University of São Paulo
"Luiz de Queiroz" College of Agriculture

Hurdles and potentials in value-added use of peanut and grape by-products as sources of phenolic compounds

Adriano Costa de Camargo

Thesis presented to obtain the degree of Doctor in Science.
Area: Food Science and Technology

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Adriano Costa de Camargo
Bachelor of Food Science

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This work is decidated to family, friends, and mentors,
whose support was everything I needed to make my dreams come true.

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CONTENTS

RESUMO

Desafios e potencialidades na agregação de valor a subprodutos da agroindústria do amendoim e da uva como fonte de compostos fenólicos

Estudos recentes têm demonstrado que subprodutos da indústria processadora de amendoim e uva podem ser mais ricos em compostos bioativos em comparação às suas matérias-primas. No entanto, alguns desafios tecnológicos precisam ser enfrentados antes da sua aplicação como fonte de compostos nutracêuticos ou na prevenção da oxidação lipídica em sistemas alimentares. Este estudo discute os recentes avanços na aplicação de subprodutos da indústria processadora de amendoim e uva como fontes de compostos fenólicos. Especial ênfase foi dada a sua caracterização por cromatografia líquida acoplada à espectrometria de massas, aos potenciais benefícios à saúde e à segurança microbiológica. As principais conclusões estão apresentadas nos capítulos 2, 3 e 4. O primeiro capítulo trata de compostos bioativos de subprodutos da indústria de suco de uva e da produção vinícola. A fração da qual foram extraídos os compostos fenólicos ligados à parede celular foi predominante. Em geral, esta fração também foi a mais eficaz na inibição da oxidação do LDL - colesterol *in vitro* quando comparada à fração que continha os fenólicos livres e os esterificados. Os compostos fenólicos de todas as frações inibiram o dano oxidativo ao DNA induzido por radicais peroxila. O terceiro capítulo fala sobre os efeitos da irradiação gama sobre a carga microbiana, a composição fenólica e as propriedades antioxidantes da película de amendoim. A irradiação gama (5,0 kGy) diminuiu a contagem microbiana do produto. Os compostos fenólicos totais, o teor de proantocianidinas e a capacidade dos extratos em neutralizar radicais como o ABTS, DPPH e espécies reativas de oxigênio como o peróxido de hidrogênio e radicais hidroxila, assim como o poder redutor da amostra, aumentaram devido à irradiação gama em ambas as frações (contendo fenólicos livres e ligados à parede celular). A bioatividade dos compostos fenólicos livres contra a oxidação do LDL-colesterol *in vitro* e contra os danos oxidativos ao DNA aumentou com a irradiação gama. Os compostos fenólicos foram positivamente ou tentativamente identificados, distribuindo-se entre: fenólicos livres > esterificados > ligados. Houve aumento na concentração de dímeros de procianidina A em todas as frações, enquanto a concentração de dímeros de procianidina B diminuiu. Essas alterações podem ser explicadas pela conversão molecular, despolimerização e formação de ligações cruzadas. No quarto e último capítulo, enzimas selecionadas foram aplicadas à matéria-prima inicial (experimento I) ou nos resíduos contendo apenas compostos fenólicos insolúveis (experimento II). Pronase e Viscozyme aumentaram a extração de compostos fenólicos insolúveis (ligados à parede celular). Viscozyme liberou maiores quantidades de ácido gálico, catequina e dímero de prodelfinidina A em comparação ao tratamento com Pronase. Além disso, os ácidos *p*-cumárico e ácido caféico, bem como o dímero de procianidina B, foram extraídos com Viscozyme, mas não com Pronase. A solubilidade desempenha um papel importante na biodisponibilidade de compostos fenólicos. Desta forma, o terceiro estudo oferece uma alternativa para a exploração de compostos fenólicos de subprodutos da indústria vinícola como ingredientes alimentares com propriedades funcionais ou suplementos alimentares.

Palavras-chave: Segurança microbiológica; Irradiação gama; Ácidos fenólicos; Flavonóides; Proantocianidinas; Bioatividade; Extração enzimática

ABSTRACT

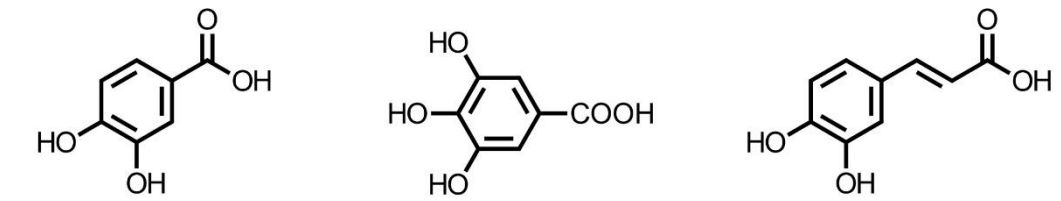
Challenges and potentials in value-added use of peanut and grape by-products as sources of phenolic compounds

Recent studies have demonstrated that peanut and grape processing by-products may be richer sources of bioactive compounds as compared to their original raw material and feedstock; however, before their application as a source of nutraceuticals or in the prevention of lipid oxidation in food systems, certain technological challenges have to be addressed. This study discusses recent advances in the application of plant food processing by-products as sources of phenolic compounds with special emphasis on the profiling and screening of phenolics using high-performance liquid chromatography-mass spectrometry, their potential health benefits, and microbiologial safety. The major findings are summarized in chapters 2, 3, and 4. The first chapter deals with phenolics from grape by-products. In general, insoluble-bound phenolics were more effective in inhibiting copper-induced human LDL-cholesterol oxidation *in vitro* than free and esterified phenolics. Phenolic extracts from all fractions inhibited peroxyl radical-induced DNA strand breakage. The third chapter brings about the effects of gamma-irradiation on the microbial growth, phenolic composition, and antioxidant properties of peanut skin. Gamma-irradiation at 5.0 kGy decreased the microbiological count of the product. Total phenolic and proanthocyanidin contents, ABTS radical cation, DPPH radical, hydrogen peroxide, and hydroxyl radical scavenging capacities as well as the reducing power of the sample were increased upon gamma-irradiation in both the free and insoluble-bound phenolic fractions. The bioactivity of the free phenolics against *in vitro* human LDL-cholesterol oxidation and copper induced DNA strand breakage was improved upon gamma-irradiation. Phenolic compounds were positively or tentatively identified and their distribution was in the decreasing order of free > esterified > insoluble-bound forms. Procyanidin dimer A was increased in all phenolic fractions, whereas procyanidin dimer B decreased. Gamma-irradiation induced changes may be explained by molecular conversion, depolymerization, and cross-linking. In the fourth chapter, the ability of selected enzymes in improving the extraction of insoluble-bound phenolics from the starting material (experiment I) or the residues containing insoluble-bound phenolics (experiment II) were evaluated. Pronase and Viscozyme improved the extraction of insoluble-bound phenolics. Viscozyme released higher amounts of gallic acid, catechin, and prodelphinidin dimer A compared to Pronase treatment. Furthermore, p-coumaric and caffeic acids, as well as procyanidin dimer B, were extracted with Viscozyme but not with Pronase treatment. Solubility plays an important role in the bioavailability of phenolic compounds, hence this study may assist in better exploitation of phenolics from winemaking by-products as functional food ingredients or supplements.

Keywords: Microbiological safety; Gamma-irradiation; Phenolic acids; Flavonoids; Proanthocyanidins; Bioactivity; Enzyme extraction

1 INTRODUCTION

The potential of plant food by-products as a source of phenolic compounds has been widely recognized. In particular, by-products from cereals, pulses, oilseeds, nuts, fresh and dried fruits, spices, coffee, and tea, to name a few, may be richer in different bioactive compounds than their original sources (SHAHIDI; AMBIGAIPALAN, 2015; CHANG; ALASALVAR; SHAHIDI, 2016). Peanut skin and grape by-products, for example, are rich in proanthocyanidins, also known as condensed tannins (DE CAMARGO et al., 2014b; DE CAMARGO et al., 2015), whereas pomegranate peels are rich in hydrolysable tannins (GARCÍA-VILLALBA et al., 2015). Citrus by-products have a high concentration of low molecular weight flavonoids (MOLINA-CALLE; PRIEGO-CAPOTE; DE CASTRO, 2015), and blueberry by-products are abundant in anthocyanins (AYOUB; DE CAMARGO; SHAHIDI, 2016; HE et al., 2016), while phenolic acids are prominent in wheat and other cereal and grain by-products (MARTINI et al., 2015). The basic structures of some common phenolic compounds are shown in Figures 1.1, 1.2, 1.3, and 1.4.



Protocatechuic acid Gallic acid Caffeic acid

Figure 1.1 – Chemical structures of major phenolic acids identified in peanut skin skin (DE CAMARGO et al., 2015) and grape by- products (DE CAMARGO et al., 2014a)

Phenolic compounds possess a myriad of health benefits. These secondary metabolites play an important role as antioxidants, scavengers of reactive species, reducers or chelators of metals ions (AYOUB; DE CAMARGO; SHAHIDI, 2016), as well as inhibitors of enzymes related to diabetes and obesity (DE CAMARGO et al., 2016a, 2015; SUN et al., 2016). Besides their health benefits, phenolics from natural resources are also being investigated for their potential in extending the shelf-life of fats and oils as well as lipid-containing foods (COMUNIAN et al., 2016; ALTUNKAYA et al., 2013; SELANI et al., 2016). Oxidation of lipids and its prevention has been of particular interest due to potential adverse effects of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ), and propyl gallate (PG). Maximum usage levels for these synthetic

antioxidants have recently been summarized (SHAHIDI; AMBIGAIPALAN, 2015). In contrast, no maximum levels have been proposed for phenolics from food by-products, which may be explained by their natural occurrence or lack of commercial availability. Even so, use of such products may happen but without a claim for their antioxidant potential as officially, only those compounds that have an RDI (required daily intake) may be used as antioxidants.

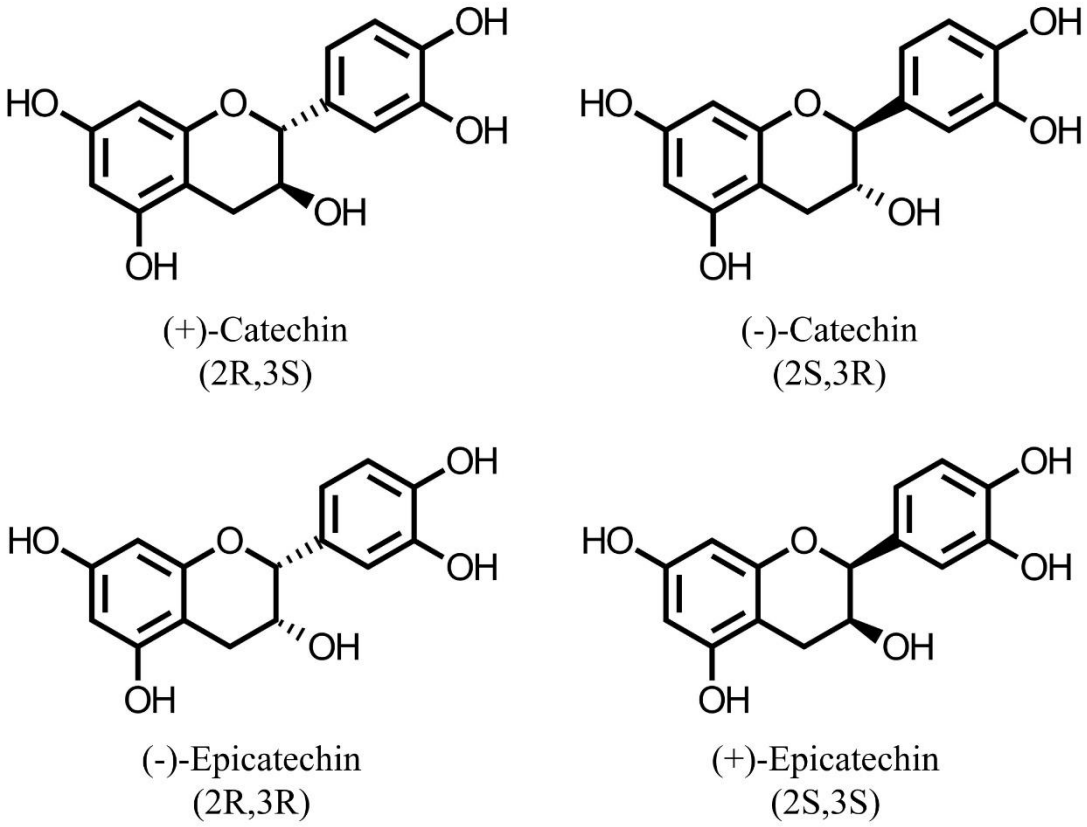


Figure 1.2 - Chemical structures of isomers of monomeric units of procyanidins (DE CAMARGO et al., 2014a; DE CAMARGO et al., 2015)

Based on the existing knowledge, it is evident that the consumption of different sources of phenolic compounds, as such or their use as natural antioxidants in food systems, is a promise for a better quality of life. This is especially true when it comes to the consumption of plant foods and their processing by-products due to their edible characteristics and as an inexpensive alternative source of important biomolecules, which may be applied in the field of functional foods, nutraceuticals, food preservation, and fortification of novel food products. Thus, this chapter briefly summarizes the hurdles and promises in the application of food processing by-products as sources of phenolic compounds.

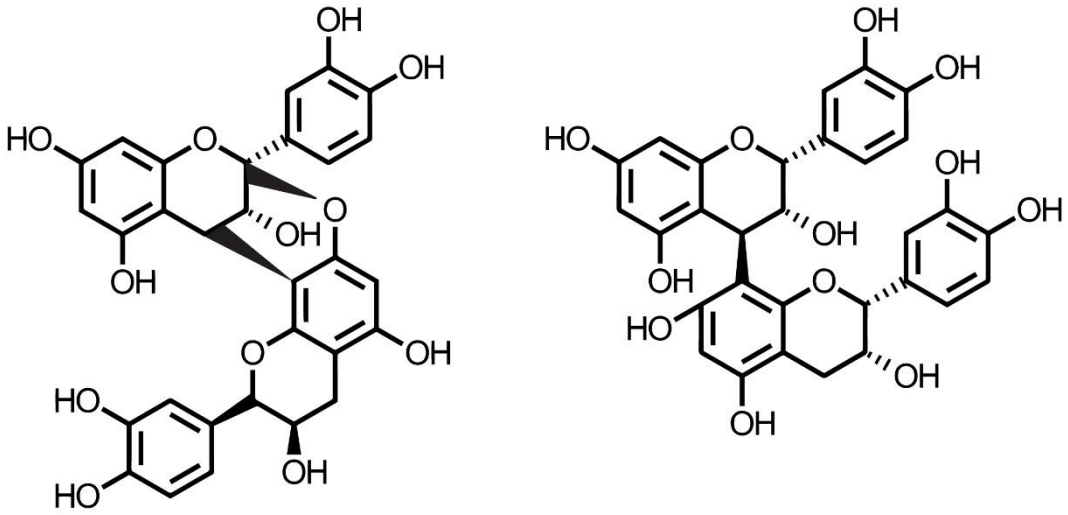


Figure 1.3 – Chemical structures of procyanidins dimer A2 and B2, which are found in peanut skin (OLDONI et al., 2016) and grape by-products (MELO et al., 2015)

1.1 Evaluation of feedstock

As already mentioned, plant food by-products are often more abundant sources of phenolics than their corresponding starting materials. Thus, it is frequently advised to consume whole grains and eat certain fruits with their peels. These peels are not only rich sources of dietary fibre, but are also important sources of phenolic compounds. The higher concentration of phenolic compounds in the outer layers of grains and seeds as well as the skin of fruits may in part be explained by the fact that they serve as plant defense against pests and pathogens. As a part of this defense, some of these phytochemicals are also known as phytoalexins (SOBOLEV, 2013). The greater concentration of phytoalexins in the peels and skins of plant foods is related to their environmental adaptation; as this part of plants is more exposed to pests and microorganisms than the inner part. Therefore, the use of plant food by-products should be carefully examined.

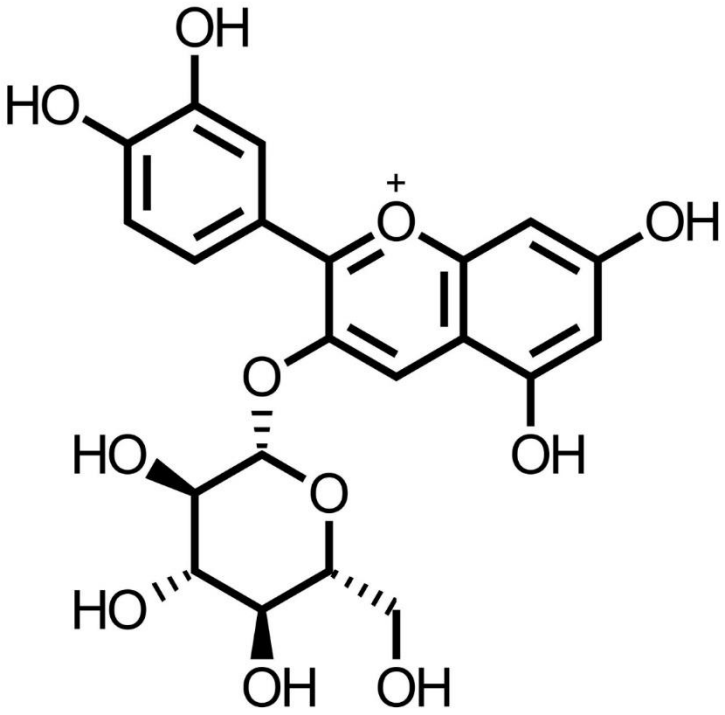
Peanuts, also known as groundnuts, have their skin removed, if subjected to the blanching process (DE CAMARGO et al., 2012a, 2016b). Several reports have substantiated the role of the peanut skin as a rich source of phenolic compounds (DE CAMARGO et al., 2012a, 2014b, 2015; MA et al., 2014a; 2014b; DE CAMARGO et al., 2015; OLDONI et al., 2016). Because of constant contact with the soil and post-harvest conditions, peanuts and their skin may not fit microbiological standards for use in producing nutraceuticals, in food fortification or as a source of natural antioxidants (e.g. food additives)(DE CAMARGO et al., 2015). Although

peanut skin is used as an example here, the same concept may be extended to different plant food by-products, especially those generated from processing of certain nuts, grains and seeds and other non-perishable food, for which storage conditions may not be adequately considered by the producers and the industry. Therefore, the microbiological status of food by-products should be checked and strategies to prevent contamination and to manage their quality standards be contemplated.

Gamma-irradiation, an ionizing radiation, has long been used to inhibit or eliminate microorganism (bacteria and fungi) in food products (DE CAMARGO et al., 2012b, 2015; FANARO et al., 2015; AYARI et al., 2016); however, due to induced free radical generation, detrimental effects towards vitamin C and liposoluble compounds such as tocopherols have brought a concern about its effect towards phenolic compounds (DE CAMARGO et al., 2012a, 2015). The literature; however, has demonstrated that induced changes are dependent on the nature of the compounds involved. Anthocyanins have been found to degrade and their concentration decrease upon gamma-irradiation (KOIKE et al., 2015), but proanthocyanidins, monomeric flavonoids, and phenolic acids increased in the fraction containing free and insoluble-bound phenolics (DE CAMARGO et al., 2015). Although gamma-irradiation may induce negative effects on anthocyanins, the same changes have also been observed upon pasteurization (MARSZAŁEK; MITEK; SKĄPSKA, 2015). Both methods have been used to decrease the microbial load of food products, but gamma-irradiation has been found effective not only towards bacteria but also against their toxins, which is not the case for heat treatment, in which enterotoxin A has been found to be resistant (GRANT; NIXON; PATTERSON, 1993).

