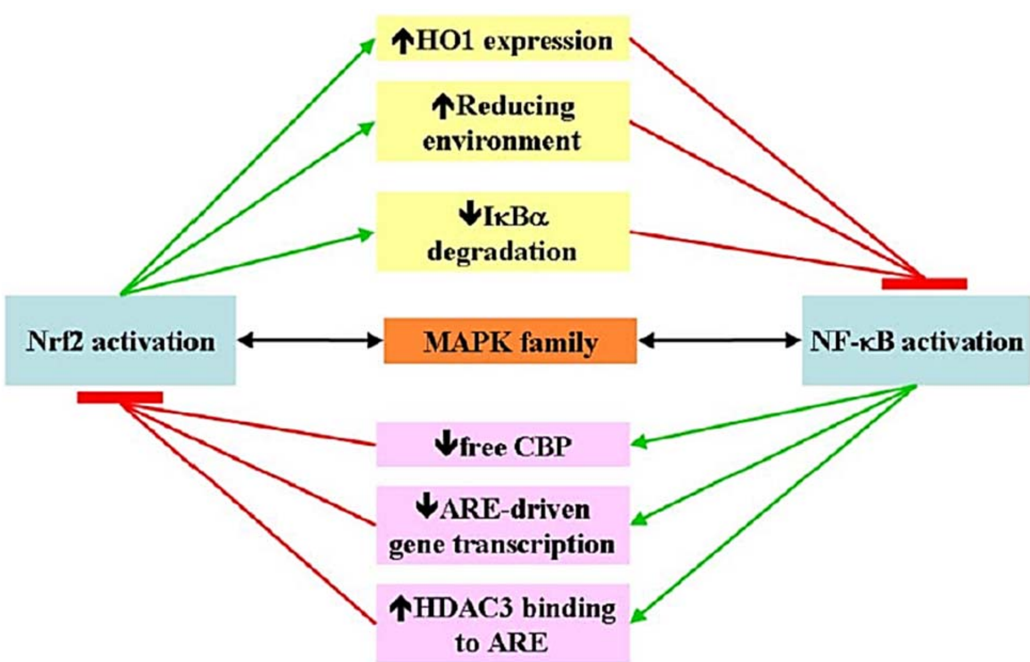


Figure 20. Proposed cross-talk between for Nrf2 and NF-κB.
Green lines: induced events; Red lines: resulting inhibitory events; Black lines: concerned modulation.
Adapted from Bellezza et al. (2010).

2.3. Oxidative Damage to Biomolecules and Biomarkers of Oxidative Stress

Depleted antioxidant defences can lead to oxidative stress, which is an important mediator of oxidative damage to cell structures, including DNA, lipids, proteins and membranes (Valko et al. 2007; Lobo et al. 2010).

Damaged biomolecules have two sides: they may propagate damage to surrounding biological structures as "second cytotoxic messengers", but may be useful as indicators of oxidative stress (Dalle-Donne et al. 2006).

Biomarkers of oxidative stress can be classified as: oxidised products of biomolecules that are modified by interactions with RONS; and, molecules of the antioxidant system that change in response to increased oxidative stress (Ho et al. 2013). These biomarkers allow monitoring overall health and oxidative stress status, as well as the degree of oxidative damage/stress in a local microenvironment. As such, oxidative stress biomarkers may lead to improved understanding of the pathogenesis of numerous illnesses. Some of these indicators can also be used to assess disease prognosis (if they have demonstrated predictive value) and progression. In addition, oxidative stress biomarkers can help to develop new therapeutic strategies and evaluate responses to treatments (Dalle-Donne et al. 2006).

The validity of biomarkers needs to be established, as it is a prerequisite for their scientific and clinical use. According to Griffiths et al. (2002), a valid biomarker should be:

- A major product of oxidative modification that may be implicated directly in the development of disease.
- A stable product, not susceptible to be lost during storage or artefactual induction.
- Representative of the balance between generation and clearance of oxidative damage.
- Determined by a reliable analytical procedure that is specific, sensitive, robust and reproducible.
- Measurable within the limits of detection of the validated analytical procedure.
- Free of being confounded with factors from dietary intake.
- Accessible either in a target tissue or in a valid surrogate tissue/biological sample of easier collection. If possible, non-invasive techniques are preferred, such as urine collection. Plasma and serum are also widely used as biological sources of biomarkers, as their collection usually requires minimally invasive techniques.

- **Oxidative DNA damage**

DNA damage caused by RONS, mainly $^{\cdot}OH$ and $ONOO^{-}$, includes a large variety of lesions covering from sugar and nitrogenous base damage (more than 60 different base lesions have been identified) to DNA breaks (single- or double-strand breaks) and DNA-protein cross-links (Evans et al. 2004).

The majority of studies on base DNA damage have focussed upon the guanine modifications, mainly in the 8-hydroxylated guanine species (Muñiz et al. 1995; Oliva et al. 1997; Evans et al. 2004). These products of oxidised guanine/2'deoxyguanosine lesions include several compounds (**Figure 21**), such as:

- Nitrogenous base derivatives: 7,8-dihydro-8-hydroxyguanine (8-OHG) and 7,8-dihydro-8-oxoguanine (8-oxoG), which are keto-enol tautomers.
- Nucleoside derivatives: 7,8-dihydro-8-hydroxy-2'-deoxyguanosine (8-OHdG) and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG).

Figure 21. RONS-mediated oxidation of guanine/2'deoxiguanosine.

First evidence about the formation of 8-OHdG by RONS was reported by Kasai & Nishimura (1984). Later, the mutagenic potential of this oxidised nucleoside, being a potential source of guanine to thymine (G > T) transversions, was proposed by Kuchino et al. (1987). Since then, 8-hydroxylated guanine species have been the focus of extensive research and are currently the most popular specific biomarkers of DNA damage.

All these 8-hydroxylated derivatives of guanine have been analysed in various biological samples, including tissue, saliva, blood, and urine (Muñiz et al. 2000; Griffiths et al. 2002; Halliwell & Whiteman 2004; Mitjavila et al. 2013). Different techniques can be used for their quantification, such as:

- High performance liquid chromatography with electrochemical detection (HPLC-EC) (Floyd et al. 1986; Muñiz et al. 1995).
- Capillary electrophoresis with electrochemical detection (CE-EC) (Arnett et al. 2005).
- Enzyme-linked immunosorbent assays (ELISA) (Yin et al. 1995).

As regards DNA strand breaks, the major techniques for this type of oxidative DNA damage is based on electrophoretic techniques:

- Electrophoresis in agarose gels of DNA isolated from several cells/whole tissue.
- Alkaline single cell gel electrophoresis, frequently referred to as the 'comet assay': this is a sensitive technique for detecting DNA strand breaks that can be applied to any cell population, provided they have been isolated from tissue without degradation. DNA damage can be correlated with the tail length of the 'comets', which are formed during electrophoresis after cell lysis and migration of DNA possessing breaks in the agarose gel (Tice et al. 2000; Griffiths et al. 2002). The comet assay has been widely used to evaluate whether endogenous and dietary antioxidants are able to protect the integrity of the genetic material (Cemeli et al. 2009; Del Pino-García et al. 2012).

Oxidative DNA damage is mainly related to aging and to an increased risk of cancer (Muñiz et al. 1995; Oliva et al. 1997; Dalle-Donne et al. 2006), although it has been also associated with a number of non-cancerous diseases, such as neurodegenerative diseases, cardiovascular diseases, HIV infection, hepatitis, diabetes mellitus, ischemia-reperfusion injury, etc. (Evans et al. 2004).

- **Lipid peroxidation**

Lipid peroxidation is the major representative of free radical chain reactions in living organisms, affecting mainly to LDL-C and polyunsaturated fatty acids of biological membranes (Griffiths et al. 2002; Montuschi et al. 2004; Niki 2008).

The process of lipid peroxidation comprises the following steps (Girotti 1998; Lee et al. 2012):

1) Abstraction of an $H^{\cdot}$ from polyunsaturated fatty acids, concretely, from a methylene group next to a double bond, producing a lipid carbon-centred radical ($L^{\cdot}$). This step is known as 'initiation'.

2) The remaining carbon radical is stabilised by a rearrangement of the double bond to form a conjugated diene.

3) This conformation can easily react with O_2, which is present at high concentration in the membrane of the cell, forming the peroxyl radical ($LOO^{\cdot}$).

4) The peroxyl radical further reacts with another lipid species, abstracting an $H^{\cdot}$ and then forming a lipid hydroperoxide (LOOH), which is the primary product of lipid peroxidation, and a new carbon-centred radical ($L^{\cdot}$). This step is usually known as 'propagation' of chain reaction.

5) The lipid hydroperoxide may undergo further reactions to form cyclic peroxides, cyclic endoperoxides, and finally aldehydes. These reactions may be catalysed by transition metals, such as free Fe^{++}.

Peroxidation of membrane lipids can be very damaging for cells (Dalle-Donne et al. 2006), as it leads to:

- Alteration/Loss of membrane fluidity and elasticity.
- Impaired cellular functioning due to inactivation of membrane-bound receptors or enzymes.
- Increased membrane permeability.
- Amplification of cellular oxidative damage, resulting from generation of oxidised products, some of which are chemically reactive and covalently modify critical macromolecules (DNA, proteins, and other lipids).

Cell responses to lipid peroxidation may range from cell survival at low lipid peroxidation (due to the balance between the pro-oxidants and antioxidants present at basal conditions or those induced by the oxidant insult) to cell death at higher oxidative stress pressure (induction of apoptosis at moderate lipid peroxidation, and necrosis due to membrane lysis at very high oxidative stress pressure) (Girotti 1998).

Lipid peroxidation generates a variety of relatively stable decomposition end products, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2-propenal (acrolein), glioxal, and isoprostanes (IsoPs), which can then be measured in plasma and urine as an indirect index of oxidative stress/damage (Griffiths et al. 2002; Dalle-Donne et al. 2006) (**Figure 22**).

Figure 22. Pathways of lipid peroxidation leading to the formation of F_2-isoprostanes (F_2-IsoPs), malondialdehyde (MDA), and other oxidised end lipid products.

Two of the most well studied biomarkers of lipid peroxidation are:

- Malondialdehyde (MDA): this is a reactive carbonyl compound generated via peroxidation of polyunsaturated fatty acids. MDA interacts with proteins and DNA, being itself potentially atherogenic, mutagenic and carcinogenic. This reactivity is believed to contribute significantly to cardiovascular diseases, diabetes and cancers linked to lifestyle and dietary factors (Lee et al. 2012; Ho et al. 2013).
  MDA is usually quantified from plasma samples different body tissues, cell homogenates, but also in natural extracts and foods. There are several assays and techniques that can be applied:
  - The most popular and simplest methodology is a colorimetric assay based on the reaction between MDA and thiobarbituric acid (TBA), which is known as the TBA reacting substances (TBARS) assay, and generated a pink coloured MDA-(TBA_2) Schiff base adduct that absorbs at λ = 532 nm (Lee et al. 2012). This method is suitable for high throughput analyses, but lacks specificity, as aldehydes other than MDA can react with TBA and generate products that absorb in the same range as MDA.
  - HPLC-UV techniques allow the specific detection of MDA (Grotto et al. 2007).
  - ELISA kits are also commercially available.

- F_2-Isoprostanes (F_2-IsoPs): They are a family of prostaglandin-like compounds produced from the free radical-induced peroxidation of arachidonic acid, a polyunsaturated fatty acid present in phospholipids of cell membranes. F_2-IsoPs generation is independent of the cyclooxygenase enzyme, which catalyzes the formation of prostaglandins from arachidonic acid. F_2-IsoPs are subsequently released into the circulation by phospholipases, where they undergo partial metabolism and exert potent biological effects in the organism before being excreted in urine (Montuschi et al. 2004; Ho et al. 2013). In fact, increased levels of isoprostanes in biological fluids (plasma, serum, and urine) and tissues have been shown to correlate with a number of diseases risk factors (mainly related to cardiovascular diseases) such as diabetes, hypercholesterolemia, obesity, etc. (Davies & Roberts 2011; Lee et al. 2012).

  F_2-IsoPs are considered the most reliable biomarkers of lipid peroxidation and, in general, of oxidative stress status because they are chemically stable and specific products of *in vivo* peroxidation (unaffected by lipid content in the diet), and also due to the specificity of the assays applied for their quantification (Montuschi et al. 2004). The 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is one of the most abundant and the most thoroughly investigated F_2-IsoPs.

F_2-IsoPs can be measured using several techniques (Dalle-Donne et al. 2006; Ho et al. 2013), such as:

- o Gas chromatography-mass spectrometry (GC-MS), which is the most sensitive, highly specific, and reliable method, especially if coupled with negative-ion chemical ionization (Mori et al., 1999). Despite the forms of F_2-IsoPs present in plasma and urine are not coincident, with higher variety of F_2-IsoP metabolites being known to be excreted in urine (Niki 2010), the GC-NCI-MS method measures total F_2-IsoPs rather than levels of a specific metabolite (Mori et al., 1999), so a relationship between the trend of circulating and excreted F_2-IsoPs is often well expected.
- o Liquid chromatography-mass spectrometry (LC-MS).
- o ELISA kits
- o Radioinmmunoassays (RIAs).

- **Protein oxidation**

Proteins are key targets for RONS attack because of their high overall abundance in biological systems and because they are chiefly responsible for most functional processes within cells. Exposure of proteins to RONS, and to secondary by-products of oxidative stress, causes major physical changes in protein structure, and may also alter every level of protein structure, from primary to quaternary (Griffiths et al. 2002).

Oxidative damage, most of which is irreparable, include: peptide backbone oxidation and/or cleavage, formation of protein-protein cross-linkages, and/or modification of the side chain of practically every amino acid. In this regard, the process of protein oxidation frequently introduces new functional groups, such as carbonyls and hydroxyls, and leads to the formation of oxidised amino acids, such as: L-hydroxyphenylalanine and bityrosine from tyrosine; *o*-tyrosine from phenylalanine; sulphoxides and disulphides from methionine and cysteine, respectively; and kynurenines from tryptophan (Griffiths et al. 2002; Halliwell & Whiteman 2004). These oxidative changes can have a wide range of downstream functional consequences (Dalle-Donne et al. 2006), which include:

- Loss of function or inhibition of enzymatic and binding activities, affecting, for instance, the functionality of several enzymes, receptors, and transport proteins.
- Increased susceptibility to aggregation and proteolysis.
- Altered immunogenicity.
- Secondary damage to other biomolecules (e.g. inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA).

If the proteolytic mechanisms responsible for protein degradation do not function properly, altered proteins accumulate in the cell and may contribute to the development of pathological conditions.

The main biomarkers of oxidative protein damage are: protein carbonyl groups, L-hydroxyphenylalanine, bityrosine, and *o*-tyrosine (Griffiths, 2002).

<u>Protein carbonyl groups</u> are ubiquitous products of protein oxidation arising on amino acid side chains due to direct attack of RONS (including many lipid peroxidation products, with the formation of both oxo-acids and aldehydes), and protein glycation by sugars. Despite carbonyls are not entirely specific markers of protein oxidation as may arise also in lipids and sugars, the measurement of carbonyl groups is a commonly used indicator of the 'average' extent of protein oxidative modification (Levine et al. 1990; Halliwell & Whiteman 2004). As such, there are several methods to measure protein carbonyl groups, including:

- Spectrophotometric determination.
- Analysis by HPLC-UV techniques.
- Immunodetection by ELISA or Western blot.

In most cases, the methods are based on the reaction between protein carbonyls and 2,4-dinitrophenylhydrazine, which generates a hydrazone chromophore, with a maximum of absorbance at 360 nm (**Figure 23**). This product can be also detected using several antibodies (Griffiths et al. 2002). Among the above-mentioned techniques, spectrophotometric determination using the carbonyl assay described by Levine et al. (1990) is a simple and low-cost technique widely applied to cell, tissue and plasma samples (Halliwell & Whiteman 2004).

$$RR'C{=}O + H_2N{-}NH{-}C_6H_3(NO_2)_2 \xrightarrow{H^+} RR'C{=}N{-}NH{-}C_6H_3(NO_2)_2$$

Aldehyde or ketone — 2,4-dinitrophenylhydrazine — 2,4-dinitrophenylhydrazone

Figure 23. Reaction between protein carbonyls and 2,4-dinitrophenylhydrazine.

2.4. Oxidative Stress: a Frequent Cause and Consequence of Human Diseases

Increasing evidence supports the implication of oxidative stress, both as a primary cause and as a down-stream consequence, in numerous pathophysiological processes. Disruption of normal RONS signalling and increased RONS generation may lead to several illnesses. In addition, the accumulation of oxidative damage to biomolecules with ageing and under such detrimental disorders is thought to cause some of the characteristic symptoms of each disease and degenerative process (Dröge 2002; Valko et al. 2007; Sen et al. 2010).

The relationship of RONS and oxidative stress with some of the most important chonic pathophysiological conditions is briefly described below:

2.4.1. Cardiovascular Diseases

Cardiovascular diseases are the leading cause of death in the world. RONS-induced oxidative stress constitutes a unifying mechanism of injury of various cardiovascular diseases and related risk factors (Higashi et al. 2009). ROS are produced by all vascular cell types, including endothelial, smooth muscle and adventitial cells, with NOXs, uncoupled NOS, xanthine oxidases and the mitochondrial electron transport chain being the main sources (Pennathur & Heinecke 2007). In addition, increased vascular ROS production is tightly linked with the action of Ang II, a vasoconstrictor peptide synthesised by the angiotensin I-converting enzyme (ACE) (Virdis et al. 2011).

Excess ROS lead to changes in membrane permeability, membrane lipid bilayer disruption, functional modification of various cellular enzymes, and lipid, lipoprotein (mainly LDLs) and protein oxidative damage (Valko et al. 2007). In addition, RONS have been implicated as playing an important role in vascular events such as: endothelial dysfunction, inflammation, extracellular matrix deposition, fibrosis, cell proliferation, migration, activation and angiogenesis; all important processes contributing to cardiovascular remodelling in hypertension, diabetes, atherosclerosis, cardiac failure, myocardial ischemia-reperfusion, etc. (Rodrigo & González 2014).

Endothelial dysfunction refers to the loss of effective regulation of homeostatic functions of endothelial cells. Under physiological conditions, the endothelium does not only provide a structural barrier between the circulation and surrounding tissues, but contributes to the regulation of vascular tone and blood flow by controlling the flow of nutrient substances, blood cells, and diverse biologically active molecules secreted by endothelial cells themselves (Cines et al. 1998). Endothelial dysfunction results from a shift in these actions toward reduced vasodilation, a proinflammatory state, and prothrombotic properties, being characterised by the imbalance between endothelium-derived vasodilators (especially $^{\cdot}$NO, endothelium-derived hyperpolarizing factor, and prostacyclin) and vasoconstrictors (such as

Ang II, endothelin-I, platelet-activating factor, norepinephrine, various prostanoids, and certain RONS). RONS are implicated both as cause and consequence of the impairment of endothelial function, which is a common hallmark of most cardiovascular diseases (Félétou & Vanhoutte 2006; Versari et al. 2009).

- *Diabetes mellitus (DM)*

DM is one of the most prevalent diseases worldwide and a major risk factor for cardiovascular diseases. Most recent statistics of the International Diabetes Federation (IDF) indicate that, in 2015, around 9.1% of the world population – 415 million people – had DM, and this number is estimated to rise beyond 642 million people in the next 25 years (IDF, 2015).

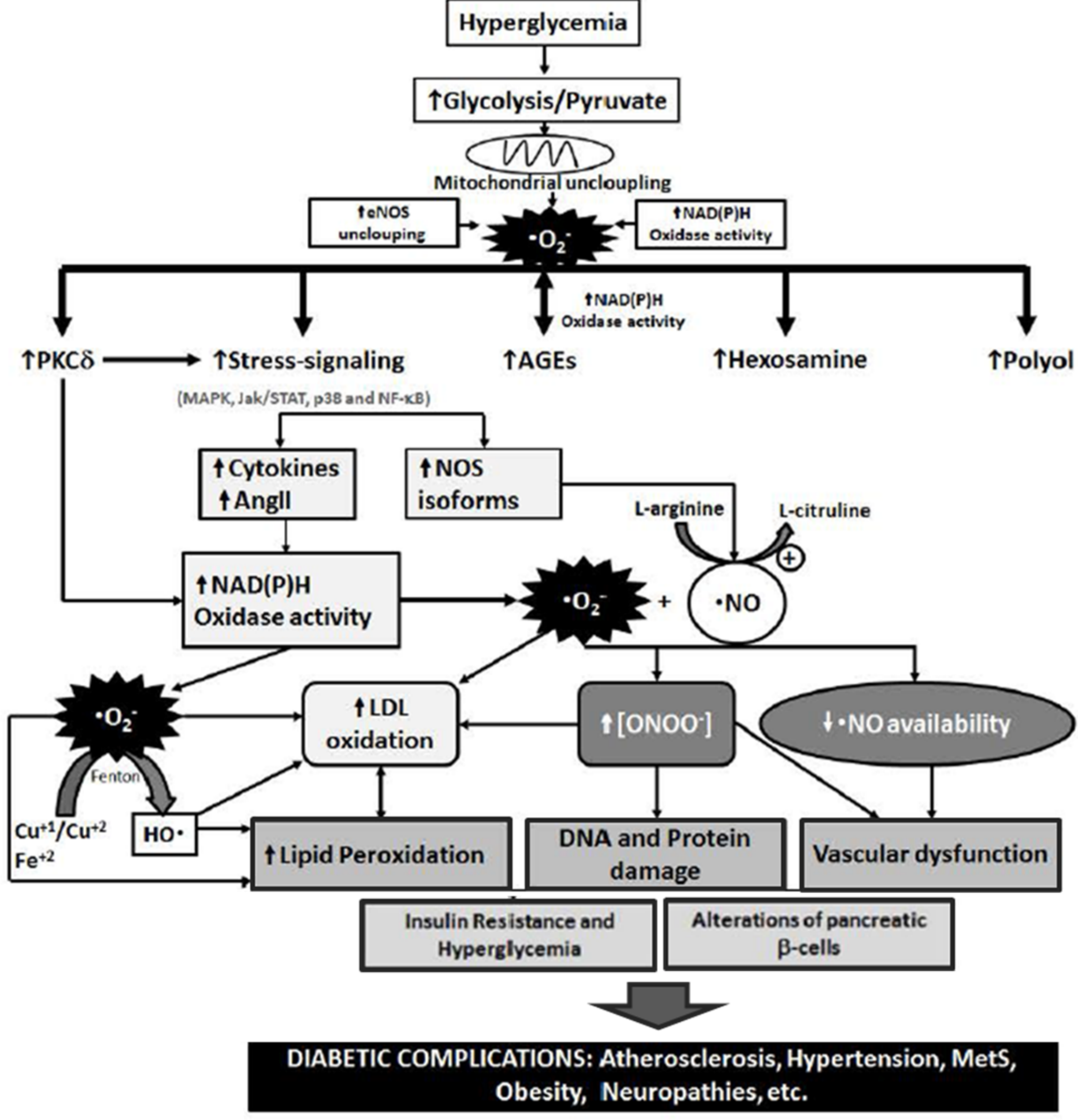


Figure 24. **Participation of hyperglycaemia in triggering the multiple oxidative stress pathways in the course of diabetes mellitus**.
Adapted from Bandeira et al. (2013).

Hyperglycaemia is the common characteristic of both insulin-dependent diabetes mellitus (Type 1 DM) and non-insulin-dependent diabetes mellitus (Type 2 DM), being well-known that the risk of developing vascular complications rises linearly with increased glycaemia. In fact, cardiovascular diseases are responsible for up to 80% of deaths in DM carriers. Type 2 DM represents approximately 90-95 % of the DM cases, occurs usually in adult people, and is mainly related to the ineffectiveness of the cells of the body to make use of glucose due to insulin resistance. Type 1 DM accounts for 5-10% of the DM cases, affects primarily children and adolescents, and happens because the β-cells in the pancreas cannot synthesize enough insulin.

Elevated glucose levels in DM have been associated with increased production of RONS by several complex and multifactorial mechanisms (**Figure 24**), which seem to be interrelated (Brownlee 2005; Stirban et al. 2008; Bandeira et al. 2013) and include:

- Activation of the poly(ADP-ribose) polymerase (PARP), PKC, and NF-κB, leading to increased stress signalling.
- Increased formation of advanced glycation end products (AGEs) and expression of their receptor (RAGE).
- Increased hexosamine pathway flux and subsequent over-modification of proteins by N-acetylglucosamine.
- Increased polyol pathway activity.

Hypertension

The burden of morbidity and mortality from hypertension and related non-communicable diseases is currently one of the most urgent public health problems worldwide (WHO 2012b). Hypertension is defined as systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg. According to the Global Status Report on Non-communicable Diseases 2010 (WHO 2014), the overall prevalence of high blood pressure in adults aged 25 and over is around 40%, causing around 13% of the total of deaths globally.

The aetiology of ROS-induced oxidative stress in the pathogenesis of hypertension is quite well established (Paravicini & Touyz 2006; Majzunova et al. 2013). In hypertensive individuals, neurohumoral stimuli such as Ang II, endothelin-I and norepinephrine activate their respective receptors, located on cell membrane. The function of these receptors is coupled to G proteins, which activate NOXs.

The activated NOXs will produce ROS, particularly $O_2^{\cdot-}$ and H_2O_2, which function as second messengers and, in turn, activate cell phosphorylation pathways:

- Tyrosine kinases
- Mitogen-activated protein kinases (MAPKs).
- Protein kinase C (PKC)
- Phosphatidylinositol-3-kinase/Akt (PI3K/Akt) pathway.

Once activated, these phosphorylation pathways further activate transcription factors (such as AP-1, NF-κB, p53, and Nrf2) which specifically induce gene transcription of pro-oxidant and antioxidant enzymes (**Figure 25**).

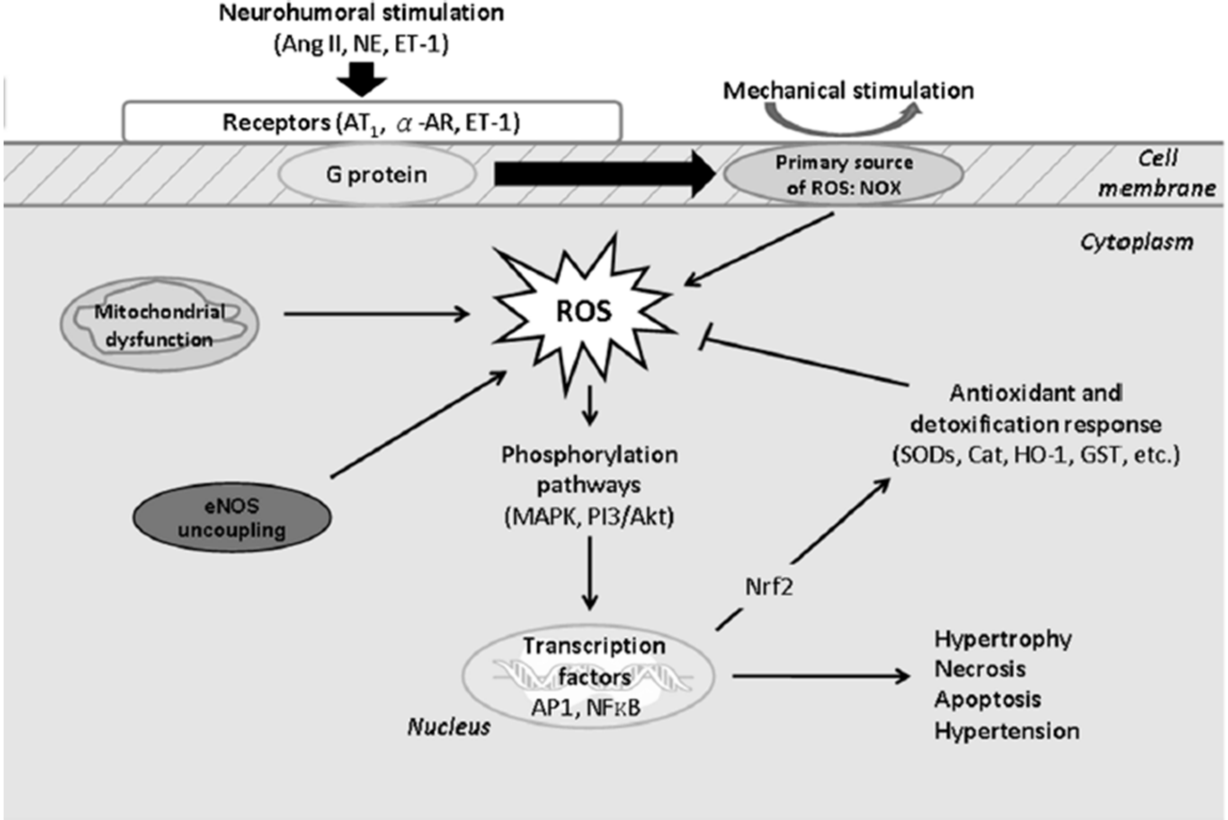


Figure 25. **Redox-sensitive intracellular signalling in endothelial cells in hypertension**.
Majzunova et al. (2013).

The formation of $ONOO^-$ due to the interaction of $O_2^{\cdot-}$ and $^{\cdot}NO$ is also involved in the process of hypertension (Chen et al. 2010). RONS overproduction may exert vasoactive functions, modulate ion channels, and release of pro-inflammatory adhesion molecules, which control vascular functions and underlie vascular damage and structural remodelling in hypertension (Touyz & Briones 2011).

2.4.2. Cancer

RONS are potential carcinogens because they facilitate mutagenesis, tumour promotion, and progression. The redox imbalance observed under oxidative stress result in oncogenic stimulation due to the RONS effects on redox-responsive signalling cascades, together with the modifications of genetic material resulting from oxidative DNA damage. In fact, the role of free radicals as primary species causing DNA damage and mutation is a critical step in the carcinogenic process, which can be described as an imbalance between cell proliferation and cell death shifted towards cell proliferation (Valko et al. 2006). Exogenous RONS (such as tobacco smoke) and chronic inflammatory conditions, as well as lipid peroxidation and protein oxidation, are also implicated in the mechanism of carcinogenesis (Valko et al. 2007; Federico et al. 2007). In addition, certain types of cancer cells produce substantial amounts of endogenous RONS and secondary mediators of oxidative stress, such as prostaglandins, cytokines, chemokines, and oxidation products, thus promoting further oxidative damage to biomolecules (Oliva et al. 1997; Dröge 2002; Federico et al. 2007; Santiago-Arteche et al. 2012).

On the other hand, high levels of RONS can also be toxic to cancer cells and could potentially induce cell proliferation arrest or even cell death (**Figure 26**). To balance the state of oxidative stress and prevent oxidative damage, certain cancer cells increase their antioxidant capacity to keep ROS levels under the cytotoxic limit (Valko et al. 2006). Thus, cancer cells can be considered to be both ROS- and antioxidant-addicted (Glasauer & Chandel 2014).

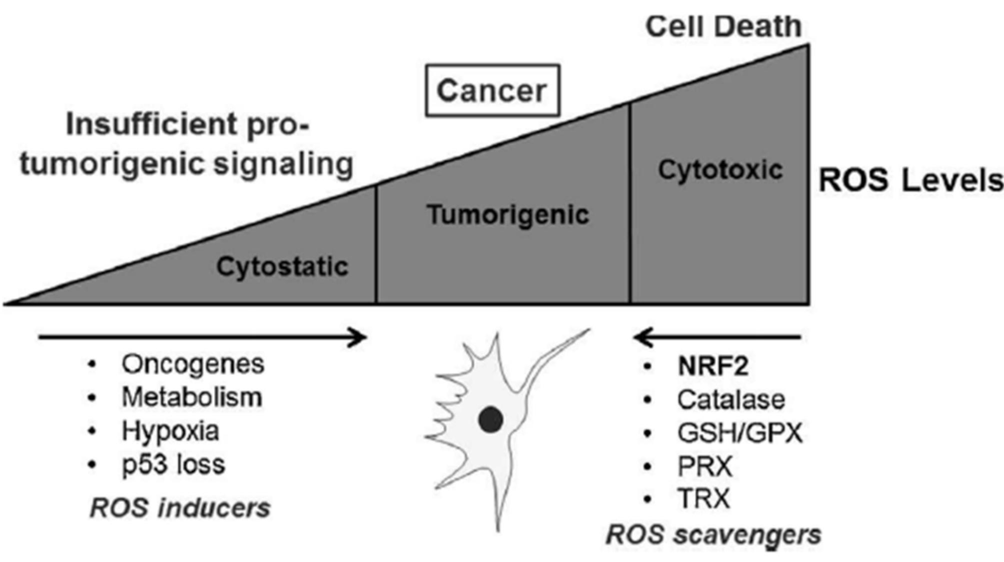


Figure 26. **Association between oxidative stress and cancer**.
(Glasauer & Chandel 2014).

2.4.3. Gastrointestinal Mucosa Diseases

The cells lining the gastrointestinal tract are prone to RONS attack as they are exposed to numerous detrimental dietary factors, including pro-oxidants, carcinogens, oxidation products in food-derived lipids and proteins, etc. In addition, gut mucosal immune cells and resident intestinal microbiota are also potential sources of RONS. Main enzymatic systems that generate RONS in the gastrointestinal tract are NOXs, xantine oxidases, iNOS and COXs.

Excessive levels of RONS damage cellular proteins, including cytoskeletal proteins, and disrupt gastrointestinal tract barrier, thus increasing gut permeability which contributes to inflammation in a variety of gastrointestinal diseases. Thus, oxidative stress is implicated in the initiation and promotion of gastric and colorectal cancers, as well as such inflammatory gastrointestinal disorders as ulceration, gastritis, and inflammatory bowel illnesses (ulcerative colitis and Crohn's disease) (Bhattacharyya et al. 2014).

2.4.4. Neurodegenerative Disorders

The brain is particularly vulnerable to oxidative damage due to its high rate of oxygen utilisation, the presence of considerable levels of transition metals (Cu, Fe), and its high content of oxidisable polyunsaturated fatty acids. As oxidative stress increases with age, increased RONS levels is considered as an important causative factor in several neurological chronic illnesses (Valko et al. 2007).

- *Alzheimer's Disease*

Alzheimer's disease causes gradual deterioration of memory and other cognitive functions in the elderly, which eventually leads to a complete incapacity and death.

This neurodegenerative pathology is characterised by the formation of senile plaques in brain due to the accumulation of amyloid-beta (Aβ) peptide, which derived from the larger Aβ precursor protein. Both Aβ and its precursor have strong Cu^{++}-reductase activity, generating Cu^{+} and releasing H_2O_2 as a by-product. Cu^{+} is a potent mediator of the highly reactive $^{\cdot}OH$ through the Fenton reaction, thus contributing to the elevated oxidative stress characteristic of the brain of Alzheimer's disease patients. At the same time, RONS may augment Aβ production and aggregation (Zhao & Zhao 2013).

Parkinson's Disease

This progressive neurodegenerative illness involves a selective loss of dopaminergic neurons in the 'substantia nigra' region of the midbrain, which use dopamine as a neurotransmitter to communicate with the nerve fibres in the striatum region. These neurons control voluntary movements and their degeneration is the cause of Parkinson's disease symptoms (resting tremor, muscular rigidity, bradykinesia, and postural imbalance).

Several studies have demonstrated the capacity of oxidative stress to induce nigral cell degeneration. Dopamine metabolism itself results in ROS production. Significant mitochondrial dysfunction, neuroinflammation, depletion in substantia nigra levels of GSH, and increased Fe^{++} levels in midbrain being observed in Parkinsonian patients (Hwang 2013).

2.4.5. Ageing

The ageing process implies a progressive decline in physiological functions of an organism after the reproductive phase of life.

There are two main theories describing this process: damage-accumulation theories and genetic theories. Damage-accumulation theories involves several possible approaches, among which, the "free radical theory" is the most complex (Valko et al. 2007). The concept that free radicals play a critical role in ageing was first proposed by in 1956 by Denham Harman (Harman 1956) and is based on the random deleterious effects provoked by free radicals during aerobic metabolism, resulting in damage to DNA, lipids and proteins, and its accumulation over time. This contribute to changes in the antioxidant response with aging, impaired physiological function, increased incidence of disease, and a reduction in life span (Valls et al. 2005; Kregel & Zhang 2007).

3. Polyphenols: Dietary Antioxidants and Beyond

Over the past 20 years, researchers and food manufacturers have become increasingly interested in plant-derived phenolic compounds, which constitute one of the most numerous and ubiquitous groups of secondary plant metabolites. In plant tissues, polyphenols are generally involved in defence against ultraviolet radiation or aggression by pathogens. Despite humans and animals cannot synthesize these molecules, phenolic compounds contained in edible plants are an integral part of their diets, and are known to exert several biological activities once ingested (Manach et al. 2004).

Most of the several hundreds of phenolic compounds present in edible plants display common biosynthetic pathways. Simple phenolic acids derive from the 'polyketide' acetate/malonate pathway (Cheynier et al. 2013), while the pentose phosphate/shikimate/ phenylpropanoid pathways give rise to cinnamic acids, which are considered as the precursors of the majority of non-volatile phenolic compounds (Heleno et al. 2015). Numerous enzymatic and non-enzymatic reactions (hydroxylation, conjugation, condensation, hydrolysis, etc.) are then responsible for the great diversity of phenolics characteristic of each plant, which are even differently distributed within the same species (Bravo 1998; Andersen & Markham 2005). Conjugation plays an important role in such diversity as, in nature, sugar residues are usually linked to hydroxyl groups or, less frequently, to an aromatic carbon atom of the phenolic compounds. The associated sugars can be present as monosaccharides, disaccharides, or oligosaccharides, with glucose being the most common sugar residue, although galactose, rhamnose, xylose and arabinose can also be found. Associations with glucuronic, galacturonic, carboxylic and organic acids, amines and lipids, as well as linkages with other phenols, are rather typical as well (Heim et al. 2002; Manach et al. 2004).

3.1. Main Phenolic Classes in Wine Pomace

In wine pomace, phenolic compounds derive from those molecules present in grapes and/or wine (Fontana et al. 2013; Yu & Ahmedna 2013). The common compositional difference is that grape pomace is richer in non-extractable phenolic compounds (many of which are polymeric structures that remain attached to the solid matrices after pressing), whereas grapes and, especially, wines contain much higher amounts of soluble and volatile phenolics.

In addition, compared with raw grapes, the chemical phenolic composition of wine pomace can be considerably more complex due to the formation of new compounds during the fermentative stage of winemaking.

On the other hand, several reactions take place during wine maturation and aging (condensation, co-pigmentation, decarboxylation, dehydration and oxidation reactions, among others), leading to the formation and/or degradation of several polyphenols. This is a determining factor for the organoleptic properties of wines (Monagas et al. 2005; Santos-Buelga & de Freitas 2009; Garrido & Borges 2013) and is also responsible for many of the phenolic composition differences between wine and wine pomace (Anastasiadi et al. 2010; Teixeira et al. 2014).

Furthermore, the qualitative and quantitative distribution of phenolic compounds in wine pomace shows significant differences depending on several factors, such as: varietal of the grapes, geographic location of the crops, soil, climatic conditions, seasonal influences, maturity, viticultural techniques, winemaking procedures, etc. (Garrido & Borges 2013; Teixeira et al. 2013; Ribeiro et al. 2015). Hence, marked divergences in the chemical structures and concentrations reported by different authors are frequently found. In this regard, comments about the specific phenolic composition found in *Vitis Vinifera* L. red grapes and wine have been indicated, which is one of the main species used to produce wine.

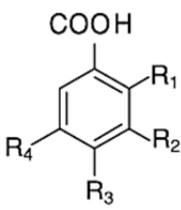
In general, the main and most studied phenolic compounds in wine pomace, as well as in grapes and wines, can be classified into four groups:

3.1.1. Phenolic Acids

Phenolic acids represent an important fraction of grape derived phenolics. They exist predominantly as hydroxybenzoic and hydroxycinnamic acids, either in their free or conjugated forms.

- **Hydroxybenzoic Acids**

These phenolic compounds derive from benzoic acid, being thus characterised by a C_6-C_1 skeleton. The most abundant hydroxybenzoic acids in grapes, wine and wine pomace are gallic, syringic, salicylic, *p*-hydroxybenzoic, protocatechuic, gentisic and vallinic acids (Garrido & Borges 2013; Ribeiro et al. 2015) (**Figure 27**).



Hydroxybenzoic acids	R_1	R_2	R_3	R_4
p-OH-benzoic acid	H	H	OH	H
Salicylic acid	OH	H	H	H
Protocatechuic acid	H	OH	OH	H
Gentisic acid	OH	H	H	OH
Gallic acid	H	OH	OH	OH
Vallinic acid	H	OCH_3	OH	H
Syringic acid	H	OCH_3	OH	OCH_3

Figure 27. Structures of hydroxybenzoic acids.

These compounds are mainly found in their free form or covalently linked to polysaccharides in the plant cell wall (Monagas et al. 2005; Acosta-Estrada et al. 2014), and are synthesised in both grape skins and seeds (Teixeira et al. 2014). In wines, ethyl esters of these phenolic acids have been also identified (Moreno-Arribas & Polo 2009; Garrido & Borges 2013).

The importance of gallic acid should be marked since this phenolic acid is the precursor of many hydrolysable tannins and can also be part of some flavan-3-ols and condensed tannins (Garrido & Borges 2013).

- **Hydroxycinnamic Acids**

Hydroxycinnamic acids present a C_6-C_3 skeleton and formally belong to the group of phenylpropanoids. Among these phenolic acids, *p*-coumaric, caffeic, ferulic and sinapic acids are some of the most representative compounds in grapes, wine and wine pomace (Garrido & Borges 2013; Teixeira et al. 2013; Ribeiro et al. 2015) (**Figure 28**).

Hydroxycinnamic acids	R_1	R_2	R_3
Caffeic acid	OH	H	H
Caftaric acid	OH	H	Tartaric acid
p-Coumaric acid	H	H	H
p-Coutaric acid	H	H	Tartaric acid
Ferulic acid	OCH_3	H	H
Fertaric acid	OCH_3	H	Tartaric acid

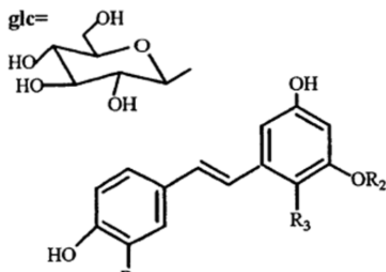
Figure 28. **Structures of hydroxycinnamic acids**.

Hydroxycinnamic acids may display *cis* or *trans* isomeric forms, which are convertible, but the latter is more stable and thereby more prevalent in nature. In grapes, these phenolics usually appear as tartaric esters or diesters, which can be present at higher concentrations than the non-esterified forms (Buiarelli et al., 2010). *trans*-Caftaric and *trans*-fertaric acids are mainly localized in the pulp, and during the grape pressing, they are quickly released into the wine must. In contrast, the *trans* and *cis* isomers of *p*-coutaric acid are mostly localized in the grape skin so they are less extractable. Nevertheless, during the wine fermentation process, the partial hydrolysis of these esters yields free hydroxycinnamic acids and ethyl ester derivatives (Garrido & Borges 2013), which are then also found in wine pomace (Alonso et al. 2002; Kammerer et al. 2004).

Additionally, hydroxycinnamates are important constituents of acylated anthocyanins (Moreno-Arribas & Polo 2009).

3.1.2. Stilbenes

Stilbenes are phenolic compounds comprising two aromatic rings linked by an ethene bridge (C_6–C_2–C_6). Resveratrol (3,5,4'-trihydroxystilbene) is undoubtedly the stilbene most referenced as present in grapes and wine, and important biological activities have been attributed to this and related molecules, mainly regarding cardiovascular beneficial effects (Kalantari & Das 2010). This stilbene is principally contained in grape skins (Teixeira et al. 2014), chiefly in its *trans*-isoform, and also as its *O*-glucoside derivative, which is known as piceid (**Figure 29**).



Stilbenes	R_1	R_2	R_3
Trans-resveratrol	OH	H	H
Trans-resveratrol-3-*O*-glucoside (piceid)	OCH_3	H	H

Figure 29. **Structures of stilbenes**.

Stilbenes are synthesised in response to a number of physiological stressing factors (fungal infections, ozone, UV-C radiation, etc.) which might increase the concentration of these phenolics in grapes up to several hundred-folds. In fact, grapes and wine are considered the most important dietary source of stilbenes. However, as considerable quantities of these molecules are transferred from grape to wine, their concentrations in wine pomace, despite remain significant, are much lower. Thus, the contribution of resveratrol and other stilbenes to the potential beneficial effects of wine pomace might be limited (Teixeira et al. 2014).

3.1.3. Flavonoids

Flavonoids constitute the most important single group of phenolic compounds, with more than 5,000 structures described. They can be further subdivided into 13 classes (Bravo 1998), although more recent classifications usually establish only 7 main classes: flavan-3-ols, flavonols, flavanonols, flavones, flavanones, anthocyanidins, and isoflavones (Heim et al. 2002; Kumar & Pandey 2013). Flavonoids display a basic structure of 15 carbon atoms comprising two aromatic rings (A and B rings) connected by a bridge of 3 carbons (C_6–C_3–C_6) that is part of a third pyran ring (C ring) for the most common classes (**Figure 30**). Then, the various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Kumar & Pandey 2013).

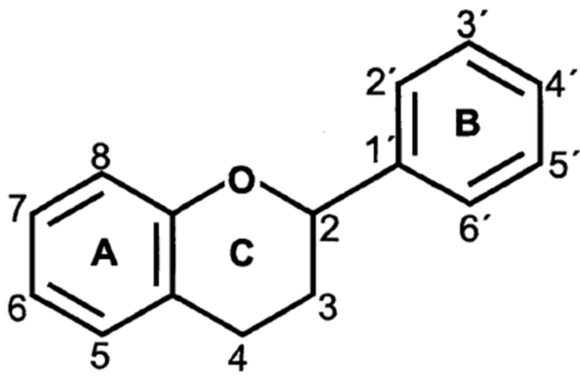


Figure 30. **Basic structure and numbering system of flavonoids**.

In nature, flavonoids can occur either in the free or conjugated form, being often esterified to one or two sugar molecules through at least one hydroxyl group (*O*-glycoside). Another type of glycosides has been described, in which the connection to the sugar molecule occurs directly in the flavonoid skeleton through a C-C bond (*C*-glycosides). These derivatives are more resistant to hydrolysis than their *O*-analogs. In addition, several condensation reactions may take place between flavonoid monomers, giving rise to dimers and polymers, which further explains the chemical diversity of flavonoids (Andersen & Markham 2005; Garrido & Borges 2013).

The main flavonoids present in wine pomace belong to three classes:

- **Flavanols (flavan-3-ols)**

Flavanols, which are usually referred as flavan-3-ols, are benzopyranes that have a saturated carbon chain between C2 and C3, a hydroxyl function in C3, and no carbonyl group in C4. The C2 and C3 positions of these molecules are two asymmetry centres.

Flanan-3-ols	R_1	R_2	R_3
(+)-Catechin	H	H	OH
(−)-Epicatechin	H	OH	H
(−)-Epicatechin 3-gallate	H	Gallic acid	H
(+)-gallocatechin	OH	H	OH
(−)-Epigallocatechin	OH	OH	H
(+)-Gallocatechin 3-gallate	OH	Gallic acid	H
(−)-Epigallocatechin 3-gallate	OH	OH	Gallic acid

Figure 31. **Structures of flavan-3-ols**.

In grapes, wine and wine pomace, (+)-catechin and its isomer (−)-epicatechin are the most abundant flavan-3-ols, which are orthohydroxylated in C3′ and C4′ positions of the B ring. In addition, (+)-gallocatechin and (−)-epigallocatechin, which have a third hydroxyl group in C5′ position, have been also identified. All these molecules can be further esterified by gallic acid at C-3 position, being (−)-epicatechin-3-*O*-gallate, (+)-gallocatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate the main gallates in grape-derived products (Monagas et al. 2005; Monagas et al. 2003; Scola et al. 2010; Chamorro et al. 2012) (**Figure 31**).

Grape flavan-3-ols are detectable in the highest concentration in the seeds, but they are also present at considerable quantities in vacuoles of the hypodermal skin cells (Teixeira et al. 2014). They can be found as monomers, and as oligomers and polymers which are also referred to as condensed tannins.

- **Flavonols**

Flavonols are yellow pigments characterised by the presence of a double bond between atoms C2 and C3, and a hydroxyl group in C3, with 90% of the flavonols being hydroxylated in C3, C5 and C7, and further hydroxylated in their B ring. Concretely, in *V. Vinifera* L. red grapes and wines, the 4′-hydroxy, the 3′,4′-dihydroxy, and the 3′,4′,5′- trihydroxy flavonols (known as kaempferol, quercetin, and myricetin, respectively) are the most important flavonols (Mattivi et al. 2006; Garrido & Borges 2013) (**Figure 32**).

Flavonols	R_1	R_2	R_3
Kaempferol	H	H	H
Kaempferol-3-*O*-glycoside	H	H	Glycoside
Quercetin	OH	H	H
Quercetin-3-*O*-glycoside	OH	H	Glycoside
Myricetin	OH	OH	H
Myricetin-3-*O*-glycoside	OH	OH	Glycoside
Isorhamnetin	OCH_3	H	H
Isorhamnetin-3-*O*-glycoside	OCH_3	H	Glycoside

Figure 32. **Structures of flavonols**.

Flavonols in grapes exist only as 3-*O*-glycosides (mainly located in the vacuoles of the skin tissues, and bound to the cell wall of skins and seeds), whereas the corresponding free aglycons can be found in wines and wine pomace, together with the 3-*O*-glycosides, as a result of acidic hydrolysis that occurs during winemaking and aging (Castillo-Muñoz et al. 2007; Teixeira et al. 2014). Different sugars can be bound to the flavonols, producing several monoglycosides (mainly glucosides, glucuronides, and rhamnosides). Diglycosides can also be found, such as quercetin-3-*O*-rutinoside (commonly known as rutin) which contains the disaccharide rutinose (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose).

- **Anthocyanidins**

Anthocyanidins have a structure based on the flavylium cation (2-phenylbenzopyrylium). This is a chromophore of eight conjugate double bonds carrying a positive charge on the heterocyclic oxygen ring. In nature, these pigments are almost always present as anthocyanidin glycosides (anthocyanins), whereas the corresponding aglycons (anthocyanidins) are obtained by hydrolysis (**Figure 33**).

Anthocyanidins and Anthocyanins	R_1	R_2	R_3
Cyanidin	OH	H	H
Cyanidin-3-*O*-glycoside	OH	H	Glycoside
Delphinidin	OH	OH	H
Delphinidin-3-*O*-glycoside	OH	OH	Glycoside
Peonidin	OCH_3	H	H
Peonidin-3-*O*-glycoside	OCH_3	H	Glycoside
Petunidin	OCH_3	OH	H
Petunidin-3-*O*-glycoside	OCH_3	OH	Glycoside
Malvidin	OCH_3	OCH_3	H
Malvidin-3-*O*-glycoside	OCH_3	OCH_3	Glycoside

Figure 33. **Structures of anthocyanidins and anthocyanins**.

Anthocyanins are essentially located in grape skins, concretely, in cell layers below the epidermis where they are present as soluble phenolics but confined into vacuoles or other cytoplasmic vesicles (Teixeira et al. 2014). These molecules are largely responsible for the colour of grapes and wine. This characteristic is determined by their chemical structure, namely their degree of hydroxylation, methylation and/or glycosylation. In red grapes and wine, five main anthocyanidins have been identified: cyanidin (orange red), peonidin (red), malvidin, petunidin and delphinidin (bluish red) (He et al. 2010). However, their actual colour is dependent on the pH, as anthocyanins occur in solution as a mixture of different secondary structures that coexist in equilibrium: the red flavylium cation, the colourless carbinol pseudo-base, the violet quinoidal base, and the yellow chalcone. Such equilibrium is responsible for the intense red-orange to blue-violet colour produced by anthocyanins under acidic conditions (Bueno et al. 2012).

In *V. vinifera* L. and other grape varieties, malvidin is the most representative anthocyanidin due to the strong 3'- and 5'-*O*-methyltransferase activities observed in the majority of grapes (Mattivi et al. 2006). However, the 3-*O*-monoglucosides of the five anthocyanidins previously referred have been identified in grapes, as well as some 3,5- and 3,7-diglycosides and 3,5,7-diglycosides derivatives (He et al. 2010).

In addition, each anthocyanidin can be also acylated by aromatic or aliphatic acids, yielding numerous different anthocyanins (Bueno et al. 2012). Acylated anthocyanins can account for more than 60% of the total anthocyanin content in some *V. vinifera* L. cultivars, with acylation taking place in the C6'' position of the glucosyl groups (He et al. 2010).

3.1.4. Tannins

Tannins have been defined as 'polyphenolic compounds of high molecular weight having the ability to complex strongly with carbohydrates and proteins' (Serrano et al. 2009).

Tannins are usually divided into two classes:

- **Hydrolysable Tannins**

Hydrolysable tannins are complex polyphenols that can be degraded into smaller fragments, mainly sugars and phenolic acids, through pH changes and enzymatic or non-enzymatic hydrolysis. The basic units of hydrolysable tannins of polyester type are gallic acid, hexahydroxydiphenic acid, and their derivatives (mainly ellagic acid), which are usually esterified with D-glucose yielding compounds of high molecular weight. Hydrolysable tannins that hydrolyse in gallic acid are termed gallotannins (**Figure 34**, A), and those that hydrolyse in ellagic acid are called ellagitannins (**Figure 34**, B) (Craft et al. 2012).

Hydrolysable tannins have been found naturally only in some grape varieties. In *V. vinifera* L. grapes, galloyl-glucose and digalloyl-glucose have been reported, but not larger hydrolysable tannins. In contrast, significant quantities of hydrolysable tannins are present in red wines because these compounds are indeed derived from the oak (or different woods) barrels where wine is aged (Narduzzi et al. 2015).

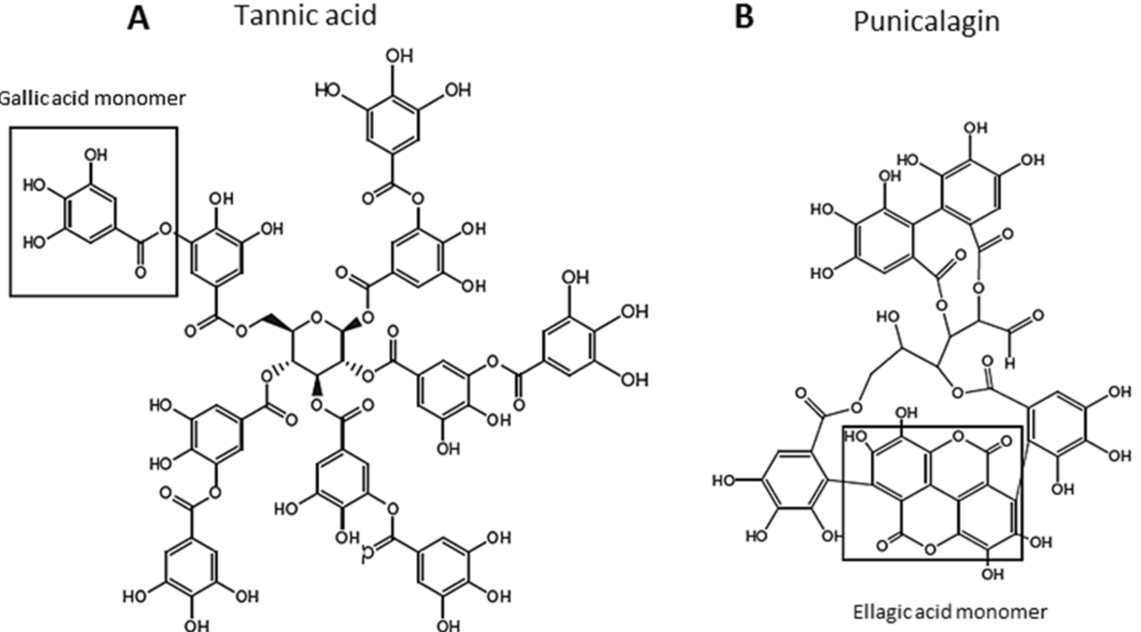


Figure 34. **Structures of hydrolysable tannins: gallotannins (A) and ellagitannins (B)**.

- **Condensed Tannins (proanthocyanidins)**

Condensed tannins are polymeric compounds conventionally known as proanthocyanidins because they release anthocyanidins when heated under acidic conditions.

These compounds are flavan-3-ol oligomers or polymers where monomers are typically linked by C4-C8 and/or C4-C6 bonds (B-type proanthocyanidins) (**Figure 35**, A). In some occasions, monomers are linked by an additional C2-C7 and/or C2-C5 ether bonds (A-type proanthocyanidins) (**Figure 35**, B), although B-type ones are much more prevalent (Craft et al. 2012). The degree of polymerization varies over a broad range from dimers up to compounds with more than 100 monomeric units (Serrano et al. 2009). The term 'oligomer' usually corresponds to proanthocyanidins presenting a mean degree of polymerization (mDP) between 2 and 5 units, and 'polymer' denotes those molecules with a mDP > 5 units (Monagas et al. 2005). It is also worth noting that oligomers are frequently named as procyanidins, with dimers referred as B- or A-series, and trimmers as C-series.

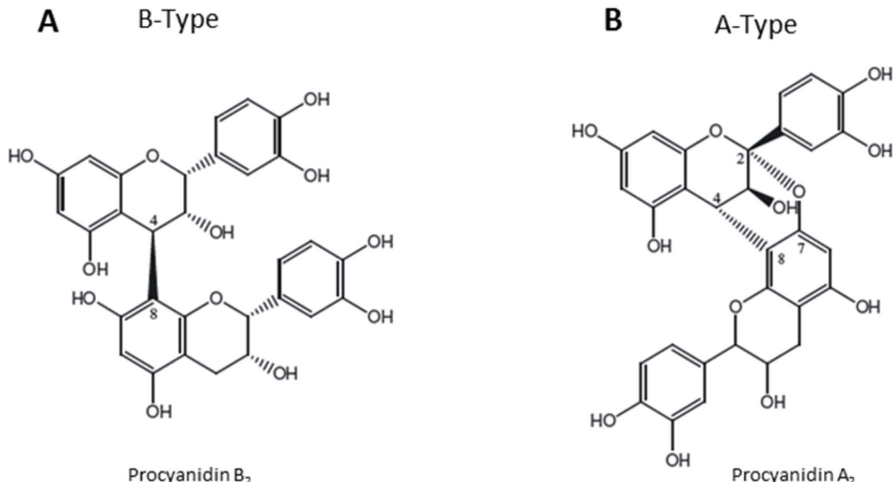


Figure 35. **Structures of condensed tannins: B-type procyanidin (A) and A type procyanidin (B)**.

Proanthocyanidins are found in considerable amounts in the solid components of grapes, mainly in seeds, but there are also substantial quantities in skins, and even in the pulp. Proanthocyanidin concentration, structure and degree of polymerization depend on such particular localization in the grape tissues. In *V. vinifera* L. grapes, grape seed tannins are mainly B-type polymers of (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-*O*-gallate monomers, whereas grape skin tannins also contain (−)-epigallocatechin and trace amounts of (+)-gallocatechin and (−)-epigallocatechin-3-*O*-gallate. Thus, proanthocyanidins in grape seeds are mainly procyanidins, whereas both procyanidins and prodelphinidins are present in grape skins (Monagas et al. 2005; He et al. 2008; Garrido & Borges 2013). Some of these compounds are transferred into the must during wine-making operations, but many of them remain attached to wine pomace (Khanal et al. 2010; Ky et al. 2014).

The great importance of tannins in grapes, wines and wine pomace is partly due to their chief contribution to sensory properties such as the astringency and bitterness. The intensity of these sensations is determined by the structural characteristics of tannins, as their ability to interact with salivary proteins (with the subsequent possible formation of insoluble precipitates) seems to be the mechanism responsible for such effects. In this regard, seed tannins show larger proportions of galloylated units than skin tannins, whereas the average molecular weight is higher in tannins of the skins than seeds. Due to these specific features, seeds are perceived as bitterer than skins, while both seeds and skins appear to be equal in astringency (Brossaud et al. 2001; Ma et al. 2014).

3.2. Antioxidant Mechanisms of Actions of Polyphenols

The interest in dietary phenolics has greatly increased in the last two decades. While polyphenols were initially considered antinutrient molecules because of the adverse effect of condensed tannins in protein digestibility, more recently, the antioxidant effects of these bioactive compounds and, subsequently, their possible food technological applications and human health beneficial consequences have received prominent attention (Scalbert et al. 2005; Pandey & Rizvi 2009).

In food systems, polyphenols are regarded as natural antioxidant agents that present appealing properties, since they can act as free-radical scavengers, electron or hydrogen donors and strong chelators of transition metals, hence inhibiting and/or delaying lipid peroxidation and other oxidative processes that may reduce the shelf-life of food items (Miller & Ruiz-Larrea 2009; Rivero-Pérez et al. 2007; Bonilla et al. 2011).

Once ingested, polyphenol protective effects were originally thought to result from their direct antioxidant properties, which were able to decrease the levels of reactive oxygen and nitrogen species (RONS) present/generated in the body, and thereby prevent against lipid peroxidation and oxidative damage to other biomolecules such as proteins and DNA. However, there is now emerging evidence that dietary phenolic metabolites further exert modulatory effects in cells through selective actions on different components of the intracellular signalling cascades. These effects are vital for cellular functions such as growth, proliferation and apoptosis. The intracellular concentrations required to affect cell signalling pathways are considerably lower than those required to impact on antioxidant capacity (Crozier et al. 2009).

A better knowledge about the nature and biological consequences of the interactions of phenolic compounds with cell components will certainly contribute to develop nutritional and pharmacological strategies intended to prevent the onset and/or the consequences of human disease. Studies that carry out structure activity relationship (SAR) analyses are thus interesting to determine the chemical structure and molecule physical properties leading to a desire activity or property (Rice-Evans et al. 1996; Shukor et al. 2013).

The number of mechanisms underlying the potential antioxidant effects of phenolic compounds in the body can be divided in:

- **Direct Antioxidant Effects**
    - Reaction with RONS: These reactions include scavenging of free radicals, chain-breaking of lipid peroxidation, etc., yielding a less toxic/reactive product, thus protecting labile biomolecules from oxidation (López-Alarcón & Denicola 2013). The main chemical features responsible for the efficiency of polyphenols as direct antioxidants are: their phenolic hydroxyl groups, which are able to reduce free

radicals through one-electron donation; and, their aromatic structure, which allows the stabilization by resonance of the resultant aroxyl radicals (the stability of this aroxyl radical will determine the potency of the parent polyphenol as direct antioxidant) (Fraga et al. 2010).

- Chelation of transition metals: Numerous phenolic compounds are able to sequester Fe^{++}, Cu^{+}, and other transition metals. This capacity depends on their chemical structure, with catechol moieties and combinations of hydroxyl and carbonyl groups being centres of high affinity for metal ions (Hider et al. 2001). This is a very important antioxidant mechanism of action by preventing the formation of free radicals through the Fenton and Haber-Weiss reactions.

The potential action of phenolic compounds as free radical scavengers and metal chelators may be important in the gastrointestinal tract and, perhaps, the blood, as they are exposed to considerable polyphenol concentrations, but is unlikely to be physiologically relevant in most other organs (Fraga et al. 2010).

Several chemical-based antioxidant assays have been developed to assess, in a simple experimental way, the direct antioxidant capacity of antioxidants *in vitro*. These methods can be applied both in food and biological samples, and have been extensively reviewed by several authors, indicating their mechanisms of actions (electron transfer and/or hydrogen atom transfer), advantages and shortcomings, and many other practical features (Sanchez-Moreno 2002; Prior et al. 2005; Rivero-Pérez et al. 2007; Ndhlala et al. 2010; López-Alarcón & Denicola 2013). Most of them consist on UV-visible or fluorescence spectroscopy methods, and are based on diverse strategies aimed to evaluate:

- *The consumption of stable free radicals by antioxidants*: this is the case of the 2,2’-azinobis-(3-ethylbenzothiazole-6-sulphonate) (ABTS) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.
- *The capacity of antioxidants to reduce ferric or cupric ions*: the ferric reducing antioxidant power (FRAP) assay, which is also referred to as the ‘ferric reducing ability of plasma’ if applied in plasma samples; and, the cupric ion-reducing antioxidant capacity (CUPRAC) method, are the main representatives of this type of assays.
- *The ability of antioxidants to protect a target molecule exposed to a free radical source*: the ‘competitive method’ most widely used is the oxygen radical absorbance capacity (ORAC) assay, followed by the total radical-trapping antioxidant parameter (TRAP) assay.

- *The capacity of antioxidants to inhibit the oxidation of LDLs*: the main advantage of this approach is that makes used of a biological relevant target easily obtainable from plasma samples.
- *The scavenging activity toward RONS formed in vivo*: these methodologies are known as 'free radical scavenger capacity' (FRSC) methods, and include: superoxide radical scavenger capacity (SRSC) assay; hydroxyl radical scavenger capacity (HRSC) assay; lipid peroxyl scavenger capacity (LRSC) assay; and other methods to evaluate $ONOO^-$ and hypochlorite ion (ClO^-), although the latters are less frequently used.

Traditionally, these antioxidant assays have been used to measure standard compounds and plant/food extracts (classical approach). Recently, a new methodological approach to measure antioxidant capacities in powdered samples has been developed (Serpen et al., 2007). This approach has been named with the acronym QUENCHER, which stands for QUick, Easy, New, CHEap and Reproducible (Gökmen et al. 2009).

Going beyond the *in vitro* chemical-based assays for screening the potential antioxidant capacity of phenolic compounds, and any other antioxidant, is more laborious and costly. Even though, proceeding to studies *ex vivo* (cellular assays) and *in vivo* (animal model or human clinical trials) is essential to investigate some aspects related to the bioavailability, metabolism, mechanisms of actions, and real effectiveness of the antioxidants.

Bearing in mind that *in vivo* studies are more expensive and time-consuming, *ex vivo* assays are considered attractive intermediate testing methods to evaluate the potential bioactivity of antioxidants (López-Alarcón & Denicola 2013). In this regard, detection and quantification of RONS generated by cells can be evaluated in cell cultures using several fluorescent probes (Kalyanaraman et al. 2012). The most widely applied analytical approach for detecting intracellular oxidative stress is the use of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable molecule that is hydrolysed intracellularly to the DCFH carboxylate anion, which is retained within the cell. Oxidation of this anion results in the formation of 2',7'-dichlorofluorescein (DCF), which can be monitored by several fluorescence-based techniques, such as fluorescence spectroscopy, confocal microscopy, flow cytometry, etc. The so-called 'DCF assay' can be then used as an indirect index for evaluating both the potency of pro-oxidants and the efficacy of antioxidants against RONS in cells (Wang & Joseph 1999). However, this method does not indicate the mechanisms used by the antioxidant compounds to reduce oxidative stress, and both direct and indirect effects may be implicated.

- **Indirect Antioxidant Effects:**

  - Inhibition of oxidant enzymes: several oxidant enzymes may be inhibited by phenolic compounds, such as NOXs (Steffen et al. 2008) and ACE (Balasuriya & Rupasinghe 2011). This is translated in a decrease in RONS production.

  - Activation of endogenous antioxidant defences: SODs, CAT, and GSH-related enzymes, such as GR, GPXs and GSTs, may be activated by some phenolic compounds (Valls-Bellés et al. 2004; Han et al. 2007).

  - Interaction with redox signalling pathways: Phenolic compounds can influence several redox transduction cascades that may be finally translated in a cellular antioxidant response, partly mediated through Nrf2 activation and NF-κB inhibition (Masella et al. 2005; Han et al. 2007).

  - Increased production/regeneration of non-enzymatic antioxidant defences: For instance, phenolic compounds are known to exert indirect antioxidant protection by modulating GSH contents, both by preventing GSH oxidation and by interacting with GSH-related enzymes implicated in GSH synthesis and metabolism (Valls-Bellés et al. 2002; Masella et al. 2005). Certain polyphenols can also interact with vitamins E and C for their regeneration (Scalbert et al. 2005; Pazos et al. 2009).

The effects of antioxidants on the activity of specific enzymes (such as ACE, NOXs, SODs, CAT, GPXs, and GSTs) can be estimated using either isolated enzymes purchased for this aim (*in vitro* methods), or biological samples obtained from *ex vivo* and *in vivo* studies, which is a more realistic approach. Most of these methods are based on changes in the absorbance/fluorescence of samples due to the consumption of a substrate or the generation of a product as a consequence of the enzyme activity (Alam & Cook 2003).

In addition, western blotting techniques can be applied to determine post-translational protein modifications modulating the activity of enzymes. For example, in numerous enzymes, their activity is highly dependent on the phosphorylation pattern of particular residues, which can be evaluated using specific antibodies against the phosphorylated forms and compare its levels in biological samples with the total levels of that enzyme (Niki 2010; Schini-Kerth et al. 2011; Ho et al. 2013). Similarly, immunochemical techniques (mainly western blotting and ELISA) are useful for monitoring the effects of phenolics in redox signalling pathways at a protein level, affecting several intermediate and final effectors of transduction cascades.

The interactions of phenolic compounds with signalling pathways can be also evaluated at the level of gene expression, by determining, for instance, changes in the transcription of redox-regulated genes. One of the most widely applied methods for this aim is the quantitative real time polymerase chain reaction (qPCR), which allows determining the mRNA levels within cells. Gene arrays are also available for the simultaneous analysis of multiple genes, which allows much higher performance, although these techniques are still considerably expensive (Lee et al. 2012).

As regards changes in the levels of non-enzymatic defences, such as GSH, its concentration or the GSH/GSSG ratio are usually evaluated in biological samples to determine beneficial antioxidant effects by modulating the redox microenvironment (Moskaug et al. 2005). There are numerous protocols and kits to measure GSH and GSSG contents (Monostori et al. 2009), including spectrophotometric, spectrofluorometric, chemiluminescence, and chromatographic techniques.

In addition, oxidative stress biomarkers are commonly used as an indirect but reliable estimation of the protective effects in biological systems following antioxidant treatments/interventions in cell cultures, animal models and humans.

A schematic representation of the several antioxidant mechanisms of action of phenolic compounds described above can be seen in **Figure 36**.

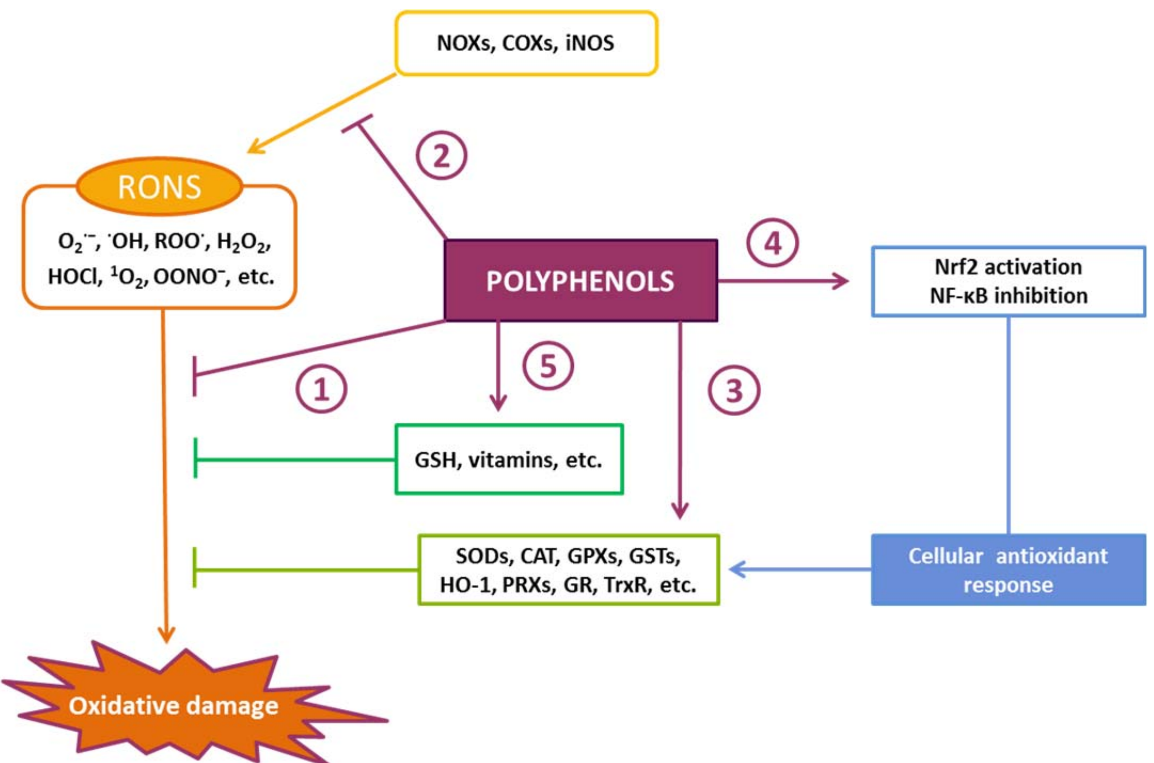


Figure 36. **Mechanisms of action of polyphenols.**
1) Direct antioxidant effects; 2) Inhibition RONS-generating enzymes;
3) Activation endogenous antioxidant enzymes; 4) Modulation redox-sensitive signalling pathways;
5) Increased production/regeneration non-enzymatic antioxidants.

3.3. Effects of Processing on Wine Pomace Polyphenol Contents and Antioxidant Activity

Drying, grinding, and heat treatment are processing techniques usually applied during management of fruit and vegetable residues for their value-adding. In order to hypothesise potential food applications and health benefits of wine pomace, understanding how these techniques and other type of food processing might alter its polyphenol concentration and antioxidant capacity is of great importance.

- **Drying Effects**

Drying is used to reduce the moisture content and, concurrently, the water activity of wine pomace in order to enhance shelf-life and prevent microbiological contamination. Several drying methods can be applied, being hot air circulating ovens the most widely used. The effect of drying temperature (60, 100, and 140 °C) on the polyphenol contents and antioxidant activity of red wine pomace peels was studied by Larrauri et al. (1997). Drying at 60°C up to a maximum moisture content of 8% did not significantly affect wine pomace characteristics, whereas significant reductions in both the phenolic concentrations and antioxidant capacity of the samples were observed at higher drying temperatures.

- **Grinding Effects**

Grinding of dried wine pomace may also result in polyphenol losses by facilitating contact between compartments rich in polyphenol oxidase, such as the cytosol, and those rich in phenolic compounds, such as vacuoles, resulting in the formation of polymeric browning products, as previously observed in plant-derived powders (Manach et al. 2004; Bohn 2014). On the other hand, the reduction of particle size will increase the total surface area, favouring the extractability of polyphenols and the enzymatic degradation of powdered matrices once ingested, which can increase their bioavailaibility and bioefficacy in the body (Moure et al. 2001; Bohn 2014). In addition, particle size determines the application of plant-derived powders as food ingredients, influencing their handling, rheological characteristics, palatability, likelihood of microbiological contamination, and other food technological properties (Oreopoulou & Tzia 2007).

- **Heat Treatment Effects**

From a general point of view, heat treatment of a plant matrix containing polyphenols may be a two-edged sword with respect to influencing wine pomace phenolic contents and bioactivity. On one hand, heating increases the risk of degradation and oxidation of phenolic compounds, with the majority of studies observing losses in phenolic concentrations and antioxidant properties due to the exposure of food matrices to high temperatures. On the other hand, contents of some compounds may increase, normally aglycons liberated from

the breakdown of more complex glycosides or esters, and new compounds might be formed upon thermal processing. Additionally, heat treatment may soften or disrupt cell walls, facilitating polyphenol release and solubilisation during digestion (Rothwell et al. 2015).

It must be further considered that chemical structural changes suffered by polyphenols during heat treatment may positively or negatively determine the bioactivity of the resultant phenolic molecules (Nicoli et al. 1999), and such structure is also a key factor that controls their rate and extent of intestinal absorption (Gonzales et al. 2015). Thus, if losses can be avoided and/or the high temperatures induce changes in the structure of phenolic molecules that promote their antioxidant capacity and their absorption in the gut, heating applied during food production may result in increased bioactivity and bioavailability of phenolic compounds (D'Archivio et al. 2010; Bohn 2014).

With regard to the effects of heat treatment on wine pomace, some studies can be found in the literature addressing the specific changes on total or individual phenolic contents of this winemaking by-product. Anthocyanins such as malvidin, petunidin, and delphinidin glycosides appear to be the most poorly retained compounds in processed food upon any heat treatment or cooking method. Indeed, a more significant degradation of anthocyanins has been found in solid wine pomace than in the extracts obtained from this winemaking by-product, suggesting a higher tendency for reactions converting monomeric anthocyanins in other molecules upon heat treatment when the solid matrix is present (Sólyom et al. 2014). Contents of flavan-3-ols in wine pomace have been also described to decrease upon heat treatment. In contrast, contents of some simple phenolic acids, such as *p*-coumaric, ferulic, sinapic, gallic, and protocatechuic acids, may increase upon processing, presumably due to their production via the breakdown of more complex polyphenols (Ross, Hoye, et al. 2011; Chamorro et al. 2012; Rothwell et al. 2015). The impact of heat treatment on the antioxidant capacity of wine has been also assessed in the previously mentioned studies, observing gains or losses of activity depending on the conditions applied and the wine pomace materials analysed.

3.4. The Journey of Polyphenols After Intake: Bioaccessibility, Bioavailability and Metabolism of Phenolic Compounds

Once ingested, the absorption of dietary phenolic compounds is a prerequisite for a causal relation between these compounds and their beneficial systemic effects. In addition, the metabolism of absorbed phenolic compounds can substantially modify their biological activity, and their body distribution and excretion are known to determine the relevance of such effects in the different tissues. Only the gastrointestinal tract might be the exception, as non-absorbed phenolics and their colonic metabolites can also exert several beneficial effects *in situ* along the gut (Rein et al. 2013; Velderrain-Rodríguez et al. 2014).

To understand the journey of polyphenols after intake (**Figure 37**), it is first important to differentiate between the terms 'bioavailability' and 'bioaccessibility', as these concepts are not the same despite being often confused.

Bioavailability can be defined as 'the fraction of a nutrient or non-nutrient that is available for the human body for physiological functions and/or storage' (Bohn 2014). In addition, different connotations of this concept can be found in the literature. From a nutritional perspective, bioavailability is the fraction of a given food that can be utilized by the body, so it is a matter of nutritional efficacy. From a pharmacological perspective, bioavailability is the rate and extent to which a bioactive compound or a drug is absorbed and becomes available at the site of action. Anyway, bioavailability addresses several distinct phases, usually separated in: liberation from food matrices, absorption, distribution, metabolism and elimination (LADME), some of which are comprised of further steps and take place at more than one location.

Bioaccessibility refers just to the first phase of bioavailability, being defined as 'the fraction of a compound which is released from the food matrix in the gastrointestinal lumen and thereby made available for intestinal absorption'. The importance of the bioaccessibility phase in the diverse bioavailability observed for dietary compounds from different sources should be stressed. Conversely, once polyphenols have been liberated from the plant/food matrix, those compounds showing similar chemical structures usually follow similar routes (Rein et al. 2013).

The different phases of the whole bioavailability process for dietary phenolic compounds are indicated below:

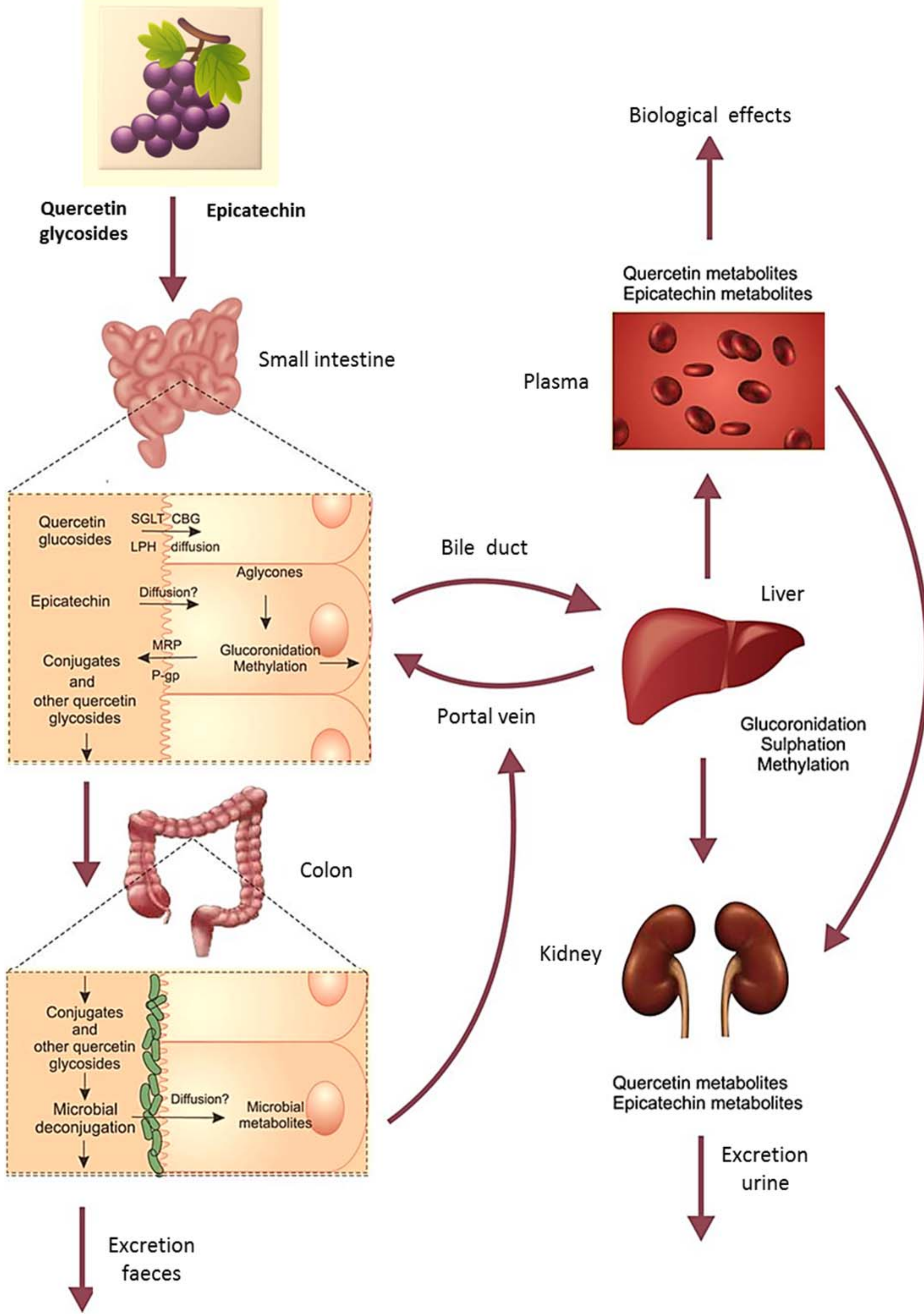


Figure 37. The journey of phenolic compounds after intake.

Quercetin glycosides represent the absorption, distribution, metabolism and excretion of flavonoid glycosides.
Epicatechin represents the absorption distribution, metabolism and excretion of simple flavonoids.
CBG, cytosolic b–glucosidase; LPH, lactase-phlorizin-hydrolase; MRP, multidrug resistance protein;
P-gp, P-glycoprotein; SGLT, sodium-dependent glucose transporter.
Adapted from Bondonno et al. (2015).

- **Liberation of polyphenols from the plant/food matrix and structural changes during digestion**

In wine pomace, some phenolic compounds (aglycons or conjugates) may be present as free forms, being either entrapped in cores of the solid matrices or, usually, accumulated in vacuoles and in the cytoplasm of plant cells not disrupted during the wine-making process. However, the majority of wine pomace polyphenols remain covalently linked to proteins and, mainly, polysaccharides (arabinose, hemicellulose, lignins, and other dietary fibre constituting molecules) in the plant cell wall of solid matrices (Touriño et al. 2011; Saura-Calixto 2011). Thus, for bioaccessibility and later bioavailability of such polyphenols after wine pomace intake, disruption of cell walls and cellular compartments, as well as cleavage of attached compounds from carbohydrates and proteins, are required (Bohn 2014).

Digestion starts in the **oral cavity** where amylase is the predominant enzyme. However, the impact of this step of digestion on polyphenols release is assumed to be low considering the short interaction time, with the exception of starch-rich foods which is not the case of wine pomace (Saura-calixto et al. 2010). Particle size reduction also takes place during mastication, which could allow higher enzyme access during the following digestion stages.

During the **gastric phase**, pepsin digestion in combination with peristaltic movements and low pH (around 2-4) result in a finely ground digesta, named chyme. A significant part of free polyphenols are released in this stage (Lafay & Gil-Izquierdo 2008)(Lafay & Gil-Izquierdo, 2008). In addition, partial depolymerisation of the proanthocyanidins of wine pomace into their constituent units (mainly epicatequin monomers and small oligomers) has been found to be promoted by the gastric milieu (Touriño et al. 2011; Mateos-Martín et al. 2012).

In the **small intestine**, the pH is approximately 7, which activates the enzymes secreted by the pancreas and bile, including phospholipase, sterol esterase, carboxypeptidase, trypsinogen, chymotrypsinogen, amylase, lipase, and bile salts, with the last two especially assisting in the digestion of the more apolar compounds such as lipids and other hydrophobic molecules (Scalbert & Williamson 2000). The movements of intestinal smooth muscles mix the chyme with digestive juices and push food particles against the intestinal mucosa to be absorbed. However, most plant-derived phenolics are present in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. Some of these compounds are hydrolysed by enzymes of the enterocytes and microbiota (10^1-10^3 microbial cells/mL in duodenum, 10^4-10^7 bacterial cells in jejunum and ileum) to cleave the conjugating moieties before they are absorbed. In the brush border of enterocytes, the enzyme lactase-phlorizin hydrolase and esterases such as carboxylesterases have been suggested as the main enzymes responsible for hydrolysis of glycosides and esters, respectively. Part of the highly polymerized proanthocyanidins remaining in the wine pomace matrix may undergo further depolymerisation mediated by the intestinal microbiota during their transit along the small bowel (Touriño et al., 2011; Mateos-Martín et al., 2012).

In the **large intestine**, deconjugation and deglycosilation of phenolic compounds continue as the colonic enterocytes and microbiota also contain esterases, β-glucosidases, and other enzymes that play a key role enhancing the bioaccessibility of those phenolic compounds non-absorbed previously (Manach et al. 2004). Further transformations of phenolic compounds playing a key role in their bioaccessibility and bioavailability take place in the large intestine due to the action of colonic microbiota, which is present at high density in this segment of the gut (10^{11}-10^{12} bacterial cells/mL). Common reactions include deglycosylation, dehydroxylation, demethylation, deconjugation, epimerization, ring cleavage (typical of C-ring, leading to the formation of hydroxylated aromatic compounds from A-ring and phenolic acids from the B-ring), hydrolysis and chain-shortening reactions. As a consequence, native phenolic compounds are degraded to several small phenolic acids, including phenylvaleric, hyppuric, phenylpropionic, phenylacetic and benzoic acid derivatives (Aura 2008).

- **<u>Absorption of aglycons and some conjugated polyphenols along the gastrointestinal tract</u>**

The **stomach** represents the preliminary absorption site for a number of free phenolic acids before the chyme passes to the small bowel (Lafay & Gil-Izquierdo 2008).

However, the **small intestine** constitutes undoubtedly the more important absorption site for most phenolic compounds, as its mucosa and submucosa are highly specialised for the absorptive process, with circular folds, villi and microvilli increasing the absorptive surface area more than 600-fold. Even though, the rate and extent of this process is determined by the chemical structure of the parent phenolic compounds, along with the several structural changes occurring throughout gastrointestinal digestion (Scalbert & Williamson 2000).

Free phenolic compounds (predominantly aglycon forms liberated in the previous phase) are potentially absorbable in the small intestine. These compounds can be taken up into the enterocytes via diverse pathways (Bohn 2014):

- *Passive diffusion*: This is the main pathway for the absorption of low-molecular-weight phenolics and certain flavonoid aglycons.
- *Active transport*: A few glycoside polyphenols are taken up actively via sodium-glucose transport proteins, such as the sodium-glucose-linked transporter 1.
- *Facilitated transport*: some polyphenols with nonpolar side chain or aromatic hydrophobic moieties, such as ferulic or caffeic acids, are absorbed through facilitated transport by monocarboxylic acid transporters.

The degree of polymerization has a significant impact on cellular uptake. Absorption of monomers is much higher than dimers whereas polymeric compounds are assumed to be unavailable, at least in the upper gastrointestinal tract (Manach et al. 2004).

In the **large intestine**, the possible uptake mechanisms of their enterocytes does not greatly differ from those described for their counterparts in the small intestine, but the mucosa of the large bowel is not such specialised in absorption, consisting on a simple columnar epithelium with lots of mucus secreting cells and being considerably less capillarised (Aura 2008).

- **Phase I/II metabolism of phenolic compounds in enterocytes and hepatocytes**

Before passing into the systemic circulation, phenolic compounds are conjugated in the enterocytes, which is the first place for phase I/II metabolism. Once in the blood stream of the portal circulation, phenolic metabolites reach the liver, which is the major site of these modifications (Rein et al. 2013).

Oxidation, reduction and hydrolysis reactions taking place in enterocytes and hepatocytes are included as phase I reactions (Cardona et al. 2013). Hydroxylation and demethylation are examples of the main cytochrome P450 mono-oxygenase-dependent phase I activities involved in the metabolism of polyphenols. However, most of these compounds do not undergo significant degree of phase I transformation, while their hydroxyl groups are readily susceptible to phase II metabolism (Day et al. 2004).

Phase II conjugation mainly includes glucuronidation, methylation and sulphation reactions, which are mediated by uridine-5'-diphosphate glucuronosyltransferase, catechol-*O*-methyltransferase and sulphotransferases, respectively, although further phase II enzymes are present in enterocytes and hepatocytes. These modifications are metabolic detoxication processes common to many xenobiotics that restrict their potential toxic effects and facilitate their biliary and urinary elimination by increasing the hydrophilicity of phenolic compounds. The conjugation mechanisms are highly efficient so, in general, aglycones are barely present or even absent in blood after consumption of nutritional doses of polyphenols (Rechner et al. 2002).

- **Transport in the bloodstream and subsequent tissue redistribution**

Phenolic metabolites that pass to the blood stream are transported either in free form or bound to proteins and lipoproteins. The majority of them appear to be bound to proteins, mainly albumin (Bohn 2014). During circulation around the body, some phenolic compounds are able to penetrate tissues, particularly those in which they are metabolized, but their ability to accumulate within specific target tissues requires further investigations (Manach et al. 2004).

- **Excretion via the kidney or re-excretion into the gut via bile and pancreatic juices**

Phenolic compounds are eliminated chiefly in urine and bile. The more polar metabolites are excreted mostly via the kidney, during which they may suffer further metabolism. The concentration of phenolics recovered in urine varies widely from one to another (Manach et al. 2005). On the other hand, when phenolics return to the duodenum via the bile, they can be subjected to the action of bacterial enzymes such as β-glucuronidase, after which they may be reabsorbed. As such, enterohepatic recycling ensures that the residence time of phenolic metabolites in plasma might be extended compared to that of their parent compounds (Manach et al. 2004).

It must be noted that a major part (around 75–99%) of the parent molecules of polyphenols ingested are not found in urine (Scalbert & Williamson 2000). This implies that they have either not been absorbed through the gut barrier; they have been absorbed and excreted in the bile; or, they have been metabolized by the colonic microbiota or different body tissues. In addition, the underestimation of bioavailability of phenolic compounds must be considered as, despite using high sensitivity techniques, often only a limited number of metabolites are analysed (Bohn 2014).

Several bioavailability studies have shown that the concentration of phenolic compounds and metabolites in human plasma rarely exceed 1 μM, maximum concentrations that are reached 1-2 h after ingestion, except for phenolic compounds that are absorbed only after their partial degradation by the colonic microbiota, which may peak in plasma around 3-6 h post-intake. However, plasma concentration then rapidly decreases for most phenolic compounds, especially for those absorbed in the small intestine, with half-lives of each phenolic varying greatly but usually ranging 2-8 h. Thus, the maintenance of a high concentration of phenolic compounds in plasma requires a repeated ingestion of polyphenols over time (Scalbert & Williamson 2000; Manach et al. 2004; Del Rio et al. 2013).

3.5. Beneficial Health Effects of Grape and Wine Polyphenols

Numerous epidemiological studies have shown an inverse association between the risk of several chronic human diseases related with oxidative stress and the consumption of grape and wine derived polyphenols (Vislocky & Fernandez 2010; Rodrigo et al. 2011). Research areas under active investigation include:

3.5.1. Cardiovascular Diseases

First observations about the positive effects of wine in human health were reported in the 1990s due to the theorised "French paradox" (Renaud & De Lorgeril 1992). It was believed that the high consumption of red wine in France reduced the prevalence of coronary heart disease even though diets contained large amounts of saturated fats. Later studies related this "paradox" with the phenolic composition of red wine, being resveratrol the most widely studied grape/wine derived-polyphenol.

Nowadays, several other compounds in the phenolic acid, flavan-3-ol, flavonol and anthocyanin classes are regarded with great interest due to their cardiovascular protective effects (Vislocky & Fernandez 2010; Andriantsitohaina et al. 2012). These health promoting effects have been attributed, among other bioactivities, to the ability of grape and wine polyphenols to (Georgiev et al. 2014; Rodrigo et al. 2011; Akaberi & Hosseinzadeh 2016):

- Improve endothelial dysfunction by modulating concentrations of vasoactive compounds.
- Decrease vascular RONS generation.
- Ameliorate LDL oxidation.
- Alter blood lipids towards a less atherogenic index.
- Attenuate hyperglycaemia.
- Reduce blood pressure.
- Reduce platelet adhesion and aggregation.
- Modulate vascular inflammatory processes.

3.5.2. Cancer

Beneficial effects of grapes and grape derived products in the several cancers, such as gastric, pancreatic, colorectal, breast, and prostate, have been described (Vislocky & Fernandez 2010; Yang & Xiao 2013; Georgiev et al. 2014). These remarkable anticarcinogenic effects are considered to be due to their unique mixture of phenolic compounds, which have been suggested to inhibit tumour initiation, promotion, and progression. Specific mechanisms by which polyphenols may prevent and/or improve cancer and its complications include (Rodrigo et al. 2011; Zhou & Raffoul 2012)(Rodrigo et al., 2011; Zhou & Raffoul, 2012):

- Preserving normal cell cycle regulation.
- Inhibiting proliferation and inducing apoptosis.
- Inhibiting tumour growth, invasion, and angiogenesis.
- Modulating the activity of transcription factors related with the pathogenesis of cancer, as NF-κB.
- Stimulating phase II detoxification enzyme activity and other effects.
- Decreasing RONS levels by acting as free radical scavengers and chelating agents.

Nonetheless, caution should be taken when using antioxidant protection therapy in cancer patients because its effects depend on the stage at which it is introduced and the concentrations used. Since elevated RONS levels cause cell apoptosis, decreased RONS concentrations due to an excessive antioxidant treatment might actually stimulate survival of damaged cells and neoplastic state proliferation. In this way, antioxidants might rather promote carcinogenesis than interrupt this pathological process (Valko et al. 2006; Glasauer & Chandel 2014) (**Figure 38**). On the other hand, phenolic compounds may induce apoptosis of cancer cells in a dose-dependent manner, resulting in toxic effects at high concentrations (Rodrigo et al., 2011).

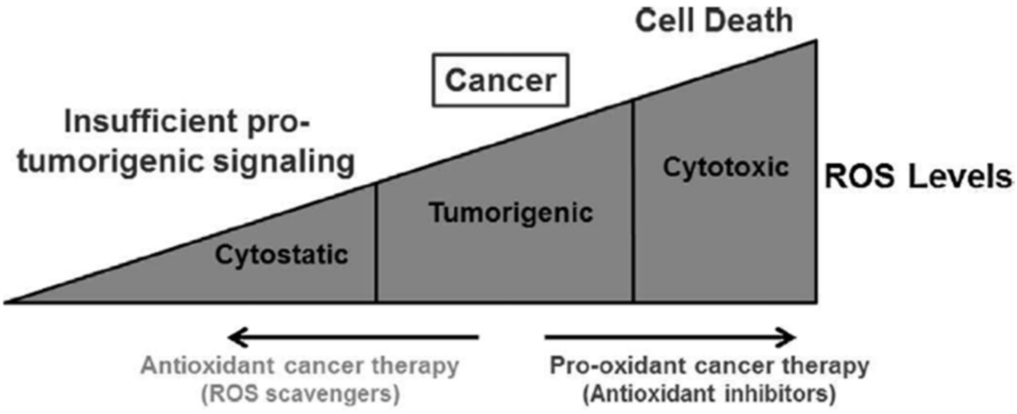


Figure 38. **Antioxidant and pro-oxidant cancer therapies**.

Adapted from Glasauer & Chandel (2014).

3.5.3. Gastrointestinal Mucosa Health

Beneficial effects in the gastrointestinal tract of antioxidant grape pomace dietary fibre rich in polyphenols have been previously described (López-Oliva et al. 2006; López-Oliva et al. 2010). These effects may be mediated through diverse mechanisms exerted by both non-absorbed phenolics that remain in the gut and compounds that can be up-taken by enterocytes. Grape and wine phenolic compounds are known to (Dolara et al. 2005; Selma et al. 2009; Cardona et al. 2013):

- Influence the growing of colonic microbiota exerting prebiotic-like effects. While certain bacterial groups can be inhibited, such as *Bacteroides*, *Clostridium* and *Propionibacterium* spp., others are promoted, including *Bifidobacterium* and *Lactobacillus* spp., altering the balance of microbial populations towards the predominance of beneficial bacteria.
- Remove dietary pro-oxidants from the microenvironment of the digestive tract, especially important in the colon, where they are retained in faeces for large periods of time.
- Stimulate signalling pathways that affect cell cycle, differentiation, and function within enterocytes of the small/large intestines, thus mediating in the homeostasis of these cells and preventing the development of gastrointestinal disorders and cancer.
- Increase levels of endogenous inducible antioxidant enzymes important in protecting against oxidative stress.
- Exert anti-inflammatory effects.
- Improve the gut immune function.

3.5.4. Neurodegenerative Diseases

Consumption of grapes and wine may have significant neuroprotective effects (Georgiev et al. 2014), preventing neurodegenerative processes (Rodrigo et al. 2011; Vislocky & Fernandez 2010; Basli et al. 2012), by the capacity of their polyphenols to:

- Decrease RONS and oxidative stress at lipid, protein and DNA levels in the brain.
- Supress neuro-inflammation.
- Attenuate or inhibit activation of microglia and/or astrocytes associated with the release of the mediators linked to neuron apoptosis.
- Modulate pro-survival genes in neurons.
- Promote anti-amyloidogenic mechanisms, preventing the formation of Aβ oligomers.
- Chelate Fe^{++}, diminishing its accumulation in brain tissues.
- Improve neural signal transduction.

3.5.5. Aging

Grape polyphenols might have beneficial effects reversing the course of ageing in body tissue and cell deterioration (Pandey & Rizvi 2009; Vislocky & Fernandez 2010; Bacalini et al. 2014) as they have been reported to:

- Prevent organs and tissues oxidative damage, decreasing the incident of lipid, protein and DNA oxidation, thus limiting the risk of degenerative illnesses.
- Regulate epigenetic modifications related with ageing by inducing changes in the DNA methylation pattern.
- Modulate longevity in normal cells by mimeting the effects of calorie restriction, thus increasing their life-span but without requiring reduced food consumption (this effect has been described for resveratrol).
- Improve cognitive and motor functions, which are inherently decreased with aging.

Justification and Objectives/

Justificación y Objetivos

JUSTIFICATION AND OBJECTIVES

Several **problems**, **needs** and **requests** of the food industry led to the initiation and development of this study:

- The high amounts of by-products generated during the winemaking process, mainly the wine pomace, pose an environmental and management problem for wineries, so it is necessary to propose new solutions for the use and value-adding of this waste.

  Also, consumers are increasingly concerned about the environment and the relationship between eating habits and their health. Thus, they demand safe and natural foods, whose production entails the least possible environmental impact, and which, in addition to their nutritional value, provide benefits to the physiological functions of the human organism.

- International and national organizations implementing 'Health' and 'Food, Diet and Nutrition' programs have reported that globally and specifically in Western countries, the average consumption of antioxidants, dietary fibre, and potassium are considerably lower than recommended intakes, whereas salt consumption is much higher, with increasing scientific evidence about the negative long-term effects for human health of these habits.

  In this sense, many food companies are trying to reformulate and adapt their products to decrease their final salt content, thus helping to reduce the risk of hypertension and other related cardiovascular diseases. Therefore, the food industry requires innovative, efficient and preferably sustainable and natural alternatives to reduce/replace common salt or current synthetic alternatives, while preserving the salty flavour and microbiological stability of products.

- In addition, nutraceutical companies continually seek new natural products with healthy functional properties that attract the attention of consumers. Nutraceuticals rich in antioxidants have been very successful in recent years. Among them, the rise of some products enriched in phenolic compounds derived from grapes, wine and wine pomace is worthy of note. However, most of these products are obtained from extracts, which are enriched in phenolic compounds but do not contain other bioactive compounds, present in grapes and derivatives, which can have high nutritional and functional value.

On the other hand, growing scientific evidence shows the preventive/protective effects of grapes, wine and wine pomace against pathological conditions related to oxidative stress, including cardiovascular disease, cancer, aging, etc. Such oxidative stress results from the ineffectiveness of the antioxidant systems within the organism to react to excessive production/presence of RONS, whose toxicity is due to their tendency to react with various biomolecules (DNA, lipids and proteins), generating permanent oxidative damage and deregulating normal physiological functions of the body.

The beneficial effects of products derived from grapes are associated with their high content of bioactive compounds, mainly: phenolic compounds, dietary fibre, and potassium.

In light of all the above, the following **hypotheses** were proposed:

- Wine pomace could be used to obtain value-added products with a high content of bioactive compounds that combine their utility as *nutraceuticals* and as *functional food ingredients*.
- The vascular system and gastrointestinal tract are highly susceptible to the damaging effects of oxidative stress, so they might benefit from the preventive and/or therapeutic strategies based on supplementation with products obtained from wine pomace. However, it is important to bear in mind that this potential will depend on the bioavailability of bioactive compounds and possible changes suffered by the products before consumption in case of processing. Furthermore, the antioxidant response promoted by these compounds can be different depending on the pathological condition, so it is interesting to study their effects on different types of disease.

The **main objective** of this PhD Thesis has been the value-adding of winery residues as powdered seasonings, evaluating their potentially beneficial health effects against endothelial dysfunction and diseases associated with oxidative stress.

To achieve this main objective, a set of *in vitro*, *ex vivo* and *in vivo* assays were planned, with the following **specific objectives**:

- Characterize the seasonings evaluating their antioxidant capacity and phenolic profile.
- Determine the bioaccessibility, bioavailability and antioxidant potential of the seasonings after intake.
- Evaluate the protective effects against oxidative stress and endothelial dysfunction of the consumption of the seasoning with the best properties to act systemically, through its study in cell cultures and in animal models of Type 1 diabetes and essential hypertension.
- Estimate the chemopreventive potential against colorectal cancer of the seasonings, evaluating their anti-proliferative, anti-genotoxic and antioxidant effects in cell cultures.

The **originality** of this PhD Thesis lies in two main aspects:

- *The object of study*: an innovative product that offers an alternative to traditional uses of pomace and meets the requirements to be patented (novelty, inventive step, and industrial application).
- *The purpose of the study*: to investigate the possible functional properties of the new seasoning after consumption (health effects and mechanisms of action involved) as support to give added value to waste wine, expanding their potential uses by industries in the wine, food and nutraceutical sectors.

JUSTIFICACIÓN y OBJETIVOS

Son varios los **problemas**, **necesidades** y **demandas** de la industria alimentaria que en su conjunto motivaron el inicio y desarrollo de este estudio:

- Las elevadas cantidades de subproductos que se generan durante el proceso de vinificación, principalmente los orujos, suponen un problema medioambiental y de gestión en las bodegas, por lo que es necesario plantear nuevas soluciones para el aprovechamiento y revalorización de los mismos.

  Asimismo, el consumidor está cada vez más preocupado por el medio ambiente y por la relación entre los hábitos alimentarios y su salud, exigiendo alimentos seguros, naturales, cuya obtención suponga el menor impacto medioambiental posible, y que, además de su valor nutritivo, aporten beneficios a las funciones fisiológicas del organismo humano.

- Organismos internacionales y nacionales que ejecutan programas de "Salud" y de "Alimentación, Dietética y Nutrición" han informado que a nivel mundial y, específicamente, en los países Occidentales, los consumos medios de antioxidantes, fibra alimentaria y potasio son considerablemente inferiores que las ingestas recomendadas, mientras que el consumo de sal es muy superior, siendo cada vez mayor la evidencia científica sobre las repercusiones negativas a largo plazo en la salud humana de dichos hábitos.

  En este sentido, muchas empresas del sector alimentario están tratando de reformular y adaptar sus productos para reducir su contenido final de sal, colaborando así a disminuir el riesgo de hipertensión y otras enfermedades cardiovasculares relacionadas. Por ello, la industria alimentaria requiere alternativas innovadoras, eficientes y, preferiblemente, sostenibles y de origen natural, que permitan reducir/reemplazar la sal común o las alternativas sintéticas actuales, a la vez que se conserve el sabor salino y la estabilidad microbiológica de los productos.

- Además, las empresas nutracéuticas buscan continuamente nuevos productos naturales con propiedades funcionales saludables que atraigan la atención de los consumidores. Los nutracéuticos ricos en antioxidantes han tenido una gran acogida en los últimos años. Entre ellos, cabe destacar el auge de algunos productos enriquecidos en compuestos fenólicos derivados de las uvas, vinos y orujos. Sin embargo, la mayoría de estos productos se obtienen a base de extractos, por lo que están enriquecidos en compuestos fenólicos pero no contienen otros compuestos bioactivos, presentes en la uva y derivados, que pueden tener gran valor nutricional y funcional.

Por otro lado, cada vez son más los trabajos científicos que evidencian los efectos preventivos/protectores de las uvas, vinos y orujos frente a enfermedades relacionadas con el estrés oxidativo como las enfermedades cardiovasculares, el cáncer, el envejecimiento, etc. Dicho estrés oxidativo se debe a la incapacidad de los sistemas de respuesta antioxidante del organismo para reaccionar frente a una excesiva producción/presencia de RONS, cuya toxicidad radica en su tendencia a reaccionar con distintas biomoléculas (DNA, lípidos y proteínas), generando daños oxidativos permanentes y desregulando las funciones fisiológicas normales del organismo.

Los efectos beneficiosos de los productos derivados de la uva se asocian a su elevado contenido en compuestos bioactivos, principalmente: compuestos fenólicos, fibra alimentaria y potasio.

En base a lo expuesto anteriormente, se plantearon las siguientes **hipótesis**:

- Los orujos podrían ser aprovechados para obtener productos con un alto contenido en compuestos bioactivos que combinen su utilidad como *nutracéuticos* y como *ingredientes funcionales alimentarios*.
- El sistema vascular y el tracto gastrointestinal son altamente susceptibles a los efectos perjudiciales del estrés oxidativo, por lo que podrían verse beneficiados por las estrategias preventivas y/o terapéuticas basadas en la suplementación con productos obtenidos de los orujos. Sin embargo, es importante tener en cuenta que dicho potencial dependerá de la biodisponibilidad de los compuestos bioactivos y de los posibles cambios sufridos antes de su consumo en caso de procesamiento. Además, la respuesta antioxidante promovida por estos compuestos puede ser diferente en función de la patología, por lo que es interesante el estudio de su efecto en diferentes tipos de enfermedad.

El **objetivo principal** de esta Tesis Doctoral ha sido la revalorización de los residuos de vinificación como sazonadores, evaluando sus efectos potencialmente beneficiosos para la salud frente a la disfunción endotelial y las enfermedades asociadas al estrés oxidativo.

Para alcanzar este objetivo principal, se plantearon un conjunto de ensayos *in vitro*, *ex vivo,* e *in viv*o con los siguientes **objetivos específicos**:

- Caracterizar los sazonadores evaluando su capacidad antioxidante y perfil fenólico.
- Determinar la bioaccesibilidad, biodisponibilidad, y potencial antioxidante de los sazonadores tras su ingesta.
- Evaluar el efecto protector frente al estrés oxidativo y la disfunción endotelial del consumo del sazonador con las mejores propiedades para actuar a nivel sistémico, mediante su estudio en cultivos celulares y en modelos animales de diabetes Tipo 1 e hipertensión esencial.
- Estimar el potencial quimiopreventivo de los sazonadores frente al cáncer colorrectal evaluando sus efectos anti-proliferativos, anti-genotóxicos y antioxidantes en cultivos celulares.

En definitiva, la **originalidad** de esta Tesis Doctoral, radica en dos aspectos principales:

- *El objeto de estudio*: un producto innovador que supone una alternativa a los usos tradicionales de los orujos y reune las características necesarias para ser patentado (novedad, actividad inventiva, y aplicación industrial).
- *El propósito del estudio*: investigar las posibles propiedades funcionales del nuevo sazonador tras su ingesta (efectos saludables y mecanismos de acción implicados) como soporte para dar un valor añadido a los residuos de vinificación, ampliando sus posibles usos por las industrias del sector vitivinícola, alimentario y nutraceútico.

Antioxidant and Phenolic Characterization of the Seasonings

CHAPTER 1:

ANTIOXIDANT AND PHENOLIC CHARACTERIZATION OF THE SEASONINGS

This chapter describes the *in vitro* studies aimed at achieving the first objective of this PhD Thesis: the antioxidant and phenolic characterization of powdered seasonings obtained from wine pomace, the main by-products of wineries.

The antioxidant properties of these seasonings (**RWPS - Red Wine Pomace Seasonings**) will be largely determined by their phenolic composition, which in turn depends qualitatively and quantitatively on several factors, including: the origin and variety of grapes; the wine pomace components used as raw materials; and, processing required for obtaining the seasonings.

The seasonings used in this PhD Thesis were obtained from a mixture of red wine pomaces collected at seven wineries from the Designation of Origin (DO) 'Ribera del Duero', all of them situated in the province of Burgos, with the aim of obtaining a representative sample of the winemaking residues generated in this important wine area of Spain. Therefore, the seasonings were obtained from wine pomace derived from *Vitis Vinifera* L. *cv. Tempranillo* grapes, also known as 'Tinta del País' or 'Tinto Fino'.

Both the variety of grape used and some technological aspects during the winemaking process are factors that influence the final ratio of the components present in wine pomace, with grape skins being estimated to represent, in average, around 65-70% of dry weight and grape seeds approximately 30-35% (Teixeira et al. 2014). Each grape tissue presents a particular distribution of polyphenols (**Figure 39**), which is the main cause that determines the preferential content of certain phenolic compounds and/or families in each of the constituents of wine pomace. There are also important differences between the skins and seeds of grapes, and consequently of the wine pomace, on their contents in other components (dietary fibre, simple carbohydrates, proteins, minerals, essential oils, etc.) which will provide important structural, nutritional and/or sensory qualities (Goñi & Serrano 2005; Xia et al. 2010; Teixeira et al. 2014; Yu & Ahmedna 2013).

Given these differences in composition between the skins and seeds of wine pomace, and that the separation of the seeds is a relatively simple and common practice in the industry, three types of powdered seasonings were developed (García-Lomillo et al. 2014), which were obtained from:

- Wine pomace, without separating its components (**W-S**).
- Seedless wine pomace, thus enriched in the skins (**Sk-S**).
- The isolated seeds (**Sd-S**).

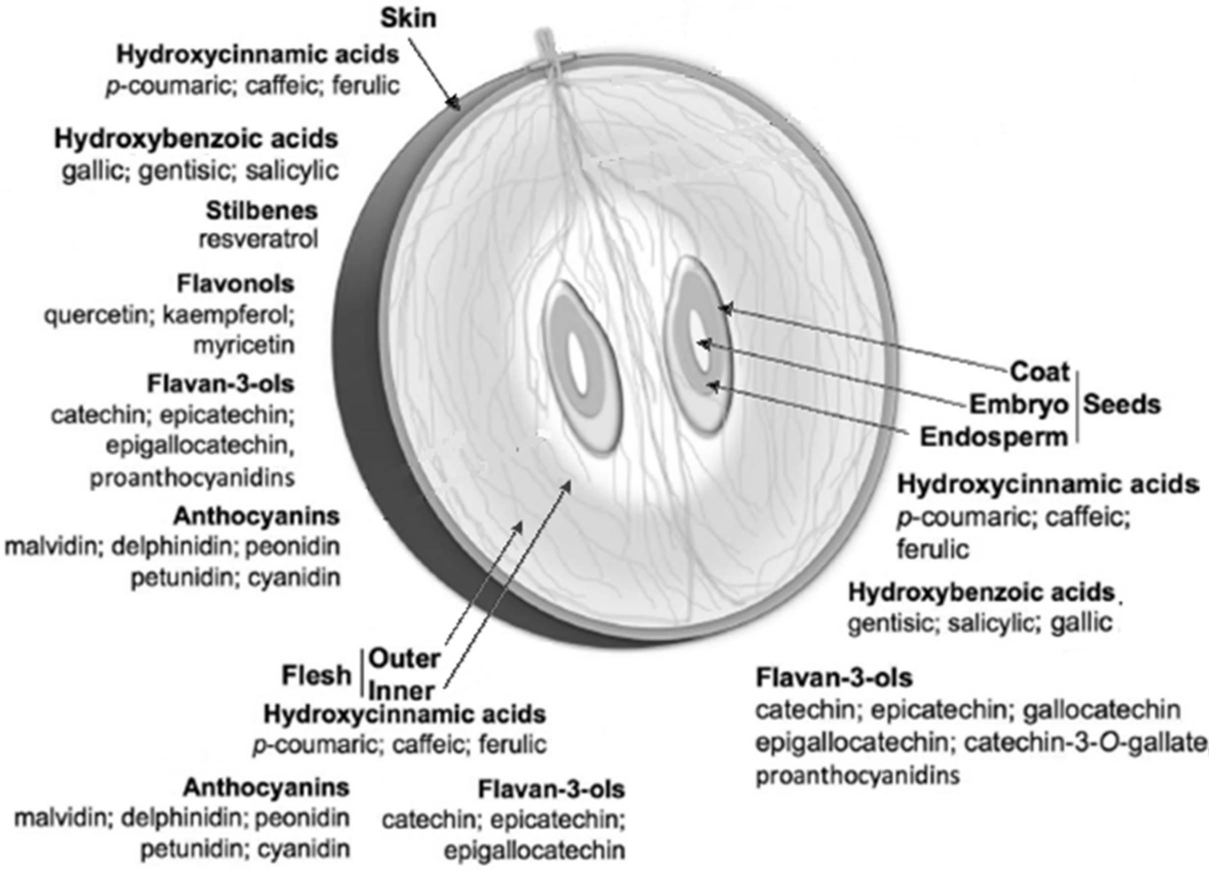


Figure 39. **Phenolic composition of the different grape tissues.**
Adapted from Teixeira et al. (2014).

Based on the literature review on the effects of drying, grinding and heat treatment on the polyphenol contents and antioxidant activity of wine pomace, and after conducting preliminary tests, the conditions to apply during the process of obtaining the seasonings were selected (García-Lomillo et al. 2014), some of which are indicated below:

- The temperature of the drying step, wherein the % moisture of wine pomace was reduced from the initial 50% to lower than 10%, did not exceed 60ºC.
- The particle sizes of the seasonings after grinding and sieving (< 250 µm for Sk and W; < 350 µm for Sd) were selected according to the food technological properties of each seasoning, trying to balance the advantages and shortcomings of very fine powdered products.
- The thermal treatment applied to the seasonings to ensure their microbiological stability was performed at 90 ºC for 90 min, managing to reduce the microbial load to the desired levels.

Seasonings obtained through this process are not extracts but solid powdered products not completely soluble in water. Therefore, is the most appropriate methodological approach to determine the antioxidant capacity of these type of samples is using QUENCHER assays (Gökmen et al. 2009), which emerged as an alternative to the conventional methods in which the antioxidant evaluation is performed in liquid samples or, in case of solid samples, after an extraction phase (Prior et al. 2005; Rivero-Pérez et al. 2007).

Accordingly, the first study conducted was the development and/or adaptation to the QUENCHER (Q-) approach of several tests to determine the total antioxidant capacity and the scavenger capacity of specific free radical ($O_2^{\cdot-}$, $^{\cdot}OH$, and $LOO^{\cdot}$). The proposed methods were validated by checking compliance with the quality criteria set for various parameters (linearity, proportionality, detection limits and precision). In addition, in order to demonstrate their suitability for analysing the antioxidant capacity of solid powder products of different nature, the proposed methods were applied to products obtained from the different components of wine pomace, as model samples whose matrices and phenolic compounds have different features and polarities.

Moreover, although the heat treatment applied to the seasonings is performed under controlled and relatively moderate conditions of temperature and duration, it is known that such type of processing can significantly alter the phenolic composition and matrices of products (Nicoli et al. 1999; Ross et al. 2011; Chamorro et al. 2012).

Therefore, when determining the phenolic profile and antioxidant capacity of the seasonings, evaluating the effects of heat treatment, in addition to the differences depending on the type of raw material used for their production (seedless wine pomace, whole wine pomace, or just the seeds), was considered as a key point in their characterization.

The following determinations were carried out:

- A detailed determination of the individualized phenolic compounds, which were identified and quantified using chromatographic techniques (HPLC-DAD), obtaining acidified methanolic extracts from the seasonings to use them as samples.
- Total antioxidant capacity characterization implementing the methods selected in the first study, which were performed from a classical approach (using the acidified methanolic extracts as samples) or from the QUENCHER approach (applied directly to the seasonings as powdered solid samples). One of the methods selected was the Folin-Ciocalteu (FC) assay, which is commonly used to estimate the concentration of total polyphenols in foods.

The major **conclusions** of these *in vitro* studies were:

- **<u>Chapter 1.1</u>**: In general, all the QUENCHER methods developed met successfully the criteria set for their validation. Among these methods, the combination of Q-FC and Q-ABTS assays (using water as reaction medium) was selected as an appropriate methodology for multisample analyses, given the simplicity of these assays and their good correlation with the other tests to evaluate the antioxidant capacity.

- **<u>Chapter 1.2</u>**: The heat treatment applied to the seasonings affected negatively their content in anthocyanins (Sk-S and W-S) and flavan-3-ols (Sd-S), while their concentration of phenolic acids and flavonols was increased. As a result, this treatment caused a slight reduction in the antioxidant capacity and total polyphenol content of the seasonings. The differences observed between the results obtained by classical and QUENCHER methods showed the importance of implementing specific methodologies for solid samples when analysing water-insoluble powdered products.

CAPÍTULO 1:

CARACTERIZACIÓN ANTIOXIDANTE Y FENÓLICA DE LOS SAZONADORES

En este capítulo se presentan los estudios *in vitro* dirigidos a la consecución del primero de los objetivos de esta Tesis Doctoral: la caracterización antioxidante y fenólica de sazonadores obtenidos a partir de orujos, los principales subproductos de las bodegas.

Las propiedades antioxidantes de estos sazonadores (**RWPS - Red Wine Pomace Seasonings**) estarán determinadas en gran medida por su composición fenólica, que a su vez dependerá cualitativa y cuantitativamente de varios factores, entre ellos: el origen y la variedad de la uva; los componentes de los orujos empleados como materia prima; y, el procesamiento para la obtención de los sazonadores.

Los sazonadores utilizados en esta Tesis Doctoral se obtuvieron a partir de una mezcla de orujos recogidos en siete bodegas de la Denominación de Origen Ribera del Duero, todas ellas situadas en la provincia de Burgos, con el objetivo de obtener una muestra representativa de los residuos de vinificación generados en esta importante área vitivinícola de España. Por ello, los sazonadores derivan de orujos procedentes de uvas de la variedad *Vitis Vinifera* L. *cv. Tempranillo*, también conocida como Tinta del País o Tinto Fino.

Tanto la variedad de uva empleada como algunos aspectos tecnológicos del proceso de vinificación son factores que influyen en la proporción final de los componentes presentes en los orujos, estimándose que, de media, los hollejos representan en torno al 65-70% del peso seco y las pepitas alrededor del 30-35% (Teixeira et al. 2014). Cada uno de los tejidos de la uva presenta una distribución particular de polifenoles (**Figura 39**), siendo ésta la principal causa que determina el contenido preferencial de ciertos compuestos y/o familias fenólicas en cada uno de los constituyentes de los orujos. Existen también importantes diferencias entre los hollejos y las pepitas de las uvas, y en consecuencia de los orujos, respecto a su contenido en otros componentes (fibra alimentaria, carbohidratos sencillos, proteínas, minerales, aceites esenciales, etc.) que aportarán características estructurales, nutricionales y/o sensoriales importantes (Goñi & Serrano 2005; Xia et al. 2010; Teixeira et al. 2014; Yu & Ahmedna 2013).

Teniendo en cuenta estas diferencias de composición entre los hollejos y las pepitas de los orujos, y que la separación de las pepitas es una práctica relativamente sencilla y habitual en la industria, se elaboraron tres tipos de sazonadores (García-Lomillo et al. 2014), obtenidos a partir de:

- Los orujos, sin separar sus componentes (**W-S**).
- Los orujos sin pepitas, enriquecidos por lo tanto en los hollejos (**Sk-S**).
- Las pepitas aisladas (**Sd-S**).

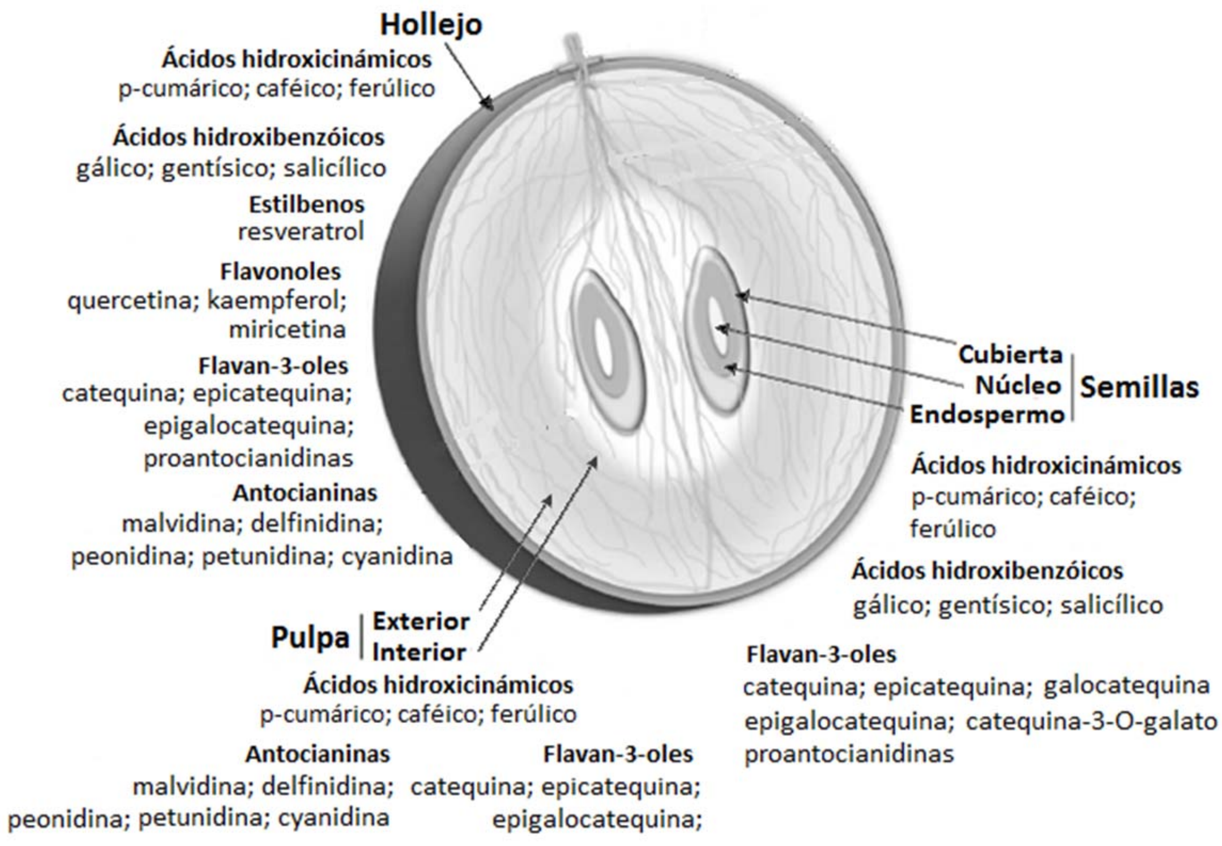


Figura 39. **Composición fenólica de los diferentes tejidos de las uvas.**
Adaptado de Teixeira et al. (2014).

En base a la revisión bibliográfica sobre los efectos del desecado, la molienda y el tratamiento térmico sobre el contenido en compuestos fenólicos y la actividad antioxidante de los orujos, y tras realizar los ensayos preliminares necesarios, se seleccionaron las condiciones a aplicar durante el proceso de obtención de los sazonadores (García-Lomillo et al. 2014), algunas de las cuales se indican a continuación:

- La temperatura de la etapa de desecado, en la que se redujo el % de humedad de los orujos del 50% inicial a valores por debajo del 10%, no excedió los 60ºC.
- Los tamaños de partícula definitivos de los sazonadores tras su molienda y tamizado (< 250 µm para Sk-S y W-S; < 350 µm para Sd-S) fueron seleccionados de acuerdo a las propiedades tecnológicas de cada sazonador, tratando de equilibrar las ventajas e inconvenientes de los productos pulverizados muy finos.
- El tratamiento térmico aplicado a los sazonadores para asegurar su estabilidad microbiológica se realizó a 90ºC durante 90 min, consiguiendo reducir la carga microbiana hasta los niveles deseados.

Los sazonadores obtenidos no son extractos, sino productos sólidos, pulverizados, y no totalmente solubles en agua. Por ello, la aproximación metodológica más adecuada para determinar la capacidad antioxidante de este tipo de muestras son los ensayos QUENCHER (Gökmen et al. 2009), que surgieron como alternativa a los métodos clásicos en los que la evaluación antioxidante se realiza en muestras líquidas o, en caso de muestras sólidas, tras una fase de extracción (Prior et al. 2005; Rivero-Pérez et al. 2007).

En consecuencia, el primero de los estudios llevados a cabo fue la puesta a punto y/o adaptación a la aproximación QUENCHER (Q-) de varios ensayos para determinar la capacidad antioxidante total y la capacidad estabilizadora de radicales libres específicos ($O_2^{\cdot-}$, $^{\cdot}OH$, y $LOO^{\cdot}$). Los métodos propuestos fueron validados, comprobando el cumplimiento de los criterios de calidad establecidos para varios parámetros (linealidad, proporcionalidad, límites de detección, y precisión). Además, con el fin de demostrar su idoneidad para analizar la capacidad antioxidante de productos sólidos pulverizados de distinta naturaleza, los métodos propuestos se aplicaron sobre productos obtenidos a partir de los diferentes componentes de los orujos, como muestras modelo cuyas matrices y compuestos fenólicos presentan diferentes características y polaridades.

Por otra parte, a pesar de que el tratamiento térmico al que son sometidos los sazonadores se lleva a cabo bajo condiciones controladas, y relativamente moderadas, de temperatura y de duración, se sabe que este tipo de procesamientos pueden alterar significativamente la composición fenólica y las matrices de los productos (Nicoli et al. 1999; Ross et al. 2011; Chamorro et al. 2012).

Por ello, a la hora de determinar el perfil fenólico y la capacidad antioxidante de los sazonadores se consideró clave evaluar, además de las diferencias en función del tipo de materia prima utilizada para su obtención (orujos sin pepitas, orujos, o sólo las pepitas), el efecto del tratamiento térmico.

Se llevaron a cabo las siguientes determinaciones:

- Determinación pormenorizada de los compuestos fenólicos individualizados, que fueron identificados y cuantificados por técnicas cromatográficas (HPLC-DAD), obteniendo para ello extractos metanólicos acidificados a partir de los sazonadores.
- Caracterización de la capacidad antioxidante total, mediante los métodos seleccionados en el primer estudio, llevados a cabo desde una aproximación clásica (utilizando como muestras los extractos metanólicos acidificados) o desde la aproximación QUENCHER (aplicados directamente en los sazonadores como muestras sólidas pulverizadas). Entre estos métodos está el ensayo de Folin-Ciocalteu (FC), empleado habitualmente para estimar la concentración de polifenoles totales en alimentos.

Las principales **conclusiones** de estos estudios *in vitro* fueron:

- **Capítulo 1.1**: En general, los métodos QUENCHER puestos a punto cumplieron satisfactoriamente con los criterios de validación establecidos. De entre estos métodos, se seleccionó la combinación de los ensayos Q-FC y Q-ABTS (utilizando agua como medio de reacción) como una metodología adecuada para el análisis de múltiples muestras, dada la simplicidad de dichos métodos y su buena correlación con los demás ensayos para evaluar la capacidad antioxidante.

- **Capítulo 1.2**: El tratamiento térmico aplicado a los sazonadores afectó negativamente a su contenido en antocianidinas (Sk-S y W-S) y flavan-3-oles (Sd-S), mientras que su concentración en ácidos fenólicos y flavonoles se vio incrementada. Como resultado, dicho tratamiento provocó una ligera reducción de la capacidad antioxidante y contenido en polifenoles totales de los sazonadores. Las diferencias observadas entre los resultados obtenidos por métodos clásicos y QUENCHER evidenciaron la importancia de aplicar las metodologías específicas para muestras sólidas cuando se analizan productos pulverizados insolubles en agua.

Chapter 1.1.

Adaptation and Validation of QUick, Easy, New, CHEap, and Reproducible (QUENCHER) Antioxidant Capacity Assays in Model Products Obtained from Residual Wine Pomace

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Abstract

Evaluation of the total antioxidant capacity of solid matrices without extraction steps is a very interesting alternative for food researchers and also for food industries. These methodologies have been denominated by QUENCHER from QUick, Easy, New, CHEap, and Reproducible assays. To demonstrate and highlight the validity of QUENCHER (Q-) methods, values of Q-method validation were showed for the first time, and they were tested with products of well-known different chemical properties. Furthermore, new QUENCHER assays to measure scavenging capacity against superoxide, hydroxyl, and lipid peroxyl radicals were developed. Calibration models showed good linearity (R^2>0.995), proportionality and precision (CV<6.5%), and acceptable detection limits (<20.4 nmol Trolox equivalents). The presence of ethanol in the reaction medium gave antioxidant capacity values significantly different from those obtained with water. The dilution of samples with powdered cellulose was discouraged because possible interferences with some of the matrices analyzed may take place.

Keywords: Antioxidant activities, ABTS, Folin-Ciocalteu, Free radical scavenging assays, Cellulose.

Abbreviations and nomenclature: **ABTS**, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; **AUC**, area under the curve; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl; **FC**, Folin-Ciocalteu; **FRAP**, ferric reducing/antioxidant power; **FRSC**, free radical scavenging capacity; **GA**, Gallic acid; **HRSC**, hydroxyl radical scavenging capacity; **LPSC**, lipid peroxyl scavenging capacity; **Q-**, QUENCHER; **OC**, oxidised controls; **ORAC**, oxygen radical absorbance capacity; **SC**, sample controls; **Sd**, product from wine pomace seeds; **Sk**, product from wine pomace skins; **SRSC**, superoxide radical scavenging capacity; **T**, Trolox; **TAC**, total antioxidant capacity; **W**, product from whole wine pomace.

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Highlights:

- QUENCHER (Q-) methods allow direct antioxidant capacity assessment of powdered samples.
- QUENCHER assays to measure scavenging capacity against free radicals have been developed.
- Q-Folin-Ciocalteu and Q-ABTS assays are suitable for multisampling analyses.
- The use of ethanol (medium) and cellulose (diluting agent) is not advised.
- Powdered products obtained from wine pomace exhibit high antioxidant capacity *in vitro*.

1. Introduction

Natural antioxidants are increasingly demanded by the food industry to prevent oxidative degradation reactions while satisfying consumers' demands. Furthermore, there is growing interest in knowing the antioxidant value of foods, food supplements, and diets. Therefore, in recent decades, several *in vitro* and *in vivo* assays have been developed to determine the total antioxidant capacity (TAC) of foods and biological samples (Prior et al. 2005; Niki 2010; López-Alarcón & Denicola 2013). It is generally recognized that effects of food materials on the redox balance *in vivo* cannot be merely extrapolated from their activities *in vitro*, with the possible exception of their antioxidant action in the gastrointestinal tract (Fraga et al. 2014; Niki 2010). However, the determination of the *in vitro* TAC can be achieved by simple and relatively fast chemical methodologies and may give more relevant information than that obtained from measuring each antioxidant compound separately, as possible synergistic interactions may not be considered (Fraga et al. 2014). Therefore, these methods have been largely used to estimate and compare the antioxidant capacity of food items (Sanchez-Moreno 2002; Pérez-Jiménez et al. 2008). Because most natural antioxidants are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems (Frankel & Meyer 2000; Karadag et al. 2009).

The most commonly applied *in vitro* TAC methodologies are based on diverse strategies to evaluate: *1)* the reducing ability of antioxidants, such as the Folin-Ciocalteu (FC) and the ferric reducing/antioxidant power (FRAP) assays; *2)* the scavenging of stable free radicals by antioxidants, including the 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays; *3)* the scavenging of short-lived radicals by competition mechanisms, such as peroxyl radicals in the oxygen radical absorbance capacity (ORAC) assay. Other less widely used methods assess the free radical scavenging capacity (FRSC) of antioxidants using radicals that are responsible for the oxidative stress damage observed *in vivo*, such as the superoxide, hydroxyl, and lipid peroxyl radicals. The respective FRSC methods have been named the superoxide radical scavenger capacity (SRSC), hydroxyl radical scavenger capacity (HRSC), and lipid peroxyl scavenger capacity (LPSC) assays.

Most of the TAC and FRSC determinations in foods and antioxidant products have been limited to their soluble compounds, and the extraction procedure has been considered a critical step (Pérez-Jiménez & Saura-Calixto 2006; Serrano et al. 2007). This reason motivated the development of the QUick, Easy, New, CHEap, and Reproducible (QUENCHER) assays to measure the antioxidant activity of food materials (Gökmen et al. 2009). These methods are a very interesting approach that avoids time-consuming solvent extraction steps of the classical protocols. The basis of QUENCHER (Q-) protocols is to place in direct contact the solid powdered food materials and the reagent solutions. Thus, the soluble antioxidants of

the sample quench the radicals present in the reaction medium according to usual liquid-liquid reactions, whereas the antioxidants bound to the insoluble particle matter exert their antioxidant activity by taking advantage of surface reactions occurring at the solid-liquid interface (Gökmen et al. 2009).

Several of the frequently used TAC methodologies (ABTS, DPPH, ORAC, FRAP, and FC) have recently been adapted to the QUENCHER approach (Serpen et al. 2007; Amigo-Benavent et al. 2010; Serpen et al. 2012b; Kraujalis et al. 2013). Up to now, these assays have been successfully applied to some foods for which the antioxidant activity is largely dependent on the insoluble part, such as cereals and bakery products, seeds, nuts, pulses, and dietary fibres (Serpen et al. 2007; Amigo-Benavent et al. 2010; Serpen et al. 2012b; Kraujalis et al. 2013; Açar et al. 2009; Serpen et al. 2008; Çelic et al. 2013; Delgado-Andrade et al. 2010). It must also be noted that a mixture of ethanol:water (50:50, v/v) has been suggested as the most appropriate reaction medium to carry out QUENCHER assays, and "neutral" powdered materials such as cellulose have been proposed as suitable diluting agents when products under study are very rich in antioxidants (Gökmen et al. 2009; Serpen et al. 2007).

Despite the advantages and previous applications of the QUENCHER methods, these assays are not yet extensively used, probably due to a lack of validation studies of these methodologies. In addition, to the best of our knowledge, the QUENCHER concept has still not been adapted to the methodologies that target biologically relevant radicals (SRSC, HRSC, and LPSC), and a statistical validation and correlations between the different Q-TAC and Q-FRSC assays have not yet been performed. These verifications are important to encourage the applicability of QUENCHER protocols as valuable tools for the *in vitro* antioxidant capacity assessment of different products such as foods, food ingredients, and others.

Therefore, to increase the use of the QUENCHER approach, the first aim of the present study was to develop new QUENCHER protocols to evaluate the scavenger capacity of some of the most biologically relevant radicals (superoxide, hydroxyl, and lipid peroxyl); the second aim was to optimize and, for the first time, validate different QUENCHER assays (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH, Q-ORAC, Q-SRSC, Q-HRSC, and Q-LPSC) using three model products of different chemical properties. Secondarily, the best combination of QUENCHER assays for routine analysis was identified and several factors that might influence the Q-TAC results, such as the presence of ethanol in the reaction medium and the use of cellulose as a diluting agent, were assessed. The study was carried out with three powdered model products, all of them rich in antioxidant compounds, but each containing antioxidants of different polarity. Powdered products were made in our pilot plant from wine pomace in order to work with familiar products for which characteristics and composition were well-known.

2. Materials and Methods

2.1. Chemicals

ABTS, cellulose, DPPH, deoxyribose (2-deoxy-D-ribose), 2,2'-diazobis-(2-aminodinopropane)-dihydrochloride (AAPH), gallic acid (GA), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 4-nitroblue tetrazolium chloride (NBT), phenazin methosulfate (PMS), 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ) and 2-thiobarbituric acid (TBA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), iron(III)-chloride acid ($FeCl_3$), iron(II)-sulphate ($FeSO_4$), FC reagent, hydrogen peroxide (H_2O_2), L-ascorbic acid ($C_6H_8O_6$), potassium persulfate ($K_2O_8S_2$), sodium acetate ($NaC_2H_3O_2$), sodium carbonate (Na_2CO_3), sodium pyrophosphate anhydrous ($Na_4P_2O_7$) and trichloroacetic acid (TCA) were purchased from Panreac Química, S.L.U. (Barcelona, Spain).

2.2. Samples

Red wine pomace was kindly supplied by different wineries located at Burgos (Spain). All red wine pomace was mixed and dehydrated until reaching final water content of < 10%. Dried materials were separated, ground and sieved, thus obtaining three different powdered products(González-Sanjosé et al. 2013) which were used as model matrices. One of them was derived from grape seeds (Sd) separated from the wine pomace; this model product represented foods with a significant content of fat and rich mainly in hydrophobic antioxidants. Another product was obtained from the wine pomace free of seeds, which was mainly constituted by grape skins (Sk); this model product represented food matrices rich in hydrophilic antioxidant and without or with very low levels of fat. The third powdered product was obtained from whole (W) wine pomace; this model product represented food materials with some fat and rich in both hydrophilic and hydrophobic antioxidants. The particle size of these model products was < 0.250 mm in the cases of Sk and W, and < 0.355 mm for Sd (García-Lomillo et al. 2014).

2.3. QUENCHER Antioxidant Capacity Methods

The adapted Q-TAC and Q-FRSC methodologies were fixed end-point procedures, with the exception of the Q-ORAC method which was a kinetic assay. Results are given as mean values ± standard deviation of three independent samples.

2.3.1. Q-FC assay

This method was adapted from the FC assay developed by Singleton & Rossi (1965). Briefly, 1 ± 0.005 mg of the tested products was weighed in a microbalance (MicroPro11, Sartorius) and mixed with 0.2 mL of MilliQ (MQ) water and 0.2 mL of FC reagent. After 5 min of reaction, 4 mL of a 0.7 M Na_2CO_3 solution was added and the final volume was made up to 10 mL with MQ water. After 1 h of incubation in an orbital shaker, the absorbance at 750 nm was measured using an UV-vis spectrophotometer (U-2000 Hitachi). The FC index was expressed as μmol of gallic acid equivalents per gram of product (GAE/g) by means of a dose-response curve for different quantities of the standard.

2.3.2. Q-FRAP assay

The Q-FRAP procedure was adapted from the method described by Benzie & Strain (1996). The reactive mixture was freshly prepared by mixing 10 mM TPTZ and 20 mM $FeCl_3$ in 300 mM $NaC_2H_3O_2$ buffer (pH 3.6) at a ratio of 1:1:10 (v/v/v) and diluted 10:1 (v/v) in MQ water. A volume of 10 mL of the FRAP solution was added to 1 ± 0.005 mg of the samples and incubated at 37 °C for 30 min with continuous stirring. The absorbance at 593 nm was measured. The results were expressed as µmol of iron(II) equivalents per gram of product (Fe(II)E/g) using linear calibration obtained with different amounts of $FeSO_4$.

2.3.3. Q-ABTS assay

The method was adapted from the assay developed by Re et al.(Re et al. 1999) and modified by Rivero-Pérez et al. (2007). The stock solution of $ABTS^{\cdot+}$ was diluted with MQ water to an absorbance of 0.70 ± 0.02 at 734 nm. 1 ± 0.005 mg of the assessed products was weighed, and the reaction was started by adding 10 mL of $ABTS^{\cdot+}$ working solution. After 30 min of incubation in darkness with continuous stirring the absorbance of the samples at 734 nm was measured and subtracted from the absorbance of the $ABTS^{\cdot+}$ working solution. The results are expressed as µmol of Trolox equivalents per gram of product (TE/g), using the dose-response curve described by different amounts of this standard.

2.3.4. Q-DPPH assay

This method is based on the scavenging of the radical $DPPH^{\cdot}$ Brand-Williams et al. (1995). A 0.1 mM $DPPH^{\cdot}$ working solution in pure methanol was prepared and diluted until an absorbance at 517 nm of 0.70 ± 0.02 Rivero-Pérez et al. (2007). Then, 1 ± 0.005 mg of the powdered products was transferred into a test tube and mixed with 10 mL of $DPPH^{\cdot}$ working solution. The absorbance at 517 nm was measured after 30 min of continuous stirring at room temperature in the samples and subtracted from the absorbance of the $DPPH^{\cdot}$ working solution. Trolox was used as standard to perform a calibration curve and the results were expressed as µmol of TE/g of product.

2.3.5. Q-ORAC assay

This assay was adapted from the method described by Ou et al. (2001). The analysis was conducted in a four-cell-fluorometre (Cary-Eclipse, Varian) with continuous stirring in the cuvette and at 37 °C. Briefly, 0.2 ± 0.005 mg of the assessed products was weighed and resuspended in 0.8 mL of 75 mM phosphate buffer (pH 7.0, warmed to 37 °C) just before use. A Trolox quality control (T10: equivalent to 10 µg of Trolox), and a 300 mM AAPH solution in phosphate buffer were also freshly prepared. At least one T10 and one blank were analyzed with every set of samples (same AAPH solution). In a glass cuvette, an aliquot of 0.4 mL of the samples (the resuspended products, the T10 control or just phosphate buffer for the blank) was mixed with 2 mL of a freshly prepared 87.5 nM fluorescein solution in phosphate buffer. The fluorescence reading (λ_{exc} = 493 nm and λ_{em} = 511 nm) was started,

and 0.1 mL of the AAPH solution was added after 2.5 min. The fluorescence decay curve was monitored for 100 min. The net area under the curve (net AUC) was calculated for each sample by subtracting the blank AUC from the sample AUC. Regression equations between net AUC and different quantities of Trolox were obtained and used for the expression of the Q-ORAC values as µmol of TE/g of product.

2.3.6. Q-SRSC assay

The Q-SRSC procedure was adapted from the method described by Liu et al.(Liu et al. 1997) and Rivero-Pérez et al.(Rivero-Pérez et al. 2007) Briefly, 1.5 ± 0.005 mg of the products was added to the test tube and mixed with a 1.5 mL final volume of 78 µM NADH, 50 µM NBT and 10 µM PMS in 16 mM buffer Tris-HCl (pH 8.0). After the mixture had been shaken for 10 s, the absorbance (A) of the samples (S) at 560 nm was measured. 'Oxidised controls' (OC; without products) and the 'sample controls' (SC; products and buffer) were also prepared. The results are expressed as oxidation inhibition percentages by the equation:

$$\text{Oxidation inhibition } \% = (A_{OC} - (A_S - A_{SC}))/ A_{OC} * 100 \quad [1]$$

2.3.7. Q-HRSC assay

The Q-HRSC assay was adapted from the method developed by Halliwell et al.(Halliwell et al. 1987) and modified by Rivero-Pérez et al. (2007). Briefly, 1 ± 0.005 mg of the powdered products was weighed and transferred into a screw-top test tube. 1 mL final reaction volume contained a mixture of 1 mM deoxyribose, 0.1 mM $C_6H_8O_6$, 1 mM H_2O_2, 0.1 mM $FeCl_3$ and 0.1 mM EDTA in 5 mM phosphate buffer (pH 7.4). OC and SC were also prepared. The tubes were incubated for 60 min at 37 °C with continuous stirring. Then, 1.5 mL of TCA (28% w/v) and 1 mL of TBA (1% w/v) were added and the capped tubes were shaken and heated at 100 °C for 15 min. The absorbance was recorded at 532 nm and the results were expressed as oxidation inhibition % following the equation [1].

2.3.8. Q-LPSC assay

This assay was adapted from the method described by Rivero-Pérez et al. (2007). The experiments were carried out in rat liver microsomal preparations (Kessler et al. 2003), and lipid peroxidation was induced using AAPH as oxidant. The total microsomal protein content was determined using the Bradford method (Bradford 1976). A microsomal solution containing 10 mg/mL protein in 0.1 M $Na_4P_2O_7$ buffer (pH 7.4) was prepared and stored at -80 °C until the day of analysis. 0.2 ± 0.005 mg of the tested products was placed into a screw-top tube, and 400 µL of 50 mM Tris-HCl buffer (pH 7.0), 50 µL of the microsomal solution and 50 µL of 100 mM AAPH solution in the Tris-HCl buffer were added. OC and SC (replacing the AAPH solution by Tris-HCl buffer) were also prepared. The tubes were incubated at 37 °C for 90 min under continuous stirring. Then, 750 µL of TCA (2.8% w/v) and 500 µL of TBA (1% w/v) were added, and the capped tubes were shaken and heated at 100

ºC for 15 min. An aliquot of 750 µL of the reaction mixture was transferred to another tube and the TBA reactive substances were extracted with the same volume of butanol. The absorbance at 532 nm of the butanol extract was measured and the results were expressed as oxidation inhibition % with respect to the OC sample following the equation [1].

2.4. Statistical Analysis

The statistical validation of the methods was performed using the Statgraphics® Centurion XVI version 16.2.04 software (Statpoint Technologies, Inc., Warranton, VA, USA). The statistical analysis of the Q-TAC data was carried out using one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) test was applied to determine the statistical significance of differences among various groups. A minimum significance level of $p < 0.05$ was considered. Pearson's correlation coefficients were determined to study linear correlations between pairs of the Q-TAC methodologies.

3. Results and Discussion

3.1. Adaptation and Optimization of the Q-TAC and Q-FRSC Methods

This study shows some modifications with respect to Q-TAC protocols previously published: Q-ABTS (Serpen et al. 2007); Q-DPPH (Serpen et al. 2007); Q-FRAP (Serpen et al. 2012b); Q-ORAC (Amigo-Benavent et al. 2010); and, Q-FC (Kraujalis et al. 2013). Moreover, three new QUENCHER methodologies were developed (Q-SRSC, Q-HRSC and Q-LPSC), which were adapted from validated classical FRSC methods (Rivero-Pérez et al. 2007).

The main modification from other Q-TAC assays was the selection of aqueous reaction mediums whenever the methodologies allowed for the use of both organic and aqueous solvents. Aqueous medium might better represent the real environment surrounding any food matrix or biological system. Therefore, MQ water or aqueous buffer was initially used in all protocols except in the Q-DPPH assay, which required an organic solvent to dissolve the radical.

The particle size of the powdered products is a factor that might influence the results of the Q-TAC and Q-FRSC measurements, and this aspect should be taken into account for comparisons among powders of different particle size. Nevertheless, Serpen et al. (2008) did not find a remarkable effect of this factor, and Gökmen et al. (2009) suggested the use of powdered samples of particle size ranging from 0.3 to 0.1 mm in QUENCHER assays.

All protocols in this study were optimized for the direct measurement of small quantities of powdered products, considering the final reaction volume and amount of sample weighed.

The Q-FC, Q-FRAP, Q-ABTS, and Q-DPPH methods are rather inexpensive and simple methodologies which allow for the adjustment of the assay volumes (maintaining the proportionality among the reagents) without a relevant increase in the cost of the analysis.

Preliminary studies were conducted to establish 1 mg as an advisable amount of product, although satisfactory results can also be obtained with smaller or larger quantities (0.2-5 mg), depending on the TAC of the products and the sensitivity of the assay.

In the case of the Q-ORAC method, the reaction volume is restricted by the maximum volume of the cuvette because the fluorescence decay needs to be continuously monitored (kinetic assay). Amigo-Benavent et al. (2010) proposed that the Q-ORAC assay be carried out in alternating stages of incubation at 37 °C in a test tube with centrifugation, transfer to a cuvette, and fluorescence reading. The Q-ORAC method proposed in the present study avoids several centrifugation/transfer steps, is more similar to classical protocols, and reproduces more effectively the advantages of the ORAC assay with respect to other TAC methods (Karadag et al. 2009; Niki 2010; Fraga et al. 2014). However, a fluorometer with a temperature control chamber and constant stirring in the cuvette is required, and the assay might not be suitable for products of very high Q-TAC.

With regard to the Q-SRSC, QHRSC, and Q-LPSC methods, the final reaction volume and amount of sample weighed must be adjusted to give results in the range of 0-100% oxidation inhibition. The Q-SRSC assay is a rather simple and quick methodology that can be directly performed in the cuvette or in a test tube. However, the Q-HRSC and the Q-LPSC assays involve more tedious protocols. It is suggested that the amount of sample used be adjusted rather than increasing the final volume, although it must be remarked that the precision of the QUENCHER methods might be compromised when < 0.2 mg of the samples is weighed.

QUENCHER fixed end-point procedures allow for transferring an aliquot of the reaction medium to a microplate for the absorbance reading step. This alternative increases the repeatability and reduces the time between different measurements, so it is especially appropriate for the simultaneous analyses of multiple samples. On the other hand, QUENCHER kinetic assays cannot be carried out directly in a microplate due to the interference of solid products on the absorbance measurement. Furthermore, the extremely small quantity of solid sample required, proportional to the small volume of wells, could put at risk the validity of this procedure.

3.2. Validation of the Q-TAC and Q-FRSC Methods

The validation study was done by the examination of the following parameters: linearity, proportionality, detection limits and precision (repeatability and intermediate precision) (García et al. 2003). In the case of Q-FRSC assays (Q-SRSC, Q-HRSC, and Q-LPSC), only the precision was evaluated because in these methods a calibration curve is not needed. In the Q-TAC methodologies (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH, and Q-ORAC), a dose-response curve was obtained by testing different amounts of the standards. The calibration was performed using linear models and the least-squares (LS) method. All of the models presented coefficient of determination (R^2) values > 0.995 (**Table 1**).

Table 1. Calibration models of the QUENCHER total antioxidant capacity (Q-TAC) methods.

Method	Standard [a]	Range (µmol)	calibration model [b]	R^2
Q-FC	GA	0.059 - 0.588	$A_{750\ nm}$ = (1.79 ± 0.009)* µmol GA + (0.005 ± 0.004)	0.999
Q-FRAP	Fe(II)	0.036 - 0.285	$A_{593\ nm}$ = (2.76 ± 0.046) * µmol Fe(II) - (0.009 ± 0.008)	0.996
Q-ABTS	T	0.083 - 0.499	$(A_{WS} - A_S)_{734\ nm}$ = (1.20 ± 0.015) * µmol T + (0.007 ± 0.005)	0.998
Q-DPPH	T	0.040 - 0.200	$(A_{WS} - A_S)_{517\ nm}$ = (3.20 ± 0.033) * µmol T + (0.007 ± 0.004)	0.999
Q-ORAC	T	0.010 - 0.050	Net AUC = (3361 ± 70.3) * µmol T + (4.78 ± 2.25)	0.996

a) GA: gallic acid; T: Trolox.

b) A: Absorbance; A_{WS}: Absorbance of the radical working solution; A_S: Absorbance of the sample; Net AUC: Net Area under the curve.

c) R^2: coefficient of determination.

Once the parameters were estimated, the models were validated by verifying the assumptions related to the residuals and the functions (Ortiz et al. 2009). The studentized residuals were normally distributed (Kolmogorov-Smirnov, chi-square, and Shapiro-Wilk tests), independent (Durbin-Watson test) and homoscedastic (Cochran and Bartlett tests) in all cases. The functional part of each linear model was first validated in a linearity test. According to the results (**Table 2**), all the models were suitable for describing the linear relationship between the standard concentration and the response of each assay. The Q-FC method obtained the best values for all of the parameters tested, whereas the Q-ORAC assay linearity response was the least satisfactory, although within the acceptance criteria. In addition, a proportionality test to determine whether the mathematical model could be used for predictive purposes was performed (**Table 2**). The conditions of proportionality were corroborated for all the methods except Q-ORAC, where the ordinate at origin did not meet any of the criteria. In this assay the Q-TAC is assessed by the net AUC determination and a lag time is observed during oxidation of the fluorescein in the presence of an antioxidant (Prior et al. 2005; Ou et al. 2001). The unsatisfactory result of the ordinate at origin is explained by the lack of proportionality of this lag time at very small amounts of Trolox. However, a linear response was observed at higher quantities of the standard, as previously observed by Ou et al. (2001) in the classical approach of this assay.

Having studied the acceptability of the linear models, the detection limits y_d (detection signal) and x_d (capability of detection) of the assessed methodologies were determined as described by Ortiz et al. (2009). In general, low limits of detection were observed (**Table 2**), which confirmed the acceptability of all methodologies to determine the TAC of very small quantities of antioxidants. Among the assays using Trolox as standard, the lowest x_d value corresponded to the Q-ORAC assay, which was corroborated as a highly sensitive methodology, and the highest limits of detection were obtained by the Q-ABTS method. A similar observation was previously reported for a comparison of classical TAC methodologies (Rivero-Pérez et al. 2007).

Table 2. Linearity Test, Proportionality and Detection limits of the QUENCHER total antioxidant capacity (Q-TAC) methods.

Method	linearity test [a]				proportionality test [b]				detection limits [c]	
	CV (Fr) (%)	CV (b) (%)	ANOVA F_{exp}	ANOVA F_{tab}	$a \pm t_{tab}$ S(a)	t_{cal}(a)	t_{cal}(b)	t_{tab}	y_d [d]	x_d [e]
Q-FC	2.99	0.48	42730	4.38	0.005 ± 0.006	1.45	207	1.73	0.016	12.0 nmol GAE
Q-FRAP	4.79	1.68	3537	4.67	-0.009 ± 0.015	1.12	59.5	1.77	0.012	15.3 nmol Fe(II)E
Q-ABTS	3.27	1.25	6328	4.49	0.007 ± 0.009	1.49	80.2	1.75	0.020	20.4 nmol TE
Q-DPPH	2.57	1.03	9384	4.67	0.007 ± 0.008	1.53	96.1	1.77	0.017	6.68 nmol TE
Q-ORAC	4.86	2.10	2271	4.97	4.78 ± 4.09*	2.12*	47.7	1.81	10.1	3.08 nmol TE

a) CV(Fr): Coefficient of variation of the response factor; CV(b): Coefficient of variation of the slope; ANOVA by means of the F-test (F_{exp}: experimental F ; F_{tab}: tabulated F(1, n-2, 0.05)). Linearity test acceptance criteria: CV(Fr) < 5 %; CV(b) < 2 %; ANOVA $F_{exp} > F_{tab}$.

b) $a \pm t_{tab}$ S(a): Confidence interval of the ordinate at origin; t_{cal}: calculated Student's t; t_{tab}: tabulated Student's t (n-2, 0.05). Proportionality acceptance criteria: interval should include 0; $t_{cal}(a) < t_{tab}$; $t_{cal}(b) > t_{tab}$. *: Results do not meet the acceptance criteria.

c) y_d: Detection signal; x_d: Capability of detection. Detection limits calculated for α and β = 0.05.

d) y_d units: Absorbance units (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH); Net area under the curve (Net AUC)(Q-ORAC).

e) GAE: gallic acid equivalents; Fe(II)E: iron(II) equivalents; TE: Trolox equivalents.

Finally, two different terms of the precision were evaluated, the repeatability (Rp) and the intermediate precision (IP), determining their estimates (S_r and S_R, respectively) and coefficients of variance (CV) (García et al. 2003). The IP refers to the precision under reproducibility conditions when only some of the factors are varied. In this study a single factor (different days) was investigated. The results of the Rp and IP experiments for each methodology are shown in **Table 3**. It can be seen that similar S_r values were obtained in both Rp and IP experiences for all of the methods. The CV of the assays was the criteria selected to evaluate their acceptability. The Rp of the longest protocols (Q-ORAC, Q-HRSC, and Q-LPSC) exceeded the CV < 5% cutoff value, whereas those assays with just one or two steps (Q-FC, Q-ABTS, and Q-DPPH) showed a high Rp between measurements. By comparinson with the Rp obtained in classical protocols validated using different wines as samples,(Rivero-Pérez et al. 2007) slightly higher CV were observed in the Q-TAC, and especially in the Q-FRSC methods, as all classical assays obtained CV < 5%. The only exception was the Q-ABTS assay, which showed better Rp than the classical approach. In terms of IP (**Table 3**), all of the methodologies presented satisfactory results, with the lowest CV (%) obtained again by two of the simplest methods (Q-FC and Q-ABTS). Therefore, the differences in the Rp and IP among the methodologies were mainly attributed to the complexity of the protocols. The sensitivity of the assays could also affect the precision observed, as might be the case for the Q-ORAC method mentioned above.

Table 3. Precision of the QUENCHER total antioxidant capacity (Q-TAC) and QUENCHER free radical scavenging capacity (Q-FRSC) methods.

Method	Units of S_r and S_R [a]	Repeatability			Intermediate Precision	
		CV (%) [b]	$S_{r\ (Rp)}$ [c]	$S_{r\ (IP)}$ [d]	CV (%) [e]	S_R [f]
Q-FC	µmol GAE/g product	2.06	1.23	1.17	2.37	1.49
Q-FRAP	µmol Fe(II)E/g product	4.98	10.8	10.7	6.48	16.0
Q-ABTS	µmol TE/g product	2.13	2.24	2.73	3.05	3.26
Q-DPPH	µmol TE/g product	3.12	1.24	1.26	3.85	1.59
Q-ORAC	µmol TE/g product	5.45*	2.94	2.79	5.48	3.01
Q-SRSC	Oxidation inhibition %	3.27	1.05	1.46	6.35	1.62
Q-HRSC	Oxidation inhibition %	5.31*	2.79	3.09	6.32	3.28
Q-LPSC	Oxidation inhibition %	5.26*	4.01	4.44	6.35	4.87

a) GAE: gallic acid equivalents; Fe(II)E: iron(II) equivalents; TE: Trolox equivalents.

b) Repeatability acceptance criteria: CV < 5%. *: Results do not meet the acceptance criteria.

c) Estimate of repeatability (S_r) determined by One-Variable analysis of seven replicates one day (Rp: Repeatability experiment).

d) Estimate of repeatability (S_r) determined by One-Way ANOVA of three replicates in three different days (IP: Intermediate precision experiment).

e) Intermediate precision acceptance criteria: CV < 10%.

f) Estimate of intermediate precision (S_R) determined by One-Way ANOVA of three replicates in three different days (IP: Intermediate precision experiment).

3.3. Q-TAC and Q-FRSC of the Model Products

To provide comprehensive information on the actual TAC of food, it is recommended that at least two TAC assays be used (Pérez-Jiménez et al. 2008; Karadag et al. 2009). Similarly, in this study, a broad overview of the antioxidant capacity of the three model products (Sk, W, and Sd) was achieved with the different QUENCHER methodologies under study.

As shown in **Table 4**, model product W (rich in both hydrophobic and hydrophilic antioxidant compounds) showed the highest Q-TAC and Q-FRSC values in most of the methods, with the exception of the Q-FRAP assay, where Sk (model product especially rich in hydrophilic antioxidant) presented the most elevated antioxidant capacity, and the Q-DPPH assay, where Sd (model product especially rich in hydrophobic antioxidant) exhibited the highest Q-TAC values. The difference between the antioxidant capacity of Sk and W was not significant in the Q-FC and Q-ORAC protocols. No significant differences were found between Sk and Sd in the Q-ABTS and Q-SRSC assays. From a comparison of the responses obtained by the three methodologies where Trolox was used as standard, Q-ORAC obtained the highest TAC values, but it must be noted that the sensitivity of this method to Trolox was also more elevated than in the Q-DPPH and Q-ABTS assays.

Table 4. QUENCHER total antioxidant capacity (Q-TAC) and QUENCHER free radical scavenging capacity (Q-FRSC) of the model products obtained from skins (Sk), seeds (Sd) and whole (W) wine pomace.[a]

Method	Units of Q-TAC [a]	Sk		W		Sd	
		Q-TAC	CV (%)	Q-TAC	CV (%)	Q-TAC	CV (%)
Q-FC	μmol GAE/g product	74.4 ± 1.1 b	1.43	78.3 ± 2.4 b	3.02	52.6 ± 2.5 a	4.80
Q-FRAP	μmol Fe(II)E/g product	275 ± 4 c	1.52	224 ± 14 b	6.05	140 ± 5 a	3.52
Q-ABTS	μmol TE/g product	120 ± 5 a	4.33	150 ± 3 b	1.78	120 ± 0.8 a	0.69
Q-DPPH	μmol TE/g product	51.9 ± 1.1 a	2.11	110 ± 4 b	3.27	174 ± 4 c	2.39
Q-ORAC	μmol TE/g product	236 ± 10 b	4.19	243 ± 4 b	1.72	181 ± 8 a	4.39
Q-SRSC	Oxidation inhibition %	31.7 ± 1.2 a	3.76	50.5 ± 1.9 b	3.72	30.1 ± 1.2 a	3.98
Q-HRSC	Oxidation inhibition %	50.1 ± 2.6 a	5.17	59.0 ± 0.6 c	1.08	53.8 ± 1.1 b	2.01
Q-LPSC	Oxidation inhibition %	52.2 ± 2.6 b	4.92	62.5 ± 3.4 c	5.41	33.7 ± 2.0 a	7.27

a) Q-TAC and Q-FRSC values are the mean ± standard deviation (n = 3). Latin letters indicate significant differences among the model products (Sk, W, Sd) within each QUENCHER methodology.

b) GAE: gallic acid equivalents; Fe(II)E: iron(II) equivalents; TE: Trolox equivalents.

The obtained results were contrasted with those obtained by classical determinations and considerable differences were found, except with the Q-DPPH method. Several studies comparing extracts from wine pomace reported a higher antioxidant capacity exhibited by seeds than skins, obtaining the same tendency using different assays: FC (Cheng et al. 2012; Ky et al. 2014; Makris et al. 2007; Rockenbach et al. 2011); FRAP (Rockenbach et al. 2011; Makris et al. 2007; Ky et al. 2014); ABTS (Ky et al. 2014); DPPH (Cheng et al. 2012; Ky et al. 2014; Makris et al. 2007; Rockenbach et al. 2011); ORAC (Ky et al. 2014); SRSC (Cheng et al. 2012); and, HRSC (Makris et al. 2007). This fact may be explained because classical protocols involve extraction with solvents, which are capable of extracting both hydrophobic and hydrophilic antioxidants. Therefore, the extracts obtained might not be representative of the antioxidant capacity that solid foods could actually exert, whereas QUENCHER assays may provide more reliable information (Gökmen et al. 2009).

3.4. Correlation Study between the QUENCHER Methodologies

Pearson's correlation coefficients (r) between pairs of the assays were determined using the Q-TAC and Q-FRSC values obtained for Sk, W, and Sd by the different QUENCHER methodologies.

In the literature, due to the wide spectrum of analytical techniques and reaction conditions available, it is difficult to find an agreement on the correlations among different TAC methodologies (Huang et al. 2005; Karadag et al. 2009; Niki 2010). To the extent of our knowledge, the correlation between different QUENCHER methodologies has not been described yet. In this study, an interesting correspondence among the assays was observed, especially for the Q-FC and Q-ABTS assays with the rest of methods. A significant (p<0.01)

positive correlation was detected between Q-FC:Q-FRAP (r = 0.847), Q-FC:Q-ORAC (r = 0.974) and Q-FC:Q-LPSC (r = 0.926). The correspondence between Q-FC:Q-FRAP is due to the fact that both methods measure the reducing capacity of the sample (Huang et al. 2005), also detected between the classical approach (Ky et al. 2014; Rockenbach et al. 2011), Furthermore, the significant correlation between Q-ORAC:Q-LPSC (r = 0.914) may be explained because peroxyl radicals are involved in both assays (Becker et al. 2004). The positive correlation of the Q-FC with the Q-ORAC and Q-LPSC assays is very convenient as they are more biologically relevant methods, but these assays are also more complicated and their validation study showed some unsatisfactory results. However, the obtained results contrast with the weaker correlation (R^2 = 0.409) found between the classical approaches of the FC and the ORAC assays by Ky et al. (2014), using also wine pomace as samples. In the present study, interesting high positive correlations (p<0.01) between Q-ABTS:Q-SRSC (r = 0.935) and Q-ABTS:Q-HRSC (r = 0.903) were also observed. According to Rivero-Pérez et al. (2007), such strong correlations were not detected in the classical approaches of these assays using wines as samples. On the other hand, negative correlations (p<0.05) between Q-DPPH:Q-FC (r = -0.781), Q-DPPH:Q-FRAP (r = -0.986), and Q-DPPH:Q-ORAC (r = -0.808) were found in the present study, and the Q-DPPH method was not significantly correlated with any of the biologically relevant Q-FRSC assays. In classical methodologies, the DPPH and HRSC assays were not found to be correlated either (Makris et al. 2007), but high positive correlations were usually detected between the DPPH and other TAC methods: FC (Ky et al. 2014; Cheng et al. 2012; Rockenbach et al. 2011); FRAP (Rockenbach et al. 2011; Makris et al. 2007); and, ABTS (Rivero-Pérez et al. 2007). It must be noted that the Q-DPPH is the only QUENCHER methodology that was performed in an organic reaction medium, and its lack of positive correlation with the rest of the protocols indicates the elevated influence of the solvent used in these assays.

The different contents of hydrophilic and lipophilic compounds (Yu & Ahmedna 2013; Arranz et al. 2010) of the three model product used in this study allow for assessing the response of each method towards matrices with compounds of high or low water solubility. Anyway, it would be advisable to confirm the correlations found in the current study by evaluating a wider range of products.

With the results of both the validation and correlation studies taken into account, the combination of both Q-FC and Q-ABTS assays is suggested as a good selection for a general Q-TAC analysis of powdered foods. The Q-SRSC, Q-HRSC, and Q-LPSC methods, first adapted to the QUENCHER approach in this study, might give a more biologically relevant overview of the antioxidant capacity of the samples. However, the Q-HRSC and Q-LPSC assays are complicated protocols for routine analysis, and it has been shown that the combination of the Q-FC and Q-ABTS methods covers the correspondence with the results obtained by the three biologically relevant Q-FRSC assays.

3.5. Effects of the Reaction Medium in the Q-FC and Q-ABTS Assays

In the QUENCHER methods there is no extraction step, but the reaction medium is a key factor that determines the solubility of the compounds present in the solid powdered product, as well as the interactions between the antioxidants and the radicals used as probes in the assays.

Serpen et al. (2012a) proposed a water:ethanol (H_2O:EtOH) 50:50 (v/v) ratio as a suitable working solution, providing accurate Q-TAC results for most of the food matrices studied. Therefore, the effect of using water or a mixture H_2O:EtOH 1:1 (v/v) as reaction medium was assessed in the current study using the two previously selected Q-TAC methods (Q-FC and Q-ABTS).

The Q-FC (H_2O:EtOH) and Q-ABTS (H_2O:EtOH) assays were validated as previously described for the rest of the methodologies and presented satisfactory results for all parameters evaluated (**Supporting Information 1**). Slight formation of white solids was observed in the Q-FC (H_2O:EtOH) method, probably due to the precipitation of Na_2CO_3 in the presence of ethanol. Nevertheless, these solids did not interfere in the measurement. The responses of the Q-FC and Q-FC (H_2O:EtOH) assays to different standard concentrations were not significantly different (similar slopes in the calibration models), whereas a 2.54 times lower response to Trolox in the Q-ABTS than in the Q-ABTS (H_2O:EtOH) method was detected. In contrast, Serpen et al. (2012a) found similar slopes of the dose-response lines obtained in the Q-ABTS assays using different solvent ratios H_2O:EtOH (0:100, 25:75, 50:50, 75:25, 100:0). A possible explanation might be that the calibration curves of Q-TAC methodologies carried out in this study were performed by weighing directly the standards, without their previous dilution in any solvent. Thus, the lower solubility of Trolox in water than in organic solvents played a critical role in the response observed in each method, as previously described by other authors (Pérez-Jiménez & Saura-Calixto 2006). This fact may lead to lower Q-TAC values in the Q-ABTS than in the Q-ABTS (H_2O:EtOH) assay due intrinsically to the methods used rather than the samples analyzed. Therefore, as the numeric Q-TAC values obtained by the Q-ABTS assays may not be directly comparable, the tendency observed among the samples (higher or lower Q-TAC values) was the main interest of these analyses.

The Q-TAC of Sk, W, and Sd obtained using the Q-FC and Q-ABTS assays can be seen in **Figure 1**. As previously described for classical methods (Becker et al. 2004; Prior et al. 2005), the reaction medium greatly influenced the Q-TAC results, with both soluble compounds and insoluble antioxidants attached to the powdered products being affected by the solvent used in the Q-TAC assays. A similar tendency was observed in both Q-FC and Q-ABTS assays when water was replaced by a mixture of H_2O:EtOH (1:1, v/v) as reaction medium.

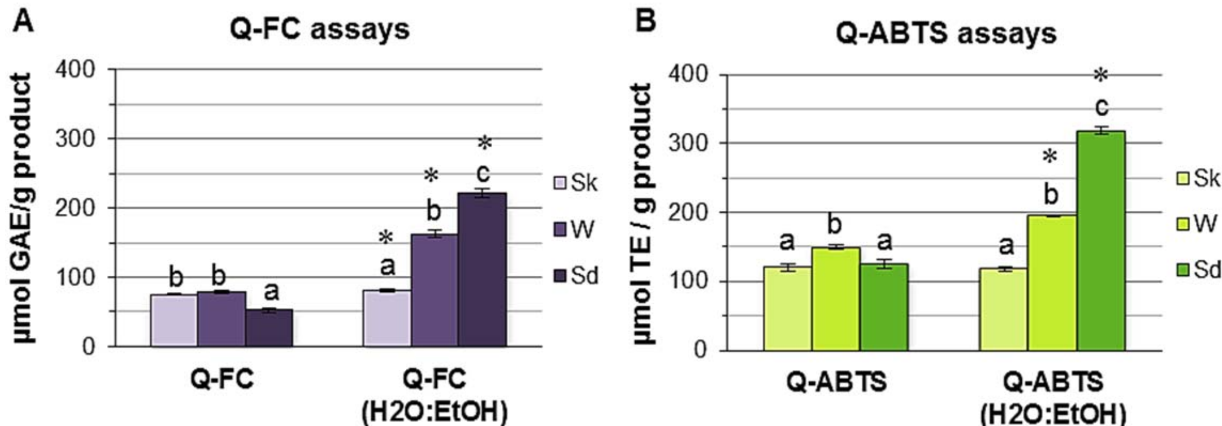

Figure 1. QUENCHER total antioxidant capacity (Q-TAC) of the model products (Skins: Sk; Whole: W; Seeds: Sd). **A**) Q-FC assays. **B**) Q-ABTS assays. GAE: gallic acid equivalents; TE: Trolox equivalents. Data expressed as mean values ± standard deviation (n=3). Roman letters indicate significant differences among the model products (Sk, W, Sd) within each assay. The asterisk (*) refers to significant differences between the Q-TAC and Q-TAC (H_2O:EtOH) approaches for each wine pomace product.

A significant increase in the Q-TAC values of Sd was observed, reaching higher antioxidant capacity than W, for which Q-TAC was also enhanced, but to a lesser extent. The Sk product showed the lowest Q-TAC in both Q-FC (H_2O:EtOH) and Q-ABTS (H_2O:EtOH) assays. Similar results were obtained in previous studies(García-Lomillo et al. 2014) carried out using conventional methodologies including extraction steps with organic solvents. Indeed, a good correlation between general results obtained using Q-TAC (H_2O:EtOH) and classical methods was observed. In contrast, this fact was only found between Q-TAC (carried out in water) and classical methods when the matrices under study were rich in hydrophilic antioxidants (**Supporting Information 2**).

Differences among results obtained for the three model products using the different Q-TAC methods can be partly explained by their chemical composition, the solubility of their antioxidant compounds in the reaction medium, and their redox reactions with the radical probes or reagents used in each assay. With regard to the chemical composition of the model products tested, it is important to have in mind that skins of grapes are a rich source of anthocyanins and hydroxycinnamic acids, although they also contain some flavanols and flavonol glycosides, whereas gallic acid, flavanols and proanthocyanidins are mainly present in the seeds of grapes (Kammerer et al. 2004; Cheng et al. 2012; Rockenbach et al. 2011). According to these facts, the Sk model product was rich mainly in anthocyanins and hydroxycinnamic acids, whereas Sd was rich in flavanols from monomers to polymers (proanthocyanins and tannins). A description of the solubility of food antioxidants, including main phenolic classes, can be found in Gökmen et al. (2009) In general, the water solubility of polyphenols increases with the number of glycosylated hydroxyl groups and with the amount of sugars constituting the carbohydrate moiety. On the other hand, in the case of

polymerized polyphenols (such as proanthocyanidins or tannins), the water solubility considerably drops with an increased number of units and with the formation of intramolecular bonds (Hemingway 1989). Thus, the higher polarity of the polyphenols found in the grape skins allows that they are well solubilized when water is used as reaction medium (Hemingway 1989), whereas the presence of ethanol seems to favour the extraction of hydrophobic antioxidants present in the grape seeds. It must be pointed out that lower H_2O:EtOH ratios are not advised because it may cause the shrinking of the major constituents of the food matrices, such as cellulose and proteins (Serpen et al. 2008; Serpen et al. 2012a). This could hamper the release of the compounds or the diffusion of the radicals into the solid matrices where they can react with inner bound insoluble antioxidants, leading to lower Q-TAC values (Gökmen et al. 2009).

The reaction medium also affects the interactions between the antioxidants and reagents involved in the methods by enhancing or decreasing the equilibrium constant of their redox reactions. In the QUENCHER approaches, when the redox equilibrium constant of an antioxidant compound in a determinate medium is higher than its solubility constant, the extraction of this antioxidant from the insoluble matrix and its solubilization in the reaction medium can be thermodynamically enhanced (Serpen et al. 2012a).

The study of the correlations between the Q-TAC assays carried out in the same reaction medium showed a strong correspondence ($p<0.01$) between Q-FC (H_2O:EtOH):Q-ABTS (H_2O:EtOH) (r = 0.971), whereas there was no significant correlation between Q-FC:Q-ABTS (r = 0.496). This finding shows that, in the Q-TAC (H_2O:EtOH) methods, the factors related to the assay reaction medium (solubilization of the antioxidant compounds) play a more critical role than those related to the method used (reaction with the probes or reagents). Therefore, the mixture H_2O:EtOH 50:50 (v/v) proposed by Serpen et al. (2012a) might be adequate to evaluate the global TAC of food materials, but the Q-TAC of hydrophobic antioxidants could be overestimated under certain conditions and, then, their real antioxidant role in food matrices.

Moreover, the correlations between the biologically relevant Q-FRSC assays and both Q-TAC (H_2O:EtOH) methods were not significant in the case for the Q-SRSC and Q-HRSC methods, and even a significant ($p<0.01$) negative correlation between Q-LPSC:Q-ABTS (H_2O:EtOH) (r = -0.716) was found. These results further support the selection of the Q-FC and Q-ABTS assays in water as preferred protocols to assess a potentially more biologically relevant Q-TAC of food products and dietary supplements.

3.6. Effects of Using Cellulose as a Diluting Agent in the Q-ABTS Assays

Cellulose has been suggested as a diluting agent in previous Q-TAC protocols (Serpen et al. 2007; Gökmen et al. 2009). However, the affinity of polyphenols to cellulose is well-known (Tang et al. 2003), so it is possible that interactions between the cellulose and these antioxidant compounds interfere in the quantification.

The possible influence of cellulose on the Q-TAC measurements was evaluated with the Q-ABTS assays (the most widely used Q-TAC assay up to now), and it was compared with the effects of weighing different amounts of sample. The two model products that presented more differences between them (Sk and Sd) were chosen to carry out this study. Two dilutions or product:cellulose ratios (2:1 and 1:2) were used. The quantities of product weighed were adapted to those required by each method to give results within their detection range. As can be seen in **Table 5**, no influence of the amount of sample or the presence of cellulose on the Q-TAC values was observed for Sk in the Q-ABTS assay and for Sd in the Q-ABTS (H_2O:EtOH) method. However, Q-ABTS results of Sd showed significantly higher Q-TAC values when lower amounts of sample were used, and this effect was more marked when the product was diluted with cellulose. In contrast, the opposite tendency was found for Sk in the Q-ABTS (H_2O:EtOH) assay. In this case, inferior Q-TAC values were obtained when a lower amount of Sk was used, which were only significant in Sk:cellulose (1:2) respect to the nondiluted Sk sample.