Independent of the decontamination process selected (e.g. gamma-irradiation or pasteurization), careful evaluation of the effects towards specific phenolics must be conducted. However, most studies on phenolic compounds have been carried out in the fraction containing soluble phenolics, ignoring the insoluble-bound fraction that are linked to the cell wall material. Furthermore, the fraction containing soluble phenolics may also be fractionated into free and soluble-conjugated molecules. The fractionation process has proven to be useful for the study of process-induced changes (DE CAMARGO et al., 2015) as well as for the classification of different feedstock in specific clusters (DE CAMARGO et al., 2014a). Additionally, more than 8000 phenolic compounds have been reported in the literature and just a few commercial standards are currently available, which demonstrates the need for hyphenated techniques such as high performance liquid chromatography-diode array detection-electrospray ionization-tandem mass spectrometry (MA et al., 2014b; ALSHIKH; DE CAMARGO; SHAHIDI, 2015) or other techniques. Finally, analyses of all fractions, including those containing soluble- and

insoluble-bound phenolics along with the use of mass spectrometry may be useful to shed further light on the initial characterization of phenolic compounds from plant food by-products as well as on the changes due to different processing which may be expressed as molecular conversion, polymerization, and cross-linking to the cell wall material (DE CAMARGO et al., 2015).



Cyanidin 3-*O*-glucoside

Figure 1.4 – Chemical structure of the phenolic compound commonly used to express the content of total anthocyanins in grapes, their products and by-products (DA SILVA et al., 2016)

In addition, conventional and non-conventional methods may be chosen for the extraction process (HE et al., 2016), and the decision must be made based on the feedstock, consumption of energy, and operational costs associated with the facility (BARBA et al., 2016). Alkaline extraction has been successfully employed for quantitative extraction of insoluble-bound phenolics (DE CAMARGO et al., 2014a, 2015; AYOUB; DE CAMARGO; SHAHIDI, 2016), but the use of enzyme-assisted extraction has also been pointed as an alternative (PAPILLO et al., 2014; DE CAMARGO et al., 2016a). Recent findings have demonstrated that enzyme treatment should be considered for the development of nutraceuticals from plant by-products as the process changes the ratio of soluble to insoluble-bound phenolics; therefore, making them physiologically readily available (DE CAMARGO et al., 2016a), whereas insoluble-bound phenolics must be metabolized by the colonic fermentation prior to absorption

(CHIOU et al., 2014). Acid hydrolysis has also been used for evaluation of phenolics such as proanthocyaninds. These molecules, which differ in their degree of polymerization (DP), are classified as polymers (DP > 10), oligomers (DP = 3-10), dimers (DP = 2) and monomers. Upon acid hydrolysis, proanthocyanidins are cleaved liberating monomers which can react with nucleophilic compounds such as phloroglucinol (KYRALEOU et al., 2016), thus allowing for the calculation of their DP. However, these molecules are no longer proanthocyanidins; therefore, the use of catechin, epicatechin, and epigallocatechin gallate has been suggested as a good alternative for preparation of novel compounds with enhanced antioxidant activity (CHEN et al., 2016).

1.2 Potential health benefits and applications in food technology

Dietitians have considered plant food by-products as a good alternative for combating malnutrition. Regardless of the source, these processing by-products are rich in carbohydrates, fibre, protein, lipid, and minerals (DE CAMARGO et al., 2014b; MA et al. 2014c; IORA et al., 2015). However, the presence of phenolic compounds should also be considered. The antioxidant potential of phenolic compounds from plant by-products has been substantiated by *in-vitro* and *in vivo* studies (AYOUB; DE CAMARGO; SHAHIDI, 2016; LINGUA et al., 2016). Free radicals are related to lipid and protein oxidation; therefore, the antiradical activity of phenolic compounds using DPPH radical, ABTS radical cation, as well as against reactive oxygen species (ROS) have been widely investigated (SHAHIDI; ZHONG, 2015). Because metal ions also participate in redox reactions methods evaluating the capacity of phenolics in neutralizing them through chelation or reduction are also desirable. These methods may follow different mechanisms of action, therefore, using just one method may not be sufficient in such evaluations (DE CAMARGO et al., 2014a, 2015). Furthremore, the results may also differ depending on the evaluation medium (e.g. solvent, buffer, pH). Thus, the data collected using different methods may be useful in anticipating the actual effects using *in vitro* biological model systems as well as *in vivo* studies.

A recent report evaluated the mechanism of antioxidant action of some phenolic acids using ABTS radical cation and ORAC (oxygen radical antioxidant capacity) methods, the last one demonstrating the ability of antioxidants in neutralizing peroxyl radicals (KOROLEVA et al., 2014). The importance of ORAC method in the field of food bioactives and associated health benefits has also been reviewed (PRIOR, 2015). Furthermore, the literature (SHAHIDI; ZHONG, 2015) has demonstrated the relevance of evaluating the antioxidant efficacy towards hydrogen

peroxide, hydroxyl radicals, as well as their reducing power and metal chelation ability as some researchers still continue to ignore them in their evaluations.

In terms of health effects, phenolics from food by-products may prevent atherosclerosis, associated cardiovascular diseases and cancers. Moreover, their role in the management or prevention of diabetes and obesity has also been reported (GUO et al., 2016). As already mentioned, identification of food phenolics is also crucial, especially when it comes to high molecular weight phenolics such as proanthocyanidins. A recent report has summarized the absorption, distribution, metabolism and excretion of procyanidins, which are quite important in providing the whole picture of their health benefits (ZHANG et al., 2016). Almond skin has also been found to be a rich source of procyanidins (TRUONG et al., 2014). Although procyanidins with degree of polymerization (DP) higher than four are not readily absorbed, the literature (CHIOU et al., 2014) has suggested that such compounds may render their benefits after colonic microbiota transformation through generation of phenolic acids as the probable metabolites. This is of special importance, for example, in the case of grape by-products which are recognized sources of proanthocyanidins (MELO et al., 2015). Furthermore, the fraction containing insoluble-bound phenolics, which is also not readily absorbable, despite being most prominent in this agro-industrial by-product (DE CAMARGO et al., 2014a), may also provide health benefits upon microbial fermentation in the colon.

The use of plant food by-products as a source of phenolic compounds has not yet been fully exploited by the industry. Among possible concerns is the microbiological safety, as mentioned earlier, but the presence of toxins produced by fungi and bacteria as well as potential presence of pesticide residues demonstrates the multitude of existing hurdles. Thus, studies involving humans may face more resistance, which substantiates the need for prior investigation on their safety, collection of data *in vitro*, evaluation in cell lines, and in animal models. Other than collecting data with *in vitro* methods such as DPPH, ABTS, and those involving antiradical activity towards ROS and the ability of phenolics in neutralizing metal ions, biological model systems are also desirable. Among the health benefits of food phenolics is the potential prevention of atherosclerosis and associated cardiovascular diseases and certain types of cancer. Methods such as the cupric ion induced human low density lipoprotein (LDL) peroxidation and peroxyl and hydroxyl radical induced supercoiled DNA strand scission have been useful in demonstrating the potential benefits of phenolic compounds in reducing the risk of atherosclerosis, associated cardiovascular diseases and cancers (AYOUB; DE CAMARGO; SHAHIDI, 2016). The former topic was recently discussed in an editorial (AMAROWICZ, 2016), highlighting its importance to the field. Meanwhile, the latter ones have also been used to anticipate potential anticancer

effect due radioprotection provided by phenolic compounds (APROTOSOAIE et al., 2014). Besides that, at a molecular level, the ability of phenolics in inhibiting the activities of alpha-glucosidase and lipase, which are enzymes related to the absorption of carbohydrates and lipids, respectively, have been shown to be a good option in pre-clinical studies (DE CAMARGO et al., 2016a; SUN et al., 2016).

Some researchers have defended the use of phenolic compounds in the form of extracts or concentrates (EVANS; WILSON; GUTHRIE, 2014) whereas some have studied the intake of plant food by-products (the starting material) as a source of phenolics (BATISTA et al., 2014). Others have dedicated their efforts in using their original sources for food fortification (DE CAMARGO et al., 2014b; MA et al., 2014) and the use of phenolics from plant food by-products as food additives to prevent or delay the onset of lipid oxidation has also been reported (MUNEKATA et al., 2015; SELANI et al., 2016). Regardless of the final use and purpose, certain hurdles must be addressed. Some research groups have questioned if the microbiological and toxicological safety is a real problem (DE CAMARGO et al., 2015); however, the final application has a critical influence in the relevance of such concern. The greater the content of by-products used in a specific delivery system the higher the concern. As for the microbiological aspects for example, if food fortification is the final purpose, processing conditions such as heat treatment as well as the pH of the medium, its water content and storage temperature may be able to provide final products within the safety standards. Besides that, other technological aspects should be considered. Bakery products have been pointed as a good option for food fortification but loss of pasting properties may occur (DE CAMARGO et al., 2014b). Furthermore, bitter taste has been reported in the use of certain plant by-products showing high content of proanthocyanidins (DE CAMARGO et al., 2014b). Appearance and colour may also be affected by the incorporation of peanut skin in peanut butter (SANDERS III et al., 2014), showing that both health benefits and sensory identity, which is important to consumers and industry (DA SILVA et al., 2014), should be considered. Finally, the same should be considered on the use of phenolics from plant food by-products to prevent oxidation in food systems, which will likely be dependent on the phenolic compounds present, the concentration used to achieve the final purpose, and the final product.

1.3 Conclusion

Plant food by-products have attracted much attention due to their potential as a source of bioactive compounds. Phenolic compounds, among others, are of special interest due to their preventive action against cardiovascular disease and certain types of cancer, which have been

linked to the antioxidant activity, reducing power, and chelation capacity of these phytochemicals. Increasing interest for their action in the management of metabolic disorders such as diabetes and obesity have also been found. For these reasons, the application in the area of functional foods, nutraceuticals as well as research and development of new products has been recommended. However, some hurdles and challenges should be addressed. This section briefly summarized some of them, which included the safety, characterization, and evaluation of potential health benefits and final application. As for the safety, more attention should be paid to the microbiological and toxicological aspects of the starting material and final product. The identification should take into account the fraction containing soluble phenolics but the insoluble-bound fraction must be included. The literature on the use of chemical versus enzymatic extraction is still scarce and the identification of phenolics still suffers from the lack of commercial standards, therefore the use of HPLC itself is not the best tool for such a purpose, which makes the use hyphenated techniques including mass spectrometry the best option. Finally, industrial food products should be sensorially accepted by consumers and demonstrate satisfatory shelf-life so that a new functional or enriched food also is developed.

References

ALSHIKH, N.; DE CAMARGO, A.C.; SHAHIDI, F. Phenolics of selected lentil cultivars: antioxidant activities and inhibition of low-density lipoprotein and DNA damage. **Journal of Functional Foods,** Amsterdam, v. 18, p. 1022-1038, 2015.

ALTUNKAYA, A.; HEDEGAARD, R. V.; HARHOLT, J.; BRIMER, L.; GOKMEN, V.; SKIBSTED, L.H. Oxidative stability and chemical safety of mayonnaise enriched with grape seed extract. **Food & Function,** Cambridge, v. 4, p. 1647-1653, 2013.

AMAROWICZ, R. Natural phenolic compounds protect LDL against oxidation. **European Journal of Lipid Science and Technology**, Weinheim, v. 118, p. 677–679, 2016.

APROTOSOAIE, A.C.; TRIFAN, A.; GILLE, E.; PETREUS, T.; BORDEIANU, G.; MIRON, A. Can phytochemicals be a bridge to develop new radioprotective agents? **Phytochemistry Reviews,** Dordrecht, v. 14, p. 555-566, 2014.

AYARI, S.; DUSSAULT, D.; HAMDI, M.; LACROIX, M. Growth and toxigenic potential of *Bacillus cereus* during storage temperature abuse in cooked irradiated chicken rice in combination with nisin and carvacrol. **LWT - Food Science and Technology,** London, v. 72, p. 19-25, 2016.

AYOUB, M.; DE CAMARGO, A. C.; SHAHIDI, F. Antioxidants and bioactivities of free, esterified and insoluble-bound phenolics from berry seed meals. **Food Chemistry,** London, v. 197, p. 221-232, 2016.

BARBA, F.J.; ZHU, Z.; KOUBAA, M.; SANT'ANA, A.S.; ORLIEN, V. Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. **Trends in Food Science & Technology,** Cambridge, v. 49, p. 96-109, 2016.

BATISTA, Â.G.; LENQUISTE, S.A.; CAZARIN, C.B.B.; DA SILVA, J.K.; LUIZ-FERREIRA, A.; BOGUSZ JR., S.; WANG HANTAO, L.; DE SOUZA, R.N.; AUGUSTO, F.; PRADO, M.A.; MARÓSTICA JR., M.R. Intake of jaboticaba peel attenuates oxidative stress in tissues and reduces circulating saturated lipids of rats with high-fat diet-induced obesity. **Journal of Functional Foods,** Amsterdam, v. 6, p. 450-461, 2014.

CHANG, S.K.; ALASALVAR, C.; SHAHIDI, F. Review of dried fruits: phytochemicals, antioxidant efficacies, and health benefits. **Journal of Functional Foods,** Amsterdam, v. 21, p. 113-132, 2016.

CHEN, J.; XU, Z.; ZHU, W.; NIE, R.; LI, C.-M. Novel proanthocyanidin dimer analogues with the C-ring-opened diaryl-propan-2-gallate structural unit and enhanced antioxidant activities. **Journal of Functional Foods,** Amsterdam, v. 21, p. 290-300, 2016.

CHIOU, Y.-S.; WU, J.-C.; HUANG, Q.; SHAHIDI, F.; WANG, Y.-J.; HO, C.-T.; PAN, M.H. Metabolic and colonic microbiota transformation may enhance the bioactivities of dietary polyphenols. **Journal of Functional Foods,** Amsterdam, v. 7, p. 3-25, 2014.

COMUNIAN, T.A.; BOILLON, M.R.G.; THOMAZINI, M.; NOGUEIRA, M.S.; DE CASTRO, I.A.; FAVARO-TRINDADE, C.S. Protection of echium oil by microencapsulation with phenolic compounds. **Food Research International**, Barking, 2016, In press.

DA SILVA, J.K.; CAZARIN, C.B.B.; CORREA, L.C.; BATISTA, Â.G.; FURLAN, C.P.B.; BIASOTO, A C.T.; PEREIRA, G.E.; DE CAMARGO, A.C.; MARÓSTICA JUNIOR, M.R.; Bioactive compounds of juices from two Brazilian grape cultivars. **Journal of the Science of Food and Agriculture**, London, v. 96, p. 1990-1996, 2016.

DA SILVA, P.P.M.; CASEMIRO, R.C.; ZILLO, R.R.; DE CAMARGO, A.C.; PROSPERO, E.T.P.; SPOTO, M.H.F. Sensory descriptive quantitative analysis of unpasteurized and pasteurized juçara pulp (*Euterpe edulis*) during long-term storage. **Food Science & Nutrition,** Malden, v. 2, p. 321-331, 2014.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; BIASOTO, A.C.T.; SHAHIDI, F. Low molecular weight phenolics of grape juice and winemaking byproducts: antioxidant activities and inhibition of oxidation of human low-density lipoprotein cholesterol and DNA strand breakage. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 12159–12171, 2014a.

_____. Enzyme-assisted extraction of phenolics from winemaking by-products: antioxidant potential and inhibition of alpha-glucosidase and lipase activities, **Food Chemistry**, Easton, 2016a. In press.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; GALLO, C.R.; SHAHIDI, F.Gamma-irradiation induced changes in microbiological status, phenolic profile and antioxidant activity of peanut skin. **Journal of Functional Foods,** Amsterdam, v. 12, p. 129-143, 2015.

DE CAMARGO, A.C.; VIDAL, C.M.M.; CANNIATTI-BRAZACA, S.G.; SHAHIDI, F. Fortification of cookies with peanut skin: effects on the composition, polyphenols, antioxidant properties and sensory quality. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 11228-11235, 2014b.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CANNIATTI-BRAZACA, S.G.; VIEIRA, T.M.F.S.; SHAHIDI, F. Chemical changes and oxidative stability of peanuts as affected by the dry-blanching, **Journal of the American Oil Chemists' Society**, Champaign, 2016b. In press.

DE CAMARGO, A C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; CANNIATTI-BRAZACA, S.G. Gamma radiation induced oxidation and tocopherols decrease in in-shell, peeled and blanched peanuts. **International Journal of Molecular Sciences,** Basel, v. 13, p. 2827-2845, 2012a.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M., CALORI-DOMINGUES, M.A.; SPOTO, M.H.F.; CANNIATTI-BRAZACA, S.G. Gamma irradiation of in-shell and blanched peanuts protects against mycotoxic fungi and retains their nutraceutical components during long-term storage. **International Journal of Molecular Sciences,** Basel, v. 13, p. 10935-10958, 2012b.

EVANS, M.; WILSON, D.; GUTHRIE, N. A randomized, double-blind, placebo-controlled, pilot study to evaluate the effect of whole grape extract on antioxidant status and lipid profile. **Journal of Functional Foods,** Amsterdam**,** v. 7, p. 680–691, 2014.

FANARO, G.B.; HASSIMOTTO, N.M.A.; BASTOS, D.H.M.; VILLAVICENCIO, A.L.C. H. Effects of γ-radiation on microbial load and antioxidant proprieties in green tea irradiated with different water activities. **Radiation Physics and Chemistry,** Oxford, v. 107, p. 40-46, 2015.

GARCÍA-VILLALBA, R.; ESPÍN, J.C.; AABY, K.; ALASALVAR, C.; HEINONEN, M.; JACOBS, G.; VOORSPOELS, S.; KOIVUMÄKI, T.; KROON, P.A.; PELVAN, E.; SAHA, S.; TOMÁS-BARBERÁN, F.A. Validated method for the characterization and quantification of extractable and nonextractable ellagitannins after acid hydrolysis in pomegranate fruits, juices, and extracts. **Journal of Agricultural and Food Chemistry,** Easton, v. 63, p. 6555-6566, 2015.

GRANT, I.R.; NIXON, C.R.; PATTERSON, M.F. Effect of low-dose irradiation on growth of and toxin producton by *Staphylococcus aureus* and *Bacillus cereus* in roast beef and gravy. **International Journal of Food Microbiology,** Amsterdam, v. 18, p. 25-36, 1993.

GUO, J.; TAO, H.; CAO, Y.; HO, C.-T.; JIN, S.; HUANG, Q. Prevention of obesity and type 2 diabetes with aged citrus peel (Chenpi) extract. **Journal of Agricultural and Food Chemistry,** Easton, v. 64, p. 2053-2061, 2016.

HE, B.; ZHANG, L.-L.; YUE, X.-Y.; LIANG, J.; JIANG, J.; GAO, X.-L.; YUE, P.-X. Optimization of ultrasound-assisted extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium ashei*) wine pomace. **Food Chemistry,** London, v. 204, p. 70-76, 2016.

IORA, S.R.F.; MACIEL, G.M.; ZIELINSKI, A.A.F.; DA SILVA, M.V.; PONTES, P.V.D.A.; HAMINIUK, C.W.I.; GRANATO, D. Evaluation of the bioactive compounds and the antioxidant capacity of grape pomace. **International Journal of Food Science & Technology,** Oxford, v. 50, p. 62-69, 2015.

KOIKE, A.; BARREIRA, J.C.M.; BARROS, L.; SANTOS-BUELGA, C.; VILLAVICENCIO, A.L.C.H.; FERREIRA, I.C.F.R. Irradiation as a novel approach to improve quality of *Tropaeolum majus* L. flowers: benefits in phenolic profiles and antioxidant activity. **Innovative Food Science & Emerging Technologies,** Amsterdam, v. 30, p. 138-144, 2015.

KOROLEVA, O.; TORKOVA, A.; NIKOLAEV, I.; KHRAMEEVA, E.; FEDOROVA, T.; TSENTALOVICH, M.; AMAROWICZ, R. Evaluation of the antiradical properties of phenolic acids. **International Journal of Molecular Sciences,** Basel, v. 15, p. 16351, 2014.

KYRALEOU, M.; KOTSERIDIS, Y.; KOUNDOURAS, S.; CHIRA, K.; TEISSEDRE, P.-L.; KALLITHRAKA, S. Effect of irrigation regime on perceived astringency and proanthocyanidin composition of skins and seeds of *Vitis vinifera* L. cv. Syrah grapes under semiarid conditions. **Food Chemistry,** London, v. 203, p. 292-300, 2016.

LINGUA, M.S.; FABANI, M.P.; WUNDERLIN, D.A.; BARONI, M.V. *In vivo* antioxidant activity of grape, pomace and wine from three red varieties grown in Argentina: its relationship to phenolic profile. **Journal of Functional Foods,** Amsterdam, v. 20, p. 332-345, 2016.