Table 5. Influence of the amount of sample weighed and the use of cellulose (C) as a diluting agent in the Q-ABTS assays evaluated in model products obtained from skins (Sk) or seeds (Sd) separated from red wine pomace.[a]

Method	Q-TAC (µmol TE/g product)				
Q-ABTS	**Sk 3 mg**	**Sk 2 mg**	**Sk 1 mg**	**Sk:C (2:1) 3 mg**	**Sk:C (1:2) 3 mg**
	116 ± 4	119 ± 3	122 ± 4	121 ± 2	118 ± 8
	Sd 3 mg	**Sd 2 mg**	**Sd 1 mg**	**Sd:C (2:1) 3 mg**	**Sd:C (1:2) 3 mg**
	126 ± 5 a	139 ± 6 b	148 ± 3 c	153 ± 3 c	167 ± 5 d
Q-ABTS (H_2O:EtOH)	**Sk 1.5 mg**	**Sk 1 mg**	**Sk 0.5 mg**	**Sk:C (2:1) 1.5 mg**	**Sk:C (1:2) 1.5 mg**
	124 ± 4 cb	131 ± 5 c	116 ± 9 b	117 ± 4 b	93 ± 5 a
	Sd 0.75 mg	**Sd 0.5 mg**	**Sd 0.25 mg**	**Sd:C (2:1) 0.75 mg**	**Sd:C (1:2) 0.75 mg**
	314 ± 5	320 ± 9	318 ± 18	323 ± 10	320 ± 17

a) Q-TAC values are the mean ± standard deviation (n = 3). TE: Trolox equivalents. Roman letters indicate significant differences among the different amounts of sample and the dilutions with cellulose tested within each method (Q-ABTS or Q-ABTS (H2O:EtOH)) for each product (Sk or Sd).

The observed effect of weighing different amounts of Sd in the Q-ABTS assay could be due to a better water extraction of its slightly soluble hydrophobic polyphenols (Ky et al. 2014; Gökmen et al. 2009) when they are at a lesser concentration. Moreover, possible hydrophobic interactions between the antioxidant compounds (favoured in the aqueous medium) (Oh et al. 1980) could be more relevant when a higher amount of sample is present, thus decreasing their ability to scavenge the soluble $ABTS^{\cdot+}$ radicals present in the reaction medium.

With regard to the influence of cellulose in the measurements, absorbance values similar to those of the $ABTS^{\cdot+}$ working solution were obtained in both methods (Q-ABTS and Q-ABTS (H_2O:EtOH)) when only cellulose was used as sample (data not shown), corroborating that cellulose alone is inert toward the $ABTS^{\cdot+}$ reagent, as has been previously described (Serpen et al. 2007). Therefore, the results obtained in this study could be due to the effects of cellulose in the solubility of the hydrophilic or lipophilic compounds present in the products and their capacity to interact with the $ABTS^{\cdot+}$ radicals, both factors influenced by the reaction medium. Hydrogen-bonding and hydrophobic interactions between cellulose and polyphenols have been described (Tang et al. 2003), which are also dependent on the solvent systems used. Hydrophobic interactions are favoured in hydrophilic solvents, whereas hydrogen bonding is favoured in the more hydrophobic ones. Thus, the affinity of polyphenols (such as gallotannins and ellagitannins) to cellulose is expected to correlate with their hydrophobicity, their number of galloyl groups, and their molecular size (Tang et al. 2003; Hemingway 1989). This evidence explains that cellulose effects are of particular importance in the hydrophilic solvents when hydrophobic compounds are tested, as it happened when Sd was assessed by the Q-ABTS assay. The monomeric and oligomeric proanthocyanidins of the grape seeds might remain insoluble in aqueous reaction mediums (Yu & Ahmedna 2013; Arranz et al. 2010). However, their interaction with cellulose through their hydrophobic moieties might raise their water solubility and leave the hydroxyl groups available to interact with the $ABTS^{\cdot+}$ radicals, leading to the increased Q-TAC values observed when the product:cellulose ratio was decreased. On the other hand, in the case of Sk in the Q-ABTS (H_2O:EtOH) assay, the contrary effect may take place. Hydrogen bonding between cellulose and the hydrophilic phenolics present in wine pomace skins could be favoured in the presence of organic solvents. Thus, the number of hydroxyl groups available in the molecules to scavenge the radical probes of the assay is reduced.

On the basis of the cited results, the use of cellulose as a diluting agent is discouraged in the Q-TAC methodologies, especially if the antioxidant products tested are a source of polyphenols. The main motive is the possible interaction of cellulose with the phenolic compounds involved in the assays, leading to an under- or overestimation of the Q-TAC of the products.

4. Conclusion

A wide range of QUENCHER analytical techniques to provide a comprehensive initial assessment *in vitro* of the TAC and FRSC of food materials have been adapted and validated (Q-FC, Q-ABTS, Q-DPPH, Q-FRAP, Q-ORAC, Q-SRSC, Q-HRSC, and Q-LPSC). The Q-TAC and Q-FRSC assays skip the extraction step of the classical antioxidant capacity protocols and are simple, quick, inexpensive, and precise methodological approaches. The QUENCHER methodologies validated in this paper working with powdered model products are suitable for application to other foods and food ingredients, both rich in fat (as seeds) and not, as skins, which are rich in fibre and minerals among others. The combination of the Q-FC and Q-ABTS assays using water as reaction medium is proposed as the best choice to analyse a large number of samples. They provide more biologically relevant information than the classical TAC methods using extracts or other Q-TAC approaches using non-aqueous solvents. It is suggested that the same amount of sample be weighed, especially when products rich in hydrophobic antioxidants are assessed. The use of cellulose as a diluting agent is not recommended in the Q-TAC methods. Finally, it must be pointed out that, despite the advantages of the proposed QUENCHER assays over other *in vitro* methodologies, the Q-TAC or Q-FRSC of food materials and dietary supplements cannot be directly translated into healthful effects provided *in vivo*.

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Conflict of interest

The authors have no conflicts of interest to disclose.

Supplementary material

Supplementary data to this article (**Supporting information 1,** Validation of Q-FC (H_2O:EtOH) and Q-ABTS (H_2O:EtOH) methods; **Supporting information 2,** Correlations between classical TAC, Q-TAC (H_2O:EtOH) and Q-TAC methods.) iare shown below the references and can be found online at:
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Supporting Information 1. Validation of Q-FC (H_2O:EtOH) and Q-ABTS (H_2O:EtOH) methods.

Table S1. Calibration models of the QUENCHER total antioxidant capacity (Q-TAC) methods.

Method	Standard [a]	Range (µmol)	calibration model [b]	R^2
Q-FC (H_2O:EtOH)	GA	0.059 - 0.588	$A_{750\ nm}$ = (1.76 ± 0.008) * µmol GA + (0.004 ± 0.004)	0.999
Q-ABTS (H_2O:EtOH)	T	0.033 - 0.200	$(A_{WS} - A_S)_{734\ nm}$ = (3.06 ± 0.036) * µmol T - (0.005 ± 0.005)	0.998

a) GA: gallic acid; T: Trolox.
b) A: Absorbance; A_{WS}: Absorbance of the radical working solution; A_S: Absorbance of the sample.
c) R^2: coefficient of determination.

Table S2. Linearity Test, Proportionality and Detection limits of the QUENCHER total antioxidant capacity (Q-TAC) methods.

Method	linearity test [a]				proportionality test [b]				detection limits [c]	
	CV (Fr) (%)	CV (b) (%)	ANOVA F_{exp}	F_{tab}	a ± t_{tab} S(a)	t_{cal}(a)	t_{cal}(b)	t_{tab}	y_d [d]	x_d [e]
Q-FC (H_2O:EtOH)	3.88	0.49	41748	4.38	0.004 ± 0.006	1.03	204	1.73	0.015	12.2 nmol GAE
Q-ABTS (H_2O:EtOH)	4.18	1.16	7491	4.49	-0.005 ± 0.008	-1.06	86.6	1.75	0.007	7.56 nmol TE

a) CV(Fr): Coefficient of variation of the response factor; CV(b): Coefficient of variation of the slope; ANOVA by means of the F-test (F_{exp}: experimental F ; F_{tab}: tabulated F(1, n-2, 0.05)). Linearity test acceptance criteria: CV(Fr) < 5 %; CV(b) < 2 %; ANOVA $F_{exp} > F_{tab}$.
b) a ± t_{tab} S(a): Confidence interval of the ordinate at origin; t_{cal}: calculated Student's t; t_{tab}: tabulated Student's t (n-2, 0.05). Proportionality acceptance criteria: interval should include 0; $t_{cal}(a) < t_{tab}$; $t_{cal}(b) > t_{tab}$.
c) y_d: Detection signal; x_d: Capability of detection. Detection limits calculated for α and β = 0.05.
d) y_d units: Absorbance units.
e) GAE: gallic acid equivalents; TE: Trolox equivalents.

Table S3. Precision of the QUENCHER total antioxidant capacity (Q-TAC) and QUENCHER free radical scavenging capacity (Q-FRSC) methods.

Method	Units of S_r and S_R [a]	Repeatability			Intermediate Precision	
		CV (%) [b]	$S_{r\ (Rp)}$ [c]	$S_{r\ (IP)}$ [d]	CV (%) [e]	S_R [f]
Q-FC (H_2O:EtOH)	µmol GAE/g product	2.74	1.88	1.92	3.52	2.50
Q-ABTS (H_2O:EtOH)	µmol TE/g product	2.78	2.68	1.76	2.51	2.61

a) GAE: gallic acid equivalents; TE: Trolox equivalents.
b) Repeatability acceptance criteria: CV < 5%.
c) Estimate of repeatability (S_r) determined by One-Variable analysis of seven replicates one day (Rp: Repeatability experiment).
d) Estimate of repeatability (S_r) determined by One-Way ANOVA of three replicates in three different days (IP: Intermediate precision experiment).
e) Intermediate precision acceptance criteria: CV < 10%.
f) Estimate of intermediate precision (S_R) determined by One-Way ANOVA of three replicates in three different days (IP: Intermediate precision experiment).

<u>Supporting Information 2.</u> Correlations between classical TAC, Q-TAC (H2O:EtOH) and Q-TAC methods.

A) Including Sk, W and Sd products.

	Q-FC (H2O:EtOH)	Q-FC	Classical ABTS	Q-ABTS (H2O:EtOH)	Q-ABTS
Classical FC	**0.969** (9) 0.000	**-0.835** (9) 0.005	**0.998** (9) 0.000	**0.997** (9) 0.000	0.023 (9) 0.954
Q-FC (H2O:EtOH)		**-0.715** (9) 0.030	**0.971** (9) 0.000	**0.971** (9) 0.000	0.214 (9) 0.580
Q-FC			**-0.824** (9) 0.006	**-0.853** (9) 0.004	0.496 (9) 0.174
Classical ABTS				**0.996** (9) 0.000	0.050 (9) 0.899
Q-ABTS (H2O:EtOH)					0.005 (9) 0.991

B) Including only Sk and W products.

	Q-FC (H2O:EtOH)	Q-FC	Classical ABTS	Q-ABTS (H2O:EtOH)	Q-ABTS
Classical FC	**0.994** (6) 0.000	**0.827** (6) 0.043	**0.993** (6) 0.000	**0.994** (6) 0.000	**0.965** (6) 0.002
Q-FC (H2O:EtOH)		0.799 (6) 0.056	**0.987** (6) 0.000	**0.995** (6) 0.000	**0.973** (6) 0.001
Q-FC			**0.833** (6) 0.039	0.774 (6) 0.071	0.755 (6) 0.083
Classical ABTS				**0.989** (6) 0.000	**0.985** (6) 0.000
Q-ABTS (H2O:EtOH)					**0.978** (6) 0.000

Pearson's correlation coefficients
(Sample size)
P-Value (Significant correlations ($p < 0.05$) highlighted in bold)

Chapter 1.2.

The Effects of Heat Treatment on the Phenolic Composition and Antioxidant Capacity of Red Wine Pomace Seasonings

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Abstract

The impact of thermal processing on the phenolic profile and total antioxidant capacity (TAC) of powdered red wine pomace seasonings (RWPSs) obtained from different sources (seedless:Sk-S; whole:W-S; seeds:Sd-S) was assessed. High contents in anthocyanins, flavonol-3-*O*-glycosides, phenolic acids and flavan-3-ols were found in Sk-S, which showed the highest quantity of phenolic compounds, as measured by HPLC-DAD analysis. Flavan-3-ols and phenolic acids (mainly gallic acid derivatives) were the main compounds identified in Sd-S. Intermediate values were obtained for W-S, although usually closer to Sk-S. Reductions in the anthocyanidin and flavonol-3-ol contents mainly determined the effects of heating on the total phenolic contents (Sk-S:-29.4%; W-S:-28.0%; Sd-S:-5.78%), although high temperatures positively affected the phenolic acid and flavonol aglycon contents. Heat treatment also caused a slight but significant decrease in the TAC of RWPSs, as determined using the Folin-Ciocalteu and ABTS assays in extracts of the RWPSs, with Sd-S being the least affected. Using directly the powdered RWPSs as samples (QUENCHER approach), higher TAC reductions were detected, but they were less marked in Sk-S. Thus, there is little evidence against submitting RWPSs to thermal processing, as heating affects differently each type of phenolic compound and does not induce very severe TAC reductions in these seasonings.

Keywords: Phenolic profile; QUENCHER; Thermal processing; Total antioxidant capacity; Total polyphenol content; Winery by-products.

Abbreviations and nomenclature: **ABTS**, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid, **C-**, classical; **FC**, Folin-Ciocalteu; **HT**, heat-treated seasonings; **NT**, non-treated seasonings; **Q-,** QUENCHER; **Sd-S**, seasoning from wine pomace seeds; **Sk-S**, seasoning from wine seedless pomace; **TAC**, total antioxidant capacity; **W-S**, seasoning from whole wine pomace; **RWPS**, red wine pomace seasoning.

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Highlights:

- Heat treatment decreases total phenolic contents in wine pomace, but not severely.

- Anthocyanidins are the phenolic compounds most severely affected by high temperatures.

- An increase in phenolic acid and flavonol contents after heat treatment was observed.

- The TAC of extracts from wine pomace seeds was the least affected by heating.

- The TAC of powders from seedless wine pomace was the least affected by heat treatment.

1. Introduction

Wine pomace comprises the skins, seeds and stems left after pressing grapes in the winemaking process. This waste is characterised by a high polyphenol content because of the only partial extraction of these compounds into wine during winemaking (Sacchi et al. 2005; Delgado Adámez et al. 2012), although the different groups of phenolic compounds are not uniformly distributed among the components of wine pomace (Kammerer et al. 2004; Pinelo et al. 2006; Rockenbach et al. 2011). The phytochemicals present in wine pomace are known to have antioxidant, preservative and health-promoting effects in different biological and food systems (Andersen & Markham 2005; Fontana et al. 2013; Teixeira et al. 2014). As such, the recycling of winery by-products represents an opportunity to provide valuable raw materials for the pharmaceutical, cosmetics, nutraceuticals, and food industries, thereby contributing to reducing the costs and environmental impact linked to the disposal of these residues in wine producing regions (Arvanitoyannis et al. 2006; Teixeira et al. 2014).

Most of the polyphenol-rich products derived from winemaking residues are obtained by applying extraction techniques (Louli et al. 2004; Monagas et al. 2006). An alternative to such extractions is to obtain powdered products derived directly from winery by-products (Saura-calixto 1998; García-Lomillo et al. 2014). This strategy is being studied by our research group to develop wine pomace products with potential uses as seasonings and dietary supplements in the food and nutraceuticals industry (González-Sanjosé et al. 2013). However, the main shortcoming of the direct use of this winery waste is the presence of undesirable microorganisms (residual yeast and bacteria from the vinification process). A drying pre-treatment is usually applied to wet wine pomace to extend its storage stability until final transformation into the desired extracts (Pedroza et al. 2012; Spigno et al. 2007) or powdered products (Larrauri et al. 1997). However, the long-term stability of these products is questionable and pre-treatment may not be sufficient to reduce the microbial loads to acceptable values to guarantee subsequent food safety when the seasonings are incorporated into fresh foodstuffs. As such, an stabilization step was included in the manufacturing process of these seasonings (González-Sanjosé et al. 2013), selecting heat treatment at 90 ºC for 90 min as the most suitable method for achieving complete microbial inactivation (García-Lomillo et al. 2014).

The sensitivity of wine pomace polyphenols to high temperatures and the effect of this on total antioxidant capacity (TAC) have been subject of numerous research studies, most of which concerned wine pomace extracts (Chamorro et al. 2012; Davidov-Pardo et al. 2011; Larrauri et al. 1998; Monrad et al. 2010; Palma et al. 2001; Pinelo et al. 2005; Sólyom et al. 2014; Volf et al. 2014). However, only a few studies have investigated the detrimental or positive impact of submitting raw wine pomace materials to heat treatment (Chamorro et al. 2012; Khanal et al. 2010; Larrauri et al. 1997; Pedroza et al. 2012; Ross et al. 2011; Sólyom et

al. 2014), and none of these works has assessed products obtained from the skins, seeds and whole wine pomace, to determine the influence of these different matrices on the impact of high temperatures. In addition, the effects of heating wine pomace and derived products have been evaluated by classical (C-) TAC methodologies for extracts, but not using QUENCHER (Q-) approaches for wine pomace powders. In this regard, Q-TAC assays have been suggested as suitable methods for the assessment of foodstuffs in which insoluble materials may play an important antioxidant role (Gökmen et al. 2009; Del Pino-García et al. 2015).

In view of the above, this study aimed to characterize and assess the impact of heat treatment on the phenolic profile (individual compounds and main classes) and antioxidant capacity (using C-TAC and Q-TAC approaches) of different wine pomace seasonings.

2. Materials and Methods

2.1. Chemicals

Pure phenolic compound standards (caffeic acid, caftaric acid, catechin, coutaric acid, ellagic acid, epicatechin, ethyl gallate, fertaric acid, ferulic acid, gallic acid, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, myricetin, myricetin-3-*O*- rhamnoside, *p*-coumaric acid, *p*-OH-benzoic acid, procyanidin B1, procyanidin B2, protocatechuic acid, quercetin, quercetin-3-*O*-rutinoside, salicylic acid, syringic acid, *t*-resveratrol, *t*-piceid and vallinic acid) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anthocyanin standards (cyanidin chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride, peonidin chloride and petunidin chloride) and pelargonidin-3-*O*-glucoside were obtained from Extrasynthese (Genay, France). Unless otherwise stated, all other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Panreac (Barcelona, Spain) or Fisher Scientific (Loughborough, UK).

2.2. Samples

2.2.1. Red wine pomace seasonings (RWPSs)

Red wine pomace from the vinification of *Vitis vinífera* L. *cv. Tempranillo* was kindly supplied by several wineries situated in Burgos (Spain). All the wine pomace was mixed and dehydrated in an oven at a temperature of less than 60 ºC to a final moisture content of less than 10%. Dried materials were then separated, milled and sieved to obtain three RWPSs: one from seedless wine pomace (enriched in skins), named Sk-S; another from whole wine pomace, named W-S; and a third derived from the isolated seeds, named Sd-S. The particle size of the powdered products was less than 0.250 (Sk-S and W-S) and 0.355 mm (Sd-S). Some of the products were then heated at 90 ºC for 90 min (García-Lomillo et al. 2014). The non-treated products were identified as NT and the heat-treated products as HT.

2.2.2. RWPS extracts

Extracts from the different RWPSs were obtained by liquid extraction using MeOH:HCl 37% (97:3, v/v) as solvent. A 1 ± 0.005 g sample of each product was weighed in triplicate, hydrated and mixed with 15 mL of the solvent. After extraction at 25 °C for 24 hour with continuous stirring (90 rpm), the mixture was filtered and the residue washed twice with 5 mL of extraction solvent. The RWPS extracts were finally made up to 25 mL with the same solvent and stored at 4 °C until used.

Different extracts were obtained for the anthocyanidin analysis using the method described in Rodríguez-Mateos et al. (2014) for the extraction of these compounds. Thus, 0.5 ± 0.005 g of each product was extracted three times with 5 mL of acidified methanol (0.1% HCl in MeOH) and pelargonidin-3-*O*-glucoside (300 µL, 1 mM) added to the samples as recovery standard. They were then vortexed for 5 min, sonicated for 5 min in an ultrasonic bath, and centrifuged for 15 min at 1800g. The supernatants were combined, diluted 1:1 with 5 M HCl and heated at 90 °C for 1 h for anthocyanin hydrolysis.

2.3. HPLC phenolic compounds analysis

2.3.1. Phenolic acids, stilbenes, flavan-3-ols and flavonols

The RWPS extracts were analyzed according to a slightly modified version of the method described by Pérez-Magariño et al. (2008). Identification and quantification of phenolic compounds was carried out using analytical reversed-phase HPLC on an Agilent 1100 series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) coupled to a diode array detector. A Spherisorb3® ODS2 reversed phase C18 column (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia S.A., Barcelona, Spain) was used. The chromatographic conditions were as follows: flow, 0.6 mL/min; injection volume, 100 µL; mobile phases: A, water:glacial acetic acid (98:2, v/v); B, water:acetonitrile:glacial acetic acid (78:20:2, v/v/v); C, acetonitrile. The solvent gradient used was: 0-25 min, linear gradient from 0-100% to 25-75% of B in A; 25-60 min, linear gradient from 25-75% to 70-30% of B in A; 60-100 min, linear gradient from 70-30% to 100-0% of B in A; 100-120 min, 100% B; 120-130 min; linear gradient from 0-100% to 100-0% of C in B; 130-140 min, 100% C; 140-150 min; linear gradient from 100-0% to 0-100% of C in A. The eluent was monitored at 254, 280, 320, 360, and 520 nm, with compound spectra being obtained between 220 and 600 nm. Samples were injected in duplicate, and calibration was performed by injecting the standards three times at five different concentrations. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards. The results were expressed in µg/g seasoning.

2.3.2. Anthocyanidins

The RWPS extracts obtained for anthocyanidin analysis were identified and quantified using an Agilent 1100 series HPLC system (Agilent Technologies) equipped with a diode array detector. The column was a Nova-Pak reverse phase C18 (250 mm x 4.6 mm (id), 4 µm particle size; 30 °C; Waters LTd., Elstree, U.K.). The chromatographic conditions were as follows: flow, 0.8 mL/min; injection volume, 50 µL; Elution solvents: A, water:formic acid (99.9:0.1, v/v); B, acetonitrile. The solvent gradient used was: 0-20 min, linear gradient from 10-90% to 30-70% of B in A; 20-25 min, linear gradient from 30-70% to 80-20% of B in A; 25-30 min, 80-20% of B in A; 30-31min, linear gradient from 80-20% to 10-90% of B in A; 31-40 min, 10-90% of B in A. The eluent was monitored at 520 nm, with compound spectra being obtained between 220 and 600 nm. Samples were injected in duplicate, and calibration curves were obtained by injecting the anthocyanidin standards three times at five different concentrations. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards. The results were expressed as µg/g seasoning.

2.4. Total antioxidant capacity (TAC)

The classical (C-) and QUENCHER (Q-) versions of TAC assays were adapted to a 10 mL final reaction volume from the FC (Folin-Ciocalteu) method developed by Singleton and Rossi (1965) and the ABTS (2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) method described by Re et al. (1999).

2.4.1. FC assays

C-FC: A volume of 0.2 mL of the RWPS extracts was mixed with 0.2 mL of FC reagent and allowed to react for 5 min. Then, 4 mL of a 0.7 M sodium carbonate solution was added and the final volume was made up to 10 mL with Milli-Q (MQ) water. The tubes were incubated for 1 hour in an orbital shaker and the absorbance at 750 nm measured using a UV-vis spectrophotometer U-2000 (Hitachi, Ltd., Hubbardston, MA, USA). The FC index was expressed as µmol of gallic acid equivalents (GAE)/g of product using a linear calibration curve obtained with different quantities of the standard.

Q-FC: The protocol described in Del Pino-García et al. (2015), which is the same as the C-FC method but substituting the volume of RWPSs extracts by 1 ± 0.005 mg of RWPSs and 0.2 mL of MQ water, was applied.

2.4.2. ABTS assays

C-ABTS: A stock $ABTS^{\cdot+}$ solution was prepared by treating a 7 mM aqueous ABTS solution with 2.45 mM $K_2O_8S_2$ in a 1:1 (v/v) ratio. Before the assay, the stock $ABTS^{\cdot+}$ solution was diluted with MQ water to obtain a working solution with an absorbance value of 0.70 ± 0.02 at 734 nm. A volume of 0.2 mL of the RWPS extracts was then mixed with the $ABTS^{\cdot+}$ working solution to reach a final volume of 10 mL. The absorbance at 734 nm was measured after incubation fro 30 min in the dark with continuous stirring. The results were expressed as µmol of Trolox equivalents (TE)/g of product by means of a dose-response curve for different amounts of Trolox.

Q-ABTS: The method described in Del Pino-García et al. (2015), which is the same protocol as the C-ABTS assay but replacing the 0.2 mL of RWPS extracts with 1 ± 0.005 mg of RWPSs, was applied.

2.5. Statistical analysis

Statistical analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies Inc., Warranton, VA, USA). Data were subjected to a one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) to detect significant differences between the products (Sk-S, W-S, and Sd-S). Student's t-test was used to determine significant differences between NT and HT for each type of seasoning. Linear correlations were established by determining Pearson's correlation coefficients. All analyses were performed in triplicate. Three levels of significance were considered for all statistics: $p < 0.05$, $p < 0.01$, and $p < 0.001$.

3. Results

3.1. Identification and quantification of phenolic compounds

3.1.1. Differences in the phenolic profile between RWPSs (Sk-S, W-S, and Sd-S)

Table 1 shows the concentration of several phenolic compounds present in seasonings obtained from different wine pomace materials (seedless, whole, and seeds) before and after being submitted to heat treatment. As can be seen, the trends in the compositional variation between Sk-S, W-S, and Sd-S found in NT were generally similar to those observed in HT.

The total phenolic compounds identified in the HPLC analysis for the RWPS are given at the end of **Table 1**. The amount of such compounds in Sk-S was almost 1.2-fold higher than in W-S, and nearly 2.4- and 1.8-fold higher than in Sd-S for NT and HT, respectively.

Table 1. Phenolic compound contents (µg/g) in non-treated (NT) and heat-treated (HT) red wine pomace seasonings (RWPSs) (seedless: Sk-S; whole: W-S; seeds: Sd-S).

Phenolic compounds	RWPSs					
	Sk-S		W-S		Sd-S	
	NT	HT	NT	HT	NT	HT
p-OH-benzoic acid	7.73 ± 0.08 c	6.27 ± 0.12 γ	4.53 ± 0.09 b	4.01 ± 0.13 β	0.755 ± 0.165 a	0.523 ± 0.124 α
Salicylic acid	5.88 ± 0.68 a	18.9 ± 0.13 γ	6.15 ± 0.76 a	11.8 ± 0.7 β	7.48 ± 0.47 a	7.72 ± 0.23 α
Protocatechuic acid	15.7 ± 0.07 b	21.3 ± 0.16 γ	14.5 ± 0.4 ab	19.6 ± 0.4 β	14.1 ± 0.08 a	16.6 ± 0.2 α
Gallic acid	62.8 ± 0.4 a	73.1 ± 0.4 α	70.8 ± 1.5 b	97.0 ± 0.4 β	95.1 ± 0.3 c	127 ± 0.1 γ
Vanillic acid	27.3 ± 0.2 c	30.4 ± 0.3 γ	17.2 ± 0.13 b	20.3 ± 0.3 β	7.19 ± 0.19 a	9.43 ± 0.08 α
Syringic acid	60.3 ± 0.6 c	74.4 ± 3.5 γ	38.2 ± 1.6 b	48.0 ± 0.8 β	1.56 ± 0.26 a	2.87 ± 0.70 α
Ellagic acid	20.4 ± 0.03 b	21.4 ± 0.06 β	8.88 ± 0.15 a	10.6 ± 0.2 α	ND	ND
Ethyl gallate	19.2 ± 0.6 a	17.1 ± 0.13 α	39.6 ± 1.2 b	33.2 ± 0.9 β	65.0 ± 2.0 c	58.7 ± 2.0 γ
Total Hydroxybenzoic acids	219 ± 0.02 b	263 ± 3 γ	199 ± 5 a	244 ± 2 β	191 ± 2 a	223 ± 1.21 α
p-Coumaric acid	3.00 ± 0.07 a	5.85 ± 0.03 α	5.40 ± 0.32 b	8.78 ± 0.34 β	9.16 ± 0.36 c	9.14 ± 0.58 β
Caffeic acid	2.55 ± 0.42 a	3.40 ± 0.42 α	3.70 ± 0.26 a	4.75 ± 0.21 α	4.97 ± 0.10 b	5.52 ± 0.60 α
Ferulic acid	0.753 ± 0.314 a	1.18 ± 0.01 γ	0.653 ± 0.225 a	0.962 ± 0.037 β	ND	0.270 ± 0.115 α
Coutaric acid	8.99 ± 0.80 c	4.59 ± 0.34 β	5.22 ± 0.21 b	2.42 ± 0.32 α	3.05 ± 0.08 a	2.99 ± 0.02 α
Caftaric acid	13.1 ± 0.3 b	13.6 ± 0.4 β	8.71 ± 1.78 a	10.9 ± 1.3 β	9.44 ± 0.15 a	9.98 ± 0.88 α
Fertaric acid	4.91 ± 0.04 b	3.76 ± 0.25 β	1.99 ± 0.18 a	1.72 ± 0.21 α	ND	ND
Total Hydroxycinnamic acids	33.3 ± 2.0 b	32.4 ± 1.6 α	25.7 ± 2.5 a	29.6 ± 1.7 α	26.6 ± 1.9 a	27.9 ± 1.9 α
Total Phenolic acids	**252** ± 2 **b**	**295** ± 5 **γ**	**226** ± 8 **a**	**274** ± 3 **β**	**217** ± 0.6 **a**	**250** ± 2 **α**
t-resveratrol	2.41 ± 0.07 b	1.73 ± 0.13 α	1.81 ± 0.06 a	1.58 ± 0.014 α	ND	ND
t-piceid	1.88 ± 0.03 b	1.42 ± 0.05 β	1.05 ± 0.06 a	0.645 ± 0.050 α	ND	ND
Total Stilbenes	**4.29** ± 0.10 **b**	**3.16** ± 0.18 **β**	**2.86** ± 0.13 **a**	**2.23** ± 0.04 **α**	**ND**	**ND**

Results expressed as the mean value ± standard deviation (n = 3).
Roman letters: Significant differences (ANOVA) between NT seasonings for each phenolic compound.
Greek letters: Significant differences (ANOVA) between HT seasonings for each phenolic compound.

Table 1. *(Continuation)*

Phenolic compounds	RWPSs					
	Sk-S		W-S		Sd-S	
	NT	HT	NT	HT	NT	HT
Catechin	56.6 ± 1.3 a	47.7 ± 2.0 α	193 ± 2 b	172 ± 1.1 β	389 ± 6 c	380 ± 15 γ
Epicatechin	24.1 ± 2.2 a	20.0 ± 1.6 α	118 ± 3 b	88.0 ± 1.2 β	311 ± 3 c	268 ± 7 γ
Total Flavan-3-ols (monomers)	80.7 ± 0.9 a	67.7 ± 3.6 α	311 ± 6 b	260 ± 0.06 β	701 ± 9 c	649 ± 21 γ
Procyanidin B1	94.8 ± 3.6 a	93.0 ± 4.3 α	107 ± 1.0 b	118 ± 5 β	131 ± 6 c	144 ± 7 γ
Procyanidin B2	60.2 ± 9.0 a	39.2 ± 1.5 α	199 ± 5 b	152 ± 7 β	407 ± 17 c	339 ± 16 γ
Total Flavan-3-ols (dimers)	155 ± 5 a	132 ± 5.8 α	306 ± 4 b	270 ± 2 β	537 ± 11 c	483 ± 9 γ
Total Flavan-3-ols	**236** ± 4 **a**	**200** ± 9 **α**	**618** ± 9 **b**	**530** ± 2 **β**	**1238** ± 20 **c**	**1131** ± 13 **γ**
Kaempferol	3.09 ± 0.06 a	3.58 ± 0.09 α	3.18 ± 0.26 a	4.23 ± 0.107 β	2.87 ± 0.05 a	6.38 ± 0.07 γ
Quercetin	14.6 ± 0.4 a	19.0 ± 0.8 α	16.9 ± 0.16 b	18.5 ± 0.6 α	25.5 ± 0.2 c	22.1 ± 1.3 β
Myricetin	59.2 ± 2.0 c	69.5 ± 2.7 γ	51.3 ± 1.3 b	61.3 ± 1.16 β	20.1 ± 1.3 a	33.0 ± 0.97 α
Total Flavonol aglycones	76.9 ± 1.7 b	92.0 ± 2.0 γ	71.4 ± 0.8 b	84.0 ± 0.7 β	48.4 ± 1.4 a	61.5 ± 0.4 α
Kaempferol-3-O-rutinoside	129 ± 1.1 c	122 ± 2 γ	74.5 ± 0.07 b	77.4 ± 1.0 β	11.5 ± 0.6 a	13.2 ± 0.5 α
Kaempferol-3-O-glucoside	55.2 ± 0.4 c	51.6 ± 0.4 γ	34.8 ± 2.2 b	35.0 ± 0.4 β	14.2 ± 0.07 a	14.2 ± 0.11 α
Quercetin-3-O-rutinoside	26.0 ± 1.8 c	24.9 ± 0.4 γ	20.9 ± 0.7 b	19.9 ± 0.7 β	11.5 ± 0.6 a	11.2 ± 0.14 α
Myricetin-3-O-rhamnoside	53.0 ± 0.9 c	52.7 ± 2.5 γ	31.0 ± 2.3 b	35.4 ± 1.9 β	8.58 ± 0.32 a	11.0 ± 0.18 α
Total Flavonol-3-O-glycosides	263 ± 0.07 c	251 ± 5 γ	161 ± 4 b	168 ± 0.2 β	45.7 ± 0.4 a	49.5 ± 0.6 α
Total Flavonols	**340** ± 2 **c**	**343** ± 3 **γ**	**233** ± 5 **b**	**252** ± 0.8 **β**	**94.2** ± 0.99 **a**	**112** ± 1.0 **α**
Delphinidin	428 ± 21 c	301 ± 8 γ	280 ± 2 b	187 ± 10 β	18.6 ± 0.7 a	11.3 ± 0.6 α
Cyanidin	12.1 ± 1.7 a	6.30 ± 1.06 α	21.2 ± 5.4 a	9.47 ± 1.65 α	41.4 ± 1.5 b	35.3 ± 1.5 β
Petunidin	273 ± 13 c	118 ± 20 β	190 ± 4 b	88.7 ± 6.6 β	9.50 ± 0.39 a	8.71 ± 0.74 α
Peonidin	66.8 ± 0.3 c	30.9 ± 2.8 β	54.5 ± 1.0 b	30.2 ± 3.9 β	0.598 ± 0.139 a	0.192 ± 0.090 α
Malvidin	2452 ± 20 c	1577 ± 109 γ	1819 ± 17 b	1108 ± 58 β	78.7 ± 0.5 a	50.1 ± 5.1 α
Total Anthocyanidins	**3231** ± 48 **c**	**2034** ± 135 **γ**	**2366** ± 26 **b**	**1424** ± 43 **β**	**149** ± 0.8 **a**	**106** ± 3 **α**
Total	**4085** ± 14 **c**	**2892** ± 124 **γ**	**3484** ± 13 **b**	**2515** ± 49 **β**	**1764** ± 19	**1657** ± 7 **α**

Results expressed as the mean value ± standard deviation (n = 3). Roman letters: Significant differences (ANOVA) between NT seasonings for each phenolic compound. Greek letters: Significant differences (ANOVA) between HT seasonings for each phenolic compound.

With regard to the total compounds grouped in phenolic classes, it can be seen that anthocyanidins were the most abundant compounds detected in Sk-S, representing around 79.6% (NT) and 72.0% (HT) of the total. The concentration of these compounds in Sk-S was around 1.4-fold higher than in W-S, with more marked differences being found when compared to Sd-S (nearly 21.7- and 19.3-fold higher in NT and HT, respectively). Furthermore, Sk-S was also richer than W-S and Sd-S in flavonols, phenolic acids, and stilbenes. In contrast, the flavan-3-ols identified in Sd-S were the main contributors in these products, representing approximately 72.5% (NT) and 70.4% (HT) of all phenolic compounds. For W-S, anthocyanidins represented around 68.6% (NT) and 57.4% (HT), whereas flavan-3-ols represented about 17.9% (NT) and 21.4% (HT) of the total.

Considering the subgroups and individual phenolic compounds within the phenolic classes, hydroxybenzoic acids contributed more to the total phenolic acid content than hydroxycinnamic acids in all RWPSs. The total hydroxybenzoic and hydroxycinnamic acids contents both followed the same trend between RWPSs as that observed for total phenolic acids (Sk-S > W-S = Sd-S for NT; Sk-S > W-S > Sd-S for HT), with the exception of the content in hydroxycinnamic acids for HT, where no significant differences between the seasonings were found. Gallic acid, and ethyl gallate were more abundant in Sd-S (Sk-S < W-S < Sd-S), whereas ellagic acid was not detected. With regard to hydroxycinnamic acids, it was also notable that ferulic acid was not found in NT for Sd-S, whereas small amounts were detected after heat treatment. The highest quantities of the rest of non-esterified forms were generally found in Sd-S whereas Sk-S showed the highest contents in the tartaric acid-derivatives.

As regards the flavan-3-ol subgroups, a smaller quantity of monomers (catechin and epicatechin) than dimers (procyanidins B1 and B2) was found for Sk-S, whereas the opposite was observed for Sd-S.

The total contents in flavonol-3-*O*-glycosides were higher than in flavonol aglycons for Sk-S and W-S. The same trend observed for the total flavonol content (Sk-S > W-S > Sd-S) was generally found for both aglycons and 3-*O*-glycosides, with the exceptions of kaempferol and quercetin.

Malvidin was the most abundant anthocyanidin for all RWPSs, although important quantities of delphinidin and petunidin were also found for Sk-S and W-S.

Table 2. Percentage (%) changes in the phenolic compound contents of heat-treated (HT) red wine pomace seasonings (RWPSs) with respect to their non-treated (NT) counterparts (seedless: Sk-S; whole: W-S; seeds: Sd-S).

Phenolic compounds	RWPSs		
	Sk-S	W-S	Sd-S
p-OH-benzoic acid	-18.9 ± 1.5 ** a	-11.5 ± 2.7 * a	-30.7 ± 16.5 a
Salicylic acid	221 ± 10 *** c	91.1 ± 11 * b	3.18 ± 3.01 a
Protocatechuic acid	35.5 ± 1.04 *** b	34.8 ± 2.6 ** b	17.5 ± 1.7 ** a
Gallic acid	16.3 ± 0.6 ** a	37.0 ± 0.6 ** c	33.8 ± 0.09 *** b
Vanillic acid	11.1 ± 1.1 ** a	17.7 ± 1.5 ** b	31.7 ± 1.1 ** c
Syringic acid	23.3 ± 5.8 * a	25.8 ± 2.2 * a	83.6 ± 44.4 a
Ellagic acid	4.67 ± 0.30 ** a	19.0 ± 2.6 * b	ND
Ethyl gallate	-11.1 ± 0.7 * a	-16.2 ± 2.4 * a	-9.80 ± 3.07 a
Total Hydroxybenzoic acids	19.7 ± 1.7 ** b	22.3 ± 0.8 ** b	16.6 ± 0.7 ** a
p-Coumaric acid	95.1 ± 8.9 ** c	62.6 ± 6.21** b	-0.205 ± 6.371 a
Caffeic acid	33.5 ± 16.7 a	28.1 ± 5.6 * a	11.1 ± 12.1 a
Ferulic acid	57.3 ± 0.6 * b	47.3 ± 5.7 * a	DHT
Coutaric acid	-48.9 ± 3.78 * a	-53.8 ± 6.2 ** a	-1.74 ± 0.64 b
Caftaric acid	3.66 ± 2.69 a	26.3 ± 14.5 a	5.71 ± 9.35 a
Fertaric acid	-23.3 ± 5.0 * a	-13.7 ± 10.4 a	ND
Total Hydroxycinnamic acids	-2.77 ± 4.89 a	15.3 ± 6.72 a	4.84 ± 0.64 a
Total Phenolic acids	**16.8** ± 2.1 ** a	**21.5** ± 1.5 * b	**15.2** ± 0.7 ** a
t-resveratrol	-28.2 ± 5.4 * a	-12.7 ± 0.8 * a	ND
t-piceid	-24.4 ± 2.5 ** a	-38.4 ± 4.8 * a	ND
Total Stilbenes	**-26.5** ± 4.1 * a	**-22.1** ± 1.3 * a	**ND**

Results expressed as the mean value ± standard deviation (n = 3).
* p<0.05; ** p<0.01; *** p<0.001: Significance (Student's t test) of the % change in contents of HT with respect to NT for each RWPS.
Roman letters: Significant differences (ANOVA) between Sk-S, W-S, and Sd-S.
ND: Not detected; DHT: Detected only in heat-treated RWPSs.

Table 2. *(Continuation)*

Phenolic compounds	RWPSs		
	Sk-S	W-S	Sd-S
Catechin	-15.7 ± 3.5 * a	-10.9 ± 0.6 ** ab	-2.36 ± 3.77 b
Epicatechin	-17.0 ± 6.8 a	-25.5 ± 0.98 ** a	-13.8 ± 2.2 * a
Total Flavan-3-ols (monomers)	-16.1 ± 4.5 * a	-16.4 ± 0.02 ** a	-7.43 ± 3.07 a
Procyanidin B1	-1.90 ± 4.5 a	10.3 ± 4.6 a	10.1 ± 5.3 a
Procyanidin B2	-34.8 ± 2.6 * a	-23.8 ± 3.6 * b	-16.7 ± 3.9 b
Total Flavan-3-ols (dimers)	-14.7 ± 3.8 a	-11.8 ± 0.7 ** a	-10.2 ± 1.7 * a
Total Flavan-3-ols	**-15.2** ± 4.0 * a	**-14.2** ± 0.3 ** a	**-8.63** ± 1.02 * a
Kaempferol	15.7 ± 2.9 * a	32.9 ± 3.4 * b	122 ± 3 *** c
Quercetin	30.1 ± 5.4 * c	9.55 ± 3.57 b	-13.3 ± 5 a
Myricetin	17.4 ± 4.5 * a	19.4 ± 2.3 * a	64.7 ± 4.8 ** b
Total Flavonol aglycones	19.7 ± 2.6 * a	17.7 ± 0.9 ** a	27.0 ± 0.8 ** b
Kaempferol-3-O-rutinoside	-5.60 ± 1.41 * a	3.83 ± 1.39 b	14.5 ± 4.2 c
Kaempferol-3-O-glucoside	-6.57 ± 0.71 * a	0.640 ± 1.204 b	0.040 ± 0.778 b
Quercetin-3-O-rutinoside	-4.27 ± 1.58 a	-5.08 ± 3.19 a	-2.44 ± 1.20 a
Myricetin-3-O-rhamnoside	-0.453 ± 4.791 a	14.30 ± 6.27 ab	28.0 ± 2.1 * b
Total Flavonol-3-O-glycosides	-4.63 ± 1.96 a	4.00 ± 0.114 * b	8.29 ± 1.37 * b
Total Flavonols	**0.872** ± 0.940 a	**8.20** ± 0.37 * b	**17.9** ± 1.1 ** c
Delphinidin	-29.7 ± 1.8 * b	-33.2 ± 3.6 ** ab	-39.1 ± 3.0 ** a
Cyanidin	-47.9 ± 8.8 a	-55.3 ± 7.8 a	-14.9 ± 3.7 b
Petunidin	-56.4 ± 7.3 * a	-53.4 ± 3.5 * a	-8.33 ± 7.82 b
Peonidin	-53.8 ± 4.2 ** a	-44.6 ± 7.1 * a	-67.9 ± 15.0 a
Malvidin	-35.7 ± 4.5 ** a	-39.1 ± 3.2 ** a	-36.4 ± 6.4 * a
Total Anthocyanidins	**-37.1** ± 4.2 ** ab	**-39.8** ± 1.8 ** a	**-29.1** ± 2.2 ** b
Total	**-29.2** ± 3.02 ** a	**-27.8** ± 1.4 ** a	**-6.04** ± 0.39 * b

Results expressed as the mean value ± standard deviation (n = 3).
* p<0.05; ** p<0.01; *** p<0.001: Significance (Student's t test) of the % change in contents of HT with respect to NT for each RWPS.
Roman letters: Significant differences (ANOVA) between Sk-S, W-S, and Sd-S. ND: Not detected; DHT: Detected only in heat-treated RWPSs.

3.1.2. Changes in the phenolic profile induced by heat treatment

The differences in the individual phenolic composition of HT with respect to NT for each type of wine pomace seasoning are presented in **Table 2** as percentage (%) concentration changes.

Heat treatment resulted in a significant decrease in the total amount of phenolic compounds detected in the analysis, with this decrease being specially marked in Sk-S and W-S (Sk-S = W-S < Sd-S, $p < 0.01$).

A significant and positive effect of the heat treatment was observed for the total phenolic acid and flavonol contents. In contrast, heating negatively affected the total stilbene, flavan-3-ol, and anthocyanidin contents. The losses of stilbenes and flavan-3-ols were similar for the three types of RWPSs. Anthocyanidins were the most severely affected compounds by the high temperatures, showing that the negative effect was around 10.7% less marked for Sd-S than for W-S.

The heat treatment had a higher positive effect on the total hydroxybenzoic acid content for Sk-S and W-S than for Sd-S (Sk-S = W-S > Sd-S, $p < 0.05$), whereas the total hydroxycinnamic acid content remained stable despite heat treatment. p-OH-benzoic acid and ethyl gallate contents decreased significantly in Sk-S and W-S. In contrast, a positive effect of exposure to high temperatures was found for the remaining hydroxybenzoic acids, with the % increase in salicylic and protocatechuic acids contents for Sk-S, and gallic acid content for Sd-S, exhibiting the most marked changes. While heat treatment negatively affected the amount of *p*-coumaric in Sd-S, this hydroxycinnamic acid was greatly increased in Sk-S and W-S. The increase in ferulic acid content was also notable in Sk-S and W-S. In contrast, significant decreases in the concentration of coutaric acid (Sk-S and W-S) and fertaric acid (Sk-S) were found.

The effects of heating on the total flavan-3-ol monomer and dimer contents were similar to the general detrimental effect found for flavan-3-ols as a whole (Sk-S = W-S = Sd-S). The most significant % decrease was observed in both catechin and epicatechin for W-S. A significant decrease of procyanidin B2 was observed for Sk-S and W-S, with this decrease being more important for Sk-S than for W-S and Sd-S.

In the case of flavonols, the aglycon content was much more positively influenced by heat treatment than the 3-*O*-glycoside content. The increase in total flavonol aglycon content was higher for Sd-S than for Sk-S and W-S ($p < 0.05$), with the increase in kaempferol and myricetin contents being also the most marked for Sd-S, while quercetin only increased significantly in Sk-S. Heat treatment had no effect in the total flavonol-3-*O*-glycoside content for Sk-S, whereas the content of these compounds rose significantly for W-S and Sd-S (Sk-S < W-S = Sd-S, $p < 0.01$). The decreases in kaemferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside contents for Sk-S, and the increase in myricetin-3-*O*-rahmnoside content for Sd-S, were the only significant % changes observed.

The most notable decreases in anthocyanidin contents upon heat treatment were found for delphinidin (W-S and Sd-S), peonidin (Sk-S) and malvidin (Sk-S and W-S). There was no significant change in cyanidin content due to the exposure to high temperatures, although the comparison of the products showed a more detrimental effect for Sk-S and W-S than for Sd-S ($p < 0.05$). An opposite effect was found for the delphinidin content, whereas no differences between the RWPSs were obtained for peonidin and malvidin. The marked reduction in malvidin content (around -37.0 %) due to heat treatment was mainly responsible for the decrease in total anthocyanidin content.

3.2. Changes in total antioxidant capacity (TAC)

The FC and ABTS assays were used to assess the TAC for RWPS extracts, using C-TAC protocols, as well as directly for the powdered products, applying the Q-TAC methods. As noted previously (Del Pino-García et al. 2015), the results obtained with each method are influenced by the solubility of the standards in the solvent or reaction medium used.

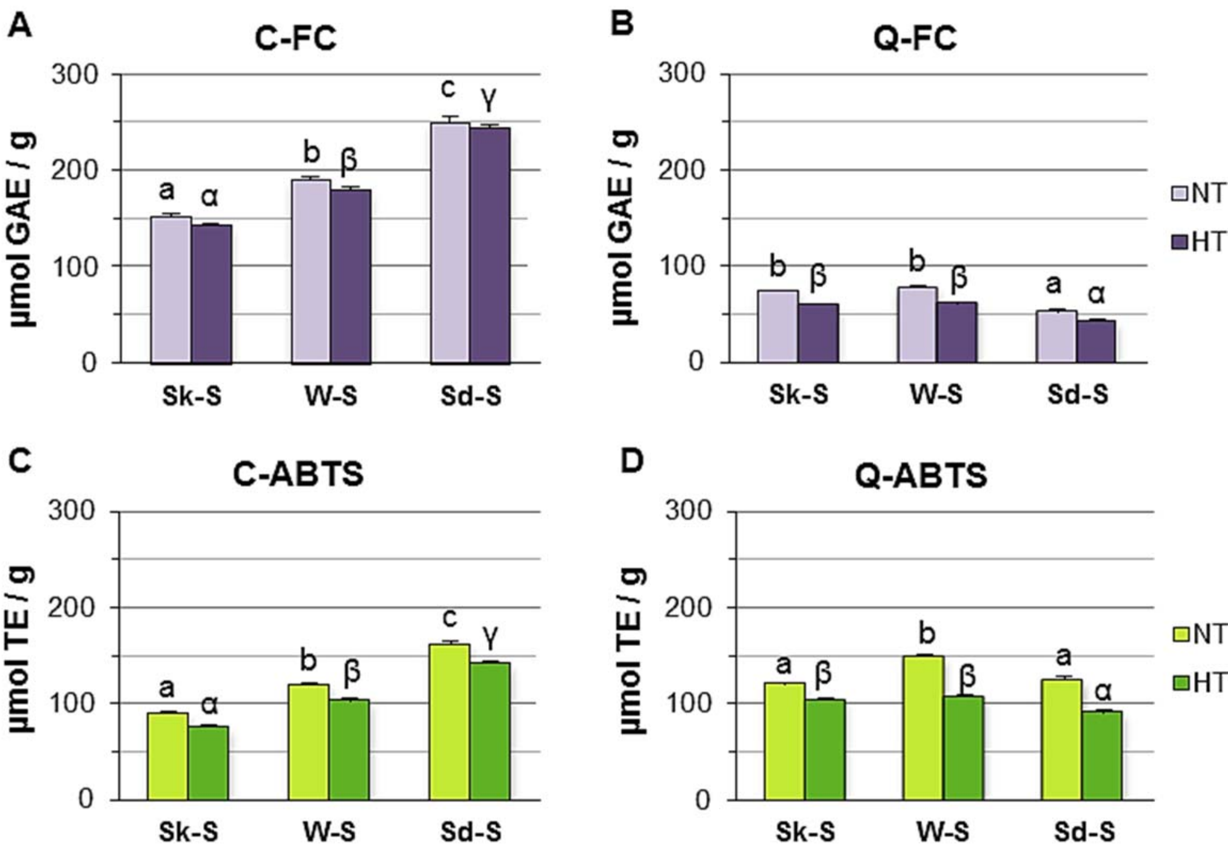

Figure 1. Total antioxidant capacity (TAC) for non-treated (NT) and heat-treated (HT) red wine pomace seasonings (RWPSs) (seedless: Sk-S; whole: W-S; seeds: Sd-S) using classical (C-) and QUENCHER (Q-) versions of the FC (Folin-Ciocalteu) and ABTS assays. GAE: gallic acid equivalents; TE: Trolox equivalents. Roman letters: Significant differences (ANOVA) between NT seasonings. Greek letters: Significant differences (ANOVA) between HT seasonings.

The C-FC and C-ABTS values (**Figure 1**A and C, respectively) showed similar trends for all RWPSs (both NT and HT), with Sd-S exhibiting the highest C-TAC and Sk-S the lowest. In contrast, this trend was not observed when using the Q-TAC methods. Similar results were obtained for Sk-S and W-S in the Q-FC assay (**Figure 1**B), with both these samples giving a higher Q-FC index than Sd-S (both NT and HT). The same trend was found for HT seasonings when using the Q-ABTS method, while W-S gave the highest value of the three NT seasonings (**Figure 1**D).

With regard to the % change in TAC between HT and NT, greater differences were found between the assays (**Table 3**). The antioxidant capacity of Sd-S was not significantly affected by heating according to the results of the C-FC assay, whereas effects of heat treatment were observed for the remaining RWPSs and when using other assays. The TAC reductions were generally more significant when using the Q-TAC than the C-TAC methods, with the most significant % change being observed for Sk-S and W-S (Q-FC assay) and W-S (Q-ABTS assay). A different trend between the RWPSs as regards the impact of heating was shown for each method.

Table 3. Percentage (%) changes in the total antioxidant capacity (TAC) of heat-treated (HT) red wine pomace seasonings (RWPSs) with respect to their non-treated (NT) counterparts (seedless: Sk-S; whole: W-S; seeds: Sd-S) determined using classical (C-) and QUENCHER (Q-) versions of the FC (Folin-Ciocalteu) and ABTS assays.

TAC assays	RWPSs		
	Sk-S	W-S	Sd-S
C-FC	-5.57 ± 0.58 ** a	-5.74 ± 1.39 ** a	-2.49 ± 0.80 b
C-ABTS	-16.2 ± 2.2 ** a	-13.7 ± 1.9 ** ab	-12.4 ± 1.3 ** b
Q-FC	-19.5 ± 2.0 *** a	-21.4 ± 2.1 *** a	-18.8 ± 5.8 * a
Q-ABTS	-13.3 ± 2.2 ** b	-28.1 ± 3.0 *** a	-26.9 ± 3.5 ** a

Results expressed as the mean value ± standard deviation (n = 3).
* p<0.05; ** p<0.01; *** p<0.001: Significance (Student's t test) of the % change in TAC of HT with respect to NT for each RWPS.
Roman letters: Significant differences (ANOVA) between Sk-S, W-S, and Sd-S for each assay.

3.3. Correlations

Two different correlation studies were performed with data obtained in this study. Firstly, Pearson's correlation coefficients were determined for the TAC values and the main phenolic class contents (**Table 4**A). Strong and positive correlations were found between the C-FC and C-ABTS methods, and also between the Q-FC and Q-ABTS assays. However, both C-TAC assays were negatively related to the Q-FC method, and unrelated to the Q-ABTS assay. The C-FC and C-ABTS methods were both highly and positively correlated with the total flavan-3-ol content, whereas a negative correspondence was detected with the remaining phenolic class and total phenolic compound contents. In contrast, the Q-FC method exhibited the opposite trend, although it was not related to the total phenolic acid content.

Secondly, correlations were calculated using the % change for HT with respect to NT (**Table 4**B). For the TAC assays, only the C-FC and C-ABTS methods were mutually positively correlated, whereas the C-ABTS assay was negatively correlated to the Q-ABTS method. The C-FC assay was strongly and positively correlated to the total flavan-3-ol and total phenolic compound contents, with a positive correlation to the total flavonol and anthocyanidin contents also being found. The positive correlation between the C-ABTS method and total stilbene and flavan-3-ol contents was also significant. In contrast, no positive correlations between the Q-TAC assays and the phenolic classes were observed.

Table 4. Correlation analyses.

A) Correlations using TAC values and main phenolic class contents.

	C-FC	C-ABTS	Q-FC	Q-ABTS
C-FC		0.978***	-0.640**	-
C-ABTS			-0.504*	-
Q-FC				0.745***
Total Phenolic acids	-0,668**	-0,786***	-	-0,609**
Total Stilbenes	-0,940***	-0,872***	0,837**	-
Total Flavan-3-ols	0,997***	0,975***	-0,672**	-
Total Flavonols	-0,995***	-0,971***	0,666**	-
Total Anthocyanidins	-0,875***	-0,790***	0,887***	-
Total Phenolic compounds	-0,798***	-0,698**	0,913***	-

B) Correlations using % changes of HT with respect to NT seasonings.

	C-FC	C-ABTS	Q-FC	Q-ABTS
C-FC		0.752*	-	-
C-ABTS				-0.713*
Q-FC				-
Total Phenolic acids	-	-	-	-
Total Stilbenes	-	0.955*	-	-0.844*
Total Flavan-3-ols	0.844**	0.736*	-	-
Total Flavonols	0,723*	-	-	-
Total Anthocyanidins	0.690*	-	-	-
Total Phenolic compounds	0.857**	-	-	-

* p<0.05; ** p<0.01; *** p<0.001: Significance (Student's t test) of the correlation.
A) n = 18 (unless Total Stilbenes: n = 12)
B) n = 9 (unless Total Stilbenes: n = 6)

4. Discussion

The interest in a more efficient exploitation of winemaking by-products has led to innovative alternatives, such as the use of wine pomace-derived products as food ingredients (González-Sanjosé et al. 2013). The heat treatment required during the microbial stabilization step in the RWPS manufacturing process made it necessary to characterize the phenolic profile and antioxidant capacity of the non-treated (NT) and heat-treated seasonings (HT) obtained from the different wine pomace constituents (Sk-S, W-S, and Sd-S).

4.1. Differences between Sk-S, W-S, and Sd-S

The individual phenolic content of wine pomace depends on numerous viticulture and wine-making factors, such as the grape variety (Sri Harsha et al. 2013), cultivation conditions (Kammerer et al. 2004), and technological parameters applied during vinification (Sacchi et al. 2005; Fontana et al. 2013). Furthermore, many of the polyphenols present in wine pomace are entrapped or bound to the cell wall matrices, especially those contained in the seed portion, and thereby are not easily extracted unless aggressive treatments are applied (Arranz et al. 2010).

In general, the distribution of phenolic classes and individual compounds in the RWPSs were in line with those published previously(Alonso et al., 2002; Chamorro et al., 2012; Delgado Adámez et al., 2012; Monagas et al., 2003; Rockenbach et al., 2011), as the skin portion of wine pomace is known to be a rich source of anthocyanins, phenolic acids, flavan-3-ols, and flavonol-3-*O*-glycosides, whereas gallic acid and flavan-3-ol monomers and oligomers are mainly present in the seed portion. The phenolic contents observed for W-S were intermediate between the values obtained for Sk-S and Sd-S, although usually closer to Sk-S. Previous studies have also reported a relative proportion of seeds ranging from 38% to 52% of wine pomace dry material (Teixeira et al. 2014).

Wine pomace is rich in phenolic acids (Alonso et al., 2002; Kammerer et al., 2004), which is supported by the considerable concentrations of many of these simple phenolics found in the RWPSs. A greater contribution of hydroxybenzoic than hydroxycinnamic acids to the total of phenolic acids was found, independently of the wine pomace material used for their production, although differences between the three types of RWPSs were detected in the distribution of most of the individual compounds.

Catechin, epicatechin, and procyanidins B1 and B2 are also present in significant amounts in wine pomace skins and seeds (González-Paramás et al. 2004; Chamorro et al. 2012). These flavan-3-ols were determined in the present study, showing that Sd-S was a richer source of flavan-3-ols than Sk-S. These results were in accordance with previous studies (Kammerer et al. 2004; Rockenbach et al. 2011; Monagas et al. 2003). Nonetheless, the polymeric fraction

(oligomers with a higher degree of polymerization and condensed tannins) are known to represent a greater proportion of the total flavan-3-ols in wine pomace (Teixeira et al. 2014), both in seeds (75-81%) and in skins (94-98%) (Monagas et al. 2003). Thus, the contribution of total flavan-3-ols to the total phenolic compounds is expected to be higher than the results obtained in the present study. Nonetheless, the differences observed between the RWPSs may be rather similar, as the total proanthocyanidin contents of the RWPSs have been previously determined (García-Lomillo et al. 2014) and good correlations ($p < 0.001$) between these results and the sums of monomers (r = 0.967), dimers (r = 0.965), and total flavan-3-ols (r =0.968) obtained in the current study were detected.

In the RWPSs containing wine pomace skins (Sk-S and W-S), flavonol-3-*O*-glycosides were more abundant than the flavonol aglycons. These differences might be even more marked as flavonols such as quercetin-3-*O*-glucuronide or quercetin-3-*O*-glucoside are also present in considerable quantities in the wine pomace skins (Kammerer et al. 2004; Teixeira et al. 2014; Sri Harsha et al. 2013) but were not analysed in this study.

Anthocyanins are also found in winery by-products mainly as numerous 3-*O*-glycosides derivatives (Kammerer et al. 2004; Pedroza et al. 2012). Malvidin was the most abundant anthocyanidin detected in the seasonings, especially in Sk-S, which agrees with previous studies describing malvidin-3-*O*-glucoside or malvidin (after acidic hydrolysis) as the predominant compounds in the skins of wine pomace (Kammerer et al. 2004; Pedroza et al. 2012). The results obtained were also well correlated (r = 0.924, $p < 0.001$) with the total anthocyanin content in RWPSs reported by García-Lomillo et al. (2014). Although a minor quantity of anthocyanidins was detected in Sd-S compared to Sk-S, their presence in wine pomace seeds might be due to their contact with the skins and pomace during winemaking. These compounds might also come from the remnants of skins left during manual separation of the seeds from wine pomace. Surprisingly, cyanidin was detected in a small but larger amount in Sd-S than in Sk-S, possibly due to release from proanthocyanidins as a consequence of the acidic hydrolysis treatment applied to the extracts.

Despite Sk-S contained the highest quantities of total identified phenols and Sd-S the lowest, the high contribution of anthocyanidins had a marked influence on these results. This fact was confirmed by comparing data from the HPLC analysis and the TAC characterization.

The FC and ABTS assays are considered among the most suitable methods for evaluating the TAC of foodstuffs in previous works, using both classical (C-) (Rivero-Pérez et al. 2007; Prior et al. 2005) and QUENCHER (Q-) approaches (Del Pino-García et al. 2015), so they were used in the present study.

As expected, important differences were observed between the C-TAC and Q-TAC results obtained in each assay (FC or ABTS). However, a positive correlation was detected upon comparing the TAC values for the assays using the same approach (C- or Q-). It must be noted that both compounds soluble in the reaction medium and insoluble antioxidants attached to the RWPS matrices can exert their antioxidant activity in Q-TAC methods, whereas only those compounds soluble in the extraction solvent are detected in C-TAC assays. However, some of the phenolic compounds removed from RWPSs during the extraction procedure may not be accessible under real conditions in the foodstuff or biological systems, where water is usually present in the surrounding medium. As a result, classical protocols might overestimate the TAC of those products containing significant quantities of insoluble antioxidants if these are released from the RWPS matrices during the extraction process. This hypothesis is supported by the lower Q-TAC generally obtained for Sd-S compared with the other RWPSs, whereas this seasoning rich in hydrophobic antioxidants gave the highest C-TAC values. In addition, the Q-TAC results obtained for W-S, especially in the Q-ABTS assay, suggest possible synergistic interactions between compounds from both wine pomace skins and seeds, as described previously in the literature (Kanner et al. 1994; Arnous et al. 2001; Yang et al. 2009). These synergisms were evident when both soluble and insoluble antioxidants attached to the RWPS matrices were present in the reaction (Q-TAC methods) but not when RWPS extracts were used (C-TAC essays). In agreement with this finding, the possible regeneration of antioxidants bound to insoluble food matter by soluble antioxidant compounds must be considered, as recently discussed by Çelic et al. (2013).

Numerous strong correlations were obtained among the TAC and the HPLC-based total phenolic content values. C-TAC assays were only positively correlated with total flavan-3-ols as Sd-S also contained the highest quantity of these compounds. This finding was consistent with previous studies (González-Paramás et al. 2004) and suggests that flavan-3-ols may be the main phytochemicals responsible for the C-TAC results for the RWPS extracts. In contrast, flavan-3-ols might have a smaller influence in the Q-TAC assays, particularly in the Q-FC results, which may be primarily determined by compounds with high water solubility and which are easily accessible or extractable from the insoluble RWPS matrices, such as anthocyanidins, stilbenes, and flavonols.

4.2. Changes induced by the heat treatment

Different mechanisms have been proposed to explain the changes in phenolic composition of products exposed to high temperatures. Thus, a decrease in phenolic content might be due to the onset of thermal degradation of certain phenolic compounds (Maillard & Berset 1995; Ross et al. 2011). In this sense, there is a general consensus regarding the loss of anthocyanins upon heating. Sólyom et al. (2014) reported that first-order kinetics can be applied to describe the concentration evolution (dC/dt) of monomeric anthocyanins during heating, although the stability of these compounds may vary considerably depending on the nature, extent, and duration of the high temperatures (Khanal et al. 2010). In the present study, the anthocyanin phenolic class was the most severely affected by heat treatment for all the RWPSs, followed by stilbenes and flavan-3-ols. The negative effect of heating was more marked for epicatechins than for catechins, and for procyanidins B2 (epicatechin-(4β→8)-epicatechin) than for B1 (epicatechin-(4β→8)-catechin). A possible explanation for this lies in the structure of grape proanthocyanidins as, at least in grape seeds, these polymeric compounds mainly comprise catechin as the terminal and epicatechin as the extension subunits, thus meaning that catechin and procyanidin dimer B1 are expected to be the major products of their breakdown (González-Paramás et al. 2004). In contrast, the increase in phenolic acid and flavonol contents of the RWPSs could be explained by the liberation of phenolic compounds bound to the food matrices and the partial degradation of lignin, which leads to the release of phenolic acid derivatives (Maillard & Berset 1995; Ross et al. 2011; Pinelo et al. 2006). In the case of gallic acid, which is one of the most widely studied phenolic acids affected by heat treatment, its increase in heat-treated products may also be due to the release of gallate groups from flavan-3-ol gallates (Davidov-Pardo et al. 2011; Chamorro et al. 2012). Moreover, heat treatment seems to induce a breakage of some esterified forms of hydroxycinnamic acids such as coutaric and fertaric acids, especially notable in Sk-S and W-S, thus increasing their contents in coumaric and ferulic acids.

The matrix structures of wine pomace constituents and the interactions of their phytochemicals with such matrices are both aspects affected by thermal processing (Mohdaly et al. 2010) and may explain the different impact of heat treatment on the phenolic contents for each type of RWPS. It should be borne in mind that some wine pomace polyphenols are free and can be found in vacuoles, whereas others are associated with cell wall compounds or polysaccharide structures in the skin cells (Pinelo et al. 2006; Arranz et al. 2010). Generally, the increases observed in the content of some phenolic compounds upon heat treatment were more marked for Sd-S, whereas the decreases were more pronounced for Sk-S and W-S. Thus, it seems that phenolic compounds are more susceptible to the detrimental effects of heating when embedded in the RWPS matrices derived from seeds than from seedless or whole wine pomace. However, no important differences were detected when considering the main phenolic classes rather than the individual phenolic

compounds, except for total flavonols and total phenolic compounds, which showed the above-mentioned RWPS matrix effect.

The negative effect of heat treatment determined in the C-TAC assays was less marked than that observed for the total phenolic content detected by HPLC, especially when evaluated using the C-FC assay. One possible explanation for the results obtained may be the easier extraction of HT phenolic compounds not identified in the HPLC analysis from the solid matrices, as well as the likely formation of new phenolic compounds from more complex compounds (such as anthocyanidins or flavan-3-ol monomers and oligomers) upon heating (Chamorro et al. 2012; Khanal et al. 2010; Ross et al. 2011). In fact, the correlations obtained suggest that changes in the flavan-3-ol content after heating might be chiefly responsible for the C-TAC changes observed.

Different results can be found in the literature as regards the impact of heating (under similar conditions to those applied to the RWPSs) on the TAC of wine pomace or derived products. Following a classical approach, Larrauri et al. (1997) found that the extractable polyphenols (determined using the FC assay) in wine pomace skins decreased significantly (about 20%) in samples dried at 100 ºC to a moisture content of around 8%. However, Pedroza et al. (2012) determined the total polyphenol index (FC assay) in skins of three wine pomaces and found different effects of heat treatment at 90 ºC (positive, no effect, and negative) depending on the source grape variety. Working with whole wine pomace, Chamorro et al. (2012) found a slight increase in the TAC of samples heated at 100 ºC for 60 min, this increase being more marked when using the ABTS (around 15%) than the FC and ferric reducing/antioxidant power (FRAP) assays (around 4%). Similarly, Sólyom et al. (2014) observed a slight increase in the antioxidant capacity determined using the FC and oxygen radical absorbance capacity (ORAC) assays after the first hour of heating whole wine pomace to 100 ºC. In contrast, Ross et al. (2011) reported different effects of heating wine pomace seeds to 120 ºC for 90 min, with no significant TAC changes being observed when using the FC and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, but minor reductions in the antioxidant capacity being detected using the FRAP assay (about 6%) and much more marked reductions when using the ABTS method (about 40%).

As noted in the results of the current study, the Q-TAC approach may better represent the real situation in food or biological systems, where water is the primary medium and many of the phenolic compounds extracted from RWPSs using organic solvents may be unable to exert their antioxidant capacity. According to the Q-ABTS assay, Sk-S was less affected than W-S and Sd-S by heat treatment. This finding prompted us to consider that the possible synergisms between compounds present in the wine pomace (increasing their antiradical activity) might be altered by heating, with the phenolic compounds derived from wine pomace seeds playing a more important role in these interactions, or being more negatively affected, than those from skins.

5. Conclusions

In light of all the above, this study provides a detail characterization of the phenolic profile and antioxidant properties of products obtained from different wine pomace materials (seedless, whole, seeds). The heat treatment applied to these products resulted in a slight decrease in their total phenolic content and TAC. However, there is little evidence against this thermal processing as different heat-related effects, as well as RWPS matrix effects on the impact of heating, were observed depending on the assay used. The decrease in anthocyanidin (Sk-S and W-S) and flavan-3-ol contents (Sd-S) appear to be the main factors governing the detrimental effect of high temperatures on the phenolic composition of the RWPSs. In contrast, heat treatment was found to positively affect the phenolic acid and flavonol contents. Indeed, heat treatment generally led to the same or more beneficial changes in the phenolic profile and TAC of extracts from Sd-S than from Sk-S or W-S, whereas the opposite trend was observed when TAC was evaluated directly for powdered products using Q-TAC assays. Thus, this study highlights important differences in the TAC results obtained using the classical and QUENCHER approaches, thereby suggesting the need to apply Q-TAC methods for samples that will be used as directly powdered products, rather than as extracts, by the food and nutraceuticals industry.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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Chapter 2

Bioaccessibility, Bioavailability and Antioxidant Potential of the Seasonings

CHAPTER 2:

BIOACCESSIBILITY, BIOAVAILABILITY AND ANTIOXIDANT POTENTIAL OF THE SEASONINGS

The bioavailability and bioavailability of RWPSs after intake, and the factors that may alter them, are key issues that will determine their possible beneficial effects against oxidative stress and related pathologies in the body. Therefore, this chapter gathers the *in vitro* and *in vivo* studies conducted to achieve the second of the specific objectives of this PhD Thesis.

Given the ethical restrictions and complexity associated with *in vivo* studies, numerous *in vitro* protocols have been developed to simulate the digestive process experimentally, predict possible changes in food matrices and bioactive compounds released during digestion, and evaluate a large number of samples (Hur et al. 2011; Alminger et al. 2014).

The most common ***in vitro*** **digestion models** to study bioaccessibility of compounds are the static ones (with pre-fixed volumes and concentrations of digested materials, enzymes, salts, etc.), presenting as the main advantage their low cost and relative simplicity. Furthermore, once the parameters have been optimized, it has been observed a good correlation of these static models with the results of *in vivo* studies (Hur et al. 2011). Continuous models have been also developed, which allow a better simulation of the secretion of enzymes and digestive fluids, as well as of changes in conditions along digestion (Alminger et al. 2014; Carbonell-Capella et al. 2014). The main disadvantage of most *in vitro* digestion methods, both static and dynamic, is the impossibility to mimic the active transport mechanisms that occur in the stomach and intestines, and the metabolism of compounds during their absorption through the intestinal epithelium.

In this study, structural changes, digestibility, and release of the bioactive components of the seasonings were estimated first *in vitro*, carrying out the static protocol developed by the group of Saura-Calixto (Saura-Calixto et al. 2007; Saura-calixto et al. 2010). The steps of this protocol can be gathered into two phases:

- Gastrointestinal enzymatic digestion.
- Colonic microbial fermentation.

The method chosen for this thesis consists of two phases because it was expected that a number of phenolic compounds present in the seasonings showed low bioaccessibility and bioavailability in the upper digestive tract, but they were highly metabolized by the resident colonic microbiota. Thus, by using this *in vitro* model of complete digestion, it was possible to investigate separately the impact of each of these phases in the release of bioactive compounds from the seasonings.

Then, an adaptation of the GAR ('Global Antioxidant Response') methodology proposed by Delgado-Andrade et al. (2010) was performed, so that the QUENCHER methods selected in *Chapter 1* (Q-FC and Q-ABTS) were used to evaluate the antioxidant capacity of the different fractions obtained during the *in vitro* complete digestion protocol, selecting those fractions remaining in the intestinal tract. This procedure allowed comparing the gain or loss of antioxidant activity of the bioactive compounds released from the seasonings during the digestive process.

However, there is no doubt that *in vivo* studies are the best way to evaluate the bioavailability of phenolic compounds along the gastrointestinal tract. A large number of studies have shown that the percentage of ingested flavonoids that is absorbed as intact molecules is relatively small, while a substantially higher percentage is absorbed in the form of phenolic metabolites, mainly phenolic acids, released from the food matrices after digestion or generated by intestinal microbiota metabolism. During and after absorption, these phenolic acids are further metabolized by phase I and phase II enzymes, and after a residence time in the body and/or enterohepatic recirculation, they are excreted by different ways: urine, bile, and faeces (Manach et al. 2004; Del Rio et al. 2013; Bondonno et al. 2015; Croft 2016) (**Figure 40**).

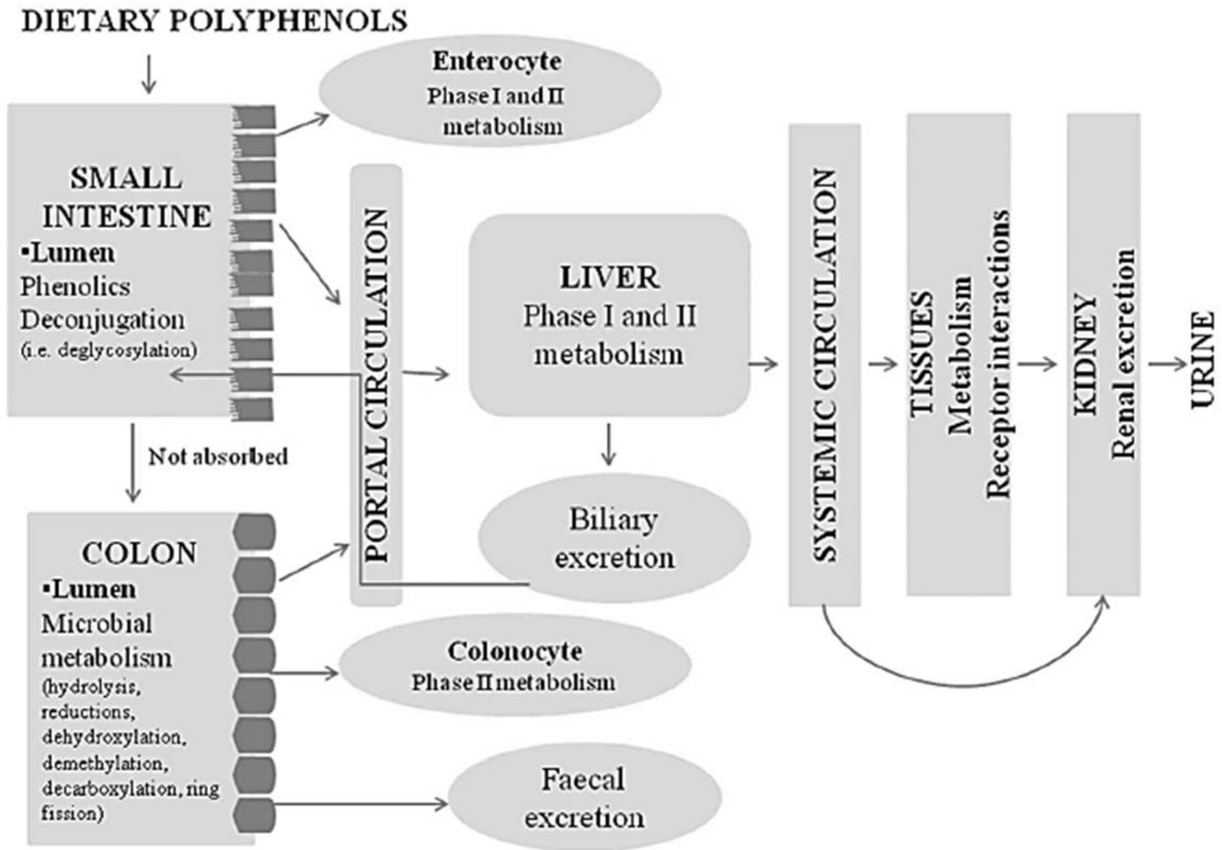

Figure 40. Routes for dietary polyphenols and their metabolites in humans.
Adapted from Cordona et al. (2013).

In vivo studies to assess the bioavailability of phenolic compounds should be performed preferably in humans as, depending on the species, the digestive and metabolic processes are slightly different. However, studies in animal models are usually simpler experiments of lower cost and with fewer ethical restrictions, being commonly used as a first approximation to evaluate the hypotheses during the initial stages of research and provide scientific support to future human clinical trials to test these hypotheses.

Therefore, in this thesis, the bioavailability of bioactive compounds released after acute intake of one of the seasonings (which presented the best characteristics to act systemically in previous studies) has been estimated *in vivo* using Wistar rats as an animal model. Briefly, plasma and urine samples were obtained at baseline and after ingestion of the seasoning, and the concentration of various phenolic acids was determined by using chromatographic techniques (GC-MS). The bioavailability of phenolic compounds was also estimated indirectly by assessing changes in plasma TAC applying the FC, FRAP and ABTS assays. These methods are much simpler and cheaper than chromatographic techniques, but their unspecificity must be always taken into account as they may be affected by other compounds present in plasma. Furthermore, biomarkers of oxidative stress and endothelial function were studied in parallel, evaluating its relation with the bioavailability of the phenolic acids studied. Moreover, the same biomarkers were analysed in biological samples collected from fasted-Wistar rats after supplementing their diet with the seasoning for 4 weeks, comparing the results obtained with the effects observed in the acute study.

It should be noted that, in all supplementation studies, the dose of seasoning used was 300 mg/kg of body weight, which corresponds to about 1.5% of their total diet. To select this concentration (as well as the duration of the supplementation in the case of the short-medium term study) it was first performed a literature review of several studies conducted in rodents administered polyphenolic extracts (Hogan et al. 2010; Mansouri et al. 2011; Pajuelo et al. 2012; Badavi et al. 2011; Belviranlı et al. 2012; Quiñones et al. 2013) or solid products (Alia et al. 2003; Goñi & Serrano 2005; López-Oliva et al. 2006; Khanal et al. 2011; Choi et al. 2012) obtained from grapes, wine and wine pomace.

The main **conclusions** of the studies presented in this chapter were:

- **Chapter 2.1**: The *in vitro* studies conducted allowed estimating that transformations taking place during the digestive process in the matrices and bioactive compounds present in the seasonings could increase their antioxidant potential. However, the fraction that was not bioaccessible after the whole digestive process also retained an important antioxidant capacity, which could help offset the effects of dietary pro-oxidant agents in the colon. The seasoning showing the best features of digestibility and antioxidant capacity in the upper digestive tract was Sk-S.

- **Chapter 2.2**: Acute consumption of the selected seasoning resulted in higher concentrations of various phenolic acids in plasma and urine of Wistar rats, detecting a substantial increase of metabolites generated mainly by the action of colonic microbiota. In parallel, a temporary improvement of vascular redox state, a decrease of lipid peroxidation, and a higher bioavailability of $^{\bullet}NO$ were observed. However, no beneficial effects were perceived after 4 weeks of supplementation with the seasoning, suggesting that, at least under normal physiological conditions, the effects observed in the acute study should be only transitory.

CAPÍTULO 2:

BIOACCESIBILIDAD, BIODISPONIBLIDAD Y POTENCIAL ANTIOXIDANTE DE LOS SAZONADORES

La bioaccesibilidad y biodisponibilidad de los RWPSs tras su ingesta, así como los factores que puedan alterarlas, son aspectos clave que determinarán sus posibles efectos beneficiosos frente al estrés oxidativo y patologías relacionadas en el organismo. Por ello, en este capítulo se recogen los estudios *in vitro* e *in vivo* llevados a cabo para la consecución del segundo de los objetivos específicos de la tesis.

Dadas las restricciones éticas y complejidad asociadas a los estudios *in vivo,* se han puesto a punto numerosos protocolos *in vitro* que permiten simular el proceso digestivo a nivel experimental, predecir posibles cambios en las matrices alimentarias y en los compuestos bioactivos que van siendo liberados durante dicho proceso, y evaluar un gran número de muestras (Hur et al. 2011; Alminger et al. 2014).

Los **modelos de digestión *in vitro*** más habituales en estudios de bioaccesibilidad son los estáticos (con volúmenes y concentraciones establecidos de los materiales digeridos, enzimas, sales, etc.), presentando como principal ventaja su bajo coste y relativa sencillez. Además, una vez que los parámetros han sido optimizados, se ha observado una correlación aceptable de estos modelos estáticos con los resultados de estudios *in vivo* (Hur et al. 2011). Existen también modelos continuos que permiten una mejor simulación de la secreción de las enzimas y fluidos digestivos, así como de los cambios en las condiciones a lo largo de la digestión (Alminger et al. 2014; Carbonell-Capella et al. 2014). La principal desventaja de la mayoría de los métodos *in vitro*, tanto estáticos como dinámicos, es la imposibilidad de simular los mecanismos activos de transporte que ocurren en el estómago y los intestinos, y el metabolismo de los compuestos durante su absorción al atravesar el epitelio intestinal.

En este estudio, los cambios estructurales, la digestibilidad, y la liberación de los componentes bioactivos de los sazonadores se estimaron en primer lugar *in vitro*, llevando a cabo el **protocolo estático** desarrollado por Saura-Calixto et al. (Saura-Calixto et al. 2007; Saura-calixto et al. 2010). Los pasos de dicho protocolo pueden agruparse en **dos fases**:

- Digestión gastrointestinal enzimática.
- Fermentación por la microbiota colónica.

El método elegido para esta tesis consta de estas dos fases porque se esperaba que varios de los compuestos fenólicos presentes en los sazonadores presentaran baja bioaccesibilidad y biodisponibilidad en el tracto digestivo superior, pero que fueran altamente metabolizados por la microbiota residente en el colon. Así, mediante este modelo *in vitro* de digestión completa, fue posible investigar por separado el impacto de cada una de estas fases en la liberación de los compuestos bioactivos de los sazonadores.

A continuación, se llevó a cabo una adaptación de la metodología GAR (acrónimo originado de su nombre en inglés "global antioxidant response" – respuesta antioxidante global) propuesta por Delgado-Andrade et al. (2010), de forma que se emplearon los métodos QUENCHER seleccionados en el *Capítulo 1* (Q-FC y Q-ABTS) para evaluar la capacidad antioxidante de las distintas fracciones obtenidas durante el protocolo *in vitro* de digestión completa, seleccionando aquellas fracciones que permanecerían en el tracto intestinal. Este procedimiento permitió comparar la ganancia o pérdida de actividad antioxidante de los compuestos bioactivos liberados a partir de los sazonadores debido al proceso digestivo.

Sin embargo, no cabe duda de que los estudios *in vivo* son la mejor forma de evaluar la biodisponibilidad de los compuestos fenólicos a lo largo del tracto gastrointestinal. Se ha demostrado en numerosos estudios que, de los flavonoides ingeridos, el porcentaje que es absorbido como moléculas intactas es relativamente pequeño, mientras que un porcentaje bastante mayor es absorbido en forma de metabolitos fenólicos, principalmente ácidos fenólicos, liberados de las matrices alimentarias durante la digestión o generados tras el metabolismo de la microbiota intestinal. Durante y tras su absorción, estos ácidos fenólicos son adicionalmente metabolizados por las enzimas de fase I y fase II, y tras un tiempo de residencia en el organismo y/o recirculación enterohepática, son excretados por diferentes vías: orina, bilis y heces (Manach et al. 2004; Del Rio et al. 2013; Bondonno et al. 2015; Croft 2016) (**Figura 40**).

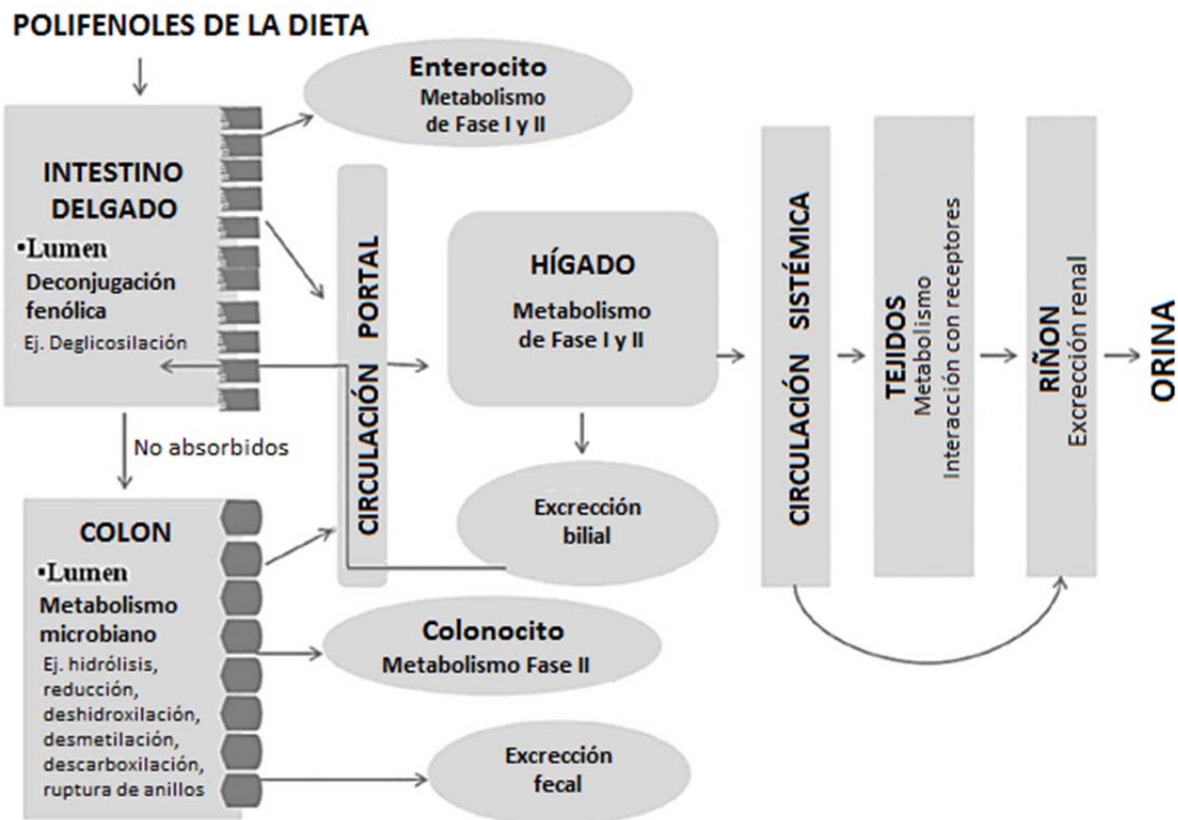

Figura 40. Rutas de los polifenoles ingeridos en la dieta y sus metabolitos en humanos.
Adaptado de Cordona et al. (2013).

Preferiblemente, los estudios *in vivo* para estudiar la biodisponibilidad de los compuestos fenólicos han de realizarse en humanos, ya que los procesos digestivos y metabólicos son ligeramente diferentes en función de las especies. Sin embargo, los estudios en modelos animales son por lo general experimentos más sencillos, de menor coste, y con menos restricciones éticas, siendo empleados habitualmente como una primera aproximación durante las etapas iniciales de las investigaciones para evaluar las hipótesis planteadas y dar soporte científico a futuros estudios clínicos en humanos que permitan comprobar dichas hipótesis.

Por ello, en esta tesis se ha estimado la biodisponibilidad *in vivo* de los compuestos bioactivos liberados tras la ingesta aguda de uno de los sazonadores (el que presentó las mejores características para actuar a nivel sistémico en los estudios previos) utilizando Ratas Wistar como modelo animal. En concreto, se obtuvieron muestras de plasma y orina en situación basal y tras la ingesta del sazonador y se determinó la concentración de varios ácidos fenólicos mediante el empleo de técnicas cromatográficas (GC-MS). También se estimó de forma indirecta la biodisponibilidad de los compuestos fenólicos evaluando los cambios en la TAC del plasma mediante los métodos FC, FRAP y ABTS. Estos métodos son mucho más simples y económicos que los métodos cromatográficos, pero hay que tener siempre en cuenta su inespecificidad, pudiendo verse afectados por otros compuestos presentes en el plasma. Además, se estudiaron en paralelo marcadores de estrés oxidativo y de función endotelial, evaluando su relación con la biodisponibilidad de los ácidos fenólicos estudiados. Por otra parte, se analizaron los mismos marcadores en muestras biológicas, recogidas en ayunas, tras la suplementación con el sazonador de ratas Wistar durante 4 semanas, comparando los resultados obtenidos con los efectos observados en el estudio agudo.