MA, Y.; KOSIŃSKA-CAGNAZZO, A.; KERR, W.L.; AMAROWICZ, R.; SWANSON, R.B.; PEGG, R.B. Separation and characterization of phenolic compounds from dry-blanched peanut skins by liquid chromatography–electrospray ionization mass spectrometry. **Journal of Chromatography A,** Amsterdam, v. 1356, p. 64-81, 2014a.

_____. Separation and characterization of soluble esterified and glycoside-bound phenolic compounds in dry-planched peanut skins by liquid chromatography–electrospray ionization mass spectrometry. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 11488-11504, 2014b.

MA, Y.Y.; KERR, W.L.; SWANSON, R.B.; HARGROVE, J.L.; PEGG, R.B. Peanut skins-fortified peanut butters: effect of processing on the phenolics content, fibre content and antioxidant activity. **Food Chemistry,** London, v. 145, p. 883-891, 2014c.

MARSZAŁEK, K.; MITEK, M.; SKĄPSKA, S. The effect of thermal pasteurization and high pressure processing at cold and mild temperatures on the chemical composition, microbial and enzyme activity in strawberry purée. **Innovative Food Science & Emerging Technologies,** Amsterdam, v. 27, p. 48-56, 2015.

MARTINI, D.; D'EGIDIO, M.G.; NICOLETTI, I.; CORRADINI, D.; TADDEI, F. Effects of durum wheat debranning on total antioxidant capacity and on content and profile of phenolic acids. **Journal of Functional Foods,** Amsterdam, v. 17, p. 83-92, 2015.

MELO, P.S.; MASSARIOLI, A.P.; DENNY, C.; DOS SANTOS, L.F.; FRANCHIN, M.; PEREIRA, G.E.; VIEIRA, T.M.F.S.; ROSALEN, P.L.; ALENCAR, S.M. Winery by-products: extraction optimization, phenolic composition and cytotoxic evaluation to act as a new source of scavenging of reactive oxygen species. **Food Chemistry,** London, v. 181, p. 160-169, 2015.

MOLINA-CALLE, M.; PRIEGO-CAPOTE, F.; LUQUE DE CASTRO, M D. Development and application of a quantitative method for determination of flavonoids in orange peel: influence of sample pretreatment on composition. **Talanta,** London, v. 144, p. 349-355, 2015.

OLDONI, T.L.C.; MELO, P.S.; MASSARIOLI, A.P.; MORENO, I.A.M.; BEZERRA, R.M.N.; ROSALEN, P.L.; DA SILVA, G.V.J.; NASCIMENTO, A.M.; ALENCAR, S.M. Bioassay-guided isolation of proanthocyanidins with antioxidant activity from peanut (*Arachis hypogaea*) skin by combination of chromatography techniques. **Food Chemistry,** London, v. 192, p. 306-312, 2016.

PAPILLO, V.A.; VITAGLIONE, P.; GRAZIANI, G.; GOKMEN, V.; FOGLIANO, V. Release of antioxidant capacity from five plant foods during a multistep enzymatic digestion protocol. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 4119-4126, 2014.

PRIOR, R.L. Oxygen radical absorbance capacity (ORAC): new horizons in relating dietary antioxidants/bioactives and health benefits. **Journal of Functional Foods,** Amsterdam, v. 18, p. 797-810, 2015.

SANDERS III, C.T.; DEMASIE, C.L.; KERR, W.L.; HARGROVE, J.L.; PEGG, R.B.; SWANSON, R.B. Peanut skins-fortified peanut butters: effects on consumer acceptability and quality characteristics. **LWT - Food Science and Technology,** London, v. 59, p. 222-228, 2014.

SELANI, M.M.; SHIRADO, G.A.N.; MARGIOTTA, G.B.; RASERA, M.L.; MARABESI, A.C.; PIEDADE, S.M.S.; CONTRERAS-CASTILLO, C.J.; CANNIATTI-BRAZACA, S.G. Pineapple by-product and canola oil as partial fat replacers in low-fat beef burger: effects on oxidative stability, cholesterol content and fatty acid profile. **Meat Science,** Barking, v. 115, p. 9-15, 2016.

SHAHIDI, F.; AMBIGAIPALAN, P. Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects – a review. **Journal of Functional Foods,** Amsterdam, v. 18, p. 820–897, 2015.

SHAHIDI, F.; ZHONG, Y. Measurement of antioxidant activity. **Journal of Functional Foods,** Amsterdam, v. 18, p. 757-781, 2015.

SOBOLEV, V.S. Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms. **Journal of Agricultural and Food Chemistry,** Easton, v. 61, p. 1850-1858, 2013

SUN, S.; KADOUH, H. C.; ZHU, W.; ZHOU, K. Bioactivity-guided isolation and purification of α-glucosidase inhibitor, 6-O-D-glycosides, from Tinta Cão grape pomace. **Journal of Functional Foods,** Amsterdam, v. 23, p. 573-579, 2016.

TRUONG, V.-L.; BAK, M.-J.; JUN, M.; KONG, A.-N. T.; HO, C.-T.; JEONG, W.-S. Antioxidant defense and hepatoprotection by procyanidins from almond (*Prunus amygdalus*) skins. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 8668-8678, 2014.

ZHANG, L.; WANG, Y.; LI, D.; HO, C.-T.; LI, J.; WAN, X. The absorption, distribution, metabolism and excretion of procyanidins. **Food & Function,** Cambridge, v. 7, p. 1273-1281, 2016.

2 LOW MOLECULAR WEIGHT PHENOLICS OF GRAPE JUICE AND WINEMAKING BY-PRODUCTS: ANTIOXIDANT ACTIVITIES AND INHIBITION OF OXIDATION OF HUMAN LOW-DENSITY LIPOPROTEIN CHOLESTEROL AND DNA STRAND BREAKAGE

Adapted with permission from DE CAMARGO, A. C.; REGITANO-D'ARCE, M. A. B.; BIASOTO, A. C. T.; SHAHIDI, F. Low molecular weight phenolics of grape juice and winemaking byproducts: antioxidant activities and inhibition of oxidation of human low-density lipoprotein cholesterol and DNA strand breakage. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 12159–12171, 2014. Copyright 2014 American Chemical Society

Abstract

Bioactive compounds belonging to phenolic acids, flavonoids, and proanthocyanidins of grape juice and winemaking by-products were identified and quantified by HPLC-DAD-ESI-MS^n. The concentration of phenolic compounds in different grape cultivars was in the order Tempranillo > Cora > Syrah > Isabel. The insoluble-bound fraction was most prominent, contributing 63 and 79% to the total for Isabel and Tempranillo, respectively. Juice-processing by-products had a higher content of free than esterified phenolics, but the opposite was noted for winemaking by-products. Insoluble-bound phenolics were up to 15 and 10 times more effective as antioxidants than those of free and esterified fractions, respectively, as evaluated by the DPPH, ABTS, and H_2O_2 scavenging activities and reducing power determinations. In general, insoluble-bound phenolics (100 ppm) were more effective in inhibiting copper-induced human LDL-cholesterol oxidation than free and esterified phenolics, exhibiting equal or higher efficacy than catechin. Phenolic extracts from all fractions inhibited peroxyl radical-induced DNA strand breakage. These findings shed further light for future studies and industrial application of grape by-products, which may focus not only on the soluble phenolics but also on the insoluble-bound fraction.

Keywords: Processing byproduct; Phenolic acids; Flavonoids; Proanthocyanidin; LDL-cholesterol; DNA

2.1 Introduction

Fruits, vegetables, nuts, and cereals have been in the spotlight due to extensive literature support demonstrating their health benefits. Other than providing carbohydrate, protein, lipid, minerals, and vitamins, a balanced diet also provides a wide range of bioactive compounds. Polyphenols are recognized for rendering several health benefits such as potential anticancer, antimicrobial, and antioxidative effects (CHANDRASEKARA; SHAHIDI, 2011a; CHENG et al., 2012). However, commercial products have not always been considered as a viable source of bioactives. In this regard, several studies have demonstrated the high content of polyphenols in different commercial food products such as chocolate, tomato sauce, grape juice, and wine.

The processing of foods and beverages generates a large amount of by-products. The juice industry is one of the major suppliers of by-products as a consequence of fruit seasonality as well as different climates and soil adaptation of the feedstock. Many tons of grapes are produced each year, and a large part of their final consumption is through grape juice and wine. Consequently, a considerable amount of by-products (e.g., grape skin and seeds) is generated, creating an environmental burden.

Several by-products have been studied as a source of polyphenols, and the information available demonstrates their potential for being exploited (LEE et al., 2006; SHAHIDI; ALASALVAR; LIYANA-PATHIRANA, 2007; DE CAMARGO et al., 2012a). Grape by-products have also been shown to serve as a good source of dietary fiber (LLOBERA; CANELLAS, 2007) and for extending the shelf life of high-lipid foods due to their antioxidant activity (SHIRAHIGUE et al., 2010). The potential health benefits of such products/by-products stems from their high polyphenol content, which has been evidenced by both *in vitro* and *in vivo* studies (JARA-PALACIOS et al., 2013; EVANS; WILSON; GUTHRIE, 2014).

Polyphenols are water-soluble and found in the free, esterified, and insoluble-bound forms, the latter fraction being linked to the cell walls of source materials. Numerous studies have evaluated the phenolic profile, antioxidant properties, and potential biological activities of grapes and their by-products (SANDHU; GU, 2010; CHENG et al., 2012; GONZÁLEZ-CENTENO et al., 2012); however, there are clear gaps in the existing knowledge about the contribution of the free, esterified, and insoluble-bound fractions to the total phenolic/polyphenolic contents that affect the antioxidant properties of grape by-products. Moreover, grape by-products are generated by both juice- and winemaking operations.

The grape variety, its maturation stage, and crop production area are some of the crucial factors influencing the different phenolic profiles found in grape juice, wine, and their by-products. Grape juice and winemaking have different processes, among them the fermentation process, which leads to alcohol generation, as the main one. For red wines, this stage is conducted in two steps called primary and secondary fermentations. The primary fermentation may take 1-2 weeks in the presence of grape skins, which are responsible for color development of red wines. Furthermore, the primary fermentation is carried out by yeast cells, and their ability to absorb phenolic compounds may result in a higher or lower phenolic content in the wine and in its byproduct. The secondary step is conducted by bacterial fermentation, in the absence of grape skins, which are removed in the process. Thus, grape skins have no influence in the secondary fermentation.

Reactive oxygen species (ROS) are detrimental to health as they take part in oxidative processes both *in vivo* and *in vitro* (WETTASINGHE; SHAHIDI, 2000). ROS are involved in cell damage, cancer development, inflammation, and heart disease. Furthermore, ROS play an important role in aging and the development of Parkinson's disease. ROS are constantly generated via mitochondrial metabolism, which can worsen with unhealthy habits such as smoking. High-lipid foods are also affected by ROS generation, which can be influenced by heating, UV light, and gamma-irradiation, leading to sensory changes, decreasing the shelf life of products and becoming an economic burden (DE CAMARGO et al., 2012b). Hydrogen peroxide generates hydroxyl radicals in the presence of ferrous ion or via UV light dissociation. Although generation of the highly reactive short-lived hydroxyl radicals is of much concern as it induces DNA damage and lipid and protein oxidation, the presence of H_2O_2 itself causes enzyme inactivation and cell damage. The scavenging activity of phenolics against H_2O_2 is attributed to electron donation, but neutralization of H_2O_2 to H_2O is also contemplated (WETTASINGHE; SHAHIDI, 2000). Moreover, ferric ion catalyzes the oxidation of proteins and lipids, thus being detrimental to food and biological systems.

The present study focused on the unraveling of the phenolic profile of grape by-products, and the contribution of each fraction to potential bioactivity as well as differences between by-products generated by the juice- and winemaking industries. Total phenolic content (TPC), proanthocyanidin content (PC), scavenging activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and H_2O_2, and reducing power (RP) were evaluated. The detailed phenolic profile was investigated using HPLC-DAD-ESI-MS^n, and the potential health benefits of the extracts were evaluated against copper-induced LDL-cholesterol oxidation and peroxyl radical induced DNA strand breakage.

2.2 Materials and Methods

Grape juice (BRS Cora and Isabel) by-products were donated by Embrapa Semiárido (Petrolina, Pernambuco state, Brazil). Syrah and Tempranillo (winemaking by-products) were donated by Ouro Verde Farm, Grupo Miolo (Casa Nova, Bahia state, Brazil) and Santa Maria winery (Lagoa Grande, Pernambuco state, Brazil), respectively. Original grapes used in the grape juice- and winemaking processes had 20 and 22 ºBrix, respectively. Brazilian grape juice is mainly produced using the Isabel cultivar, due to its large production. BRS Cora, hereafter named Cora, is a Brazilian variety developed by Embrapa through crossing between ‘Muscat Belly A’ and ‘H.65.9.14’ varieties. Cora and Isabel grapes were grown in Petrolina,

Pernambuco state, Brazil. Syrah grape was grown in Casa Nova, Bahia state, Brazil, and Tempranillo was grown in Lagoa Grande, Pernambuco state, Brazil. The microbiological evaluation (*Salmonella* spp., yeasts and molds, coliform bacteria, and coagulase-positive *Staphylococcus*) demonstrated that grape by-products were safe to use as a functional ingredient and/or supplement (data not shown).

Folin-Ciocalteu's reagent, vanillin, DPPH, ABTS, mono- and dibasic potassium phosphates, hydrogen peroxide, potassium ferricyanide, ferric chloride, copper sulfate, human LDL-cholesterol, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), tris acetate, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), agarose, bromophenol blue, xylene cyanol, glycerol, trolox, caffeic, gallic, protocatechuic, and *p*-coumaric acids, (+)-catechin, and (−)-epicatechin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium carbonate, sodium hydroxide, sodium chloride, potassium persulfate, trichloroacetic acid, diethyl ether, ethyl acetate, hexane, acetone, methanol, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide, and pBR 322 from *Escherichia coli* were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). SYBR safe gel stain was purchased from Probes (Invitrogen, Eugene, OR, USA).

2.2.1 Extraction of phenolic compounds

Grape by-products were freeze-dried at -48 ºC and 30 x 10^{-3} mbar (Freezone 6, model 77530, Labconco, Co., Kansas City, MO, USA) and ground using a coffee bean grinder (model CBG5 series, Black & Decker, Canada, Inc., Brockville, ON, Canada). The powder so obtained was passed through a mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA) sieve. Ground samples were defatted three times using hexane (solid/solvent, 1:5, w/v) in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT, USA). Defatted samples were then stored at -20 ºC until used for the extraction of phenolic compounds within 1 week.

Defatted samples (2.5 g) were extracted with 70% acetone (100 mL) in a gyratory water bath shaker (model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA) at 30 ºC for 20 min. After centrifugation at 4000g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the upper layer was collected and extraction was repeated twice. The combined supernatants were evaporated to remove the organic solvent, and the residue in water was acidified to pH 2 using 6 M HCl. Free phenolic compounds were extracted five times with diethyl ether and ethyl acetate (1:1, v/v). Combined supernatants were evaporated in vacuo at 40 ºC (Buchi, Flawil, Switzerland). The remaining water phase was mixed with 4 M NaOH

and hydrolyzed while stirring under nitrogen for 4 h at room temperature (23-25 ºC) to release esterified phenolics. This was followed by acidification to pH 2 using 6 M HCl, five extractions with diethyl ether and ethyl acetate (1:1, v/v), and evaporation of combined supernatants in vacuo at 40 ºC. Free phenolics and those released from their esterified form were separately reconstituted in HPLC grade methanol and stored at -20 ºC until used for further analysis within 3 months.

To the solid residue remaining after the first set of extractions, 4 M NaOH was added and hydrolyzed with stirring under nitrogen for 4 h at room temperature (23-25ºC). The resulting slurry was acidified to pH 2 with 6 M HCl. Phenolics released from their insoluble-bound form were then extracted with diethyl ether and ethyl acetate (1:1, v/v) and reconstituted in HPLC grade methanol, as explained above.

2.2.2 Total phenolic contents

TPC were determined according to the Folin-Ciocalteu method (SWAIN; HILLIS, 1959) with slight modifications, as previously described by de Camargo et al. (2012c). The phenolic extracts were used in concentrations ranging from 3 to 30 mg/mL. First, the extracts with appropriate dilutions (0.5 mL), deionized water (4 mL), and Folin Ciocalteu's reagent (0.5 mL) were added into flasks and mixed thoroughly. After 3 min, a saturated solution of sodium carbonate (0.5 mL) was added, and the mixture was kept in the dark at room temperature (23-25 °C) for 2 h. Finally, the absorbance was read at 760 nm using an Agilent diode array spectrophotometer (Agilent 8453, Palo Alto, CA, USA). The results were expressed as milligram gallic acid equivalents per gram of dry weight of defatted sample.

2.2.3 Proanthocyanidin content

Total proanthocyanidins (condensed tannins) were determined according to the method of Price et al. (1980) as explained by de Camargo et al. (2012a) . Briefly, phenolic extracts were diluted in methanol (10–100 mg/mL), and 1 mL of the extracts so obtained was added to 5 mL of a 0.5% (w/v) vanillin solution prepared in 4% (v/v) HCl in methanol. The mixture was incubated in a gyratory water bath shaker (model G76, New Brunswick Scientific Co., New Brunswick, NJ, USA) at 30 °C for 20 min. Finally, the absorbance was read at 500 nm using an Agilent diode array spectrophotometer (Agilent 8453). The results were expressed as milligram catechin equivalents per gram of dry weight of defatted sample.

2.2.4 ABTS radical cation scavenging activity

The ABTS assay (RE et al., 1999) was performed using a modified version of the method described by de Camargo et al. (2012a). The ABTS radical, which was generated by oxidation with potassium persulfate, was prepared in 100 mM phosphate buffer saline solution (PBS) (pH 7.4, 0.15 M sodium chloride). The ABTS stock solution consisted of potassium persulfate (2.45 mM) and ABTS (7.00 mM) in PBS. At the time of analysis, the working solution of ABTS was prepared by diluting its stock solution in PBS to reach an absorbance value of 0.70 (734 nm). Phenolic extracts were diluted in PBS (10–30 mg/mL). Diluted phenolic extracts (20 μL) were added to 2 mL of ABTS radical cation solution, and the absorbance was read at 734 nm after 6 min using an Agilent diode array spectrophotometer (Agilent 8453). ABTS radical scavenging activity was calculated using the equation.

$$\text{ABTS radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of ABTS radical + PBS and Abs_{sample} is the absorbance of ABTS radical cation + phenolic extract or Trolox. The results were expressed as micromoles of Trolox equivalents per gram of dry weight of defatted sample.

2.2.5 DPPH radical scavenging activity (DRSA)

The DPPH assay was carried out using a modified version of the method explained by Chandrasekara and Shahidi (2011c). The phenolic extracts were used at different concentrations of 10–50 mg/mL. Two milliliters of a methanolic solution of DPPH (0.5 mM) were added to 500 μL of the extracts diluted in methanol. After 10 min, the mixture was passed through the capillary tubing that guides the sample through the sample cavity of a Bruker e-scan electron paramagnetic resonance (EPR) spectrophotometer (Bruker E-Scan, Bruker Biospin Co., Billercia, MA, USA). The spectrum was recorded using the following parameters: 5.02×10^2 receiver gain, 1.93 G modulation amplitude, 2.62 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G center field, 5.12 ms time constant, 9.793220 GHz microwave frequency, and 86.00 kHz modulation frequency. The DPPH scavenging activity of the extracts was calculated using the equation.

$$\text{DPPH scavenging activity (\%)} = [(EPR_{control} - EPR_{sample})/(EPR_{control})] \times 100$$

where $EPR_{control}$ is the signal intensity of DPPH radical + methanol and EPR_{sample} is the signal intensity of DPPH radical + phenolic extract or trolox. The results were expressed as micromoles of Trolox equivalents per gram of dry weight of defatted sample.

2.2.6 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of extracts of grape by-products was evaluated as explained elsewhere (WETTASINGHE; SHAHIDI, 2000). Phenolic extracts (4–40 mg/mL) and 0.4 mM hydrogen peroxide solution were prepared in 0.1 M phosphate buffer (pH 7.4). The extracts (0.4 mL) were mixed with hydrogen peroxide solution (0.6 mL), and the final volume was made to 2 mL with the same buffer. The samples were kept in a gyratory water bath shaker (model G76, New Brunswick Scientific Co. Inc.) for 40 min, and the absorbance was read at 230 nm using an Agilent diode array spectrophotometer (Agilent 8453). Blanks devoid of hydrogen peroxide (added by phosphate buffer) were prepared for background corrections. The results were expressed as micromoles of Trolox equivalents per gram of dry weight of defatted sample. The scavenging activity was calculated with the equation.

$$H_2O_2 \text{ scavenging activity } (\%) = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of H_2O_2 radical + phosphate buffer and Abs_{sample} is the absorbance of H_2O_2 + phenolic extract extract or trolox.