Destacar que, para todos los estudios de suplementación, la dosis de sazonador empleada fue de 300 mg/kg de peso corporal, que corresponde aproximadamente a un 1,5% del total de su dieta. Para seleccionar esta concentración (así como la duración de la suplementación en el caso del estudio a corto-medio plazo) se realizó una revisión bibliográfica de diversos estudios en roedores a los que se administraron tratamientos basados en extractos polifenólicos (Hogan et al. 2010; Mansouri et al. 2011; Pajuelo et al. 2012; Badavi et al. 2011; Belviranlı et al. 2012; Quiñones et al. 2013) o en productos sólidos (Alia et al. 2003; Goñi & Serrano 2005; López-Oliva et al. 2006; Khanal et al. 2011; Choi et al. 2012) obtenidos a partir de uvas, vino u orujos.

Las principales **conclusiones** de los estudios presentados en este capítulo fueron:

- **Capítulo 2.1**: Los estudios *in vitro* realizados permiten estimar que las transformaciones producidas durante el proceso digestivo en las matrices y compuestos bioactivos presentes en los sazonadores podrían aumentar su potencial antioxidante. Sin embargo, la fracción no bioaccesible después de todo el proceso digestivo también retuvo una importante capacidad antioxidante, por lo que podría ayudar a contrarrestar los efectos de agentes pro-oxidantes dietéticos a nivel del colon. El sazonador que presentó mejores características de digestibilidad y capacidad antioxidante en el tracto digestivo superior fue Sk-S.

- **Capítulo 2.2**: El consumo agudo del sazonador seleccionado resultó en una mayor concentración de varios ácidos fenólicos en plasma y orina de las ratas Wistar, detectando el incremento importante de metabolitos originados principalmente por la acción de la microbiota colónica. En paralelo, se observó una mejora temporal del estado redox vascular, una disminución de la peroxidación lipídica y una mayor biodisponibilidad de $^{\cdot}NO$. Sin embargo, no se apreciaron efectos beneficiosos tras la suplementación con el sazonador durante 4 semanas, sugiriendo que, al menos bajo condiciones fisiológicas normales, los efectos observados en el estudio agudo debían ser sólo transitorios.

Chapter 2.1.

Total Antioxidant Capacity of New Natural Powdered Seasonings after Gastrointestinal and Colonic Digestion

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Abstract

New powdered seasonings, rich in natural antioxidant compounds, have successfully been applied recently in different food matrices. Once ingested, the antioxidants contained in these seasonings may exert protective effects against oxidative stress along the gastrointestinal tract. This fact was evaluated by submitting the different seasonings under study to simulated digestion followed by assessing the reducing and antiradical capacities of the digested fractions. Enzymatic gastrointestinal digestion enhanced 2-3 times both antioxidant activities and colonic fermentation increased more than 10-fold the radical scavenging ability of digested fractions compared with undigested seasonings. Digested fractions derived from the seedless wine pomace seasoning presented generally the highest antioxidant properties. The results were evaluated considering bioaccessibility factors to have a more realistic overview of the potential antioxidant capacities of the seasonings and of the probable beneficial effects of their consumption on the prevention of oxidative damage along the gut.

Keywords: ABTS; Antioxidant capacities; Colonic microbial fermentation; Enzymatic gastrointestinal digestion; Folin index; QUENCHER; Wine pomace; Seasoning.

Abbreviations and nomenclature: **ABTS**, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid*;* **CF**, colonic fermented; **CFr**, colonic fermented residue; **CFs**, colonic fermented supernatant; **FC**, Folin-Ciocalteu; **GAR**, global antioxidant response; **GID**, gastrointestinal digested; **GIDD**, gastrointestinal digested+dialyzed; **Q-**, QUENCHER; **RWPS**, red wine pomace seasoning; **Sd-S**, seasoning obtained from wine pomace seeds; **Sk-S**, seasoning obtained from seedless wine pomace; **TAC**, total antioxidant capacity; **UD**, undigested; **W-S**, seasoning obtained from whole wine pomace.

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Highlights:

- Antioxidant capacity of wine pomace seasonings following in vitro digestion evaluated.
- Higher TAC values were exhibited by digested fractions than undigested seasonings.
- Enzymatic gastrointestinal digestion enhanced both reducing and antiradical abilities.
- Compounds solubilized during colonic fermentation displayed high antiradical activity.
- Seedless wine pomace seasoning showed high digestibility and antioxidant capacities.

1. Introduction

Epidemiological studies and associated meta-analyses strongly suggest that long term consumption of fruits and vegetables plays a pivotal role in the prevention against numerous chronic diseases such as cancer (Sun et al. 2002; Pandey & Rizvi 2009). In the gastrointestinal tract, these health-protective effects are partially attributed to their antioxidant properties (Halliwell et al. 2000), which have been associated with their high phytochemical (mainly phenolic compounds and carotenoids) and antioxidant dietary fibre contents (Saura-calixto et al. 2010; Pérez-Jiménez et al. 2013).

An adequate bioavailability of bioactive substances is a prerequisite for potential systemic effects *in vivo* (Manach et al. 2004). However, many antioxidants remain in the intestinal luminal contents and may exert a local beneficial effect within the gut by protecting possible oxidisable molecules and the intestinal epithelium from oxidative damage occurring during digestion (Halliwell et al. 2000; Goñi & Serrano 2005). In this regard, the chemical alterations and the bioaccessibility of antioxidant compounds in the gastrointestinal tract are key aspects that determine their bioavailability (Rein et al. 2013; Carbonell-Capella et al. 2014), especially for those foods rich in antioxidant dietary fibre due to its low digestibility (Palafox-Carlos et al. 2011). Gastrointestinal digestion is able to release, from food matrices, some entrapped antioxidants that might be absorbed in the small intestine, whereas other antioxidants remain enclosed in the indigestible fraction and reach the large intestine (Scalbert & Williamson 2000). These bioactive substances and the metabolites formed after their fermentation by gut microbiota could exert their antioxidant activity in situ or, to some extent, be absorbed in the lower regions of the colon (Delgado-Andrade et al. 2010; Saura-calixto et al. 2010). Similarly, the insoluble matter in ingested food, which remains in the gastrointestinal tract for a long time, may help to counteract the free radicals that are continuously formed in the gut (Tabernero et al. 2011; Pérez-Jiménez et al. 2013).

In view of the above, the effects of the digestive process on the phytochemicals contained in foods, and on their antioxidant activity, have attracted great attention from the scientific community over the last years (Rein et al. 2013; Heim et al. 2002). Thus, several in vitro digestion models to assess bioaccessibility that allow the study of changes in dietary components during the gastric and intestinal stages have been implemented (Hur et al. 2011; Carbonell-Capella et al. 2014; McDougall et al. 2005). Despite the limitations of in vitro digestion models, especially those comprising only a static simulated digestion, the good correlation of the results obtained with those from several animal and human studies has been established (Saura-calixto et al. 2010; Alminger et al. 2014). Furthermore, the combination of in vitro digestion models with total antioxidant capacity (TAC) assays for the digested fractions obtained has been suggested as a first approach to predict the *in vivo* antioxidant activity of foods (Goñi et al. 2005; Rufián-Henares & Delgado-Andrade 2009).

Consequently, Delgado-Andrade et al. (2010) have proposed a methodology to determine the global antioxidant response (GAR) of food, which is defined as the sum of the antioxidant activities of the soluble and insoluble fractions obtained after a simulated gastrointestinal digestion. According to this method, the TAC of these digested fractions is measured separately, using classical and QUENCHER (Gökmen et al. 2009) assays, respectively, and then combined to estimate the GAR of foodstuffs. Thus far, several plant-based foods have been assessed following the GAR method, and important variations in the antioxidant activities exhibited by the different food matrices tested have been detected (Papillo et al. 2014; Pastoriza et al. 2011).

The promising use as food ingredients of new seasonings obtained from red wine pomace (RWPSs) has recently been demonstrated (García-Lomillo et al. 2014). The new powdered vegetal seasonings are antioxidant-rich products, containing mainly phenolic compounds, which may contribute to the intake of exogenous natural antioxidants and reinforce the endogenous redox environment once ingested. In this regard, it has been suggested that consumption of wine pomace may help prevent colon cancer (López-Oliva et al. 2006) and its high antioxidant content certainly play an important role in this protective effect.

On the basis of the previous considerations, the present study was conducted to evaluate the effects of the digestive process on the antioxidant activity of three of these new seasonings, targeting the antioxidant capacities of digested fractions which can mimic those produced in the small and the large intestine after intake of each studied seasoning. For this purpose, the TAC of in vitro digested fractions (including both gastrointestinal and colonic phases) was measured using QUENCHER methodologies.

2. Materials and Methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), 2,2’-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), porcine bile extract, calcium chloride dihydrate ($CaCl_2.2H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), L-cysteine hydrochloride, gallic acid, hydrochloric acid (HCl), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), maleic acid, porcine pancreas pancreatin, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), resazurin sodium salt, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium sulphide nonahydrate ($Na_2S.9H_2O$), Tris hydrochloride (Tris), tryptone, enzymes used in enzymatic digestion α-amylase, amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1), and cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocalteu (FC) reagent and sodium carbonate (Na_2CO_3) were purchased from Panreac Química S.L.U. (Barcelona, Spain).

2.2. Materials

The seasonings used in this study were made in the pilot plant of the Food Technology Department of University of Burgos (Spain) as previously described (García-Lomillo et al. 2014), applying the process patented by González-Sanjosé et al. (2013). Three different types of powdered seasonings were used, which were labelled as W-S, corresponding to the seasonings obtained from whole red wine pomace; Sk-S, representing the seasonings obtained from seedless red wine pomace, in which grape skins are the main component; Sd-S, referring to the seasonings obtained from the seeds separated from red wine pomace. Three different batches of each type of RWPS were used in this study.

The inoculums used for colonic fermentation were obtained at the animal-housing unit of the University Hospital of Burgos (Spain) by mixing the caecal content from 5 male Wistar rats (body weight of 250 ± 5 g) fed with standard maintenance diet. All aspects of this procedure were conducted in accordance with the guidelines established by the Ethics Committee at both the University Hospital of Burgos and the University of Burgos.

2.3. *In vitro* gastrointestinal digestion and colonic fermentation of the seasonings

Simulated complete digestion of the three RWPSs (Sk-S, W-S, and Sd-S) was performed according to the method describe by Saura-Calixto et al. (2007), with only slight modifications in the dialysis step. This *in vitro* static digestion model allows the estimation of the bioaccessibility of dietary antioxidants, and mainly comprises two consecutive stages, an enzymatic gastrointestinal digestion first phase, followed by a colonic microbial fermentation phase (**Figure 1**). Briefly, each powdered seasoning, labelled as “undigested” (UD), was successively incubated with digestive enzymes, as described in detail by Saura-Calixto et al. (2007), yielding the so-called "gastrointestinal digested" (GID) fractions. Each GID was centrifuged (3,000 g, 15 min, 25 °C) to separate the supernatant and the solid residue. This centrifugation was repeated twice, washing the residue with 5 mL of Milli-Q water. All the supernatants obtained were then combined, transferred into cellulose membrane dialysis tubing, and dialyzed against a total of 2 L of water for 24 h (changing the water twice). The dialysis retentate was mixed with the GID solid residue to obtain the so-called “gastrointestinal digested+dialyzed" (GIDD) fraction, which contained the compounds hypothetically non-absorbed in the small intestine that may reach the large intestine. The GIDD fraction was the substrate for the action of colonic microbiota, obtaining the "colonic fermented" (CF) fraction. Finally, the CF fraction was centrifuged (2,500 g, 10 min, 25 °C) to collect the supernatant (CFs) and the residual solid (CFr) respective fractions. All fractions isolated along the simulated digestive procedure were lyophilized, weighed, and stored at -20 °C until their analysis.

Each digested fraction (GID, GIDD, CF, CFs, and CFr) were obtained by triplicate from each batch of each type of RWPSs under study.

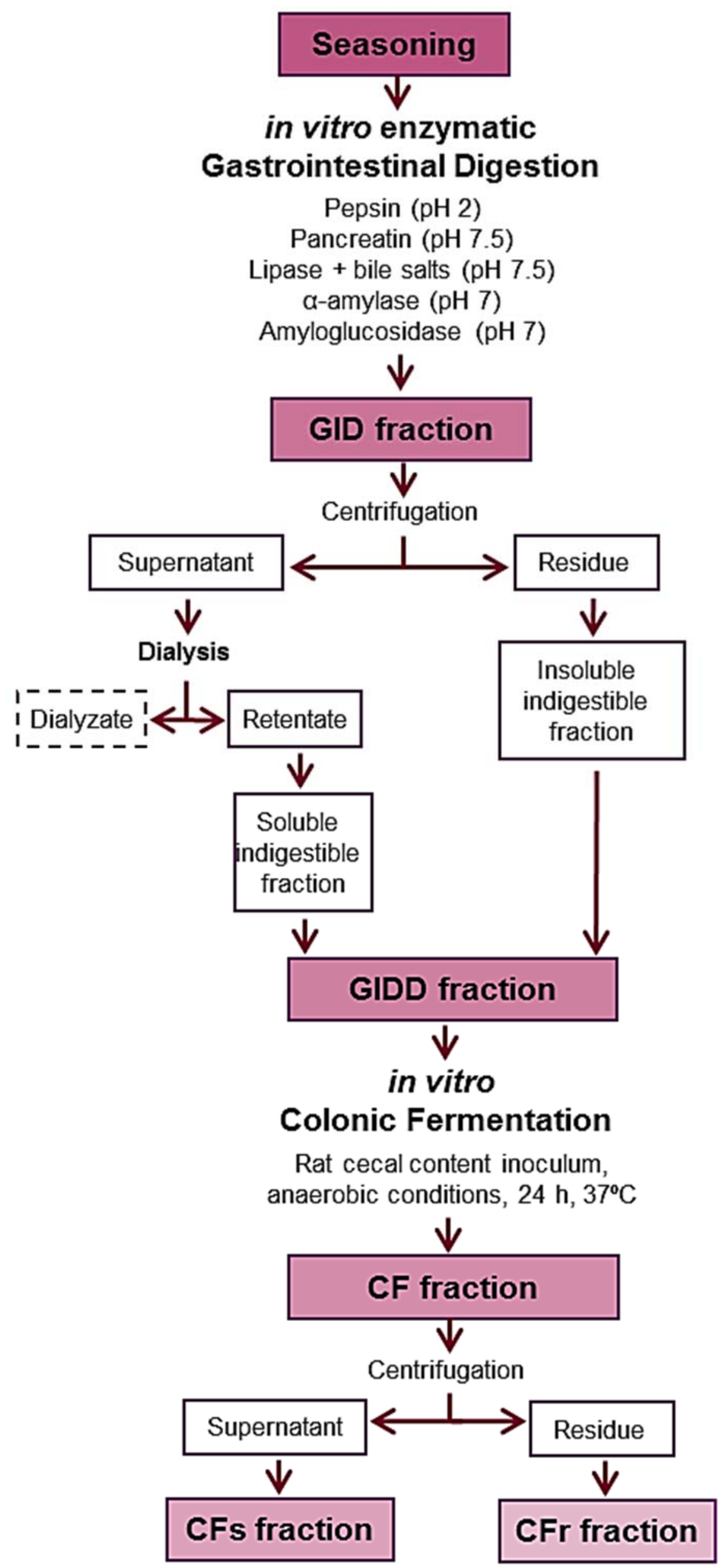


Figure 1. Diagram of the main steps performed during the complete *in vitro* digestion of the seasonings. The different fractions under study were: GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue.

2.4. Yields (%, w/w) of the in vitro digested fractions

The yields of each of the digested fractions (GID, GIDD, CF, CFs, and CFr) were expressed as percentages (%, w/w) with respect to the corresponding initial quantity of each UD UD fraction.

Taking into account that the chemicals, enzymes, and colonic inoculums added to perform the *in vitro* pre- and post-colonic digestion also contributed to the mass of the lyophilized digested fractions, control digestive procedures were run in parallel in absence of seasonings. As such, digested control fractions were also obtained in triplicate. The mass of these control fractions was used to calculate the “real” yield of each corresponding digested fraction.

2.5. Total antioxidant capacity (TAC)

QUENCHER (Q-) versions of two classical total antioxidant capacity (TAC) assays (Folin-Ciocalteu index and ABTS) were selected to evaluate the TAC of such fractions.

2.5.1. QUENCHER Folin-Ciocalteu assay (Q-FC)

A sample mass of each lyophilized digested fraction (1 ± 0.005 mg) was mixed with 0.2 mL of Milli-Q water and 0.2 mL of FC reagent, and allowed to react for 5 min. A 4 mL aliquot of 0.7 M Na_2CO_3 solution was then added and the mixture made up to a final volume of 10 mL with MQ water. After incubation for 1 h in the dark with continuous stirring, the supernatant was separated and the absorbance at 750 nm was measured in an UV-vis spectrophotometer U-2000 (Hitachi, Ltd., Hubbardston, MA, USA) (Del Pino-García et al. 2015). A dose-response curve was plotted using different quantities of gallic acid as the standard.

2.5.2. QUENCHER ABTS assay (Q-ABTS)

A sample mass of each lyophilized fraction (1 ± 0.005 mg) was weighed and mixed with 10 mL of the $ABTS^{\cdot+}$ working solution, which was prepared in Milli-Q water following the procedure described by Del Pino-García et al. (2015). After incubation for 30 min in the dark in an orbital shaker, the supernatant was separated and the absorbance at 734 nm was measured. A linear calibration curve was obtained with different amounts of Trolox as the standard.

2.5.3 Total antioxidant capacity expressions

The Q-TAC values were expressed in two different ways:

- *Absolute approach*: in this case Q-TAC values were expressed with respect to the mass of each fraction analysed. Thus, final values were expressed as µmol of standard equivalents (GAE or TE) per gram of GID, GIDD, CF, CFs, and CFr fractions, and per gram of UD fractions.

As mentioned above, chemicals, enzymes, and colonic inoculums used for the *in vitro* digestion protocol may contribute to the mass of each digested fraction. Similarly, they can contribute to the antioxidant activity measured on the lyophilized digested fractions. So, "real" TAC values (absolute approach) for each studied fraction were calculated considering both the Q-TAC values and the mass contribution of the respective digested control fractions.

- *Relative approach*: in this case Q-TAC values of each of the digested fraction (GID, GIDD, CF, CFs, and CFr) were expressed as μmol of standard equivalents (GAE or TE) per gram of undigested seasoning. These values were calculated taking into account the "real" yields of such fractions. In this way, the relative approach tries to give a more accurate estimation of the potential TAC of each seasoning after intake, considering the potential bioaccessibility of their bioactive compounds along the gastrointestinal tract.

2.6. Data presentation and statistical analysis

The results were expressed as mean ± standard deviation (n=3). Real replicates from three different batches of each seasoning were used. Furthermore, analytical parameters were measured also in triplicate.

Statistical data analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). A one-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was applied to establish significant differences among the values of each digested fraction for the same seasoning, and among the values of each studied seasonings for the same fraction. Significance level of $p < 0.05$ was considered.

3. Results and Discussion

A methodology that combines a complete *in vitro* pre- and post-colonic digestion of foodstuffs with the analysis of the total antioxidant capacity of the obtained digested fractions by QUENCHER assays is proposed.

This methodology has been used to study the potential antioxidant capacities of three new seasonings derived from red wine pomace, which have been satisfactorily applied in food matrix. Yields of each simulated digestion phases were determined, and the potential antioxidant ability of the obtained fractions was estimated applying Q-TAC assays. Since few data are available about the effects of the digestive process on the antioxidant activity of wine pomace and related products, this study can contribute to the state of the art and point out new interesting data.

3.1. Yields of each *in vitro* digested fractions

Digestive and colonic fermentative processes lead to the release and modification of food components, thus determining their bioaccessibility and bioavailability. The extent, intensity and location of these transformations depend, among other parameters, on the foodstuff composition (Palafox-Carlos et al. 2011; Rein et al. 2013; Acosta-Estrada et al. 2014). This fact was evidenced by the differences observed among the obtained yields for each seasoning (Sk-S, W-S, and Sd-S) (**Table 1**).

Table 1. **Yields of each digested fraction obtained after *in vitro* pre- and post-colonic digestion of the studied seasonings.**

Digested fractions [a]	RWPSs [b]		
	Sk-S	W-S	Sd-S
GID	92.5 ± 1.4 d / α	92.5 ± 1.1 d / α	88.1 ± 1.3 bc /α
GIDD	68.4 ± 0.7 c / α	71.6 ± 1.5 b / β	82.1 ± 0.9 b / γ
CF	69.3 ± 2.3 c / α	77.6 ± 1.5 c / α	89.4 ± 7.6 c / β
CFs	34.9 ± 0.8 a / α	41.6 ± 0.5 a / β	48.6 ± 1.9 a / γ
CFr	40.8 ± 0.9 b / α	43.1 ± 1.2 a / α	47.9 ± 2.1 a / β

Yields (%, w/w) with respect to undigested seasonings (100%, w/w) are given as the mean ± standard deviation (n = 3).

a) Digested fractions: GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue.

b) Red wine pomace seasoning (RWPSs) which were obtained from: seedless wine pomace (Sk-S); whole wine pomace (W-S); and seeds (Sd-S).

Significant differences ($p < 0.05$) between fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) between seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

Fractions obtained after submitting plant food to such gastrointestinal digestive phase contain different types of soluble and insoluble compounds (Saura-Calixto et al. 2007): bioaccessible and absorbable molecules (which are known as bioavailable compounds), bioaccessible but non-absorbable molecules, and non-bioaccessible particles that remain attached to the food matrices. GID products represent all these potentially bioactive compounds which derive primarily from several precursors of higher size (oligomers and polymers) present in the studied seasonings, such as phenolic compounds, proteins, and the different constituents of the dietary fibre fraction (García-Lomillo et al. 2014).

The observed decrease in the yields of the GIDD fractions compared to those of GID fractions noted that significant amounts of compounds were solubilized during the enzymatic gastrointestinal digestion, and some of them were able to diffuse out of the dialysis tubing. Although only mechanical forces are considered in the simulated digestion (Alminger et al.

2014), in a simplified manner, the dialyzed compounds could represent the constituents of the seasonings hypothetically bioavailable in the small intestine. The significantly higher decrease from GID to GIDD fractions detected for Sk-S than for W-S and Sd-S pointed out the higher digestibility and putative bioavailability of the compounds contained in the first RWPS. This fact was evidenced by comparing, in percentage, the amount of GIDD fractions with respect to the corresponding GID fractions (Sk-S: 73.9%, W-S: 77.4%, and Sd-S: 93.2%), with the higher percentage indicating the lower estimated digestibility and bioavailability. In general terms, the yields obtained for GIDD fractions were in agreement with the values reported by Bravo & Saura-Calixto (1998) and Goñi et al. (2005), who found that the indigestible fraction of grape pomace represented around 80% of dry matter, with dietary fibre (including associated non-extractable polyphenols, being the major constituent of this fraction (ranging from 72-79%). Consequently, the lower dietary fibre content in Sk-S (48.6%) than in Sd-S (58.9%) (García-Lomillo et al. 2014) might partly explain the higher digestibility of Sk-S and the lower yields of its GIDD fraction.

The yields obtained for CF fractions were similar or higher than for GIDD fractions. Concretely, significant increases were detected in W-S and Sd-S, but not in Sk-S. Previous research has reported that grape seed flavan-3-ol monomers can promote the growth of certain beneficial gut bacterial groups (Cueva et al. 2013). As W-S and Sd-S contain wine pomace seeds and are richer in flavan-3-ol derivatives than Sk-S (García-Lomillo et al. 2014), it is possible that the release of some monomers during the fermentation of their GIDD fractions exerted a positive effect on the growth of colonic microbiota. In this case, the higher microbial population in CF fractions of W-S and Sd-S could explain the observed increase in their mass.

The overall fermentability of each RWPS by colonic microbiota was estimated by comparing, in percentage, the amount of solubilized colonic fermented compounds (CFs fractions) with respect to the corresponding GIDD fractions. In this way, interferences due to the possible different amount of gut microbiota acting in each case were partially eliminated. Lower quantities of CFs fractions were obtained after colonic fermentation of GIDD fractions derived from Sk-S (51.0%) than from W-S and Sd-S (58.1% and 59.2%, respectively). Bravo & Saura-Calixto (1998) reported that insoluble dietary fibre represented more than 90% of the total dietary fibre in grape pomace, with no important differences between grape skin- or seed-enriched samples. Therefore, taking into account the low colonic fermentability of insoluble dietary fibre (Bravo et al. 1994; Saura-calixto et al. 2010), the results of the current study may be explained considering other compounds present in the GIDD fraction. Goñi et al. (2005) noted that proteins are the second highest constituent of the indigestible fraction of grape pomace (about 14%), which is due to their low digestibility (about 12%, with no differences between grape pomace constituents). However, proteins are metabolized to a large extent in the colon, with higher yields observed for seed proteins (around 70%) than

for skin proteins (around 60%). In light of the above, and considering that the protein content of the RWPSs was rather similar, ranging from 12-14% (García-Lomillo et al. 2014), the fermentation of proteins might play an important role to explain the estimated lower fermentability of Sk-S compared with W-S and Sd-S. In addition, the fermentation of proanthocyanidins, most abundant in Sd-S than Sk-S (García-Lomillo et al. 2014), and other non-absorbed phenolic compounds by gut microbiota will certainly contribute to increase the contents of soluble compounds liberated in the CF fractions (Saura-Calixto et al., 2010), thus increasing the fermentability observed.

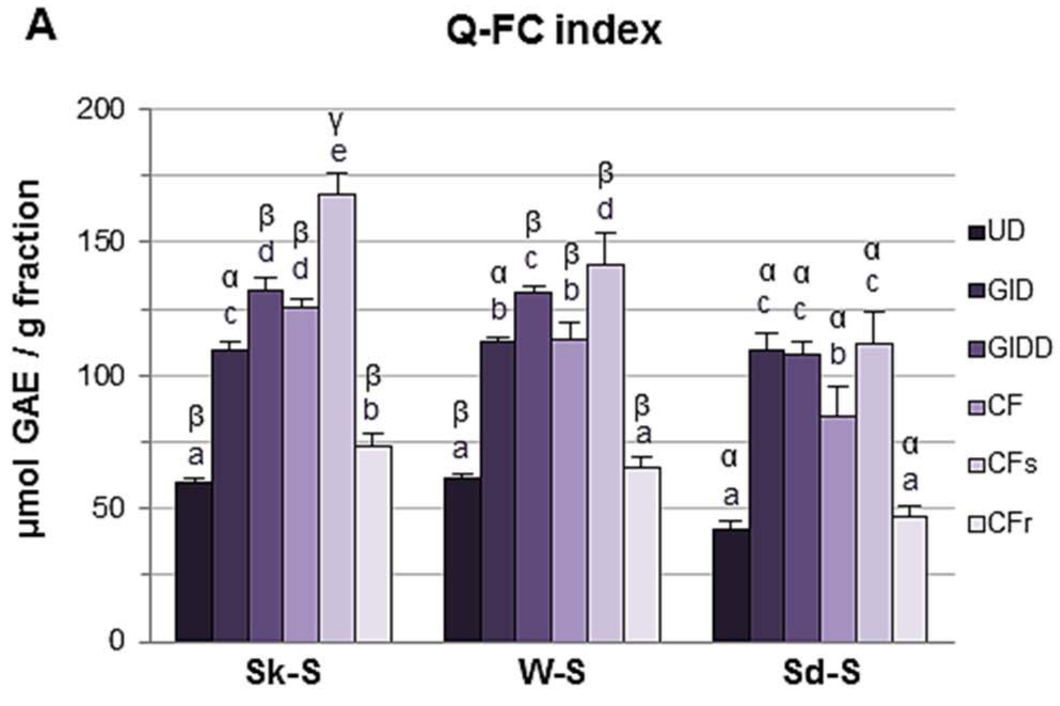
3.2. Potential antioxidant activities of each *in vitro* digested fractions

The ability of the different RWPSs, once ingested, to reduce reactive species (Q-FC assay) and to quench free radicals (Q-ABTS assay) along the gastrointestinal tract was estimated from the Q-TAC data of the fractions isolated throughout the *in vitro* digestion procedure. These Q-TAC values were evaluated under two points of view, considering the antioxidant capacity of each gram of the digested fractions (absolute approach), and regarding the antioxidant capacity expressed per gram of UD seasonings (relative approach). Furthermore, two comparative studies were carried out, one among seasonings (Sk-S, W-S, and Sd-S), and other among digested fractions (GID, GIDD, CF, CFs, and CFr), considering also the UD fractions in the case of absolute approach data.

3.2.1. Total antioxidant capacity of each digested fractions: absolute approach

The results obtained from the Q-FC and Q-ABTS assays (**Figure 2**, **A** and **B**, respectively) showed significant variations regarding the two factors under study, the seasonings and the digested fractions analysed.

Concerning the differences among the three RWPSs before digestion (UD fractions), Sk-S and W-S exhibited greater antioxidant capacity than Sd-S. These results were almost certainly due to the differences among wine pomace constituents in terms of content, antioxidant capacity, hydrophilicity, solubility, and accessibility of their antioxidant compounds (Del Pino-García et al. 2015). GID fractions showed similar Q-TAC values for the different seasonings. Therefore, despite the lower digestibility of Sd-S (*Section 3.1.*), a higher increase in the “absolute” antioxidant activity displayed by Sd-S than by Sk-S and W-S was observed following enzymatic gastrointestinal digestion. The rest of digested fractions (GIDD, CF, CFs, and CFr) generally showed a similar trend between the three RWPSs as found prior digestion. Thus, after the liberation and absorption of bioavailable antioxidants in the small intestine, the net antioxidant capacity displayed by the compounds that reach the colon might again be higher for Sk-S and W-S than for Sd-S, both before and after the action of colonic microbiota. In addition, it must be noted that Sk-S gave the highest Q-TAC values in CFs products, despite the slightly lower fermentability estimated for this RWPS (*Section 3.1.*).



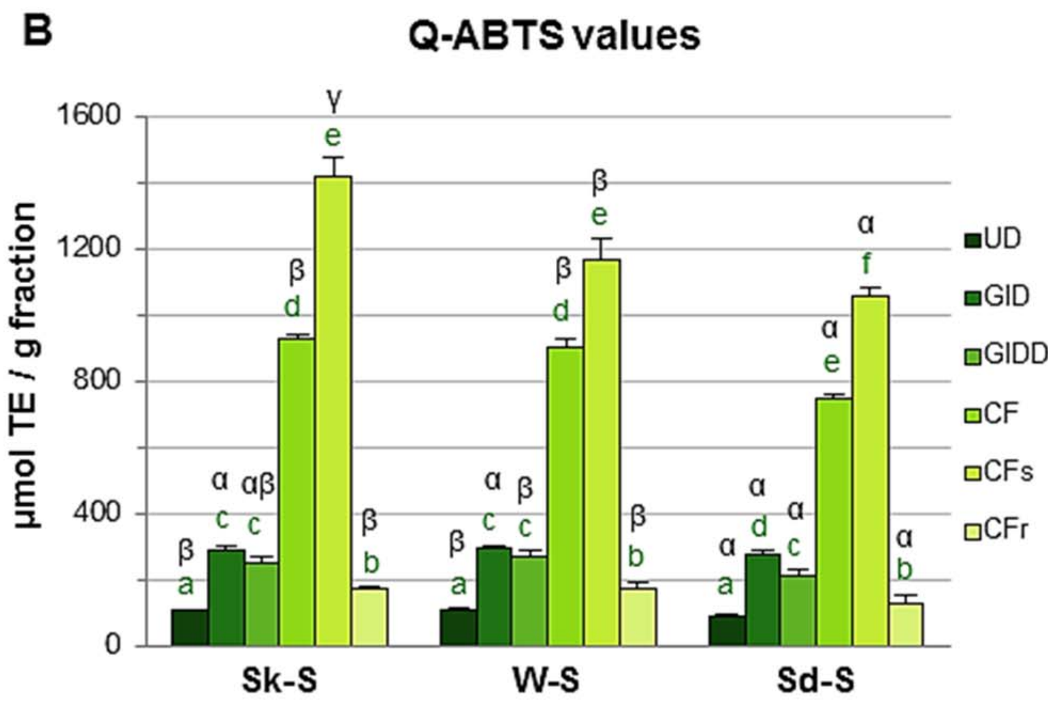


Figure 2. **Antioxidant activities of the *in vitro* digested fractions derived from red wine pomace seasonings: absolute approach.** Total antioxidant capacities determined using the Q-FC (Folin-Ciocalteu) (**A**) and Q-ABTS (**B**) assays. Sk-S: seasoning obtained from seedless red wine pomace; W-S: seasoning obtained from whole red wine pomace; Sd-S: seasoning obtained from seeds. UD: undigested; GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue. Q-TAC values are expressed as mean ± standard deviation (n = 3). GAE: gallic acid equivalents. TE: Trolox equivalents. Significant differences ($p < 0.05$) among fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) among seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

Regarding the effects of the simulated digestion, in general, the different digested fractions showed significantly higher Q-TAC values than their respective undigested seasonings. Concretely, the Q-FC and Q-ABTS values for GID fractions were around two- and three-fold higher than for UD fractions, respectively. Therefore, the enzymatic gastrointestinal digestion phase produced a marked increase in the antioxidant capacity of the RWPSs. These results generally agree with those reported by Rufián-Henares & Delgado-Andrade (2009). These authors demonstrated that *in vitro* gastrointestinal digestion was essential to allow the release of a large quantity of antioxidant compounds, showing an increase of up to four-fold in the ABTS values for the soluble digested fraction with respect to the original products. However, the small variations detected between the TAC values of GID and GIDD fractions suggested that the absorption of compounds in the small intestine did not severely affect the "absolute" antioxidant capacity of GIDD fractions. The observed opposite tendencies between the values of both Q-TAC assays could be explained considering the possible elimination, during dialysis, of compounds with free radical scavenging capacities, as well as with capacity to interfere in the measure of the FC index.

The colonic fermentation phase led to marked increases in the Q-ABTS values, but less noticeable effects on the Q-FC results. Concretely, the values of $ABTS^{\cdot+}$ scavenging capacity displayed by CF fractions exceeded that obtained for UD fractions by almost 10-fold. These results appear to point out that the action of colonic microbiota induced important chemical changes on the compounds retained in GIDD fractions, releasing metabolites with high free radical scavenging ability but not with significant reducing properties. The Q-TAC values for CFs fractions appear to show that metabolites solubilized during the colonic fermentation had higher antioxidant capacity than the global CF fractions. These compounds had both reducing and free radical scavenging capacities. On the contrary, CFr products gave marked lower Q-FC and Q-ABTS values than their respective CF fractions. These findings suggest that the soluble antioxidant compounds generated and released due to the action of gut microbiota were the main responsible for the results obtained in CF fractions, which was especially important with regard to the potential antiradical capacity of the digested seasonings.

Differences observed between the absolute antioxidant capacities of the digested fractions seem to be, at least in part, due to the structural changes occurring in the RWPS matrices during digestion. These alterations might lead to the exposure of functional groups on the surface or somewhere inside the solid matrices, thus improving the accessibility for the radicals (Rufián-Henares & Delgado-Andrade 2009). This fact could partially explain the increased antioxidant capacity of the digested fractions in comparison with the UD fractions.

In parallel, it must be noted that the type, amount, and activity of any antioxidant compound present in the digested fractions may greatly differ from the initial situation of their precursors in the seasonings. Indeed, some antioxidants, such as anthocyanins and other

phenolic compounds, might be lost or transformed (isomerized, hydrolysed, etc.) by gastrointestinal enzymes, or due to their instability under the pH conditions of the simulated digestion (McDougall et al. 2005; Tagliazucchi et al. 2010; Velderrain-Rodríguez et al. 2014). In contrast, different new bioactive molecules with antioxidant properties can be simultaneously formed in the gastrointestinal tract. Some of them may consist on bioactive metabolites generated from modifications of antioxidant compounds, and others become from metabolic reactions associated to other food components.

Saura-Calixto et al. (2007) estimated that around 32% of the ingested fruit-derived phenols were bioaccessible in the small intestine, and around 56% became bioaccessible in the large intestine. The most hydrophilic phenolic forms, such as glycosylated flavonols or quinic acid derivatives of hydroxycinnamic acids, may readily solubilize in the aqueous phase in the upper gastrointestinal tract, whereas less soluble flavonoid aglycones or procyanidins may be strongly bound to dietary fibre and proteins (Le Bourvellec & Renard 2012; Palafox-Carlos et al. 2011; Rein et al. 2013). In the case of wine pomace, certain complex phenolics, such as polymeric proanthocyanidins bound to antioxidant dietary fibre, represent about 15-30% of dry matter (Bravo & Saura-Calixto 1998). In addition, acidic pH and proteolytic enzymes (such as pepsin) in the gastric tract play an important role in polyphenols bioaccessibility by releasing phenolic compounds bound to solid matters (Tagliazucchi et al. 2010; Rufián-Henares & Delgado-Andrade 2009; Alminger et al. 2014). This fact is in agreement with the increase of Q-TAC values observed after the enzymatic gastrointestinal digestion phase in the present study. Furthermore, according to the high Q-ABTS values observed after the colonic fermentation phase, the action of microbial enzymes (such as esterase and xylanase) must be regarded as another factor that could contribute to increase the pool of free phenolic acids, thereby enhancing the antioxidant capacity of the fermented fractions (Kroon et al. 1997; Acosta-Estrada et al. 2014). Similarly, the action of β-glucosidases of enteric bacterial origin might hydrolyse phenolic glycosides, thus liberating the corresponding aglycones which usually display higher antioxidant activity than the glycoside forms (Aura et al. 2005).

Finally, it should be noted that, in the large intestine, several antioxidant phenolic compounds may be newly formed as a result of the extensive transformation of phenols retained into the residual undigested fractions by gut microbiota. Simple soluble compounds can thereby be generated, being phenylacetic, phenylpropionic and benzoic acid derivatives the main phenolic bacterial metabolites (Acosta-Estrada et al. 2014; Pérez-Jiménez et al. 2013; Sánchez-Patán et al. 2012; Fernández-Panchón et al. 2008). Consequently, non-extractable phenolic compounds that reach the large intestine may significantly contribute to the antioxidant capacity in the colonic contents (Touriño et al. 2011).

3.2.2. Total antioxidant capacity of each digested fractions: relative approach

The results of the relative Q-TAC values of each digested fractions evidence that RWPS intake may considerably increase the pool of exogenous antioxidants in the entire gastrointestinal tract and, thereby, contribute to the antioxidant capacity of the intestinal luminal contents. Relative Q-TAC values of the gastrointestinal digested fractions showed the highest Q-FC values, while colonic fermentation fractions exhibited the highest Q-ABTS values (**Figure 3, A** and **B**, respectively). Furthermore, Q-TAC values of CFr fractions were the lowest, although the Q-TAC values of these fractions indicated certainly important role in the antioxidant capacity of the colonic contents.

Independently of the Q-TAC methodology used, the results showed that most of the digested fractions may display considerably higher antioxidant capacities once they are present in the small or large bowels than before being ingested.

With regard to differences between the seasonings (Sk-S, W-S, and Sd-S), similar antioxidant capacity profiles were obtained for the three RWPSs in each Q-TAC assay. A slight tendency toward lower values was observed in the products derived from Sd-S, but differences were statistically significant in only a few cases.

A significant decrease of about 11% between the Q-FC values of GID and GIDD fractions of the seasonings with higher digestibility (Sk-S and W-S) was observed. However, marked falls (ranged from 27-36%) in the Q-ABTS values were detected for the three seasonings. This finding suggests a higher antiradical activity than reducing capacity of those compounds potentially absorbable through the intestinal barrier in the small intestine. Nevertheless, the most notable difference between the values obtained by the two Q-TAC assays was found comparing the values after the colonic fermentation. Significant decrease (about 8-19%) between the Q-FC values of GIDD and CF fractions were observed. These results contrasted with the marked increase (around 3.6-fold) showed by the Q-ABTS values of these fractions. The soluble compounds in the large intestine (CFs fractions) represented around 70-76% of the reducing capacity of CF fractions, and about 73-81% of their $ABTS^{\cdot+}$ scavenging ability, whereas the insoluble compounds that remained in the residue (CFr fractions) represented around 32-36% of the Q-FC indexes obtained for CF fractions but only about 10-11% of their Q-ABTS values. As these contributions clearly show, the Q-ABTS values for CF fractions are higher than those obtained when adding the values for their soluble and insoluble components, which represent 91.4% (Sk-S), 84.3% (W-S), and 91.0% (Sd-S) of all colonic fermented products. This finding indicates that possible synergistic interactions might take place between soluble and insoluble antioxidants in CF fractions, which has previously been suggested (Çelic et al. 2013). Moreover, these synergisms appear to be specially marked in W-S, suggesting that the presence of compounds from both wine pomace skins and seeds might promote the synergistic effects. However, these interactions do not appear to occur with regard to the reducing power of CF fractions.

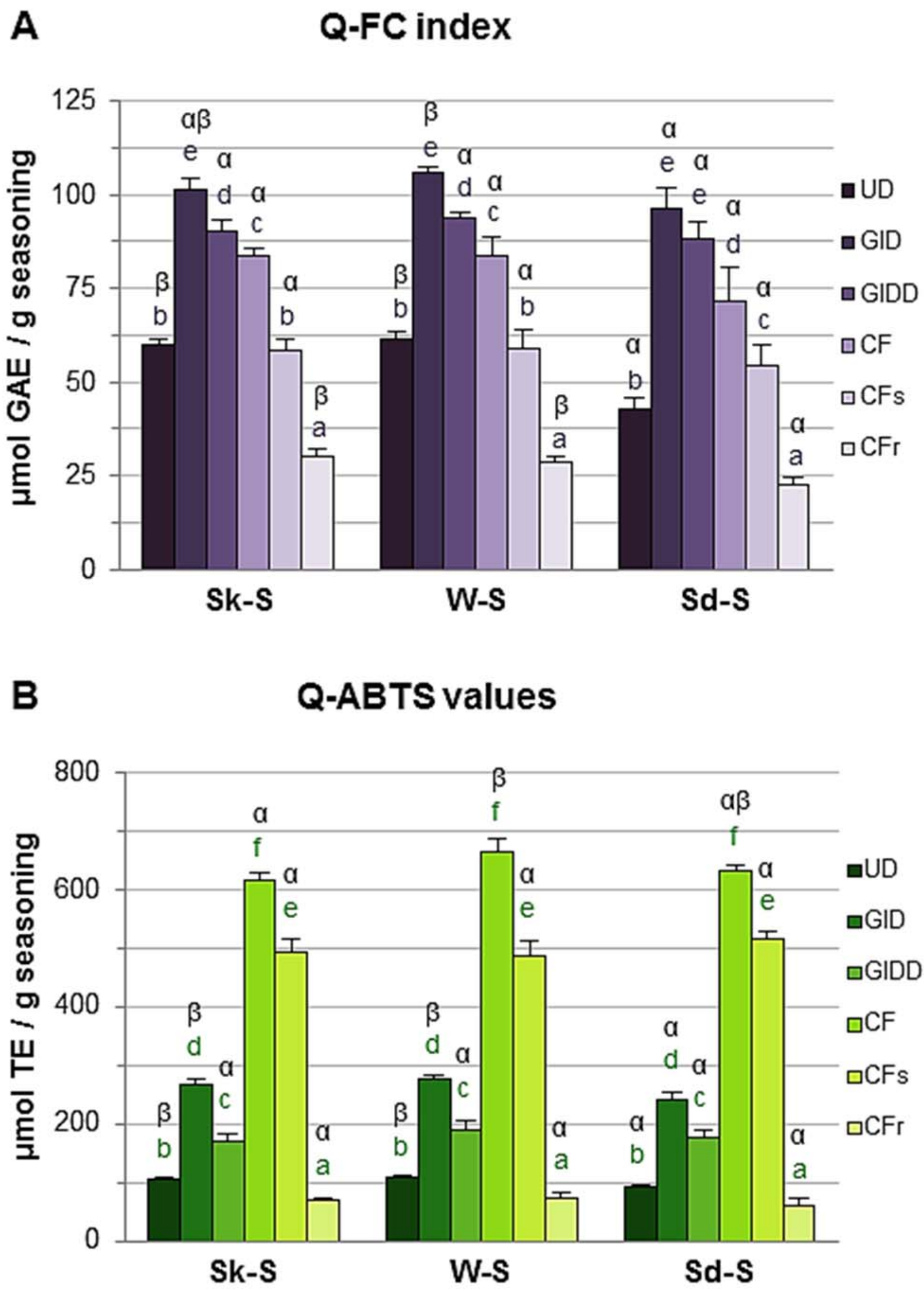


Figure 3. **Antioxidant activities of the *in vitro* digested fractions derived from red wine pomace seasonings: relative approach.** Total antioxidant capacities determined using the Q-FC (Folin-Ciocalteu) (**A**) and Q-ABTS (**B**) assays. Sk-S: seasoning obtained from seedless red wine pomace; W-S: seasoning obtained from whole red wine pomace; Sd-S: seasoning obtained from seeds. UD: undigested; GID: gastrointestinal digested; GIDD: gastrointestinal digested+dialyzed; CF: colonic fermented; CFs: colonic fermented supernatant; CFr: colonic fermented residue. Q-TAC values are given as mean ± standard deviation (n = 3). GAE: gallic acid equivalents. TE: Trolox equivalents. Significant differences ($p < 0.05$) among fractions (UD, GID, GIDD, CF, CFs, and CFr) for each seasoning are indicated by Roman letters. Significant differences ($p < 0.05$) among seasonings (Sk-S, W-S, and Sd-S) for each digested fraction are indicated by Greek letters.

Compounds contained in the CFr fractions showed the lowest potential antioxidant capacities of all digested products analysed, although they still retained around half of the Q-TAC exhibited by the UD fractions.

However, a higher antioxidant capacity in the colonic residual contents can be expected following the consumption of RWPSs than of other plant-based foods with lower quantities of highly polymerized phenolic compounds bound to and/or entrapped in the food matrices. The easier digestibility of such foods certainly results in higher bioaccessibility of their antioxidant compounds in the upper intestine (Carbonell-Capella et al. 2014; Papillo et al. 2014). In this regard, the higher Q-FC index obtained for CFr fractions derived from Sk-S and W-S than from Sd-S must finally be pointed out. These results can be partly explained by the much higher degree of polymerization of the proanthocyanidins present in skins than seeds of *Vitis vinífera* L. *cv. Tempranillo* grapes (Monagas et al. 2003), which might restrict their fermentability (Serrano et al. 2009) and contribute to the higher reducing capacity exhibited by those compounds retained in the colonic contents after intake of RWPSs containing wine pomace skins.

4. Conclusions

Gastrointestinal digestion and colonic fermentation may certainly produce important positive effects on the total antioxidant capacities of seasonings obtained from red wine pomace (RWPSs). This fact is evidenced by the considerably higher antioxidant activities exhibited by most of the digested fractions isolated throughout *in vitro* digestion.

Enzymatic gastrointestinal digestion enhanced both the reducing and the antiradical activities, whereas colonic fermentation produced a marked increase in the free radical scavenging capacity, mainly due to the contribution of the colonic fermented solubilized compounds. However, insoluble residues that might pass thought the gut still retained considerable antioxidant capacity, so they may help to counteract the effects of dietary pro-oxidants in the gastrointestinal tract.

A general tendency to higher antioxidant capacity was observed for the digested fractions of the seasonings obtained from seedless and whole wine pomace (Sk-S and W-S, respectively) than for those of the seasonings derived from seeds (Sd-S). Furthermore, the higher digestibility of the former seasoning enabled the release of large quantities of bioaccessible antioxidants which could possibly be absorbed in the small intestine. This fact, in addition to the slightly lower fermentability of Sk-S, may balance the antioxidant activity that the different seasonings evaluated might potentially display along the gastrointestinal tract.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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Chapter 2.2.

Bioavailability of Phenolic Compounds and Antioxidant Effects of Wine Pomace Seasoning after Oral Administration in Rats

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Abstract

Winery by-products, such as wine pomace, retain a high content of polyphenols and may have potential as part of functional foods or dietary supplements. The bioavailability of phenolics contained in a powdered red wine pomace seasoning (RWPS) and its beneficial effects after acute and short-term supplementation in healthy rats were investigated. In Study 1, a single oral-dose of RWPS (300 mg/kg of body weight) was administered to Wistar rats (n=6). Several phenolic acids that circulated in plasma at 0h, 2h and 4h post-RWPS intake, and those excreted in urine between -3–0h, 0–3h and 3–6h intervals, were determined by GC-MS analysis. Concurrently, the potential protective effects of this intervention were evaluated using plasma and urinary F_2-isoprostanes, plasma total antioxidant capacity, and urinary contents of nitric oxide metabolites (nitrites and nitrates) as biomarkers of oxidative stress and endothelial function. Higher prevention of lipid peroxidation and improved nitric oxide bioavailability were observed in samples collected at 4h and between 3–6h ($p<0.05$). This suggests an important antioxidant role of the metabolites generated by the action of colonic microbiota, such as dihydroferulic and 4-hydroxyphenylacetic acids, although indirect effects of phenolics released and absorbed in the small intestine may be also implicated in the observed protection. In contrast, following 4-weeks of daily RWPS consumption (Study 2), no significant differences were detected between supplemented and control groups for any of the biomarkers assessed, which indicates that acute beneficial effects of RWPS intake might be only transient under normal physiological conditions for this rat model.

Keywords: Bioavailability; Isoprostanes; Nitric oxide; Polyphenols; Oxidative stress; Wine pomace.

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Abbreviations and nomenclature: ABTS, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; **eNOS**, endothelial nitric oxide synthase; **F_2-IsoPs**, F_2-isoprostanes; **FC**, Folin-Ciocalteu; **FRAP**, ferric reducing ability of plasma; **GC-EI-MS**, gas chromatography/electron ionization mass spectrometry; **GC-NCI-MS**, gas chromatography/electron capture-negative chemical ionization mass spectrometry; **NO_2^-**, nitrite; **NO_3^-**, nitrate; **ROS**, reactive oxygen species; **RWPS**, red wine pomace seasoning; **TAC**, total antioxidant capacity.

Highlights:

- Healthy Wistar rats were supplemented a red wine pomace seasoning (RWPS).
- Bioavailability and acute and short-term beneficial effects were assessed.
- Acute RWPS-intake decreased oxidative stress and nitric oxide inactivation.
- 4-week-intake did not improve lipid peroxidation or nitric oxide production.
- Acute benefits of RWPS consumption may be transient in healthy subjects.

1. Introduction

Wine pomace potentially constitutes an abundant and relatively inexpensive source of a wide range of grape derived phenolic compounds, including phenolic acids, stilbenes, flavan-3-ols (mono-, oligo- and polymers) and anthocyanins (Kammerer et al. 2004). While most wine pomace-derived products are obtained applying extractive processes, those produced avoiding any kind of extraction present several advantages as carriers of dietary polyphenols (Saura-Calixto 2011).

Red wine pomace seasonings (RWPSs) are value-added products obtained directly from winemaking by-products. The capacity of the phenolic compounds contained in these powdered seasonings to prevent detrimental effects of oxidative reactions in biological systems represents an interesting opportunity for nutraceutical and food industries to use RWPSs as dietary supplements and functional food ingredients (García-Lomillo et al. 2014; Yu & Ahmedna 2013). In addition, the potential for beneficial properties of RWPSs is supported by their high content in dietary fibre (García-Lomillo et al. 2014). RWPSs show high in vitro antioxidant capacities (Del Pino-García et al. 2015), and promising antioxidant protective effects demonstrated *ex vivo* in endothelial cell cultures (Del Pino-García et al. 2016d). However, when dietary polyphenols are consumed, their bioavailability in vivo should be always considered.

The actual bioactivity and antioxidant function of plant-derived phenolic compounds in the organism is clearly affected by their chemical structural changes due to the extensive digestive and metabolic transformations, and by the interactions with other molecules in the plant or food matrices that determine their intestinal absorption (Heim et al. 2002; Fernández-Panchón et al. 2008; Bohn 2014). As such, health effects consequent of polyphenol intake depend on their bioavailability (Manach et al. 2004). Certain phenolic compounds are highly bioavailable whereas others are poorly or not absorbed in the small intestine (Manach et al. 2005) and reach the colon, where they are metabolized by the intestinal microbiota into various phenolic acids, mainly phenylpropionic, phenylacetic, and benzoic acid derivatives (Rechner et al. 2002; Aura 2008). Nevertheless, long-term consumption of polyphenol-rich foods does not generally result in accumulation of significant amounts of these metabolites in plasma (Lotito & Frei 2006). In fact, half-lives of circulating phenolic compounds and metabolites are short, usually in the range of a few hours, with their levels varying from low to high nanomolar concentrations in plasma and within the micromolar range in urine (Manach et al. 2005; Lafay & Gil-Izquierdo 2008; Aura 2008; Del Rio et al. 2013). Consequently, the antioxidant efficacy of phenolic compounds *in vivo* may be limited by their digestive and metabolic alterations, rather low bioavailability, and rapid elimination from the organism.

In light of the above, expanding our knowledge towards the metabolic fate of wine pomace phenolic compounds is critical to understand the actual benefits of these potential bioactive compounds in preventing and protecting against oxidative stress. Therefore, the current study aimed to quantitatively investigate the phenolic acid metabolites that circulate in plasma and are excreted in urine after administration to healthy rats of a single-dose of RWPS. The effect of this dietary supplement, obtained directly from seedless red wine pomace, to modulate biomarkers of oxidative stress and endothelial function under normal physiological conditions in both acute and short-term intervention experiments was also investigated.

2. Materials and Methods

2.1. Chemicals

Acetonitrile anhydrous (99,8%), 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), N,O-bis(trimethylsilyl)trifuoroacetamide with 1% trimethylchlorosilane (BSTFA+TMCS), butylated hydroxytoluene (BHT), bovine serum albumin (BSA), diethylene triamine pentaacetic acid (DTPA), N,N-diisopropylethylamine (DIPEA), 2,4-dinitrophenylhydrazine (DNFH), ethanol, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), gallic acid (GA), glutathione (GSH), guanidine hydrochloride, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), iodine (I_2), isooctane, methanol, 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), phosphoric acid solution (H3PO4), potassium hydroxide (KOH), pyridine anhydrous (99,8%), potassium iodide (KI), sodium acetate, sodium bicarbonate (NaHCO3); sodium hydroxide (NaOH), sodium nitrite (NaNO2), sodium [15N] nitrate, sodium [15N] nitrite, 1,1,3,3-tetramethoxypropane (TMP), tetraoctylammonium bromide (TOA-Br), 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ), and all phenolic acids standards were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Acetone, hexane, and glacial acetic acid were provided by Univar Australia Pty. Ltd. (Ingleburn, NSW, Australia). Certify II cartridges for solid phase extraction (SPE) were from Varian Australia, Pty. Ltd. (Mulgrave, VIC, Australia). Ferric(III)-chloride acid (FeCl3), ferrous(II)-sulphate (FeSO4), Folin-Ciocalteau (FC) reagent, potassium persulfate (K2O8S2), sodium acetate (NaC2H3O2), and sodium carbonate (Na2CO3) were obtained from Panreac Quimica S.L.U. (Barcelona, Spain). 8-iso-PGF2α-d4 (Cat. No. 316351), 8-iso-PGF2α (Cat. No. 16350), and 8,12-iso-iPF2α-VI-d11 (Cat. No. 10006878) standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). All solvents were HPLC grade.

2.2. Composition of red wine pomace seasoning (RWPS)

The wine pomace-derived product used as a dietary supplement in this study was prepared in the pilot plant of the Food Technology Department of University of Burgos (Spain) (González-Sanjosé et al. 2013). Red wine pomace from the vinification of *Vitis vinifera* L. *cv Tempranillo* was kindly supplied by several wineries situated in Burgos (Spain). Seedless wine pomace was used as raw material to produce the powdered seasoning, which was finally submitted to a heat treatment as a microbial stabilization step for its safety use as a food ingredient. The nutrient composition, total antioxidant capacity, and phenolic contents of this seedless red wine pomace seasoning (RWPS) have been previously reported (García-Lomillo et al. 2014) and are summarized in **Table 1**.

Table 1. **Composition and antioxidant capacity of the seedless red wine pomace seasoning.**

Nutrient/Compound	Concentration
Moisture (%)	6.78 ± 0.43
Dietary fibre (g/100 g)	**48.6 ± 0.70**
Fat (g/100 g)	3.69 ± 0.07
Protein (g/100 g)	14.4 ± 0.81
Ash (g/100 g)	14.37 ± 0.27
Minerals (mg/g)	
Potassium	**43.3 ± 2.53**
Sodium	1.31 ± 0.11
Calcium	1.82 ± 0.11
Phosphorus	1.93 ± 0.11
Phytochemicals	
Total polyphenols (mg GAE/g)	**24.4 ± 0.15**
Total anthocyanins (mg Malv-3GE/g)	2.47 ± 0.10
Total proanthocyanidins (mg P-B1E/g)	39.6 ± 1.03
Total catechins (mg D-CatE/g)	8.78 ± 0.22
Total Antioxidant Capacity	
FRAP (µmol Fe(II)E/g)	244 ± 5.25
ABTS (µmol TE/g)	103 ± 3.63
ORAC (µmol TE/g)	215 ± 5.61
DPPH (µmol TE/g)	39.4 ± 1.54

Concentration results expressed per gram of red wine pomace seasoning (RWPS) as mean values ± standard deviation (n=3).

GAE: Gallic acid equivalents; Malv-3GE: Malvidin-3-*O*-glucoside equivalents; P-B1E: Procyanidin B1 equivalents; D-CatE: D-Catechin equivalents; Fe(II)E: Iron(II) equivalents; TE: Trolox equivalents.

a) Total antioxidant capacity (TAC) determined using the following assays: FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; ORAC: Oxygen radical absorbance capacity; DPPH: 2,2-Diphenyl-1-picrylhydrazyl.

2.3. Animals and experimental design

The protocol for this rat study was approved by the Ethics Committee for Experimental Animal Care at the University Hospital of Burgos and the Ethics Committee at the University of Burgos. All animal procedures were carried out in accordance with the current Spanish and European laws (Royal Decree 53/2013 of the Spanish Ministry of Agriculture, Food and Environment and Ministry of Economy and Competitiveness, and European Directive 2010/63/EU).

Male Wistar rats weighing 307±12 g were purchased from the Animal Research and Welfare Service of Valladolid (SIBA, Valladolid, Spain). Animals were housed at 23 °C with an alternating light/dark cycle of 12h, and they received a standard rodent diet (A04 Safe Iberica-Panlab, Barcelona, Spain) and drinking water *ad libitum* during the experiments.

2.3.1. Study 1: Acute supplementation.

RWPS at a dose of 300 mg/kg of body weight (2 mL in water) was administered by oral gavage to Wistar rats (n = 6) after food deprivation for 12h with free access to water. Rats were placed in metabolic cages and cumulative urine was collected in 3 hour-periods before (-3–0h) and post-RWPS administration (0–3h and 3–6h). Aliquots of these urine samples were stored frozen at -80 °C until biochemical analyses. Blood samples were withdrawn at baseline (0h) and post-RWPS consumption (2h and 4h). These samples were collected by tail venipuncture into Vacutainer® tubes containing sodium and lithium heparin (17 I.U./mL blood) as anticoagulant (Becton Dickinson, Co., Madrid, Spain). In order to prevent oxidation of lipids on storage, those blood samples for F_2-IsoP analysis were collected in ice-cold tubes containing a GSH/BTH/EDTA mixture, as previously suggested (Barden et al. 2014). To recover plasma, all blood samples were centrifuged at 1,500 g for 10 min at 4 °C. Plasma aliquots were kept frozen at -80 °C until analyses.

2.3.2. Study 2: Short-term supplementation.

Wistar rats were randomly divided into two groups (n = 5): the control (C) group and the RWPS-supplemented (+RWPS) group. During 4 weeks, rats were given a daily single dose of the seasoning (300 mg/kg of body weight) dissolved in 2 mL water (+RWPS group) or 2 mL of water (C group), which was orally administered by gavage between 9 and 10 a.m. Food and drink consumption and the animal body weights were recorded every day. There were no significant differences among the groups for any of these determinations (data not shown). Two days before the end of the study, rats were placed in metabolic cages 6h post-RWPS intake and urine was collected during 5h. The last day of the experiment, 12-h fasted animals (which had received the last RWPS dose approximately 24h prior to biological sample collection) were anesthetized and sacrificed to collect blood samples by cardiac puncture. Urine and blood samples were collected and treated as described above (Study 1) and urine and plasma aliquots stored at -80 °C for further analyses.

2.4. Identification and quantification of phenolic metabolites

2.4.1 Phenolic acids in plasma

Concentration of phenolic acids were measured in plasma samples by using a gas chromatography coupled to electron ionization mass spectrometry (GC-EI-MS) method previously described (Caccetta et al. 2000), with slight modifications. Plasma samples collected at all-time points from each rat were assayed together on the same day. Briefly, 100 µL of plasma were acidified with 2 mL of 0.1 M acetate buffer (pH 4.6) and then 50 ng of the internal standard (IS: 1-hydroxy-2-naphthoic acid), 20 µL of β-glucuronidase (4,000 U) with sulfatase activity (200 U), and 10 µL of β-glucosidase (16 U) were added. This solution was then incubated for 4 h at 37 °C to hydrolyze the glucuronide, sulfate and glucoside metabolites. The mixture was further acidified with 6 mM HCl to a pH of < 3 and then extracted with 4 mL of ethyl acetate. The ethyl acetate layer was extracted with $NaHCO_3$ (5% w/v, 3 mL), the bottom layer immediately acidified with 6 mol HCl/L, extracted again with 2 mL ethyl acetate, and dried under nitrogen. These dried extracts were derivatized with 50 µL of BSTFA and 50 µL of dry pyridine, mixed and heated at 40 °C for 30 min. The trimethylsilyl (TMS) derivatives obtained were analyzed on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies, Inc., Palo Alto, CA) coupled to an Agilent 5973B Series Mass Selective Detector and fitted with an DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies) using helium as the carrier gas with an inlet pressure of 30 kPa. Injections were made in the splitless mode. The initial column temperature of 120 °C was held for 1 min and then increased at 20 °C/min to 300 °C, at which it was held for 5 min. The mass spectrometer was operated in the electron-impact mode (70 eV). Mass chromatograms in the full scan mode were recorded in the mass range of 50-500 atomic mass units. Selected ion monitoring (SIM) was carried out to monitor the major characteristic ion for each compound, and peak identification was based on retention time and mass spectra compared with authentic standards. Control samples in which plasma was substituted by Milli-Q water were also analyzed for noise signal correction. For quantification, calibration curves were established by measuring peak areas versus response in comparison with the internal standard over a range of each analyte concentrations. Characteristic ions of the TMS derivatives of each compound, their retention times and the calibration curves obtained can be seen in **Supplementary Table S1**. The concentration of phenolic acids in plasma was finally expressed in µM.

2.4.2. Phenolic acids in urine

Phenolic acids present in urine samples were quantified according to a previously reported protocol (Ward et al. 2004). This method follows almost the same procedure as described above for plasma samples, but β -glucosidase was not added to the samples and 200 µL of urine was used for each analysis. To normalize all urine determinations, urinary creatinine concentration was determined for each urine sample at the Core Clinical Laboratory at Royal Perth Hospital, using a Technicon Axon analyser (Bayer Diagnostics, Scoresby, VIC, Australia). Levels of phenolic acids in urine were hence expressed in µmol /mmol creatinine.

2.5. Assessment of plasma total antioxidant capacity (TAC)

Three different colorimetric methods were used to estimate plasma TAC, following the protocols as previously reported (Santiago-Arteche et al. 2012).

- *Folin-Ciocalteu (FC) method:* This assay determines the total reducing capacity of samples and is generally used to estimate the total phenols in foods and biological samples. The blue-colored phosphomolybdic/phosphotungstic acid complexes generated following reaction absorbs light at 750 nm. Gallic acid (GA) was used as the standard to prepare the calibration lines. The results were expressed as millimolar of GA equivalets (mM GAE).

- *Ferric reducing ability of plasma (FRAP) method:* This assay also evaluates the existence of plasma reducing agents but is based on their ability to reduce Fe(III) to Fe(II). The absorbance at 593 nm is determine as the blue-colored Fe(II)-TPTZ complex formed absorbs light at this wavelength. Aqueous solutions of $FeSO_4$ at different concentrations were prepared for calibration. The results were finally expressed as micromolar of Fe(II) equivalents (µM Fe(II)E).

- *ABTS method:* The ability of plasma antioxidants to scavenge free radical was assessed by their capacity to quench the $ABTS^{\cdot +}$ radical, which absorbs at 734 nm. A linear dose-response curve was obtained using Trolox as the standard, thus expressing final results as millimolar of Trolox equivalets (mM TE).

A PowerWave XS2 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) was used for the absorbance measurements.

2.6. Determinations of F_2-isoprostanes (F_2-IsoPs)

2.6.1. F_2-IsoPs levels in plasma

The concentration of F_2-IsoPs in plasma samples were determined by gas chromatography /electron capture-negative chemical ionization mass spectrometry (GC-NCI-MS), following a previously published method (Mori et al. 1999) with slight modifications. In brief, an IS solution (0.1 mg/L in methanol) of the deuterium-labeled standard (8-iso-$PGF_{2\alpha}$-d_4, also known as 15-F_{2t}-IsoP-d_4) was firstly obtained. Plasma samples were thawed and a 0.2 mL aliquot mixed with 50 µL of the IS solution. Then, 250 µL of KOH (1 M in methanol) was added to the samples, vortexed, incubated at 40 °C for 30 min, acidified to a pH 4.5-4.7 with HCl (1 M), and applied to SPE cartridges. These cartridges were conditioned with 2 mL of methanol/water (1:1, v/v) and 2 mL of hexane/ethyl acetate (3:1, v/v). F_2-IsoPs were then collected with 2 mL of ethyl acetate/methanol (9:1, v/v) and dried under a stream of nitrogen. Samples were derivatized with 40 µL of PFBBr (10% (v/v) in acetonitrile) and 20 µL of DIPEA (10% (v/v) in acetonitrile) by incubating at 25 °C for 30 min. Samples were dried with nitrogen and the trimethylsilyl-derivatives of F_2-IsoPs obtained by treating with 20 µL of BSTFA+TMCS and 10 µL of anhydrous pyridine and incubating at 45 °C for 20 min. After drying again under nitrogen, 30 µL of isooctane was added to reconstitute samples, which were analyzed by GC-NCI-MS. Briefly, the analyses were carried out in an Agilent 6890N Network Gas Chromatograph coupled to an Agilent 5975B Series Mass Selective Detector and fitted with a DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies) by using helium as the carrier gas. NCI used methane as the reagent gas at an ion source pressure of 1.8 torr. The injector temperature was maintained at 250 °C, the transfer line at 280 °C, and the ion source and quadrupole temperatures were 200 and 100 °C, respectively. Injections were made using an HP 6890 Series Injector autosampler (Hewlett–Packard) in a splitless mode for the first 1 min. The initial column temperature of 160 °C was held for 1 min, then increased at 20 °C/min to 300 °C and maintained at this temperature for 17 min, giving a total run time of 25 min. Selected ion monitoring (SIM) was performed to monitor the carboxylate anion (M-181; loss of pentafluorobenzyl group) at m/z 569 and m/z 573 for F_2-IsoPs and the IS (8-iso$PGF_{2\alpha}$-d_4), respectively. Peak identification was based on comparison of retention times with the standard 8-iso-$PGF_{2\alpha}$. A 5-point calibration curve obtained from different solutions of this standard was used for quantitative analysis. Plasma F_2-IsoPs results were expressed as concentration values in nM.

2.6.2. F_2-IsoPs concentrations in urine

The concentration of F_2-IsoPs in urine samples was also assessed by GC-NCI-MS according to almost the same method as indicated above for plasma, but the IS was 8,12-iso-i$PF_{2\alpha}$-VI-d_{11} (*m/z* 580) and alkaline hydrolysis was not performed. Urinary F_2-IsoPs results were normalized with respect to the creatinine concentration in urine, expressing F_2-IsoPs concentration values in nmol/mmol creatinine.

2.7. Measurement of the concentrations of nitrates ($NO3^-$) and nitrites ($NO2^-$) in urine

The nitric oxide breakdown products NO_3^- and NO_2^- were quantified in urine samples as an indirect method to estimate the nitric oxide production. Both nitric oxide metabolites were converted to their stable pentafluorobenzyl derivatives directly from aqueous acetone medium, using TOA-Br as catalyst, and then analyzed using GC-NCI-MS in the selected-ion monitoring (SIM) mode (NO_3^- m/z = 62; NO_2^- m/z = 46), as previously reported (Yang et al. 2013). The equipment used was an Agilent 6890N Network Gas Chromatograph coupled to an Agilent 5973B Series Mass Selective Detector and fitted with a DB-225MS column (30 m x 0.25 mm, 0.25 µm film thickness, Agilent Technologies). Peak identification was based on retention time and mass spectra compared with ^{15}N-labeled internal standards ($^{15}NO_3^-$ m/z = 63; $^{15}NO_2^-$ m/z = 47). Each nitric oxide breakdown product was quantified using calibration lines obtained from authentic NO_3^- and NO_2^- standards and labeled standards. Final results for NO_3^-, NO_2^-, and the sum of both NO_3^-+NO_2^- were normalized with the urinary levels of creatinine and expressed in µmol/mmol creatinine.

2.8. Data presentation and statistical analysis

All results from Studies 1 and 2 are expressed as mean values ± standard error of the mean (SEM). The Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies Inc., Warranton, VA, USA) software was used for statistical analyses. In the acute experiment (Study 1), data obtained at the three determination times were subjected to paired-samples Student’s t-test to detect whether the results at one time could be paired with the values of the rest. Linear correlations between each individual phenolic acid and the biomarkers studied at each collection time were also established by determining Pearson's correlation coefficients, with the significance of the correlation being determined using Student's t-test. In the short-term experiment (Study 2), a standard Student's t-test was used to determine significant differences between experimental groups. A value of $p < 0.05$ was considered for all analyses.

3. Results

3.1. Phenolic metabolic fate in plasma and urine after acute RWPS supplementation

A total of fourteen phenolic acids were identified and quantified, which are shown in **Table 2** ordered according to their time of maximum concentration in plasma, as indicative of which compounds may be preferentially absorbed at either the gastrointestinal or the colonic phases.

Table 2. Concentration of phenolic acids in plasma and urine of rats from Study 1[a], following acute administration of red wine pomace seasoning (RWPS).

	PLASMA (μM)				URINE (μmol/mmoL creatinine)			
Phenolic acids	**0h**	**2h**	**4h**	**Trend**	**-3–0h**	**0–3h**	**3–6h**	**Trend**
4-*O*-methylgallic acid	**0.056** ± 0.031 a	**0.094** ± 0.020 b	**0.061** ± 0.029 a		**0.041** ± 0.032 a	**0.379** ± 0.227 a	**0.092** ± 0.041 a	
Gentisic acid	**0.717** ± 0.196 a	**1.19** ± 0.24 b	**1.25** ± 0.28 ab		**0.044** ± 0.041 a	**0.705** ± 0.157 b	**0.584** ± 0.125 b	
Caffeic acid (*trans*-)	**0.564** ± 0.141 a	**0.972** ± 0.268 b	**1.30** ±0.44 ab		**0.081** ± 0.073 a	**1.01** ± 0.17 b	**0.460** ± 0.183 ab	
4-Hydroxyphenylacetic acid	**3.07** ± 0.55 a	**4.95** ± 0.68 b	**5.35** ± 0.85 b		**1.38** ± 0.74 a	**6.60** ± 0.84 c	**4.37** ± 0.76 b	
Homovallinic acid	**0.876** ± 0.249 a	**1.67** ± 0.33 b	**1.61** ± 0.32 b		**1.09** ± 0.36 a	**1.14** ± 0.44 a	**0.698** ± 0.233 a	
Vallinic acid	**0.162** ± 0.092 a	**0.590** ± 0.217 b	**0.539** ± 0.202 b		**0.249** ± 0.166 a	**3.01** ± 0.50 c	**0.876** ± 0.158 b	
Syringic acid	**0.464** ± 0.097 a	**1.00** ± 0.23 b	**0.765** ± 0.149 b		**0.144** ± 0.087 a	**1.34** ± 0.24 b	**0.501** ± 0.167 a	
Isoferulic acid	**0.091** ± 0.042 a	**0.296** ± 0.109 b	**0.286** ± 0.117 b		**0.038** ± 0.023 a	**0.477** ± 0.152 b	**0.148** ± 0.030 b	
Dihydroferulic acid	**2.02** ± 0.54 a	**5.75** ± 0.93 b	**8.59** ± 1.44 c		**0.421** ± 0.320 a	**4.24** ± 0.85 b	**3.50** ± 0.80 b	
Protocatechuic acid	**0.526** ± 0.198 a	**1.57** ± 0.41 b	**2.21** ± 0.62 c		**0.048** ± 0.028 a	**0.329** ± 0.042 c	**0.197** ± 0.037 b	
3-Hydroxyphenylacetic acid	**0.376** ± 0.064 a	**0.468** ± 0.064 ab	**0.569** ± 0.100 b		**0.594** ± 0.189 a	**0.849** ± 0.237 b	**0.857** ± 0.241 b	
Dihydro-3-coumaric acid	**0.774** ± 0.219 a	**0.707** ± 0.246 a	**1.04** ± 0.38 a		**0.169** ± 0.086 a	**2.58** ± 0.79 b	**1.59** ± 0.57 ab	
Ferulic acid (*trans*-)	**0.452** ± 0.082 a	**0.571** ± 0.133 a	**0.508** ± 0.086 a		**0.176** ± 0.125 a	**1.70** ± 0.12 a	**0.620** ± 0.153 a	
Homoprotocatechuic acid	**0.149** ± 0.067 a	**0.266** ± 0.082 a	**0.339** ± 0.102 a		**0.138** ± 0.047 a	**0.157** ± 0.062 a	**0.093** ± 0.032 a	

Results expressed as mean ± SEM values (n = 6). Letters indicate significant differences ($p < 0.05$) between either plasma or urine samples for each phenolic acid.
a) Samples were collected at the indicated hours (plasma) or time intervals (urine) pre- and post-supplementation of rats with RWPS (300 mg/kg of body weight).
b) Trends of each phenolic acid concentration along time are represented by sparklines obtained using Microsoft Office's Excel 2010 software.

In plasma samples, dihydroferulic, followed by 4-hydroxyphenylacetic, were the phenolic acids present at the highest concentration post-RWPS consumption, considerable levels of these metabolites were also present under basal conditions. Substantial concentrations of homovallinic, protocatechuic, syringic, gentisic, caffeic, dihydro-3-coumaric and ferulic acids were also detected following the seasoning administration, whereas the concentrations of the rest of phenolics were within the nM range. Plasma concentration of phenolic acids at 2h and 4h was dependent on the compound. Some metabolites reached maximum levels 2h post-RWPS intake, and then tended to decrease or leveled out at 4h. The most marked fall at 4h was observed for 4-*O*-methylgallic, whereas more variability was found for gentisic and caffeic acids, and similar concentrations at 2h and 4h were obtained for 4-hydroxyphenylacetic, homovallinic, vallinic, syringic and isoferulic acids. Interestingly, plasma levels of dihydroferulic, protocatechuic and 3-hydroxyphenylacetic acids gradually increased post-RWPS consumption. Concentration of dihydro-3-coumaric, ferulic and homoprotocatechuic acids did not change significantly following the seasoning intake.

In urine samples, 4-hydroxyphenylacetic and dihydroferulic were also the phenolic acids excreted in the highest concentrations after RWPS consumption (they reached low µmol/mmol creatinine values). High contents of dihydro-3-coumaric, vallinic, ferulic and syringic acids were also found in urine. Levels of several phenolics were increased in samples collected between 0–3h post-RWPS administration, but their concentration slightly (dihydro-3-coumaric and caffeic acids) or significantly (4-hydroxyphenylacetic, protocatechuic, vallinic and syringic acids) decreased between 3–6h, with levels of syringic acid returning to basal values. For dihydroferulic, isoferulic, gentisic and 3-hydroxyphenylacetic acids, similar concentrations were detected between samples collected at 0–3h and 3–6h intervals following RWPS intake. As regards homovallinic, 4-*O*-methylgallic, and ferulic acids, no significant increase in their cumulative excretion was detected with time in comparison with their basal urinary levels.

3.2. Plasma lipid peroxidation and total antioxidant capacity (TAC) following acute RWPS supplementation

Following the acute administration of RWPS to rats (Study 1), lipid peroxidation was estimated from the concentration of plasma F_2-isoprostanes (F_2-IsoPs), and total antioxidant capacity (TAC) of plasma was assessed using the FC index, FRAP and ABTS assays (**Figure 1**). Plasma F_2-IsoPs levels decreased by 23% at 4h post-RWPS intake compared to samples collected before the intervention. A significant increase of around 10% in the FC index was observed in plasma collected both at 2h and 4h post-RWPS consumption compared to basal conditions, whereas FRAP and ABTS values were only significantly higher in the latest collected samples. The increment was considerably more marked for FRAP results (about 24%) than for ABTS values (about 3%).

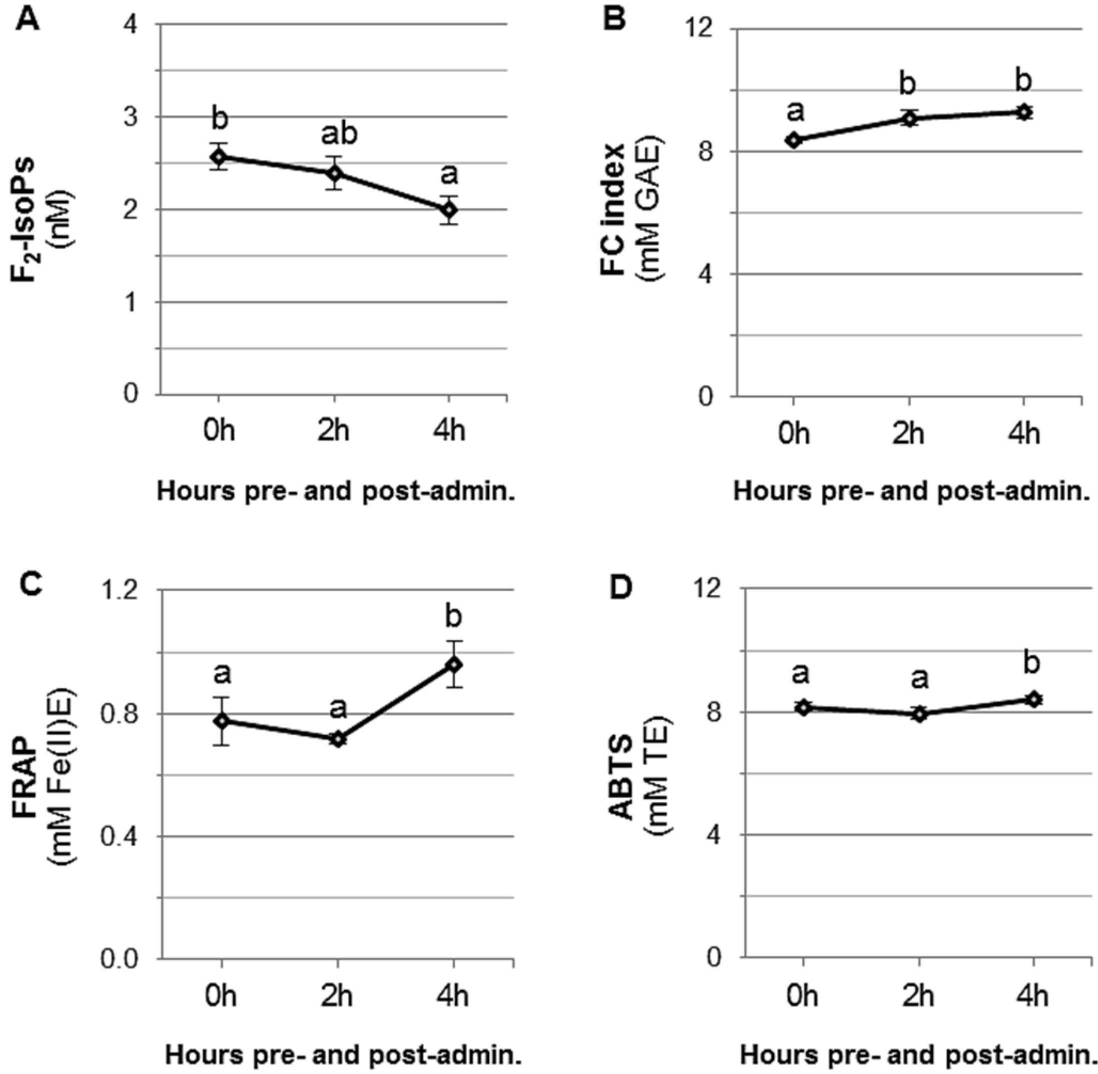

Figure 1. Plasma F_2-isoprostanes levels (A) and total antioxidant capacity, as determined using the FC index (B), FRAP (C) and ABTS (D) assays, in rats from Study 1. These oxidative stress biomarkers were evaluated in plasma samples collected at basal conditions (0h) and following acute administration of seedless red wine pomace seasoning (RWPS) (2h and 4h). F_2-IsoPs: F_2-isoprostanes; FC: Folin-Ciocalteu; FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid. Data expressed as mean ± SEM values (n = 6). Letters indicate significant changes ($p < 0.05$) between hours pre- and post-administration.

3.3. Urinary markers of oxidative damage to lipids and nitric oxide bioavailability following acute RWPS consumption.

Excretion of F_2-IsoPs in urine was lower at both collection times after acute RWPS intake than at basal conditions ($p < 0.05$), with no difference between the levels of this biomarker excreted at 0–3h and 3–6h post-RWPS consumption (**Figure 2**, A). Urinary concentrations of both nitric oxide metabolites (NO_3^- and NO_2^-) followed similar trends, showing a marked raise post-RWPS intake only in the urine accumulated between 3–6h ($p < 0.01$) (**Figure 2**, B).

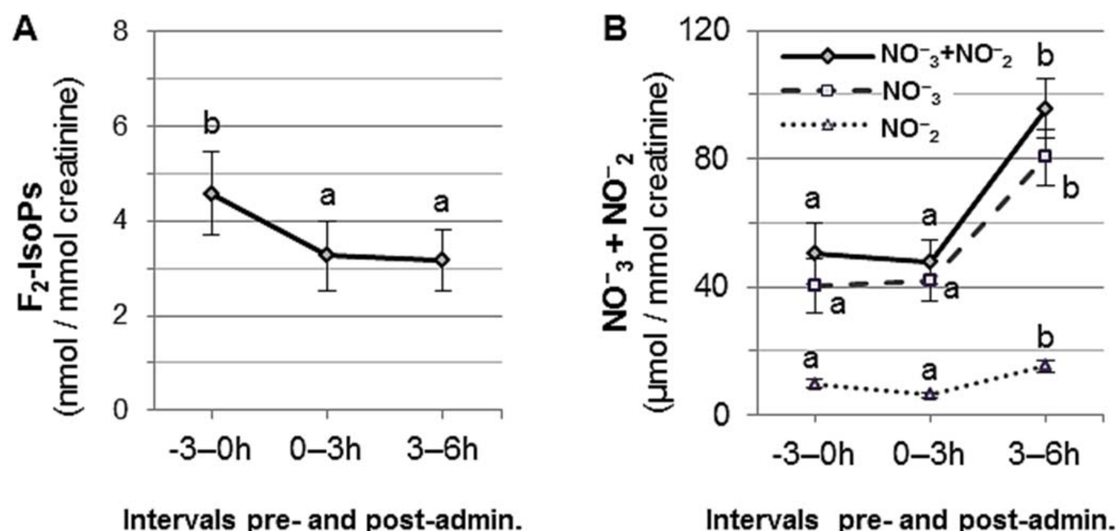

Figure 2. **Urine levels of F_2-isoprostanes (A) and nitric oxide metabolites (B) in rats from Study 1.** These biomarkers were determined in urine samples collected at basal conditions (-3–0h) and at the indicated time intervals following acute administration of seedless red wine pomace seasoning (RWPS) (0–3h and 3–6h). F_2-IsoPs: F_2-isoprostanes; Nitrites: NO^-_2; Nitrates: NO^-_3; Nitrates and Nitrites: NO^-_3+NO^-_2). Data expressed as mean ± SEM values (n = 6). Letters indicate significant changes ($p < 0.05$) between time intervals pre- and post-administration.

3.4. Correlations between biomarkers and phenolic acid contents in the acute study

In plasma collected during the acute study (Study 1), significant ($p < 0.05$) and positive correlations between the FC index and the concentration of three phenolic acids were detected (FC:dihydroferulic, r = 0.496; FC:4-hydroxyphenylacetic, r = 0.487; FC:gentisic, r = 0.478).

In urine, F_2-IsoPs values were significantly ($p < 0.05$) and negatively correlated with the contents of seven phenolic acids (F_2-IsoPs : gentisic, r = -0.765; F_2-IsoPs : 3-hydroxyphenylacetic, r = -0.739; F_2-IsoPs : dihydroferulic, r = -0.697; F_2-IsoPs : 4-hydroxyphenylacetic, r = -0.613; F_2-IsoPs:syringic, r = -0.593; F_2-IsoPs : protocatechuic, r = -0.577; F_2-IsoPs : vallinic, r = -0.547).

3.5. Plasma and urinary biomarkers of oxidative stress and endothelial function following short-term RWPS consumption.

As shown in **Table 3**, none of the biomarkers (plasma F_2-IsoPs and TAC; urinary F_2-IsoPs and nitric oxide metabolites) that were evaluated in biological samples collected from 12h-fasted rats at the end of the Study 2 (daily RWPS consumption during 4 weeks) showed significant differences between control (C) and supplemented (+RWPS) rats.

Table 3. Levels of biomarkers of oxidative stress and endothelial dysfunction in plasma and urine of rats from Study 2 [a].

Biological Sample	Biomarker [b]	Units [c]	C [d]	+RWPS [e]
Plasma	**F_2-IsoPs**	nM	**2.27** ± 0.16	**2.15** ± 0.17
Plasma	**FC index**	mM GAE	**9.47** ± 0.29	**9.27** ± 0.16
Plasma	**FRAP**	mM Fe(II)E	**0.874** ± 0.041	**0.810** ± 0.030
Plasma	**ABTS**	mM TE	**8.01** ± 0.19	**7.99** ± 0.15
Urine	**F_2-IsoPs**	nmol/mmol creatinine	**3.69** ± 0.25	**3.63** ± 0.48
Urine	**$NO^-_3 + NO^-_2$**	µmol/mmol creatinine	**57.9** ± 4.3	**57.0** ± 2.0
Urine	**NO^-_3**	µmol/mmol creatinine	**46.6** ± 3.6	**46.4** ± 1.5
Urine	**NO^-_2**	µmol/mmol creatinine	**11.3** ± 0.7	**10.6** ± 0.5

Results expressed as mean ± SEM values (n = 6).
a) Biomarkers were analyzed in samples collected from 12h-fasted rats at the end of 4-week-administration of red wine pomace seasoning (RWPS).
b) F_2-IsoPs: F_2-isoprostanes; FC: Folin-Ciocalteu; FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; NO^-_2: Nitrites; NO^-_3: Nitrates; NO^-_3+NO^-_2: Nitrates and Nitrites.
c) GAE: gallic acid equivalents; Fe(II)E: iron(II) equivalents; TE: Trolox equivalents.
d) C: control rats.
e) +RWPS: Rats supplemented with RWPS.
No significant differences ($p < 0.05$) were detected between C and +RWPS groups.

4. Discussion

The high phenolic contents of wine pomace suggests its potential to positively influence the redox environment and vascular function following consumption of foods or dietary supplements derived from this product (Pérez-jiménez et al. 2009; Yu & Ahmedna 2013).

The metabolic fate of phenolic compounds following acute administration of a seedless red wine pomace seasoning (RWPS) was first investigated in plasma and urine of healthy rats. The phenolic acids determined in this study represent both the free forms and the phenolic backbones of phase II metabolites, as the conjugating moieties of such metabolites were cleavage during the phenolic extraction protocol by the incubation of biological samples with different phase II deconjugation enzymes. Most of the phenolic compounds analyzed have

been proposed to arise from the biotransformation of dietary polyphenols once ingested (Rechner et al. 2002; Manach et al. 2005; Aura 2012).

Acute RWPS intake significantly altered plasma and urinary profiles of phenolic acids. A reasonable relationship between the absorption and excretion of most metabolites was observed. In general, phenolic acid levels increased in plasma at 2h and/or 4h post-intake, and concentrations found in urine were higher in the first collection interval (0–3h samples) and then tended to fall (3–6h samples), with a few exceptions for which a maintained excretion with time was observed.

The phenolic composition of the seedless RWPS has been described in a previous work (Del Pino-García et al. 2016d). Some of these compounds may be directly released from the wine pomace matrix throughout the entire gastrointestinal tract. Free phenolic acids and those linked weakly to the cell wall or contained in vacuoles were probably liberated and rapidly absorbed in the stomach and the upper intestine (Bohn 2014), being thereby present at high concentrations in the first samples collected post-RWPS administration (2h for plasma and 0–3h for urine). In contrast, those phenolics physically trapped within the matrix microstructure but released in the lower intestine most likely peaked primarily in samples collected at 4h in plasma and at 3–6h in urine.

In addition, numerous new phenolic acids are generated following cleavage of ester and glycoside bonds, ring-fission, and extensive biotransformations of more complex precursors carried out by gut microbiota. As microbial population density is much higher in the large than the small intestine, most of these conversions are known to occur once non-assimilated compounds reach the colon and/or following the return to the lumen, via bile, of previously absorbed molecules (enterohepatic recirculation) (Aura 2008; Landete 2012). The resultant metabolites are thus expected to reach maximum concentrations primarily in the latest collected samples. However, microbial transformations were certainly much faster for extractable phenolics than for those compounds strongly attached to insoluble components of the wine pomace matrix (Rechner et al. 2002; Aura 2012). This fact may partly explain the different intensity and timing of each metabolite appearance, and why the concentrations of some phenolics were largely maintained over time. The latter was observed for dihydroferulic, isoferulic, gentisic, protocatechuic and 3-hydroxyphenylacetic acids (in both plasma and urine), as well as for 4-hydroxyphenylacetic, vallinic, homovallinic and syringic acids (only in plasma).

Health benefits of phenolic metabolites can be ascribed to their pleotropic bioactivities, which may include antioxidant properties due to modulation of the endogenous antioxidant defenses (enzymatic and non-enzymatic). The resultant protective effects prevent oxidative damage of such vulnerable biomolecules as lipids (Pandey & Rizvi 2009; Fraga et al. 2010).

Acute RWPS supplementation (Study 1) led to a significant protection of lipids from oxidative damage, as evidenced by the F_2-IsoPs levels in both the plasma and the urine samples. These products of polyunsaturated fatty acids (arachidonic acid) are recognized as a sensitive and reliable index of *in vivo* lipid peroxidation (Mori et al. 1999; Montuschi et al. 2004). F_2-IsoPs in plasma have a short half time (approximately 18 min) and are rapidly excreted in urine, which means that they must be formed constantly to maintain a steady-state concentration (Griffiths et al. 2002). Since the concentration of F_2-IsoPs decreased in plasma from 2h to 4h post-intake, whereas levels of this biomarker were similar in urine collected at both 3-hour-intervals post-administration, the maximum level of lipid protection might be achieved between 2h and 4h following RWPS consumption. In addition, the concentration of F_2-IsoPs in urine was negatively correlated with several phenolic acids, and a number of phenolic metabolites detected in both plasma and urine followed an opposite trend than F_2-IsoPs levels. These findings support the important role that those metabolites generated principally by colonic microbiota might play following acute RWPS intake in the prevention and/or protection against lipid peroxidation.

The assessment of changes in plasma TAC after the intake of food antioxidants is a non-specific approach to examine their effects in the redox balance of the organism. Although it has been proposed as a simple biomarker of the overall absorption of such antioxidants (Prior & Cao 1999; Fernández-Panchón et al. 2008), it is unlikely to represent true *in vivo* direct antioxidant actions of phenolic compounds (Hollman et al. 2011; Sies 2007; Croft 2016). Hence, the changes observed in TAC, especially at 4h post-RWPS intake, might be partly due to indirect antioxidant effects of phenolic metabolites although could be also influenced by fluctuations in other circulating antioxidants such as uric acid following RWPS consumption.

Oxidative stress appears to be a common denominator underlying endothelial dysfunction, a pathological condition which is a target for prevention of numerous cardiovascular diseases. The fundamental feature of endothelial dysfunction is the impaired bioavailability of nitric oxide, a vasodilator molecule that exerts several vasoprotective effects. Nitric oxide can be inactivated by reactive oxygen species (ROS) or can be produced at lower levels by the endothelial nitric oxide synthase (eNOS), which are both the most frequent causes of endothelial dysfunction (Versari et al. 2009). Therefore, dietary approaches that enhance nitric oxide bioavailability are regarded as beneficial strategies to protect the endothelium and prevent cardiovascular events (Andriantsitohaina et al. 2012; Papageorgiou et al. 2013; Chistiakov et al. 2015). The potential ability of RWPS consumption to increase nitric oxide production is supported by the increased excretion of urinary nitrate and nitrite observed. Our results further revealed that such increase in nitric oxide bioavailability was not noticeable until, at least, 3h post-RWPS intake. These findings evidence that colonic metabolites derived from RWPS may play a substantial direct and/or indirect role enhancing

nitric oxide bioavailability. Direct effects resulting in higher nitric oxide could be due to the ability of phenolic compounds to scavenge superoxide anion, which is the main ROS that reacts with nitric oxide to generate peroxynitrite, a potent vasoconstrictor and oxidant molecule that targets mainly lipid oxidation (O'Donnell & Freeman 2001). However, indirect effects are more likely, these include: i) activation/up-regulation of eNOS and inhibition of eNOS uncoupling); ii) inhibition of ROS generation (such as inactivation/down-regulation of NADPH oxidases and xanthine oxidases); iii) reduction of ROS levels (such as the activation/up-regulation of the antioxidant defense enzymes) (Montezano & Touyz 2011; Zinkevich & Gutterman 2011). Nitric oxide and ROS themselves can also induce several redox signaling pathways that control the expression of vascular antioxidant defense enzymes, including heme oxygenase-1and extracellular superoxide dismutase (Gewaltig & Kojd 2002; Finley et al. 2011; Zinkevich & Gutterman 2011).

In contrast to acute treatment, longer term (4-weeks) of RWPS consumption (Study 2), did not improve oxidative stress and endothelial function biomarkers analyzed in plasma and urine of 12h-fasted rats. This finding may be due to the disappearance of many phenolic metabolites during the fasting period and suggests a transient effect of these compounds. These findings need to be confirmed in humans, in agreement with previous authors (Pérez-Jiménez & Saura-Calixto 2008; Rodrigo et al. 2005; Hollman et al. 2011), our results using animal models support the hypothesis that healthy individuals, which in general are not submitted to high chronic oxidative insults, might be less susceptible to benefit from dietary antioxidant supplements than patients with pathological conditions associated to oxidative stress.

In summary, acute consumption of a seasoning product derived from seedless red wine pomace (RWPS) temporally improved the vascular redox status, decreased lipid peroxidation, and improved nitric oxide bioavailability in healthy rats. These potentially beneficial effects were parallel in time with significant increments in the concentrations of several plasma and urinary phenolic metabolites derived from the seasoning but, in general, they could not be ascribed to specific compounds as indirect effects of bioactive metabolites may prevail over their direct antioxidant activities. The phenolic acids derived from the action of gut microbiota (mainly dihydroferulic, followed by gentisic, 3- and 4-hydroxyphenylacetic, syringic, protocatechuic and vallinic acids) may have a predominant role.

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Conflict of interest

The authors have no conflicts of interest to disclose.

Supplementary material:

Supplementary data to this article (**Table S1**) is shown below the references.

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Table S1. Phenolic acids, retention times, molecular mass and characteristics ions used for quantitative analysis.

Common name [a]	Chemical nomenclature	t_R (min)	MW [b] (g/mol)	$[M-H]^-$ (m/z) [c]	2nd ion (m/z) [d]	Fragment ions (m/z) [e]
3-Hydroxyphenylacetic acid	3-Hydroxyphenylacetic acid	5.91	152	296	164	73, **164**, 147, 281, **296**, 252
4-Hydroxyphenylacetic acid	4-Hydroxyphenylacetic acid	6.10	152	296	179	73, **179**, 252, **296**, 281, **164**, 147
Dihydro-3-coumaric acid	3-(3-Hydroxyphenyl)propionic acid	6.65	166	310	205	**205**, 192, 73, **310**, 177, 147, 105
Vallinic acid	4-hydroxy-3-methoxybenzoic acid	6.78	168	312	193	297, 267, **312**, 73, **223**, **253**, 126, 282, **193**
Homovallinic acid	4-Hydroxy-3-methoxyphenylacetic acid	6.82	182	326	209	73, **209**, **326**, **179**, 267, 311, **296**, 149
Gentisic acid	2,5-Dihydroxybenzoic acid	6.87	154	355	223	**355**, 73, **223**, 267, 281, 147, 297
Protocatechuic acid	3,4-Dihydroxybenzoic acid	7.10	154	370	193	**193**, **370**, 73, **355**, 311, 281, **223**, 165
Homoprotocatechuic acid	3,4-Dihydroxyphenylacetic acid	7.15	168	384	179	73, **384**, **179**, 267, **221**, 237, 147, 369
4-O-methylgallic acid	3,5-dihydroxy-4-methoxybenzoic acid	7.42	184	400	370	73, **370**, **400**, 385, **355**, 170, 311, 341
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	7.51	198	342	253	327, **342**, **312**, 297, 73, **253**, 141, **223**, 283
Dihydroferulic	3-(4-Hydroxy-3-methoxyphenyl)propionic acid	7.56	196	340	209	**340**, **209**, 192, 73, **310**, 325, **179**, 222
Dihydrocaffeic acid	3,4-Dihydroxyhydrocinnamic acid	7.79	182	398	179	**398**, **179**, 73, 267, 280, 283, **193**
(IS) 1-Naphthol-2-carboxylic acid	1-Hydroxy-2-maphthoic acid	8.11	188	317	243	**317**, 73, 147, 185, **243**
Isoferulic acid	3-Hydroxy-4-methoxycinnamic acid	8.43	194	338	207	**338**, 73, **207**, 308, 323, **249**, 293, 146, 191
Ferulic acid (*trans*-)	4-Hydroxy-3-methoxycinnamic acid	8.52	194	338	249	**338**, 73, 323, 308, **249**, 293, **219**, 146, 279
Caffeic acid (*trans*-)	3,4-Dihydroxycinnamic acid	8.70	180	396	219	73, **219**, **396**, 381, 191, 307, **249**

a) Phenolic compounds ordered regarding their retention time (t_R). IS: internal standard.
b) MW: molecular weight.
c) Mass to charge ratio (m/z) of the molecular ion $[M-H]^-$ of each trimethylsilylated phenolic acid.
d) Mass to charge ratio (m/z) of the second ion considered for analyses.
e) Mass to charge ratio (m/z) of the molecular (indicated in green color) and several fragment ions (ordered according to their relative abundance, from higher to lower) determined from the analysis in scan mode with the standards. Those ions detected during selected ion monitoring (SIM) are indicated in bold numbers.

Chapter 3

Protective Effects against Oxidative Stress and Endothelial Dysfunction of the Seasoning

CHAPTER 3:

PROTECTIVE EFFECTS AGAINST OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION OF THE SEASONING

Endothelial dysfunction and oxidative stress are closely related, and in turn they are both cause and consequence of numerous cardiovascular pathologies (Kojda & Harrison 1999; Friedman et al. 2003; Versari et al. 2009). Therefore, to meet the third specific objective of this PhD Thesis, a series of *ex vivo* and *in vivo* studies were carried out to evaluate the healthy potential of the seasoning made from seedless wine pomace in cases of oxidative stress and endothelial dysfunction.

Many promising results on the health effects of phenolic compounds or other bioactive compounds have been obtained from experiments testing non-achievable dietary doses or, in the case of *ex vivo* studies, after exposure of cultured cells to extracts, products or standards in molecular forms that are not to be present *in vivo* at the site of action. These facts make it difficult to extrapolate the positive outcomes obtained *ex vivo* to consistent and relevant evidence of the potential of dietary phenolic compounds for disease prevention (Núñez-Sánchez et al. 2015). Therefore, the combination of *in vitro* digestion protocols with cell culture assays has been proposed as a useful approach to partly solve these problems (Scharlau et al. 2010), and this has been the strategy used in the present thesis for all the experiments performed *ex vivo*.

The immortalised cell line of human endothelial cells from the umbilical vein (HUVEC EA.hy926) cultured in medium with high glucose concentration (25 mM) was chosen as an ***ex vivo* model of endothelial dysfunction**, as such hyperglycaemic conditions induce a pro-oxidant environment due to an increased intracellular production of $O_2^{\cdot-}$ and other RONS (Patel et al. 2013). Digested fractions obtained from the seasoning after the pre-colonic and colonic phases of *in vitro* digestion (collecting the fractions passing through a dialysis membrane) were used as treatments for cell incubation during their exposure to hyperglycaemic conditions. These fractions were evaluated first *in vitro* to determine their TAC and inhibitory capacity of the angiotensin-converting enzyme I (ACE). Based on previous studies of polyphenol bioavailability in which their plasma concentration after ingestion has been determined (Scalbert & Williamson 2000) and the results of *in vitro* studies, the concentration of the treatments were selected. Then, it was checked that the viability of cells was not affected by the treatments, and the cellular redox state, several markers of oxidative damage to biomolecules, and some of the underlying mechanisms were assessed by analysing the gene expression of antioxidant enzymes important in the vascular system and the ACE activity of cultured cells.

After the *ex vivo* studies, the potential health effects of the seasoning *in vivo* was investigated in animal models, selecting Type 1 diabetes mellitus (DM) and essential hypertension as diseases associated with oxidative stress and endothelial dysfunction (Friedman et al. 2003; Hadi & Suwaidi 2007; Higashi et al. 2009; Silva et al. 2012).

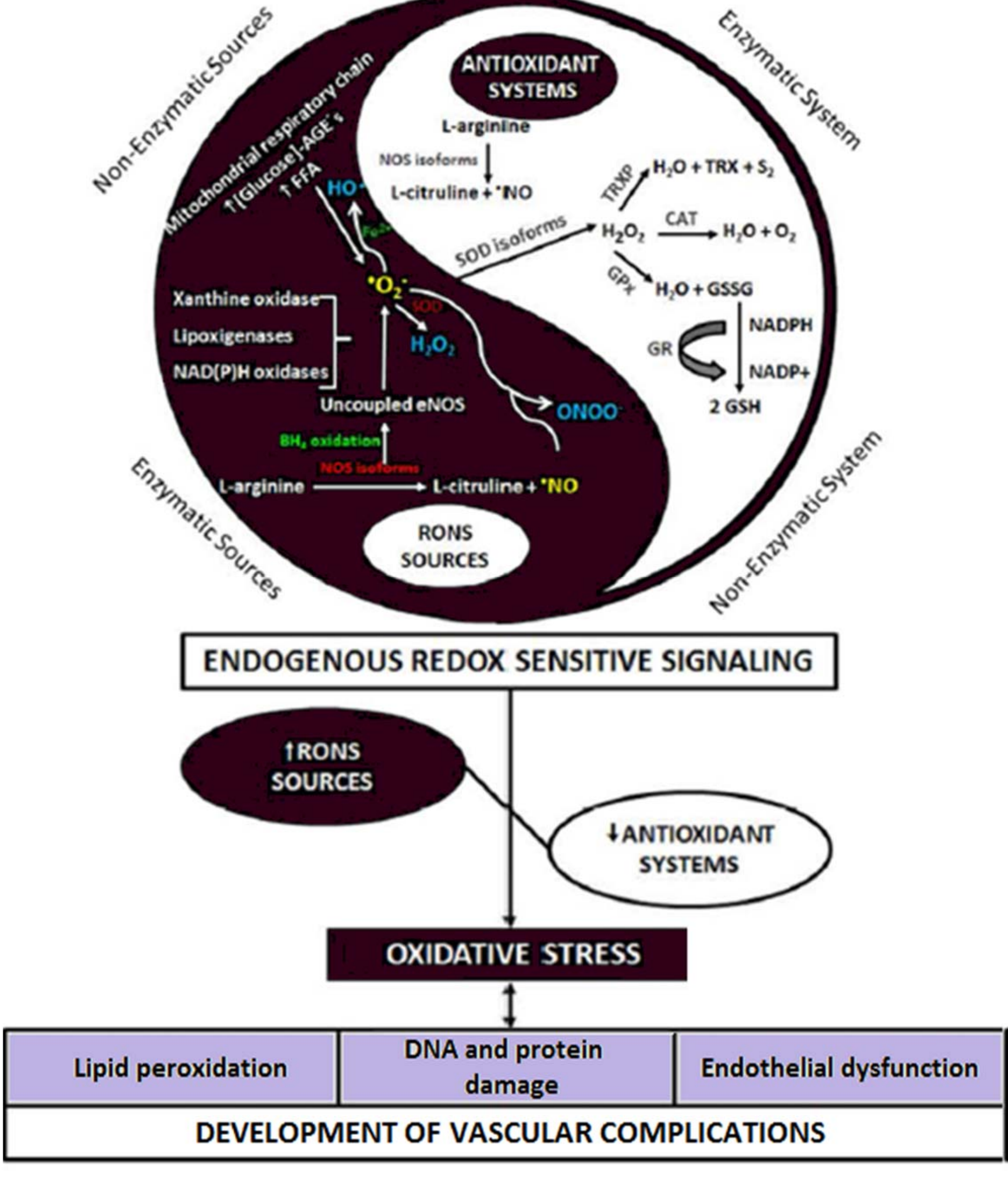

Figure 42. **Mechanisms implicated in regulation of redox balance under physiological conditions, deregulation under oxidative stress conditions, and consequences of such imbalance.**
Adapted from Bandeira et al. (2013).

Both DM and hypertension are considered major risk factors for cardiovascular disease. The oxidative stress characteristic of both diseases is associated with an increased production of endogenous RONS, and reducing enzymatic and non-enzymatic antioxidant defences (SODs, GSH, Vitamin E, etc.), resulting in an accumulation of RONS which promote endothelial dysfunction, oxidative damage to biomolecules in the organism, and the appearance of several complications (Cohen & Tong 2010). A schematic representation of the imbalance in the intracellular redox state that occurs during endothelial dysfunction is shown in **Figure 42**. However, many food interventions, including increased consumption of natural dietary antioxidants, have been proposed as possible strategies for the management of several cardiovascular diseases.

Main mechanisms of action identified for the beneficial effects of phenolic compounds in case of DM include (Hanhineva et al. 2010; Thomas & Pfeiffer 2012; Bahadoran et al. 2013; Babu et al. 2013):

- Modulation of the circulating concentrations of vasoactive products, such as $^{\bullet}$NO and RONS, by activation of eNOS and inhibition of NOXs.
- Decrease of inflammation and oxidative damage in several tissues.
- Improvement of hyperglycaemia through:
    - Influencing carbohydrates digestion and glucose intestinal absorption, transport and uptake in skeletal muscle and white adipose tissue.
    - Regulating glucose metabolism in hepatocytes.
    - Reducing apoptosis and promoting proliferation of pancreatic β-cells, promoting their proliferation.
    - Enhancing insulin secretion.
    - Insulin-mimetic effects mediated by interactions with the insulin receptors of cells.
    - Decreasing insulin resistance.

Major mechanisms of action proposed for phenolic compounds against hypertension include (Rodrigo et al. 2012; Majzunova et al. 2013):

- Inhibition of RONS-producing enzymes (NOXs and xanthine oxidases).
- Inhibition of ACE activity/expression, thus suppressing Ang II-stimulation of NOXs.
- Anti-inflammatory effects by inhibiting NF-kB, AP-1, COX-2 and iNOS activity/expression.
- Stimulation of the antioxidant response through the Nrf2/AREs pathway.
- Activation of eNOS and modulation of its expression.
- Reduction of LDL oxidation.

There are several animal models of experimental Type 1 DM (Rees & Alcolado 2005) and essential hypertension (Dornas & Silva 2011), some genetically manipulated or selected, and others in which DM is induced through nutritional, surgical or chemical interventions.

The **animal models** chosen for the studies of this thesis were:

- **Type 1 DM**: induction by administration of streptozotocin, one of the chemicals most often used by the scientific community for this purpose. Streptozotocin acts specifically destroying pancreatic β cells when administered in diabetogenic doses, causing in the animal similar symptoms to those characteristic of patients with uncontrolled Type 1 DM (hyperglycaemia, polydipsia, polyphagia, and polyuria), as well as most of the complications associated with DM, such as cardiomyopathy, coronary dysfunctions, macro- and microvascular complications, liver disorders, etc. (Rees & Alcolado 2005).
- **Essential Hypertension**: spontaneously hypertensive rats (SHRs) were selected, using the original controls of this strain, the Wistar-Kyoto (WKY) rats as control normotensive rats. The SHRs are one of the most widely used models for the study of this disease. This is a genetically selected model, since these rats develop hypertension spontaneously after their week 4-6 of age, being pre-hypertensive between the week 6-8, and evolving in the next few weeks towards a state of stable hypertension with a similar pathophysiology of essential hypertension in humans, occurring various cardiovascular complications with the progress of hypertension (Dornas & Silva 2011). In this study, supplementation of SHRs began at their week 12 of age, detecting an average systolic blood pressure (sBP) of 212 mmHg, which is similar to the values described by other authors in these rats (Duarte et al. 2001; Quiñones et al. 2013) and roughly doubles the sBP determined in the control groups.

Moreover, it is worth noting the main features of the **seasoning obtained from seedless wine pomace** that justify the interest to evaluate the beneficial effects of its supplementation in cases of Type 1 DM and essential hypertension:

- Its <u>phenolic compounds</u> are relatively bioaccessible, and thereby potentially bioavailable, since the early stages of digestion.
- It has high content in <u>dietary fibre</u> (45.3% in wet weight) which can be fermented by colonic microflora to yield short chain fatty acids (SCFAs), whose beneficial effects against diabetes and other cardiovascular diseases are well established (Pérez-Jiménez et al. 2008; Puddu et al. 2014).
- It has a high content of <u>potassium</u> (40.4 mg/g in wet weight), which is well above the levels of this mineral present in the seasoning derived from seeds (García-Lomillo et al. 2014), a feature of great interest for its potential benefits in case of hypertension (Workman & Paller 1985; Zicha & Kunes 1999).

To check the healthy potential against the pathologies above-indicated of this seasoning, it was administered as a dietary supplement to the respective animal models at a dose of 300 mg/kg of body weight for 4 weeks. Throughout this period, the evolution of animals was monitored and, at the end of the study, various biomarkers of oxidative stress, the $^{\cdot}$NO bioavailability, the lipid profile, the insulin levels, etc. were assessed, also investigating possible mechanisms of action of the seasoning by determining the gene expression of certain antioxidant enzymes in the aorta.

The main **conclusions** of these studies were:

- **Chapter 3.1**: The *ex vivo* assays showed the potential of the seasoning-derived bioactive compounds, after digestion, to restore endothelial function and improve the redox state under hyperglycaemic conditions. The modulation of gene expression of some enzymes deregulated by hyperglycaemia (NOX4, SOD2 and HO-1) and the inhibitory effect in the activity of ACE, together with possible direct antioxidants effects, were identified as mechanisms of action probably involved in the beneficial effects of the seasoning, detecting an increase in the bioactivity of its compounds after their metabolism by colonic microbiota.

- **Chapter 3.2**: The consumption of the seasoning for 4 weeks by Type 1 DM rats improved several of the symptoms and complications of this disease (polyphagia, polydipsia, weight loss, fasting hyperglycaemia, atherogenic cholesterol profile, and oxidative damage to plasma biomolecules). An increase in the insulin levels and SOD2 gene expression, along with slight modulations in the expression of other genes, were detected as possible mechanisms of action of the bioactive compounds derived from this seasoning, including phenolic metabolites and SCFAs.

- **Chapter 3.3**: The study in SHRs suggests that consumption of the seasoning has hypotensive and beneficial properties against oxidative stress and endothelial dysfunction characteristic of essential hypertension. Significant reductions in sBP (~11%) and in oxidative damage to biomolecules in plasma and urine, and an increase in $^{\cdot}$NO bioavailability, were observed. eNOS, HO-1 and SOD2 gene up-regulation, together with ACE down-regulation, may be involved in the effects observed.

CAPÍTULO 3:

EFECTO PROTECTOR DEL SAZONADOR FRENTE AL ESTRÉS OXIDATIVO Y LA DISFUNCIÓN ENDOTELIAL

La disfunción endotelial y el estrés oxidativo están íntimamente relacionados, y a su vez ambos son causa y consecuencia de numerosas patologías cardiovasculares (Kojda & Harrison 1999; Friedman et al. 2003; Versari et al. 2009). Por ello, para cumplir con el tercer objetivo específico de esta Tesis Doctoral, se llevaron a cabo un conjunto de estudios *ex vivo* e *in vivo* para evaluar el potencial saludable del sazonador obtenido a partir de los hollejos (orujos sin pepitas) en casos de estrés oxidativo y disfunción endotelial.

Muchos de los prometedores resultados sobre los efectos saludables de los compuestos fenólicos u otros compuestos bioactivos se han obtenido mediante experimentos en los que las dosis ensayadas no serían alcanzables a través de su incorporación con la dieta o, en el caso de estudios *ex vivo*, tras la incubación de las células con extractos, productos o estándares en formas moleculares que no son las que estarán presentes *in vivo* en el lugar de acción. Estos hechos dificultan la extrapolación de los resultados positivos obtenidos *ex vivo* en evidencias consistentes y relevantes sobre el potencial para prevenir enfermedades del consumo de compuestos fenólicos en la dieta (Núñez-Sánchez et al. 2015). Por ello, se ha propuesto la combinación de protocolos de digestión *in vitro* con ensayos en cultivos celulares como una aproximación útil para tratar de solucionar estos problemas (Scharlau et al. 2010), y ha sido la estrategia empleada en esta tesis en los experimentos *ex vivo*.

Como **modelo de disfunción endotelial *ex vivo*** se seleccionó el cultivo de una línea inmortalizada de células endoteliales de vena umbilical humana (HUVEC EA.hy926) en medio con alta concentración de glucosa (25 mM), ya que estas condiciones hiperglicémicas inducen un ambiente pro-oxidante debido a un incremento en la producción intracelular de $O_2^{\cdot-}$ y otros RONS (Patel et al. 2013). Como tratamientos con los que incubar las células durante la exposición a las condiciones hiperglicémicas, se emplearon fracciones digeridas obtenidas a partir del sazonador tras la fase pre-colónica y colónica del proceso digestivo *in vitro* (recogiendo las fracciones capaces de atravesar una membrana de diálisis). Estas fracciones fueron evaluadas en primer lugar *in vitro*, para determinar su TAC y capacidad inhibitoria de la ACE. En base a estudios previos de biodisponibilidad de polifenoles en los que se ha determinado su concentración en plasma tras la ingesta (Scalbert & Williamson 2000) y los resultados de los estudios *in vitro*, se seleccionó la concentración de los tratamientos. A continuación, se comprobó que dichos tratamientos no afectaban a la viabilidad de las células y se evaluó el estado redox celular, varios marcadores de daño oxidativo a biomoléculas, y algunos de los mecanismos implicados, analizando la expresión génica de enzimas antioxidantes importantes en el sistema vascular y la actividad de la ACE de las células en cultivo.

Tras los estudios *ex vivo*, se procedió a evaluar el potencial saludable del sazonador *in vivo*, en modelos animales, seleccionando la diabetes mellitus (DM) Tipo 1 y la hipertensión esencial como enfermedades asociadas con el estrés oxidativo y la disfunción endotelial (Friedman et al. 2003; Hadi & Suwaidi 2007; Higashi et al. 2009; Silva et al. 2012).

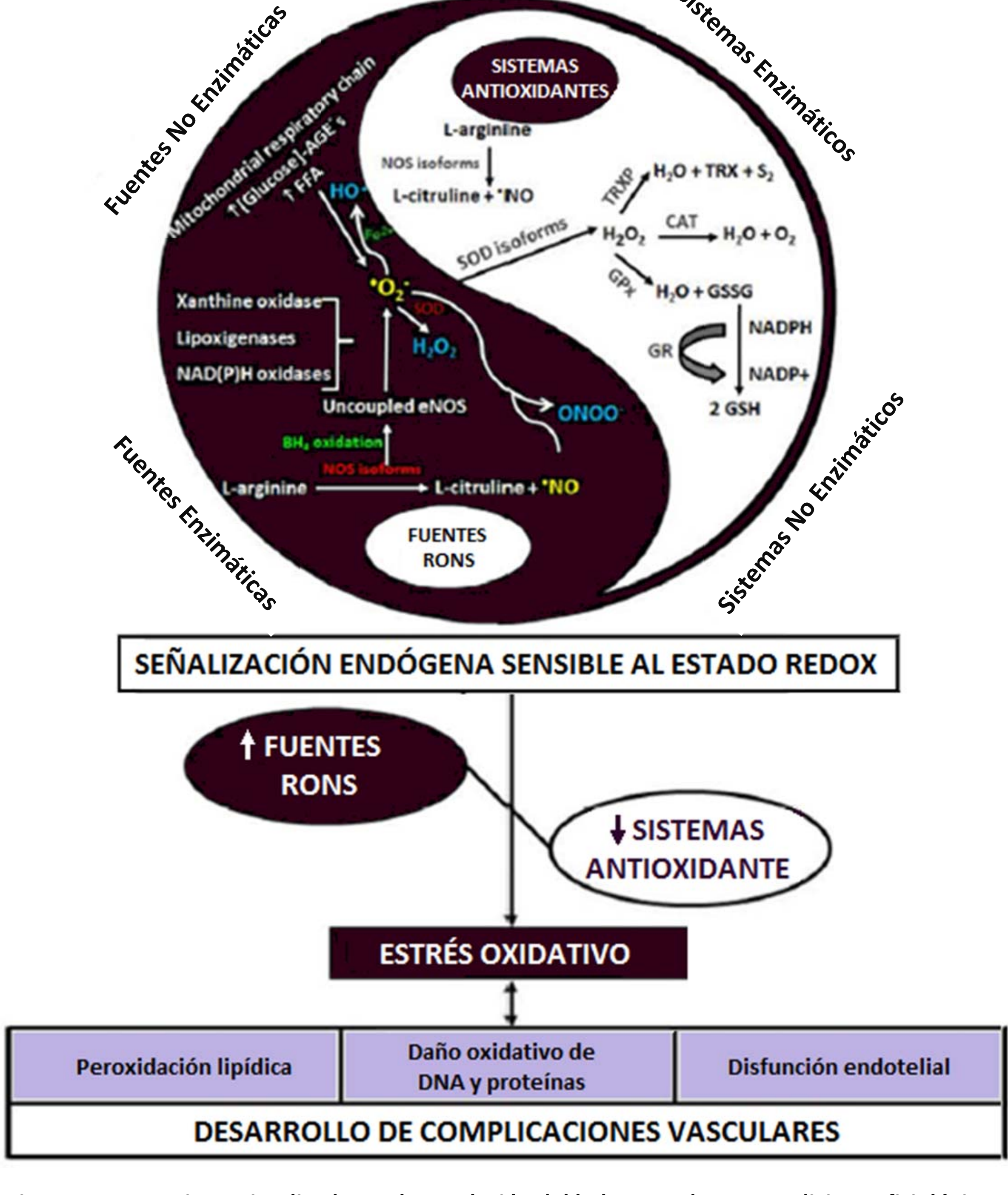

Figura 41. **Mecanismos implicados en la regulación del balance redox en condiciones fisiológicas, desregulación en condiciones de estrés oxidativo y consecuencias de dicho desbalance**.
Adaptado de Bandeira et al. (2013).

Tanto la DM como la hipertensión arterial están consideradas principales factores de riesgo de sufrir enfermedades de tipo cardiovascular. El estrés oxidativo característico de ambas patologías está relacionado con un incremento en la producción de RONS endógenos, y con la reducción de las defensas antioxidantes enzimáticas y no enzimáticas (SODs, GSH, Vitamina E, etc.), lo que resulta en una acumulación de RONS que promueven la disfunción endotelial, el daño oxidativo a biomoléculas del organismo, y la aparición de complicaciones (Cohen & Tong 2010). Una representación esquemática del desbalance en situación redox intracelular que se produce durante la disfunción endotelial puede verse en la **Figura 41**. Sin embargo, numerosas intervenciones alimentarias, entre ellas el incremento del consumo de antioxidantes naturales con la dieta, se han propuesto como posibles estrategias para el manejo de varias enfermedades cardiovasculares.

En caso de DM, los principales mecanismos de acción identificados para los efectos beneficiosos de los compuestos fenólicos incluyen (Hanhineva et al. 2010; Thomas & Pfeiffer 2012; Bahadoran et al. 2013; Babu et al. 2013):

- Modulación de las concentraciones de sustancias vasoactivas, como el $^{\cdot}$NO y los RONS, mediante la activación de la eNOS e inhibición de las NOXs.
- Disminución de la inflamación y daño oxidativo en diversos tejidos.
- Mejora de la hiperglicemia a través de:
    - Alteración de la digestión de carbohidratos y de la absorción intestinal, transporte y asimilación por el tejido muscular y adiposo de la glucosa.
    - Regulación del metabolismo de la glucosa en hepatocitos.
    - Disminución de la apoptosis de las células β del páncreas, promoviendo su proliferación.
    - Incremento en la secreción de insulina.
    - Efectos insulinomiméticos mediante la interacción con los receptores de insulina en las células del organismo.
    - Disminución de la resistencia a la insulina.

Entre los mecanismos de acción propuestos para los compuestos fenólicos frente a la hipertensión destacan (Rodrigo et al. 2012; Majzunova et al. 2013):

- Inhibición de las enzimas productoras de RONS (NOXs y xantina oxidasas).
- Inhibición de la actividad/expresión de la ACE, disminuyendo la estimulación de las NOXs mediada por la Ang II.
- Efectos antiinflamatorios a través de la inhibición de la actividad/expresión de NF-kB, AP-1, COX-2 e iNOS.
- Estimulación de la respuesta antioxidante a través de la vía Nrf2/AREs.
- Activación de eNOS e incremento de su expresión génica.
- Reducción de la oxidación de LDLs.

Existen varios modelos animales de DM Tipo 1 (Rees & Alcolado 2005) y de hipertensión esencial (Dornas & Silva 2011) a nivel experimental, algunos de ellos manipulados o seleccionados genéticamente y otros en los que el cuadro diabético se induce de manera nutricional, quirúrgica o química.

Los **modelos animales** elegidos para los estudios de esta tesis fueron:

- **DM Tipo 1**: inducción mediante la administración de estreptozotocina, uno de los compuestos químicos más empleados por la comunidad científica con esta finalidad. La estreptozotocina actúa específicamente destruyendo las células β pancreáticas al ser administrado en dosis diabetogénicas, provocando en el animal un estado similar al característico de los pacientes con DM Tipo 1 no controlada (hiperglicemia, polidipsia, polifagia, y poliuria), así como la mayoría de las complicaciones asociadas a la DM, tales como: cardiomiopatías, disfunciones coronarias, complicaciones macro- y microvasculares, alteraciones hepáticas, etc. (Rees & Alcolado 2005).

- **Hipertensión esencial**: se eligieron las ratas espontáneamente hipertensas (SHRs - spontaneously hypertensive rats), utilizando los controles originales de esta cepa, las ratas Wistar-Kyoto (WKY), como ratas controles normotensas. Las SHRs son uno de los modelos más empleados para el estudio de esta enfermedad. Se trata de un modelo seleccionado genéticamente, ya que estas ratas desarrollan hipertensión de manera espontánea a partir de las 4-6 semanas de edad, siendo pre-hipertensas entre las semanas 6-8, y evolucionando en las siguientes semanas hacia un estado de hipertensión estable, similar a la patofisiología de la hipertensión esencial humana, produciéndose diversas complicaciones cardiovasculares con el progreso de la hipertensión (Dornas & Silva 2011). En este estudio, la suplementación de las SHRs comenzó en la semana 12 de edad, detectando una presión sistólica media de 212 mmHg, la cual es similar a la descrita por otros autores en estas ratas (Duarte et al. 2001; Quiñones et al. 2013) y corresponde aproximadamente con el doble de la determinada en los grupos control.

Por otra parte, merece la pena destacar las principales características del **sazonador obtenido a partir de hollejos** que justifican el interés para evaluar los efectos beneficiosos de su suplementación en casos de DM Tipo 1 e hipertensión esencial:

- Sus <u>compuestos fenólicos</u> son relativamente bioaccesibles, y por la tanto potencialmente biodisponibles, ya desde las primeras etapas de la digestión.

- Tiene un elevado contenido en <u>fibra alimentaria</u> (45.3% en peso húmedo) que puede ser fermentada por la microbiota colónica para dar lugar a ácidos grasos de cadena corta (AGCCs), de reconocido efecto beneficioso frente a la diabetes y otras enfermedades de tipo cardiovascular (Pérez-Jiménez et al. 2008; Puddu et al. 2014).

- Presenta un elevado contenido de potasio (40.4 mg/g en peso húmedo), muy superior a los niveles de este mineral presentes en el sazonador derivado de las semillas (García-Lomillo et al. 2014), característica de gran interés por su potencial beneficioso en caso de hipertensión (Workman & Paller 1985; Zicha & Kunes 1999).

Para comprobar el potencial saludable frente a las patologías indicadas de este sazonador, se administró como suplemento alimenticio a los respectivos modelos animales a una dosis de 300 mg/kg de peso corporal durante 4 semanas. A lo largo este periodo, se monitorizó la evolución de los animales y, al final del estudio, se evaluaron diversos biomarcadores de estrés oxidativo, la biodisponibilidad de ˙NO, el perfil lipídico, los niveles de insulina, etc. y se investigaron posibles mecanismos de acción del sazonador determinando la expresión génica de algunas enzimas antioxidantes en la aorta.

Las principales **conclusiones** de estos estudios fueron:

- **Capítulo 3.1**: Los ensayos *ex vivo* evidenciaron el potencial de los compuestos bioactivos derivados del sazonador, tras su digestión, para restaurar la función endotelial y mejorar el estado redox bajo condiciones hiperglicémicas. La modulación de la expresión génica de algunas enzimas desregulados por la hiperglicemia (NOX4, SOD2 y HO-1) y el efecto inhibitorio de la actividad de la ACE, junto con posibles efectos antioxidantes directos, se identificaron como mecanismos de acción probablemente implicados en los efectos beneficiosos del sazonador, detectando un incremento en la bioactividad de sus compuestos tras ser metabolizados por la microbiota colónica.

- **Capítulo 3.2**: El consumo del sazonador durante 4 semanas por ratas diabéticas Tipo 1 mejoró varios de los síntomas y complicaciones de esta enfermedad (polifagia, polidipsia, pérdida de peso, hiperglicemia en ayunas, perfil de colesterol aterogénico, y daño oxidativo a biomoléculas del plasma). Como posibles mecanismos de acción de los compuestos bioactivos generados tras la ingesta del sazonador, entre ellos metabolitos fenólicos y AGCCs, se detectó un incremento en los niveles de insulina y en la expresión génica de SOD2, además de ligeras modulaciones en la expresión de otros genes.

- **Capítulo 3.3**: El estudio realizado en SHRs sugiere que el consumo del sazonador tiene propiedades hipotensoras y beneficiosas frente al estrés oxidativo y disfunción endotelial característicos de la hipertensión esencial. Se observó una reducción significativa de la presión sistólica (~11%) y del daño oxidativo a biomoléculas en plasma y orina, así como un incremento en la biodisponibilidad del ˙NO. El incremento en la expresión génica de eNOS, SOD2 y HO-1, junto con la menor expresión de la ACE, podrían estar involucrados en los efectos observados.

Chapter 3.1.

Wine Pomace Seasoning Attenuates Hyperglycaemia-Induced Endothelial Dysfunction and Oxidative Damage in Endothelial Cells

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Abstract

Dietary antioxidants such as phenolic phytochemicals may prevent/improve endothelial function impairment observed in cardiovascular diseases. This study investigates the specific protective effects of a vegetal seasoning obtained from seedless wine pomace against hyperglycaemia-induced oxidative damage and dysfunction in human umbilical vein endothelial cells EA.hy926. Non-cytotoxic doses of the bioactive compounds obtained following *in vitro* digestion of the seasoning were used as treatments. Digested compounds, especially colonic bacterial metabolites, restored a more balanced redox environment, prevented lipid peroxidation and cell membrane damage, ameliorated protein oxidation, and improved the balance between endothelial reactive oxygen species and nitric oxide production in hyperglycaemic cells. Reduction of angiotensin I-converting enzyme activity and gene modulation of superoxide dismutase, heme oxygenase-1, β-nicotinamide adenine dinucleotide phosphate oxidase-4, and endothelial nitric oxide synthase are proposed as the mechanisms underlying this protection. These results support the potential benefits of functional wine pomace seasonings in vascular complications associated with oxidative stress.

Keywords: Colonic metabolites; Dietary antioxidants; Endothelial function; Hyperglycaemia; Redox signalling; Wine pomace.

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Abbreviations and nomenclature: **ABTS**, 2,2'-azinobis 3-ethylbenzothiazoline-6-sulphonic acid; **ACE**, angiotensin I-converting enzyme; **Ang**, angiotensin; **AREs**, antioxidant responsive elements; **BCF**, potentially bioavailable fraction obtained after simulated colonic fermentation; **BGID**, potentially bioavailable fraction obtained after simulated gastrointestinal digestion; **CGs**, carbonyl groups; **eNOS**, endothelial nitric oxide synthase; **FC**, Folin-Ciocalteu; **GAE**, gallic acid equivalents; **GL**, glucose; **GSH**, glutathione reduced; **GSSG**, glutathione oxidised; **HO**, heme oxygenase; **HUVECs**, human umbilical vein endothelial cells; **LDH**, lactate dehydrogenase; **MDA**, malondialdehyde; **mDP**, mean degree of polymerization; **NAD(P)H**, β-nicotinamide adenine dinucleotide phosphate; **NF-κB**, nuclear factor-kappa B; **NO**, nitric oxide; **NOX**, NAD(P)H oxidase; **Nrf2**, nuclear factor-erythroid 2-related factor 2; **Q-**, QUENCHER; **RAAS**, renin-angiotensin aldosterone system; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TE**, Trolox equivalents.

Highlights:

- HUVECs treated with *in vitro* digested products from a seed-free wine pomace seasoning.
- Lower oxidative damage and endothelial dysfunction in hyperglycaemic treated cells.
- Observed benefits partly mediated by HO-1, NOX4 and SOD2 gene expression modulation.
- Digested products decreased ACE activity *in vitro* and *ex vivo* in hyperglycaemic cells.
- Key role of polyphenol colonic metabolism in the observed endothelial protection.

1. Introduction

Oxidative stress plays a critical role in the pathogenesis and development of cardiovascular disorders, with a growing body of evidence indicating that impaired endothelial function is a common cause and/or consequence of diverse risk factors relating to cardiovascular disease such as hypertension, diabetes mellitus, and hypercholesterolemia (Versari et al. 2009). An increased production of reactive oxygen species (ROS) and the subsequent decrease in vascular bioavailability of nitric oxide ($^{\cdot}NO$) have long been proposed as one of many complex and multifactorial causes that contribute to endothelial dysfunction (Higashi et al. 2009).

The most relevant sources of ROS in vascular cells, mainly superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are NAD(P)H oxidases (NOXs), uncoupled endothelial NO synthase (eNOS), xanthine oxidase, and the enzymes of the mitochondrial electron transport chain (Pennathur & Heinecke 2007). The rapid reaction of $O_2^{\cdot-}$ with NO leads to an excessive formation of peroxynitrite ($ONOO^-$), a reactive nitrogen specie that may act as a vasoconstrictor, and most importantly, as a cytotoxic molecule, causing direct structural damage to proteins, lipids, and DNA, as well as further ROS generation (Münzel et al. 2010).

A reduction in NO production has also been described as one of the mechanisms leading to the lower bioavailability of NO observed in the pathogenesis of several vascular complications. The main causes reported to underlie this decreased formation of NO are reduced eNOS mRNA levels and/or stability, and decreased synthesis, activation, and/or activity of eNOS enzymes (Versari et al. 2009; Schmitt & Dirsch 2009).

Another factor implicated in the development of oxidative stress and injury in the vascular system is the over-activation of the renin-angiotensin aldosterone system (RAAS). Angiotensin I-converting enzyme (ACE) is crucial in the regulation of RAAS, as it cleaves angiotensin I (Ang I) to produce the potent vasoconstrictor angiotensin II (Ang II) (McFarlane et al. 2003; Hsueh & Wyne 2011). The major mechanisms by which Ang II causes vascular damage include ROS generation and the stimulation of redox-dependent signalling pathways in endothelial and other vascular cells (Virdis et al. 2011). Therefore, RAAS is also involved in the control of NO bioavailability in vasculature. However, the inhibition of ACE activity has been suggested as a promising therapeutic approach for the prevention and treatment of several cardiovascular diseases and associated complications (Balasuriya & Rupasinghe, 2011; McFarlane et al., 2003).

Living organisms have developed several effective mechanisms to protect themselves from the detrimental effects of ROS. The endogenous antioxidant-defence system includes enzymes such as superoxide dismutases (SOD), catalase, glutathione peroxidases, heme oxygenases (HO), and others, as well as non-enzymatic antioxidants such as glutathione (GSH) (Lee et al. 2012).

In addition, several exogenous interventions using antioxidant dietary compounds have been proposed to prevent endothelial dysfunction and to restore the redox status in the vascular microenvironment, with certain phenolic compounds being highlighted among the most promising natural antioxidants with beneficial cardiovascular effects (Stoclet et al. 2004). In this regard, a number of studies have been initiated dealing with the antioxidant, hypotensive, and vascular health-promoting effects of phenolic compounds in grapes, wine, and wine pomace. The antioxidant capacity of these phytochemicals is not simply related to their direct ROS scavenging ability, but also to the inhibition of enzymatic sources of oxidative stress, such as NOX and ACE, as well as the stimulation of endogenous antioxidant enzymes (Nijveldt et al. 2001; Eriz et al. 2011; Münzel et al. 2010).

In relation to wine pomace, this winemaking by-product has traditionally been treated as a winery residue, but there is growing industrial and scientific interest nowadays in alternative uses for its value adding properties (Dwyer et al. 2014). In this context, new powdered seasonings obtained from wine pomace have recently been developed (García-Lomillo, González-Sanjosé, Del Pino-García, Rivero-Pérez, & Muñiz, 2014). These seasonings represent an innovative strategy to mitigate problems related to the disposal of wine pomace in wineries, while taking advantages of the bioactive phytochemicals, including phenolic antioxidants, remaining in such by-products. Taking into account that wine pomace seasonings have demonstrated significant antioxidant properties *in vitro* (Del Pino-García et al. 2015), their use as natural additives or ingredients in functional foods is expected to enhance exogenous antioxidant dietary pool and contribute to maintaining/restoring a healthy internal redox status.

However, one of the principal topics concerning the beneficial effects of phenolic compounds is their bioavailability and metabolic fate, which are dependent upon their digestive stability, their bioaccessibility (release from the food matrix), the efficiency of their trans-epithelial passage, and their further metabolism in enteric and hepatic tissue, as well as by gut microbiota (Scalbert & Williamson 2000; Manach et al. 2004; Tagliazucchi et al. 2010). Therefore, despite the limitations of simulated digestion systems (Alminger et al. 2014), the combination of *in vitro* digestion protocols with cell assays has recently been proposed as a suitable approach to determine the antioxidant activity of phenolic compounds once they have been ingested (Huang et al. 2014).

A frequently used model to investigate endothelial function impairment and associated pro-oxidative environment *ex vivo* is the incubation of vascular cells such as human umbilical vein endothelial cells (HUVECs) under high glucose concentrations (Inoguchi et al. 2000; Koziel et al. 2012; Patel et al. 2013; Zhou et al. 2012). The rationale behind this model lies in the significant oxidative stress observed in the vascular system of diabetic patients, which

may lead to important vascular complications and is well-known to be caused, at least in part, by the hyperglycaemic conditions that characterize diabetes (Hadi & Suwaidi 2007; Fatehi-Hassanabad et al. 2010).

In view of the above, this paper is focused on the potential protective effects against endothelial dysfunction and oxidative damage in the vasculature of new natural food ingredients, trying to bring together the results of fundamental and applied research into functional foods. Its major aim was to evaluate the specific effects of a red wine pomace-derived seasoning under hyperglycaemia-induced oxidative stress in the endothelial cell line HUVECs EA.hy926. To achieve this objective, cell treatments consisted of those compounds released after simulated gastrointestinal digestion and colonic fermentation of the powdered seasoning under study. In this way, the bioaccessibility and potential passive absorption of bioactive molecules was broadly considered.

2. Materials and Methods

2.1. Chemicals

2,2'-Azinobis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 2,4-dinitrophenylhydrazine (DNFH), DMEM containing either 5.6 mM (Low GL) and 25 mM D-glucose (High GL), foetal bovine serum (FBS), gallic acid, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), L-glutamine solution (200 mM), 4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), N-hippuryl-L-histidyl-L-leucine (HHL) tetrahydrate, β-nicotinamide adenine dinucleotide reduced (NADH) disodium salt hydrate, 10,000 U/mL penicillin and 100 mg/mL streptomycin solution (P/S), 85% (v/v) phosphoric acid solution (H_3PO_4), *o*-phthaldialdehyde, porcine bile extract, porcine pancreas pancreatin, potassium chloride (KCl), sodium chloride (NaCl), sodium pyruvate, 1,1,3,3-tetramethoxypropane (TMP), 2-thiobarbituric acid (TBA), thiazolyl blue tetrazolium bromide (MTT), trifluoroacetic acid (TFA), 2-vinylpyridine, cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off), and the enzymes used in the simulated gastrointestinal digestion (α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1)) were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). Folin-Ciocalteu (FC) reagent, 70% (v/v), perchloric acid (PCA) and trichloroacetic acid (TCA) were purchased from Panreac Quimica S.L.U. (Barcelona, Spain). Dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) tablets were purchased from Merck Millipore, Co. (Darmstadt, Germany).

2.2. Seasoning obtained from wine pomace

The powdered seasoning examined in this study was made at the University of Burgos according to a previously described method (González-Sanjosé et al. 2013). This product was obtained from seedless red wine pomace using a heat treatment as stabilization process, and its main characteristics and composition (dietary fibre, fat, protein, minerals and phenolic classes) have been previously reported (García-Lomillo et al. 2014).

2.3. *In vitro* gastrointestinal digestion and colonic fermentation of the seasoning

The wine pomace seasoning underwent *in vitro* digestion following the method described by Saura-Calixto et al. (2010). This procedure mainly comprised two sequential phases that simulate conditions along the gut: a) enzymatic gastrointestinal digestion; and b) colonic microbial fermentation. A schematic representation of the main steps performed and the fractions obtained along simulated digestion can be seen in **Supplementary Figure S1**, which provides further information on this process and its conditions. Briefly, the raw seasoning was incubated with pepsin, pancreatin, lipase, bile salts, α-amylase, and amyloglucosidase, which are digestive enzymes and agents secreted in the stomach, pancreas, liver and intestine. The enzymatic gastrointestinal digested fraction was centrifuged to separate supernatant and residue. The residue was washed twice and stored at 4 ºC, whereas all supernatants were mixed. A volume of 7.5 mL of the combined supernatant was then transferred into cellulose membrane dialysis tubing and dialysed against a total of 50 mL of Milli-Q water for 4 h (changing the water twice). The dialysis step was chosen to model the mechanical aspects of the epithelial barrier (Fernández & Labra, 2013). The dialysate (solution extracted from the dialysis tubing) was collected, lyophilized, weighed, and stored at -20 ºC. This fraction was labelled as 'BGID fraction' (potentially bioavailable fraction after gastrointestinal digestion). Then, the dialysis retentate was mixed with the residue from the previous centrifugation step, obtaining the gastrointestinal digested and dialyzed fraction which was the substrate for the action of colonic microbiota in the next digestion phase. The microbial inoculum was obtained following the method described in Saura-Calixto et al. (2010), by mixing the caecal content from 5 male Wistar rats (250 ± 5 g body weight). All procedures with these animals were performed following the guidelines established by the Ethics Committee of both the University Hospital of Burgos and the University of Burgos. After *in vitro* colonic fermentation, the resultant colonic fermented fraction was separated by centrifugation to collect the supernatant, which was then dialyzed by following the same procedure described above. The dialysate obtained was named 'BCF fraction' (potentially bioavailable fraction after colonic fermentation). Three replicates were carried out for each fraction. Negative digested controls (without seasoning) for both types of fractions were also prepared.

2.4. Analysis of BGID and BCF treatments

2.4.1. Total antioxidant capacity

The *in vitro* antioxidant activities of each treatment were directly evaluated in the lyophilized powdered fractions using QUENCHER (Q-) methods, as previously described (Del Pino-García et al., 2015).

The reducing capacity was assessed using the Q-Folin-Ciocalteu (Q-FC) assay. After 1 hour of sample reaction with the FC reagent in basic medium, absorbance at 750 nm was measured in an UV-vis spectrophotometer U-2000 (Hitachi, Ltd., Hubbardston, MA, USA). The results were expressed as µmol of gallic acid equivalents per gram of fraction (GAE/g fraction).

Radical scavenging ability was determined using the Q-ABTS assay. Following incubation of samples with the $ABTS^{\cdot+}$ working solution for 30 min, absorbance at 734 nm was evaluated. The results were expressed as µmol of Trolox equivalents per gram of fraction (TE/g fraction).

2.4.2. In vitro determination of angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the BGID and the BCF fractions was measured using the methodology described by Cinq-Mars and Li-Chan (2007). This method is based on the liberation of hippuric acid from the synthetic substrate HHL catalysed by ACE. Each digested fraction (inhibitor-containing sample) was mixed with the ACE solution and incubated for 1 h at 37 ºC, after which HHL solution was added and further incubated for 1 h at 37 ºC. Hippuric acid was extracted with ethyl acetate, which was then evaporated, and the remaining hippuric acid residue was redissolved in Milli-Q water. Spectrophotometric absorbance was read at 228 nm in a quartz cuvette. As ACE activity is depressed in the presence of ACE inhibitors, less hippuric acid is generated, yielding a lower absorbance value. Percentage (%) ACE inhibitory activity was calculated, as described in the following equation [1]:

$$\%\ \text{ACE inhibitory activity} = [1 - (A_{\text{inhibitor-containing sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})] \times 100 \qquad [1]$$

where, *'A'* refers to the absorbance value; the positive control (inhibitor-containing sample replaced by the buffer) represented a sample with 0% ACE inhibitory activity; and, the negative control (ACE added only after reaction termination) was taken as a sample with 100% ACE inhibitory activity.

2.5. Cell culture and treatment

The immortalized cell line HUVEC EA.hy926 was kindly provided by Dr. Diana Hernández-Romero from the research group 'Arterial thrombosis and interstitial, vascular and myocardial remodelling' (IMIB-Arrixaca/University of Murcia, Murcia, Spain).

The concentration of the BGID and the BCF digested fractions used during cell treatment is based in previous bioavailability studies (Scalbert & Williamson 2000). They have shown that, after intake of 500 mg of polyphenols, the concentration of total phenolic metabolites in human plasma may reach about 50 µM, whilst the total phenol contents of the BGID and the BCF treatments used in the present study may be around 24.0 and 15.4 µM GAE, respectively, as calculated considering the Q-FC data of both types of digested fractions and their final concentration (0.5 mg/mL) in the cell medium.

Cells were maintained in DMEM Low GL supplemented with 10% FBS, 1% P/S, and 1% L-glutamine solution at 37 °C in a humidified incubator with 5% CO_2. The medium during the treatment incubation period was DMEM, either Low or High GL, supplemented with 1% FBS, 1% P/S, 1% L-glutamine solution, and 0.1% DMSO. HUVECs EA.hy926 for experiments were taken from exponential phase cultures at passages 4–8 and seeded at a 2 x 10^4 cells/cm^2 density in all assays. Cells were grown for 24 h under basal conditions. Then, the treated-cells were exposed for 24 h to either the BGID or the BCF treatments (0.5 mg of fraction/mL of cell medium) or the respective negative digested control treatments. Non-treated control cells were only incubated with Low GL (normoglycaemic control) and High GL (hyperglycaemic control) mediums. All experiments were carried out as three independent assays. Those assays to determine cell viability, intracellular ROS, and NO production were performed in 96-well cell culture multiplates and the remaining experiments in 25 cm^2 cell culture flasks. After the treatment period, the cells were scraped and centrifuged (1,500 g, 5 min, 25 °C). Supernatant was collected and frozen at -80 °C for further analyses. The cell pellets were resuspended in 1 mL of PBS and used in the analyses, as explained below.

2.6. Cell viability assessment

Cell viability was analysed using the MTT method (Twentyman & Luscombe 1987). In this assay, MTT is reduced in the mitochondria of metabolically active cells (viable cells) by succinate dehydrogenase, which cleaves the tetrazolium ring yielding water-insoluble purple formazan crystals. After incubation for 4 h with the MTT solution, the medium was carefully aspirated, MTT formazan crystals were dissolved in DMSO, and absorbance at 550 nm was measured in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The results were expressed as % cell viability with respect to control cells grown in the Low GL medium.

2.7. Glutathione reduced/oxidised (GSH/GSSG) ratio analysis

Aliquots of the HUVECs EA.hy926 suspensions collected after the treatment period were immediately acidified with PCA (2% final concentration), centrifuged (6,500 g, 5 min, 4 ºC), and the supernatants were frozen at -80 ºC until their use as samples. GSH and GSSG levels in these samples were determined using the Cayman's GSH assay kit (Cayman Chemical, Co., Ann Arbor, MI, USA). This kinetic spectrophotometric assay evaluates total GSH (reduced+oxidised), by measuring absorbance at 410 nm at 2 min intervals for 20 min. Quantification of GSSG was performed following GSH derivatisation with 2-vinylpyridine, and GSH was estimated by subtracting GSSG from total GSH. All these assays followed the manufacturer's instructions. The results were finally expressed as a GSH/GSSG ratio.

2.8. Malondialdehyde (MDA) determination

Lipid peroxidation was estimated from the MDA levels according to the method described by Grotto et al. (2007), with slight modifications to perform the assay in cell homogenates obtained by sonication of the HUVEC EA.hy926 suspensions collected after the treatment period. In this chromatographic method, the MDA-TBA_2 complex generated by the reaction of MDA with TBA is then separated from other interfering compounds that may also react with TBA using reverse phase high-performance liquid chromatography (HPLC). Briefly, a volume of 75 µL of cell homogenate was added to 25 µL of Milli-Q water and 25 µL of 3 M NaOH and incubated at 60 ºC for 30 min in a shaking water bath system. After this, 125 µL of 6 % (v/v) H_3PO_4 and 125 µL of 0.8% (w/v) TBA were added and the mixture was heated at 90 ºC for 45 min. Then, the mixture containing the MDA-TBA_2 complex was cooled and extracted with 300 µL of *n*-butanol by vortex-mixing for 1 min and centrifugation at 3,000 *g* for 10 min. The butanol layer was collected and a volume of 20 µL was injected into an Agilent 1100 Series HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a diode array detector. The column was a Spherisorb3® ODS2 reversed phase C18 (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia, S.A., Barcelona, Spain). The mobile phase was a mixture of Milli-Q water:methanol (1:1, v/v). The flow rate was maintained isocratically at 0.6 ml/min, the absorbance at 532 nm of the eluent was monitored, and the total run time was 8 min. Concentrations of MDA were calculated from calibration curves obtained using TMP as standard. MDA levels were normalized using each cell homogenate total protein concentration, which was determined according to Bradford (1976). The results were finally expressed as nmol MDA equivalents /mg of protein.

2.9. Carbonyl groups (CGs) determination

Changes in cell proteins due to oxidative stress were determined in sonicated cell lysates using the spectrophotometric method described by Levine et al. (1990) to quantify protein CGs. In this assay, cell homogenates were mixed with DNFH acidified solution. After incubation for 1 h, proteins were precipitated with TCA and the samples were centrifuged to discard the supernatant. The pellets were washed 3 times with ethanol:ethyl acetate (1:1, v/v) to remove free reagent. Finally, the pellets were redissolved in a guanidine acidified solution and spectrophotometric absorbance at 373 nm was measured in quartz cuvettes. The CGs concentration was calculated using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$. The CG levels were then normalized by the protein content of each cell homogenate, expressing the final results as nmol CGs/mg of protein.

2.10. Lactate dehydrogenase (LDH) leakage assessment

The LDH activity was measured in the extra- and intracellular medium using the method reported by Vassault (1983), with some modifications. Briefly, a reaction mixture containing 100 mM potassium phosphate buffer, 0.7 mM sodium pyruvate, and 0.48 mM NADH was freshly prepared. A volume of 1.25 mL of the reaction mixture was transferred to a quartz cuvette and mixed with 50 µL of the extra- or intracellular medium. The absorbance decay at 340 nm was monitored for 2 min every 15 s. The units of extra- and intracellular LDL activity (1 unit = 1 µmol NADH/min) were determined considering a molar absorption coefficient for NADH of 6,220 $M^{-1}cm^{-1}$. Finally, cell membrane integrity was estimated from LDH activity, in terms of % LDH release to the extracellular medium with respect to total LDH (intracellular + extracellular).

2.11. Intracellular reactive oxygen species (ROS) measurement

Overall intracellular ROS production in HUVECs EA.hy926 was measured by the 2’,7’-dichlorofluorescein (DCF) assay (Wang & Joseph 1999), with certain modifications. Following their treatment and incubation, the cells were washed with a colourless external medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, and 10 mM HEPES. They were then added 100 µL of 20 µM DHCF-DA solution in external medium and placed in the chamber reader of a Cary Eclipse Fluorescence Spectrophotometer (Varian Australia, Pty. Ltd., Mulgrave, VIC, Australia) with a microplate reader accessory to take the initial fluorescence reading (t_0) using 480 nm excitation/530 nm emission filters. Fluorescence emission was also recorded after incubation in the dark at 37 °C for 30 min (t_{30}) and the variations in relative fluorescence units (t_{30} - t_0) were calculated for each sample. The MTT assay plates were run in parallel and the % cell viability results were used to normalize the recorded increase in fluorescence intensity, thereby taking into account the amount of viable cells per well. ROS levels were then estimated as DCF fluorescence increases and the final results were expressed as folds of the control cells grown in the Low GL medium.

2.12. Extracellular nitric oxide (NO) determination

The quantification of NO breakdown products, nitrite (H), in the culture medium was used as an indirect method to assess the cellular production of NO. Total nitrite and nitrate (NO_2^-+NO_3^-) was determined using the Cayman's Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the supplier's instructions. This method is based on the Griess reaction assay, with a previous conversion of NO_3^- to NO_2^- by the action of nitrate reductase (EC 1.7.1.2), and spectrophotometric measurement of absorbance at 540 nm. The % viability determined by the MTT assays run in parallel was used to normalize cellular production of NO, which was finally expressed as µM NO_2^-+NO_3^-.

2.13. Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from HUVECs Ea.hy926 suspensions using TRI Reagent solution (Applied Biosystems, Foster City, CA, USA). After treatment with DNase I (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µg of total RNA was reverse-transcribed using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and finally amplified using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, S.A., Madrid, Spain). All the procedures were performed according to the manufacturers' protocols. Primers were designed using Primer3 v.0.4.0 software (Untergasser et al., 2012) and synthesised by Metabion (Metabion International AG., Steinkirchen, Germany). The sequences of primer sets (forward and reverse) were:

- NOX4, 5'-GGAAGAGCCCAGATTCCAAG-3' and 5'-AGTCTTTCGGCACAGTACAG-3'
- eNOS, 5'-GAAGCTGCAGGTGTTCGAT-3' and 5'-CGGTTGGTGGCATACTTGAT-3'
- SOD2, 5'-GGAACGGGGACACTTACAAA-3' and 5'-ACTGAAGGTAGTAAGCGTGC-3'
- HO-1, 5'-GCCAGCAACAAAGTGCAAG-3' and 5'-AAAGCTGAGTGTAAGGACCC-3'
- GAPDH, 5'-GCTCTCCAGAACATCATCCC-3' and 5'-GTCCACCACTGACACGTTG-3'

qPCR was carried out with an iCycler iQ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle of initial denaturing and enzyme activation at 95 °C for 3 min; 45 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The proper product amplification was verified by melting curve analysis. Amplification efficiencies were calculated for each pair of primers and quantification was performed using the efficiency-corrected $\Delta\Delta C_t$ method, with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene. Relative gene expression was finally expressed as folds of change compared to control cells grown in the Low GL medium.

2.14. *Ex vivo* determination of angiotensin I-converting enzyme (ACE) activity

After the treatment and incubation of HUVECs EA.hy926, the ACE activity in sonicated cell lysates was measured using the ACE substrate HHL following the spectrofluorometric method reported by Hermanns, Müller, Tsokos, & Kirkpatrick (2014). The fluorescence of the *o*-phthaldialdehyde-histidyl-leucine adduct generated in the assay was measured in the Cary Eclipse Fluorescence Spectrophotometer using 365 nm excitation/500 nm emission filters. ACE activity was considered proportional to fluorescence intensity and was normalized to the protein concentration of cell homogenates. The final results were expressed as % ACE activity compared to control cells grown in the Low GL medium.

2.15. Data presentation and statistical analysis

Data were expressed as means ± standard deviation of independent experiments (n=3). Statistical analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was used to determine significant differences ($p < 0.05$) between data from cells incubated with the different treatments (control, BGID, and BCF) in the same medium (represented with Roman or Greek letters), and between the results of cells cultured in different mediums (Low and High GL) for each of the treatments (represented as % of change of hyperglycaemic with respect to normoglycaemic cells).

3. Results and Discussion

Powdered seasonings obtained from winery by-products could be used by the food industry as innovative condiments and ingredients with antioxidant functional effects. Thus, several experiments were conducted to evaluate the properties of a red wine pomace-derived seasoning and its potential protective effects against oxidative stress and endothelial dysfunction induced by high glucose conditions in vascular cells.

Considering the transformations that would take place in bioactive compounds following ingestion of the seasoning, a simulated gastrointestinal digestion and colonic fermentation of this functional ingredient was performed, obtaining two fractions that were dialysed to emulate trans-epithelial passage and then used as cell treatments.

Important modifications in the structure and activity of antioxidants, such as phenolic compounds, are known to occur during colonic microbial metabolism (Aura 2008). Therefore, the two types of digestion fractions obtained (BGID and BCF, respectively) were evaluated separately, as this approach represents a fast and easy way to distinguish between the bioactivity of compounds that are generated and potentially absorbed in either

the small or the large intestine after intake of the seasoning, thereby allowing us to assess changes in bioactivity that are the consequence of colonic microbiota fermentation. Specifically, both the BGID and the BCF fractions were first characterised *in vitro* and then used *ex vivo* for treating HUVECs EA.hy926.

3.1. Analysis of the seasoning-derived digestion fractions

The digestion fractions evaluated in this study derived from a seedless wine pomace seasoning that has been characterised in previous works. This seasoning has a high content of dietary fibre (48.6 ± 0.7 %), and is a rich source of phenolic compounds (24.4 ± 0.1 mg GAE/g), especially proanthocyanidins and anthocyanins (García-Lomillo et al. 2014). More detailed data about its individual phenolic contents are provided as **Supplementary TableS1**. In addition, the undigested seasoning presents high *in vitro* total antioxidant capacities (Q-FC index: 59.9 ± 1.5 µmol GAE/g; Q-ABTS values: 103 ± 3 µmol TE/g), which was determined directly in the powdered product, as previously described (Del Pino-García et al. 2015).

In the present study, the antioxidant and ACE inhibitory activities the digestion fractions derived from such seasoning (BGID and BCF) were firstly assessed *in vitro*. The Q-FC index and Q-ABTS values for the BGID fraction were 48.0 ± 1.3 µmol GAE/g fraction and 153 ± 21 µmol TE/g fraction, respectively, whereas the results for the BCF fraction were 30.8 ± 8.7 µmol GAE/g fraction and 435 ± 48 µmol TE/g fraction. Therefore, both fractions showed an interesting but different *in vitro* total antioxidant capacity profile. Q-FC values were around 35% higher before the action of colonic microbiota than after it, but the $ABTS^{\cdot+}$ antiradical capacity of the metabolites that could be absorbed in the colon was around 3-fold higher than those antioxidants present in the BGID fraction. These antioxidant capacities shown by the BGID and the BCF fractions are certainly due to their phenolic composition. Some of such phenolic compounds were individually determined (**Supplementary Table S2)**. Proanthocyanidins from wine pomace are known to be partly depolymerized into their flavan-3-ols monomers during transit along the digestive tract, and further transformed into free or conjugated microbial-derived phenolic metabolites in the colon (Touriño et al. 2011). This fact might explain the important content of epicatechin found in the BGID fractions, and the high content of simpler phenolics, such as gallic acid, in the BCF fractions. In this regard, the extensive microbial catabolism of main flavonoids (anthocyanins and flavan-3-ols) and phenolic acids in red wine extracts has been reported to result in the formation of numerous metabolites, such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acid derivatives (Sánchez-Patán et al. 2012). Many of these metabolites still contain free hydroxyl groups and could thereby contribute to enhance antioxidant capacity *in vivo* (Scalbert & Williamson 2000; Fernández-Panchón et al. 2008; Pérez-Jiménez & Saura-Calixto 2008).

Regarding the ability of the seasoning-derived compounds to act as ACE inhibitors *in vitro*, higher capacity was shown by the BGID (32.9 ± 0.9%) than the BCF (11.2 ± 4.5%) treatment. Several studies have confirmed the inhibitory power of certain polyphenols against ACE activity by competing with the substrate (Ang I) for the active sites of the enzyme (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Guerrero et al., 2012; Hidalgo et al., 2012). The ACE inhibitory capacity of flavonoid-rich foods appears to be especially related to their procyanidin and total flavan-3-ol content, and may depend on the mean degree of polymerization (mDP) of proanthocyanidins. An IC_{50} in the 100 µM range for dimer and trimer fractions, and within the mM range for monomeric flavan-3-ols and other phenolic compounds have been described (Actis-Goretta, Ottaviani, & Fraga, 2006). Recently, the effect of *in vitro* gastrointestinal digestion on the ACE inhibitory activity of a grape skin extract was studied (Fernández & Labra 2013). These authors noted that 80% of polymeric proanthocyanidins (mDP~ 19) were degraded during digestion in the small intestine to low-molecular-weight (mDP~ 2) oligomers, which crossed the dialysis membrane and efficiently inhibited around 60% of ACE activity. However, to the best of our knowledge, the present study has for the first time evaluated the ACE inhibitory power of fractions obtained after simulating the action of colonic microbiota. Structural changes of flavonoids can have an important impact on their potential as ACE inhibitors (Guerrero et al. 2012). According to the phenolic composition of the treatments (**Supplementary Table S2**), the higher *in vitro* ACE inhibitory ability observed for the BGID fraction, rather than the BCF fraction, may be due to the higher content of flavan-3-ol monomers and dimers in the former, together with the simpler molecules generated following depolymerisation and transformation of phenolic precursors by colonic microbiota. These small phenolic acid metabolites, contained in the BCF fraction, may exhibit lower capacity to depress ACE activity than their parent molecules, as previously suggested by other authors using chemically synthesised anthocyanidin-3-*O*-glucosides and several standard compounds of their putative gut breakdown metabolites (Hidalgo et al. 2012).

3.2. Cell viability and biomarkers of oxidative stress and endothelial dysfunction

Reduced cell proliferation and viability have been reported after short-term (McGinn et al. 2003) and long-term (Varma et al. 2005) exposure of HUVECs to high glucose concentrations, independently of osmotic changes. Likewise, for all type of cells (control, BGID-treated, and BCF-treated) evaluated in the current 24-hour study, around 15% lower cell viability was observed under hyperglycaemic than normoglycaemic conditions (**Figure 1**). Furthermore, the effects of the treatments on cell survival were assessed to ensure that non-cytotoxic doses of the digested fractions were used. Previous studies have considered concentrations of phenolic compounds resulting in higher than 80% cell viability as non-cytotoxic (Müller et al. 2009; Margina, et al. 2013; Stagos et al. 2014).

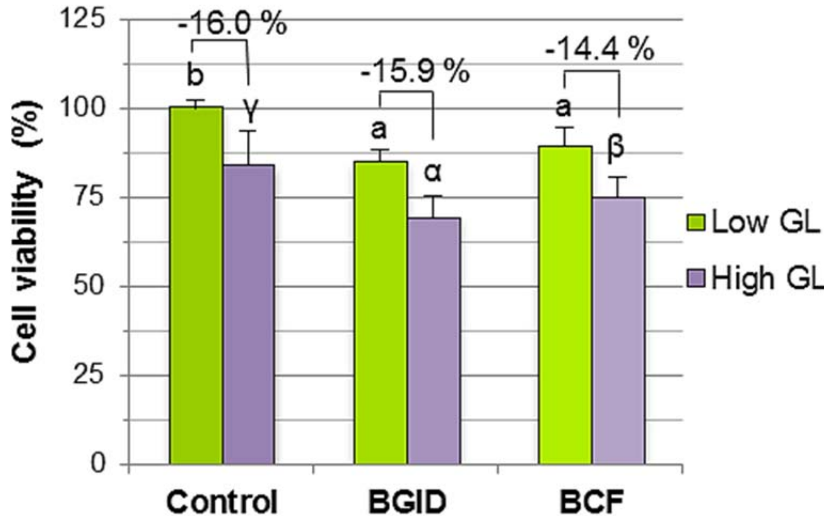

Figure 1. Cell viability of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the digested fractions obtained from the seasoning. BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed in % as mean values ± standard deviation (n=3) with respect to the control normoglycaemic cells (100%). Significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells are also indicated in % values. Significant changes (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

In the current study, the results presented in **Figure 1** correspond to the 0.5 mg/mL concentration, which was evaluated together with other doses (0.25 and 1 mg/mL) in preliminary MTT assays, being found as the highest non-cytotoxic concentration, among those tested, for both treatments. Concretely, comparing between HUVECs EA.hy926 incubated in the Low GL medium, the % viability of treated cells with respect to control cells was around 85% (BGID-treated) and 92% (BCF-treated). Similar non-cytotoxic effects were observed in comparisons of treated with respect to control cells incubated in the High GL medium.

The state of oxidative stress *in vivo* is governed by the intricate interplay between enzymatic pathways accountable for ROS production and the various endogenous or exogenous anti-oxidant mechanisms responsible for ROS elimination (Lee et al. 2012). Intracellular GSH was selected as representative of the endogenous non-enzymatic mechanisms protecting against oxidative damage, so the GSH/GSSG ratio served as a marker of the overall cell redox environment (Moskaug et al. 2005). The results showed a decrease higher than 40% in the GSH/GSSG ratio of control cells in response to high glucose conditions (**Figure 2**). This finding indicates an enhanced use of GSH to scavenge free radicals, either directly or acting as a substrate for GSH-dependent enzymes (GSH peroxidases and transferases) to protect against hydrogen peroxides, lipid hydroperoxides and electrophilic toxic compounds (Masella et al. 2005). Furthermore, the reduced GSH/GSSG ratio may be due to the decreased availability of

NAD(P)H under oxidative stress induced by hyperglycaemia, with NAD(P)H being required for the activity of glutathione reductase to transform GSSG into GSH (Srivastava et al. 1989). Under hyperglycaemic conditions, the incubation of HUVECs EA.hy926 with the BCF compounds was able to improve the intracellular redox environment significantly with respect to control cells. These results suggest that bioavailable colonic fermented metabolites derived from the seasoning may have a great capacity to restore a balanced redox state in hyperglycaemic endothelial cells. Phenolic metabolites might exert direct protection in biological systems by scavenging ROS, thereby decreasing the need for endogenous GSH. Nonetheless, several lines of research support an indirect action as a more probable mechanism, as exogenous antioxidants may activate the endogenous defence systems through antioxidant responsive elements (AREs), which are known to stimulate the transcription of the rate-limiting enzyme for GSH synthesis in a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent manner (Masella et al. 2005; Moskaug et al. 2005; Christensen & Christensen 2013).

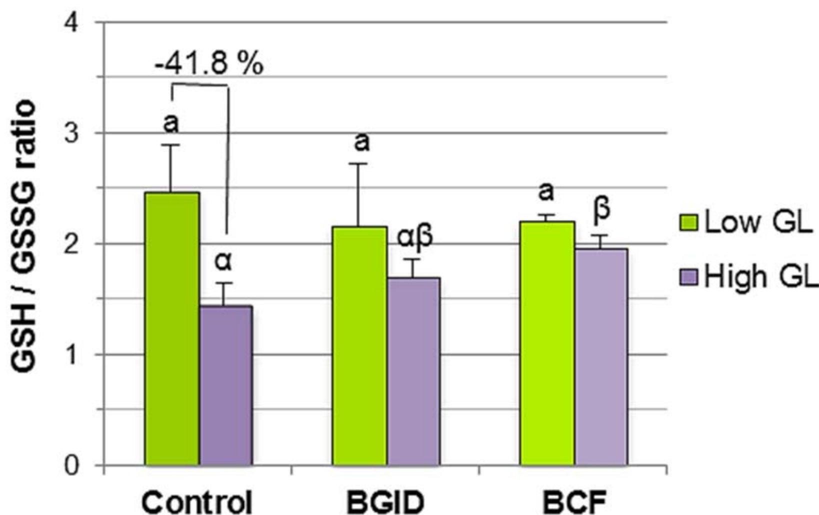

Figure 2. **Intracellular redox status (GSH/GSSG ratio) of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the digested fractions obtained from the seasoning.** BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed as mean values ± standard deviation (n=3). Percentage (%) values indicate significant differences ($p<0.05$) between hyperglycaemic and normoglycaemic cells. Significant differences ($p<0.05$) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

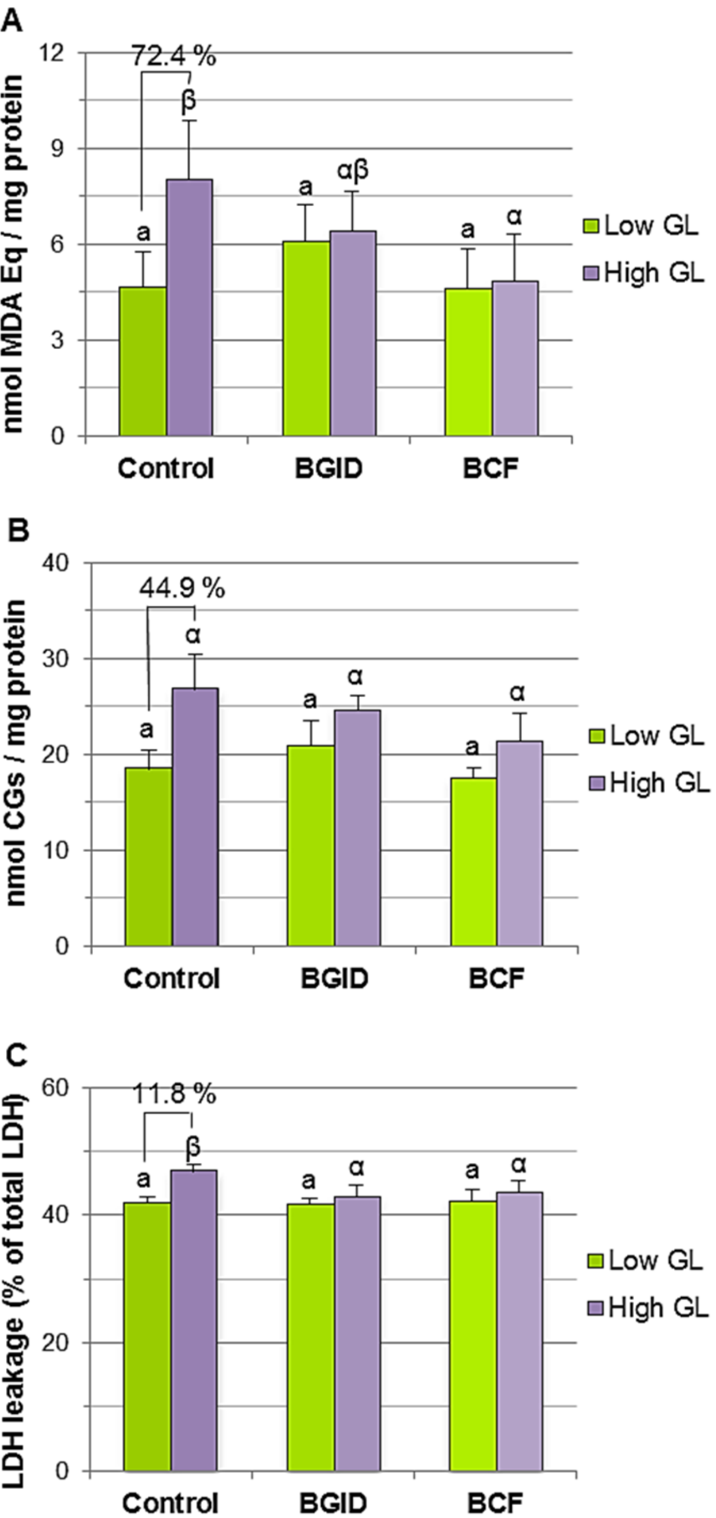

Figure 3. **Biomarkers of lipid, protein and cell membrane oxidative damage of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested fractions. A)** Malondialdehyde (MDA). **B)** Carbonyl groups (CGs). **C)** % lactate dehydrogenase (LDH) leakage. Cells for all assays were treated with the potentially bioavailable compounds obtained after the *in vitro* gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning under evaluation. Data expressed as mean values ± standard deviation (n=3). Significant variations (p<0.05) between cells cultured in different mediums are indicated as % of hyperglycaemic with respect to normoglycaemic cells. Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL). Eq: equivalents.

The oxidation products of biological molecules are also widely used as biomarkers of oxidative damage and provide specific evidence of cellular oxidative stress status and changes induced by exposure to antioxidant nutrients (Ho et al. 2013). Oxidative stress and subsequent lipid peroxidation are known to disturb the integrity of cellular membranes, leading to the release of cytoplasmic enzymes and pro-inflammatory factors to the extracellular medium (Margina et al. 2013). These aspects were assessed using MDA and CG levels (**Figure 3**A and B) as biomarkers of oxidative damage to lipids and proteins, respectively, and LDH leakage (**Figure 3**C) as an indicator of cell membrane damage (Li & Lau, 1993). In the control cells, hyperglycaemic conditions caused a significant increase in the levels of MDA and protein CGs, as well as in the plasma membrane permeability (LDH leakage). The BGID and the BCF treatments tended to ameliorate high glucose-induced protein oxidation and, more markedly, lipid peroxidation and LDH leakage. Concretely, a significant protection of lipids in the BCF-treated cells, and of the cell membrane in both the BGID- and the BCF-treated cells, was observed with respect to the control HUVECs EA.hy926 incubated in the High GL medium. The results suggested that the BGID and especially the BCF compounds helped to preserve endothelial cell membrane integrity under hyperglycaemia-induced oxidative conditions. This finding agrees with previous studies reporting the ability of certain polyphenols to restore the cell membrane fluidity and transmembrane potential in hyperglycaemic HUVECs (Margina et al. 2013).

Oxidative stress implicated in the cell membrane and biomolecules damage is also linked to functional alterations in vascular cells. Endothelial function is controlled by a complex network that regulates ROS production and clearance, concurrently with NO generation and inactivation, but this network is impaired under hyperglycaemic conditions (Inoguchi et al. 2000; Hadi & Suwaidi 2007). In the current study, the exposure of control cells to the High GL medium caused a marked 76.4% increase in ROS levels and, simultaneously, a significant 34.8% decrease in the concentration of NO breakdown products (**Figure 4**). This endothelial dysfunction was in line with previous studies reporting that the exposure of HUVECs to high glucose concentrations more or less caused ROS generation to double (Koziel et al. 2012; Chao et al. 2009) and led to a significant decrease in NO bioavailability (Yang et al. 2010; Zhou et al. 2012). It is widely recognized that NOXs represent the primary source of ROS ($O_2^{\cdot-}$ and H_2O_2) in the vascular wall, production of which is triggered by stimulation of vasoconstrictor agents such as Ang II and endothelin-1 (Virdis et al. 2011). A number of additional sources of ROS have been identified under conditions of oxidative stress and hyperglycaemia (Pennathur & Heinecke 2007; Münzel et al. 2010; Zhou et al. 2012), including uncoupled eNOS, which implies that the physiological activity of the enzyme for NO production is decreased and switched to the deleterious $O_2^{\cdot-}$ generation (Virdis et al. 2011; Roe & Ren 2012). NO bioavailability also depends on its rapid inactivation by reaction with $O_2^{\cdot-}$ to form $ONOO^-$ (Chen et al., 2010), which can further explain the decreased NO levels under the hyperglycaemic conditions observed in this study.

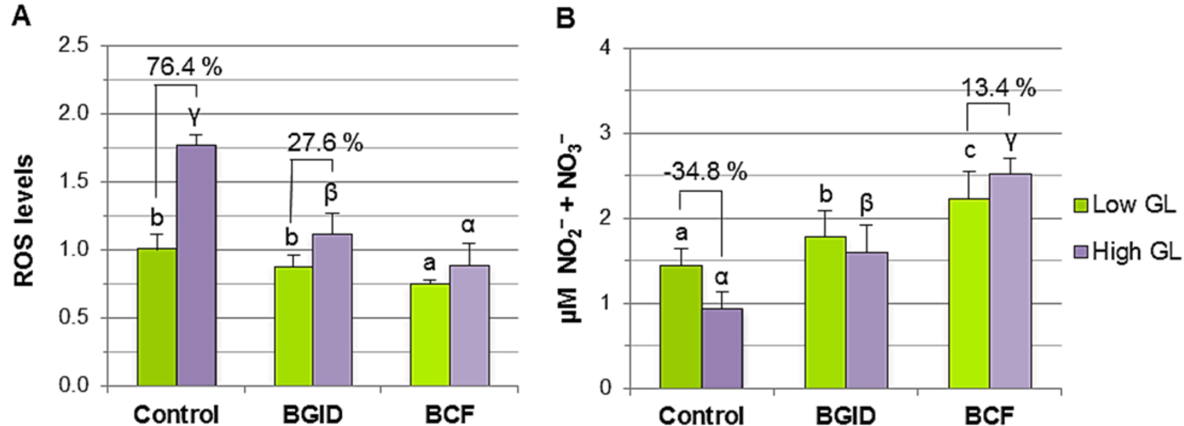

Figure 4. **Intracellular ROS and extracellular NO levels of normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested fractions.** Overall intracellular ROS production by HUVECs EA.hy926 was estimated by folds of 2,7-dichlorofluorescein (DCF) fluorescence increases with respect to the control normoglycaemic cells. **B)** NO generation by HUVECs EA.hy926 was estimated as the concentration (μM) in the culture medium of the total sum of NO breakdown products (NO_2^- + NO_3^-). Cells for both assays were treated with the potentially bioavailable compounds obtained after the *in vitro* gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning assessed. Data expressed as mean values ± standard deviation (n=3). Significant differences ($p<0.05$) between hyperglycaemic and normoglycaemic cells are indicated in % values. Significant differences ($p<0.05$) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

Interestingly, the BGID and the BCF treatments were able to partly restore the ROS levels detected in hyperglycaemic cells to levels found in normoglycaemic cells. Furthermore, incubation with these treatments prevented hyperglycaemia-impaired NO bioavailability in HUVECs EA.hy926, with significantly higher NO concentrations found in the medium of the treated rather than the control cells. The seasoning-derived BCF compounds showed a higher potential than the BGID compounds to improve endothelial function. Similar trends in these beneficial treatment effects were observed in cells exposed to the Low and the High GL mediums, but more markedly under hyperglycaemic conditions. According to previous studies, the results could be due to the anti-ROS effects of those phenolic compounds and metabolites present in the seasoning-derived treatments (**Supplementary Table S2**), such as numerous phenolic acids, flavan-3-ols, flavonols, anthocyanidins, and their degradation metabolites (Ruijters et al. 2013; Steffen et al. 2008; Edwards et al. 2015). Although only a limited number of individual phenolic metabolites were able to enhance the endothelial NO levels (Appeldoorn et al. 2009), the NO-preserving actions of these compounds have been suggested to rely mainly on NOX inhibition and perhaps partially in combination with their $O_2^{\cdot-}$ scavenging capacity (Steffen et al. 2008). In fact, the lower ROS levels and reduced NO inactivation detected in the BCF-treated cells were in accordance with the higher antiradical activity (ABTS values) for the BCF than the BGID fraction observed *in vitro*. In addition, grape and wine derived phenolic compounds may increase NO production through eNOS protein activation mediated by the phosphatidylinositol 3-kinase/Akt pathway (Schmitt & Dirsch 2009; Schini-Kerth et al. 2010).

3.3. Possible mechanisms of action implicated in the protection against oxidative stress and endothelial dysfunction under hyperglycaemia

3.3.1. Changes in NOX4, eNOS, SOD2 and HO-1 gene expression

In physiological terms, controlled ROS production plays an important role in cell signalling and maintains normal vascular contraction-relaxation and cell growth. The enhanced ROS production under pathological conditions is also known to stimulate several redox-sensitive signalling pathways (Zinkevich & Gutterman 2011; Virdis et al. 2011). In the present study, changes were therefore assessed in the mRNA levels of four genes involved in the endothelial cells ROS and NO balance (NOX4 and eNOS) and the endogenous antioxidant system (SOD2 and HO-1) (**Figure 5**).