2.2.7 Reducing power

The RP assay (OYAIZU, 1986) was conducted according to the method described by Alasalvar et al. (2009). The extracts (2–20 mg/mL) were diluted in phosphate buffer (pH 6.6, 0.2 mM). Extracts (1 mL) were then further mixed with phosphate buffer (2.5 mL) and 1% (w/v) potassium ferricyanide solution (2.5 mL), followed by their incubation in a gyratory water bath shaker (model G76, New Brunswick Scientific Co. Inc.) at 50 ºC for 20 min, after which a 10% (w/v) solution of trichloroacetic acid was added (2.5 mL). The mixture was subsequently centrifuged at 1750g for 10 min, and the supernatant (2.5 mL) was added to distilled water (2.5 mL) and 0.1% (w/v) ferric chloride solution (0.5 mL). The absorbance was read at 700 nm using an Agilent diode array spectrophotometer (Agilent 8453). A calibration curve was prepared using Trolox as a standard, and results were expressed as Trolox equivalents per gram of dry weight of defatted sample.

2.2.8 Copper-induced human LDL-cholesterol oxidation

The LDL-cholesterol oxidation method described by Shahidi et al. (2007) was slightly modified to evaluate the potential inhibitory effect of phenolic extracts of grape by-products. The solution of LDL-cholesterol was dialyzed overnight against PBS (10 mM, 0.15 M NaCl, pH 7.4) at 4 ºC under a flow of nitrogen. The resulting ethylenediaminetetraacetic acid (EDTA)-free LDL-cholesterol was diluted in PBS to reach a concentration of 0.04 mg/mL. Methanol was removed from grape byproduct extracts under a stream of nitrogen followed by their resuspension in PBS to obtain 100 ppm of total phenolic equivalents in relation to the final volume assay (1 mL). This was calculated on the basis of TPC as evaluated by HPLC-DAD-ESI- MS^n. Phenolic extracts (100 µL) and LDL-cholesterol (800 µL) were added into Eppendorf tubes and incubated at 37 ºC for 15 min, after which the peroxidation was induced by the addition of a 100 µM solution of $CuSO_4$ (100 µL). The reaction was incubated for 24 h at 37 ºC, and the conjugated dienes (CD) were assayed at 234 nm using an Agilent diode array spectrophotometer (Agilent 8453). Blanks devoid of LDL-cholesterol and $CuSO_4$ were prepared for background subtraction. A positive control was prepared with catechin (100 ppm), and the results were expressed as percent inhibition according to the equation.

$$\text{Inhibition of formation of CD (\%)} = [(Abs_{oxidized} - Abs_{sample})/(Abs_{oxidized} - Abs_{native})] \times 100$$

where $Abs_{oxidized}$ is the absorbance of LDL-cholesterol with $CuSO_4$, Abs_{sample} is the absorbance of LDL-cholesterol with extract or catechin and $CuSO_4$, and Abs_{native} is the absorbance of LDL-cholesterol without $CuSO_4$.

2.2.9 Inhibition of peroxyl radical induced supercoiled plasmid DNA strand breakage

The supercoiled plasmid DNA strand breakage inhibition was evaluated using a slightly modified version of the method explained by Shahidi et al. (2007). Methanol was removed from phenolic extracts under a stream of nitrogen followed by resuspension in water to achieve a concentration of 1-5 mg/mL. An aliquot (2 µL) was pipetted in Eppendorf tubes, and the remaining reagents were added in the following order: 2 µL of PBS (0.5 M, pH 7.4, 0.15 M sodium chloride), 2 µL of supercoiled plasmid DNA pBR 322 from *Escherichia coli* RRI diluted in PBS (50 µL/mL), and 4 µL of a 7 mM AAPH solution. The mixture was incubated at 37 °C for 1 h in the dark, after which 1 µL of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in distilled water) was added. The samples were

loaded onto 0.7 (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer consisting of 40 mM Tris acetate and 1 mM EDTA, pH 8.5. The gel was stained with SYBR safe (100 μL/L). The procedure was conducted at 80 V for 90 min using a submarine gel electrophoresis apparatus (VWR, Radnor, PA, USA). The images were acquired with a Sony digital camera under UV light and analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA, USA). The inhibition percentage was calculated as follows: inhibition of DNA strand breakage = [(intensity of supercoiled DNA in the presence of oxidant and extract/intensity of supercoiled DNA devoid of oxidant and extract) x 100]. Results were expressed as percentage retention of supercoiled DNA achieved with 1 mg/mL of defatted sample.

2.2.10 HPLC-DAD-ESI-MS^n analysis

Identification of major phenolics in the free, esterified, and insoluble-bound fractions of grape by-products was carried out using an Agilent 1100 system equipped with a G1311A quaternary pump, a G1379A degasser, a G1329A ALS automatic sampler, a G1130B ALS Therm, a G1316 Colcom column compartment, A G1315B diode array detector (DAD), and a system controller linked to a ChemStation Data handling system (Agilent). Separations were conducted with a SUPERLCOSIL LC-18 column (4.6 × 250 mm × 5 μm, Merck, Darmstadt, Germany). The binary mobile phase consisted of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The flow rate of mobile phase was 0.5 mL/min, and the elution gradient used was as follows: 0 min, 100% A; 5 min, 90% A; 35 min, 85% A; 45 min, 60% A; held at 60% A from 45 to 50 min. Afterward mobile phase A was increased to 100% at 55 min, followed by column equilibration from 55 to 65 min. The compounds were detected at 280 nm, and the samples were filtered before injection using a 0.45 μm PTFE membrane syringe filter (Thermo Scientific, Rockwood, TN, USA).

HPLC-ESI-MS^n analysis was carried out under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography-mass selective detector (LC-MSD) ion trap system in electrospray ionization (ESI) in the negative mode. The data were acquired and analyzed with Agilent LC-MSD software. The scan range was set from m/z 50 to 1200, using smart parameter setting, drying nitrogen gas at 350 °C, flow of 12 L/min, and nebulizer gas pressure of 70 psi. Phenolic acids, namely, protocatechuic, p-coumaric, gallic, ferulic, and ellagic acids, and flavonoids (+)-catechin, (−)-epicatechin, and quercetin were identified by comparing their retention times and ion fragmentation patterns with coded and authentic standards under the same conditions as the samples. Hydroxycaffeic and caftaric acids

and monogalloyl glucose as well as isorhamnetin, epicatechin gallate, kaempferol hexoside, quercetin hexoside, quercetin glucuronide, isorhamnetin hexoside, myricetin hexoside, and dimers through tetramers of proanthocyanidins were tentatively identified using tandem mass spectrometry (MS^n), UV spectral, and literature data.

2.2.11 Statistical analysis

The experimental design was randomized with three replications, and the results were analyzed using Tukey's test ($p < 0.05$) and SAS software. The correlation analysis ($p < 0.01$ and 0.05) was carried out using the ASSISTAT 7.6 program.

2.3 Results and Discussion

2.3.1 HPLC-DAD-ESI-MS^n analysis

In the present study, phenolics from grape juice- and winemaking by-products were extracted in different fractions (free, esterified, and insoluble-bound) to fill the apparent gap in the existing knowledge and to offer new information on the subject. The MS spectra of free, esterified, and insoluble-bound phenolic fractions of grape by-products are shown in Table 2.1. Compounds 1-5, 7, and 10-12 were identified by comparison of their retention times and ion fragmentation patterns with authentic standards. Compound 6 gave deprotonated ions at m/z 197 and MS^2 at m/z 179, due to loss of water $[M-H-18]^-$, and at m/z 135. This ion fragmentation pattern is typical for caffeic acid, as confirmed using a commercial standard, and thus tentatively identified as hydroxycaffeic acid. In the present study, hydroxycaffeic was found only in the esterified fraction of the Tempranillo variety. Hydroxycaffeic acid, which is found in blackberries and blueberries (ZADERNOWSKI; NACZK; NESTEROWICZ, 2005), has not been reported in grapes, possibly because few varieties may contain it or it may be esterified to other components present. Compound 8, which was present in the free fraction of Cora, Isabel, and Tempranillo varieties, showed an m/z at 311 in MS followed by 179 and 135 in MS^2 and was tentatively identified as caftaric acid (LAGO-VANZELA et al., 2013).

Monogalloyl glucose (compound 9) was also tentatively identified due to its m/z of 331 in MS and m/z of 169 and 125 in MS^2, both typical of gallic acid (JARA-PALACIOS et al., 2013). Furthermore, monogalloyl glucose was present in all fractions of all samples tested. Compound 13 gave deprotonated ion $[M-H]^-$ at m/z 315 and at m/z 271 in MS^2, which is compatible with the isorhamnetin dissociation pattern (HANHINEVA et al., 2008). Compound 14 was tentatively identified as epicatechin gallate (galloylated flavan-3-ol) due to its deprotonated molecular ion at m/z 441 followed by m/z at 289 and 245 in MS^2, showing

decarboxylation of epicatechin $[M-H-44]^-$, at m/z 169 and 125, the last one from decarboxylated gallic acid (SANDHU; GU, 2010).

Compound 15 with a deprotonated ion at m/z 447 gave product ions at m/z 285 (kaempferol) in MS^2, showing loss of hexose $[M-H-162]^-$, thus being tentatively identified as kaempferol hexoside (JARA-PALACIOS et al., 2013). Compound 16 (m/z 463) also showed loss of hexoside and its typical quercetin dissociation at m/z 179 and 151 in MS^2, which was confirmed by quercetin standard, indicating its identity as quercetin hexoside.

Table 2.1 - Phenolic compounds identified in grape by-products

	juice by-products		winemaking by-products					
	Cora	Isabel	Syrah	Tempranillo	Phenolic Acids	MW	$[M-H]^-$	Other product ions (m/z)
1	E, B	B	F, B	F, E, B	Protocatechuic acid*	154	153	109
2	F, E, B	F, E, B	E, B	F, E, B	*p*-Coumaric acid*	164	163	119
3	F, E, B	F, E, B	F, E, B	F, E, B	Gallic acid*	170	169	125
4	F, E, B	F, E, B	E, B	E, B	Caffeic acid*	180	179	135
5	E	F, E		F	Ferulic acid*	194	193	177, 149, 134
6				E	Hydroxycaffeic acid	198	197	179, 135
7	E	E, B	F	B	Ellagic acid*	302	301	283, 257
8	F	F		F	Caftaric acid	312	311	179, 135
9	F, E, B	F, E, B	F, E, B	F, E, B	Monogalloyl glucose	332	331	169, 125
					Flavonoids			
10	F, E, B	F, E, B	F, E, B	F, E, B	(+)-Catechin*	290	289	245, 205, 179
11	F, E, B	F, E, B	F, E, B	F, E, B	(−)-Epicatechin*	290	289	245, 205, 179
12	F, B	F	F, B	F, B, E	Quercetin*	302	301	179, 151, 107
13	E	B	E, B	F, E, B	Isorhamnetin	316	315	271
14	F, B	F, B, E	F, B, E	F, B, E	Epicatechin gallate	442	441	289, 169, 125
15	F, B, E	F, B, E	F	F	Kaempferol hexoside	448	447	285
16	F, E, B	F, E, B	F, E, B	F	Quercetin hexoside	464	463	301, 179, 151
17	F, E, B	F, E, B	F, E, B	F, E, B	Quercetin glucuronide	478	477	301, 179, 151
18	F, B	F	F, E, B	F, E, B	Isorhamnetin hexoside	478	477	315, 271
19	F, B	F, E	F	F	Myricetin hexoside	480	479	317, 179, 151
20	B	B		B	Procyanidin dimer A	576	575	449, 423, 407, 289, 285
21	F, B	F, E, B	F, E, B	F, E, B	Procyanidin dimer B	578	577	451, 425, 289
22	F		B	B	Prodelphinidin dimer A	592	591	573, 465, 451, 421, 303, 285
23	F, E	F, E, B	E, B	B	Prodelphinidin dimer B	594	593	575, 456, 449, 423, 303, 289, 285
24		F, E, B	F, E, B	F, E, B	Galloyled procyanidin dimer	730	729	577, 425, 407, 289
25	B	F, E, B	F, E	F, E	Procyanidin trimer C	866	865	739, 695, 575, 407, 289, 287

F, E, and B are free, esterified, and insoluble-bound phenolics, respectively. * Compounds identified with authentic standards

Quercetin glucuronide was tentatively identified as compound 17, which gave a deprotonated ion at m/z 477 and MS^2 at 301, 179, and 151 (JARA-PALACIOS et al., 2013). Compound 19 showed $[M-H]^-$ at m/z 477, such as quercetin glucuronide, but its MS^2 gave product ions at m/z 315 and 271, enabling its tentative identification as isorhamnetin hexoside (JARA-PALACIOS et al., 2013). Myricetin hexoside was also tentatively identified according to its deprotonated ion at m/z 479, followed by loss of hexose (SANDHU; GU, 2010), which gave signals at m/z 317, 179, and 151, the last ones confirmed by using an authentic standard.

With the exception of galloylated procyanidin dimer (JARA-PALACIOS et al., 2013), the remaining proanthocyanidins were tentatively identified according to the studies of Sarnoski et al. (2012) and de Camargo et al. (2014). Procyanidins B1-B8 are currently known (SAINT-

CRICQ DE GAULEJAC; PROVOST; VIVAS, 1999); thus, some peaks were identified with the same ionization pattern, which has been common for proanthocyanidins isomers. However, accurate stereoisomer identification requires nuclear magnetic resonance (NMR) analysis; thus, for quantification purposes total value was reported.

The quantification data for the phenolics identified are shown in Table 2.2. Although anthocyanins have been reported in grape by-products, the HPLC-DAD acquisition data set at 520 nm, which is typical for anthocyanins, did not show major compounds (data not show); thus, the present study focused on phenolic acids, flavonoids, and proanthocyanidins. Varietal differences in phenolics are well documented in the literature (CHENG et al., 2012; GONZÁLEZ-CENTENO et al., 2012; WEIDNER et al., 2012); thus, statistical treatment was carried out among the free, esterified, and insoluble-bound fractions. With very few exceptions, the insoluble-bound fraction had the highest content of individual phenolics. The HPLC-DAD-ESI-MS^n evaluation demonstrated that gallic and caffeic acids were the major phenolic acids accounting for up to 20 and 14% of the total, respectively. Gallic acid has previously been reported as being the major phenolic acid in grape seeds (WEIDNER et al., 2013). Catechin was the major monomeric flavonoid (up to 17%), and procyanidin dimer B was the main proanthocyanidin, with a content of up to 52%. Several factors such as climatic and stress conditions as well as soil quality play important roles in the content of phenolics; thus, a direct comparison with the literature data is not possible, but some trends may be discernible.

The present study reveals similarities with the data reported by Ćurko et al. (2014), in which catechin and procyanidin B were the major monomeric flavonoid and proanthocyanidin, respectively. Interestingly, a high concentration of procyanidin dimer A, which has not been commonly reported for grape and its by-products, was found in the present study. However, Cheng et al. (2012) also reported the presence of procyanidin dimer A in grape by-products (skins, seeds, and pomace). Furthermore, according to these authors, the highest procyanidin dimer A concentration was found in the seeds.

Total phenolics, as evaluated by HPLC-DAD-ESI-MS^n, hereafter named HPLC TPC, are shown in Figures 2.1 and 2.2. The HPLC TPC were present in the order insoluble-bound > free > esterified for both juice by-products and insoluble-bound > esterified > free for both winemaking by-products, which suggests that the process type influences this distribution. The HPLC TPC were in the decreasing order Tempranillo > Cora > Syrah > Isabel (Figure 2.2), with insoluble-bound fraction representing up to 12 and 23 times the content of the free and esterified fractions, respectively. This is important because most of the available information is focused only on the soluble phenolics (free plus esterified). Finally, Figure 2.3 shows the

percent contribution of soluble and insoluble-bound phenolics in the samples. Insoluble-bound phenolics accounted for 63-79% of the total, thus demonstrating their dominance as a major source of bioactive compounds in grape by-products.

Table 2.2 - Contents (μg/g of dry weight) of free, esterified and insoluble-bound phenolic acids and derivatives of grape by-products[a]

	juicemaking by-products		winemaking by-products	
	Cora	Isabel	Syrah	Tempranillo
		Protocatechuic acid		
free	nd	nd	tr	3.01 ± 0.0b
esterified	9.96 ± 0.2b	nd	nd	4.24 ± 0.3b
insoluble-bound	44.0 ± 1.2a	41.5 ± 0.8	33.5 ± 0.8	226 ± 8.0a
		p-Coumaric acid		
free	tr	3.71 ± 0.3c	nd	0.70 ± 0.0b
esterified	69.5 ± 0.5b	81.4 ± 2.2b	168 ± 3.8b	176 ± 3.3a
insoluble-bound	192 ± 4.6a	193 ± 3.3a	243 ± 7.4a	172 ± 12a
		Gallic acid		
free	7.06 ± 0.7b	5.73 ± 0.6c	8.39 ± 0.2c	16.9 ± 0.3c
esterified	13.8 ± 0.2b	165 ± 1.2b	130 ± 0.5b	236 ± 0.2b
insoluble-bound	1081 ± 44a	799 ± 21a	343 ± 17a	301 ± 20a
		Caffeic acid		
free	6.54 ± 0.7c	6.86 ± 0.8c	nd	nd
esterified	54.1 ± 0.1b	56.5 ± 1.3b	95.7 ± 2.6b	75.3 ± 1.2b
insoluble-bound	628 ± 19a	636 ± 7.7a	449 ± 0.9a	234 ± 3.4a
		Ferulic acid		
free	nd	tr	nd	tr
esterified	tr	4.19 ± 0.2	nd	nd
insoluble-bound	nd	nd	nd	nd
		Hydroxycaffeic acid[b]		
free	nd	nd	nd	nd
esterified	nd	nd	nd	8.73 ± 0.4
insoluble-bound	nd	nd	nd	nd
		Ellagic acid		
free	nd	nd	1.75 ± 0.2	nd
esterified	0.70 ± 0.1	tr	nd	nd
insoluble-bound	nd	0.54 ± 0.1	nd	8.00 ± 0.7
		Caftaric acid[b]		
free	152 ± 14	101 ± 5.8	nd	1.83 ± 0.1
esterified	nd	nd	nd	nd
insoluble-bound	nd	nd	nd	nd
		Monogalloyl glucose[c]		
free	10.9 ± 0.7c	8.14 ± 0.4c	2.27 ± 0.2b	2.13 ± 0.0b
esterified	15.7 ± 0.3b	21.1 ± 0.9b	3.20 ± 0.3ab	4.73 ± 0.5a
insoluble-bound	27.5 ± 1.6a	27.1 ± 1.3a	3.61 ± 0.6a	4.00 ± 0.4a

[a] Data represent mean values for each sample ± standard deviations (n = 3). Means followed by the same letter within a column part are not significantly different ($p > 0.05$). nd, non-detected; tr, trace. [b]Compounds quantified as caffeic acid equivalents. [c]Compound quantified as gallic acid equivalent

As can be noted by both identification and quantification, a wide range of phenolics was present in the samples. Furthermore, each fraction and grape variety had its own fingerprint; thus, in the following sections, one should bear in mind that this difference may affect each assay to different extents, depending on the operative mechanisms.

Table 2.3 - Contents (μg/g of dry weight) of free, esterified and insoluble-bound monomeric flavonoids and derivatives of grape by-products[a]

	juicemaking by-products		winemaking by-products	
	Cora	Isabel	Syrah	Tempranillo
			Catechin	
free	190 ± 11b	114 ± 15b	12.8 ± 0.5c	21.6 ± 0.6c
esterified	9.66 ± 0.9c	82.1 ± 10c	111 ± 1.8b	209 ± 5.8b
insoluble-bound	896 ± 48a	530 ± 2.8a	874 ± 28a	506 ± 34a
			Epicatechin	
free	89.4 ± 0.2b	149 ± 11a	10.8 ± 1.7c	15.3 ± 0.6c
esterified	tr	26.3 ± 2.3b	46.0 ± 7.8b	73.0 ± 2.6b
insoluble-bound	173 ± 10a	157 ± 25a	162 ± 2.3a	105 ± 12a
			Quercetin	
free	2.29 ± 0.0b	3.45 ± 0.4	1.29 ± 0.3b	6.85 ± 0.3b
esterified	nd	nd	nd	2.39 ± 0.5c
insoluble-bound	4.77 ± 0.2a	nd	14.6 ± 1.6a	9.07 ± 0.6a
			Isorhamnetin[b]	
free	nd	nd	tr	11.1 ± 0.2b
esterified	53.4 ± 0.8	nd	nd	tr
insoluble-bound	nd	tr	tr	515 ± 48b
			Epicatechin gallate[b]	
free	38.7 ± 2.3b	35.3 ± 2.8b	45.0 ± 1.7c	25.7 ± 4.2c
esterified	nd	16.4 ± 2.9c	64.5 ± 5.7b	65.3 ± 3.3b
insoluble-bound	163 ± 1.1a	144 ± 5.4a	136 ± 1.6a	253 ± 3.7a
			Kaempferol hexoside[d]	
free	13.3 ± 1.0b	6.13 ± 0.9	11.4 ± 0.8	6.46 ± 0.5
esterified	tr	tr	nd	nd
insoluble-bound	37.7 ± 6.0a	tr	nd	nd
			Quercetin hexoside[b]	
free	94.2 ± 5.4a	187 ± 7.2a	20.9 ± 1.1b	35.0 ± 1.9
esterified	11.2 ± 0.2c	15.1 ± 0.6c	29.9 ± 3.4a	nd
insoluble-bound	64.2 ± 4.0b	48.3 ± 4.1b	9.46 ± 1.9c	nd
			Quercetin glucuronide[b]	
free	242 ± 2.0a	187 ± 7.2a	20.9 ± 1.1b	35.0 ± 1.6a
esterified	6.10 ± 0.8c	5.49 ± 0.2c	28.3 ± 2.6a	28.7 ± 1.5b
insoluble-bound	84.5 ± 4.5b	48.3 ± 4.1b	26.7 ± 1.7a	13.3 ± 1.1c
			Isorhamnetin hexoside[b]	
free	17.5 ± 1.2a	11.6 ± 2.3	29.4 ± 0.7b	30.1 ± 0.6a
esterified	nd	nd	25.6 ± 1.3a	9.92 ± 0.8b
insoluble-bound	70.6 ± 2.1b	nd	tr	tr
			Myricetin hexoside[b]	
free	190 ± 0.9a	16.2 ± 1.1a	tr	3.97 ± 0.08
esterified	nd	8.20 ± 0.1b	nd	nd
insoluble-bound	143 ± 7.9b	nd	nd	nd

[a] Data represent mean values for each sample ± standard deviations (n = 3). Means followed by the same letter within a column part are not significantly different ($p > 0.05$). nd, non-detected; tr, trace. [b]Compounds quantified as catechin equivalents

Table 2.4 - Contents (μg/g of dry weight) of free, esterified and insoluble-bound proanthocyanidins and derivatives of grape by-products[a]

	juicemaking by-products		winemaking by-products	
		Procyanidin dimer B[b]		
free	384 ± 26b	313 ± 22a	127 ± 12c	206 ± 1.1b
esterified	tr	24.2 ± 2.9c	333 ± 25b	254 ± 7.2b
insoluble-bound	687 ± 26a	124 ± 3.0c	2126 ± 3.5a	4108 ± 46a
		Procyanidin dimer A[b]		
free	nd	nd	nd	nd
esterified	nd	nd	nd	nd
insoluble-bound	915 ± 18	63.2 ± 7.4	nd	376 ± 12
		Prodelphinidin A[b]		
free	23.2 ± 3.9	nd	nd	tr
esterified	nd	nd	nd	nd
insoluble-bound	nd	nd	518 ± 12	16.7 ± 1.4
		Prodelphinidin B[b]		
free	tr	tr	nd	nd
esterified	tr	74.8 ± 5.1a	26.8 ± 3.2b	nd
insoluble-bound	nd	47.2 ± 3.0b	126 ± 10a	263 ± 20
		Galloyled procyanidin[b]		
free	nd	17.8 ± 1.3	66.2 ± 6.1c	50.4 ± 0.8b
esterified	nd	tr	105 ± 6.9b	151 ± 7.1a
insoluble-bound	nd	tr	147 ± 17a	38.0 ± 4.4c
		Procyanidin trimer C[b]		
free	nd	50.8 ± 7.3b	81.7 ± 12a	115 ± 14a
esterified	nd	15.5 ± 1.4c	28.7 ± 2.1b	tr
insoluble-bound	491 ± 49	269 ± 19a	nd	123 ± 1.6a

[a] Data represent mean values for each sample ± standard deviations (n = 3). Means followed by the same letter within a column part are not significantly different ($p > 0.05$). nd, non-detected; tr, trace. [b]Compounds quantified as catechin equivalents

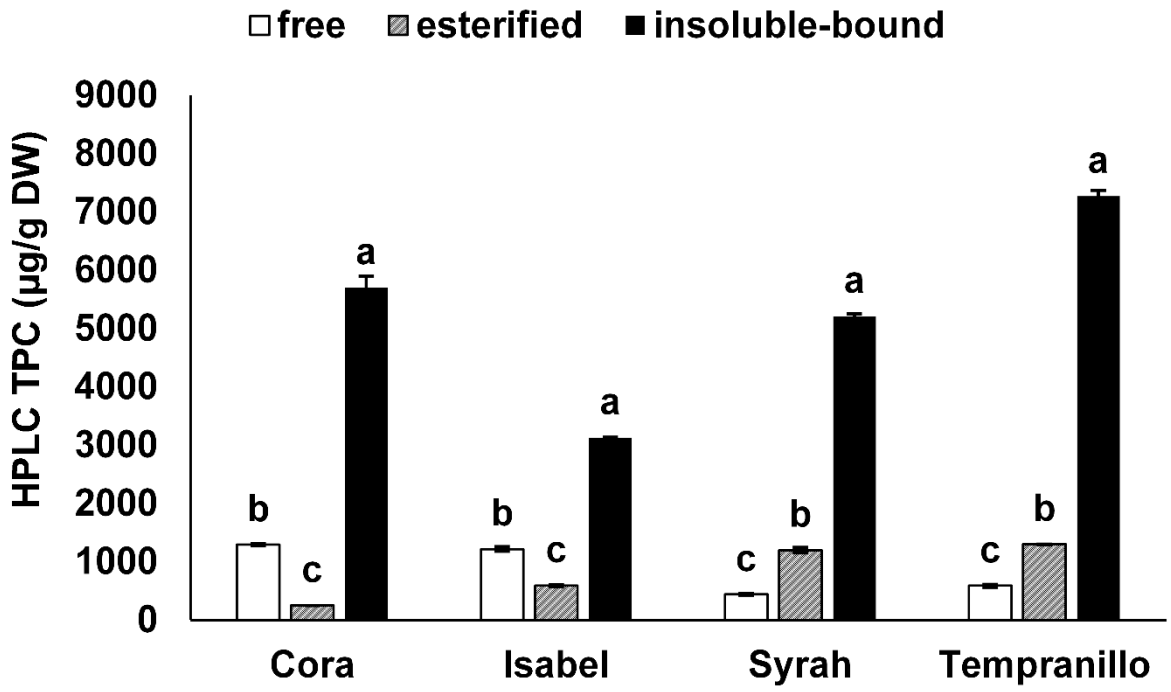


Figure 2.1 - HPLC-DAD-ESI-MS^n total phenolic content (HPLC TPC) of different fractions of juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products. Data represent the mean ± standard deviation of each sample (n = 3). Means with different letters indicate significant differences ($p < 0.05$) among fractions within the same grape variety

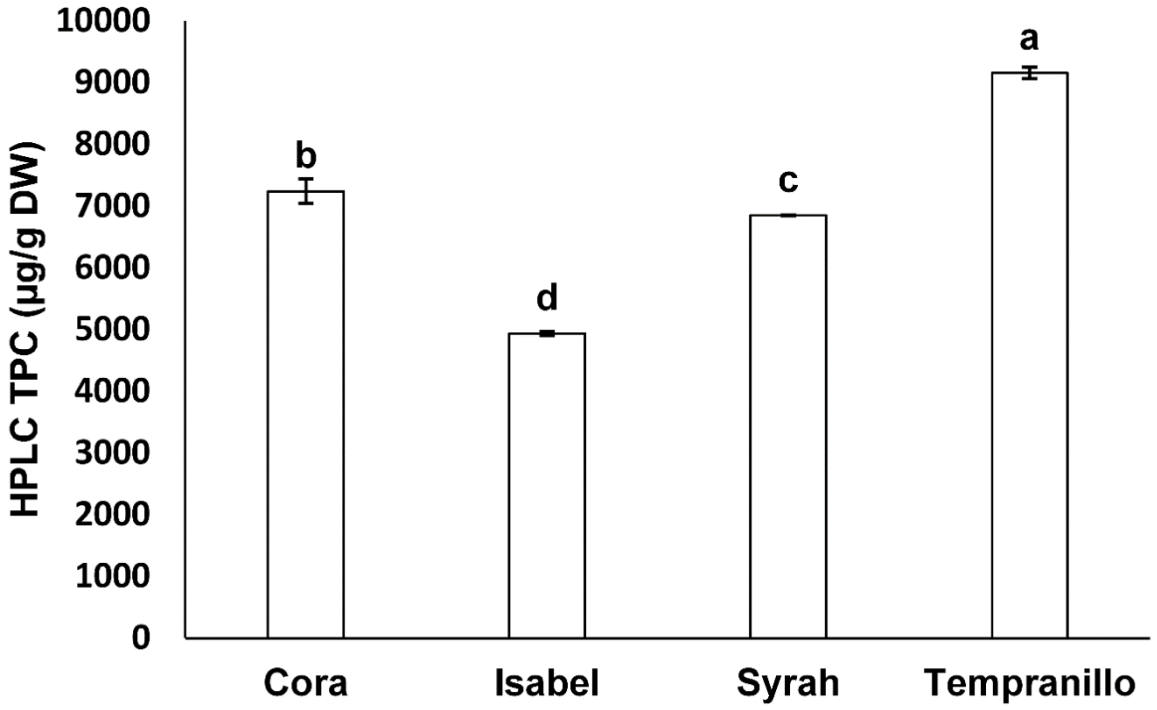


Figure 2.2 - HPLC-DAD-ESI-MS^n total phenolic content (HPLC TPC) representing free plus esterified plus insoluble-bound phenolics of juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products. Data represent the mean ± standard deviation of each sample (n = 3). Means with different letters indicate significant differences ($p < 0.05$)

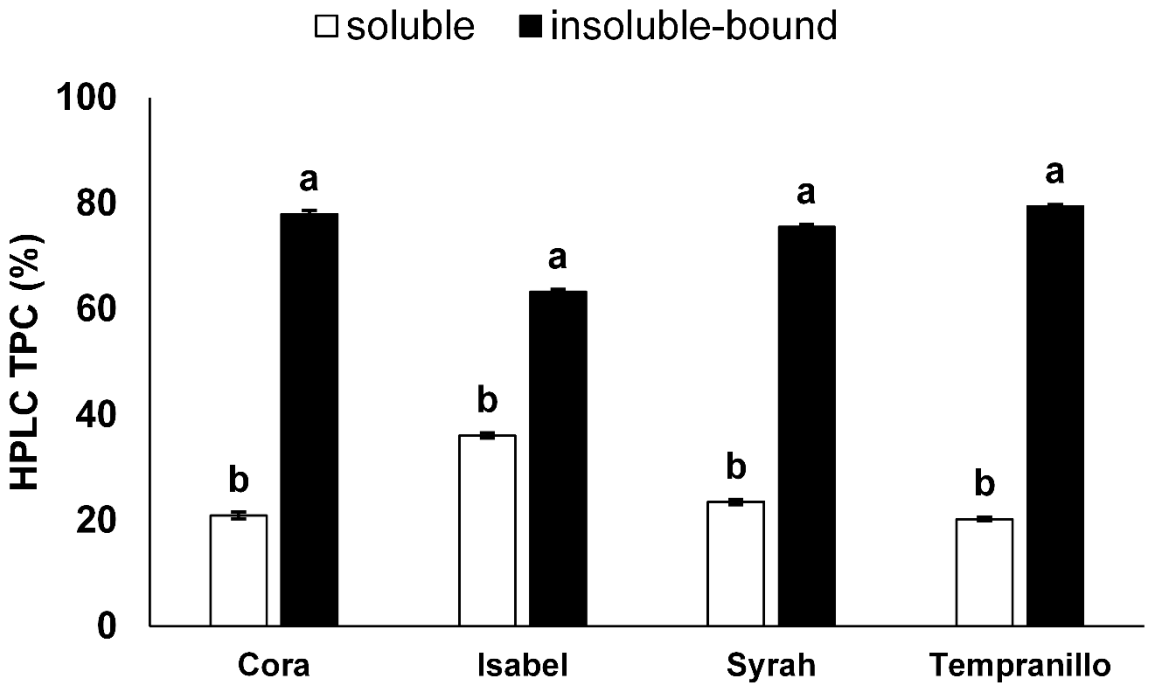


Figure 2.3 - HPLC-DAD-ESI-MS^n total phenolic content (HPLC TPC) of soluble and insoluble-bound fractions of juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products. Means with different letters indicate significant differences ($p < 0.05$) within the same grape variety

2.3.2 Total phenolic contents

The TPC of grape juice- and winemaking by-products are given in Table 2.3. The differences in the TPC, due to different grape varieties, are well documented (CHENG ET AL., 2012; GONZÁLEZ-CENTENO et al., 2012). The TPC of free, esterified, and insoluble-bound fractions were in the ranges of 45.8-124, 48.6-124, and 171-339 mg GAE/g dry weight of fat-free sample, respectively. These data are in accordance with a previous study on soluble phenolics (free plus esterified) from winemaking by-products, in which 10 grape varieties were evaluated (GONZÁLEZ-CENTENO et al., 2012).

Table 2.3 - Total phenolic content, proanthocyanidin content, antiradical scavenging capacity and reducing power of free, esterified and insoluble-bound phenolic compounds from juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products [a]

	juice by-products		winemaking by-products	
	Cora	Isabel	Syrah	Tempranillo
Total phenolic content (mg GAE/g DW)				
Free	124 ± 1.3Ab	112 ± 3.4Bb	45.8 ± 0.2Db	69.5 ± 1.9Cc
Esterified	48.6 ± 1.4Cc	69.3 ± 2.6Bc	65.0 ± 2.7Bb	124 ± 4.2Ab
Insoluble-bound	209 ± 5.9BCa	171 ± 12Ca	213 ± 14Ba	339 ± 23Aa
Proanthocyanidin content (mg CAT/g DW)				
Free	26.6 ± 0.9Bb	68.1 ± 2.3Ab	20.2 ± 2.2Cc	23.0 ± 0.7BCc
Esterified	7.22 ± 0.3Dc	17.1 ± 0.9Cc	25.0 ± 1.4Bb	45.6 ± 0.8Ab
Insoluble-bound	142 ± 5.7Aa	91.3 ± 4.0Ba	85.7 ± 1.8Ba	145 ± 14Aa
ABTS radical cation scavenging activity (μmol TE/g DW)				
Free	75.5 ± 6.2Ab	74.3 ± 8.2Ab	31.7 ± 1.5Dc	50.8 ± 0.4Cc
Esterified	34.6 ± 2.8Cc	52.5 ± 1.7Bc	53.5 ± 3.9Bb	99.7 ± 4.2Ab
Insoluble-bound	283 ± 7.9Ca	175 ± 8.4Da	561 ± 9.3Ba	738 ± 14Aa
DPPH radical scavenging activity (μmol TE/g)				
Free	137 ± 4.7Ab	79.3 ± 3.6Bb	36.9 ± 1.3Dc	56.2 ± 1.6Cc
Esterified	55.0 ± 1.0Cc	72.3 ± 1.1Bb	61.0 ± 3.1Cb	91.4 ± 4.1Ab
Insoluble-bound	233 ± 3.9Ca	185 ± 7.8Da	554 ± 13Ba	801 ± 18Aa
H_2O_2 scavenging activity (μmol TE/g)				
Free	143 ± 0.2Bb	167 ± 0.3Ab	75.8 ± 1.2Cb	43.2 ± 0.6Dc
Esterified	95.9 ± 1.6Bc	111 ± 1.7Ac	71.7 ± 0.1Cb	60.2 ± 0.1Db
Insoluble-bound	304 ± 3.1Aa	224 ± 14Ba	261 ± 28Ba	250 ± 1.7Ba
Reducing power (μmol TE/g DW)				
Free	56.1 ± 14ABb	67.5 ± 1.9Ab	39.9 ± 1.0BCc	33.7 ± 3.2Cc
Esterified	43.0 ± 1.4Db	52.9 ± 1.6Cc	90.3 ± 2.7Ab	71.5 ± 1.0Bb
Insoluble-bound	276 ± 10Ca	186 ± 7.0Da	484 ± 26Aa	358 ± 19Ba

[a] Data represent the mean values for each sample ± standard deviations (n = 3). Means followed by the same capital letters within a row are not significantly different ($p > 0.05$). Means followed by the same lower case letters within a column part are not significantly different ($p > 0.05$). CE, catechin equivalents; TE, Trolox equivalents; DW, dry weight of defatted sample

In the present study, by-products of the juice industry (Cora and Isabel) showed the highest TPC in the free phenolic fraction in comparison with the winery by-products (Syrah and Tempranillo). The lower TPC in the free fraction of winemaking by-products in comparison with those of grape juice by-products may be explained by different grape variety, their maturation, and crop production area; however, the process influence is also possible.

Polyphenols have been shown to provide a better affinity to alcoholic solvents; thus, grape skins and seeds of the winemaking process may lose their soluble phenolics to the liquid medium, which mimics a solvent system. Moreover, a clear cluster can be noted in relation to free and soluble esterified phenolics (e.g., grape juice- vs winemaking by-products). Cora and Isabel, which are grape juice by-products, had free phenolics in the range of 62-72%, in relation to total soluble phenolics (free plus esterified), whereas Syrah and Tempranillo, which are winery by-products, had those in the range of 36-41%. The opposite trend was found for the TPC of the fraction containing phenolics released from their esterified form. This can also be noted when the TPC is evaluated by HPLC (Figure 2.1). TPC of the insoluble-bound fractions did not indicate the same influence of the process as statistical analysis did not allow their grouping into different clusters (e.g., grape juice- or winemaking by-products), suggesting that the noted differences were due to varietal influences.

The extraction of insoluble-bound phenolics was conducted via hydrolysis using 4 M NaOH for 4 h. Meanwhile, a simple contact with the medium (e.g., grape juice or wine) would hardly be sufficient to release insoluble-bound phenolics during the grape juice- or winemaking manufacture. Only this would enable their loss to the liquid medium. As this is unlikely, it is possible to suggest that the differences found in the fraction containing insoluble-bound phenolics are due to grape variety, their maturation, and crop production area. The distribution of phenolic compounds among all forms (free, esterified, and insoluble-bound) depends not only on the feedstock but also on the variety and field conditions. Although further studies are necessary to confirm this assumption, the present study indicates a possible influence of the process (e.g., grape juice vs wine) in their distribution.

2.3.3 Proanthocyanidin content

Proanthocyanidins, also known as condensed tannins, consist of flavan-3-ol units, ranging from dimers to higher oligomers. Grapes have a high content of proanthocyanidins, especially procyanidin dimer B, which consists exclusively of catechin and epicatechin units; however, prodelphinidins, which have (epi)gallocatechin in their structures, are also found in grapes, as demonstrated before (Table 2.1). The method for estimating the PC used here showed better correlation with low molecular weight proanthocyanidins (2-4 units) (VRHOVSEK; MATTIVI; WATERHOUSE, 2001), however, the PC showed a significant and positive correlation ($p < 0.01$) with all antioxidant assays carried out in the present study (Table 2.4), indicating the quality of the results.