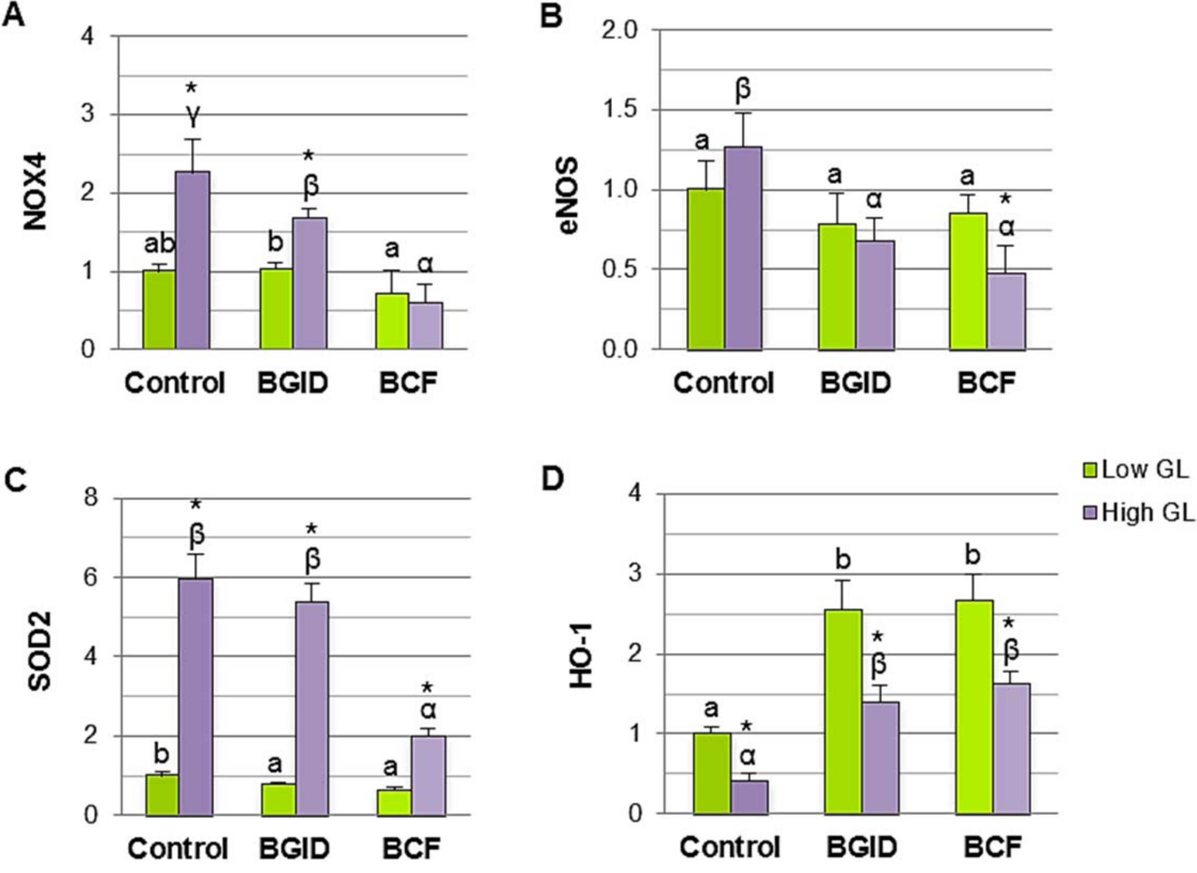

Figure 5. **Gene expression in normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the seasoning digested fractions.** Genes analysed: **A**) NOX4, **B**) eNOS, **C**) SOD2, and **D**) HO-1. Cells were treated with the potentially bioavailable compounds obtained after the *in vitro* gastrointestinal digestion (BGID) or colonic fermented (BCF) of the seasoning under study. mRNA levels of the genes under assessment were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the control normoglycaemic cells. Data expressed as mean values ± standard deviation (n=3). Significant changes (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated with an asterisk (*). Significant differences (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

This assessment was done to elucidate some of the mechanisms underlying the observed protective effects of the potentially bioavailable compounds derived from the seasoning. In the hyperglycaemic control cells, gene expression of the endothelial ROS generating enzyme NOX4 increased more than 2-fold, whereas no changes in the eNOS mRNA levels were detected (**Figure 5**A and B, respectively). The observed NOX4 up-regulation agrees with previously published works and has been described as mediated by the activation of phosphatidylinositol 3-kinase/Akt (Zhou et al. 2012) and protein kinase C pathways (Chai et al. 2008), and by the stimulation of Ang II (Selemidis et al. 2008). However, it is unclear how hyperglycaemia affects eNOS gene expression in HUVECs, which might be up-regulated (Ding et al., 2004; Li et al., 1998), down-regulated (Sun & Liao 2004) or, in accordance with our observations, not significantly affected (Patel et al. 2013). The results of the present study suggest that the decreased NO levels observed in the hyperglycaemic control cells were unlikely to be due to down-regulation of eNOS gene expression. It might therefore involve other previously cited mechanisms, such as ROS-mediated NO inactivation and eNOS uncoupling.

Interestingly, the treatment of hyperglycaemic HUVECs EA.hy926 with the BGID and the BCF compounds both resulted in a significant decrease in the levels of NOX4 and eNOS mRNA. In line with other authors working with resveratrol (Spanier et al. 2009) and grape pomace extracts rich in procyanidins (Álvarez et al. 2012), the current results could be associated with the ability of the seasoning-derived phenolic compounds to down-regulate NOX4 gene expression, with a higher potential observed for the potential colonic fermented metabolites. This capacity may be a key mechanism to explain the lower ROS levels found in the BGID and the BCF-treated cells. In addition, our results appear to suggest a compensatory decrease in the eNOS gene expression in response to the enhanced NO levels described above for hyperglycaemic cells treated with the BGID and especially the BCF fractions. Despite some reports supporting the up-regulation of the eNOS gene by red grapes and wine polyphenols such as resveratrol, in numerous cases their effective concentrations were higher than in the present study (Schini-Kerth et al. 2010). Furthermore, resveratrol is present at low concentrations in both the BGID and the BCF treatments (**Supplementary Table S2**), and many other wine compounds were found not to modify eNOS expression and they even exerted an inhibitory action at increasing concentrations, as in the case of quercetin (Wallerath et al. 2005).

In parallel to the mRNA level changes observed for the ROS and NO generating enzymes, the gene expression of the mitochondrial antioxidant enzyme SOD2 was 6-fold up-regulated, whereas the HO-1was 0.6-fold down-regulated, in the hyperglycaemic control cells (**Figure 5**C and D). These results are in agreement with other authors (Chen et al., 2012; Koziel et al., 2012) and they may reflect the endothelial cell response under hyperglycaemic conditions in an attempt to counteract over-production of $O_2^{\cdot -}$ (Patel et al. 2013). The

vascular up-regulation of SOD2 is attributed to the presence of response elements in its promoter for the redox-sensitive transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (Faraci & Didion 2004), which are known to be activated under hyperglycaemia (Chen, Mukherjee, Chakraborty, & Chakrabarti, 2003). However, controversial results can be found in the literature on the modulation of HO-1 gene expression by glucose in the vasculature, as this modulation has been suggested to depend upon levels of glucose (Iori et al. 2008).

SOD2 mRNA levels were considerably decreased in the hyperglycaemic BCF-treated cells, and also significantly reduced in normoglycaemic cells after incubation with both treatments. In contrast, the HO-1 gene was over-expressed to a similar extent by the BGID and the BCF treatments, but the effects were more marked for hyperglycaemic (3.7-fold) than normoglycaemic cells (2.6-fold). These results are consistent with several lines of evidence suggesting that some phenolic compounds and their metabolites have the capacity to improve endothelial function by HO-1 gene over-expression, mainly through the Nrf2/ARE pathway activation (Ungvari et al. 2010; Edwards et al. 2015). The observed HO-1 up-regulation almost certainly contributed to the decreased ROS levels observed in the treated cells, a fact that may further influence other redox-sensitive signalling pathways and enzymes that were over-expressed due to the increased oxidative environment in cells, such as might be the case of SOD2. The direct ROS scavenging capacity of the seasoning-derived phenolic compounds and their ability to inhibit the high glucose-induced NF-kB translocation to the nucleus, as previously reported for certain flavan-3-ols (Yang et al. 2013), might also explain the decreased requirement for SOD2 gene expression in the treated cells.

3.3.2. Changes in *ex vivo* ACE activity

Renin-angiotensin aldosterone system (RAAS) and ACE activation are associated with alterations in endothelial function observed in diabetes and hypertension (Hadi & Suwaidi 2007; Hsueh & Wyne 2011). In this regard, the Ang II over-production observed in hyperglycaemic cells is known to contribute to NOXs over-expression and activation, increased ROS generation, and subsequent endothelial dysfunction (McFarlane et al. 2003; Selemidis et al. 2008; Virdis et al. 2011). In addition, Ang II significantly up-regulates mRNA expression of renin, ACE, and angiotensinogen (the Ang II precursor), thus resulting in a vicious circle of RAAS activation in endothelial cells (Ide et al. 2008). Interestingly, there is accumulating evidence that flavonoids and polyphenol-enriched plant extracts are effective ACE inhibitors, although most of them have been shown to be more effective *in vitro* than *ex vivo* or *in vivo* (Balasuriya & Rupasinghe, 2011). Consequently, the *ex vivo* capacity of the BGID and the BCF fractions to down-regulate ACE activity in HUVECs EA.hy926 was also evaluated in this study.

The results showed that ACE activity was around 21% higher in lysates obtained from hyperglycaemic rather than from normoglycaemic cells (**Figure 6)**. This significant increase in ACE activity was in accordance with the enhanced ACE-1 expression previously reported in HUVECs under hyperglycaemia (Chen et al., 2014). However, *ex vivo* ACE activity was significantly decreased in the hyperglycaemic treated cells by about 13.6% (BGID) and 9.7% (BCF). These findings reveal that the BGID and the BCF treatments interfered in high glucose-induced RAAS activation. This effect may be partly explained by the direct capacity of the phenolic compounds released from the seasoning along digestion to inhibit ACE activity, which is supported by our *in vitro* ACE results, and by previous experiments *in vitro* and in HUVECs that demonstrated the ACE inhibitory capacity of certain flavonol metabolites (Balasuriya & Rupasinghe, 2012). In addition, the decreased Ang II production resulting from this direct ACE inhibition might lead to an indirect effect of the BGID and the BCF compounds down-regulating ACE expression in hyperglycaemic cells during the incubation period with these treatments (Ide et al. 2008).

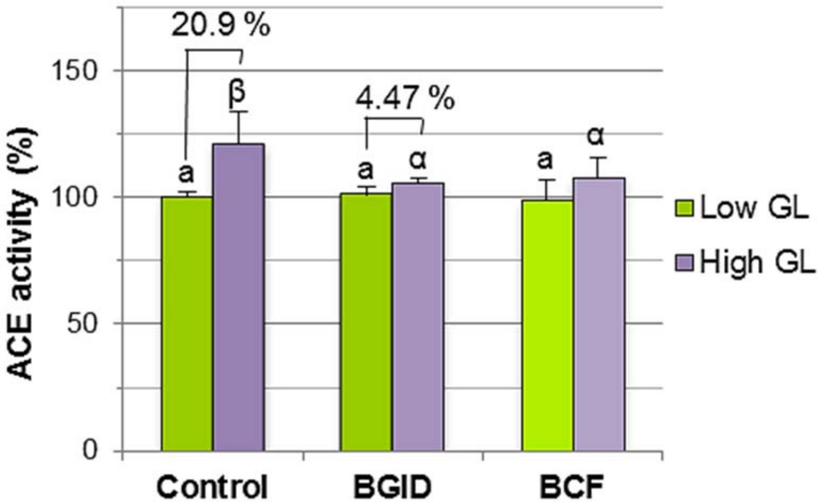

Figure 6. **ACE activity determined *ex vivo* in normoglycaemic (Low GL) and hyperglycaemic (High GL) HUVECs EA.hy926 treated with the digested fractions obtained from the seasoning evaluated.** BGID: potentially bioavailable samples after simulated gastrointestinal digestion; BCF: potentially bioavailable samples after simulated colonic fermentation. Data expressed in % as mean values ± standard deviation (n=3) with respect to the control normoglycaemic cells (100%). Significant differences (p<0.05) between hyperglycaemic and normoglycaemic cells are indicated in % values. Significant variations (p<0.05) between the control, the BGID-, and the BCF-treated cells are expressed in Roman (Low GL) and Greek letters (High GL).

4. Conclusions

The findings of this study have provided novel and compelling evidence of the potential protective effects of pre- and post-colonic digested compounds derived from a seasoning obtained from wine pomace against hyperglycaemia-induced endothelial dysfunction and oxidative damage.

Probable mechanisms of action by which these compounds restore the redox environment and endothelial function in HUVECs EA.hy926 are: direct ROS scavenging; modulation of NOX4, SOD2 and HO-1 gene expression by inducing different redox-sensitive signalling pathways; and, their capacity to decrease ACE activity.

The metabolism of bioactive compounds by colonic microbiota was found to play a key role along the digestive process in the beneficial vascular effects observed for the seasoning that has been assessed.

Altogether, this *ex vivo* study encourages the incorporation of seasonings derived from wine pomace in functional foods and sets the stage for future animal and clinical trials in order to confirm *in vivo* the promising healthy properties of such seasonings in cases of diabetes, hypertension, and other cardiovascular diseases.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary data to this article (**Figure S1**, **Table S1**, and **Table S2**) is shown below the references and can be found online at:
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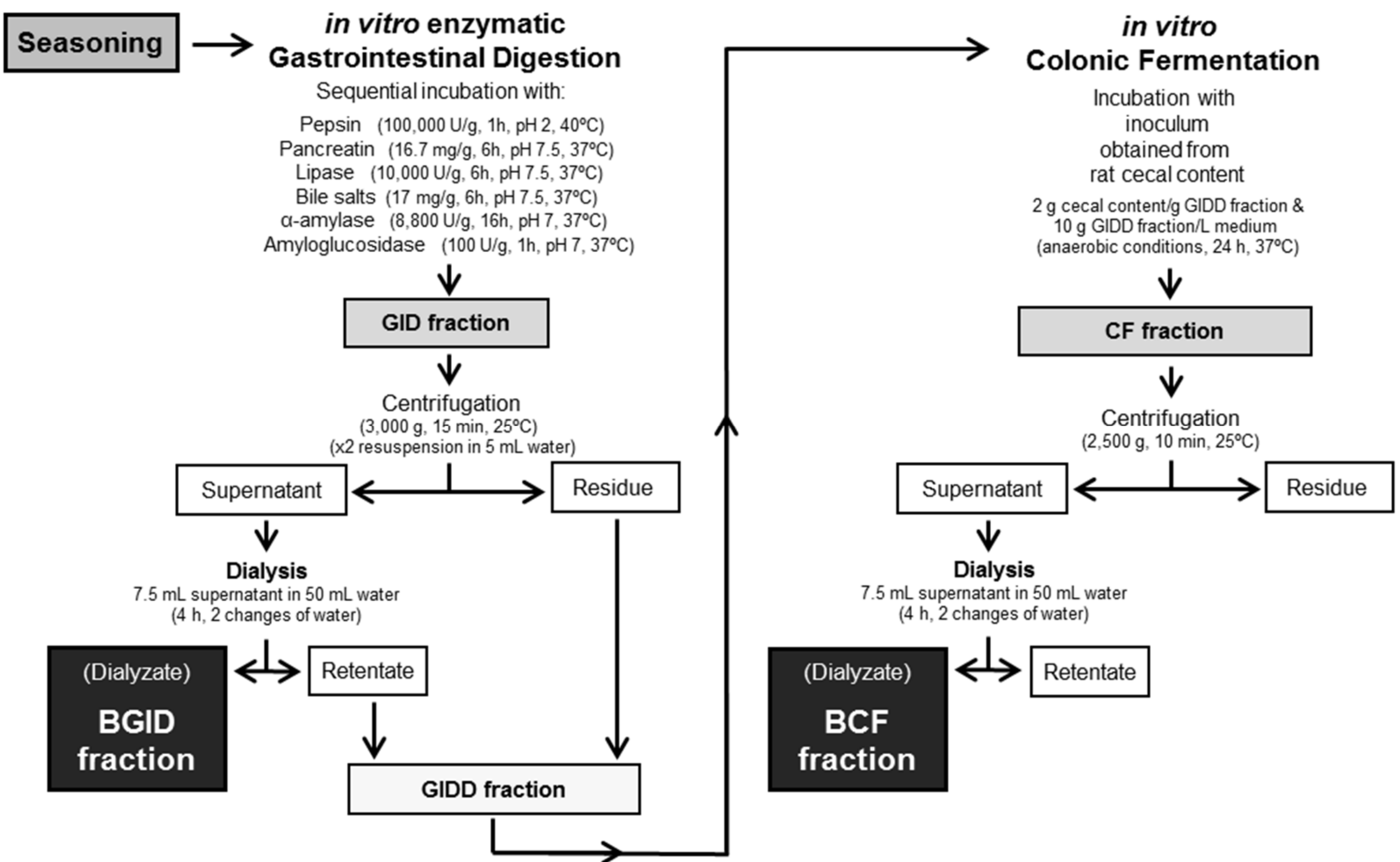

Figure S1. **Schematic representation of the main steps of *in vitro* digestion.** GID fraction: gastrointestinal digested fraction; BGID fraction: potentially bioavailable fraction obtained after simulated gastrointestinal digestion; GIDD: gastrointestinal digested and dialysed fraction: CF: colonic fermented fraction; BCF: potentially bioavailable fraction obtained after simulated colonic fermentation. BGID and BCF were the fractions used as cell treatments.

Table S1. **HPLC-DAD characterisation of the phenolic composition of the seasoning.**

Phenolic compounds	Concentration (µg/g)[a]
Phenolic acids	
Hydroxybenzoic acids	
p-OH-benzoic acid	6.27 ± 0.12
Salicylic acid	18.9 ± 0.6
Protocatechuic acid	21.3 ± 0.2
Gallic acid	73.1 ± 0.4
Vanillic acid	30.4 ± 0.3
Syringic acid	74.4 ± 3.5
Ellagic acid	21.4 ± 0.1
Ethyl gallate	17.1 ± 0.1
Hydroxycinnamic acids	
p-Coumaric acid	5.85 ± 0.27
Caffeic acid	3.40 ± 0.42
Ferulic acid	1.18 ± 0.01
Coutaric acid	4.59 ± 0.34
Caftaric acid	13.6 ± 0.35
Fertaric acid	3.76 ± 0.25
Stilbenes	
t-resveratrol	1.73 ± 0.13
t-piceid	1.42 ± 0.05
Flavan-3-ols	
Flavan-3-ols (monomers)	
Catechin	47.7 ± 2.0
Epicatechin	20.0 ± 1.7
Flavan-3-ols (dimers)	
Procyanidin B1	93.0 ± 4.3
Procyanidin B2	39.2 ± 1.6
Flavonols	
Flavonol aglycones	
Kaempferol	3.58 ± 0.09
Quercetin	19.0 ± 0.8
Myricetin	69.5 ± 2.7
Flavonol-3-*O*-glycosides	
Kaempferol-3-*O*-rutinoside	122 ± 2
Kaempferol-3-*O*-glucoside	51.6 ± 0.4
Quercetin-3-*O*-rutinoside	24.9 ± 0.4
Myricetin-3-*O*-rhamnoside	52.7 ± 2.5
Anthocyanidins	
Delphinidin	301 ± 8
Cyanidin	6.30 ± 1.06
Petunidin	118 ± 20
Peonidin	30.9 ± 2.8
Malvidin	1577 ± 109

a) Concentration of phenolic compounds expressed in µg/g of seasoning (mean value ± standard deviation, n=3).

Table S2. HPLC-DAD characterisation of the phenolic contents in digested fractions obtained from the wine pomace seasoning.

Phenolic compounds	BGID[a]	BCF[b]
Phenolic acids		
Hydroxybenzoic acids		
p-OH-benzoic acid	45.2 ± 3.9	11.8 ± 0.3
Salicylic acid	1.08 ± 0.27	4.71 ± 0.99
Protocatechuic acid	54.6 ± 9.8	1.59 ± 0.16
Gallic acid	21.9 ± 3.5	577 ± 18.4
Vanillic acid	50.9 ± 2.84	9.06 ± 0.70
Syringic acid	49.2 ± 5.4	29.4 ± 11.9
Ellagic acid	1.65 ± 1.41	7.63 ± 1.31
Ethyl gallate	ND	12.5 ± 3.2
Hydroxycinnamic acids		
p-Coumaric acid	0.428 ± 0.041	0.353 ± 0.034
Caffeic acid	2.35 ± 0.26	0.539 ± 0.165
Ferulic acid	0.165 ± 0.270	0.270 ± 0.025
Coutaric acid	2.29 ± 0.17	ND
Caftaric acid	1.25 ± 0.31	1.22 ± 0.21
Fertaric acid	7.53 ± 0.10	0.539 ± 0.165
Stilbenes		
t-resveratrol	0.766 ± 0.108	ND
t-piceid	ND	ND
Flavan-3-ols		
Flavan-3-ols (monomers)		
Catechin	5.19 ± 1.40 a	62.5 ± 3.64
Epicatechin	1033 ± 17	2.41 ± 2.11
Flavan-3-ols (dimers)		
Procyanidin B1	27.8 ± 5.47	12.1 ± 7.8
Procyanidin B2	ND	ND
Flavonols		
Flavonol aglycones		
Kaempferol	ND	ND
Quercetin	ND	ND
Myricetin	4.64 ± 0.66	ND
Flavonol-3-*O*-glycosides		
Kaempferol-3-*O*-rutinoside	ND	ND
Kaempferol-3-*O*-glucoside	1.18 ± 0.34	ND
Quercetin-3-*O*-rutinoside	15.7 ± 0.1	ND
Myricetin-3-*O*-rhamnoside	ND	ND

Concentration of phenolic compounds expressed in µg/g of digested fraction (mean value ± standard deviation, n=3).

a) BGID: potentially bioavailable fraction obtained after simulated gastrointestinal digestion.

b) BCF: potentially bioavailable fraction obtained after simulated colonic fermentation.

ND: not detected

Chapter 3.2.

Attenuation of Oxidative Stress and Endothelial Dysfunction in Type 1 Diabetic Rats Supplemented with a Seasoning obtained from Red Wine Pomace

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Abstract

Type 1 diabetes mellitus (DM) is characterised by hyperglycaemia resulting from insulin deficiency. This is usually accompanied by a pro-oxidative environment, dyslipidemia and endothelial dysfunction, thus leading to several micro- and macro-vascular complications. This study investigated the potential benefits of a seasoning obtained from seedless red wine pomace (RWPS) in protecting against oxidative damage and preserving endothelial function in Type 1 DM, and the underlying mechanisms involved at the level of gene expression. The diet of streptozotocin (45 mg/kg)-induced diabetic (DB) and control (CN) male Wistar rats (n=5 rats/group) was supplemented with RWPS (300 mg/kg/day) or vehicle for 4 weeks. Characteristic indicators of DM such as increased food and water intakes and weight loss were significantly ameliorated in DB+RWPS rats, with a notable normalization in their fasting glycemic control and cholesterol profile. Plasma total antioxidant capacity (TAC) was substantially increased, and biomarkers of oxidative damage to lipids (F_2-isoprostanes, 24.9%; malondialdehyde, 28.4%) and proteins (carbonyl groups, 5.91%) were significantly decreased. Nitric oxide availability was improved in plasma of DB+RWPS compared with DB rats. Insulin levels were increased (1.51-fold) and aortic tissue antioxidant enzymes such as mitochondrial superoxide dismutase (SOD2, 1.93-fold) were up-regulated. Other important genes for endothelial function, including endothelial β-nicotinamide adenine dinucleotide phosphate oxidase (NOX4), endothelial and inducible nitric oxide synthases (eNOS, iNOS), and angiotensin-converting enzyme I (ACE), were non-significantly modulated, although certain potentially positive trends were observed. These results indicate that RWPS supplementation might be a useful nutritional approach to manage Type 1 DM and ameliorate its vascular complications.

Keywords: Antioxidant; Diabetes mellitus; Endothelial dysfunction; Lipid peroxidation; Oxidative stress, Wine pomace.

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Abbreviations and nomenclature: ABTS, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; **ACE**, angiotensin-converting enzyme I; **CGs**, carbonyl groups; **DM**, diabetes mellitus; **eNOS**, endothelial nitric oxide synthase; **F_2-IsoPs**, F_2-isoprostanes; **FC**, Folin-Ciocalteu; **FRAP**, ferric reducing ability of plasma; **HO**, heme oxygenase; **HUVECs**, human umbilical vein endothelial cells; **iNOS**, inducible nitric oxide synthase; **MDA**, malondialdehyde; **NF-κB**, nuclear factor-kappa B; **NO**, nitric oxide; **NO_2^-**, nitrite; **NOX**, β-nicotinamide adenine dinucleotide phosphate oxidase; **RONS**, reactive oxygen and nitrogen species; **RXNOs**, nitros(yl)ated species; **RWPS**, seedless red wine pomace seasoning; **SOD**, superoxide dismutase; **STZ**, streptozotocin; **TAC**, total antioxidant capacity.

Highlights:

- Type 1 diabetic (DB) rats were given a wine pomace seasoning (RWPS) for 4 weeks.
- RWPS consumption ameliorated polyphagia, polydipsia and vascular oxidative stress.
- RWPS supplementation increased insulin levels and lowered fasting hyperglycaemia.
- RWPS consumption raised aortic SOD2 and tended to decrease iNOS gene expression.
- RWPS phenolic and dietary fibre contents may explain its anti-diabetic effects.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder of multiple etiology characterised by chronic hyperglycaemia resulting from defects in insulin secretion and/or insulin action (American Diabetes Association 2014).The elevated prevalence of DM and its increasing incidence worldwide, with an estimate number of 387 million carriers in 2014 that is expected to reach 592 million carriers in 2035 (International Diabetes Federation 2015), is becoming a public health issue of great relevance.

Macro- and microvascular disease are considered as major DM complications (Stirban et al. 2008; Bandeira et al. 2013), with the risk of developing vascular complications rising linearly with increased glycaemia. In this context, oxidative stress has been proposed as a key mechanism reinforcing this relationship (Bandeira et al. 2013), which is supported by the increased levels of markers of oxidative stress demonstrated in diabetic individuals suffering from vascular complications (Jakus 2000; Rahimi et al. 2005; Rösen et al. 2001).

Chronic hyperglycaemia and cumulative episodes of high blood glucose concentrations are associated with overproduction of reactive oxygen and nitrogen species (RONS) and with their insufficient removal due to a diminished endogenous antioxidant capacity, which further contributes to the establishment and maintenance of oxidative stress. This situation results in reduced nitric oxide (NO) bioavailability, which is the major hallmark of the endothelial dysfunction identified as playing a central role in DM micro- and macro-vascular disease pathogenesis (Erejuwa 2012; Kolluru et al. 2012; Sena et al. 2013).

In addition to glucose autoxidation, four cellular enzyme systems seem to predominate as potential sources of RONS in vascular cells (Kojda & Harrison 1999; Fatehi-Hassanabad et al. 2010; Pennathur & Heinecke 2007): β-nicotinamide adenine dinucleotide phosphate oxidases (NOXs), xanthine oxidase, uncoupled NO synthases (NOSs), and some mitochondrial respiratory chain complexes. RONS can directly damage lipids, proteins and DNA, and indirectly modulate intracellular signaling pathways, such as mitogen activated protein kinases and certain redox sensitive transcription factors causing several changes in gene/enzyme expression (Jakus 2000; Johansen et al. 2005).

In light of the above, understanding and treating the impaired endothelial function characteristic of DM is a major focus in the prevention of its associated vascular complications (Ceriello 2003; Sena et al. 2013). In this regard, emerging evidence suggests that several foods, nutrients or individual dietary compounds, including antioxidant phytochemicals such as polyphenols, may effectively improve and/or protect against endothelial dysfunction associated to DM (Stoclet et al. 2004; Schini-Kerth et al. 2010).

Grapes, wine, and winemaking grape pomace, also termed wine pomace, are well-known as significant sources of phenolic compounds and have been proposed to exhibit several cardiovascular protective properties (Dohadwala & Vita 2009; Pérez-Jiménez & Saura-Calixto 2008). Wine pomace is also a great source of dietary fibre, thus being a phenolic-rich dietary fibre matrix with numerous potential benefits on human health (Zhu et al. 2015).

In the present study, a natural seasoning obtained from seedless red wine pomace (RWPS), whose potential vascular protection under hyperglycemic conditions has been suggested from previous studies in human umbilical vein endothelial cells (HUVECs) (Del Pino-García et al. 2016d), was given as a dietary supplement to a rat model of Type 1 DM. The main purpose was to: firstly, evaluate the potential hypoglycemic and protective effects of RWPS against oxidative stress and associated endothelial dysfunction; and, secondly, provide mechanistic insight into the gene expression modulation of key genes involved in vascular homeostasis.

2. Materials and Methods

2.1. Chemicals

Acetonitrile anhydrous (99,8%), 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), N,O-*bis*(trimethylsilyl)trifuoroacetamide with 1% trimethylchlorosilane (BSTFA+TMCS), butylated hydroxytoluene (BHT), bovine serum albumin (BSA), diethylene triamine pentaacetic acid (DTPA), N,N-diisopropylethylamine (DIPEA), 2,4-dinitrophenylhydrazine (DNFH), ethanol, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), gallic acid (GA), glutathione (GSH), guanidine hydrochloride, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), iodine (I_2), isooctane, methanol, 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), phosphoric acid solution (H_3PO_4), potassium hydroxide (KOH), pyridine anhydrous (99,8%), potassium iodide (KI), sodium acetate, sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), streptozotocin (STZ), 1,1,3,3-tetramethoxypropane (TMP), thiobarbituric acid (TBA), 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ) and analytical standard solutions (≥99,8%) of short chain fatty acids (acetic acid, butyric acid, propionic acid, and valeric acid) were purchased from Sigma–Aldrich, Co. (St. Louis, MO, USA). Acetone, hexane, and glacial acetic acid were provided by Univar Australia, Pty. Ltd. (Ingleburn, NSW, Australia). 8-iso-$PGF_{2\alpha}$-d_4 (Cat. No. 316351) and 8-iso-$PGF_{2\alpha}$ (Cat. No. 16350) standards were purchased from Cayman Chemical, Co. (Ann Arbor, MI, USA). Certify II cartridges for solid phase extraction (SPE) were from Varian Australia, Pty. Ltd. (Mulgrave, VIC, Australia). Ferric(III)-chloride acid ($FeCl_3$), ferrous(II)-sulphate ($FeSO_4$), Folin-Ciocalteau (FC) reagent, potassium persulfate ($K_2O_8S_2$), sodium acetate ($NaC_2H_3O_2$), sodium carbonate (Na_2CO_3), and trichloroacetic acid (TCA) were obtained from Panreac Química, S.L.U. (Barcelona, Spain). All solvents were HPLC grade.

2.2. Seedless red wine pomace seasoning (RWPS)

Red wine pomace collected after winemaking of red grapes (*Vitis vinifera* var. Tempranillo) was kindly provided by several wineries located in Burgos (Spain). All wine pomace was mixed and dehydrated in an oven at 60 ºC for 4 h to a final moisture content < 10%. Then, seeds were separated from wine pomace and the remaining seedless dried material was milled and sieved, obtaining a product of particle size < 0.250 mm. This powdered product was submitted to a heat treatment at 90 ºC for 90 min to ensure the microbial reduction degree required to obtain a stable and safe potential food ingredient (González-Sanjosé et al. 2013).

2.3. Animals and experimental procedures

All animal procedures followed the principles of laboratory animal care and protection outlined in the current European and Spanish laws (European Directive 2010/63/EU and Royal Decree 53/2013 of the Spanish Ministry of Agriculture, Food and Environment and Ministry of Economy and Competitiveness). The experimental protocol was approved by the Ethics Committee for Experimental Animal Care at the University Hospital of Burgos and the Ethics Committee at the University of Burgos.

Twenty adult male Wistar Kyoto rats weighing 306 ± 13 g were randomly assigned to the following groups (n=5): control (CN), control supplemented (CN+RWPS), diabetic (DB), or diabetic supplemented (DB+RWPS). Rats were housed at 23 ºC with 12-h light/dark cycles, were fed with a standard diet (A04 Safe Iberica-Panlab, Barcelona, Spain) and had access to drinking water *ad libitum* during the experiments. After a week of acclimatization, diabetes was induced in overnight fasted rats of DB groups by a single intraperitoneal injection of STZ (45 mg/kg) freshly dissolved in 0.1 M citrate buffer (pH 4.6). Rats of CN groups were injected with citrate buffer. The diabetic status was confirmed 3 days later in all DB rats by blood glucose levels >350 mg/dL. RWPS supplementation started one week after the induction of Type 1 DM. The seasoning product under study was dissolved in 2 mL water and orally administered by gavage to RWPS-supplemented rats (300 mg/kg of body weight) between 9 and 10 a.m., whereas rats of control groups received vehicle (just water) alone. The nutrient composition, total polyphenol contents and antioxidant capacities of the supplement have been previously determined (García-Lomillo et al. 2014; Del Pino-García et al. 2015). A detailed characterization of the individual phenolic composition of RWPS has been also published (Del Pino-García et al. 2016d).

Food and water consumption were recorded every day and the mean daily intake was calculated each week, the body weight of each rat was measured every week, and postprandial blood glucose levels were also monitored weekly using a StatStrip™ Xpress glucometer (Nova Biomedical, Co., Waltham, MA, USA). Faecal samples from each rat were randomly collected the first and the last day of the study, immediately stored under CO_2

atmosphere and frozen at -20 °C until analyses. The study was terminated after 4 weeks of supplementation. On the last study day, blood glucose levels were measured in 12-hour fasted rats, which were then anesthetized and sacrificed.

Blood samples were collected by cardiac puncture in Vacutainer® tubes (Becton Dickinson, Co., Madrid, Spain) containing sodium and lithium heparin (17 I.U./mL blood) as anticoagulant. As previously described (Mori et al. 1999), those blood samples for F_2-IsoP analysis were collected in ice-cold tubes containing a mixture of GSH/BTH/EDTA in order to prevent oxidation of lipids on storage. All blood samples were centrifuged at 1,500 g for 10 min at 4 °C and the plasma obtained was then divided into aliquots for analyses. Thoracic aortic tissue from each rat was also harvested and immediately frozen in liquid nitrogen. All these samples were kept at -80 °C until biochemical assays.

2.3. Biochemical analyses

2.3.1. Faecal contents of short chain fatty acids (SCFAs)

The concentration of acetic, propionic and butyric acids in rat feces were measured by a previously described method (Zhao et al., 2006), with slight modifications. Briefly, faecal samples were thawed, weighted, and suspended in Milli-Q water (1 g feces/5 mL water). These samples were homogenized for 2 min in an Ultra-turrax T-25 (IKA®-Werke GmbH & Co. KG, Staufen, Germany), and then centrifuged for 5 min at 5,000 rpm, giving a clear supernatant. A volume of 50 µL of the internal standard solution (valeric acid at 1 mM concentration in 0.1% phosphoric acid solution) was spiked into 50 µL of the previously collected supernatant, resulting in samples with a pH of 2-3 which were injected in an Agilent 7890A Series gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA) equipped with a flame ionization detector (FID) and fitted with a capillary column with a free fatty acid phase DB-FFAP 125-3237 (30 m x 0.53 mm, 0.50 µm film thickness, Agilent Technologies). The conditions indicated by Zhao et al., (2006) were used for the chromatographic analysis of samples. Peak identification was based on comparison of retention times with the respective SCFA standards. Individual 9-point calibration curves were obtained for each SCFA standard and used for quantitative analysis. For calibration and samples analyses, the areas obtained for acetic, propionic and butyric acids were corrected by the area obtained for the internal standard. The results were expressed as concentration values in millimol of SCFA per gram of feces (mmol SCFA/g feces).

2.3.2. Plasma insulin levels

The insulin concentration in plasma of fasted rats was assessed by ELISA, using the Mercodia Rat Insulin Kit (Ref: 10-1250-01, Mercodia, AB., Uppsala, Sweden), according to the manufacturer's instructions.

2.3.3. Plasma cholesterol analysis

Fasting plasma total (TC) and high-density lipoprotein (HDL) cholesterol concentrations were determined in a semi-automated MC-15 analyzer (RAL técnica para el laboratorio, S.A., Barcelona, Spain) using Gernon Reagents (RAL) and standard procedures. The TC/HDL cholesterol ratio was calculated for each rat as a parameter to estimate their cholesterol profile.

2.3.4. Plasma total antioxidant capacity (TAC) assessment

Plasma TAC was estimated using three different methods, following the procedures previously described (Santiago-Arteche et al. 2012).

Folin-Ciocalteu (FC) assay: This method is commonly used to estimate total phenols based on the total reducing capacity of samples, thus was used to assess plasma TAC. The absorbance at 750 nm of samples, containing the blue-colored phosphomolybdic/phosphotungstic acid complexes formed during reaction, was measured in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Gallic acid (GA) was used as standard to obtain a calibration curve and results were expressed as millimolar of GA equivalets (mM GAE).

Ferric reducing ability of plasma (FRAP) assay: This analysis determine the presence of reducing agents in plasma by their ability to reduce Fe(III) to Fe(II). A blue-colored Fe(II)-TPTZ complex is formed, which absorbs light at 593 nm. $FeSO_4$ aqueous solutions of known concentration were used to construct a dose-response curve and the FRAP value for each sample was expressed as micromolar of Fe(II) equivalents (µM Fe(II)E).

ABTS assay: This method determines the capacity of plasma antioxidants to scavenge the $ABTS^{\cdot +}$ radical by measuring the inhibition of absorbance at 734 nm. A linear calibration curve was obtained using Trolox as standard and results were expressed as millimolar of Trolox equivalets (mM TE).

2.3.5. Plasma F_2-isoprostanes (F_2-IsoPs) determination

F_2-IsoPs levels in plasma samples were determined by GC and electron capture negative chemical ionization mass spectrometry (GC-NCI-MS), according to a previously reported method (Mori et al. 1999) with slight modifications. Briefly, a 0.1 mg/L internal standard solution of 8-iso-$PGF_{2\alpha}$-d_4 (also known as 15-F_{2t}-IsoP-d_4) was prepared and 50 µL of internal standard solution were added to 0.2 mL of plasma. Then, samples were mixed with 250 µL of 1 M KOH in methanol by vortexing, and incubated at 40 °C for 30 min. After acidification (pH 4.5–4.7) with 1 M HCl, F_2-IsoPs were separated from samples using SPE cartridges. The isolated F_2-IsoPs were then derivatized, dried under nitrogen, reconstituted in isooctane, and analyzed by GC-NCI-MS using an Agilent 6890N Network gas chromatograph (Agilent

Technologies, Inc., Palo Alto, CA) coupled to an Agilent 5975B Series Mass Selective Detector (Agilent Technologies) and fitted with a DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies), as previously reported (Mori et al. 1999). F_2-IsoPs were detected by monitoring *m/z* 569 and the corresponding ion for the deuterium-labeled 8-iso-$PGF_{2\alpha}$ (*m/z* 573), with peaks being identified by comparison of retention times with standards. Quantification was performed using a dose-response curve obtained from a native 8-iso-$PGF_{2\alpha}$ standard. Plasma F_2-IsoPs results were finally expressed as concentration values in nM.

2.3.6. Plasma malondialdehyde (MDA) levels measurement

Plasma MDA levels were assessed following a previously reported chromatographic method (Grotto et al. 2007). An Agilent 1100 series HPLC system (Agilent Technologies) equipped with a diode array detector (DAD) and a Spherisorb3® ODS2 reversed phase C18 (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia, S.A., Barcelona, Spain) column were used for analyses. MDA levels were calculated from calibration curves constructed with the standard (TMP), and results for each plasma sample were normalized by their total protein concentration, which was determined according to the Bradford method (Bradford 1976). Final results were expressed as nmol MDA/mg protein.

2.3.7. Plasma protein carbonyl groups (CGs) assessment

A previously described spectrophotometric method (Levine et al. 1990) was used to quantify the concentration of protein CGs in plasma samples. The protein concentration of each sample was used to normalize the CGs levels, thereby expressing the results as nmol CGs/mg protein.

2.3.8. Plasma nitrite and nitros(yl)ated species (NO_2^-+RXNOs) levels measurement

Nitrite (NO_2^-) and several nitros(yl)ated species (RXNOs: the sum of S-nitrosothiols, N-nitrosamines and nitrosylhemes), derived from the rapid NO metabolism in plasma, were both quantified in these analyses as an indirect approach to estimate the NO production in the vasculature. A mixture of iodine/tri-iodide in glacial acetic acid was used for the reductive denitrosation of plasma samples. The NO released reacted with ozone and was then detected by gas-phase chemiluminescence (Feelisch et al. 2002) in a NO analyzer (Eco Physics-CLD 60, ANRI Instruments & Controls, Pty.Ltd. VIC, Australia). Raw data obtained was processed using PowerChrom software. NO peaks were integrated for each sample after baseline corrections and signal smoothing to eliminate high-frequency noise. A calibration curve was obtained using $NaNO_2$ standard solutions and the final NO_2^-+RXNOs results were expressed as concentration values in µM.

2.3.9. Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA from rat aortic tissue was extracted using TRI Reagent solution (Applied Biosystems, Foster City, CA, USA) and treated with DNase I (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, reverse-transcription was carried out with 1 µg of total RNA by the First Strand cDNA Synthesis kit (Thermo Fisher Scientific) and the cDNA obtained was finally amplified with the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, S.A., Madrid, Spain) on the iCycler iQ Real-Time PCR Detection System (Bio-Rad). All these procedures were performed following the manufacturers' instructions. The expression of the following genes was assessed: β-nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), inducible NOS (iNOS), endothelial NOS (eNOS), angiotensin-converting enzyme I (ACE), mitochondrial superoxide dismutase (SOD2), and heme oxygenase-1 (HO-1). Primer3 v.0.4.0 software (Untergasser et al., 2012) was used for designing the primer sets, which were synthesised by Metabion (Metabion International, AG, Steinkirchen, Germany) according to the following sequences (forward and reverse):

- NOX4, 5'-CAGAGGGAAAACAGTTGGAGT-3' and 5'-CCCATATGAGTTGTTCCGGT-3'
- iNOS, 5'-CTTGCCCCTGGAAGTTTCTC-3' and 5'-TGTTGTTGGGCTGGGAATAG-3'
- eNOS, 5'-TATTTGATGCTCGGGACTGC-3' and 5'-AACGAAGATTGCCTCGGTTT-3'
- ACE, 5'-TGGAGCATCTCTACCACCAA-3' and 5'-TGTATTTGTCCCCATAGCGG-3'
- SOD2, 5'-AAGGTCGCTTACAGATTGCC-3' and 5'-CTCCCACACATCAATCCCC-3'
- HO-1, 5'-GTGGCAGTGGGAATTTATGC-3' and 5'-AAGAGAGCCAGGCAAGATTC-3'
- GAPDH, 5'-ACTCCCTCAAGATTGTCAGC-3' and 5'-CTTCCACGATGCCAAAGTTG-3'

The cycling conditions consisted of an initial denaturing and enzyme activation at 95 ºC (3 min), followed by 45 alternating cycles of denaturing at 95 ºC (15 s) and annealing/extension at 60 °C (30 s). Specificity of the reaction was checked by a melting curve. Amplification efficiencies were determined using dilution series of a control aortic tissue sample. Final gene expression results were evaluated applying the efficiency-corrected ΔΔCt method with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference gene and mRNA levels fold change was calculated relative to the CN group.

2.4. Data presentation and statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) of measurements, which were performed in duplicate for each rat. For statistical analyses, we used Statgraphics® Centurion XVI software version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). One-way analysis of variance (ANOVA) followed by the Fisher's Least Significant Difference (LSD) test was applied to evaluate significant differences among experimental groups. For all comparisons, differences were considered statistically significant at a value of $p < 0.05$.

3. Results

3.1. Food and water intakes, body weight, and glycaemia

The daily food and water intake (**Figure 1**A and B) was comparable and rather constant in the normoglycemic control (CN) groups along the experimental period. At the beginning of the study, these intakes were approximately 1.5-fold (food) and 6.5-fold (water) significantly higher in the diabetic (DB) than CN groups. The amount of food ingested (**Figure 1**A) increased gradually in DB rats until consuming more than twice than CN rats at week 4, whereas the increase in DB+RWPS rats was less pronounced from the first week of supplementation, with a 15.2% lower ($p < 0.001$) food intake than DB rats at the end of the study. There was a marked higher daily water intake by DB rats during the study (**Figure 1**B). Conversely, the volume of water consumed by DB+RWPS rats decreased significantly from week 1 and was a 25.4% lower ($p < 0.001$) than that of their DB counterparts at the end of the supplementation period.

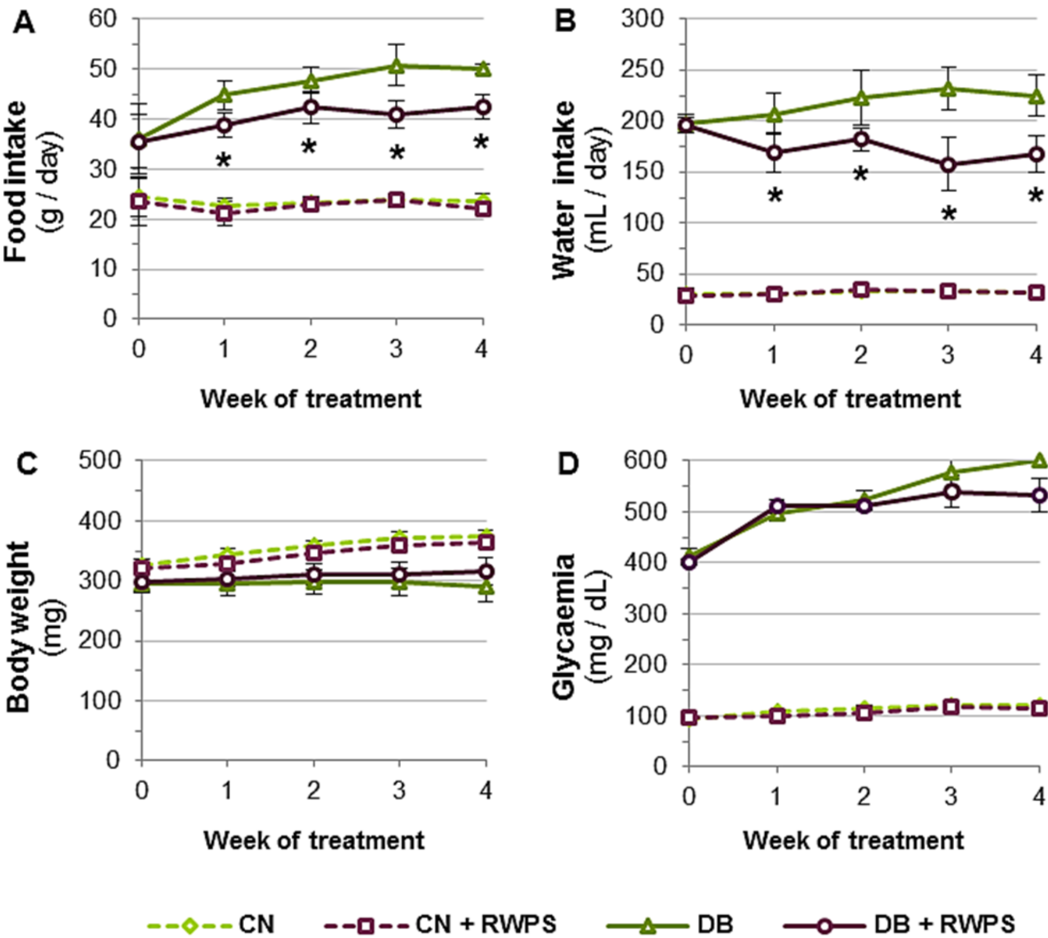

Figure 1. Food and water intakes (A and B), body weight (C), and glycaemia (D). Determinations were performed weekly along the 4-week-supplementation study of Type 1 diabetic rats with a powdered seedless red wine pomace seasoning (RWPS). Experimental rat groups: CN, control; CN+RWPS, control supplemented; DB, diabetic; DB+RWPS, diabetic supplemented. Data expressed as mean ± SEM values (n = 5). Asterisks (*) indicate significant differences ($p < 0.05$) between DB and DB+RWPS.

Although the body weight of all rats was similar before inducing DM, at the beginning of the supplementation (week 0) the DB groups weighed close to 10% less ($p < 0.01$) than CN rats (**Figure 1**C). A progressive gain of weight was observed in CN groups along the study, whereas the body weight in DB groups did not increase. There were no significant differences due to the supplementation in either the normoglycemic or the diabetic groups with a trend for the body weight to decrease in CN+RWPS with respect to CN rats, while it slightly increased in DB+RWPS compared to DB rats.

Postprandial blood glucose levels (**Figure 1**D) showed a marked hyperglycaemia in both DB groups at the beginning of the study (around 400 mg/dL). This high blood glucose concentration increased gradually in the DB rats whereas glycaemia appeared to stabilize from week 2 in the DB+RWPS counterparts, but the differences were not significant between these groups.

3.2. Pre- and post-RWPS supplementation faecal contents of short chain fatty acids (SCFAs)

The faecal contents of SCFAs changed significantly from the beginning (day 0) to the end (day 28) of the study in the experimental groups supplemented with RWPS, showing more than 20% of increase (**Figure 2**). This was mainly due to the higher butyric acid concentration observed in feces of both RWPS supplemented groups, which increased approximately 50% (CN+RWPS) and 64% (DB+RWPS) from day 0 to day 28 of the study ($p < 0.05$).

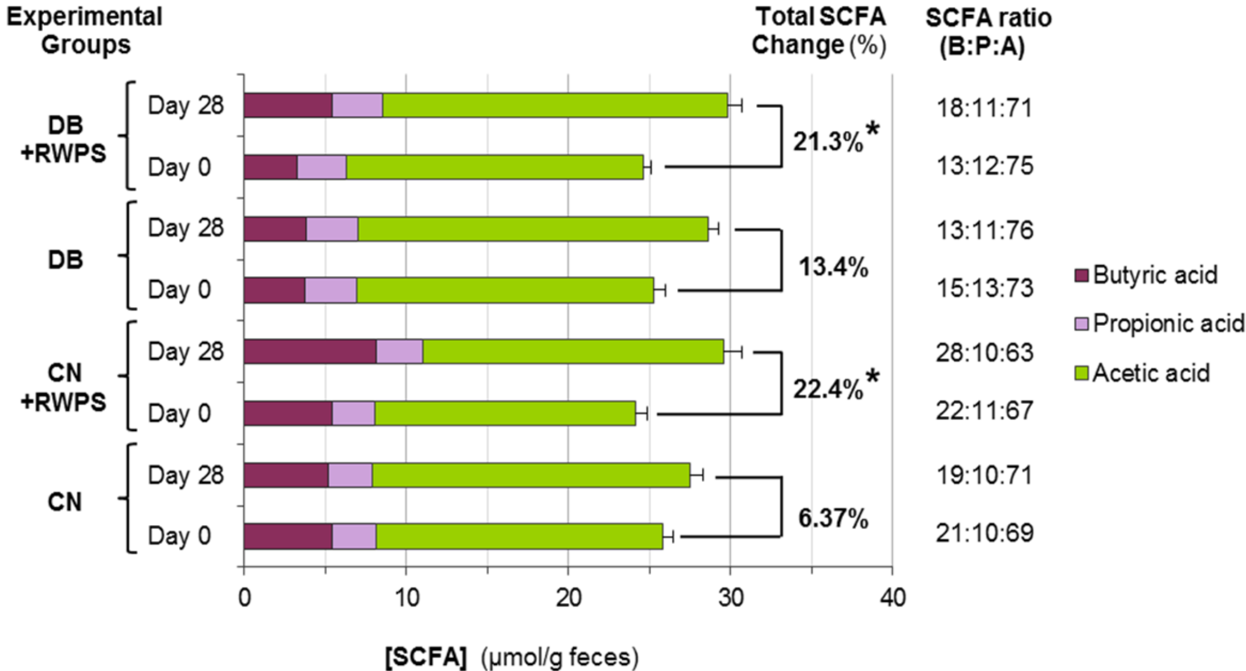

Figure 2. **Faecal short chain fatty acid (SCFA) concentration.** Samples were collected at the beginning (day 0) and at the end (day 28) of the supplementation study of Type 1 diabetic rats with a powdered seedless red wine pomace seasoning (RWPS). Experimental rat groups: CN, control; CN+RWPS, control supplemented; DB, diabetic; DB+RWPS, diabetic supplemented. Total SCFA change (%) represents the differences between the sum of butyric, propionic and acetic acid faecal contents at day 28 compared to day 0 for each experimental rat group, with asterisks (*) indicating significant differences ($p<0.05$). SCFA ratio (B:P:A) represents the molar ratio of butyric (B), propionic (P) and acetic (A) acids in each of the faecal samples.

The molar ratio of butyric:propionic:acetic acids (B:P:A) obtained revealed lower relative excretion of butyric acid in feces of DB than CN groups pre-RWPS supplementation (day 0). In the CN rats, the B:P:A molar ratio was maintained rather similar from the beginning to the end of the study. In the CN+RWPS group, the final relative faecal content of butyric acid was increased, and that of acetic acid was decreased. The DB rats showed a trend toward lower relative content of butyric acid, and higher acetic acid concentration, from day 0 to day 28 of the study. In contrast, after 4 week of seasoning consumption, the relative faecal content of butyric acid in DB+RWPS rats was increased, and that of acetic acid was decreased, obtaining a similar B:P:A molar ratio compared to CN rats.

3.3. Fasting blood glucose and plasma insulin levels, cholesterol profile and total antioxidant capacity

Several analyses performed in blood and plasma collected the last day of the study are shown in **Table 1**. Fasting blood glucose levels in DB rats were approximately double those observed in the other groups, whereas a marked decrease was detected in the DB+RWPS rats ($p < 0.05$), reaching similar values to those of the CN groups.

Table 1. **Fasting blood glucose, plasma insulin concentration, atherogenic index, and plasma total antioxidant capacity (TAC) determinations.**

Biochemical Analyses[a]	Experimental Groups[b]			
	CN	CN + RWPS	DB	DB + RWPS
Fasting blood glucose (mg/dL)	72.8 ± 2.06 a	81.0 ± 3.61 a	147.5 ± 19.1 b	86.3 ± 5.57 a
Plasma insulin levels (μg/L)	0.162 ± 0.008 c	0.216 ± 0.017 d	0.080 ± 0.009 a	0.121 ± 0.007 b
Plasma TC/HDL ratio (relative units)	2.95 ± 0.082 ab	3.00 ± 0.075 ab	3.13 ± 0.090 b	2.77 ± 0.071 a
Plasma TAC[c]				
FC index (GAE mM)	9.47 ± 0.287 b	9.27 ± 0.162 b	7.66 ± 0.237 a	9.27 ± 0.264 b
FRAP (Fe(II)E mM)	0.486 ± 0.041 b	0.454 ± 0.030 b	0.270 ± 0.008 a	0.452 ± 0.035 b
ABTS (mM TE)	8.01 ± 0.188 b	7.99 ± 0.151 b	7.16 ± 0.140 a	8.23 ± 0.324 b

Data expressed as mean ± SEM values (n=5). Letters indicate significant variations ($p<0.05$) between the experimental groups for each analysis.

a) Biochemical analyses performed in samples collected from 12 h-fasted rats at the end of the study, following 4-week-supplementation with a seedless red wine pomace seasoning (RWPS).

b) Experimental rat groups: control (CN), control supplemented (CN+RWPS), diabetic (DB), and diabetic supplemented (DB+RWPS).

c) Plasma TAC determined using the following methods: FC index: Folin-Ciocalteu index expressed in mM of gallic acid equivalents (mM GAE); FRAP: Ferric reducing ability of plasma expressed in mM of iron(II) equivalents (mM Fe(II)E); ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid expressed in mM of Trolox equivalents (mM TE).

The supplementation with RWPS resulted in a significant increase in the insulin levels of both normoglycemic (1.34-fold) and diabetic rats (1.50-fold) with respect to their non-supplemented counterparts. The TC/HDL ratio was significantly lower in DB+RWPS rats than DB rats, with both CN groups showing intermediate values. All the TAC analyses showed lower values in plasma of DB rats than the rest of groups (p < 0.01), whereas significant TAC increases of approximately 21% (FC index), 67% (FRAP), and 15% (ABTS) were observed in plasma of DB+RWPS compared to DB rats.

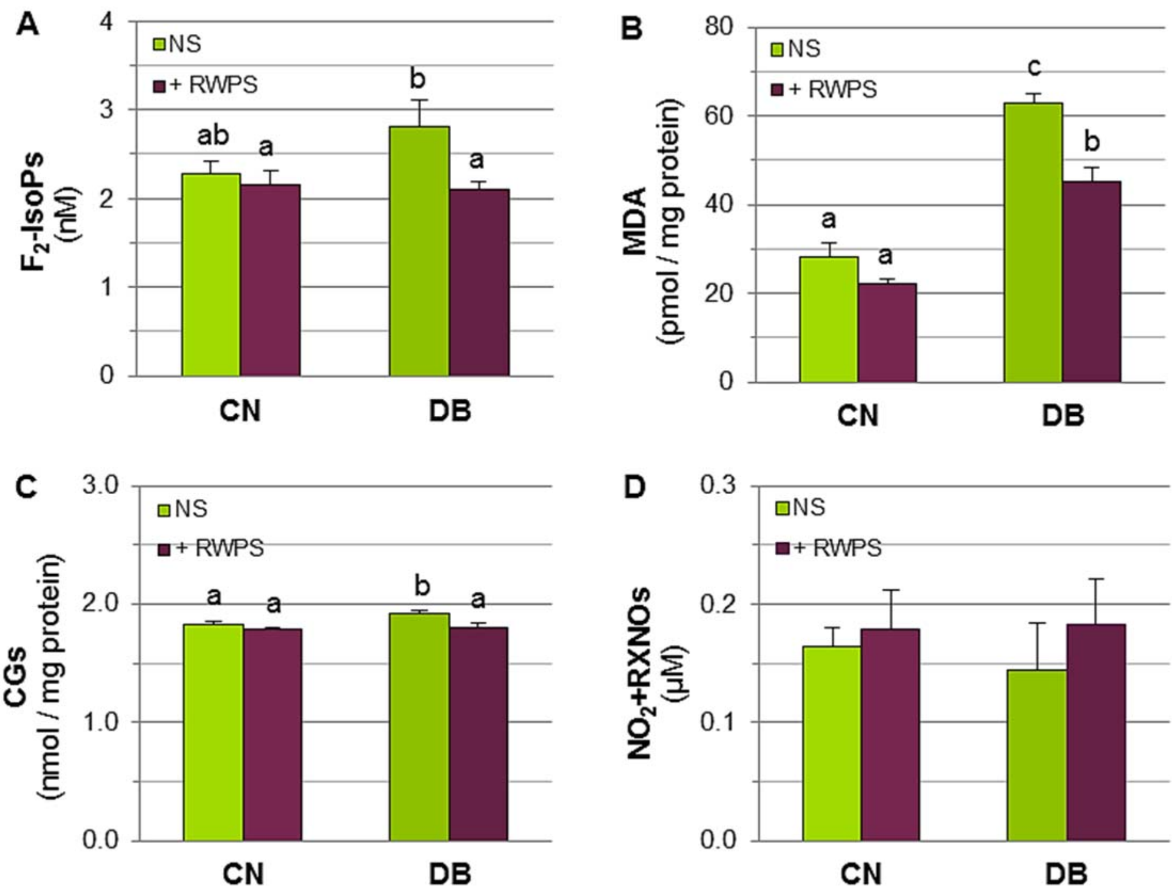

Figure 3. **Lipid and protein oxidative damage biomarkers and nitric oxide (NO) bioavailability.** Lipid peroxidation estimated from the F_2-isoprostanes (F_2-IsoPs) and malondialdehyde (MDA) concentrations in plasma (**A** and **B**, respectively). Protein oxidative damage estimated from the carbonyl groups (CGs) levels in plasma (**C**). NO bioavailability estimated from the contents of nitrite and nitros(yl)ated species (NO_2^-+RXNOs) in plasma (**D**). Samples were collected at the end of the supplementation study of Type 1 diabetic rats with a powdered seedless red wine pomace seasoning (RWPS). NS: non-supplemented; +RWPS: supplemented with RWPS during 4 weeks. Experimental rat groups: CN, control; CN+RWPS, control supplemented; DB, diabetic; DB+RWPS, diabetic supplemented. Data expressed as mean ± SEM values (n=5). Letters indicate significant variations (p<0.05) between the experimental groups.

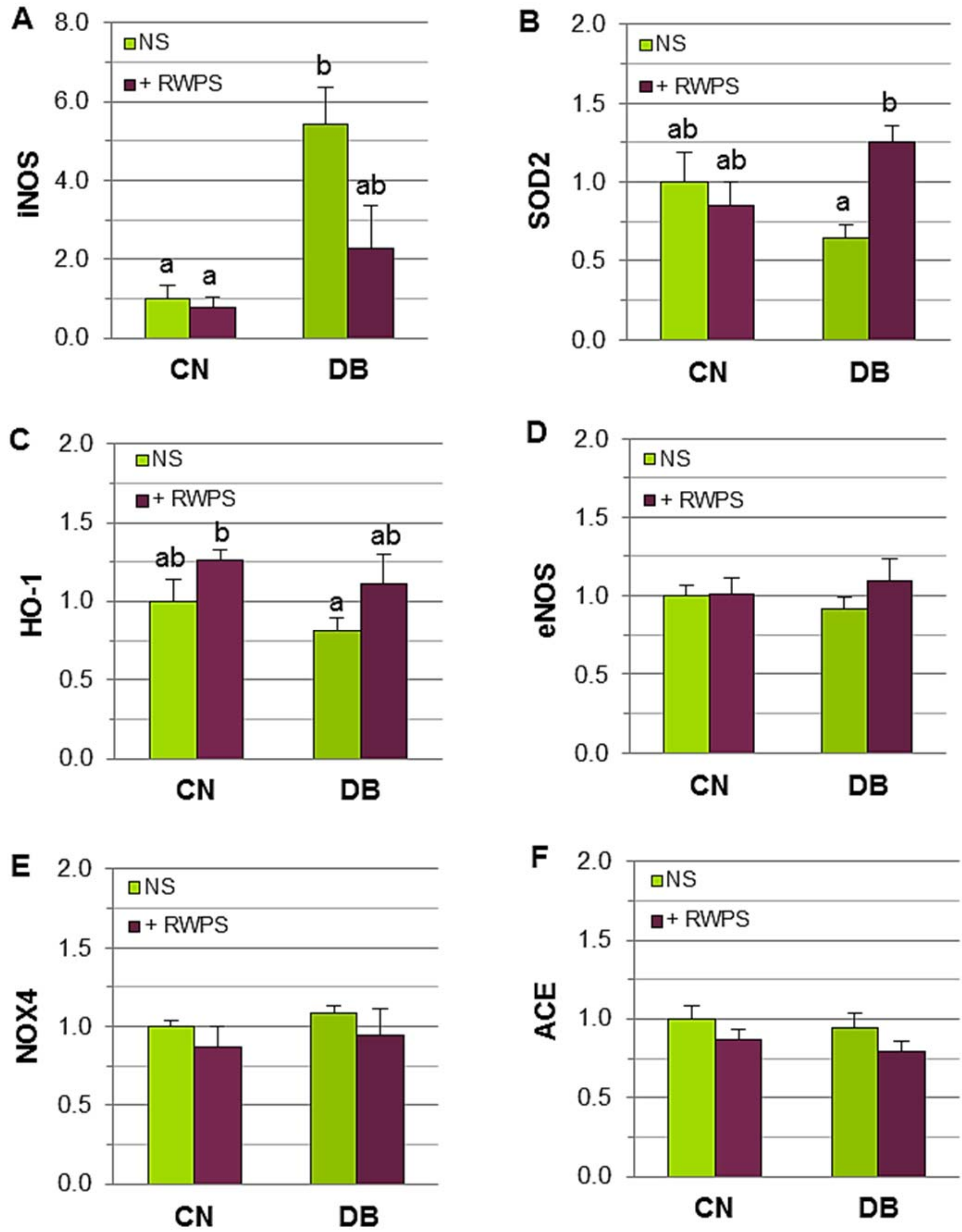

Figure 4. **Gene expression in the rat aorta.** Genes assessed: **A**) iNOS, **B**) SOD2, **C**) HO-1, **D**) eNOS, **E**) NOX4 and **E**) ACE. Thoracic aortic tissue was collected at the end of the supplementation study of Type 1 diabetic rats with a powdered seedless red wine pomace seasoning (RWPS). NS: non-supplemented; +RWPS: supplemented with RWPS during 4 weeks. Experimental rat groups: CN, control; CN+RWPS, control supplemented; DB, diabetic; DB+RWPS, diabetic supplemented. Target mRNA levels were determined by quantitative real-time PCR (qPCR) and normalized to the GAPDH gene expression. Results were represented as relative fold-change with respect to the control non-supplemented (CN) rats. Data expressed as mean ± SEM values (n=5). Letters indicate significant differences (*p*<0.05) between the experimental groups.

3.4. Biomarkers of lipid peroxidation, protein oxidation, and endothelial dysfunction

In plasma of rats collected at the end of the study, F_2-IsoPs concentration was approximately 25% lower ($p < 0.05$) in DB+RWPS than DB rats (**Figure 3**A). MDA levels were increased in the DB compared to the CN groups ($p < 0.001$) with these levels being 28% lower ($p < 0.05$) in DB+RWPS than in DB rats (**Figure 3**B). Similar trends were observed for the CG contents, being significantly higher in DB compared to CN rats, and more than 6% lower in DB+RWPS than in DB rats (**Figure 3**C; $p < 0.01$). The NO_2^-+RXNOs levels in plasma were used as a surrogate index of endothelial function in order to estimate vascular NO bioavailability. While there was a trend toward increased NO bioavailability in rats treated with RWPS, these differences were not statistically significant (**Figure 3**D).

3.5. Aortic gene expression

The relative mRNA levels of certain key genes involved in the endothelial function and the antioxidant defense system were determined in the rat thoracic aorta. iNOS mRNA levels were significantly increased in DB rats compared to CN rats, with intermediate levels between these groups being observed for their DB+RWPS counterparts (**Figure 5**A). Most important gene expression changes due to RWPS supplementation were detected for SOD2, as its mRNA levels were significantly higher in DB+RWPS compared with DB rats (**Figure 5**, B). Certain modulation of the HO-1 mRNA levels was also observed. A trend toward increased HO-1 gene expression due to RWPS supplementation was seen, but differences with respect to the non-supplemented counterparts were not significant (**Figure 5**C). Despite RWPS supplementation tended to increase eNOS mRNA levels in diabetic rats and down-regulate NOX4 and ACE gene expression in both normoglycemic and diabetic rats, no significant differences between the experimental groups were observed for these genes (**Figure 5**D, E, and F, respectively).

4. Discussion

Diabetes mellitus (DM) is not only a metabolic disorder but also considered as a vascular disease because of the injurious DM outcomes on macro- and microvascular vessels (Sena et al. 2013). The present study investigated the potential benefits of supplementing the diet of Type 1 DM rats with a powdered seasoning obtained from seedless red wine pomace (RWPS).

Uncontrolled hyperglycaemia caused by insulin deficiency is known to produce polyphagia (increased hunger), polydipsia (increased thirst), polyuria (frequent urination) and weight loss (Thrower & Bingley 2010; Kolluru et al. 2012). These classical Type 1 DM symptoms were clearly seen in the DB groups of this study, and were progressively ameliorated following treatment with RWPS. Fasting plasma glucose levels collected on the last day of the study

evidenced a facilitated glucose uptake in DB+RWPS compared to DB rats. This fact was supported by the plasma insulin measurements, which showed that impaired insulin secretion from β-cells, characteristic of Type 1 DM (Vehkavaara et al. 1999), was less marked in DB+RWPS rats. Therefore, RWPS supplementation substantially increased systemic insulin levels and normalized glucose homeostasis in diabetic rats.

These positive effects could be due to the high content of bioactive components present in RWPS (García-Lomillo et al. 2014), such as phenolic compounds and dietary fibre. RWPS is rich in anthocyanins, flavan-3-ols (oligomers and polymers), flavonols, and several phenolic acids (Del Pino-García et al. 2016d). Growing evidence indicates that numerous of these compounds can regulate carbohydrate metabolism and significantly reduce blood glucose (Hanhineva et al. 2010), showing β-cell protection (Sun et al. 2012), insulinomimetic activities (Pinent et al. 2004; Su et al. 2006), and increasing serum insulin concentration and tissue glucose uptake (Nizamutdinova et al. 2009). In addition, a number of potential anti-diabetic mechanisms of action for dietary fibre have been suggested, such as: beneficial effects on satiety and weight loss; reduction of macronutrients absorption and alteration of intestinal transit time, thus decreasing postprandial glycaemia; improvement of insulin sensitivity; and, healthy effects mediated by the SCFAs generated through colonic fermentation of indigestible dietary fibre (Giacco et al. 2002; Thomas & Pfeiffer 2012). Among SCFA, butyrate seems to exert primarily positive effects in the gastrointestinal tract (Hamer et al. 2008) and be responsible of numerous of the systemic helpful effects of dietary fibre in the management of diabetes (Puddu et al. 2014; Weickert & Pfeiffer 2008; Kumar et al. 2002), partly by enhancing β-cell proliferation and expression of insulin (Khan & Jena 2014). Therefore, the higher production of butyrate observed after 4 weeks of RWPS supplementation may have played a substantial role increasing plasma insulin contents and promoting the recovery of normal fasting blood glucose levels in DB+RWPS rats. Furthermore, higher plasma acetate concentration has been reported in diabetic compared to normal subjects due to impaired circulating glucose and non-esterified fatty acid metabolism (Todesco et al. 1993; Akanji et al. 1989), which may explain the trend towards increased faecal content of acetic acid observed in DB rats.

Hypocholesterolemic effects have been also reported in diabetic patients after consumption of soluble dietary fibre (Nuttall 1993) and grape pomace derived polyphenols (Pérez-Jiménez & Saura-Calixto 2008). Elevated low-density lipoprotein (LDL) cholesterol and oxidised LDLs, together with low HDL cholesterol, are considered important risk factors directly contributing to endothelial dysfunction (Sena et al. 2013), and DM is usually characterised by these blood lipid abnormalities (Erejuwa 2012). Therefore, the decrease in TC/HDL ratio observed in DB+RWPS rats demonstrated an improved lipoprotein cholesterol profile in diabetic rats after RWPS supplementation, thus providing evidence supporting the potential benefits of wine pomace-derived seasoning products to attenuate the risk of cardiovascular disease in DM.

The reduced antioxidant capacity in the vascular system typically observed in DM was corroborated in this study by the decreased plasma TAC detected in DB rats, in accordance with numerous previous studies (Rösen et al. 2001; Stirban et al. 2008; Vessby et al. 2002). Interestingly, the three different assays used to estimate the overall reducing (FC and FRAP) and free radical quenching (ABTS) capacities of plasma showed similar results for RWPS supplementation to significantly improve the oxidative environment observed under diabetic conditions. Although these assays of total antioxidant capacity are difficult to interpret, we also measured more specific markers of oxidative damage. F_2-IsoPs and MDA are both biomarkers of lipid peroxidation and have been suggested as main indicators of the oxidative damage associated with DM (Rahimi et al. 2005; Ho et al. 2013). The importance of F_2-IsoP concentrations should be noted, as they have been proposed to correlate with the impaired glycemic control and oxidative stress status in diabetic patients, as well as with the lipoprotein status (lower HDL and higher LDL levels) in hypercholesterolemic individuals (Mezzetti et al. 2000). Products of protein oxidation tend to accumulate in plasma and tissues of diabetic subjects, with heavily carbonylated proteins mediating further hyperglycaemia-induced detrimental effects (Jakus 2000; Sena et al. 2013; Stirban et al. 2014). The results of this study indicated a small but significant protective effect of RWPS supplementation on protein carbonyls in diabetic animals. These findings are in agreement with others, following administration of grape seed extracts in plasma (Chis et al. 2009) and kidneys (Mansouri et al. 2011), and of grape and red wine phenolic compounds such as resveratrol in plasma (Silan 2008), liver, and spleen (Chang et al. 2012) of diabetic rats.

The major manifestation of endothelial dysfunction is an impaired endothelium-dependent vasodilatation, which has been demonstrated in both clinical and experimental DM and is largely associated to a reduced NO bioavailability (Fatehi-Hassanabad et al. 2010; Kolluru et al. 2012; Sena et al. 2013). RWPS intake might positively affect endothelial function, as suggested by the trend toward higher plasma NO availability observed after 4 weeks of RWPS supplementation, especially under hyperglycemic conditions.

Changes in the mRNA levels of the NO generating enzymes expressed in the vasculature (eNOS and iNOS) were investigated in the rat aortic tissue. Overexpression of iNOS was detected in DB rats, results that agree with previous reports (Olukman et al. 2010; Nagareddy et al. 2005). Interestingly, iNOS mRNA levels tended to decrease after RWPS intake, probably due to the anti-inflammatory and antioxidant bioactivities of its polyphenols (Mukai et al. 2011; Dias et al. 2005) and the anti-inflammatory response associated to increased dietary fibre intake and SCFA production (Puddu et al. 2014). With regard to eNOS, reduced activity and/or expression of this constitutive NO generating enzyme have been proposed among causes that potentiate the diabetes-impaired NO biavailability (Kolluru et al. 2012; Sena et al. 2013), but its transcriptional and post-transcriptional modulation under hyperglycaemia is still unclear (Chatterjee & Catravas

2008). In contrast with other authors that have associated, at least in part, the increased NO production mediated by grape and wine derived polyphenols to their ability to up-regulate eNOS gene expression (Schmitt & Dirsch 2009), our results did not support a significant transcriptional modulation of this gene by RWPS supplementation. This finding, along with the fact that the increased iNOS expression under hyperglycemic conditions was not translated into a higher plasma NO content, suggest that NO might be produced in lower amounts in DB rats, which could be explained by eNOS uncoupling (Ceriello 2003; Kolluru et al. 2012) or by a decreased activation of this enzyme (Chatterjee & Catravas 2008). In addition, NO may be inactivated after its synthesis (with the subsequent formation of peroxynitrite) due to the augmented RONS levels under hyperglycaemia (El-Remessy et al. 2010; Kojda & Harrison 1999; Stirban et al. 2014).

Among mechanisms resulting in enhanced RONS production and oxidative stress status in DM, the activation of protein kinase C and the generation of advanced glycation endproducts play a central role, with NOXs being strongly implicated in both cases (Spitaler & Graier 2002; Fatehi-Hassanabad et al. 2010; Stirban et al. 2014). The dominant NOX isoform that controls basal levels of $O_2^{\cdot-}$ production in endothelial and smooth muscle cells is NOX4 (Gao & Mann 2009). The observed tendency towards NOX4 down-regulation in RWPS-supplemented rats was in line with previous studies in endothelial cells (Del Pino-García et al. 2016d) and with the effects detected in aortic tissue of STZ-induced diabetic rats after lignin-derived lignophenols intake (Mukai et al. 2011). The ability of grape derived polyphenols to inhibit NOX at the enzymatic level has also been described previously (Álvarez et al. 2012).

Oxidative stress and endothelial dysfunction in DM might be also promoted by activation of the renin-angiotensin aldosterone system due to the metabolic abnormalities associated with this disease (Hayashi et al. 2010). The tendency toward decreased ACE gene expression in the rats supplemented with RWPS, which could be prompted by its ACE inhibitory ability (Del Pino-García et al. 2016d), might favour the vascular protective effects of this seasoning.

Furthermore, a decline of certain endogenous antioxidant systems, such as SODs, catalase, and thioredoxin, has been described under diabetic conditions, thus resulting in decreased scavenging of RONS and increased oxidative damage (Fatehi-Hassanabad et al. 2010; Erejuwa 2012). Excess production of reactive species from mitochondria is highly implicated in DM and related complications because hyperglycaemia stimulates oxidative phosphorylation, causing $O_2^{\cdot-}$ overproduction (Brownlee 2005; Ceriello 2003). This free radical accumulates if its generation exceeds the dismutation capacity of SOD2, or if such capacity is diminished, as may occur in DM (Jakus 2000; Stirban et al. 2008). Interestingly, SOD2 was up-regulated in DB+RWPS rats compared to their non-supplemented counterparts. The relevance of this result should be pointed out as SOD2 overexpression has been found to reverse endothelial dysfunction in the aorta of diabetic animal models

(Zanetti et al. 2001) and reduce protein kinase C activation, advanced glycation endproduct formation, and nuclear factor kappa B activation in endothelial cells (Nishikawa et al. 2000). A tendency toward HO-1 up-regulation was also observed in RWPS-supplemented rats. As this important cytoprotective/antioxidant enzyme for the cardiovascular system seems to be impaired in DM (Ahmad et al. 2005), HO-1 inducers have been proposed as potent anti-diabetic agents (Tiwari & Ndisang 2014). Thus, the trends detected in the present study should be further explored, as the reinforcement of the endogenous antioxidant system may be an important mechanism by which RWPS mediates the observed anti-hyperglycemic and vascular protective effects.

In conclusion, this study suggests potential health effects of using RWPS as a dietary supplement for tackling Type 1 DM symptoms and associated vascular complications. Polyphagia, polydipsia, weight loss, fasting hyperglycaemia, hypoinsulinemia, atherogenic cholesterol profile and vascular oxidative damage observed in STZ-induced diabetic rats were significantly alleviated after supplementation with this seasoning derived from seedless wine pomace, rich in polyphenols and dietary fibre. Probable mechanisms underlying these anti-diabetic and antioxidant effects include a marked increase in plasma insulin levels and SOD2 gene overexpression, along with small transcriptional modulation of other key vascular enzymes. Accordingly, further research in healthy and diabetic subjects given RWPS as a dietary supplement, as well as incorporated as an ingredient of functional foods, is required to confirm the promising RWPS protective effects in the management of Type 1 DM.

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Conflict of interest

The authors have no competing financial interest.

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Chapter 3.3.

Antihypertensive and Antioxidant Effects of a Powdered Seasoning obtained from Red Wine Pomace in Spontaneously Hypertensive Rats

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Abstract

Purpose Hypertension is associated with enhanced vascular oxidative stress and impaired endothelial function, which is related to an imbalance between reactive oxygen species and nitric oxide bioavailability. Short-term supplementation with a polyphenol-rich powdered seasoning obtained from red wine pomace (RWPS) was investigated in a model of essential hypertension.

Methods Male spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto rats were administered a daily dose of RWPS (300 mg/kg) or vehicle by gavage.

Results In SHRs, RWPS supplementation progressively decreased blood pressure, reaching 11.5% reduction at the end of the study. RWPS consumption also increased the ferric reducing ability of plasma and attenuated oxidation of plasma lipids and proteins. Moreover, nitric oxide production was 1.5-fold higher in SHRs+RWPS compared to SHRs. RWPS-derived bioactive compounds modulated aortic gene expression, observing eNOS, SOD2 and HO-1 over-expression and ACE down-regulation.

Conclusion These results suggest the potential of wine pomace-derived seasonings to help in the management of hypertension.

Keywords: Endothelial dysfunction; Hypertension; Nitric oxide; Oxidative stress; Redox signaling; Wine pomace.

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Abbreviations and nomenclature: ACE, angiotensin I-converting enzyme; **Ang II**, angiotensin II; **AREs**, antioxidant response elements; **CGs**, carbonyl groups; **eNOS**, endothelial nitric oxide synthase; **F_2-IsoPs**, F_2-isoprostanes; **GC-NCI-MS**, gas chromatography/electron capture-negative chemical ionization mass spectrometry; **HO**, heme oxygenase; **MDA**, malondialdehyde; **NO**, nitric oxide; **NO_2^-**, nitrite; **NO_3^-**, nitrate; **NOX**, β-nicotinamide adenine dinucleotide phosphate oxidase; **ROS**, reactive oxygen species; **Nrf2**, nuclear factor-erythroid 2-related factor 2; **RAAS**, renin-angiotensin aldosterone system; **RXNOs**, nitros(yl)ated species; **RWPS**, red wine pomace seasoning; **sBP**, systolic blood pressure; **SHRs**, spontaneously hypertensive rats; **SOD**, superoxide dismutase; **TAC**, total antioxidant capacity; **WKY**, Wistar-Kyoto.

Highlights:

- Spontaneously hypertensive rats (SHRs) were given a red wine pomace seasoning (RWPS).
- Elevated blood pressure in SHRs decreased gradually along 4 weeks of RWPS consumption.
- Oxidative damage of vascular lipids and proteins was ameliorated in SHRs+RWPS.
- RWPS intake increased NO bioavailability, which may alleviate endothelial dysfunction.
- Enhanced eNOS, SOD2 and HO-1 and reduced ACE gene expression in aorta of SHRs+RWPS.

1. Introduction

Hypertension is characterised by a chronically elevated systemic arterial blood pressure and, as a major risk factor for cardiovascular diseases, represents the leading cause of death worldwide, especially in developed countries (Montezano & Touyz 2012).

Hypertension involves complex interactions between genetic, environmental, and demographic factors. Despite extensive research into the etiology of this multifactorial pathological condition, about 90-95% of human cases of hypertension are of unknown cause, which is referred to as 'essential' or 'primary' hypertension (Oparil et al. 2003). However, endothelial dysfunction and oxidative stress have been suggested as common and strong underlying factors in the physiopathology of hypertension (Cai & Harrison 2000). Endothelial dysfunction results from the imbalanced release of endothelium-derived relaxing and contracting factors, in favour of the latter (Félétou & Vanhoutte 2006), and oxidative stress occurs when there is a disproportion between the generation of reactive oxygen species (ROS) and the antioxidant defense systems, so that the latter become overwhelmed (Ogita & Liao 2004). As such, the increased ROS production and impaired nitric oxide (NO) bioavailability characteristic of endothelial dysfunction are associated with inadequate vasodilatation and elevated blood pressure (Montezano & Touyz 2012). In parallel, hypertension itself may contribute to increased oxidative stress in the vascular system, as evidenced by the increased plasma levels of oxidative stress markers being found in patients with essential and other types of hypertension (Touyz & Briones 2011). Therefore, strategies to improve endothelial dysfunction and vascular oxidative stress may result in decreased morbidity and mortality in hypertensive patients.

A large array of direct and indirect antioxidant biological actions are attributed to plant-derived phenolic compounds, such as free radical-scavenging, metal chelating, enzyme modulation, and regulation of cell signaling pathways and gene expression (Stoclet et al. 2004; Rodrigo et al. 2012). In the last decades, consumption of polyphenol containing foods has been associated with cardiovascular protection. In this context, several studies indicate that the "Mediterranean diet" and other specialized diets rich in fruit, vegetables, and low-fat dairy products, such as the DASH (Dietary Approaches to Stop Hypertension) diet and the DASH low-sodium diet, may prevent or ameliorate hypertension (Rodrigo et al. 2012).

There is currently a great interest in value adding of winemaking residues, especially of wine pomace, to obtain polyphenolic extracts and other products (Moure et al. 2001; Fontana et al. 2013). As such, our research group is studying the use of powdered products obtained directly from wine pomace, without extraction steps, as functional natural seasonings (González-Sanjosé et al. 2013). These red wine pomace seasonings (RWPSs) are rich in polyphenolic compounds, dietary fibre, and also have a high potassium/sodium ratio, especially those products enriched in wine pomace skins (García-Lomillo et al. 2014). We

hypothesize that developed seasonings may exert cardiovascular and antihypertensive beneficial actions when used as food ingredients or dietary supplements. The protective effects on endothelial function and oxidative stress markers of the seedless RWPS enriched have been recently evaluated *ex vivo* in endothelial cells. Modulation of the expression of genes involved in the endogenous antioxidant system and the ROS/NO balance and a decreased renin-angiotensin aldosterone system (RAAS) activity indicate probable vascular protective mechanisms (Del Pino-García et al. 2016d). However, the beneficial effects of wine pomace-derived products have not yet been assessed in hypertensive humans or animal models.

The aim of the present study was to investigate the potential of a powdered seasoning product obtained directly from red wine pomace (RWPS) to decrease blood pressure and improve endothelial dysfunction and oxidative stress biomarkers in a well-established rat model of essential hypertension.

2. Materials and Methods

2.1. Chemicals

Acetonitrile anhydrous (99,8%), N,O-*bis*(trimethylsilyl)trifuoroacetamide with 1% trimethylchlorosilane (BSTFA), butylated hydroxytoluene (BHT), bovine serum albumin (BSA), diethylene triamine pentaacetic acid (DTPA), N,N-diisopropylethylamine (DIPEA), 2,4-dinitrophenylhydrazine (DNFH), ethanol, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), glutathione (GSH), guanidine hydrochloride, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), iodine (I_2), isooctane, methanol, 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), phosphoric acid solution (H_3PO_4), potassium hydroxide (KOH), pyridine anhydrous (99,8%), potassium iodide (KI), sodium acetate, sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), sodium [^{15}N] nitrate, sodium [^{15}N] nitrite, 1,1,3,3-tetramethoxypropane (TMP), tetraoctylammonium bromide (TOA-Br), thiobarbituric acid (TBA), and 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Acetone, hexane, and glacial acetic acid were provided by Univar Australia, Pty. Ltd. (Ingleburn, NSW, Australia). 8-iso-$PGF_{2\alpha}$-d_4 (Cat. No. 316351), 8-iso-$PGF_{2\alpha}$ (Cat. No. 16350), and 8,12-iso-$iPF_{2\alpha}$-VI-d_{11} (Cat. No. 10006878) standards were purchased from Cayman Chemical, Co. (Ann Arbor, MI, USA). Certify II cartridges for solid phase extraction (SPE) were from Varian Australia, Pty. Ltd. (Mulgrave, VIC, Australia). Ferric(III)-chloride acid ($FeCl_3$), ferrous(II)-sulphate ($FeSO_4$), sodium acetate ($NaC_2H_3O_2$), and trichloroacetic acid (TCA) were obtained from Panreac Química, S.L.U. (Barcelona, Spain). Diazepam (Valium®) was purchased from Roche Diagnostics, S.L. (Barcelona, Spain). Atropine sulphate and heparin sodium injections (5,000 I.U./mL) were obtained from Grupo B. Braun España (Barcelona, Spain). Ketamine (Ketalar®) was purchased from Par Sterile Products, L.L.C. (Parsippany, NJ, USA).

2.2. Seedless red wine pomace seasoning (RWPS)

Red wine pomace from the winemaking of *Vitis vinífera* L. *cv. Tempranillo* was kindly supplied by several wineries situated in Burgos (Spain). Following the process indicated for the production of seasonings of vegetable origin (González-Sanjosé et al. 2013), dried wine pomace free of seeds, thus enriched in skins, was transformed into a powdered product (particle size < 0.250 mm) following a heat treatment at 90 ºC for 90 min in order to achieve the microbial stabilization degree required for its safety use as a food ingredient. The main characteristics, nutrient composition, polyphenol content and *in vitro* antioxidant capacity of the seedless red wine pomace seasoning (RWPS) have been described in previous works (García-Lomillo et al. 2014; Del Pino-García et al. 2015; Del Pino-García et al. 2016d).

2.3. Animals, experimental groups and general procedures

All procedures with rats were performed as authorized for scientific research (European Directive 2010/63/EU and Royal Decree 53/2013 of the Spanish Ministry of Agriculture, Food and Environment and Ministry of Economy and Competitiveness) and in accordance with the guidelines established by the Ethics Committee for Experimental Animal Care at the University Hospital of Burgos and the Ethics Committee at the University of Burgos (BU28U13-CEBA13/University Health Complex of Burgos).

In this study, 12-week-old male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) control rats weighing 307±12.0 g were assigned to 4 groups (n = 5 rats/group): normotensive (WKY), normotensive supplemented (WKY+RWPS), hypertensive (SHR), or hypertensive supplemented (SHR+RWPS) groups. The animals were maintained at 23 ºC with 12-h light/dark cycles and were given tap water and a standard diet (A04, Panlab, S.L.U., Barcelona, Spain) *ad libitum* during the experiment. Rats were supplemented with the studied RWPS (300 mg/kg body weight, dissolved in water) or vehicle (2 mL of water), which was orally administered by gavage between 9 and 10 a.m. Food and drink consumptions of rats were registered every day, and the body weight and arterial blood pressure were measured weekly. Two days before the end of the supplementation period, rats were placed in metabolic cages 8 hours post-administration of RWPS, and urine was collected during 5 hours. Aliquots of total urine collected were stored frozen at -80 ºC, without any preservative, until further analyses. The last day of the study, 12-hour fasting animals were anesthetized with intraperitoneal injection of ketamine HCl (20 mg/kg), diazepam (2 mg/kg) and atropine (0.05 mg/kg) and sacrificed to collect blood samples and the thoracic aortic tissue for the biochemical analyses. Blood samples were collected by cardiac puncture in Vacutainer® tubes (Becton Dickinson, Co., Madrid, Spain) containing sodium and lithium heparin (17 I.U./mL blood) as anticoagulant. In order to prevent oxidation of lipids on storage, those blood samples for F_2-IsoP and MDA analysis were collected in ice-cold tubes containing a mixture of GSH/BTH/EDTA, as previously described (Mori et al. 1999). All blood

samples were centrifuged at 1,500 g for 10 min at 4 ºC to obtain the plasma, which was then divided into aliquots and stored at -80 ºC until analyses. The aortic tissues were snap frozen in liquid nitrogen, and stored at -80 ºC for further analyses.