The PC is shown in Table 2.3; the results presented are in good agreement with those reported for soluble phenolics (free plus esterified) of winemaking by-products (GONZÁLEZ-CENTENO et al., 2012). In accordance with TPC in the present study, the highest PC was found in the insoluble-bound fraction, and its content was up to 6-fold higher than that found in the free fraction. Furthermore, winemaking by-products (Syrah and Tempranillo) had the highest esterified PC in comparison with the juice by-products (Cora and Isabel), suggesting that the grape juice process influence on PC takes place in the esterified fraction.

The juice process in the present study was carried out at 85°C, and pH 3.5 for 60 min. Meanwhile, no heat was applied during the winemaking process. Acid hydrolysis (95°C, 45 min) has been used to release phenolics from their glycosidic bonds (CHANDRASEKARA; SHAHIDI, 2011b). Flavanone 3-hydroxylase is the first enzyme in the biosynthesis of proanthocyanidins. Its optimum temperature is 30-40°C (OWENS et al., 2008; FLACHOWSKY et al., 2012). The remaining enzymes also have low optimum temperature (e.g., dihydroflavonol reductase, 30-45°C; leucoanthocyanidin reductase, 30-37°C; and anthocyanidin reductase, 45-55°C) (XIE et al., 2004; XIE; SHARMA; DIXON, 2004; PFEIFFER et al., 2006). High temperature and time used during the juice process may denature all enzymes present. Thus, heating and acidic condition may release phenolics from their esters during juicemaking. Furthermore, enzyme inactivation probably occurs, which affects further biosynthesis. Thus, both factors lower the content of esterified proanthocyanidins and the remaining phenolics in the juice by-products as compared to the winemaking ones.

Supporting the findings of the present study, higher contents of soluble proanthocyanidins (free plus esterified) were found in grape skin and seeds produced in winemaking with a longer fermentation process (CERPA-CALDERÓN; KENNEDY, 2008). It is noteworthy that the highest PC in the esterified fraction of winemaking by-products is supported by the highest procyanidin B content, the major proanthocyanidin present, as evaluated by HPLC-DAD-ESI-MS^n (Table 2.2). Interestingly, both catechin and epicatechin, which are subunits of procyanidins, were found in higher concentrations in the esterified fraction of the winemaking by-products in the present study.

2.3.4 ABTS radical cation scavenging activity

In the present study, no difference was found between the ABTS antioxidant activity (Table 2.3) of the free phenolic extracts from Cora and Isabel (juicemaking by-products), which had the highest scavenging activities in comparison with those of the winemaking by-products (Syrah and Tempranillo). On the other hand, the Tempranillo variety showed the highest

scavenging activity for esterified and insoluble-bound fractions. Furthermore, the Syrah cultivar exhibited the highest difference between the scavenging activity of the soluble (free plus esterified) and insoluble-bound fractions, the latter contributing up to 6.5-fold higher scavenging activity than the soluble counterpart. Gallic acid is a potent antioxidant, and grapes contain a high amount of gallic acid and polyphenols bearing galloyl groups (Table 2.2), which may explain the large difference in scavenging activity between soluble and insoluble-bound fractions, despite the relatively low difference found in their TPC, which was not higher than 1.9-fold (Table 2.3). In accordance with TPC and PC, the highest scavenging activity for all grape by-products was found for the insoluble-bound fraction.

2.3.5 DPPH radical scavenging activity using electron paramagnetic resonance

The DRSA results (Table 2.3) showed a trend similar to those for the free phenolic fraction in both ABTS and TPC determinations, for which grape juice by-products (Cora and Isabel) demonstrated higher values. However, Cora had the highest DRSA, different from that observed in the ABTS assay, which exhibited no significant difference between both juice by-products. Different results, depending on the method used, may be obtained due to different chemical structures as well as synergistic, additive, or antagonistic interaction effects. Furthermore, pro-oxidant effects are also possible depending on the reaction mechanism and polyphenol concentration, among other factors (MEYER; HEINONEN; FRANKEL, 1998; WANASUNDARA; SHAHIDI, 1998).

2.3.6 Hydrogen peroxide scavenging activity

The scavenging activity of hydrogen peroxide by grape by-products is shown in Table 2.3. Similar to other antioxidant assays, the insoluble-bound fraction exhibited a higher efficacy in scavenging hydrogen peroxide. Interestingly, for the first time in the present study, insoluble-bound phenolics from Cora variety exhibited the most antioxidant effectiveness, suggesting the presence of a particular polyphenol or a synergistic effect among some polyphenols that renders a higher protective influence to Cora against ROS. The remaining results for the free fraction followed a similar trend to TPC, rendering a different cluster, grape juice by-products (Cora and Isabel) being the most effective in the free phenolic fraction. Furthermore, different from other antioxidant assays, the esterified fraction of grape juice by-products also displayed the highest effect against H_2O_2. Grape by-products have shown scavenging activity against superoxide anion and peroxyl and hydroxyl radicals (YILMAZ; TOLEDO, 2004; FALCHI et al., 2006; CHENG et al., 2012; JARA-PALACIOS et al., 2013).

2.3.7 Reducing power

The RP of grape by-products is summarized is Table 2.3, indicating the ability of polyphenols present to reduce the ferric ion to the ferrous ion. The comparison of reducing power within the free fraction showed that Cora and Isabel, which are grape juice by-products, had the highest efficacy in reducing ferric to ferrous ions, compared to winemaking by-products (Syrah and Tempranillo). On the other hand, in the esterified fraction, the winemaking by-products accounted for the highest RP in comparison to grape juice by-products, which was also found for the insoluble-bound fraction. Finally, the comparison among free, esterified, and insoluble-bound phenolic fractions confirmed the highest potential bioactivity for the insoluble-bound fraction in all grape varieties. Other studies (GONZÁLEZ-CENTENO et al., 2012; JARA-PALACIOS et al., 2013) provide information supporting the reducing ability of grape by-products; however, a direct comparison is not possible due to different units used to report the results and the methods employed in these determinations. Most studies have been using ferric reducing antioxidant power assay (FRAP) at pH 3.6, which is different from the pH 6.6 used in this study. The antioxidant/reducing power of phenolic compounds is known to be dependent on the pH of the medium (PAIVA-MARTINS; GORDON, 2002).

2.3.8 Copper-induced LDL-cholesterol oxidation

The presence of oxidized LDL-cholesterol at high levels constitutes a risk factor for atherosclerosis. In the present study, copper-induced oxidation of human LDL-cholesterol was inhibited by all phenolic extracts (Figure 2.4) and the highest effect was rendered by the insoluble-bound fraction. A recent study demonstrated that nonextractable polyphenols are the major contributors to the Spanish diet (ARRANZ; SILVÁN; SAURA-CALIXTO, 2010).

Consumption of commodities containing phenolic acids, monomeric flavonoids, and proanthocyanidins has been correlated with a decrease in the level of oxidized cholesterol in high-risk cardiovascular patients (KHAN et al., 2012). Grapes have a high content of procyanidin B and monomeric flavonoids as well as gallic acid as their major phenolics, the latter being an antioxidatively potent phenol. The presence of this broad range of bioactive compounds may be responsible for the ability of grape products to prevent LDL-cholesterol oxidation and potentially the development of heart disease.

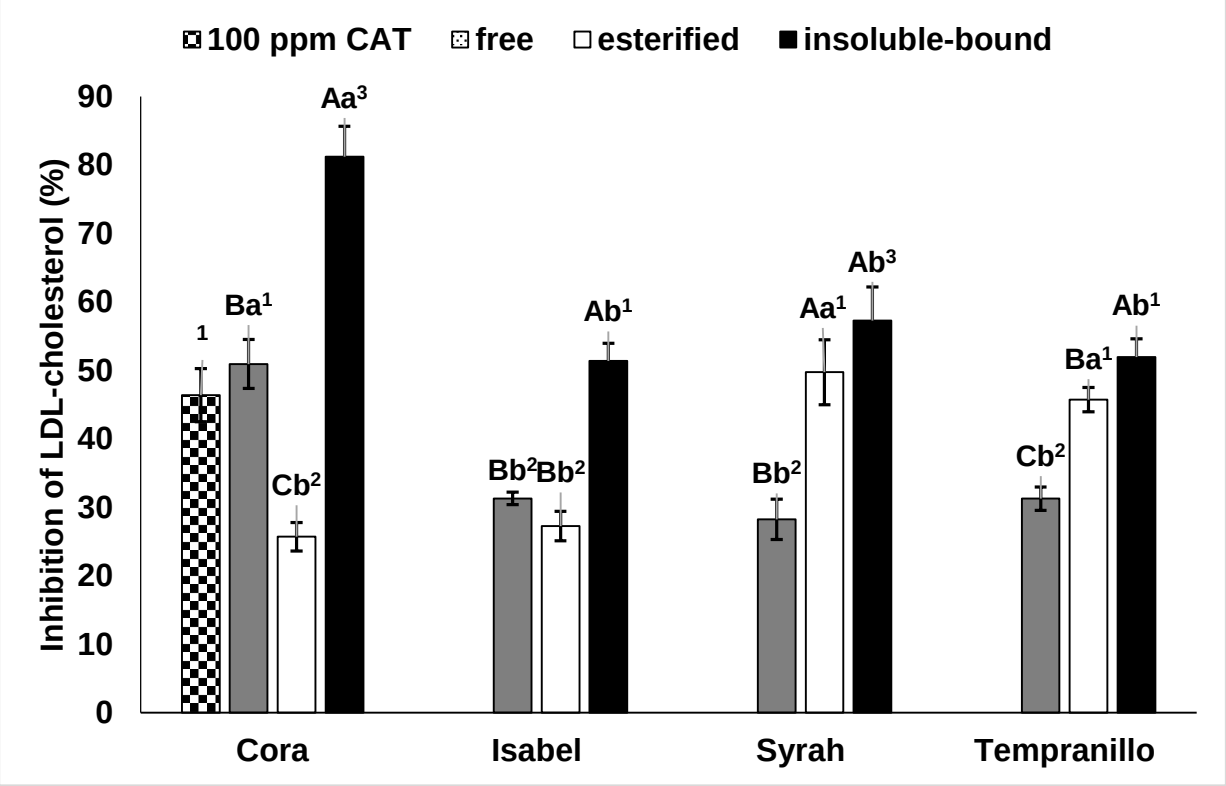


Figure 2.4 - Biological activity of free, esterified and insoluble-bound phenolics of juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products against LDL-cholesterol induced oxidation. Data represent the mean ± standard deviation of each sample (n = 3). Means with different superscript figures indicate significant differences ($p < 0.05$) compared to 100 ppm catechin (CAT). Extracts were assayed in concentration of 100 ppm total phenolic content equivalent (as evaluated by HPLC-DAD-ESI-MS^n). Means with different capital letters indicate significant differences ($p < 0.05$) among fractions within the same grape variety. Means with different lower case letters indicate significant differences ($p < 0.05$) among grape varieties within the same fraction

2.3.9 Inhibition of peroxyl radical induced supercoiled plasmid DNA strand breakage

ROS oxidize the native form of DNA, which can be evaluated by its conversion to a nicked circular or linear form via single- or double-strand breaks, respectively. Such a process affects DNA replication and transcription, causing mutagenesis and cancer initiation. The present study evaluated the ability of different grape processing by-products in inhibiting peroxyl radical induced supercoiled plasmid DNA strand breakage (Figure 2.5). Peroxyl radicals have a relatively long half-life, and therefore their detrimental effects may not only take place at a cellular level but also be extended to biological fluids. All grape extract phenolics rendered a DNA protective effect against peroxyl radicals.

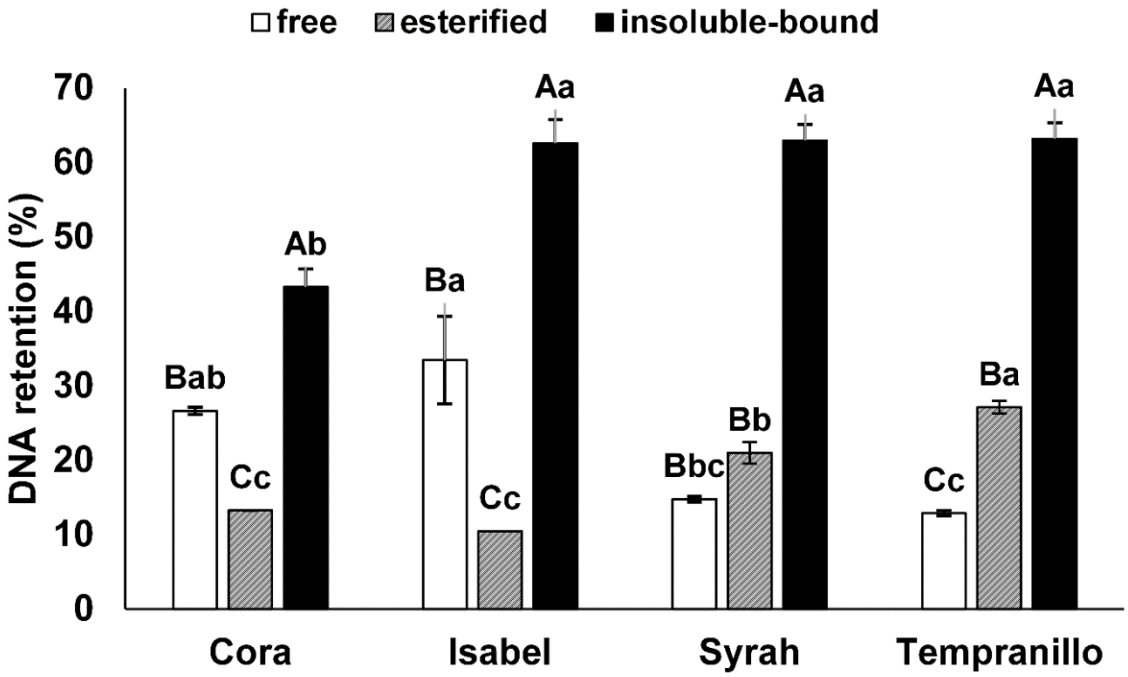


Figure 2.5 - Supercoiled plasmid DNA strand breakage inhibition of free, esterified, and insoluble phenolic fractions from juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products. Data represent the mean ± standard deviation of each sample (n = 3). Means with different capital letters indicate significant differences ($p < 0.05$) among fractions within the same grape variety. Means with different lower case letters indicate significant differences ($p < 0.05$) among grape varieties within the same fraction. Results were transformed for concentrations of 1 mg/mL of defatted sample. Representative raw data are given in Figure 2.5

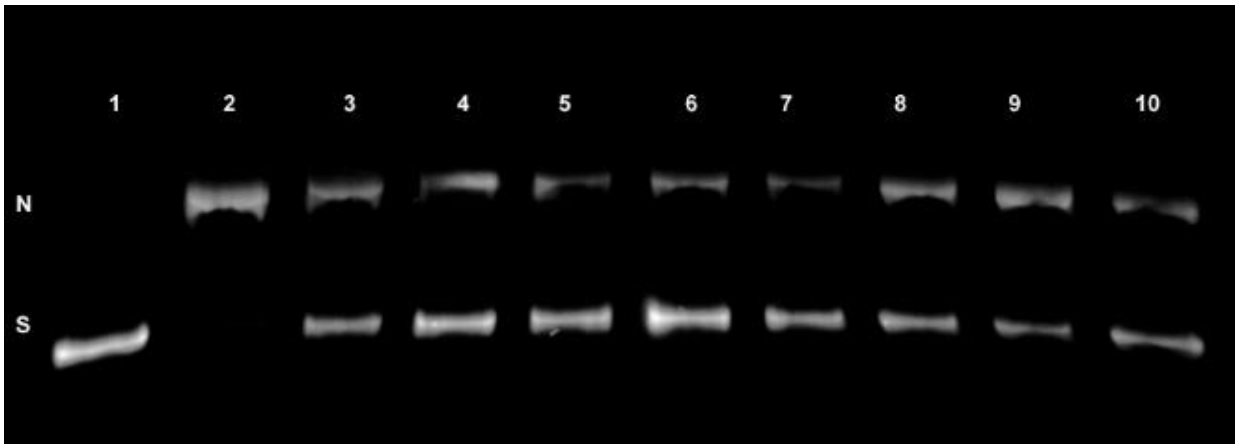


Figure 2.6 - Representative raw data of supercoiled plasmid DNA strand breakage inhibition of the free and esterified phenolic fraction of juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products. Lane 1: Control (DNA only); lane 2: DNA + AAPH; lane 3: free phenolics from Cora (2.5 mg/mL; 53 ± 1 % DNA retention); lane 4: free phenolics from Isabel (2.5 mg/mL; 67 ± 12 % DNA retention); lane 5: free phenolics from Syrah (5.0 mg/mL; 74 ± 2 % DNA retention); lane 6: free phenolics from Tempranillo (5.0 mg/mL; 64 ± 2 % DNA retention); lane 7: esterified phenolics from Cora (5.0 mg/mL; 66 ± 0.3 % DNA retention); lane 8: esterified phenolics from Isabel (5.0 mg/mL; 52 ± 0.2 % DNA retention); lane 9: esterified phenolics from Syrah (2.5 mg/mL; 42 ± 3 % DNA retention); lane 10: esterified phenolics Tempranillo (2.5 mg/mL; 54 ± 2 % DNA retention).Data represent the mean ± standard deviation of each sample (n = 3). S and N are supercoiled and nicked plasmid DNA strands, respectively

Insoluble-bound phenolics had the highest protective action against peroxyl radical induced supercoiled plasmid DNA strand breakage, showing supercoiled DNA retention of up to 63%. The concentration used for this fraction was equivalent to 1 mg/mL of defatted sample (8 ppm of phenolic compounds as evaluated by HPLC-DAD-ESI-MS^n), showing that even a very low concentration of insoluble-bound phenolics of grape by-products may have health benefits.

Minor differences among cultivars were found in the insoluble-bound fraction, thus suggesting little varietal influence on polyphenols liberated from cell walls via hydrolysis. Varietal differences in the free and esterified fractions were in accordance with other antioxidant assays, showing a clear cluster between juice (Cora and Isabel) and winemaking (Syrah and Tempranillo) by-products, in which, generally, juice by-products had higher free phenolic content than winemaking by-products and the opposite was found for their esterified counterparts.

2.3.10 Correlation of analyses

Correlation analyses were carried out between TPC, PC, and the bioactivity evaluations (Table 2.4). Correlations of individual phenolics quantified were also evaluated to better understand their contribution on each activity. All antioxidant assays were correlated with either TPC and PC, and only the correlation between TPC and inhibition of LDL-cholesterol oxidation demonstrated a lower significance ($p < 0.05$), whereas the remaining results exhibited a higher significance ($p < 0.01$). Most antioxidant assays evaluated (ABTS, DPPH, RP, and supercoiled plasmid DNA strand breakage inhibition) demonstrated a higher correlation with TPC, suggesting that phenolic compounds other than proanthocyanidins may play an important role in preventing oxidation-related diseases.

Catechin and epicatechin gallate were the only compounds showing significant correlations with all antioxidant assays. *p*-Coumaric acid, caffeic acid, epicatechin, quercetin, kaempferol hexoside, and procyanidin dimer B had correlations in four of six assays conducted, being positively correlated to 67% of the methods used. In general, epicatechin gallate had higher correlations with the antioxidant assays as compared with epicatechin itself. This suggests that galloyl groups had positive effects on the antioxidant activities of the grape by-products studied here. Epicatechin has only four hydroxyl groups, whereas epicatechin with a gallic acid attached (epicatechin gallate) has eight. In fact, both the stereochemistry and number of hydroxyl groups may influence the activity of phenolic compounds. *p*-Coumaric acid, gallic acid, caffeic acid, catechin, epicatechin, and epicatechin gallate were positively and

significantly correlated with both biological model systems (inhibition of copper-induced human LDL-cholesterol oxidation and peroxyl radical-induced DNA strand breakage), whereas some compounds correlated only with the inhibition of peroxyl-induced DNA strand breakage (quercetin and procyanidin dimer B) or inhibition of copper-induced human LDL-cholesterol oxidation (kaempferol hexoside and procyanidin trimer C). Catechin and epicatechin have also been found to protect against N-nitrosodibutylamine- and N-nitrosopiperidine-induced DNA damage in human hepatoma cells *in vitro* (DELGADO et al., 2009). Catechin and caffeic acid also prevented human LDL-cholesterol oxidation (MEYER; HEINONEN; FRANKEL, 1998).

Table 2.4 - Pearson's correlation between total phenolic content (TPC), proanthocyanidin content (PC), or phenolic compounds and scavenging activities, reducing power or induced LDL-cholesterol oxidation inhibition[a]

	ABTS	DPPH	H_2O_2	RP	LDL	DNA
TPC	0.9319**	0.9239**	0.8181**	0.8384**	0.6606*	0.8807**
PC	0.7945**	0.7496**	0.8887**	0.7807**	0.7495**	0.8506**
Protocatechuic acid	0.8213*	0.8581*	0.4973ns	0.5212ns	0.2419ns	0.5876ns
p-Coumaric acid	0.5819*	0.5382ns	0.5622ns	0.7322**	0.7720**	0.6815*
Gallic acid	0.3700ns	0.2925ns	0.7417**	0.5395ns	0.8010**	0.6128*
Caffeic acid	0.4517ns	0.3727ns	0.7939**	0.6696**	0.7308**	0.7338**
Ellagic acid	0.9500*	0.9527*	0.5826ns	0.8390ns	0.5178ns	0.5058ns
Monogalloyl glucose	-0.1151ns	-0.1460ns	0.4534ns	0.0279ns	0.3199ns	0.1654ns
Catechin	0.7323**	0.6650*	0.9066**	0.8983**	0.8634**	0.8337**
Epicatechin	0.5034ns	0.4530ns	0.8703**	0.6642*	0.6904*	0.8212**
Quercetin	0.7839*	0.7540*	0.5373ns	0.8589**	0.3045ns	0.7494*
Epicatechin gallate	0.8867**	0.8662**	0.7819**	0.8186**	0.6653*	0.8605**
Kaempferol hexoside	0.9529*	0.9050*	0.8583ns	0.9620**	0.9500*	0.7157ns
Quercetin hexoside	-0.1648ns	-0.1637ns	0.1946ns	-0.1860ns	0.0434ns	0.1357ns
Isorhamnetin hexoside	0.7958*	0.6877ns	0.6917ns	0.8500*	0.7192ns	0.4240ns
Myricetin hexoside	0.5157ns	0.7752ns	0.5610ns	0.4714ns	0.7710ns	0.5710ns
Procyanidin dimer B	0.9544**	0.9733**	0.5560ns	0.7706**	0.3296ns	0.6692*
Prodelphinidin B	0.9113*	0.9360*	0.6348ns	0.6413ns	0.2539ns	0.5229ns
Galloyled procyanidin	0.0529ns	0.0071ns	-0.0578ns	0.2869ns	0.5994ns	0.1229ns
Procyanidin trimer C	0.2864ns	0.1921ns	0.7534*	0.5935ns	0.8543**	0.5192ns

[a]**, is significant at $p < 0.01$; *, significant at $p < 0.05$; ns is nonsignificant. ABTS, ABTS radical cation scavenging activity; DPPH, DPPH radical scavenging activity; H_2O_2, H_2O_2 scavenging activity; RP, Reducing power; LDL, copper-induced LDL-cholesterol oxidation, DNA; supercoiled plasmid DNA strand breakage inhibition. Correlations for ferulic acid, hydroxycaffeic acid, caftaric acid, procyanidin dimer A, and prodelphinidin A were not calculated due to insufficient data

2.4 Conclusions

The present contribution demonstrated the distribution of free, esterified, and insoluble-bound phenolics in juice- and winemaking by-products. Although further studies are necessary for confirmation, it is possible to assume that differences in the distribution of soluble free and esterified phenolics (Figure 2.1) are due to the process (juice- vs winemaking). Insoluble-bound phenolics must be included in all studies due to their major contribution and efficacy in the by-products tested. Twenty-five phenolic compounds were positively or tentatively identified by HPLC-DAD-ESI-MS^n. Gallic and caffeic acids were the major phenolic acids, catechin was the

major monomeric flavonoid, and procyanidin dimer B was the major proanthocyanidin. Procyanidin dimer A was found only in the insoluble-bound fraction, which contained the highest concentration of phenolics, reflecting a higher scavenging activity, reducing power, and inhibition of copper-induced human LDL-cholesterol oxidation and peroxyl-induced DNA strand breakage. Catechin and epicatechin gallate showed significant and positive correlations with all antioxidant assays.

References

ALASALVAR, C.; KARAMAC, M.; KOSINSKA, A.; RYBARCZYK, A.; SHAHIDI, F.; AMAROWICZ, R. Antioxidant activity of hazelnut skin phenolics. **Journal of Agricultural and Food Chemistry,** Easton, v. 57, p. 4645-4650, 2009.

ARRANZ, S.; SILVÁN, J.M.; SAURA-CALIXTO, F. Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: a study on the Spanish diet. **Molecular Nutrition & Food Research,** Weinheim, v. 54, p. 1646–1658, 2010.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. **Official methods of analysis of AOAC International.** 17th ed. Gaithersburg: AOAC International, 2000. 937 p.

_____. **Official methods of analysis of AOAC International**. 18th ed. Gaithersburg: AOAC International, 2005. 1v.

CERPA-CALDERÓN, F.K.; KENNEDY, J.A. Berry integrity and extraction of skin and seed proanthocyanidins during red wine fermentation. **Journal of Agricultural and Food Chemistry,** Easton, v. 56, p. 9006-9014, 2008.

CHANDRASEKARA, A.; SHAHIDI, F. Antiproliferative potential and DNA scission inhibitory activity of phenolics from whole millet grains. **Journal of Functional Foods,** Amsterdam, v. 3, p. 159-170, 2011a.

_____. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. **Journal of Functional Foods,** Amsterdam, v. 3, p. 144-158, 2011b.

_____. Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels, and testa. **Journal of Agricultural and Food Chemistry,** Easton, v. 59, p. 5006-5014, 2011c.

CHENG, V.J.; BEKHIT, A.E.A.; MCCONNELL, M.; MROS, S.; ZHAO, J. Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. **Food Chemistry,** London, v. 134, p. 474-482, 2012.

ĆURKO, N.; KOVAČEVIĆ GANIĆ, K.; GRACIN, L.; ĐAPIĆ, M.; JOURDES, M.; TEISSEDRE, P.L. Characterization of seed and skin polyphenolic extracts of two red grape

cultivars grown in Croatia and their sensory perception in a wine model medium. **Food Chemistry,** London, v. 145, p. 15–22, 2014.

DE CAMARGO, A.C.; VIDAL, C.M.M.; CANNIATTI-BRAZACA, S.G.; SHAHIDI, F. Fortification of cookies with peanut skin: effects on the composition, polyphenols, antioxidant properties and sensory quality. **Journal of Agricultural and Food Chemistry**, Easton, v. 62, p. 11228–11235, 2014.

DE CAMARGO, A.; VIEIRA, T.; REGITANO-D'ARCE, M.; CALORI-DOMINGUES, M.; CANNIATTI-BRAZACA, S. Gamma radiation effects on peanut skin antioxidants. **International Journal of Molecular Sciences,** Basel, v. 13, p. 3073-3084, 2012a.

DE CAMARGO, A. C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; CANNIATTI-BRAZACA, S.G. Gamma radiation induced oxidation and tocopherols decrease in in-shell, peeled and blanched peanuts. **International Journal of Molecular Sciences,** Basel, v. 13, p. 2827-2845, 2012b.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; SPOTO, M.H.F.; CANNIATTI-BRAZACA, S.G. Gamma irradiation of in-shell and blanched peanuts protects against mycotoxic fungi and retains their nutraceutical components during long-term storage. **International Journal of Molecular Sciences,** Basel, v. 13, p. 10935-10958, 2012c.

DELGADO, M.E.; HAZA, A.I.; GARCÍA, A.; MORALES, P. Myricetin, quercetin, (+)-catechin and (−)-epicatechin protect against N-nitrosamines-induced DNA damage in human hepatoma cells. **Toxicology in Vitro,** Oxford, v. 23, p. 1292-1297, 2009.

DOWNES, F.P.; ITO, K. **Compendium of methods for the microbiological examination of food**. 4th ed. Washington: American Public Health Association, 2001. 676 p.

EVANS, M.; WILSON, D.; GUTHRIE, N. A randomized, double-blind, placebo-controlled, pilot study to evaluate the effect of whole grape extract on antioxidant status and lipid profile. **Journal of Functional Foods,** Amsterdam, v. 7, p. 680–691, 2014.

FALCHI, M.; BERTELLI, A.; LO SCALZO, R.; MORASSUT, M.; MORELLI, R.; DAS, S.; CUI, J.; DAS, D.K. Comparison of cardioprotective abilities between the flesh and skin of grapes. **Journal of Agricultural and Food Chemistry,** Easton, v. 54, p. 6613-6622, 2006.

FLACHOWSKY, H.; HALBWIRTH, H.; TREUTTER, D.; RICHTER, K.; HANKE, M.-V.; SZANKOWSKI, I.; GOSCH, C.; STICH, K.; FISCHER, T. C. Silencing of flavanone-3-hydroxylase in apple (*Malus* × *domestica* Borkh.) leads to accumulation of flavanones, but not to reduced fire blight susceptibility. **Plant Physiology and Biochemistry,** Paris, v. 51, p. 18-25, 2012.

GONZÁLEZ-CENTENO, M.R.; JOURDES, M.; FEMENIA, A.; SIMAL, S.; ROSSELLÓ, C.; TEISSEDRE, P.-L. Proanthocyanidin composition and antioxidant potential of the stem winemaking byproducts from 10 different grape varieties (*Vitis vinifera* L.). **Journal of Agricultural and Food Chemistry,** Easton, v. 60, p. 11850-11858, 2012.

HANHINEVA, K.; ROGACHEV, I.; KOKKO, H.; MINTZ-ORON, S.; VENGER, I.; KÄRENLAMPI, S.; AHARONI, A. Non-targeted analysis of spatial metabolite composition in strawberry (*Fragaria* × *ananassa*) flowers. **Phytochemistry,** New York, v. 69, p. 2463–2481, 2008.

JARA-PALACIOS, M.J.; GONZALEZ-MANZANO, S.; ESCUDERO-GILETE, M. L.; HERNANZ, D.; DUENAS, M.; GONZALEZ-PARAMAS, A.M.; HEREDIA, F.J.; SANTOS-BUELGA, C. Study of Zalema grape pomace: phenolic composition and biological effects in *Caenorhabditis elegans*. **Journal of Agricultural and Food Chemistry,** Easton, v. 61, p. 5114-5121, 2013.

KHAN, N.; MONAGAS, M.; ANDRES-LACUEVA, C.; CASAS, R.; URPI-SARDA, M.; LAMUELA-RAVENTOS, R.M.; ESTRUCH, R. Regular consumption of cocoa powder with milk increases HDL cholesterol and reduces oxidized LDL levels in subjects at high-risk of cardiovascular disease. **Nutrition Metabolism and Cardiovascular Diseases,** Rome, v. 22, p. 1046-1053, 2012.

LAGO-VANZELA, E.S.; REBELLO, L.P.G.; RAMOS, A.M.; STRINGHETA, P.C.; DA-SILVA, R.; GARCÍA-ROMERO, E.; GÓMEZ-ALONSO, S.; HERMOSÍN-GUTIÉRREZ, I. Chromatic characteristics and color-related phenolic composition of Brazilian young red wines made from the hybrid grape cultivar BRS Violeta ("BRS Rúbea" × "IAC 1398-21"). **Food Research International,** Barking, v. 54, p. 33–43, 2013.

LEE, N.; JO, C.; SOHN, S.; KIM, J.; BYUN, M. Effects of gamma irradiation on the biological activity of green tea byproduct extracts and a comparison with green tea leaf extracts. **Journal of Food Science,** Chicago, v. 71, p. 269-274, 2006.

LLOBERA, A.; CANELLAS, J. Dietary fibre content and antioxidant activity of Manto Negro red grape (*Vitis vinifera*): pomace and stem. **Food Chemistry,** London, v. 101, p. 659-666, 2007.

MEYER, A.S.; HEINONEN, M.; FRANKEL, E.N. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. **Food Chemistry,** London, v. 61, p. 71–75, 1998.

OWENS, D.K.; CROSBY, K.C.; RUNAC, J.; HOWARD, B.A.; WINKEL, B.S.J. Biochemical and genetic characterization of Arabidopsis flavanone 3β-hydroxylase. **Plant Physiology and Biochemistry,** Paris, v. 46, p. 833-843, 2008.

OYAIZU, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. **The Japanese Journal of Nutrition and Dietetics,** Tokyo, v. 44, p. 307-315, 1986.

PAIVA-MARTINS, F.; GORDON, M.H. Effects of pH and ferric ions on the antioxidant activity of olive polyphenols in oil-in-water emulsions. **Journal of the American Oil Chemists Society,** Chicago, v. 79, p. 571-576, 2002.

PFEIFFER, J.; KÜHNEL, C.; BRANDT, J.; DUY, D.; PUNYASIRI, P.A.N.; FORKMANN, G.; FISCHER, T.C. Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (*Vitis vinifera* L.), apple (*Malus* x *domestica* Borkh.) and other crops. **Plant Physiology and Biochemistry,** Paris, v. 44, p. 323-334, 2006.

PRICE, M.L.; HAGERMAN, A.E.; BUTLER, L.G. Tannin content of cowpeas, chickpeas, pigeon peas, and mung beans. **Journal of Agricultural and Food Chemistry,** Easton, v. 28, p. 459-461, 1980.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.; RICE-EVANS, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine,** New York, v. 26, p. 1231-1237, 1999.

SAINT-CRICQ DE GAULEJAC, N.; PROVOST, C.; VIVAS, N. Comparative study of polyphenol scavenging activities assessed by different methods. **Journal of Agricultural and Food Chemistry,** Easton, v. 47, p. 425-431, 1999.

SANDHU, A.K.; GU, L.W. Antioxidant capacity, phenolic content, and profiling of phenolic compounds in the seeds, skin, and pulp of *Vitis rotundifolia* (muscadine grapes) as determined by HPLC-DAD-ESI-MS^n. **Journal of Agricultural and Food Chemistry,** Easton, v. 58, p. 4681-4692, 2010.

SARNOSKI, P.J.; JOHNSON, J.V.; REED, K.A.; TANKO, J.M.; O'KEEFE, S.F. Separation and characterisation of proanthocyanidins in Virginia type peanut skins by LC-MS^n. **Food Chemistry,** London, v. 131, p. 927-939, 2012.

SHAHIDI, F.; ALASALVAR, C.; LIYANA-PATHIRANA, C.M. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. **Journal of Agricultural and Food Chemistry,** Easton, v. 55, p. 1212-1220, 2007.

SHIRAHIGUE, L.; PLATA-OVIEDO, M.; DE ALENCAR, S.; D'ARCE, M.; VIEIRA, T.; OLDONI, T.; CONTRERAS-CASTILLO, C. Wine industry residue as antioxidant in cooked chicken meat. **International Journal of Food Science and Technology,** Oxford, v. 45, p. 863-870, 2010.

SWAIN, T.; HILLIS, W.E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. **Journal of the Science of Food and Agriculture,** London, v. 10, p. 63-68, 1959.

VRHOVSEK, U.; MATTIVI, F.; WATERHOUSE, A.L. Analysis of red wine phenolics: comparison of HPLC and spectrophotometric methods. **Vitis,** Siebeldingen, v. 40, p. 87-91, 2001.

WANASUNDARA, U.N.; SHAHIDI, F. Antioxidant and pro-oxidant activity of green tea extracts in marine oils. **Food Chemistry,** London, v. 63, p. 335-342, 1998.

WEIDNER, S.; POWAŁKA, A.; KARAMAĆ, M.; AMAROWICZ, R. Extracts of phenolic compounds from seeds of three wild grapevines: comparison of their antioxidant activities and the content of phenolic compounds. **International Journal of Molecular Sciences,** Basel, v. 13, p. 3444-3457, 2012.

WEIDNER, S.; RYBARCZYK, A.; KARAMAĆ, M.; KRÓL, A.; MOSTEK, A.; GRĘBOSZ, J.; AMAROWICZ, R. Differences in the phenolic composition and antioxidant properties between *Vitis coignetiae* and *Vitis vinifera* seeds extracts. **Molecules,** Basel, v. 18, p. 3410-3426, 2013.

WETTASINGHE, M.; SHAHIDI, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. **Food Chemistry,** London, v. 70, p. 17-26, 2000.

XIE, D.-Y.; SHARMA, S.B.; DIXON, R.A. Anthocyanidin reductases from Medicago truncatula and Arabidopsis thaliana. **Archives of Biochemistry and Biophysics,** New York, v. 422, p. 91-102, 2004.

XIE, D.-Y.; JACKSON, L.A.; COOPER, J.D.; FERREIRA, D.; PAIVA, N.L. Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from *Medicago truncatula*. **Plant Physiology,** Bethesda, v. 134, p. 979–994, 2004.

YILMAZ, Y.; TOLEDO, R.T. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. **Journal of Agricultural and Food Chemistry,** Easton, v. 52, p. 255-260, 2004.

ZADERNOWSKI, R.; NACZK, M.; NESTEROWICZ, J. Phenolic acid profiles in some small berries. **Journal of Agricultural and Food Chemistry,** Easton, v. 53, p. 2118–2124, 2005.

3 GAMMA-IRRADIATION INDUCED CHANGES IN MICROBIOLOGICAL STATUS, PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF PEANUT SKIN

Reprinted with permission from DE CAMARGO, A. C.; REGITANO-D'ARCE, M. A. B.; GALLO, C. R.; Shahidi, F. Gamma-irradiation induced changes in microbiological status, phenolic profile and antioxidant activity of peanut skin. **Journal of Functional Foods**, Amsterdam, v. 12, p. 129-143, 2015.

Abstract

The effects of gamma-irradiation on the microbial growth, phenolic composition, and antioxidant properties of peanut skin were evaluated. Gamma-irradiation at 5.0 kGy decreased the microbiological count of the product. Total phenolic and proanthocyanidin contents, ABTS radical cation, DPPH radical, H_2O_2, and hydroxyl radical scavenging capacities as well as the reducing power of the sample were increased upon gamma-irradiation in both the free and insoluble-bound phenolic fractions. However, a decrease in the esterified phenolics was noticed. The bioactivity of the free phenolics against *in vitro* human LDL-cholesterol oxidation and copper induced DNA strand breakage was improved upon gamma-irradiation. Phenolic acids, flavonoids, and proanthocyanidins were positively or tentatively identified by HPLC-DAD-ESI-MS^n and their distribution was in the decreasing order of free > esterified > insoluble-bound forms. Procyanidin dimer A was increased in all phenolic fractions, whereas procyanidin dimer B decreased. Gamma-irradiation induced changes may be explained by molecular conversion, depolymerization, and cross-linking.

Keywords: Microbiology; LDL-cholesterol oxidation; DNA strand breakage inhibition; HPLC-DAD-ESI-MS^n; Polyphenol; Proanthocyanidin

3.1 Introduction

The role of food phenolics and polyphenolics in the prevention of cardiovascular disease and certain types of cancer is well recognized. Polyphenols have also been reported as having positive *in vivo* effect in reducing obesity and visceral fat, as potential anti-inflammatory compounds (TERRA et al., 2007), and in the management of pre-diabetic and/or diabetic conditions (ROOPCHAND et al., 2013). The antioxidant properties of phenolic compounds have been extensively reported. Studies on vegetable oils, fruits, cereals, spices, teas, and nuts, among other foods and beverages, have highlighted the potential health benefits of polyphenols. By-products such as the skin of Brazil nut (JOHN; SHAHIDI, 2010), hazelnut (ALASALVAR et al., 2009), almond (WIJERATNE; ABOU-ZAID; SHAHIDI, 2006) and peanuts (SARNOSKI et al., 2012) also serve as a rich source of antioxidants.

Peanut skin has almost 20-fold higher total phenolics than whole peanuts and more than 100-fold free radical scavenging capacity (DE CAMARGO et al., 2012a, 2012c), which explains the interest of the peanut industry in exploring the potential applications of this low-cost feedstock. However, there is a concern about the microbiological status of peanut and its by-products due to possible presence of mycotoxinogenic fungi. Worldwide regulations for aflatoxins limit their level in food to less than 20 µg/kg (20 ppb). In addition, it is difficult and sometimes impossible to attain such low values due to environmental conditions in most places where peanuts are produced and stored, making this an additional economic burden (DORNER, 2008).