2.4. Systolic blood pressure (sBP) measurement

The sBP was recorded every week between 8-10 h post-administration of RWPS in conscious, pre-warmed, restrained rats by the tail-cuff plethysmographic method (Buñag 1973) using a LE 5002 Storage Pressure Meter (Panlab) and a pulse transducer for rats model LE5160R (Panlab). At least eight determinations were made on each rat in every session and the mean of the lowest five values within 10 mmHg was taken as the sBP level.

2.5. Plasma total antioxidant capacity (TAC) assessment

Plasma TAC was estimated as the ferric reducing ability of plasma (FRAP) according to the method previously described (Benzie & Strain 1996), which is based on the increase in absorbance at 595 nm of the Fe(II)-TPTZ complex in the presence of plasma reducing agents. A calibration curve was constructed with $FeSO_4$ aqueous solutions of known concentration and the FRAP value of each plasma sample was expressed as millimolar of Fe(II) equivalents (mM Fe(II)E).

2.6. F_2-isoprostanes (F_2-IsoPs) determinations

2.6.1. Plasma F_2-IsoPs levels

F_2-IsoPs concentration in plasma samples was measured by gas chromatography coupled with electron capture negative chemical ionization mass spectrometry (GC-NCI-MS), according to a previously reported method (Mori et al. 1999) with slight modifications. Briefly, 8-iso-$PGF_{2\alpha}$-d_4 (also known as 15-F_{2t}-IsoP-d_4) was used as internal standard (IS) and dissolved in methanol to obtain a 0.1 mg/L IS solution. Plasma samples (0.2 mL) were thawed and mixed with the IS solution (50 µL). Then, 1 M KOH in methanol (250 µL) was added to the samples, vortexed, and incubated at 40 ºC for 30 min. After acidification (pH 4.5–4.7) with 1 M HCl, samples were applied to SPE cartridges. The isolated F_2-IsoPs were then derivatized, dried under nitrogen, reconstituted in isooctane, and analyzed by GC-NCI-MS using an Agilent 6890N Network Gas Chromatograph (Agilent Technologies, Inc., Palo Alto, CA) coupled to an Agilent 5975B Series Mass Selective Detector (Agilent Technologies) and fitted with a DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies), as previously described (Mori et al. 1999). The F_2-IsoPs were detected by monitoring *m/z* 569 and the corresponding ion for the deuterium-labeled 8-iso-$PGF_{2\alpha}$ (*m/z* 573). Peak identification was based on comparison of retention times with standards. Quantification was performed using calibration curves obtained from a native 8-iso-$PGF_{2\alpha}$ standard. The plasma F_2-IsoPs final results were expressed as concentration values in nM.

2.6.2. Urinary F_2-IsoPs levels

Urinary F_2-IsoPs were also measured by GC- NCI-MS following the procedure outlined above for plasma samples, but using 8,12-iso-i$PF_{2\alpha}$-VI-d_{11} (*m/z* 580) as internal standard and excluding the addition of KOH and the incubation at 40 ºC steps prior to the acidification of samples. Creatinine concentration of each urine sample was analyzed at the Core Clinical Laboratory at Royal Perth Hospital using a Technicon Axon analyser (Bayer Diagnostics Aust., Scoresby, VIC, Australia). The urinary F_2-IsoPs final results were expressed as nmol/mmol creatinine.

2.7. Plasma malondialdehyde (MDA) concentration measurement

Plasma MDA levels were assessed according to the chromatographic method described by Grotto *et al*. (Grotto et al. 2007) to determine lipid peroxidation. An Agilent 1100 Series HPLC system (Agilent Technologies) equipped with a diode array detector and a Spherisorb3® ODS2 reversed phase C18 (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia, S.A., Barcelona, Spain) column were used. MDA concentration was calculated from calibration curves obtained using TMP as standard, and normalized using each plasma sample total protein concentration, which was determined by the Bradford method (Bradford 1976). The results were finally expressed as pmol MDA/mg protein.

2.8. Plasma protein carbonyl groups (CGs) concentration assessment

Protein CGs were quantified in plasma using the spectrophotometric method described by Levine et al. (Levine et al. 1990) to determine protein oxidative stress damage. The CGs levels were normalized by the protein concentration in plasma, expressing the final results as nmol CGs/mg protein.

2.9. Nitric oxide (NO) production determination

Due to the short life-span of NO in biological samples, indirect methods based on the quantification of NO breakdown stable products nitrite (NO_2^-), nitrate (NO_3^-) and nitros(yl)ated species (RXNOs) in biological samples were used as an approach to estimate endothelial and systemic NO production (Lee et al. 2012).

2.9.1. Plasma NO_2^- + RXNOs levels

The concentration of plasma NO_2^- and various RXNOs (S-nitrosothiols, N-nitrosamines, and nitrosylhemes) were quantified after their reductive cleavage using a mixture of iodine/tri-iodide in glacial acetic acid and subsequent detection of the NO released by gas-phase chemiluminescence following reaction with ozone, as previously described (Feelisch et al. 2002). Raw data from the chemiluminescence NO analyzer (Eco Physics-CLD 60, ANRI Instruments & Controls, Pty. Ltd., VIC, Australia) was processed using PowerChrom software. The area under the curve of NO peaks was calculated and the integration results were quantitated against $NaNO_2$ standard. The results were expressed as NO_2^-+RXNOs levels in µM.

2.9.2. Urinary NO_2^- and total NO_2^-+NO_3^- levels

Levels of NO_2^- and NO_3^- in urine were simultaneously analyzed using GC-NCI-MS, after the conversion of these NO metabolites to their stable pentafluorobenzyl derivatives directly from aqueous acetone medium, using TOA-Br as catalyst, as previously reported by Yang *et al*. (Yang et al. 2013). Analyses were carried out in an Agilent 6890N Network Gas Chromatograph (Agilent Technologies) coupled to an Agilent 5973B Series Mass Selective Detector (Agilent Technologies) and fitted with a DB-225MS column (30 m x 0.25 mm, 0.25 µm film thickness, Agilent Technologies). Peak identification was based on retention time and mass spectra compared with ^{15}N-labeled internal standards by selected-ion monitoring at *m/z* 46 (NO_2^-), 47 ($^{15}NO_2^-$), 62 (NO_3^-), and 63 ($^{15}NO_3^-$), and quantification of each NO metabolite was performed using calibration curves obtained from authentic NO_2^- and NO_3^- standards and labeled standards. The NO_2^- final results were expressed as µmol NO_2^-/mmol creatinine, and the total NO_2^- and NO_3^- results as µmol NO_2^-+NO_3^-/mmol creatinine.

2.10. Quantitative real-time polymerase chain reaction (qPCR) analysis

A quantitative real-time RT-PCR technique was used to analyze the mRNA expression in rat aortic tissue of the endothelial NO synthase (eNOS), mitochondrial superoxide dismutase (SOD2), heme oxygenase-1 (HO-1), angiotensin-I converting enzyme (ACE) and β-nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4). In brief, total RNA was isolated by the use of TRI Reagent solution (Applied Biosystems, Foster City, CA, USA), treated with DNAse I (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and reverse transcribed using the First Strand cDNA Synthesis kit (Thermo Fisher Scientific), following in all cases the manufacturer's instructions. Primers were designed using Primer3 v.0.4.0 software (Untergasser et al., 2012) and synthesised by Metabion (Metabion International, AG., Steinkirchen, Germany).The sequences of primer sets (forward and reverse) were:

- eNOS, 5'-TATTTGATGCTCGGGACTGC-3' and 5'-AACGAAGATTGCCTCGGTTT-3'
- SOD2, 5'-AAGGTCGCTTACAGATTGCC-3' and 5'-CTCCCACACATCAATCCCC-3'
- HO-1, 5'-GTGGCAGTGGGAATTTATGC-3' and 5'-AAGAGAGCCAGGCAAGATTC-3'
- ACE, 5'-TGGAGCATCTCTACCACCAA-3' and 5'-TGTATTTGTCCCCATAGCGG-3'
- NOX4, 5'-CAGAGGGAAAACAGTTGGAGT-3' and 5'-CCCATATGAGTTGTTCCGGT-3'
- GAPDH, 5'-ACTCCCTCAAGATTGTCAGC-3' and 5'-CTTCCACGATGCCAAAGTTG-3'

cDNA amplification was performed using iQ™ SYBR®Green Supermix (Bio-Rad Laboratories, S.A., Madrid, Spain) and the iCycler iQ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle of initial denaturing and enzyme activation at 95 °C for 3 min; 45 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The fidelity of qPCR amplification was verified by melting temperature analysis. Amplification efficiencies were determined by performing dilution series of a control aortic

tissue sample. The efficiency-corrected ΔΔCt method using the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference gene was applied to determine the relative expression of the rest of genes. Measurements were carried out in duplicate wells and repeated three separate times. Relative gene expression was finally expressed as fold-change with respect to the non-supplemented WKY group.

2.11. Data presentation and statistical analysis

All values are shown as mean ± standard error of the mean (SEM), with measurements for each rat conducted in duplicate. Statistical analyses were performed using Statgraphics® Centurion XVI software, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). Significant differences among experimental groups were evaluated by one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Food and water consumptions, body weight, and systolic blood pressure (sBP)

Daily food intake was similar in all groups and remained more or less constant during the 4 weeks of supplementation period (**Figure 1**A). Water consumption behavior in WKY groups was comparable and did not significantly change along the study. At the beginning of data recording, daily water intake in SHRs and SHRs+RWPS was around 1.5-fold higher compared to WKY rats. In the SHR group, this difference increased up across the weeks until reaching 1.7-fold higher water intake at week 4. As such, these rats drank a significant 13.9% enhanced volume of water than SHRs+RWPS at that time (**Figure 1**B). Supplementation with the powdered seasoning did not alter body weight during the study either in hypertensive or normotensive rats (**Figure 1**C). At the beginning of the study, the sBP in 12-week-old SHRs was around 2-fold higher with respect to their age-matched WKY counterparts, with this difference remaining steady in non-supplemented SHRs over the 4 weeks. However, a progressive decrease in the sBP of SHRs+RWPS was observed, which was significant at week 2 and reached an 11% decrease compared with SHRs at week 4 ($p < 0.001$), thus suggestive of the hypotensive effects of RWPS supplementation in cases of essential hypertension (**Figure 1**D).

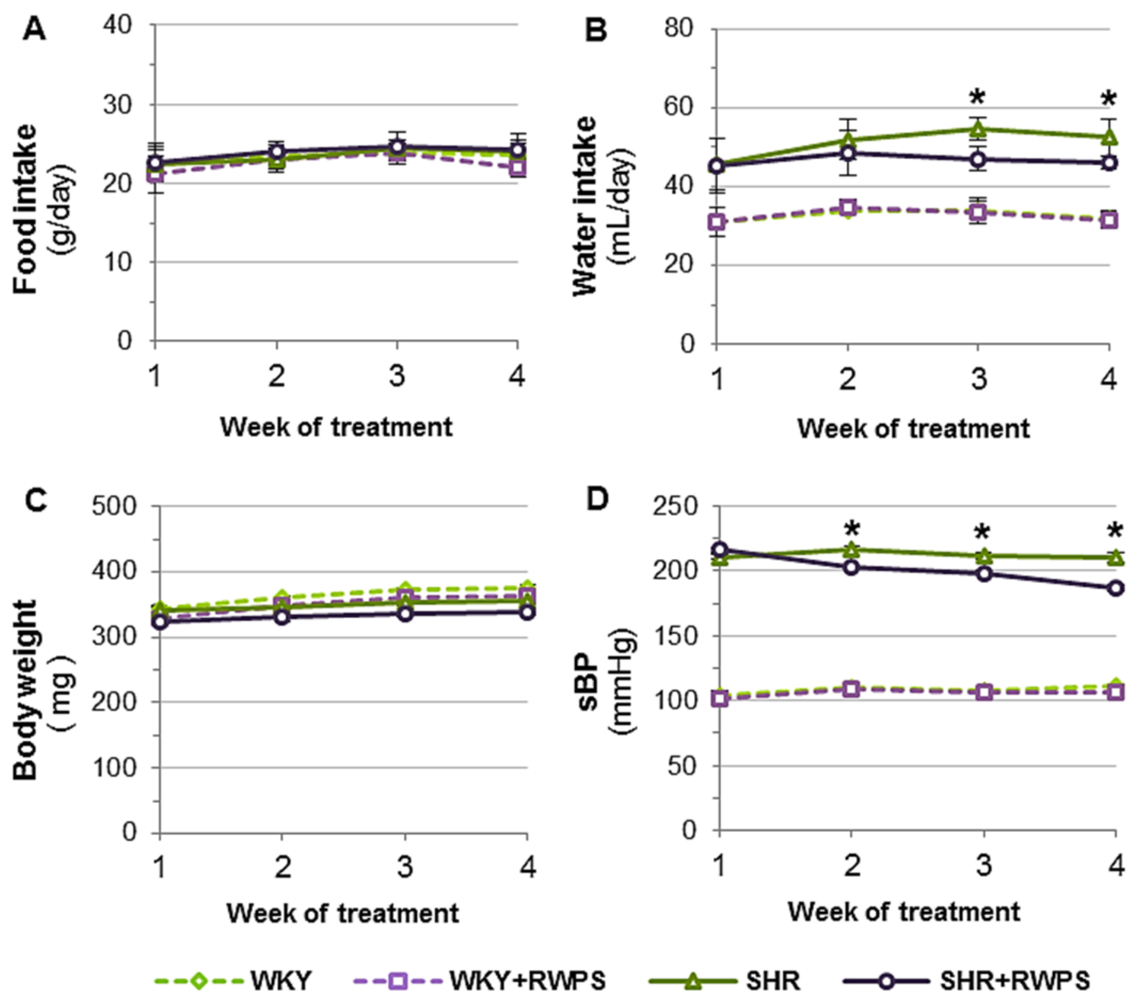

Figure 1. **Food and water intakes (A and B), body weight (C), and systolic blood pressure (sBP) (D) progression along the study using a powdered red wine pomace seasoning (RWPS) as a dietary supplement**. Experimental groups: WKY, normotensive rats; WKY+RWPS, normotensive supplemented rats; SHR, hypertensive rats; SHR+RWPS, hypertensive supplemented rats. Data expressed as mean ± SEM values (n=5). Asterisks (*) indicate significant differences (p<0.05) between SHR and SHR+RWPS.

3.2. Plasma TAC and biomarkers of oxidative damage to lipids and proteins

FRAP values of samples collected at the end of the study were around 31% significantly higher in SHRs+RWPS than SHRs, although neither of these plasma TAC results were significantly different from FRAP values obtained in the WKY groups (**Figure 2**A). As evidenced by the F_2-IsoPs and MDA concentration in plasma, a considerable protection against lipid oxidative damage was observed due to RWPS supplementation in hypertensive rats. Plasma F_2-IsoPs levels were approximately 37% higher in SHRs than SHRs+RWPS ($p < 0.05$), with levels of this lipid peroxidation biomarker in SHRs+RWPS being comparable to those in the WKY groups (**Figure 2**B). A similar trend was observed for the urine F_2-IsoPs concentration but did not reach statistical significance between the experimental groups (**Figure 2**C). Plasma MDA levels were 28% higher in SHRs than SHRs+RWPS ($p < 0.05$).

The concentration of MDA in plasma was also reduced in normotensive WKY animals treated with RWPS (**Figure 2**D). Plasma protein oxidative damage was around 5% higher in SHRs compared with WKY rats ($p < 0.05$), and slight non-significant decreases were detected in the protein CGs levels of both hypertensive and normotensive rats that consumed RWPS (**Figure 2**E).

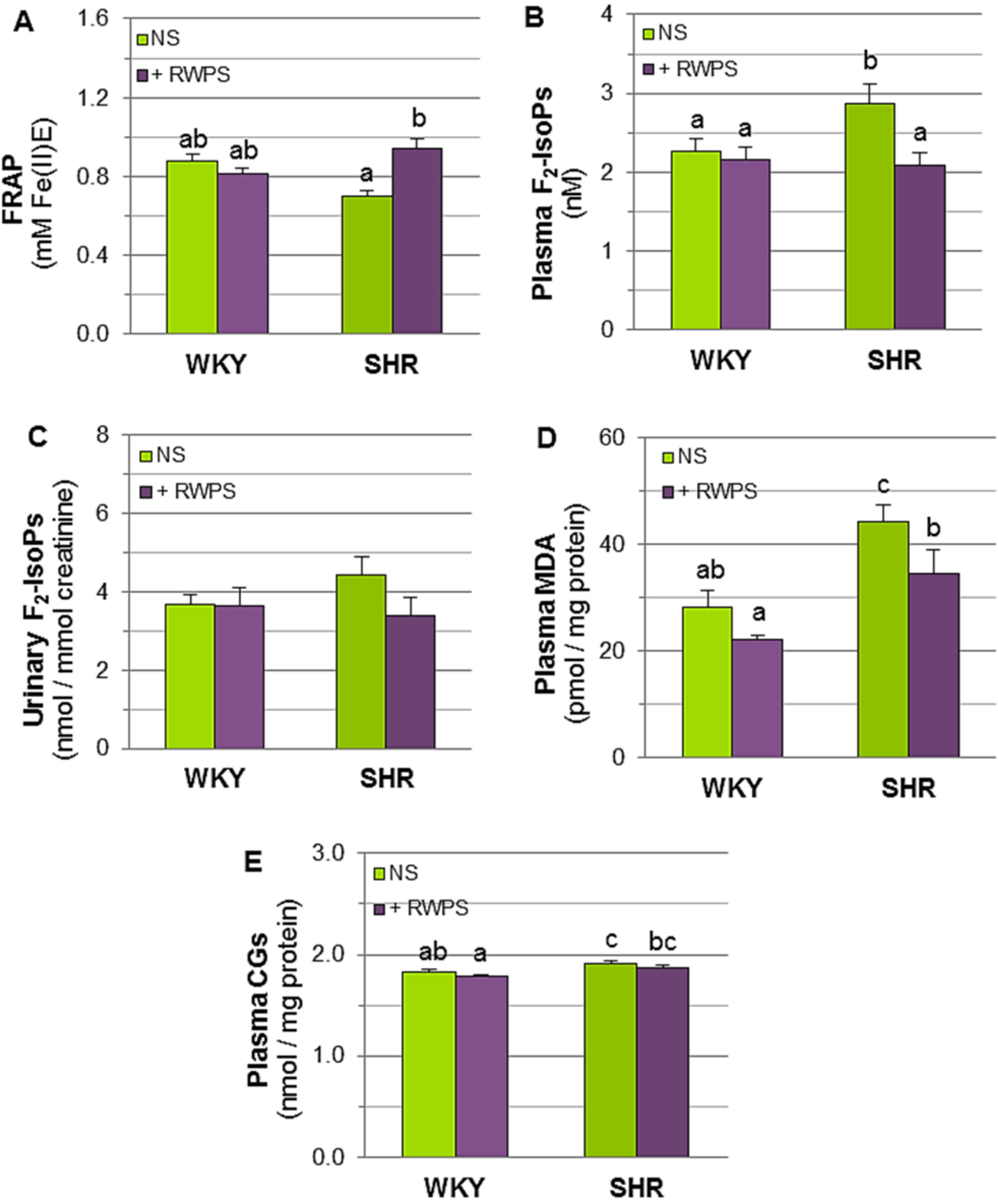

Figure 2. **Total antioxidant capacity (TAC) and biomarkers of lipid and protein oxidative damage in samples collected at the end of the study**. TAC estimated as the Ferric Reducing Capacity of Plasma (FRAP) (**A**); Lipid peroxidation estimated from the F_2-isoprostanes (F_2-IsoPs) levels in plasma (**B**) and urine (**C**), and malondialdehyde (MDA) concentration in plasma (**D**); Protein oxidative damage estimated from the carbonyl groups (CGs) levels in plasma (**E**). NS: non-supplemented; +RWPS: supplemented with a powdered red wine pomace seasoning during 4 weeks. Experimental groups: WKY, normotensive rats; WKY+RWPS, normotensive supplemented rats; SHR, hypertensive rats; SHR+RWPS, hypertensive supplemented rats. Data expressed as mean ± SEM values (n=5). Letters indicate significant variations (p<0.05) between the experimental groups.

3.3. Nitric oxide (NO) bioavailability

Plasma and urine samples collected after 4 weeks of supplementation with RWPS were assessed for NO content by measuring the concentration of plasma NO_2^-+RXNOs and urinary NO_2^- and total NO_2^-+NO_3^-. In plasma, NO_2^-+RXNOs levels were 36% lower in SHRs than in their WKY counterparts ($p < 0.05$). RWPS intake increased 1.4-fold the endothelial NO production in supplemented SHRs compared with the non-supplemented SHRs (**Figure 3**A). In urine, NO_2^- and NO_2^-+NO_3^- contents showed a similar response, with concentrations of these NO metabolites being around 50% higher in SHRs+RWPS than untreated SHRs ($p < 0.05$) (**Figure 3**B and C). RWPS supplementation had no effect on NO production in normotensive WKY rats. Together, these data indicate an increased NO bioavailability in hypertensive rats following 4 weeks of RWPS consumption.

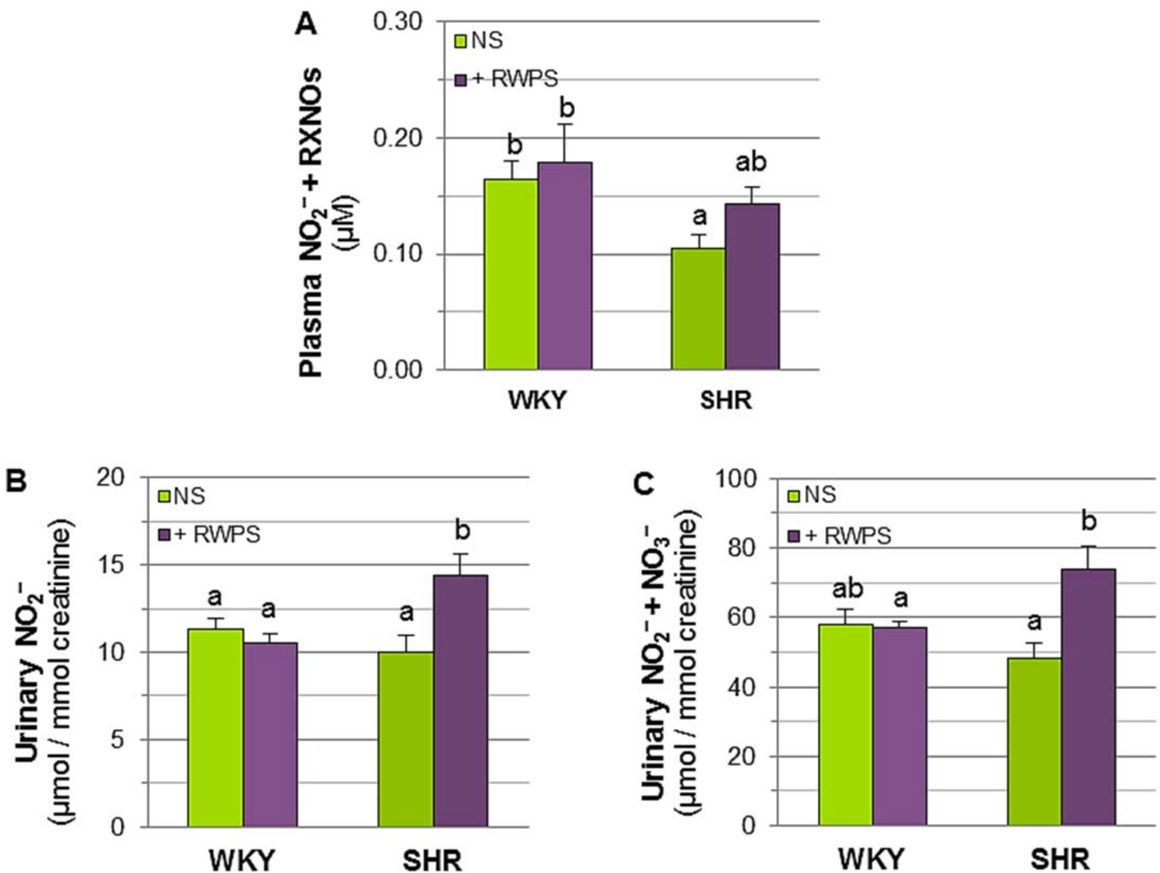

Figure 3. **Nitric oxide (NO) metabolites in samples collected at the end of the study**. Plasma nitrite and nitros(yl)ated species (NO_2^-+RXNOs) levels were used as an estimation of NO availability in the vascular system (**A**); Main NO breakdown products nitrite (NO_2^-) and nitrate (NO_3^-) excreted in urine were determined and represented as concentration of urinary NO_2^- (**B**) and both urinary NO_2^- and NO_3^- (**C**). NS: non-supplemented; +RWPS: supplemented with a powdered red wine pomace seasoning over 4 weeks. Experimental groups: WKY, normotensive rats; WKY+RWPS, normotensive supplemented rats; SHR, hypertensive rats; SHR+RWPS, hypertensive supplemented rats. Data expressed as mean ± SEM values (n=5). Letters indicate significant changes (p<0.05) between the experimental groups.

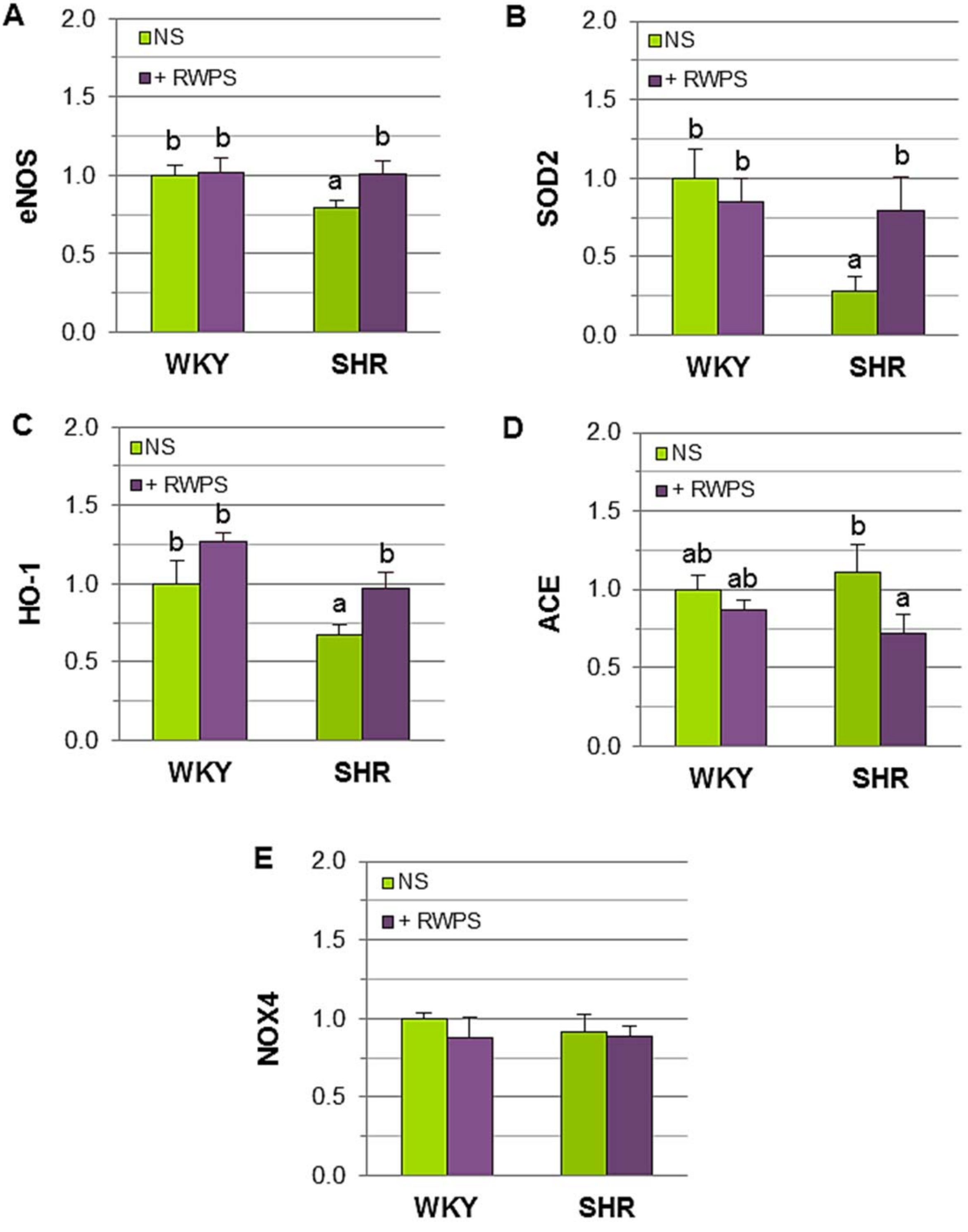

Figure 4. **Gene expression in the aortic tissue of rats.** Genes evaluated: **A**) eNOS, **B**) SOD2, **C**) HO-1, **D**) ACE and **E**) NOX4. NS: non-supplemented; +RWPS: supplemented with a powdered red wine pomace seasoning during 4 weeks. Experimental groups: WKY, normotensive rats; WKY+RWPS, normotensive supplemented rats; SHR, hypertensive rats; SHR+RWPS, hypertensive supplemented rats. Target mRNA levels were determined by quantitative real-time PCR (qPCR) and normalized to the GAPDH gene expression. Results were represented as relative fold-change with respect to the normotensive non-supplemented (WKY) rats. Data expressed as mean ± SEM values (n=5). Letters indicate significant differences ($p<0.05$) between the experimental groups.

3.4. Aortic gene expression

The relative aortic mRNA levels of several key genes implicated in endothelial function and the endogenous antioxidant system are shown in **Figure 4**. The results revealed a significant down-regulation of certain genes in SHRs, with their mRNA levels representing the 79.5% (eNOS), 28.2% (SOD2), and 67.4% (HO-1) of those found in WKY rats, whereas ACE gene expression tended to increase. RWPS treatment induced an increased expression of eNOS (1.3-fold), SOD2 (2.8-fold), and HO-1 genes (1.4-fold), together with an approximately 35% lower ACE gene expression under hypertensive conditions. No significant differences between WKY and WKY+RWPS rats were seen in any case. There was no effect on NOX4 gene expression among any of the experimental groups.

4. Discussion

Several lines of research have established associations between hypertension, endothelial dysfunction, and oxidative stress (Félétou & Vanhoutte 2006; Ogita & Liao 2004; Touyz & Briones 2011). There is some evidence that consumption of grapes, wine and derived products offer cardiovascular protection (Leifert & Abeywardena 2008; Pérez-Jiménez & Saura-Calixto 2008; Rodrigo et al. 2012; Guilford & Pezzuto 2011). The present suggests that a powdered seasoning product obtained directly from residual seedless red wine pomace (RWPS) has potential to reduce blood pressure and exert a beneficial vascular protection in a model of essential hypertension.

The diet of SHRs was supplemented for 4 weeks with RWPS (300 mg/kg) resulting in a significantly lower blood pressure compared to untreated SHRs. The US National High Blood Pressure Education program estimated that a 5 mmHg decrease of sBP in the population could result in high reductions in mortality due to cardiovascular disorders such as stroke (14%) or coronary heart disease (9%) (Chobanian et al. 2003). Therefore, the approximately 30 mmHg decrease of sBP observed in the SHR+RWPS group at the end of the study indicates promising results that support the hypotensive properties of the natural seasoning tested as a dietary supplement, although these findings need to be confirmed in humans.

The antihypertensive effects of grape and wine derived polyphenols have been demonstrated in hypertensive subjects and animal models (Feringa et al. 2011; López-Sepúlveda et al. 2008; Pérez-Jiménez & Saura-Calixto 2008; Quiñones et al. 2013; Sarr et al. 2006), with beneficial properties of these phytochemicals being attributed to different biological activities that result in a reduced oxidative stress damage (Duarte et al. 2001; Pérez-Jiménez et al. 2008) and endothelial dysfunction (Stoclet et al. 2004; Schini-Kerth et al. 2010), enhanced NO-mediated vasodilation (Török 2008; Barona et al. 2012; Galleano et al. 2013), and RAAS inhibition (Balasuriya & Rupasinghe 2011), among others

(Andriantsitohaina et al. 2012). The considerably high potassium levels and dietary fibre content of the studied powdered seasoning (García-Lomillo et al. 2014) may also have a positive influence in its hypotensive properties. An elevated potassium intake has been seen to lower blood pressure and even counteract the hypertensive effects of excess sodium consumption in humans and animals, including SHRs (Workman & Paller 1985; Zicha & Kunes 1999). Soluble dietary fibre intake is mainly related with hypocholesterolemic effects, but its hypotensive potential has been also suggested (Pérez-Jiménez et al. 2008).

SHR is an animal model with increased activation of the brain sympathetic system and enhanced centrally release of angiotensin II (Ang II), which is related to a higher blood pressure and concomitant increased water intake (Szczepańska-Sadowska 1996). Lower water consumption was observed in SHRs+RWPS compared with SHRs from the third week of supplementation, which was almost simultaneous with the blood pressure reduction detected. This fact suggested a decreased RAAS activation (due to lower ROS levels) or inhibitory interactions with some of the RAAS components as possible direct or indirect mechanisms of action of the seasoning bioactive compounds leading to the decreased water intake in SHRs+RWPS.

Hypertension is associated with enhanced vascular ROS production and oxidative damage (Touyz & Briones 2011). A decreased plasma TAC has been observed in patients with cardiovascular disease and hypertension (Montezano & Touyz 2012), although TAC assessment is a non-specific tool for evaluating the effects of antioxidant therapeutics strategies in the vascular system (Lee et al. 2012). In our study, RWPS supplementation increased plasma FRAP suggesting improved oxidative stress status in hypertensive rats. These results agree with previous observations in hypertensive animal models reporting higher plasma TAC values after supplementation with grapes or wine, which has been generally associated with their polyphenolic content (Cook & Samman 1996; Tsang et al. 2005; Pérez-Jiménez & Saura-Calixto 2008). However, these measures are unspecific and difficult to interpret and we therefore analyzed more specific biomarkers of oxidative damage (Griffiths et al. 2002; Lee et al. 2012).

In line with previous studies in hypertensive patients and animal models (Bhatt et al. 2011; Duarte et al. 2001; Jiménez et al. 2007; López-Sepúlveda et al. 2008; Simic et al. 2006; Török 2008), levels of plasma F_2-IsoPs, MDA and CGs were increased in SHRs compared with WKY rats. Interestingly, a decrease in the oxidative damage to lipids and proteins was observed in hypertensive rats after 4 weeks of RWPS intake, especially regarding markers of lipid peroxidation. F_2-IsoPs are the biomarkers of choice for endogenous oxidative stress assessment, but levels of these stable end-products of lipid peroxidation may not be equivalent in different body fluids, so measurements in both plasma and urine have previously been suggested (Halliwell & Lee 2010). In the current study, results obtained in both types of biological samples showed a similar trend, although differences between

experimental groups were less marked in urine. It should be noted that F_2-IsoPs participate as mediators of oxidant injury and have other biological effects, including vasoconstriction actions and platelet activation (Montuschi et al. 2004). As such, strategies that reduce lipid peroxidation have a great beneficial potential in hypertension and other cardiovascular diseases (Davies & Roberts 2011; Duarte et al. 2001).

Nitric oxide (NO) is one of the most important regulatory molecule in the vasculature, with a reduced NO bioavailability being a hallmark of the impaired vasodilatation characteristic of hypertension (Heiss et al. 2006; Kleinbongard et al. 2006). Our study evidenced decreased NO levels in SHRs compared to normotensive WKY rats. The impaired NO production observed in SHR was in line with previous studies (Félétou & Vanhoutte 2006). Reduced NO-mediated vasodilatation in other hypertensive animal models and patients has been also described, but it is still not clear whether this is mainly due to a decreased NO synthesis or an enhanced NO consumption through its interaction with excess superoxide anion (Chatterjee & Catravas 2008; Montezano & Touyz 2012; Montezano & Touyz 2011). Interestingly, an increase in NO bioavailability following RWPS supplementation was observed in hypertensive rats. It is also possible that diet-derived NO_3^- intake may influence the values of NO metabolites measured in biological samples (Lee et al. 2012; Rocha et al. 2011). Nonetheless, grapes are known to contain low levels of NO_3^- (in the range of 0.3 mg/kg) (Susin et al. 2006), so RWPS-supplemented rats were unlikely to be greatly affected by the NO_3^- contents of the seasoning itself. Our results thereby suggest that a higher endogenous NO production and/or decreased NO inactivation were responsible of the increased levels in NO breakdown products observed in SHRs+RWPS.

Among the number of variables that determine endothelial NO generation and its physiological action (Sessa 2004), eNOS expression is known to be subject to significant degrees of transcriptional and post-transcriptional regulation (Searles 2006). In this study, decreased eNOS mRNA levels were observed in the aorta of SHRs once hypertension was established compared to age-matched WKY counterparts. However, eNOS gene expression was restored in SHRs+RWPS to similar levels found in normotensive rats. This is an interesting finding considering that an increased eNOS transcription has been proposed as an effective strategy to ameliorate hypertension (Yang et al. 2011). In agreement, emerging evidence indicates that many dietary polyphenols, including grape and wine derived anthocyanins and flavanol-3-ol oligomeric condensed tannins, cause an enhanced endothelial NO synthesis and endothelium-dependent relaxation in isolated arteries by increasing eNOS activity and/or expression (Quiñones et al. 2014; Andriambeloson et al. 1998; Mendes et al. 2003; Schini-Kerth et al. 2010).

NOXs are primary sources of ROS in the vascular wall (Brandes et al. 2010). NOX4 gene over-expression was found in basilar arteries of SHRs versus WKY rats (Paravicini et al. 2004) and we have also recently observed that NOX4 is up-regulated under hyperglycaemia-induced

endothelial dysfunction in HUVECs, whereas the exposure of cells to the seasoning-derived treatments causes down-regulation of this ROS generating enzyme (Del Pino-García et al. 2016d). However, the current study showed that NOX4 mRNA levels were not significantly modulated in the aorta of SHRs compared with their WKY counterparts, either in RWPS-supplemented or non-supplemented rats. Similar findings were previously reported in vascular cells co-incubated with Ang II and anthocyanin metabolites (Edwards et al. 2015).

Elevated levels of Ang II are involved in many forms of hypertension (Hsueh & Wyne 2011). In fact, a vicious cycle for RAAS over-activation due to Ang II-induced gene up-regulation of several components of this system, including ACE, has previously been suggested (Shiota et al. 1992; Dzau 2001). We observed a trend toward enhanced aortic ACE gene expression in SHRs, whereas 4-week intake of RWPS induced a decrease in the mRNA levels of this gene. Thus, our results suggest the potential of RWPS consumption to alter the positive feedback for ACE up-regulation in essential hypertension, which was in line with previous studies in SHRs fed with fruit juices rich in phenolic compounds (Kivimäki et al. 2012). The hypotensive effects of flavonoids are also partly due to their direct ACE inhibition capacity (Balasuriya & Rupasinghe 2011) and subsequent lower Ang II generation, which could further explain the decreased ACE gene expression observed post-supplementation with the polyphenol-rich RWPS.

Modulation of redox-sensitive transcription factors that contribute to high blood pressure (Zinkevich & Gutterman 2011; Montezano & Touyz 2012), as well as stimulation of others implicated in the antioxidant defense response, such as the nuclear factor-erythroid 2-related factor 2 (Nrf2), have been proposed as useful strategies to alleviate ROS accumulation and protect against oxidative stress and endothelial dysfunction in hypertension (Paravicini & Touyz 2006; Majzunova et al. 2013). In this regard, SOD2 and HO-1are antioxidant enzymes whose gene transcription is particularly responsive to oxidative challenges and antioxidant stimulus.

In the mitochondria, SOD2 rapidly dismutates superoxide anions to hydrogen peroxide, thus protecting against superoxide mediated-cytotoxicity and preventing NO degradation by reaction with this ROS (Faraci & Didion 2004). Our results revealed a significant depletion of SOD2 mRNA levels in the aorta of SHRs, whereas RWPS intake restored this gene expression to comparable levels as in WKY groups. Over-expression of SOD2 is known to reduce oxidative stress in mitochondria, attenuate Ang II-induced endothelial dysfunction, improve NO bioavailability, and promote antihypertensive effects (Dikalova et al. 2011; Faraci & Didion 2004; Majzunova et al. 2013; Higashi et al. 2009). In line with these results, several polyphenols have been shown to up-regulate the expression/activity of SODs as a mechanism to exert cardiovascular benefits (Andriantsitohaina et al. 2012; Bhatt et al. 2011).

HO-1 has a pivotal protective role in the vasculature due to its indirect antioxidant effects through breakdown of a potent oxidant (free heme) and the subsequent formation of molecules with antioxidant properties (biliverdin and bilirubin) and carbon monoxide, which is a vasodilatory gas. As such, HO-1 may play an important role in the regulation of vascular function and blood pressure (Chen et al. 2003). The transcription of HO-1 gene is induced by Nrf2 binding to the antioxidant response element (ARE) sequences contained in the promoter region. Nrf2 is known to induce the transcription of ARE genes during acute oxidative insults, but increasing evidence indicates that the Nrf2/ARE signaling pathway can be attenuated following long term oxidative stress, as may occur in hypertension (Majzunova et al. 2013). In this regard, inconsistent results have been reported about HO-1 expression in different rat and human tissues in the hypertensive state, with levels of HO-1 protein found enhanced (Ishizaka et al. 1997) or decreased (Ndisang & Wang 2003). In the present study, HO-1 gene was significantly down-regulated in SHRs compared with normotensive WKY rats, whereas its expression was up-regulated following RWPS consumption. This is in agreement with previous studies showing improved NO bioavailability and vascular function in mice supplemented with quercetin (Shen et al. 2013) and ellagic acid (Ding et al. 2014) which was critically related to the arterial induction of HO-1. Certain anthocyanin metabolites also demonstrated their ability to enhance HO-1 gene expression in cultured vascular cells ñ, thus alleviating oxidative stress and conferring endothelial protection.

In conclusion, this study provides evidence that consumption of a powdered seasoning obtained from seedless red wine pomace (RWPS) reduces arterial blood pressure, ameliorates oxidative damage to lipids and proteins, and increases NO bioavailability in SHRs. Such results support the vasorelaxing and protective potential of this seasoning against endothelial dysfunction associated with essential hypertension. Restoration of eNOS, SOD2 and HO-1 gene expression, in combination with ACE down-regulation, were both seen to be involved in the hypotensive and vascular protective effects of RWPS supplementation.

Therefore, our findings evidence the promising healthful properties of RWPS intake in the management of hypertension, which strongly supports the probable benefits of using this product of vegetable origin as a natural dietary supplement and as a food ingredient, especially convenient for low-salt diets and foods. This study lays the ground work for future human clinical studies with this wine pomace value-added alternative.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

Chemopreventive Potential against Colorectal Cancer of the Seasonings

CHAPTER 4:

CHEMOPREVENTIVE POTENTIAL AGAINST COLORECTAL CANCER OF THE SEASONINGS

In this chapter, the *ex vivo* studies aimed at achieving the last objective of this PhD Thesis are presented: assessing the potential of the seasonings as chemopreventive agents against colorectal cancer.

Colorectal cancer is the third most common cancer worldwide in males, and the second in females. An estimated 1.4 million cases and nearly 700,000 deaths occurred in 2012, showing the highest incidence rates in developed countries (Torre et al. 2015).

Colorectal cancer is causally related to both genes and environment, with diet playing a key role among the environmental factors. This high dietary influence on colorectal cancer is associated with the contribution of free radicals in its aetiology (Babbs 1990) and its pathogenesis (Perše 2013), as there are several pro-inflammatory and pro-oxidant dietary factors which, together with free radicals derived from the intestinal microbiota metabolism, lead to increased oxidative stress in the colonic microenvironment and inside the colonocytes themselves. Large amounts of generated RONS cause oxidative damage to DNA, lipids and proteins of these cells, contributing to the onset of tumour changes (Hussain et al. 2003; Itzkowitz & Yio 2004). In the later stages, cancer cells have RONS production and elimination rates higher than normal cells, thus achieving their redox homeostasis under conditions that stimulate cell proliferation and inhibit apoptosis (Glasauer & Chandel 2014). This pro-oxidant environment within cells contributes to cause further damage to biomolecules and to the progression of cancer (Valko et al. 2006; Perše 2013).

Colorectal carcinogenesis is a multistage process, beginning with a small dysplasia or polyp (Stage 0, according to the ‘Astler and Coller classification’) and eventually leading to the appearance of distant metastases (Stage IV) (Ballinger & Anggianasah 2007). Thus, there are a number of possible stages at which the process can be slowed down, arrested, or even reversed, with chemopreventive strategies showing a great potential to achieve such goals (**Figure 42**).

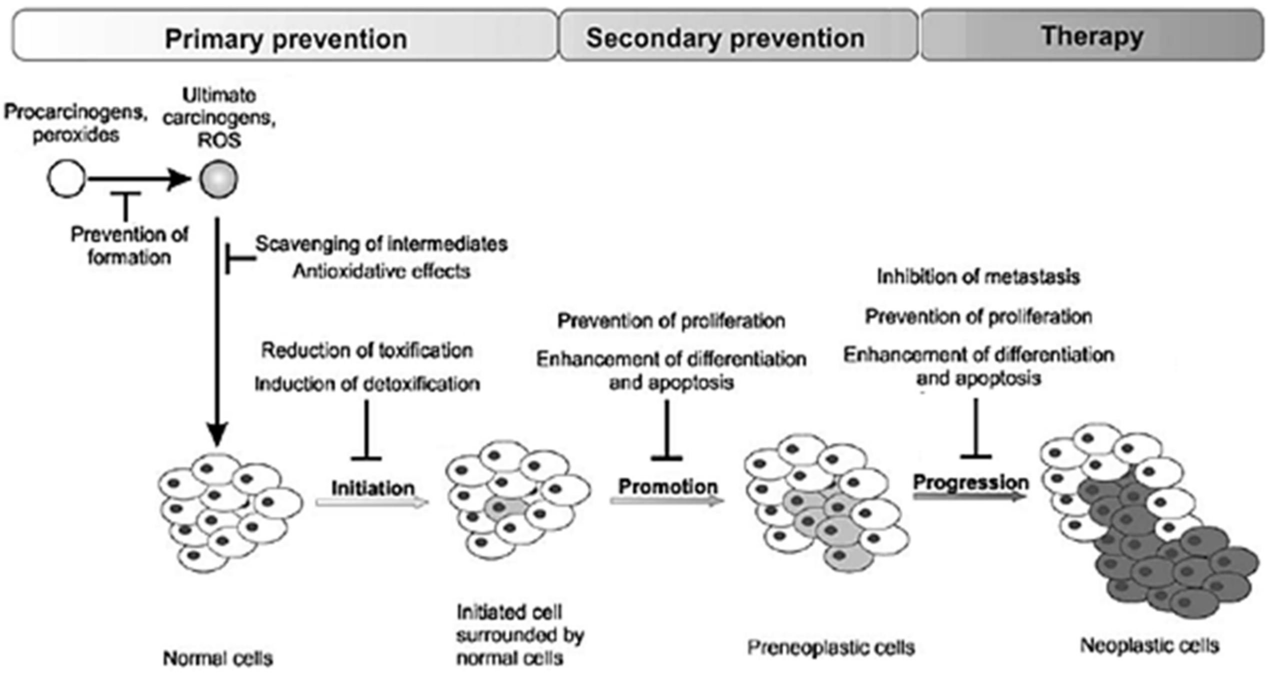

Figure 42. **Different phases of chemoprevention**

Adapted from Scharlau et al. (2009).

Chemoprevention strategies can be divided in three types (Scharlau et al. 2009; Mehta et al. 2010; Steward & Brown 2013):

- **Primary chemoprevention**: It involves inhibition of tumour initiation, either by reducing toxicity or by inducing detoxification mechanisms. This may be achieved through several mechanisms, such as: preventing the formation of ultimate RONS and carcinogens, inducing DNA repair mechanisms, and direct and/or indirect antioxidant effects.
Agents that act in this manner are called 'tumour-blocking agents'.

- **Secondary chemoprevention**: It consists in inhibition of the promotion of initiated cells to preneoplastic cells, for instance, by reducing cell growth and/or increasing differentiation and apoptosis in initiated cells. Typically, these effects involve modulation of signal transduction pathways that control such cellular processes.
Agents acting in this way are termed 'tumour-supressing agents'.

- **Tertiary chemoprevention**: It refers to the blockage of the progression of preneoplastic cells to neoplastic cells, which occurs in comparably late during carcinogenesis.
This type of chemoprevention includes therapeutic approaches.

Among dietary compounds with chemopreventive potential at the gastrointestinal level, dietary fibre and phenolic compounds have received great attention, especially in primary and secondary chemoprevention (Scheppach et al. 1995; Russo 2007; Ramos 2008).

Given the important antioxidant capacities of the fractions which may be present in the colon after the intake of the three types of seasonings (*Chapter 2.1*), all of them were initially evaluated (Sk-S, W-S, y Sd-S). The contents in some of their bioactive compounds that could contribute to the chemopreventive potential of these fractions were first determined, using chromatographic techniques to evaluate their concentration in phenolic acids (HPLC-DAD) and SCFAs (GC-FID).

Next, several *ex vivo* assays were carried out in the cell line HT-29, one of the most widely used as a model of human colon adenocarcinoma. This cell line is characterised by showing mutations in p53, one of the key transcription factors regulating cell cycle and apoptosis that is also mutated in many cases of clinical colorectal cancer.

The tumour-suppressive potential of the seasonings and their digested fractions was evaluated using different concentrations of the treatments. From these studies, a non-cytotoxic concentration of 200 µg of fraction/mL of medium was used to evaluate the potential of the seasonings as anticancer blocking agents against genotoxic compounds. This concentration can be considered physiologically relevant, since in all cases would be < 5 mg GAE/mL of medium (value estimated from the results of their Folin-Ciocalteu index) and previous studies have estimated that a concentration of phenolic compounds < 50 mg GAE/mL would be achievable doses in the colonic contents (Brown et al. 2012).

The protective effects of the seasoning derived from seedless wine pomace against endogenous and exogenous oxidative stress were further evaluated, analysing the possible indirect tumour-blocking mechanisms involved. Specifically, the regulation of the transcription factors NF-KB and Nrf2 and some of their target antioxidant genes was assessed (**Figure 43**).

The oxidative stress-inducing exogenous agents used were:

- Menadione: a quinone that induces single- and double-strand DNA breaks in a RONS-dependent way, as it induces the production of $^{\bullet}$OH within cells (Nutter et al. 1992). Hence, this compound was employed as a genotoxic agent.
- *tert*-Butylhydroperoxide (*t*-BOOH): an organic peroxide which decomposes in other alkoxy and peroxyl radicals through a reaction mediated by transition metals, generating RONS that accelerate lipid peroxidation and other cellular processes that increase intracellular oxidative stress (Goya et al. 2009).

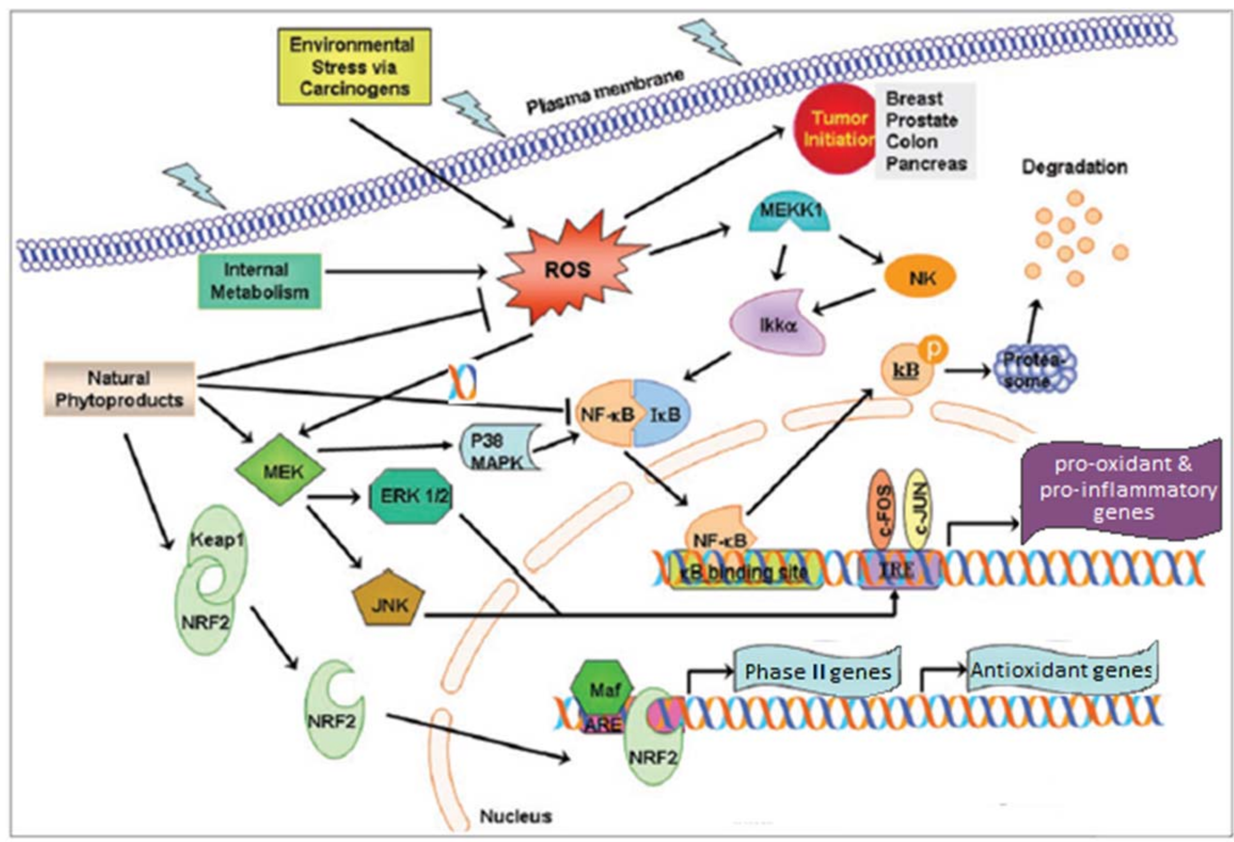

Figure 43. **A putative model for oxidative stress induced interactions among signalling pathways mediated by NF-κB and Nrf2 in carcinogenesis**.

Adapted from Acharya et al. (2010).

The main **conclusions** of these studies were:

- **Chapter 4.1**: All the seasonings showed an interesting chemopreventive potential against colorectal cancer, being able to act both as tumour-suppressing agents through their anti-proliferative effects, and as tumour-blocking agents given their anti-genotoxic effects against putative exogenous pro-oxidant agents. The digestive process did not affect the overall activity exhibited by bioactive compounds derived from Sk-S, which was the most effective prior to digestion, while in Sd-S a gradual improvement of its chemopreventive properties throughout the process digestive was observed, so that all the seasonings could have similar chemopreventive potential in the colon after their intake.

- **Chapter 4.2**: The seasoning derived from seedless wine pomace reduced lipid peroxidation detected in colorectal cancer cells under basal conditions, and was able to protect against the oxidation of biomolecules (lipids and proteins) and cell membranes after the exposure of cells to an exogenous pro-oxidant insult. The treatments were able to modulate the transcription factors NF-kB and Nrf2 and several of their target genes encoding antioxidant enzymes. However, it was difficult to establish specific mechanisms of protection, likely due to the involvement of other signalling pathways and enzymes in the observed effects. Nonetheless, the results suggest the chemopreventive potential of the seasoning under study as a blocking agent against colorectal cancer through its antioxidant effects.

CAPÍTULO 4:

POTENCIAL QUIMIOPREVENTIVO DE LOS SAZONADORES FRENTE AL CANCER COLORRECTAL

En este capítulo se presentan los estudios *ex vivo* dirigidos a la consecución del último de los objetivos de esta Tesis Doctoral: evaluar en el potencial de los sazonadores como agentes quimiopreventivos frente al cáncer colorrectal.

A nivel mundial, el cáncer colorrectal es el tercero más común en hombres y el segundo en mujeres. Se estima que aproximadamente 1,4 millones de casos y 700.000 muertes tuvieron lugar en 2012, observándose las mayores tasas de mortalidad en los países desarrollados (Torre et al. 2015).

Las causas que dan lugar a este cáncer están asociadas tanto a factores genéticos como ambientales, jugando la dieta un papel fundamental entre los ambientales. Esta alta influencia de la dieta en el cáncer colorrectal está asociada a la contribución de los radicales libres en su etiología (Babbs 1990) y su patogénesis (Perše 2013), ya que son varios los factores dietéticos pro-inflamatorios y pro-oxidantes que, junto con los radicales libres derivados del metabolismo de la microbiota intestinal, dan lugar a un incremento del estrés oxidativo en el microambiente colónico y en el interior de los propios colonocitos. Las grandes cantidades de RONS generados provocan daños oxidativos en el ADN, lípidos y proteínas de estas células, contribuyendo al inicio de los cambios tumorales (Hussain et al. 2003; Itzkowitz & Yio 2004). En las últimas etapas, las células cancerosas presentan tasas de producción y eliminación de RONS más elevadas que las células normales, consiguiendo así alcanzar su homeostasis bajo condiciones redox que estimulan la proliferación celular e inhiben la apoptosis (Glasauer & Chandel 2014). Este ambiente pro-oxidante en el interior celular contribuye a provocar un mayor daño a biomoléculas y a la progresión del cáncer (Valko et al. 2006; Perše 2013).

La carcinogénesis colorrectal comprende varias etapas, comenzando con una pequeña displasia o pólipo (*Estadio 0,* según la “clasificación de Astler y Coller”) y, finalmente, pudiendo llegar a provocar metástasis a distancia (*Estadio IV*) (Ballinger & Anggianasah 2007). Por ello, el proceso puede ser ralentizado, parado, o incluso invertido, en distintas etapas, presentando las estrategias quimiopreventivas un gran potencial para lograr dichos objetivos (**Figura 42**).

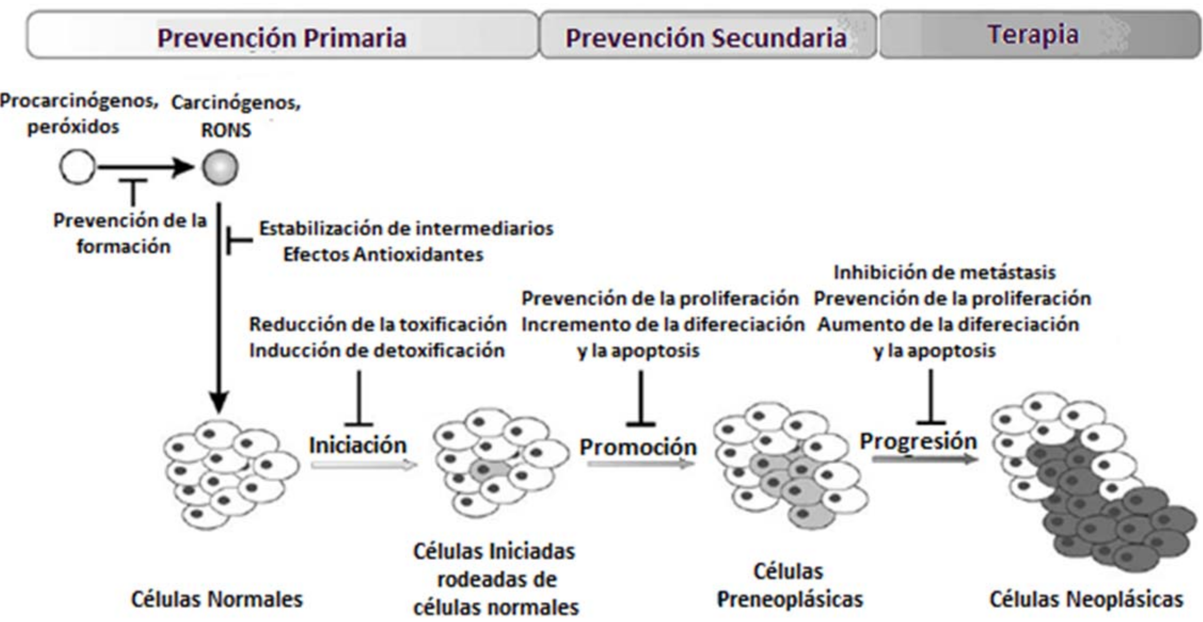

Figura 42. **Diferentes etapas de la quimioprevención.**
Adaptado de Scharlau et al. (2009).

Las estrategias de quimioprevención se puedan dividir por lo tanto en tres tipos (Scharlau et al. 2009; Mehta et al. 2010; Steward & Brown 2013):

- **Quimioprevención primaria**: implica la inhibición de la iniciación del tumor, bien reduciendo la toxicidad o induciendo mecanismos de detoxificación. Esto puede conseguirse, por ejemplo, mediante la prevención de la formación de RONS y carcinógenos, la inducción de mecanismos de reparación del DNA, y a través de efectos antioxidantes directos o indirectos.
  Los agentes que actúan de esta manera se denominan "agentes bloqueantes del tumor”.

- **Quimioprevención secundaria**: consiste en la inhibición de la promoción de las células del estado iniciado al preneoplásico, por ejemplo, mediante la reducción de crecimiento celular y/o incremento de la diferenciación y la apoptosis en las células iniciadas. Normalmente, estos efectos implican la modulación de las vías de transducción de señales que controlan dichos procesos celulares.
  Los agentes que actúan de esta forma se denominan "agentes supresores del tumor”.

- **Quimioprevención terciaria**: se refiere a la obstrucción de la progresión de las células del estado preneoplásico al neoplásico, que se produce en las últimas etapas de la carcinogénesis. Esto tipo de quimioprevención incluye los enfoques terapéuticos.

Entre los componentes ingeridos en la dieta con potencial quimiopreventivo a nivel gastrointestinal, la fibra alimentaria y los compuestos fenólicos han recibido una gran atención, principalmente en la quimioprevención primaria y secundaria (Scheppach et al. 1995; Russo 2007; Ramos 2008).

Teniendo en cuenta la importante capacidad antioxidante de las fracciones que pueden estar presentes en el colon tras la ingesta de los tres tipos de sazonadores (*Capítulo 2.1*), inicialmente fueron evaluados todos ellos (Sk-S, W-S, y Sd-S). En primer lugar se evaluó el contenido de algunos de los compuestos bioactivos que podrían contribuir al potencial quimiopreventivo de dichas fracciones, evaluando mediante técnicas cromatográficas su contenido en ácidos fenólicos (HPLC-DAD) y ácidos grasos de cadena corta (GC-FID).

A continuación, se llevaron a cabo ensayos en la línea celular HT-29, una de las más empleadas como modelo de adenocarcinoma de colon humano. Esta línea celular se caracteriza por presentar mutaciones en p53, uno de los principales factores de transcripción de regulación del ciclo celular y la apoptosis que está mutado en muchos casos de pacientes con cáncer colorrectal.

El potencial supresor de tumores de los sazonadores y sus fracciones digeridas se evaluó utilizando diferentes concentraciones de los tratamientos. A partir de estos estudios, se seleccionó una concentración no citotóxica de 200 µg de fracción/mL de medio de cultivo estudió para evaluar el potencial de los sazonadores como agentes anticancerígenos bloqueantes frente a compuestos genotóxicos. Esta concentración puede considerarse fisiológicamente relevante, ya que en todos los casos sería < 5 µg GAE/mL de medio (valor estimado a partir de sus resultados en el ensayo de Folin-Ciocalteau) y estudios previos han estimado que una concentración de compuestos fenólicos de < 50 µg GAE/mL serían dosis alcanzables en los contenidos colónicos (Brown et al. 2012).

Los efectos protectores del sazonador derivado de los hollejos frente al estrés oxidativo endógeno y exógeno fueron evaluados más a fondo, analizando los posibles mecanismos indirectos bloqueantes de tumores implicados. En concreto, se evaluó la regulación de los factores de transcripción NF-κB and Nrf2 y algunos de sus genes antioxidantes diana (**Figura 43**).

Como agentes inductores de estrés oxidativo exógeno se utilizó:

- Menadiona: una quinona que induce rupturas simples o dobles en las cadenas del DNA de manera dependiente de la generación de RONS, al inducir la producción de $^{\cdot}OH$ en las células (Nutter et al. 1992). Este compuesto fue por tanto empleado como agente genotóxico.
- *tert*-Butil hidroperóxido (*t*-BOOH): un peróxido orgánico que se descompone en otros radicales alcoxi y peroxilo a través de una reacción mediada por metales de transición, generando RONS que aceleran la peroxidación lipídica y otros procesos celulares que incrementan el estrés oxidativo intracelular (Goya et al. 2009).

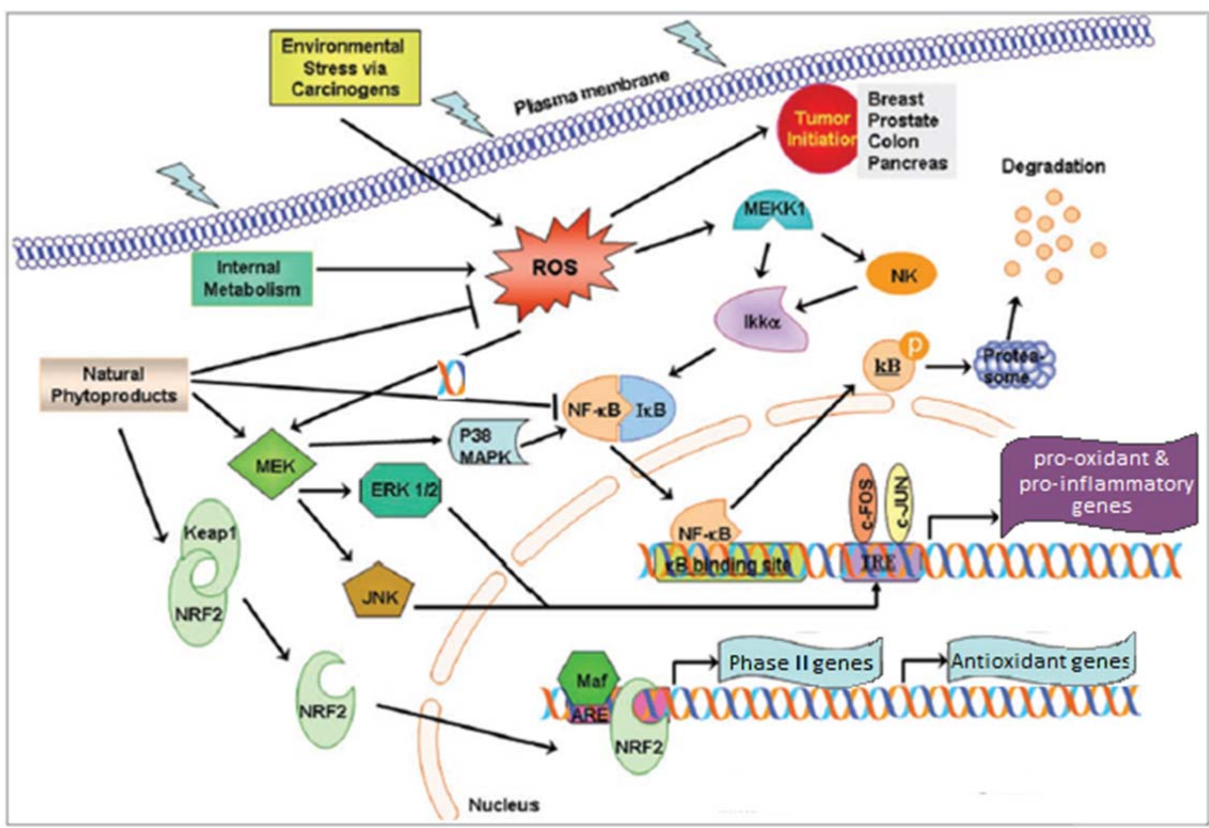

Figura 43. **Posible modelo de las interacciones entre las vías de señalización mediadas por NF-κB and Nrf2 inducidas por el estrés oxidativo durante la carcinogénesis**.
Adaptado de Acharya et al. (2010).

Las principales **conclusiones** de los estudios realizados fueron:

- **<u>Capítulo 4.1</u>**: Todos los sazonadores presentaron un interesante potencial quimiopreventivo frente al cáncer colorrectal, pudiendo actuar como agentes supresores de tumores mediante sus efectos anti-proliferativos, y como agentes bloqueantes de tumores gracias a sus efectos anti-genotóxicos frente a posibles agentes oxidantes exógenos. El proceso digestivo no afectó a la actividad global exhibida por los compuestos bioactivos derivados de Sk-S, que fue el más efectivo antes de su digestión, mientras que en Sd-S se observó una mejora gradual de sus propiedades quimiopreventivas a lo largo del proceso digestivo, de forma que todos los sazonadores podrían presentar un potencial quimiopreventivo similar en el colon tras su ingesta.

- **<u>Capítulo 4.2</u>**: El sazonador derivado de los hollejos redujo la peroxidación lipídica detectada en células de cáncer colorrectal bajo condiciones basales, y fue capaz de proteger frente la oxidación de biomoléculas (lípidos y proteínas) y de las membranas celulares al exponer las células a un agente pro-oxidante exógeno. Los tratamientos fueron capaces de modular los factores de transcripción NF-κB y Nrf2, así como varios de sus genes diana que codifican enzimas antioxidantes. Sin embargo, fue difícil establecer mecanismos específicos de protección, siendo probable la implicación de otras vías se señalización y enzimas en los efectos observados. Aun así, estos resultados sugirieren el potencial quimiopreventivo del sazonador estudiado como agente bloqueante frente al cáncer colorrectal a través de sus efectos antioxidantes.

Chapter 4.1.

Potential of Red Wine Pomace Seasonings as Chemopreventive Agents through the Anti-proliferative and Anti-genotoxic Effects of its Bioaccessible Fractions in Colon Cancerous Cells

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Abstract

Intake of grapes and grape derived products has attracted prominent attention in terms of gastrointestinal health and chemoprevention of colorectal cancer. This study evaluates the putative anti-proliferative and anti-genotoxic actions of new seasonings obtained from different parts of red wine pomace (Sk-S: seedless, W-S: whole, Sd-S: seeds) which are rich in polyphenols and dietary fibre. The influence of the digestive process in the bioaccessibility and bioactivities of these seasonings until reaching the colon were considered, obtaining *in vitro* gastrointestinal digested and colonic fermented fractions. Their characterization showed that phenolic acids from Sk-S were the most bioaccessible in the small intestine, but higher quantities of these phenolics may be present in the microenvironment of colonocytes after intake of Sd-S, followed by W-S. Sk-S was the best substrate for production of short chain fatty acids by colonic microbiota, both quantitatively and qualitatively due to the increased relative production of butyrate. The treatment concentration that inhibited 50% colon cancerous cell viability (IC_{50} values) ranged from 845 µg/mL (Sk-S) to 1085 µg/mL (Sd-S) prior digestion, while all the digested fractions showed similar anti-proliferative activities (mean IC_{50}=814 µg/mL), suggesting the cancer suppressing potential of the seasonings once in the colon. Moreover, all the seasonings and derived digested fractions pre-incubated at non-cytotoxic concentrations (200 µg/mL of medium) for 24 h were able to attenuate oxidative DNA damage both in their absence and in their presence during an oxidative insult. The anti-genotoxic activity of the digested fractions compared to the undigested seasonings did not change for Sk-S but increased for Sd-S, with all digested fractions showing rather similar genoprotective actions in the colon. In conclusion, this study evidences the potential of red wine pomace seasonings as chemopreventive agents in colorectal cancer through their anti-proliferative and anti-genotoxic effects.

Keywords: Anti-proliferative activity; Chemoprevention; Colorectal cancer; Genoprotection; Oxidative stress; Wine pomace.

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Abbreviations and nomenclature: **CF**, colonic fermented; **GIDD**, gastrointestinal digested+dialyzed; **HT-29**, human colorectal adenocarcinoma; **IC_{50}**, concentration of each treatment that inhibited 50% of cell viability; **RONS**, reactive oxygen/nitrogen species; **RWPS**, red wine pomace seasoning; **SCFAs**, short chain fatty acids; **Sd-S**, seasoning obtained from the seeds isolated from wine pomace; **Sk-S**, seasoning obtained from seedless red wine pomace, in which grape skins are the main component; **UD**, undigested; **W-S**, seasoning obtained from whole red wine pomace.

Highlights:

- Seasonings obtained from wine pomace (Sk-S: seedless, W-S: whole, Sd-S: seeds).
- RWPS are sources of phenolic acids and short chain fatty acids in the colon.
- High chemopreventive potential of Sk-S in colon cancer pre- and post-digestion.
- W-S and Sd-S might exhibit higher anti-proliferative action post-digestion.
- Anti-genotoxic effects of W-S and chiefly of Sd-S may be promoted by digestion.

1. Introduction

Oxidative stress is undoubtedly associated with colorectal cancer. The colonic epithelium is greatly expose to several oxidants incorporated with the diet and reactive oxygen/nitrogen species (RONS) generated in faecal materials, which has been suggested to play a significant role in the aetiology of colon cancer (Babbs 1990; Hague et al. 1993). In addition, inflammatory cells and cancer cells themselves overproduce free radicals and soluble mediators which lead to further RONS production. All the exogenous and endogenous deleterious reactive species generated can directly oxidize DNA and interfere with mechanisms of DNA repair, triggering DNA chain breaks, base modification, and other oxidative DNA lesions (Federico et al. 2007; Valko et al. 2006; Oliva et al. 1997). Permanent alteration of genetic material as a result of such oxidative damage is involved in mutagenesis, carcinogenesis, and ageing (Khansari et al. 2009).

In this framework, attenuation of RONS-induced DNA damage is included as a first line of defence against carcinogenesis as it could prevent the initiation phase of this multistage process (primary cancer prevention). In addition, other important anti-cancer mechanisms consist on inhibiting cancerous cell proliferation, which could arrest cell promotion and progression (secondary cancer prevention) (Manson et al. 2000; Landis-Piwowar & Iyer 2014). These effects can be achieved by the chronic administration of synthetic, natural or biological agents to reduce or delay the occurrence of cell malignancy, strategy that is known as cancer chemoprevention (Steward & Brown 2013).

Numerous studies support the beneficial effects of some dietary constituents as chemopreventive agents against colorectal cancer. Consistent evidence indicates that high intake of dietary fibre from fruit, vegetables and whole grains is inversely associated with the risk of this type of cancer (Murphy et al. 2012). Such benefits have been partly attributed to the production of short chain fatty acids (SCFAs) in the large intestine during fibre fermentation (Scheppach et al. 1995; Topping & Clifton 2001). Also the presence of significant amounts of phenolic antioxidants in fruits and vegetables is believed to largely contribute to their chemopreventive effects in the colon (Araújo et al. 2011; Donaldson 2004; Ramos 2008; Santiago-Arteche et al. 2012).

Previous research in polyphenol-rich extracts from wines and grapes has demonstrated the ability of their phytochemicals to modulate colonocyte mutagenesis and prevent tumour initiation and promotion (Dolara et al. 2005; Duthie & Dobson 1999; Sgambato et al. 2001). Beneficial effects of wine pomace upon the large intestinal mucosa have been also suggested (López-Oliva et al. 2006; López-Oliva et al. 2010) and mainly related to the high content of both polyphenolic molecules and dietary fibre in this winemaking residue (Saura-Calixto 2011; Zhu et al. 2015).

Recently, new seasonings obtained directly from different parts of red wine pomace have been developed, avoiding any extractive processes during its manufacture. These powdered red wine pomace seasonings (RWPSs) represent an innovative strategy to reduce/replace salt added to foods while increasing the intake of dietary fibre and natural antioxidants, mainly polyphenols (García-Lomillo et al. 2014). Such high contents in sources of bioactive compounds further suggest possible health benefits of RWPSs in the gastrointestinal tract.

On the basis of these considerations, the major aim of this study was to investigate the potential of different RWPSs in chemoprevention by assessing their anti-proliferative and anti-genotoxic effects against oxidative stress in human colorectal adenocarcinoma (HT-29) cells, taking into account the biaccessibility and metabolism of the RWPS polyphenols and dietary fibre along the gut, until reaching the colon.

2. Materials and Methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), calcium chloride dihydrate ($CaCl_2.2H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), L-cysteine hydrochloride, hydrochloric acid (HCl), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), Low-Melting-point agarose, Normal-Melting-point agarose, menadione, Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Tritton X-100, magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), 10,000 U/mL penicillin and 100 mg/mL streptomycin solution (P/S), phosphoric acid solution (H_3PO_4), porcine bile extract, porcine pancreas pancreatin, potassium chloride (KCl), resazurin sodium salt, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), sodium-lauryl-sarcosineate, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), sodium sulphide nonahydrate ($Na_2S.9H_2O$), thiazolyl blue tetrazolium bromide (MTT), Tris hydrochloride (Tris), tryptone, enzymes used in enzymatic digestion (α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1)), pure phenolic acid standards (caffeic acid, caftaric acid, coutaric acid, ellagic acid, ethyl gallate, fertaric acid, ferulic acid, gallic acid, *p*-coumaric acid, *p*-OH-benzoic acid, protocatechuic acid, salicylic acid, syringic acid, and vallinic acid), analytical standard solutions of SCFAs (acetic acid, butyric acid, propionic acid, and valeric acid) and cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), and sodium carbonate (Na_2CO_3) were purchased from Panreac Química S.L.U. (Barcelona, Spain). Dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) tablets were purchased from Merck (Darmastadt, Germany).

2.2. Red wine pomace seasonings (RWPSs)

The seasonings used in this study were made in the pilot plant of the Food Technology Department of University of Burgos (Spain) as previously described (García-Lomillo et al. 2014). Three different types of powdered seasonings were obtained, which were labelled as W-S, representing the seasonings obtained from whole red wine pomace; Sk-S, referring to the seasonings obtained from seedless red wine pomace, in which grape skins are the main component; Sd-S, corresponding to the seasonings obtained from the previously isolated seeds.

2.3. *In vitro* gastrointestinal digestion and colonic fermentation of the seasonings

Simulated complete digestion of the three RWPSs (Sk-S, W-S, and Sd-S) was performed according to the method describe by Saura-Calixto et al. (2007), with the modifications indicated in Del-Pino García et al. (2016b). This *in vitro* static digestion model allows the estimation of the bioaccessibility of dietary antioxidants, and mainly comprises two successive stages, an enzymatic gastrointestinal digestion first phase, followed by a colonic microbial fermentation phase. Briefly, each powdered seasoning, labelled as 'undigested' (UD), was successively incubated with digestive enzymes, as described in detail by Saura-Calixto et al. (2007). The gastrointestinal digested fraction was centrifuged (3,000 g, 15 min, 25 ºC) to separate the supernatant and the solid residue, repeating this step twice (washing the residue with 5 mL of Milli-Q water). The solid residue was stored at 4 ºC while all the supernatants were combined, transferred into cellulose membrane dialysis tubing, and dialyzed against a total of 2 L of water for 24 h (changing the water twice). The dialysis retentate was mixed with the previously-stored solid residue to obtain the so-called 'gastrointestinal digested+dialyzed' (GIDD) fraction. This fraction was the substrate for the action of colonic microbiota during colonic fermentation, obtaining the 'colonic fermented' (CF) fraction. The inoculums containing colonic microbiota were obtained at the animal-housing unit of the University Hospital of Burgos (Spain) by mixing the caecal content from 5 male Wistar rats (body weight of 250 ± 5 g) fed with standard maintenance diet. All aspects of this procedure were conducted in accordance with the guidelines established by the Ethics Committee at both the University Hospital of Burgos and the University of Burgos. GIDD and CF fractions were lyophilized, weighed, and stored at -20 ºC until their analysis. For each type of RWPS, three replicates were performed for each fraction. Negative digested controls (without seasoning) for both types of fractions were also prepared.

2.4. Identification and quantification of phenolic acids (HPLC-DAD analysis)

The contents of several phenolic acids were determined using reverse-phase HPLC-DAD analysis, according to the method reported by Pérez-Magariño et al. (2008). The analyses were performed in MeOH:HCl 37% (97:3, v/v) extracts of the RWPSs, obtained as previously

described (Del Pino-García et al. 2016a) and in the digested fractions derived from the seasonings, which were filtered through 0.45 µm filters before being injected in an Agilent 1100 series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) coupled to a diode array detector (DAD) and a Spherisorb3® ODS2 reversed phase C18 column (250 mm x 4.6 mm, 3 µm particle size; Waters Cromatografia S.A., Barcelona, Spain).Samples were injected in duplicate, and calibration was performed from solutions of the standards at five different concentrations, which were injected in triplicate. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards of each phenolic acid. The results were expressed in ug/g of fraction.

2.5. Identification and quantification of short chain fatty acids (SCFAs) (GC-FID analysis)

The concentration of butyric, propionic and acetic acids in the CF fractions were measured following a previously described method (Zhao et al. 2006) slightly modified to perform it directly in the CF fractions. Briefly, these fractions were centrifuged for 5 min at 5,000 rpm, giving a clear supernatant. A volume of 50 µL of the internal standard solution (valeric acid at 1 mM concentration in 0.1% phosphoric acid solution) was spiked into 50 µL of the previously collected supernatant, resulting in samples with a pH of 2-3. Aliquots of 2 µL of these samples were injected in an Agilent 7890A Series gas chromatograph (Agilent Technologies) equipped with a flame ionization detector (FID) and fitted with a capillary column with a free fatty acid phase DB-FFAP 125-3237 (30 m x 0.53 mm, 0.50 µm film thickness, Agilent Technologies). The conditions indicated by Zhao et al. (2006) were used for the chromatographic analysis of samples. Peak identification was based on comparison of retention times with the respective SCFA standards. Individual 9-point calibration curves were obtained for each SCFA standard and used for quantitative analysis. For calibration and samples analyses, the areas obtained for acetic, propionic and butyric acids were corrected by the area obtained for the internal standard. The results were expressed as concentration values in mM of SCFAs.

2.6. Cell culture and treatment

The HT-29 cell line was purchase from the European Collection of Authenticated Cell Cultures (ACACC, Salisbury, UK). These human colorectal adenocarcinoma cells were maintained under basal conditions in DMEM containing 25 mM D-glucose supplemented with 10% FBS, 1% P/S, and 1% L-glutamine solution at 37 °C in humidity saturated 5% CO_2 atmosphere, splitting the cells 1:3 twice a week. HT-29 cells for experiments were taken from exponential phase cultures at passages 10–20. For the treatments, DMEM supplemented with 1% FBS, 1% P/S, 1% L-glutamine solution and 0.1% DMSO was used to reconstituted the RWPS-derived fractions, which were then filtered through 0.45 µm filters before being added to cells. Citotoxycity assays were performed in Corning®Costar® 96-well multiplates (Corning Incorporated, Corning, NY, USA). HT-29 cell cultures for the comet assays were incubated in 25 cm tissue culture flasks with 5 mL of medium/treatments.

2.7. Anti-proliferative activity evaluation (MTT assay)

The ability of the RWPSs and their respective digested fractions to inhibit cell proliferation was evaluated by the MTT colorimetric method, which was used to estimate cell viability (Twentyman & Luscombe 1987). Briefly, HT-29 cells were incubated in 96-well multiplates ($1.0x10^4$ cells/well filled with 200 µL of medium) for 24 h under basal conditions and then exposed for other 24 h to different concentrations of the treatments (100, 250, 500, 750, 1000, 1250, 2500 µg of each fraction (UD, GIDD, and CF) per mL of medium) or just DMEM for the control (C) cells. Then, MTT reagent dissolved in PBS was added to the wells at a final concentration of 0.5 mg/mL of DMEM and the plate was maintained in the incubator for 2 h. The medium was then removed, and the intracellular formazan crystals were dissolved in 200 µL of DMSO. The absorbance of each well at a wavelength of 550 nm was measured in a PowerWave XS2 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). The experiments were repeated three times in four parallel wells. The % of viable cells was calculated with respect to the C cells and the concentration of each treatment that inhibited 50% of cell viability (IC_{50}) was determined, expressing the IC_{50} values for each fraction in µg/mL of medium.

2.8. Anti-genotoxic activity assessment (Comet assay)

Single-cell gel electrophoresis under alkaline conditions, also known as the comet assay, was performed as previously reported (Tice et al. 2000; Del Pino-García et al. 2012) to determine the effect of RWPS and their derived fractions on colonocyte DNA damage, using menadione as oxidation agent. HT-29 cells were seeded in 25 cm^2 cell culture flasks ($1x10^4$ cells/cm^2, flask filled with 5 mL of medium), pre-incubated with basal DMEM for 24 h, and exposed for other 24 h to each fraction at a final concentration of 200 µg/mL of medium. Non-oxidised control (C) and oxidised control (O C) cells were incubated just with DMEM. Two types of experiments were performed:

a) O-RWPS: the treatments were first removed and oxidation was then induced by adding 5 mL of DMEM containing menadione (0.2 µM of final concentration) and incubating for 5 h.

b) O+RWPS: an aliquot of 50 µL of menadione (20 µM, diluted in DMEM) was directly added to the flasks containing the treatments and oxidation was maintained for 5 h.

The possible genotoxic effect of the fractions was also evaluated by treating pre-incubated cells with PBS instead of menadione. Following oxidation, HT-29 cells were scraped, centrifuged (3,000 g, 5 min, 4 ºC), resuspended in pre-heated 1% Low-Melting-point agarose, and added to frosted microscope slides precoated with 1% Normal-Melting-point agarose. After the agarose solidified, slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium-lauryl-sarcosineate, 1% Tritton X-100, 10% DMSO, pH 10) overnight at 4 ºC. The next day, all slides were placed in an electrophoresis tank

containing alkaline electrophoresis buffer (1 mM EDTA, 0.3 N NaOH, pH 10) and the DNA allowed to unwind for 40 min. Electrophoresis was conducted at 4 °C, 25 V, 500 mA and 150 W. The slides were subsequently wash three times for 2 min with neutralizing buffer (0.4 M Tris, pH 7.5), stained with ethidium bromide (20 µg/mL, 20µL/slide), observed using a ZEISS axioplan fluorescence microscope (Carl Zeiss A.G., Oberkochen, Germany), and photographed with a Nikon CoolPix L31 16MP digital camera (Nikon Europe B.V., Amsterdam, The Netherlands). The images were analysed using the program *CometScore*™ Version 1.5 (TriTek, Corp., Sumerduck, VA, USA). For each analysis, 60 randomly selected individual cells were calculated and their tail length evaluated. Each experiment was repeated independently three times. DNA damage was expressed as mean % tail length in relation to the oxidised control ([T/O C]%).

2.9. Statistical analysis

Analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies Inc., Warranton, VA, USA). Statistical significance of the data was tested by one-way analysis of variance (ANOVA), using the Fisher's least significant difference (LSD) test to compare the means that showed significant variation ($p < 0.05$).

3. Results

3.1. Phenolic acid contents of RWPSs and derived digested fractions

The sum of phenolic acids determined in the UD, GIDD and CF fractions derived from the RWPSs is presented in **Figure 1**. In the three types of RWPSs, higher concentrations of phenolic acids were detected in the digested than the UD fractions, but important differences between the seasonings were detected. For Sk-S the most concentrated fraction was GIDD, showing about three- and two-folds higher ($p < 0.001$) contents than UD and CF, respectively. For W-S both digested fractions were around three times more concentrated than the UD fraction, with GIDD showing slightly but significantly higher values than CF. The opposite trend was observed for Sd-S where the CF fraction contained the highest quantity of phenolic acids, being almost five- and four-folds more concentrated ($p < 0.001$) than UD and GIDD, respectively. Comparing between the RWPSs, the sum of phenolic acids was significantly higher in Sk-S than Sd-S for both the UD and the GIDD fractions whereas the contrary was observed for the CF fractions, with W-S obtaining intermediate values in all cases. Further information about the contents in the individual phenolics acids can be seen as **Supplementary Table S1**, which reflects the higher contribution of hydroxybenzoic than hydroxycinnamic acids to the sum of phenolic acids, as well as the most representative compounds determined for each fraction.

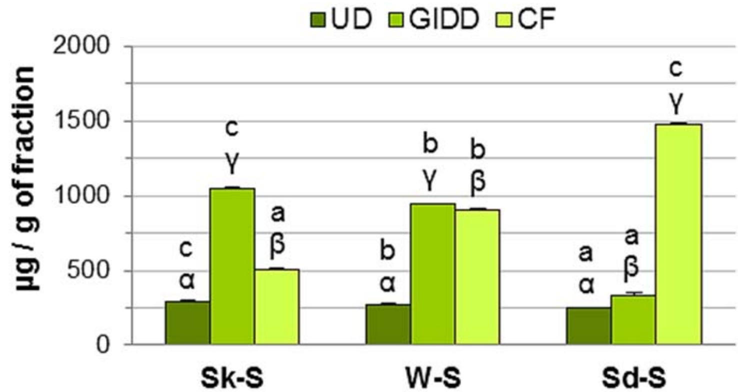


Figure 1.**Phenolic acid contents of the red wine pomace seasonings (RWPSs) and their respective digested fractions**. RWPS obtained from seedless wine pomace (Sk-S), whole wine pomace (W-S), and isolated seeds (Sd-S). Fractions analysed during simulated digestion: undigested (UD), gastrointestinal digested and dialysed (GIDD), and colonic fermented (CF). Results expressed in µg/g of fraction and represented as the mean value ± standard deviation (n=3) of the total sum of phenolic acids quantified in each fraction. Roman letters indicate significant differences (p<0.05) between the RWPSs (Sk-S, W-S, Sd-S). Greek letters show significant differences (p<0.05) between the fractions (UD, GIDD, CF).

3.2. SCFAs levels of CF fractions

The highest levels of SCFAs were detected in Sk-S, and the lowest in Sd-S, both evaluating the individual (butyric, acetic and propionic acids) and the total SCFA contents (**Figure 2**). Acetic acid was the main SCFA produced in the three RWPSs, and butyric acid the lowest. The molar SCFA ratio obtained for each seasoning was different, with Sk-S showing the highest relative production of butyric acid, W-S of propionic acid, and Sd-S of acetic acid.

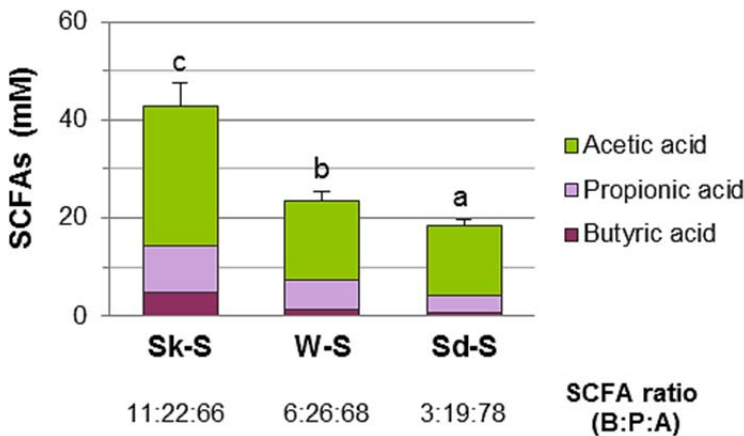


Figure 2. **Short chain fatty acid (SCFA) contents of the colonic fermented (CF) fractions**. CF fractions derived red wine pomace seasonings (RWPSs) obtained from seedless wine pomace (Sk-S), whole wine pomace (W-S), and isolated seeds (Sd-S). Results expressed as mM of total SCFAs and represented as the mean value ± standard deviation (n=3), also indicating the mean values of each individual SCFA. CFA ratio (B:P:A) represents the molar ratio of butyric (B), propionic (P) and acetic (A) acids in each CF fraction. Roman letters indicate significant differences (p<0.05) between the RWPSs (Sk-S, W-S, Sd-S).

3.3. Anti-proliferative effects of RWPSs and their respective digested fractions

Following incubation of HT-29 cells with different concentrations of the treatments for 24 h, a dose-dependent inhibition of cell viability was observed for all the RWPSs and derived digested fractions (data not shown), allowing to calculate the IC_{50} value of each fraction (**Figure 3**). The concentration of treatment capable to inhibit cell viability by 50% was used to compare the anti-proliferative actions between the different fractions and seasonings. For Sk-S, all fractions elicited similar anti-proliferative activities (mean IC_{50} around 833 µg of fraction/mL of medium). For W-S the GIDD fraction was the most effective, showing around 25% higher ($p < 0.05$) capacity to reduce the number of viable cells than the respective UD treatment. As regards Sd-S, both of its digested fractions obtained similar IC_{50} values and displayed around 23% more potent anti-proliferative effects than prior digestion.

Comparing the same type of fractions, differences between the RWPSs were observed only for the UD treatments where Sk-S exhibited the highest ability to reduce cell viability and Sd the lowest.

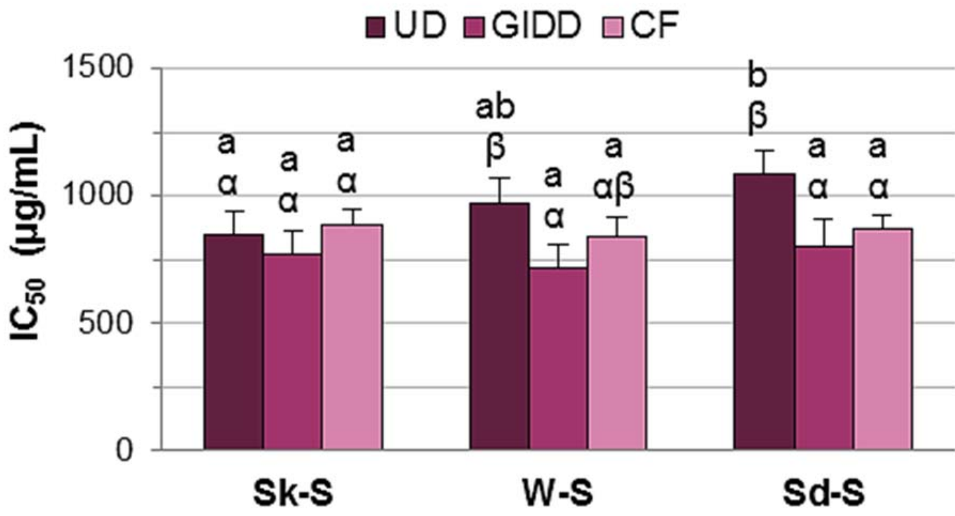
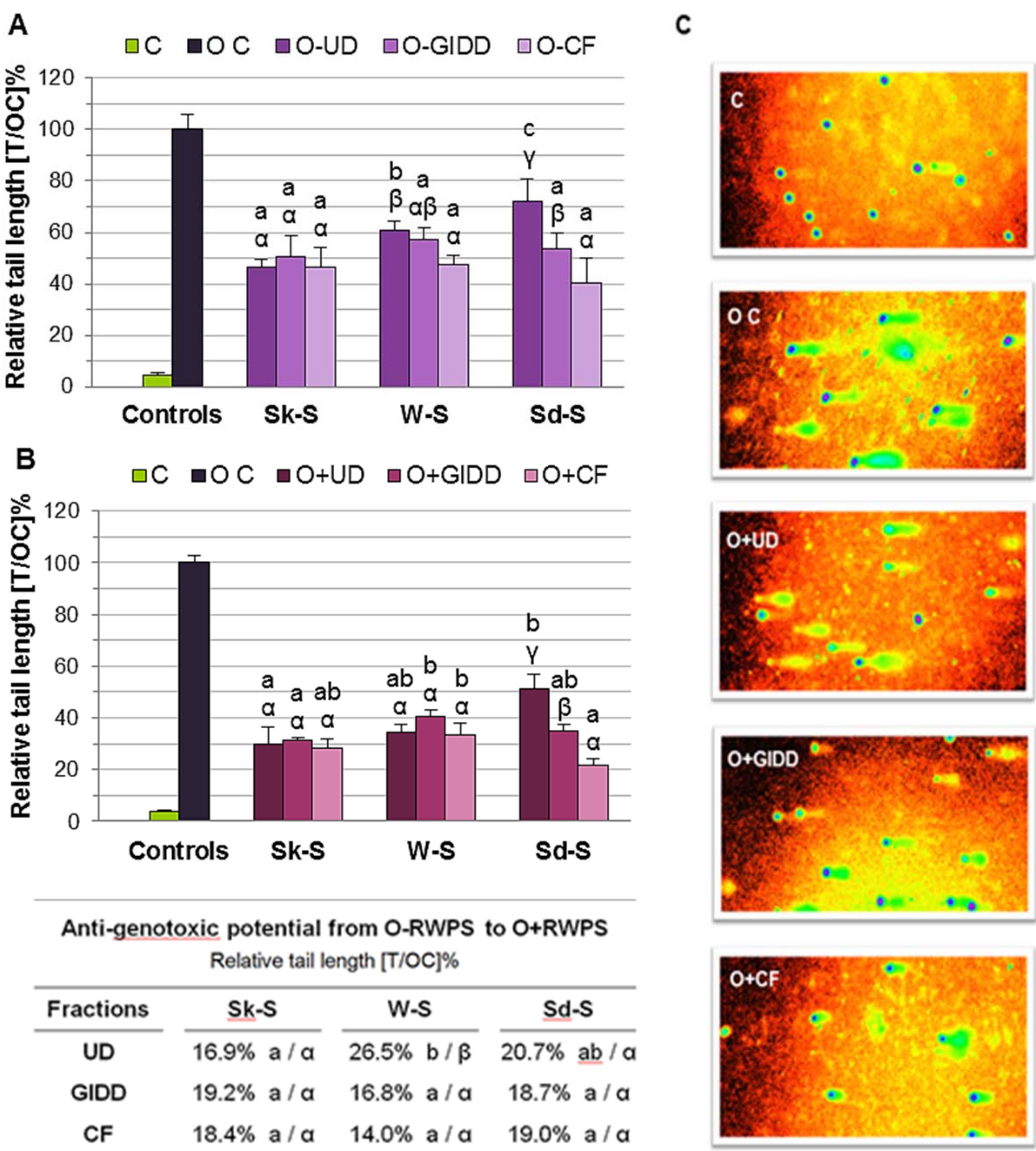


Figure 3. **Anti-proliferative activity of the RWPSs and their respective digested fractions in HT-29 cells**. Red wine pomace seasonings (RWPSs) obtained from seedless wine pomace (Sk-S), whole wine pomace (W-S), and isolated seeds (Sd-S). Fractions analysed: undigested (UD), gastrointestinal digested and dialysed (GIDD), and colonic fermented (CF). Results expressed as the concentration of the treatments giving 50% inhibition (IC_{50}) relative to the viability of control cells and represented as the mean value ± standard deviation (n=3). Roman letters point out significant differences (p<0.05) between the RWPSs (Sk-S, W-S, Sd-S). Greek letters indicate significant differences (p<0.05) between the fractions (UD, GIDD, CF).



Anti-genotoxic potential from O-RWPS to O+RWPS

Relative tail length [T/OC]%

Fractions	Sk-S	W-S	Sd-S
UD	16.9% a / α	26.5% b / β	20.7% ab / α
GIDD	19.2% a / α	16.8% a / α	18.7% a / α
CF	18.4% a / α	14.0% a / α	19.0% a / α

Figure 4. Anti-genotoxic effects of the RWPSs and their respective digested fractions in HT-29 cells. Red wine pomace seasonings (RWPSs) obtained from seedless wine pomace (Sk-S), whole wine pomace (W-S), and isolated seeds (Sd-S). Fractions analysed: undigested (UD), gastrointestinal digested and dialysed (GIDD), and colonic fermented (CF). These treatments were used at a non-cytotoxic concentration (200 µg of fraction/mL of medium). Protective effects against oxidative DNA damage induced by menadione were assessed when the treatments were absent (**A**, O-RWPS) and present (**B**, O+RWPS) during the oxidation. DNA migration evaluated by the comet tail length (n = 60 cells for each sample). The results are expressed as [T/OC]% = % of relative tail length with respect to the oxidised control and represented as mean values ± standard deviation (n=3). The differences between the anti-genotic effects of O-RWPS and O+RWPS experiences are presented as % in the table below the comet test results. Roman letters show significant differences (p<0.05) between the RWPSs (Sk-S, W-S, Sd-S). Greek letters refer to significant differences (p<0.05) between the fractions (UD, GIDD, CF). **C**) Photographs obtained during the comet tests using the fractions derived from Sd-S and analysed using the *CometScore*™ programme.

3.4. Anti-genotoxic effects of RWPSs and their respective digested fractions

The protection of the treatments against oxidative DNA damage in HT-29 cells was assessed using the comet assay. When the treatments were absents during the oxidative insult (**Figure 4**A) the genopreventive effect of Sk-S before *in vitro* digestion was preserved after the whole process, while the capacity to prevent oxidative DNA damage for the other RWPSs increased progressively along digestion, showing significant differences from UD to CF fractions for W-S, and between all the fractions for Sd-S ($p < 0.05$). Significant differences in the anti-genotoxic effect observed between the RWPSs were only found before digestion, with Sk-S showing the highest efficacy and Sd-S the lowest. In the presence of the treatments during the oxidation (**Figure 4**B) the trends between the fractions derived from both Sk-S and Sd-S were similar as described above in their absence, but the tendency observed for W-S changed, not detecting significant differences between its fractions in the O+RWPS experience. The seasoning showing the highest anti-genotoxic action was Sk-S preceding colonic fermentation (UD and GIDD fractions) and Sd-S after the action of gut microbiota (CF fraction). The treatments alone were not genotoxic towards the HT-29 cells at the concentrations used.

The table accompanying **Figure 4** shows the increase in the anti-genotoxic effect from O-RWPS to O+RWPS. The differences ranged around 20% higher ($p < 0.001$) protection due to the presence of the treatments during the oxidation for most types of fractions and seasonings. Nonetheless, the % change detected for the UD fraction derived from W-S was the highest, both in comparisons between the different fractions of this seasoning and between the UD fractions of the three RWPSs.

4. Discussion

There is increasing interest in strategies for cancer prevention, with a number of dietary constituents being regarded as promising chemopreventive agents (Mehta et al. 2010; Landis-Piwowar & Iyer 2014). In this study, three new powdered seasonings derived from different parts of red wine pomace (seedless: Sk-S, whole: W-S, seeds: Sd-S) have been investigated for their anti-genotoxic and anti-proliferative effects in cancerous colonocytes as surrogate indexes of their potential in primary and secondary cancer chemoprevention (Manson et al. 2000; Steward & Brown 2013; Scharlau et al. 2009).

Our results showed that Sk-S and W-S contained high levels of weakly bound and/or free phenolic acids that were readily liberated during the gastrointestinal phase of digestion. In the organism, some of these phenolic acids will pass through the epithelial cells of the small intestine and reach the blood stream whereas non-absorbed ones will reach the large bowel. Part of these compounds are represented by the phenolic acids contained in the GIDD fractions (Saura-calixto et al. 2010), which were found at substantial increased concentrations than before digestion in both Sk-S and W-S.

Once compounds bound to dietary fibre of wine pomace reach the colon, they may be liberated from non-digested matrices and degraded by colonic microbiota (Saura-Calixto et al. 2010; Touriño et al. 2011; Sánchez-Patán et al. 2012). Monomeric flavonoids and soluble proanthocyanidins of high molecular weight are susceptible to extensive microbial fermentation, suffering de-polymerisation and degradation of monomers and resulting in the accumulation of simple phenolic acids and metabolites (Bravo & Saura-Calixto 1998; Mateos-Martín et al. 2012; Goodrich & Neilson 2014). Hence, the high concentration in phenolic acids, predominantly gallic acid, detected in the CF fractions derived from W-S and especially from Sd-S was probably due to the large contents in mono- and oligomeric flavan-3-ols and galloylated molecules of wine pomace seeds (Yu & Ahmedna 2013), which can be degraded to gallic acid in the colon (Selma et al. 2009).

The colonic microbiota can also break down dietary fibre contained in RWPSs (García-Lomillo et al. 2014), leading to the production of SCFAs which are chiefly represented by acetic, propionic and butyric acids (Bravo & Saura-Calixto 1998). Qualitative and quantitative differences were detected in the pattern of SCFAs resultant from colonic fermentation of the different RWPSs. The differences in the type of carbohydrates constituting dietary fibres and the peptides retained to the insoluble matrices, as well as the interactions between them and their influence in gut microbiota (Titgemeyer & Bourquin 1991; Taciak et al. 2015), might partly explained the different SCFA levels and molar proportion found for each RWPS. In addition, the higher contents of proanthocyanidins in wine pomace seeds than skins may well contribute to the lower SCFA contents detected in colonic fermented fractions derived from Sd-S than from Sk-S, as these complex phenolic compounds can inhibit SCFA formation (Aura et al. 2013).

The potential of the RWPS-derived bioactive compounds to exert beneficial actions against colon cancer were assessed in the HT-29 cell line, which is a widely employed model of transformed neoplastic colorectal cells in studies of cell cycle events and genotoxicity (Coates et al. 2007). The chemopreventive effects described for some phenolic compounds (Russo 2007; Ramos 2008) and SCFAs (Scheppach et al. 1995; Scharlau et al. 2009) are mediated both by their blocking activities, which are mostly related to primary cancer prevention, and by their suppressing activities, which can provide secondary cancer prevention (Manson et al. 2000).

The IC_{50} values obtained for RWPS and their respective fractions showed that, before digestion, Sk-S may display the highest anti-proliferative activity in colon cancerous cells. However, the transformations taking place along the digestive process appear to increase the overall capacity to inhibit cell viability of the compounds derived from W-S and Sd-S, but not from Sk-S. Therefore, our results are consistent with the previous studies reporting anti-proliferative effects of grape phytochemicals in numerous cancer cells, which have been

associated with molecular mechanisms that result in the arrest of the cell cycle, and the induction of apoptosis, among other cancer supressing effects (Derry et al. 2014; Hsu et al. 2009; Yang & Xiao 2013; Jara-Palacios et al. 2015).

Considering the phenolic composition of the undigested RWPSs (García-Lomillo et al. 2014, Del-Pino García et al. 2016_*Chapter 1.2*), the highest efficacy of Sk-S to inhibit HT-29 cell growth preceding digestion can be explained by its high content in anthocyanins, whose cytotoxic and anti-proliferative effects in colon cancerous cells have been reported by several authors (Jing et al. 2008; Forester & Waterhouse 2010; Núñez-Sánchez et al. 2015). In fact, previous research in extracts obtained from grapes has demonstrated that those fractions rich in anthocyanins, followed by flavonols and tannins, displayed the highest anti-proliferative activity in HT-29 cells, while the phenolic acid-enriched fractions were the least effective (Yi et al. 2005). In the fractions collected just after gastrointestinal digestion (GIDD fractions), our results suggested that some phenolic acids such as salicylic, syringic, and protocatechuic acids could contribute to the observed anti-proliferative effects, particularly in Sk-S and W-S, where these hydroxybenzoic acids are present at high concentrations. Salicylic could be particularly responsible bearing in mind that its pro-apoptotic effects on colon carcinoma cells have been previously demonstrated (Zitta et al. 2012), whereas none or low anti-proliferative and cytotoxic activities have been seen for protocatechuic, syringic and vallinic acids (Forester & Waterhouse 2010; Correa-Betanzo et al. 2014). In addition, non-absorbed flavonoids liberated from the RWPS matrices may also play a substantial cancer suppressing role (Jara-Palacios et al. 2015), mainly in Sd-S where phenolic acids were poorly bioaccessible until reaching the colon.

After colonic fermentation, gallic acid could be implicated in the observed reduction of HT-29 cell viability, mainly in W-S and Sd-S, as this phenolic acid has been described as a potent inhibitor of cancerous cell proliferation (Forester & Waterhouse 2010; Forester et al. 2014). The CF fraction derived from Sk-S retained its capacity to decrease cell viability to a similar extent as prior colonic fermentation and as other CF fractions, which evidenced the contribution of either non-degraded native compounds such as anthocyanins (Correa-Betanzo et al. 2014; Núñez-Sánchez et al. 2015) or colonic metabolites to the anti-proliferative effects of this treatment. Furthermore, the SCFAs formed in the large bowel may also protect against colorectal cancer (Hague et al. 1995). Previous studies have proposed that both butyrate and propionate, but not acetate, are able to counteract HT-29 cells proliferation (Gamet et al. 1992). In particular, the ability of butyrate to promote growth arrest, differentiation, and apoptosis in cancerous cells has been extensively studied (Topping & Clifton 2001; Wong et al. 2006). As the HT-29 cells carry a mutant form of the p53 gene, which is often mutated in colorectal tumours, butyrate might efficiently induce the programmed death of these cancerous cells because this SCFA has been suggested to activate p53-independent mechanisms of apoptosis (Hague et al. 1993).

The anti-genotoxic effects in HT-29 cells of the RWPSs and their respective digested fractions (used at non-cytotoxic concentrations) were assessed by the comet assay, observing that the RWPS-derived bioactive compounds were able to decreased DNA breakage under oxidative stress when they were both absent (O-RWPS) and present (O+RWPS) in the medium during the oxidative insult. As expected, the presence of the fractions during the oxidation increased their capacity to protect DNA, showing that the extent of this increase was rather similar for most of the treatments. Indirect antioxidant mechanisms leading to these anti-genotoxic effects may be mediated by the RWPS-derived bioactive compounds up-taken by the cells (Goodrich & Neilson 2014) and also by those in contact with the colonocytes. Both polyphenols from grapes and wine (Dolara et al. 2005; Yi et al. 2005; López-Oliva et al. 2010) and certain SCFAs such as butyrate and propionate (Abrahamse et al. 1999; Hamer et al. 2008; Scharlau et al. 2009) have previously demonstrated their ability to inhibit cellular processes associated with genotoxic events in colorectal cancer through a broad spectrum of indirect mechanisms that involve the modulation of several signalling pathways. On the other hand, phenolic compounds retained within cells and those present in the medium in the O+RWPS experience may also exhibit direct antioxidant activities that help to decrease RONS levels and contribute to reduce oxidative DNA damage in colon cells (Sgambato et al. 2001; Georgiev et al. 2014).

The anti-genotixic action of Sk-S preceding digestion, which was the highest between the RWPSs in both experiences, was not altered by the digestion, which agrees with previous studies using digested and fermented anthocyanin-rich extracts from different berries, also in HT-29 cells (Brown et al. 2012). On the other hand, a progressive higher anti-genotoxic potential was observed for Sd-S with the advance of the digestive process, which was parallel to the increased release/generation of phenolic acids in this RWPS. These results support the great contribution of phenolic compounds to the potential anti-genotoxicity of Sd-S, which is in accordance with the high genoprotective effects reported for gallic acid by other authors (Fan & Lou 2004). With regard to W-S, certain synergisms may occur before digestion between its compounds, derived from both grape skins and seeds (Yang & Xiao 2013, Del Pino-García et al. 2016b), but these synergistic interactions might be lost, or at least not so noticeable, after the digestive process.

Taken together, our data suggest the chemopreventive properties of seasonings directly obtained from winemaking residues against colorectal cancer. Dose-dependent anti-proliferative effects in cancerous colon cells for all the seasonings tested and their respective digested fractions, which might help to attenuate cancer progression, as well as anti-genotoxic effects in their presence or their absence during an oxidative insult, which could prevent mutagenesis and block tumour initiation and advance, were detected.

The seasoning derived from seedless wine pomace was the most effective prior digestion, but the seasonings containing wine pomace seeds were more susceptible to increase their chemopreventive capacity during digestion, with all of the seasonings tested showing similar effects once in the colon.

Finally, it should be noted that the potential supressing and blocking activities against colorectal cancer demonstrated *in vitro* in this study should be further assessed *in vivo* to confirm the interest of these seasonings as primary and secondary chemopreventive agents in colorectal cancer. However, these dual protective role of the RWPSs, along with the synergistic or antagonistic effects that chemoprevention might have with other chemotherapies, must be taking into account when designing strategies with therapeutic purposes.

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Conflict of interest

The authors declare no competing financial interest.

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Table S1. **Phenolic acid contents (µg/g) of the red wine pomace seasonings (RWPSs) in the fractions obtained during simulated digestion.**

Phenolic Compounds	RWPSs [a]								
	Sk-S			W-S			Sd-S		
	UD	GIDD	CF	UD	GIDD	CF	UD	GIDD	CF
p-OH-benzoic acid	6.27 ± 0.12 c/β	10.8 ± 0.7 c/γ	1.47 ± 0.05 a/α	4.01 ± 0.13 b/β	7.09 ± 0.19 b/γ	1.50 ± 0.08 a/α	0.523 ± 0.124 a/α	3.16 ± 0.06 a/γ	1.64 ± 0.07 a/β
Salicylic acid	18.9 ± 0.13 c/α	550 ± 8 c/γ	87.3 ± 1.4 c/β	11.8 ± 0.7 b/α	500 ± 11 b/γ	63.9 ± 2.0 b/β	7.72 ± 0.23 a/β	45.7 ± 1.1 a/γ	5.20 ± 0.42 a/α
Protocatechuic acid	21.3 ± 0.16 c/α	84.1 ± 3.8 b/β	18.7 ± 0.6 c/α	19.6 ± 0.4 b/β	87.3 ± 1.5 b/γ	15.2 ± 0.5 b/α	16.6 ± 0.2 a/β	30.8 ± 0.4 a/γ	10.2 ± 0.2 a/α
Gallic acid	73.1 ± 0.4 a/β	56.5 ± 2.15 b/α	279 ± 8 a/γ	97.0 ± 0.4 b/β	56.6 ± 1.2 b/α	745 ± 14 b/γ	127 ± 0.1 c/β	31.3 ± 0.08 a/α	1448 ± 10 c/γ
Vanillic acid	30.4 ± 0.3 c/γ	27.3 ± 0.5 c/β	12.1 ± 0.3 b/α	20.3 ± 0.3 b/γ	15.9 ± 1.2 b/β	3.84 ± 0.23 a/α	9.43 ± 0.08 a/β	1.89 ± 0.06 a/α	ND
Syringic acid	74.4 ± 3.5 c/α	130 ± 4 a/β	74.4 ± 4.5 c/α	48.0 ± 0.8 b/α	126 ± 7 a/β	47.7 ± 1.4 b/α	2.87 ± 0.70 a/α	127 ± 7 a/β	8.48 ± 0.62 a/α
Ellagic acid	21.4 ± 0.06 b/β	6.59 ± 2.12 b/α	ND	10.6 ± 0.2 a/β	3.78 ± 0.66 a/α	ND	ND	ND	ND
Ethyl gallate	17.1 ± 0.13 a/α	153 ± 9 c/β	16.1 ± 0.1 c/α	33.2 ± 0.9 b/β	120 ± 4 b/γ	13.6 ± 0.6 b/α	58.7 ± 2.0 c/β	79.6 ± 4.2 a/γ	3.83 ± 0.10 a/α
Total Hydroxybenzoic acids	263 ± 3 c/α	1019 ± 5 c/γ	489 ± 10 a/β	244 ± 2 b/α	917 ± 3 b/γ	891 ± 11 b/β	223 ± 1.21 a/α	320 ± 12 a/β	1477 ± 10 a/γ
p-Coumaric acid	5.85 ± 0.03 a/α	7.03 ± 0.23 c/α	7.25 ± 0.04 b/α	8.78 ± 0.34 b/γ	4.27 ± 0.26 b/α	6.38 ± 0.16 a/β	9.14 ± 0.58 b/β	2.80 ± 0.22 a/α	ND
Caffeic acid	3.40 ± 0.42 a/γ	2.40 ± 0.21 c/β	0.701 ± 0.087 a/α	4.75 ± 0.21 a/γ	2.44 ± 0.40 b/β	0.767 ± 0.028 a/α	5.52 ± 0.60 a/β	2.32 ± 0.34 a/α	ND
Ferulic acid	1.18 ± 0.01 c/α	3.56 ± 0.32 c/γ	2.12 ± 0.13 c/β	0.962 ± 0.037 b/α	2.84 ± 0.27 b/γ	1.59 ± 0.03 b/β	0.270 ± 0.115 a/α	2.70 ± 0.42 a/β	0.797 ± 0.091 a/α
Coutaric acid	4.59 ± 0.34 b/β	8.97 ± 0.16 c/γ	2.88 ± 0.20 c/α	2.42 ± 0.32 a/α	6.29 ± 0.19 b/β	2.20 ± 0.26 b/α	2.99 ± 0.02 a/γ	1.19 ± 0.13 a/β	0.552 ± 0.057 a/α
Caftaric acid	13.6 ± 0.4 b/γ	2.13 ± 0.04 b/α	3.06 ± 0.03 b/β	10.9 ± 1.3 b/β	2.14 ± 0.17 b/α	2.10 ± 0.48 a/α	9.98 ± 0.88 a/β	1.70 ± 0.004 a/α	1.49 ± 0.12 a/α
Fertaric acid	3.76 ± 0.25 b/β	8.07 ± 0.36 b/γ	0.159 ± 0.015 b/α	1.72 ± 0.21 a/β	7.53 ± 0.31 b/γ	0.026 ± 0.022 a/α	ND	5.67 ± 0.007 a	ND
Total Hydroxycinnamic acids	32.4 ± 1.6 a/β	32.2 ± 0.2 c/β	16.2 ± 0.014 c/α	29.6 ± 1.7 a/γ	25.5 ± 0.7 b/β	13.1 ± 0.92 b/α	27.9 ± 1.9 a/γ	16.4 ± 0.8 a/β	2.80 ± 0.08 a/α
Total Phenolic acids	**295** ± 5 **c/α**	**1051** ± 5 **c/γ**	**505** ± 10 **a/β**	**274** ± 3 **b/α**	**942** ± 2 **b/γ**	**904** ± 10 **b/β**	**250** ± 2 **a/α**	**336** ± 13 **a/β**	**1480** ± 9 **c/γ**

Results expressed as the mean value ± standard deviation (n = 3).
a) RWPS obtained from seedless wine pomace (Sk-S), whole wine pomace (W-S), and isolated seeds (Sd-S). Fractions analysed during simulated digestion: undigested (UD), gastrointestinal digested and dialysed (GIDD), and colonic fermented (CF).
Roman letters: Significant diferences (ANOVA) between the different RWPSs (Sk-S, W-S, Sd-S) for the same type of sample.
Greek letters: Significant diferences (ANOVA) between the different type of samples (UD, GIDD, CF) for the same type of RWPS.

Chapter 4.2.

Indirect Antioxidant Mechanisms of a Red Wine Pomace Seasoning to Attenuate Oxidative Damage in HT-29 Cells

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Abstract

Several endogenous and exogenous antioxidant agents may operate together in the colon and influence the constitutively hyper-activated oxidative status of colorectal cancerous cells. This study investigates the cytoprotective effects of a seedless red wine pomace seasoning (RWPS) against oxidative damage in HT-29 cells and the indirect antioxidant mechanisms implicated. Cells were pre-incubated for 24 h with non-cytotoxic doses of RWPS-derived *in vitro* digested fractions, and biomarkers of oxidative damage (malondialdehyde and carbonyl groups) and cell membrane integrity (lactate dehydrogenase release) were assessed in cells exposed or not to a pro-oxidant stimulus (*tert*-butyl hydroperoxide). The significant protection against oxidative stress observed in treated cells could be partly ascribed to the modulation of nuclear factor-kappa B (NF-κB), nuclear factor-erythroid 2-related factor-2 (Nrf2) and their down-stream antioxidant genes by the RWPS-derived bioactive compounds. In conclusion, the results suggest the chemopreventive potential of RWPS as a natural tumour-blocking agent in colorectal cancer.

Keywords: Colorectal cancer; Chemoprevention; Dietary antioxidants; Oxidative damage; Redox signalling; Wine pomace.

Abbreviations and nomenclature: AREs, antioxidant responsive elements; **CAT**, catalase; **CF**, fraction obtained after simulated colonic fermentation; **CGs**, carbonyl groups; **COX**, cyclooxygenase; **GIDD**, fraction obtained after simulated gastrointestinal digestion+dialysis; **HO**, heme oxygenase; **iNOS**, inducible nitric oxide synthase; **LDH**, lactate dehydrogenase; **MDA**, malondialdehyde; **NF-κB**, nuclear factor-kappa B; **NOX**, β-nicotinamide adenine dinucleotide phosphate oxidase; **Nrf2**, nuclear factor-erythroid 2-related factor-2; **RONS**, reactive oxygen/nitrogen species; **RWPS**, red wine pomace seasoning; **SOD**, superoxide dismutase; **UD**, undigested fraction.

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Highlights:

- A red wine pomace seasoning (RWPS) attenuated oxidative stress in HT-29 cells.
- RWPS prevented oxidative damage to biomolecules in these colon cancerous cells.
- RWPS can modulate gene expression of NF-κB and Nrf2 transcription factors.
- Lower antioxidant response was induced in treated cells by an oxidative insult.
- Pro-oxidant enzymes (NOX1, COX2, iNOS) were down-regulated in treated cells.

1. Introduction

Oxidative stress is widely recognized as a contributing factor in the development of colorectal cancer (Babbs 1990; Federico et al. 2007), one of the most common cancers worldwide (Torre et al. 2015). Chronic exposure to oxidative challenging conditions may lead to excessive and uncontrolled production of reactive oxygen/nitrogen species (RONS) and secondary mediators of oxidative stress, damage to biomolecules (DNA, lipids and proteins), redox imbalance, loss of homeostasis, and deregulation of cell cycle, with cancer initiation as a likely endpoint, along with other inflammatory gastrointestinal disorders (Federico et al. 2007; Sosa et al. 2013; Bhattacharyya et al. 2014). In addition, endogenous RONS production is well-known to participate in cancer promotion, as most cancerous cells are characterised by high RONS generation rate and altered redox state in comparison to normal cells, which stimulates cell division and support tumour growth (Valko et al. 2006; Glasauer & Chandel 2014).

The main endogenous enzymatic systems that generate RONS in the gastrointestinal tract are the cyclooxygenases (COXs), the inducible nitric oxide synthase (iNOS), and the nicotinamide adenine dinucleotide phosphate oxidases (NOXs) (Bhattacharyya et al. 2014). There are also several exogenous pro-oxidant factors that affect the extra- and intracellular environment of gut epithelial cells (Key et al. 2004; Perše 2013). To balance their redox state and cope with damaging oxidative challenges, cells have a tightly regulated endogenous antioxidant defence system, which include non-enzymatic components such as glutathione, thioredoxins, ferritin, and bilirubin, as well as several enzymes such as superoxide dismutases (SODs), catalase (CAT), glutathione-related enzymes and heme-oxygenases (HO), among many others (Valko et al. 2006).

In both cancerous and normal cells, these systems in charge of RONS production and elimination are controlled by numerous signalling events where redox sensitive transcription factors take part as key final mediators, such as the nuclear factor-erythroid 2-related factor-2 (Nrf2), which is generally considered as playing a positive/protective role in cancer and in stress-related diseases, and the nuclear factor-kappa B (NF-κB), which is strongly implicated in tumour promotion since this factor induces an oxidative environment, inflammation, proliferation and resistance to apoptosis (Acharya et al. 2010; Bellezza et al. 2010). There are several dietary compounds/products which, in addition to possible direct antioxidant actions, can indirectly increase the endogenous antioxidant defences and attenuate pro-oxidant and inflammatory stimulus in the gut by regulating redox sensitive transcription factors and, subsequently, the expression of antioxidant enzymes. Thus, a number of products and bioactive molecules incorporated with the diet have been proposed as potential chemopreventive agents to cope with RONS overproduction and oxidative damage in cancerous cells (Ramos 2008; Zhao et al. 2010; Araújo et al. 2011; Kou et al. 2013).

The potential of polyphenol- and dietary fibre-rich seasonings obtained from different parts of red wine pomace (RWPSs) as chemopreventive agents against colorectal cancer due to their anti-proliferative and anti-genotoxic effects has been recently suggested (Del Pino-García et al. 2016g). These health effects were determined in the original RWPSs but also in more physiologically relevant fractions obtained pre- and post-colonic microbiota metabolism during *in vitro* digestion. The ability of RWPS-derived bioactive compounds to indirectly modulate signalling pathways may also help to alter the oxidative status in colon cancerous cells and prevent further oxidative damage to biomolecules under basal conditions, when these cells are not submitted to exogenous oxidants.

Therefore, the present study aimed to obtain further insight into the potential of the seedless RWPS to protect against oxidative stress in colorectal cancer cells, and into the indirect antioxidant mechanism implicated. These chemopreventive antioxidant effects were assessed under two hypothetical situations: the increased oxidative stress characteristic of these cancerous cells, and the additional oxidative stress that could be induced by exogenous pro-oxidant factors.

2. Materials and Methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), calcium chloride dihydrate ($CaCl_2.2H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), L-cysteine hydrochloride, 2,4-dinitrophenylhydrazine (DNFH), Dulbecco's Modified Eagle Medium (DMEM, 25 mM D-glucose), foetal bovine serum (FBS), L-glutamine solution (200 mM), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), β-nicotinamide adenine dinucleotide reduced (NADH) disodium salt hydrate, 10,000 U/mL penicillin and 100 mg/mL streptomycin solution (P/S), phosphoric acid solution (H_3PO_4), porcine bile extract, porcine pancreas pancreatin, potassium chloride (KCl), potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), resazurin sodium salt, sodium bicarbonate ($NaHCO_3$), sodium pyruvate, sodium hydroxide (NaOH), sodium phosphate dibasic (Na_2HPO_4), sodium sulphide nonahydrate ($Na_2S.9H_2O$), *tert*-butyl hydroperoxide (*t*-BOOH), 1,1,3,3-tetramethoxypropane (TMP), 2-thiobarbituric acid (TBA), trifluoroacetic acid (TFA), tryptone, cellulose membrane dialysis tubing (12,000 Da molecular weight cut-off), and the enzymes used in the simulated gastrointestinal digestion (α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3), lipase (EC 3.1.1.3), and pepsin (E.C 3.4.23.1)) were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Panreac Quimica S.L.U. (Barcelona, Spain). Dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) tablets were purchased from Merck Millipore, Co. (Darmstadt, Germany).

2.2. Obtaining of the fractions used as cell treatments

The powdered seasoning examined in this study was obtained from seedless red wine pomace at the University of Burgos, according to a previously described method (González-Sanjosé et al. 2013). Heat treatment was used as a stabilization process to obtain the final seasoning, which was labelled as RWPS. The main characteristics and composition (dietary fibre, fat, protein, minerals and phenolic classes) of this seasoning have been previously reported (García-Lomillo et al. 2014).

RWPS was submitted to *in vitro* digestion following the method described by Saura-Calixto et al. (2010), with the modifications indicated in Del Pino-García et al. (2016b). Among the digested fractions isolated during this procedure, those used as cell treatments were the undigested (UD), the enzymatic 'gastrointestinal digested+dialysed' (GIDD), and the 'colonic fermentation' (CF) fractions. For both types of fractions obtained after simulated digestion, negative digested controls (without seasoning) were also prepared. Three replicates were carried out for each fraction.

2.3. Cell culture and treatment

The human colon adenocarcinoma cell line HT-29 (European Collection of Authenticated Cell Cultures, Salisbury, UK), which represent stage II of carcinogenesis, was grown in DMEM containing 25 mM D-glucose supplemented with 10% FBS, 1% P/S, and 1% L-glutamine solution at 37 ºC in a humidified incubator with 5% CO_2. Treatments were prepared in DMEM supplemented with 1% FBS, 1% P/S, 1% L-glutamine solution, and 0.1% DMSO, which was used to reconstitute the UD, GIDD and CF fractions at a final concentration during treatments of 0.2 mg of fraction per mL of DMEM. The fractions were then filtered through 0.45 µm filters before being added to cells. For all experiments, HT-29 cells (passage 20-30) were seeded at a 1 x 10^4 cells/cm^2 density and cultured 24 h with growing DMEM. The treated-cells were exposed for 24 h to the UD, GIDD or CF treatments or the respective negative digested control treatments, while the non-oxidised control (C) and the oxidised control (O C) cells were both incubated with just DMEM. The treatments were then removed and some of the non-oxidised cell cultures were collected at this point, whereas all the oxidised cells were subsequently exposed for 5 h to *t*-BOOH at a final concentration of 250 µM in the medium. This oxidant was replaced by just PBS in the non-oxidised cells used as C of the oxidation experiments. After either the 24-h treatment or the oxidation period, the cells were scraped and centrifuged (1,500 g, 5 min, 25 ºC). Supernatants were collected and frozen at -80 ºC for further analyses. The cell pellets were resuspended in 1 mL of PBS, aliquoted, and frozen at -80 ºC until their use for the assays. All the experiments were carried out as three independent assays.

2.4. Lactate dehydrogenase (LDH) leakage assessment

The LDH activity was measured in the extra- and intracellular medium using the method reported by Vassault (1983), with the slight modifications indicated by Del Pino-García et al. (2016d). Thus, the integrity of cell membranes was estimated from LDH activity, in terms of % LDH release to the extracellular medium with respect to total LDH (intracellular + extracellular).

2.5. Malondialdehyde (MDA) determination

Lipid peroxidation was estimated from the MDA levels in cell lysates obtained by sonication according to the method described by Grotto et al. (2007), with the slight modifications described by Del Pino-García et al. (2016d). MDA levels were normalized using each cell homogenate total protein concentration, which was determined according to Bradford (1976). The results were finally expressed as nmol of MDA equivalents /mg of protein.

2.6. Carbonyl groups (CGs) determination

Protein damage as a consequence of oxidative stress was determined from the carbonyl groups (CGs) levels in sonicated cell homogenates using the spectrophotometric method reported by Levine et al. (1990), as previously described by Del Pino-García et al. (2016d). The CG concentration was then normalized by the protein content of each cell homogenate, expressing the final results as nmol of CGs/mg of protein.

2.7. Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from HT-29 cell suspensions using TRI Reagent solution (Applied Biosystems, Foster City, CA, USA). Purity, integrity and concentration were analyzed using PowerWave XS2 microplate spectrophotometer with the Take3 micro-volume microplate adapter and the Gen5 1.10 software (BioTek Instruments Inc.). Following treatment with DNase I (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µg of total RNA was reverse-transcribed using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and finally amplified using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, S.A., Madrid, Spain). All the procedures were performed according to the manufacturers' protocols. Primers were designed using Primer3 v.0.4.0 software (Untergasser et al. 2012) and then synthesised by Metabion (Metabion International AG., Steinkirchen, Germany). The sequences of primer sets (forward and reverse) were:

- NF-κB1 (p105/p50), 5'-CCTCTGTGTTTGTCCAGCTT-3' and 5'-CTTCTGACGTTTCCTCTGCA-3'
- Nrf2 (NFE2L2), 5'-GCATCACCAGAACACTCAGT-3' and 5'-CAGGGGCACTATCTAGCTCT-3'
- HO-1, 5'-GCCAGCAACAAAGTGCAAG-3' and 5'-AAAGCTGAGTGTAAGGACCC-3'
- SOD2, 5'-GGAACGGGGACACTTACAAA-3' and 5'-ACTGAAGGTAGTAAGCGTGC-3'
- SOD1, 5'-GGCCAAAGGATGAAGAGAGG-3' and 5'-TGATGCAATGGTCTCCTGAG-3'
- CAT, 5'-TACGTCCTGAGTCTCTGCAT-3' and 5'-CCCCATTTGCATTAACCAGC-3'

- NOX1, 5'-CTTCCTGTGTGTCGCAATCT-3' and 5'-CTTGTGGAAGGTGAGGTTGT-3'
- COX2, 5'-CGGTGAAACTCTGGCTAGAC-3' and 5'-GGGACTTGAGGAGGGTAGAT-3'
- iNOS, 5'-TTACGGCTCCTTCAAAGAGG-3' and 5'-TCATCTCCCGTCAGTTGGTA-3'
- GAPDH, 5'-GCTCTCCAGAACATCATCCC-3' and 5'-GTCCACCACTGACACGTTG-3'

qPCR was carried out with an iCycler iQ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 1 cycle of initial denaturing and enzyme activation at 95 ºC for 3 min; 45 cycles of denaturing at 95 ºC for 15 s and annealing/extension at 62 ºC for 30 s. The proper product amplification was verified by melting curve analysis. Amplification efficiencies were calculated for each pair of primers (using 10 - 10^5 ng cDNA in the reaction). Quantification and normalization of the gene expression was performed using the efficiency-corrected $\Delta\Delta C_t$ method, with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene. Measurements were performed in duplicates and repeated at least three separate times. Relative gene expression was finally expressed as folds of change compared to non-oxidised C cells.

2.8. Data presentation and statistical analysis

Data were expressed as mean values ± standard deviation of independent experiments (n=3). Statistical analysis was conducted using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was used to determine significant differences between the treatments. The level of significance was established at a *p*-value of < 0.05.

3. Results and Discussion

3.1. Protective effects of RWPS against oxidative stress in cancerous cells under basal conditions

3.1.1. Changes on oxidative stress biomarkers of HT-29 cells

The suppressing anti-cancer effects of RWPS and derived digested fractions at high concentrations have been previously investigated (Del Pino-García et al. 2016g). However, the present study is focussed on the blocking antioxidant activities of the seasoning, so non-cytotoxic doses were used to treat HT-29 cells and only slight suppressing effects might be detectable.

The protective effects of RWPS against the basal oxidative conditions characteristics of colorectal cancer were first investigated by assessing biomarkers of oxidative damage to lipids, proteins and cell membranes following 24-h incubation of HT-29 cells with the undigested (UD) and digested (GIDD and CF) fractions derived from this wine pomace

seasoning (**Table 1**). In general, the results obtained between the treatments were rather similar for each biomarker, suggesting that digestion might not greatly affect the final outcomes of RWPS-derived bioactive compounds in colorectal cancerous cells. While the integrity of cell membranes was not altered by the incubation with any of the treatments, significantly lower MDA concentrations (except for UD-treated cells) and higher CG levels were detected in the treated cells compared to the control (C) cells.

Table 1. **Biomarkers of oxidative stress in colonic cancerous cells under basal conditions.**

Oxidative Stress Biomarkers	C [a]	RWPS fractions [b]		
		UD	GIDD	CF
Cell membrane damage (% LDH released of total LDH)	37.2 ± 2.3 a	37.3 ± 2.9 a	37.3 ± 3.2 a	36.6 ± 2.0 a
Lipid peroxidation (nmol of MDA equivalents / mg of protein)	2.62 ± 0.34 b	2.16 ± 0.46 ab	1.88 ± 0.35 a	2.02 ± 0.32 a
Protein oxidation (nmol of CGs / mg of protein)	12.9 ± 1.1 a	15.2 ± 1.4 b	14.9 ± 0.8 b	14.7 ± 0.7 b

Results represented as mean values ± standard deviation.
LDH: Lactate dehydrogenase; MDA: malondialdehyde; CGs: carbonyl groups.
a) Control (C) HT-29 cells.
b) HT-29 cells treated for 24 h with the red wine pomace seasoning (RWPS) derived fractions: undigested (UD), gastrointestinal digested+dialysed (GIDD), and colonic fermented (CF).
Roman letters indicate significant differences ($p < 0.05$) between the treatments (C, UD, GIDD, and CF).

Given that MDA can react with nitrogenous bases and generate DNA adducts that are mutagenic (Marnett 2000; Valko et al. 2006), the protection against lipid peroxidation achieved by RWPS after digestion could be an effective mechanism to neutralize the detrimental secondary effects of increased RONS production in tumour cells (Perše 2013). In fact, digestion in the upper gut was found to increase the efficacy of the bioactive compounds contained in RWPS to prevent oxidative damage to cell lipids, showing 28% lower ($p < 0.01$) MDA levels in GIDD-treated cells compared to C cells. Phenolic compounds liberated from the wine pomace matrix but not absorbed in the small intestine, simple bioactive molecules resultant from the transformation of more complex phenolic structures by colonic microbiota (Saura-calixto et al. 2010; Pérez-Jiménez et al. 2013; Del Pino-García et al. 2016g), and short chain fatty acids generated mainly by colonic fermentation of wine pomace dietary fibre (Bravo & Saura-Calixto 1998; Del Pino-García et al. 2016g) will be in contact with the colonic mucosa and, some of them, will be up-taken by colonic cells (Goodrich & Neilson 2014), displaying direct and/or indirect antioxidant actions that might explain the observed attenuation of lipid peroxidation in HT-29 cells.

On the other hand, increased protein oxidation in other colorectal cancer cell lines treated with grape seed extracts has been previously observed and related to the ability of their flavan-3-ols derivatives to interact with several endoplasmic reticulum stress response proteins and induce oxidative protein modification (Derry et al. 2014). This was considered as a suppressing mechanism of chemoprevention, as it could lead to cancerous cell death due to the accumulation of oxidation proteins, disruption of cell membranes and activation of apoptotic signalling pathways.

3.1.2. Indirect mechanisms of action implicated in the protection against oxidative stress in HT-29 cells

The possible indirect antioxidant mechanisms driven by the RWPS-derived bioactive compounds were assessed under basal conditions in HT-29 cells, determining first the gene expression of key redox sensitive transcription factors (NF-κB and Nrf2).

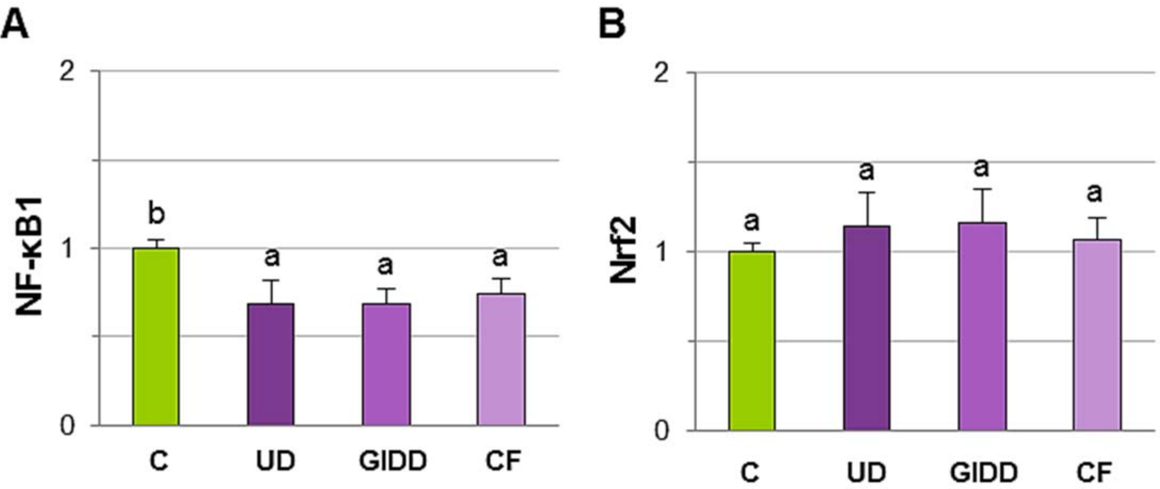

Figure 1. **Gene expression of redox-sensitive transcription factors in human colorectal cancerous HT-29 cells treated with RWPS under basal conditions of oxidative stress.** Genes analysed: **A**) nuclear factor-kappa B 1 (NF-κB1), **B**) nuclear factor-erythroid 2-related factor 2 (Nrf2). Cells were treated with the undigested (UD), gastrointestinal digested+dialysed (GIDD), and colonic fermented (CF) fractions derived from a seedless red wine pomace seasoning (RWPS) submitted to *in vitro* digestion. mRNA levels of the genes assessed were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the control (C) HT-29 cells. Data expressed as mean values ± standard deviation (n=3). Roman letters indicate significant differences (ANOVA, $p<0.05$) between cells exposed to the different treatments.

All the RWPS derived fractions decreased NF-κB1 mRNA levels ($p < 0.05$) to around 0.70-fold of control HT-29 cells (**Figure 1**A), which was consistent with previous studies using polyphenol-rich extracts (Dias et al. 2014) and SCFA-rich fermented fractions from beans (Cruz-Bravo et al. 2014) to treat colorectal cancer cells. The link between colorectal carcinogenesis and increased constitutive expression and activity of NF-κB has been well established in numerous tissues and cell lines, including the HT-29 (Kojima et al. 2004;

Sakamoto et al. 2009; Dias et al. 2014). On the other hand, a variety of anti-carcinogenic and anti-inflammatory phytochemicals have been suggested to confer cell protection by suppressing this transcription factor signalling pathway (Surh et al. 2001). Hence, the ability of RWPS to target NF-κB1 and down-regulate its gene expression even after the transformations taking place during digestion until reaching the colon was a promising result supporting the chemopreventive potential of this seasoning against colorectal cancer.

Several dietary polyphenols have been also proposed to limit cancer initiation and progression in normal and premalignant tissues by activating the Nrf2 signalling pathway, as this is an effective strategy to induce several antioxidant and phase II detoxifying enzymes with antioxidant response elements (AREs) in their regulatory regions (Surh et al. 2008; Zhao et al. 2010; Kou et al. 2013). However, over-expression and constitutive hyper-activation of Nrf2 at late stages of carcinogenesis has been observed as this adaptive response can protect cancerous cells from chemotherapeutic agents and facilitate cancer progression (Gañan-Gómez et al. 2013; Kansanen et al. 2013). In the present study, no significant effect on the gene expression of Nrf2 was detected for any of the treatments (**Figure 1**B).

The expression of down-stream genes of these redox-sensitive transcription factors was assessed to gain insight into the indirect effects mediated by RWPS in colorectal cancerous cells. HO-1, SODs and CAT are key cytoprotective/antioxidant enzymes regulated by both the NF-κB (Pahl 1999; Kinnula & Crapo 2004; Morgan & Liu 2011) and the Nrf2 (Acharya et al. 2010; Glasauer & Chandel 2014; Hayes & Dinkova-Kostova 2014) pathways. On the other hand, genes encoding RONS generating enzymes, including NOX1, which has been associated with activation of the NF-κB pathway in colorectal cancer (Wang et al. 2011), and two well-known targets of this transcription factor such as COX2 and iNOS (Kojima et al. 2004; Federico et al. 2007), were also investigated.

The results showed that HO-1 was slightly up-regulated ($p < 0.05$) by all the RWPS derived fractions, ranging from 1.5-fold (GIDD) to 1.2-fold (CF) of the control (C) HT-29 cells (**Figure 2**A). HO-1 has been described as one of the main targets of dietary antioxidants involved in their cytoprotective effects in various cell types (Ogborne et al. 2004), showing a great potential in chemoprevention. Therefore, the basal oxidative stress conditions of colorectal cancerous cells could be alleviated by the RWPS-mediated induction of HO-1, with this ability being higher pre- than post-colonic fermentation, similarly as described above for the attenuation of lipid peroxidation.

The results obtained for SOD2, SOD1 and CAT showed the significant down-regulation of these genes in HT-29 cells pre-incubated with the UD fraction, mainly for SOD2 (0.75-fold of C cells), but not with any of the digested fractions (**Figure 2**C-D).

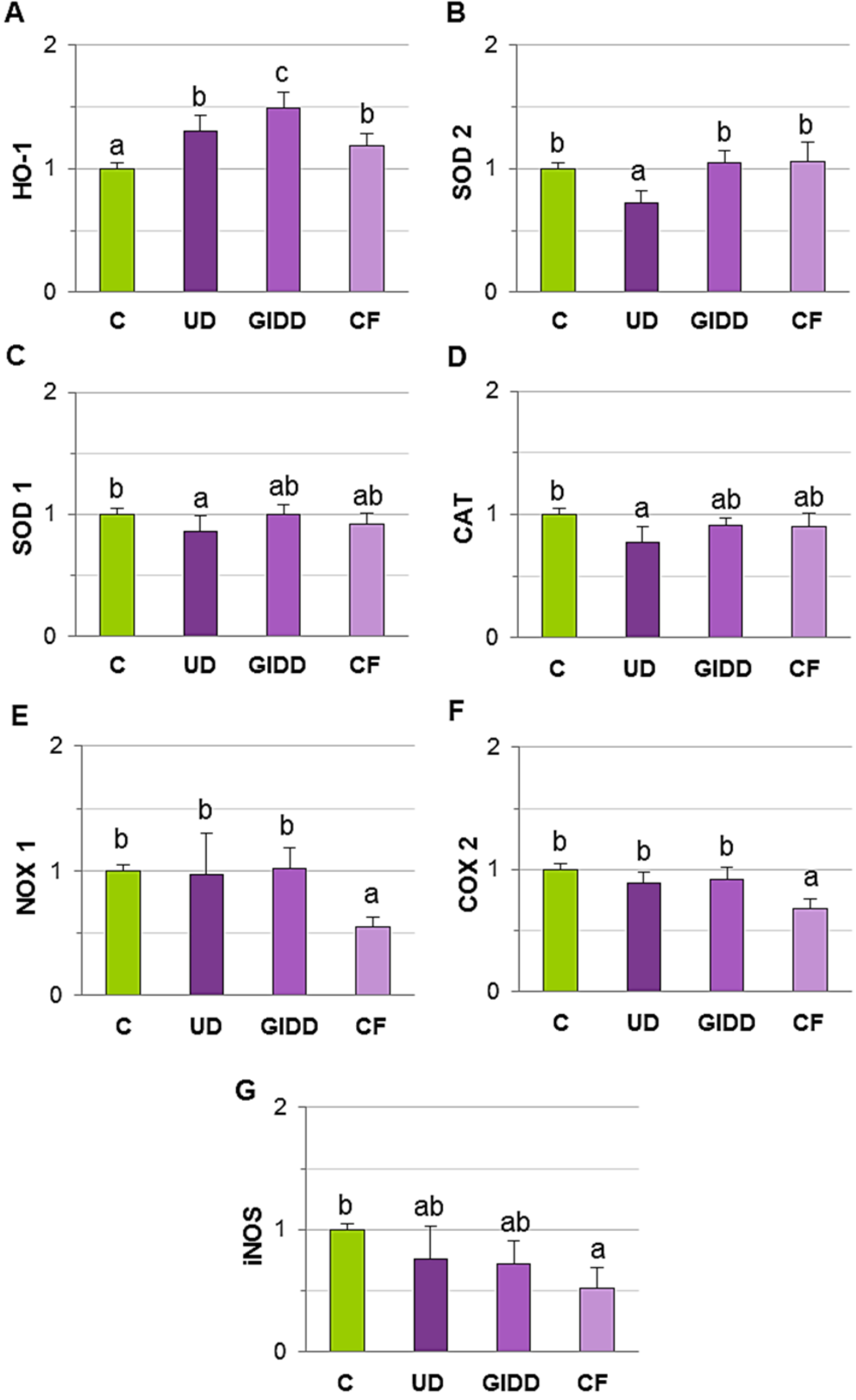

Figure 2. **Gene expression of pro-oxidant and antioxidant enzymes in human colorectal cancerous HT-29 cells treated with RWPS under basal conditions of oxidative stress.** Genes analysed: **A**) heme oxygenase-1 (HO-1), **B**) superoxide dismutase-2 (SOD2), **C**) superoxide dismutase-1 (SOD1), **D**) catalase (CAT), **E**) β-nicotinamide adenine dinucleotide phosphate oxidase-1 (NOX1), **F**) cyclooxygenase-2 (COX2), **G**) inducible nitric oxide synthase (iNOS). Cells were treated with the undigested (UD), gastrointestinal digested+dialysed (GIDD), and colonic fermented (CF) fractions derived from a seedless red wine pomace seasoning (RWPS) submitted to *in vitro* digestion. mRNA levels of the genes studied were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the control (C) HT-29 cells. Data expressed as mean values ± standard deviation (n=3). Roman letters show significant differences (ANOVA, $p<0.05$) between cells subjected to the different treatments.

Following the action of colonic microbiota, the bioactive compounds derived from RWPS could also attenuate oxidative stress through their ability to down-regulate the main RONS producing enzymes in the gut, as their mRNA levels in the CF-treated HT-29 cells were around 0.5-fold (NOX1 and iNOS) and 0.7-fold (COX2) of those in C cells (**Figure 2**E-G). Over-expression of all these pro-oxidant enzymes in colon carcinoma tissues has been detected and related to the constitutive activation of NF-κB (Kojima et al. 2004; Wang et al. 2011), which remarks the potential interest of RWPS intake in chemoprevention against colorectal cancer. Given that all the fractions decreased NF-kB expression but only CF was capable to down-regulate the RONS-generating enzymes, it is possible that the RWPS-derived colonic metabolites were more effective blocking the activation of the NF-κB pathway, ability previously described for phenolic acids such as some anthocyanin metabolites (Forester et al. 2014) and for SCFAs (Yin et al. 2001; Tedelind et al. 2007). Our results were consistent with the COX2 and iNOS down-regulation observed in colon tumours of rats supplemented with polyphenols extracted with red wine (Luceri et al. 2002) and the decreased iNOS activity detected in colon tissues of rats with ulcerative colitis treated with proanthocyanidins from grape seeds (Wang et al. 2010).

3.2. Protective effects of RWPS against exogenous pro-oxidant insult in colorectal cancerous cells

3.2.1. Changes on oxidative stress biomarkers of HT-29 cells exposed to *t*-BOOH

The potential cytoprotective actions of RWPS against such exogenous pro-oxidant conditions in the colon were assessed by the exposure of HT-29 cells to *t*-BOOH, a frequently used compound to induce pharmacological oxidative stress (Goya et al. 2009).

The oxidative insult significantly enhanced cell membrane damage (2.3-fold), lipid peroxidation (3.5-fold), and protein oxidation (3.1-fold) in the CO compared to the C colonic cancerous cells, but all the treatments succeed to protect cells ($p < 0.05$) against the induced oxidative injury (**Table 2**). The GIDD fraction showed the highest efficacy to reduce cell membrane damage and lipid peroxidation (19% lower LDH leakage and 40% lower MDA levels than the CO cells, respectively), while the UD and CF fractions were slightly less protective, obtaining similar results between them (13% lower LDH leakage and 31% lower MDA levels than the CO cells, respectively). In line with our results, protective effects of gallic acid (Pandurangan et al. 2015) and grape seed proanthocyanidins (Li et al. 2008; Wang et al. 2010) supplementation against oxidative insults leading to lipid peroxidation in experimental models of ulcerative colitis have been previously described. Attenuation of the *t*-BOOH-induced protein oxidation was also detected for all the treated cells, showing around 16% significantly lower CG levels in comparison with the CO cells. Therefore, under an oxidative insult, the protective antioxidant effects of RWPS pre- and post-digestion in cell proteins of colonic cancerous cells may predominate over the pro-oxidant outcomes detected under basal conditions.

Table 2. Biomarkers of oxidative stress in colonic cancerous cells submitted to an exogenous oxidative insult.

Oxidative Stress Biomarkers	C [a]	O C [b]	RWPS fractions [c]		
			O UD	O GIDD	O CF
Cell membrane damage (% LDH released of total LDH)	37.8 ± 1.5 a	85.3 ± 1.7 d	73.9 ± 3.6 c	69.3 ± 3.9 b	74.7 ± 2.1 c
Lipid peroxidation (nmol of MDA equivalents / mg of protein)	2.75 ± 0.39 a	9.11 ± 0.41 d	6.36 ± 0.44 c	5.56 ± 0.48 b	6.41 ± 0.57 c
Protein oxidation (nmol of CGs / mg of protein)	13.5 ± 1.2 a	39.9 ± 0.5 c	34.0 ± 1.3 b	32.4 ± 1.5 b	34.1 ± 1.1 b

Results represented as mean values ± standard deviation.
LDH: Lactate dehydrogenase; MDA: malondialdehyde; CGs: carbonyl groups.
a) Control (C) HT-29 cells.
b) Oxidised control (O C) HT-29 cells. The oxidative insult consisted on 250 µM of tert-butyl hydroperoxide.
c) Oxidised HT-29 cells pre-incubated for 24 h with the red wine pomace seasoning (RWPS) derived fractions: undigested (O UD), gastrointestinal digested+dialysed (O GIDD), and colonic fermented (O CF).
Roman letters indicate significant differences (p<0.05) between the treatments (C, OC, OUD, OGIDD, and OCF).

Altogether, these findings clearly showed the chemopreventive potential of RWPS as a blocking agent against exogenous pro-oxidant insults that act by inducing lipid peroxidation and subsequent damage to other biomolecules (Goya et al. 2009; Van der Paal et al. 2016), with the transformations suffered during digestion in the upper gut even potentiating the efficacy of its bioactive compounds, although colonic fermentation might slightly attenuate such antioxidant bioactivities. These protective effects are of great interest as may help to delay cancer promotion and progression. Nonetheless, it should be noted once again their possible antagonistic actions with certain chemotherapeutic treatments, which must be always considered (Valko et al. 2006; Glasauer & Chandel 2014).

3.1.2. Indirect mechanisms of action implicated in the protection against oxidative stress in HT-29 cells exposed to *t*-BOOH

The exposure to *t*-BOOH significantly down-regulated NF-κB (0.76-fold of C) and induced the gene expression of the Nrf2 (2.7-fold of C) in the oxidised control (O C) cells (**Figure 3**A and B, respectively), which evidenced that colon cancerous cells may response to exogenous oxidative stress insults by up-regulating their antioxidant defence system (Kansanen et al. 2013; Lau et al. 2008; Hayes & Dinkova-Kostova 2014) and simultaneously repressing the main pro-oxidant and pro-inflamatory pathway (Bubici et al. 2006). In this context, the crosstalk regulation between the NF-κB and Nrf2 networks proposed in recent years should be underlined (Buelna-Chontal & Zazueta 2013). The interplay between these networks is tumour and cell type-specific, which can strongly influence the outcomes of chemopreventive and therapeutic interventions (Bellezza et al. 2010), with our results suggesting that it might take place in the oxidised HT-29 cells.

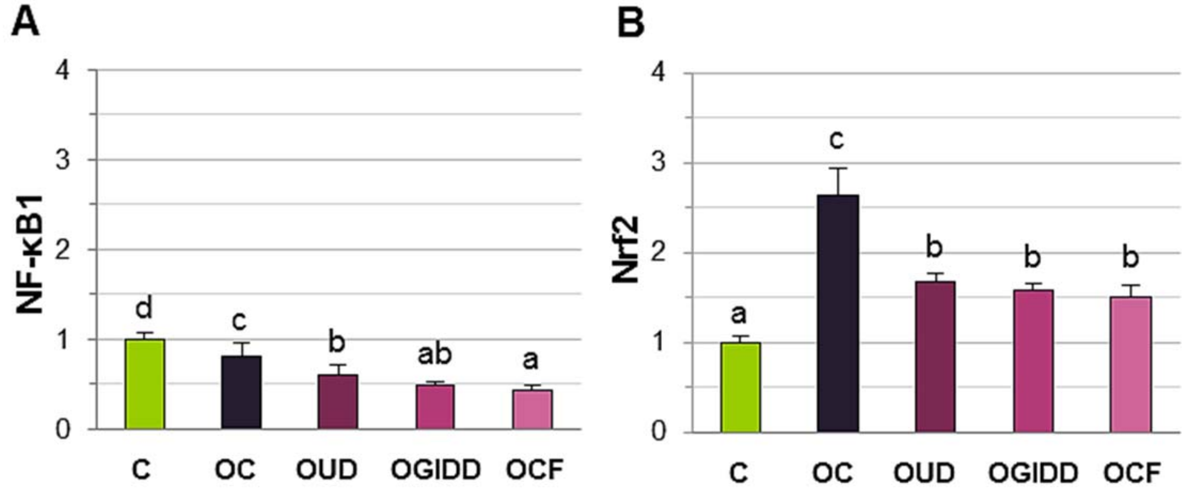

Figure 3. **Gene expression of redox-sensitive transcription factors in human colorectal cancerous HT-29 cells treated with RWPS and exposed to *t*-BOOH.** Genes analysed: **A**) nuclear factor-kappa B 1 (NF-κB1), **B**) nuclear factor-erythroid 2-related factor 2 (Nrf2). Cells were pre-incubated for 24 h with *in vitro* digested fractions derived from a seedless red wine pomace seasoning (RWPS), and then challenged with *tert*-butylhydroperoxide (*t*-BOOH) as a pro-oxidant agent. Control cells (C), Oxidised control cells (O C), Oxidised cells pre-incubated with the undigested fraction of RWPS (O UD), Oxidised cells pre-incubated with the gastrointestinal digested+dialysed fraction of RWPS (GIDD), Oxidised cells pre-incubated with the colonic fermented fraction of RWPS (CF). mRNA levels of the genes evaluated were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the C cells. Data expressed as mean values ± standard deviation (n=3). Roman letters point out significant differences (ANOVA, $p<0.05$) between cells submitted to the different treatments.

Interestingly, pre-incubation of oxidised cells with RWPS-derived fractions reduced ($p<0.001$) NF-κB1 mRNA levels to even lower values than those detected for the O C cells, ranging between 0.59-fold (O UD) and 0.43-fold of C (O CF) (**Figure 3**A). These results were in line with the ability of RWPS pre- and post-digestion to decrease the gene expression of NF-κB1 under oxidative basal conditions although, when the oxidative stress was induced, the bioactive compounds generated after the action of colonic microbiota might be slightly more effective down-regulating NF-κB1 than their precursors. In addition, all the treatments were able to attenuate the over-expression of Nrf2 detected in O C cells to around 1.6-fold of C (**Figure 3**B), indicating a lower up-regulation and probably activation of this pathway, which is in agreement with the less oxidative environment that might be present in the treated HT-29 cells due to the protective effects of the treatments.

A similar pattern as the modulation observed for the Nrf2 transcription factor was detected for some of its targeted cytoprotective genes. In the oxidised HT-29 cells, both the HO-1 and SOD2 genes were significantly up-regulated 10.7-fold (**Figure 4**A) and 2.5-fold of C (**Figure 4**B), respectively, whereas significant reductions (ranging 50%) of the mRNA levels of these genes were observed for all the RWPS-treated cells compared to the respective O C cells. The large number of AREs contained in the HO-1 enhancers can explain the high sensitivity of this gene to oxidative stress (Alam & Cook 2003). Previous studies have indicated the

interest of combining anti-cancer agents that act as RONS inducers with phytochemicals that decrease the antioxidant response of colorectal cancerous cells to such oxidative stress by inhibiting HO-1 expression and Nrf2 translocation into the nucleus (Lee et al. 2012; Yin et al. 2014; Furfaro et al. 2015). RWPS could thus promote the susceptibility of colorectal cancer cells to such type of chemotherapies in a similar way, but our results also evidenced the cytoprotective final outcome against oxidative stress in the RWPS-treated cells which, as aforementioned, could have just the opposite effect in combination with pro-oxidant therapies (Glasauer & Chandel 2014).

A different gene expression modulation was found for SOD1 (cytosolic) with respect to SOD2 (mitochondrial), showing that the cytosolic isoform might be up-regulated by the oxidative insult and also by the RWPS digested fractions, particularly after the action of colonic microbiota (**Figure 4**C). On the other hand, the expression of CAT was significantly down-regulated around 0.75-fold of C for both the control and RWPS-treated oxidised cells (**Figure 4**D). Hence, these results suggested that the enzymatic activity of SODs might be inhibited, thus producing low amounts of H_2O_2, although it is also probable that both the oxidative insult and the treatments were able to up-regulate other enzymes with peroxidase activity such as some glutathione peroxidases that are also targeted by the Nrf2 pathway (Brigelius-Flohé & Kipp 2009).

In parallel with the antioxidant response induced by the *t*-BOOH insult, NOX1 was significantly down-regulated in the O C cells (0.65-fold of C) (**Figure 4**, E), which could be implicated in the inhibition of the NF-κB pathway (Wang et al. 2011). In contrast, remarkable up-regulation (p <0.001) of COX2 (11-fold of C) and iNOS (17-fold of C) gene expression was detected (**Figure 4**, F and G, respectively), which certainly contributed to generate the pro-oxidant redox imbalance that resulted in the oxidative damage to biomolecules and membranes observed in the O C cells. Interestingly, the RWPS-derived fractions showed a great potential to hinder the gene expression of all these RONS generating enzymes compared with the O C cells. The mRNA levels of iNOS were particularly decreased, with the UD fraction achieving almost a complete inhibition of the *t*-BOOH-induced over-expression of this gene. With regards to NOX1 and COX2 genes, the colonic fermented metabolites were the most effective down-regulators of their expression.

The observed reduction in the gene expression of NF-κB1, along with the above-mentioned possible decreased activation of the NF-κB pathway and other pro-oxidant stimulus prompted by the compounds present in the CF fraction (Forester et al. 2014; Yin et al. 2001; Tedelind et al. 2007), might likely be involved in the observed down-regulation of RONS generated enzymes. These results were generally in accordance with those obtained under basal conditions but cannot explain the higher protective effect against t-BOOH-induced lipid peroxidation observed for the RWPS-derived compounds pre- than post-colonic digestion. Therefore, the implication of other antioxidant defences such as glutathione and its related enzymes, many of which are also modulated by Nrf2 (Kulinsky & Kolesnichenko 2009), and of other redox-sensitive signalling pathways, is suggested.

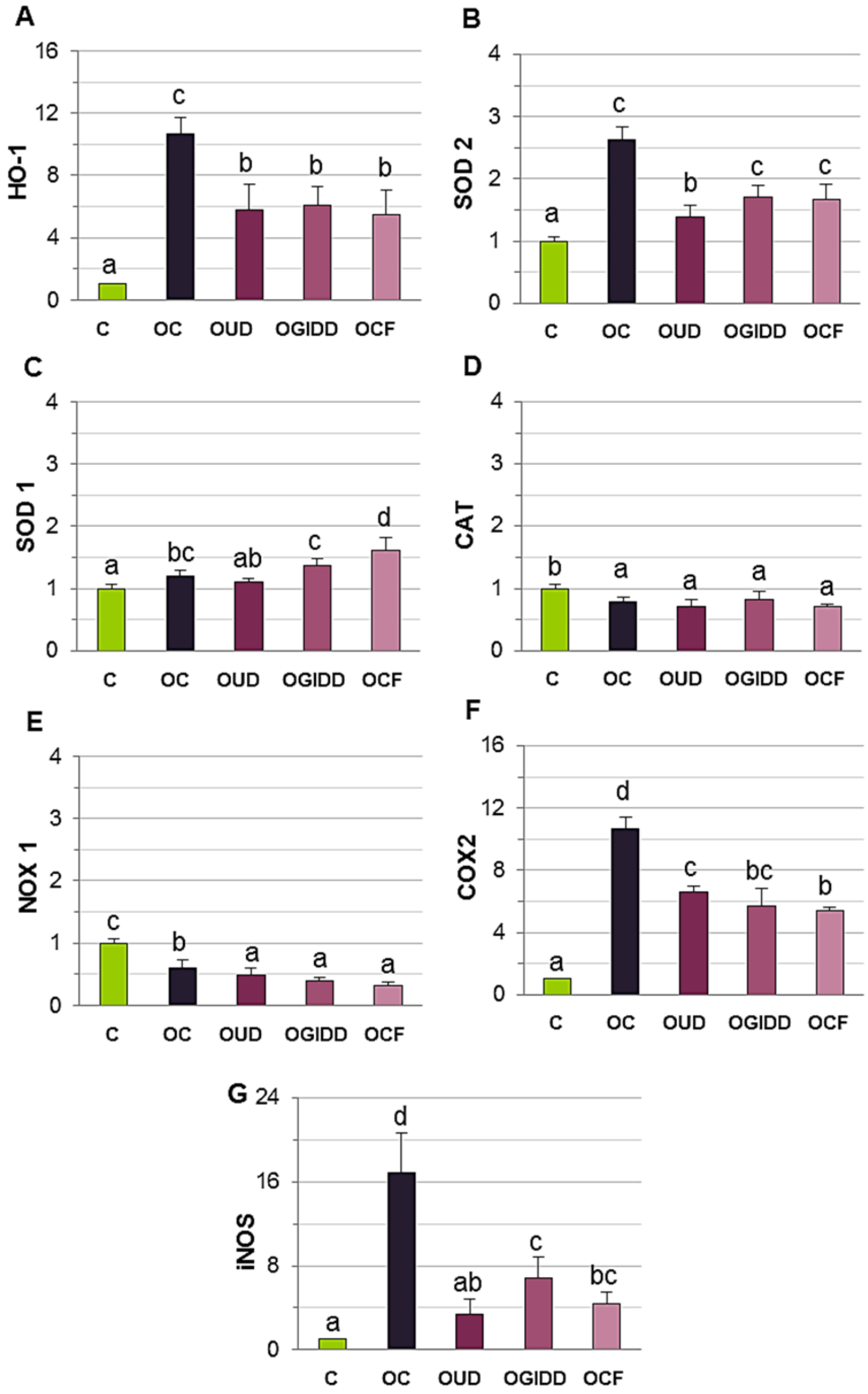

Figure 4. Gene expression of pro-oxidant and antioxidant enzymes in human colorectal cancerous HT-29 cells treated with RWPS and exposed to *t*-BOOH. Genes analysed: **A**) heme oxygenase-1 (HO-1), **B**) superoxide dismutase-2 (SOD2), **C**) superoxide dismutase-1 (SOD1), **D**) catalase (CAT), **E**) β-nicotinamide adenine dinucleotide phosphate oxidase-1 (NOX1), **F**) cyclooxygenase-2 (COX2), **G**) inducible nitric oxide synthase (iNOS). Cells were pre-incubated for 24 h with *in vitro* digested fractions derived from a seedless red wine pomace seasoning (RWPS), and then challenged with *tert*-butylhydroperoxide (*t*-BOOH) as a pro-oxidant agent. Control cells (C), Oxidised control cells (O C), Oxidised cells pre-incubated with the undigested fraction of RWPS (O UD), Oxidised cells pre-incubated with the gastrointestinal digested+dialysed fraction of RWPS (GIDD), Oxidised cells pre-incubated with the colonic fermented fraction of RWPS (CF). mRNA levels of the genes analysed were determined by quantitative real-time PCR, normalized to the GAPDH gene expression. The results are presented as relative fold changes with respect to the C cells. Data expressed as mean values ± standard deviation (n=3). Roman letters indicate significant differences (ANOVA, $p<0.05$) between cells exposed to the different treatments.

4. Conclusions

A new seasoning obtained from red wine pomace (RWPS), both pre- and post-simulated digestion, showed interesting cytoprotective effects in colorectal cancerous cells against their biomolecule oxidative damage, preventing lipid peroxidation under basal conditions, and also protein and membranes oxidative injury under exogenous pro-oxidant insults. A broad overview of several indirect antioxidant mechanisms by which RWPS may induce such protective effects is provided, evidencing the gene modulation of the main redox-sensitive transcription factors and their down-stream genes. Down-regulation of NF-κB gene expression was found as a common mechanism for all the RWPS-derived fractions and under both oxidative stress situations, observing also a lower Nrf2-mediated antioxidant response against the *t*-BOOH-induced oxidative stress in those cells pre-incubated with the treatments. Our findings also suggest the implication of additional transcription factors and endogenous antioxidant systems, which should be investigated in future studies, along with the positive or negative interactions between the intake of RWPS and anti-cancer therapeutic treatments acting thought pro-oxidant mechanisms. Even though, the antioxidant effects and mechanisms identified in the present study suggest the chemopreventive potential of RWPS as a tumour-blocking agent against colorectal carcinogenesis.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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Concluding Remarks / Conclusiones

Concluding Remarks

The most important conclusions drawn from the overall analysis of the results obtained in this PhD Thesis, focussed on the study of new seasonings (RWPSs) proposed as a solution for value-adding wine pomace, are the following:

1) RWPSs display high total antioxidant capacity *in vitro*, of interest for their use as functional food ingredients. The heat treatment applied to stabilize RWPSs induces significant changes in their phenolic profile, but their antioxidant capacity is not severely affected, with the seasoning obtained from seedless wine pomace being the most stable to such processing.

2) The antioxidant potential of the RWPSs is increased as a result of the digestive process. Specifically, after simulating this process, the seasoning obtained from seedless wine pomace shows the highest digestibility and could have greater potential to exert bioactive effects systemically.

3) The bioavailability of the phenolic compounds of the seedless RWPS in Wistar rats, after acute intake, is evidenced by the increase of phenolic acids in plasma and urine, observing also an improvement in biomarkers of oxidative stress and endothelial function. However, in healthy animals, these effects appear to be transient, not detecting significant changes in the biomarkers after 4 weeks of supplementation.

4) The seedless RWPS presents an interesting potential to exert beneficial effects against endothelial dysfunction and oxidative stress, characteristic pathophysiological states of diseases such as Type 1 diabetes and essential hypertension. This potential has been demonstrated in cell cultures and RWPS-supplemented animal models, observing gene expression changes in antioxidant enzymes deregulated during these pathologies and improvements in the characteristic symptoms and biomarkers assessed.

5) The RWPSs, both before and after simulating digestion, exhibit anti-proliferative, anti-genotoxic and antioxidant activities in colorectal cancer cells (HT-29), which demonstrates their potential in chemoprevention as tumour-suppressing and tumour-blocking agents.

In short, the main goal of this PhD Thesis has been achieved, since the results obtained permit adding value to the new seasonings obtained from winery by-products, especially the RWPS derived from seedless wine pomace, supporting its potential benefits against Type 1 diabetes, essential hypertension, and colorectal cancer.

Conclusiones Generales

Las conclusiones más importantes que se deducen del análisis global de los resultados obtenidos en esta Tesis Doctoral, en la que se han estudiado unos nuevos sazonadores (RWPSs) propuestos como solución para el aprovechamiento de los subproductos de vinificación, son las siguientes:

1) Los RWPSs presentan una elevada capacidad antioxidante total *in vitro*, de interés para su aplicación como ingredientes alimentarios funcionales. El tratamiento térmico aplicado para estabilizar los RWPSs induce cambios significativos en su perfil fenólico, pero su capacidad antioxidante no se ve severamente afectada, siendo el sazonador obtenido a partir de hollejos el más estable frente a dicho procesamiento.

2) El potencial antioxidante de los RWPSs se ve aumentado como resultado del proceso digestivo. En concreto, tras simular dicho proceso, el sazonador de hollejos parece ser el más digestible y podría tener un mayor potencial bioactivo a nivel sistémico.

3) La biodisponibilidad de los compuestos fenólicos del sazonador derivado de hollejos en ratas Wistar, tras su ingesta aguda, queda reflejada en el aumento de ácidos fenólicos en plasma y orina, observándose también una mejora en biomarcadores de estrés oxidativo y de función endotelial. Sin embargo, en animales sanos, estos efectos parecen ser transitorios, no detectándose cambios significativos en los biomarcadores tras 4 semanas de suplementación.

4) El sazonador derivado de hollejos presenta un interesante potencial para ejercer efectos beneficiosos frente a la disfunción endotelial y el estrés oxidativo, estados fisiopatológicos característicos de enfermedades como la diabetes Tipo 1 y la hipertensión esencial. Dicho potencial se ha comprobado en estudios en cultivos celulares y en modelos animales suplementados con el sazonador, observándose cambios en la expresión génica de enzimas antioxidantes desreguladas durante estas patologías y mejoras en los síntomas característicos y biomarcadores analizados.

5) Los RWPSs, tanto antes como después de simular su digestión, presentan actividad anti-proliferativa, anti-genotóxica y antioxidante en células de cáncer colorectal (HT-29), lo que evidencia su potencial quimiopreventivo como agentes supresores y bloqueantes de tumores.

En definitiva, se ha alcanzado el objetivo principal de esta Tesis Doctoral, ya que los resultados obtenidos permiten revalorizar los sazonadores obtenidos a partir de residuos de vinificación, en especial el derivado de los hollejos, avalando su potencial beneficioso frente a la diabetes Tipo 1, la hipertensión esencial, y el cáncer colorrectal.

Transference of Research Results and Future Research

TRANSFERENCE OF RESEARCH RESULTS

First it should be noted that the industrial property rights of these new vegetal seasonings, which include both their concept and manufacturing process, have been protected from the initial stages of this study by submitting on 12 June 2013 a national patent application (González-Sanjosé et al. 2013) to the the Spanish Office of Patents and Trademarkss (Oficina Española de Patentes y Marcas - OEPM). Following a premilimary examination, the patent was granted on 23 June 2015. The property rights are hold by the University of Burgos.

Preliminary cost/benefit analyses have proven the technical and economical feasibility of RWPS production at industrial scale. This project would present assumable risks that could be reasonably mitigated at initial and advances phases (industrial implementation and exploitation).

Main features gathered by the manufacturing process of RWPSs which are desirable for the industry are:

- Small initial investment.
- Minimally processed products
- Easy scalability for industrial production.
- Low time, energy and water consumption during operation.
- None extraction or use of organic solvents involved in the process.

Therefore, RWPSs are a novel alternative for wineries to the traditional consideration of wine pomace as a residue. These new seasonings present interesting advantages over other wine pomace valorization options and allow value-adding this winery by-product through an environmentally friendly and cost-efficient process.

A complete and customized feasibility analysis should be carried out to ensure the success of the exploitation of RWPSs by each specific industry interested in their manufacturing and commercialization. The characteristics of these products, such as the wine pomace constituent used as raw material, final particle size, type of process used as stabilization step, etc. should be also set and checked for each specific situation. Nonetheless, these new seasonings show several potential industrial applications:

RWPSs could be produced directly in wineries willing to invest in innovation, which could sell these new seasonings to potential intermediate customers, health food stores, and also to tourists if the wineries offer wine routes.

For wineries not interested in their own exploitation of wine pomace, a collection system could be established to deriver this by-product to other specialised or diversified companies in charge of RWPS production. For example, destileries might be interested in diversifying their lines of business as they already received wine pomace collected from wineries.

Given that RWPSs have proven antioxidant and antimicrobial effectiveness (García-Lomillo et al. 2014), before and after digestion (Del Pino-García et al. 2016b), the food industry could use these innovative products as natural functional ingredients for their incorporation in foods. From a healthy perspective, RWPSs are rich sources of several potential bioactive compounds once ingested (mainly polyphenols, dietary fibre and potassium), thus being also products of interest for the nutraceutical industry.

In this regard, if RWPS reach the market, highlighting their nutritional value and potential healthful properties through suitable marketing campaigns could be of great help to ensure their acceptance by consumers and their final success. Nutrition and health claims are important messages that can be communicated to consumers through product labelling and enable them to make an informed choice at the point of purchase. These types of labelliing and claims also bring benefits to industrial brands and increase competitiveness in the market place. Nonetheless, there are a number of challenges for industries dealing with regulatory framework around claims substantiation, especially with those related with health, as they must be approved by the national and/or international authorities in charge of nutrition and health issues. In the case of European countries, the EFSA is in charge of these authorizations, following the Regulation EC 1924/2006 on nutrition and health claims made on foods (European Parliament & Council 2006), and subsequent amendments and corrections.

To use a nutritional claim, it is required a complete nutritional characterization of the final product. In this regard, according to the composition of each RWPS (seedless: Sk-S; whole: W-S; seeds: Sd-S), they could fit well within the following nutritional claims: 'natural' (all the RWPSs), 'source of fibre' (all the RWPSs), and 'source of potassium' (Sk-S and W-S). However, there are not health claims approved for grape or wine pomace dietary fibre, neither for their polyphenols, and new health claims are really complicated to obtain. Hence, despite the RWPS obtained from seedless red wine pomace, which has been studied in more detail, could have beneficial effects in cases of diseases related with oxidative stress and endothelial dysfunction, these potential benefits cannot be used in its labelling and marketing.

If any company would be interested in using a health claim for marketing RWPS, much further studies and clinical trials should be performed following the guidance provide by the EFSA Panel of Dietetic Products, Nutrition and Allergies (NDA) about the scientific requirements for health claims related to: antioxidants, oxidative damage and cardiovascular health (EFSA NDA Panel 2011); appetite ratings, weight management, and blood glucose concentrations (EFSA NDA Panel 2012); and immune system, the gastrointestinal tract and defence against pathogenic microorganisms (EFSA NDA Panel 2016), among others.

FUTURE RESEARCH

Future studies proposed to continue the research initiated in this PhD Thesis include:

- Complete the study of the functional effects of the seasonings by assessing other biomarkers of oxidative stress and endothelial dysfunction. Concretely, it could be of great interest: determining the protection against DNA damage by analysing 8-hydroxylated guanine species (both *ex vivo* and *in vivo*); evaluating the implication of the GSH and Trx systems in the protection against oxidative damage; and, assessing endothelial function by measuring the contraction/dilation of aortic ring segments and by evaluating flow-mediated dilation (FMD) *in vivo*. Furthermore, it could be interesting in future work to study the modulation of antioxidant responses at the level of protein expression and enzymatic activity.

- Study in more depth the therapeutic potential of the seasonings when used as dietary supplements in future controlled clinical trials with human patients, in order to obtain consistent evidence about their beneficial effects against the pathologies investigated in this PhD Thesis or other cardiovascular diseases associated with oxidative stress and endothelial dysfuntion.

- Evaluate *in vivo* the chemopreventive effects of the seasonings against colorectal cancer or other gastrointestinal pathologies are encouraged as future research directions.

- From the industrial perspective, the results obtained in this PhD Thesis suggest the potential use of the new seasonings by the food industry as antioxidant and functional dietary ingredients, which could be included in the formulation of several processed foods, such as meat, bakery and dairy products, with special interest in low-salt and functional foodstuffs. In this regard, the sensorial and technological properties of the final products will need to be optimized, taking into account their influence in the colour, aroma, flavour, bitterness, astringency, texture, and other properties of the final products.

  The findings of this PhD Thesis also evidence the potential use of the seasonings by the nutraceutical industry as dietary supplements.

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