Gamma-irradiation is an ionizing radiation with high energy that removes one electron from water, creating highly reactive species including free radicals. The interaction of such species with the DNA of microorganisms brings about their death (KILCAST, 1995). Insects are known to be vectors of mycotoxin-producing fungi (NESCI; MONTEMARANI; ETCHEVERRY, 2011). Additionally, low doses of gamma-irradiation (0.2–0.8 kGy) are also efficient for killing and sterilizing insects (FARKAS, 2006). The effectiveness of gamma-irradiation in inhibiting mycotoxinogenic fungi has already been reported (DE CAMARGO et al., 2012c). Nevertheless, antioxidants or by-products intended for use as functional food ingredients need to satisfy microbiological standards for a broad spectrum of microorganisms, such as coagulase-positive *Staphylococcus, Escherichia coli,* and *Salmonella*. In addition, gamma-irradiation is detrimental to antioxidants such as tocopherol (DE CAMARGO et al., 2012b) and ascorbic acid. Thus, investigating the effects of gamma-irradiation on antioxidant compounds and their activity is necessary. Opposite to tocopherols, monophenols that are mainly found in the lipid fraction of peanuts, other phenolics and polyphenolics are concentrated in the water-soluble fraction, and phenolic acids generally exist in the free, esterified, and insoluble-bound forms, the latter being linked to the cell wall components. Soluble phenolic extracts are often defined as the crude phenolic extracts in the literature, accounting for both the free and esterified forms. Phenolics from the crude extract may be found in the glucosides as well as in the aglycone forms.

Although the effect of gamma-irradiation on the total phenolic content and antioxidant capacity of crude phenolics of peanut skin extract has already been studied (DE CAMARGO et al., 2012a), there is no information available about its effect on different fractions of phenolic extracts. Moreover, there is a lack of data on handling microbiological contamination and effects of gamma-irradiation on the individual phenolic compounds of peanut skin. Thus, the objective of the present study was to investigate the application of gamma-irradiation to

decrease the microbiological count of peanut skin and its effect on the content of phenolic compounds and antioxidant properties in the free, esterified and insoluble-bound phenolic fractions.

3.2 Materials and Methods

Peanut skin samples (cv. Runner IAC 886 and Runner IAC 505) were kindly donated by CAP—Agroindustrial, Dumont, São Paulo State, Brazil. Peptone water was purchased from Merck (Whitehouse Station, NJ, USA). TECRA Unique *Salmonella* test was purchased from 3M Microbiology Products (St. Paul, MN, USA). Potato dextrose agar and Baird-Parker agar were purchased from Difco Laboratories (Detroit, MI, USA). Egg yolk tellurite and plasma coagulase EDTA (ethylenediaminetetraacetic acid) were purchased from Laborclin (Pinhais, PR, Brazil). SimPlate coliform and *Escherichia coli* colour indicator (CEc-CI) medium were purchased from BioControl (Bellevue, WA, USA). Phenol reagent, vanillin, 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox) mono- and dibasic potassium phosphates, hydrogen peroxide, DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide), ferrous sulphate, potassium ferricyanide, trichloroacetic acid, human LDL-cholesterol, $CuSO_4$, caffeic, gallic, protocatechuic, and *p*-coumaric acids, (+)-catechin, and (−)-epicatechin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Plasmid DNA pBR 322 from *Escherichia coli* RRI, sodium carbonate, sodium hydroxide, sodium chloride, potassium persulphate, diethyl ether, ethyl acetate, hexane, acetone, methanol, acetonitrile, formic acid, hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada).

3.2.1 Irradiation process

Peanut skin samples were separated into 1.5-kg portions and placed in polyethylene plastic bags. The bags (excluding “controls”) were irradiated at tentative doses of 2.5 and 5.0 kGy at a dose rate of 3.75 kGy/h. The minimum absorbed doses were 2.5 and 5.1, respectively. Dosimetric measurements were carried out with a Harwell Perspex polymethylmethacrylate Amber 3042 dosimeter (PMMA Instruments, Harwell, UK). The irradiation process was carried out in São Paulo, São Paulo State, Brazil, using a multipurpose Cobalt-60 γ-irradiation apparatus from the Nuclear Energy Research Institute (IPEN, São Paulo, Brazil). The samples were irradiated in the air, at 20 °C. One portion was stored at room temperature and used for

microbiological evaluation (within one week) and the remaining samples were stored at -20 °C until the time of phenolic extraction and further analysis (within three months).

3.2.2 Microbiological evaluation

3.2.2.1 Sample preparation

The samples (25 g) and 225 mL of 0.1 % peptone water were crushed in a sterilized blender to obtain the stock solution. Serial 10-fold dilutions using peptone water (0.1%) were then made.

3.2.2.2 *Salmonella* spp

The TECRA Unique *Salmonella* test described by the method 2000.07 (AOAC, 2000) was used for detection of *Salmonella*. All steps needed were performed in accordance with the official method and the results were read as recommended by the supplier. Results were expressed as presence or absence in 25g of representative samples of the product.

3.2.2.3 Yeasts and molds

The samples (0.1 mL of each dilution) were analyzed in acidified potato dextrose agar medium followed by incubation at 25 °C for 3-5 days, according to the method described by Downes and Ito (2001). The results were expressed as colony forming units (CFU) per gram of sample.

3.2.2.4 Coliform bacteria

The SimPlate coliform and *Escherichia coli* colour indicator (CEc-CI) medium was used for the detection and quantification of total coliform and *E. coli* according to the AOAC (2005) method 2005.03. The SimPlate device was filled with 1 mL of each sample dilution and CEc-CI. The incubation was carried out at 35 °C for 24 h. The wells that presented colour changes from the background were counted for total coliforms. The wells with fluorescence colour change (UV light, 366 nm) were counted for *E. coli.* The results were expressed as most probable number (MPN) per gram of sample.

3.2.2.5 Coagulase-positive *Staphylococcus*

The Baird-Parker agar (BPA) medium containing egg yolk tellurite emulsion was used for enumeration of coagulase-positive *Staphylococcus* (DOWNES; ITO, 2001). The samples were incubated at 35-37 ºC for 48 h. Typical colonies (grey to black surrounded by clear zones)

and atypical (grey to black, without the clear zone) ones were tested for coagulase production using plasma coagulase EDTA. The results were expressed as CFU (colony forming units) per gram.

3.2.2.6 Determination of radio sensitivity by D_{10} value

D_{10} value, which is defined as the dose required to eliminate 90% of the initial contamination for a specific microorganism, was calculated using a linear regression equation ($y = ax + b$), where y = Log (CFU), and x = gamma-irradiation dose. D_{10} value was then calculated using the equation $a = (-1/D_{10})$, where a is the slope of the regression equation.

3.2.3 Phenolics and antioxidant evaluation

3.2.3.1 Extraction of phenolic compounds

To obtain a fine powder, peanut skin samples were ground using a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc. Brockville, ON, Canada). The powder so obtained was passed through a mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH, USA) sieve. Ground peanut skin samples were defatted three times using hexane (solid/solvent, 1:5, w/v) in a Warring blender (Model 33BL73, Warring Products Division Dynamics Co. of America, New Hartford, CT, USA). Defatted samples were stored at -20 ºC until used for the extraction of phenolic compounds within one week.

Defatted peanut skin (2.5 g) was extracted with 70% acetone (100 mL) in a gyratory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA) at 30 ºC for 20 min. After centrifugation at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA, USA), the upper layer was collected and extraction was repeated twice. The combined supernatant was evaporated to remove the organic solvent and the residue in water was acidified to pH 2 using 6 M HCl. Free phenolic compounds were extracted five times with diethyl ether and ethyl acetate (1:1, v/v). Combined supernatants (organic phase) were evaporated in vacuo at 40ºC (Buchi, Flawil, Switzerland). The remaining water phase was mixed with 4 M NaOH (1:1, v/v), and hydrolyzed while stirring under nitrogen for 4 hours at room temperature (23-25ºC) to release esterified phenolics, which was acidified to pH using 6 M HCl and extracted with the same procedure of the free fraction. Phenolic compounds were reconstituted in HPLC-grade methanol and stored at -20 ºC until used for further analysis within three months.

To extract insoluble-bound phenolics the solid residue remaining after the first set of extractions was mixed with 4M NaOH, (50 mL) and hydrolyzed while stirring under nitrogen

for 4 hours at room temperature (23-25ºC). The resulting slurry was acidified to pH 2 with 6 M HCl. Phenolic compounds liberated from insoluble-bound form were extracted with diethyl ether and ethyl acetate (1:1, v/v). The organic solvent was evaporated in vacuo at 40ºC, which was followed by suspension in HPLC-grade methanol, as explained above.

3.2.3.2 Total phenolic contents (TPC)

Total phenolic contents were determined according to the method of Swain and Hillis (1959) with slight modifications as previously described by de Camargo et al. (2012a, 2012c). The phenolic extracts were used in different concentrations (2 – 20 mg/mL). First, the extracts with appropriate dilutions (0.50 mL), deionized water (4.0 mL), and phenol reagent (0.50 mL) were added into flasks and mixed thoroughly. After 3 min, a saturated solution of sodium carbonate (0.5 mL) was added, and the mixture was kept in the dark at room temperature (23-25 °C) for 2 h. Finally, the absorbance was read at 760 nm using an Agilent UV–visible spectrophotometer (Agilent 8453, Palo Alto, CA, USA). The results were expressed as milligram catechin equivalents/g dry weight of defatted sample.

3.2.3.3 Proanthocyanidin content (PC)

Total proanthocyanidins (condensed tannins) were determined according to the method of Price, Hagerman, and Butler (1980) as explained by de Camargo et al. (2012a). Briefly, peanut skin extracts were diluted in methanol (20 – 200 mg/mL), and 1.0 mL of the extracts so obtained was added to 5.0 mL of a 0.5% (w/v) vanillin solution prepared in 4% (v/v) HCl methanolic solution. The mixture was incubated in a gyratory water bath shaker (Model G76, New Brunswick Scientific Co) at 30 °C for 20 min. Finally, the absorbance was read at 500 nm using an Agilent UV–visible spectrophotometer (Agilent 8453). The results were expressed as milligram catechin equivalents/g dry weight of defatted sample.

3.2.3.4 ABTS cadical cation scavenging activity

The ABTS assay (RE et al., 1999) was performed using a modified version of the method described by de Camargo et al. (2012a, 2012c). The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation, which was generated by oxidation with potassium persulphate, was prepared in 100 mM phosphate buffer saline solution (PBS) (pH 7.4, 0.15 M sodium chloride). The ABTS radical cation stock solution consisted of potassium persulphate (2.45 mM) and ABTS (7 mM) in PBS. At the time of analysis, the working solution of ABTS radical cation was prepared by diluting its stock solution in PBS to reach an

absorbance value of 0.7 (734 nm). Peanut skin extracts (PSE) were diluted in PBS (4 – 40 mg/mL). PSE (20 μL) was added to 2 mL of ABTS radical cation solution and the absorbance was read at 734 nm after 6 min using an Agilent UV–visible spectrophotometer (Agilent 8453). ABTS radical scavenging activity was calculated using the following equation.

$$\text{ABTS radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of ABTS radical cation + PBS; Abs_{sample} is the absorbance of ABTS radical cation + peanut skin extract or Trolox. The results were expressed as μmol of Trolox equivalents/g dry weight of defatted sample.

3.2.3.5 DPPH radical scavenging activity (DRSA)

The DPPH assay was carried out using a modified version of the method explained by Chandrasekara and Shahidi (2011b). The phenolic extracts were used at different concentrations of 20 – 200 mg/mL. Two millilitres of a methanolic solution of DPPH (0.5 mM) were added to 500 μL of peanut skin extracts diluted in methanol. After 10 min, the mixture was passed through the capillary tubing that guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-Scan, Bruker Biospin Co., Billerica, MA, USA). The spectrum was recorded with the parameters as follows: 5.02×10^2 receiver gain, 1.93 G modulation amplitude, 2.62 s sweep time, 8 scans, 100 G sweep width, 3495 G centre field, 5.12 ms time constant, 9.79 GHZ microwave frequency, and 86 kHZ modulation frequency. For quantitative measurements of radical concentration remaining after reaction with the extracts, the method of comparative determination based on the corresponding signal intensity of first-order derivative of absorption curve was used (MADHUJITH; SHAHIDI, 2006). The DPPH scavenging activity of the extracts was calculated using the following equation.

$$\text{DPPH scavenging activity (\%)} = [(EPR_{control} - EPR_{sample})/(EPR_{control})] \times 100$$

where $EPR_{control}$ signal intensity of DPPH radical + methanol; EPR_{sample} is the signal intensity of

DPPH radical + peanut skin extract or catechin. The results were expressed as μmol of catechin equivalents/g dry weight of defatted sample.

3.2.3.6 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of peanut skin extracts was evaluated as previously explained (WETTASINGHE; SHAHIDI, 2000). Peanut skin extracts (2 – 20 mg/mL) and 0.4 mM hydrogen peroxide solution were prepared in 0.1 M phosphate buffer (pH 7.4). The extracts (0.4 mL) were mixed with hydrogen peroxide solution (0.6 mL) and the final volume was made to 2.0 mL with the same buffer. The samples were kept in a gyratory water bath shaker (Model G76, New Brunswick Scientific Co. Inc.) for 40 min, and the absorbance was read at 230 nm in an Agilent UV–visible spectrophotometer (Agilent 8453). Blanks devoid of hydrogen peroxide (added by phosphate buffer) were prepared for background corrections. The results were expressed as μmol of catechin equivalents/g dry weight of defatted sample. The scavenging activity was calculated with the following equation.

$$H_2O_2 \text{ scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

where $Abs_{control}$ is the absorbance of H_2O_2 + phosphate buffer; and Abs_{sample} is the absorbance of H_2O_2 + peanut skin extract or catechin.

3.2.3.7 Hydroxyl radical scavenging activity

The ability of phenolic compounds in scavenging hydroxyl radicals generated by Fenton reaction was evaluated by electron paramagnetic resonance (EPR) spectroscopy using a slightly modified version of a method previously reported (WETTASINGHE; SHAHIDI, 2000). Phenolic peanut skin extracts were removed from the original solvent (methanol) under a stream of nitrogen and diluted in 0.1 M phosphate buffer (pH 7.4). A 0.2 mL portion of the solution so obtained was mixed with 0.2 mL of H_2O_2 (10 mM), 0.4 mL of 5,5-dimethyl-1-pyrroline-*N*-oxide 17.6 mM, and 0.2 mL of $FeSO_4$ (10 mM). After 3 min the EPR spectrum was recorded using a Bruker e-scan EPR spectrophotometer (Bruker E-Scan, Bruker Biospin Co.) The spectrum was recorded with the same parameters as for DPPH. The hydroxyl radical scavenging activity of the extracts was calculated using the following equation.

$$\text{Hydroxyl radical scavenging activity (\%)} = [(EPR_{control} - EPR_{sample})/(EPR_{control})] \times 100$$

where $EPR_{control}$ is the signal intensity of hydroxyl radical + phosphate buffer; and EPR_{sample} is the signal intensity of hydroxyl radical + peanut skin extract or catechin. The results were expressed as μmol of catechin equivalents/g dry weight of defatted sample.

3.2.3.8 Reducing power

The reducing power assay (OYAIZU, 1986) was conducted according to the method described by Alasalvar et al. (2009). The extracts (4 – 40 mg/mL), were diluted in phosphate buffer (pH 6.6, 0.2 mM). Extracts (1.0 mL) were then mixed with phosphate buffer (2.5 mL) and 1% (w/v) potassium ferricyanide solution (2.5 mL), followed by their incubation in a gyratory water bath shaker (Model G76, New Brunswick Scientific Co. Inc.) at 50 ºC for 20 min, after which 10% (w/v) trichloroacetic acid solution was added (2.5 mL). The mixture was centrifuged at 1750g for 10 min and the supernatant (2.5 mL) was added to distilled water (2.5 mL) and 0.1% (w/v) ferric chloride solution (0.5 mL). The absorbance was read at 700 nm using an Agilent UV–visible spectrophotometer (Agilent 8453). The calibration curve was prepared using Trolox and expressed as μmol of Trolox equivalents/g dry weight of defatted sample.

3.2.3.9 Copper-induced LDL-cholesterol oxidation

The LDL-cholesterol oxidation method (SHAHIDI; ALASALVAR; LIYANA-PATHIRANA, 2007) was slightly modified to evaluate the potential inhibitory effect of peanut skin extracts. The solution of LDL-cholesterol was dialyzed overnight against PBS (10 mM, 0.15 M NaCl, pH 7.4) at 4 ºC under a flow of nitrogen. The resulting EDTA-free LDL-cholesterol was diluted in PBS to reach a concentration of 0.02 mg/mL. Methanol was removed from peanut skin extracts under a stream of nitrogen followed by their resuspension in PBS to obtain a 100 ppm total phenolic content equivalent (as evaluated by HPLC-DAD-ESI-MS^n). Peanut skin extracts (100 μL) and LDL-cholesterol (800 μL) were added into Eppendorf tubes and incubated at 37 ºC for 15 min, after which the peroxidation was induced by addition of a 100 μM solution of $CuSO_4$ (100 μL). The reaction was incubated for 21 h at 37 ºC and the conjugated dienes (CD) were assayed at 234 nm using an Agilent UV–visible spectrophotometer (Agilent 8453). Blanks devoid of LDL-cholesterol and $CuSO_4$ were prepared for background subtraction. A positive control was prepared with catechin (100 ppm) and the results were expressed as inhibition percentage according to the following equation.

$$\text{Inhibition of formation of CD (\%)} = [(Abs_{oxidized} - Abs_{sample})/(Abs_{oxidized} - Abs_{native})] \times 100$$

where $Abs_{oxidized}$ is the absorbance of LDL-cholesterol with $CuSO_4$; Abs_{sample} is the absorbance of LDL-cholesterol with extract or catechin and $CuSO_4$; and Abs_{native} is the absorbance of LDL-cholesterol without $CuSO_4$.

3.2.3.10 Supercoiled plasmid DNA strand breakage inhibition

The supercoiled plasmid DNA strand breakage inhibition was evaluated with minor modifications of the previously explained method (SHAHIDI; ALASALVAR; LIYANA-PATHIRANA, 2007). Methanol was removed from PSE under a stream of nitrogen followed by resuspension in water to achieve a concentration of 2.5 mg/mL. An aliquot (5µL) was pipetted in Eppendorf tubes and the same amount of the remaining reagents was added in the following order: PBS (0.5 M, pH 7.4, 0.15 M sodium chloride), supercoiled plasmid DNA pBR 322 from *Escherichia coli* RRI diluted in PBS (50 µL/mL), H_2O_2 (0.5 mM), and $FeSO_4$ (0.5 mM). The mixture was incubated at 37 °C for 1 h in the dark, after which 2.5 µL of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in distilled water) were added. The samples were loaded onto 0.7 (w/v) agarose gel prepared in Tris-acetic acid-EDTA (TAE) buffer consisting of 40 mM Tris acetate, 1 mM EDTA, pH 8.5, containing stain SYBR safe (100 µL/L). The procedure was conducted at 80 V for 90 min using a submarine gel electrophoresis apparatus (VWR, Radnor, PA, USA). The images were acquired with a Sony digital camera under UV light and analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA, USA). The inhibition percentage was calculated as follows: inhibition of DNA strand breaking = [(intensity of supercoiled DNA in presence of oxidant and extract/intensity of supercoiled DNA devoid of oxidant and extract) x 100].

3.2.3.11 HPLC-DAD-ESI-MS^n analysis

The identification of major phenolics in the free, esterified, and insoluble-bound fractions of peanut skin was performed on an Agilent 1100 system (Agilent) equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1130B ALS Therm, a G1316 Colcom column compartment, A G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 µm, Merck, Darmstadt, Germany). The binary mobile phase consisted of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The flow rate was adjusted to 0.5 mL/min and the elution gradient used was as follows; 0 min, 100% A; 5 min, 90% A; 35 min, 85% A; 45 min, 60% A;

held at 60% A from 45 to 50 min; afterward mobile phase A was increased to 100% at 55 min, followed by column equilibration from 55 to 65 min (de Camargo et al., 2014a,b). The compounds were detected at 280 nm, and the samples were filtered before injection using a 0.45 µm PTFE membrane syringe filter (Thermo Scientific, Rockwood, TN, USA).

HPLC-ESI-MS^n analysis was carried out under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionization (ESI) in the negative mode. The data were acquired and analyzed with an Agilent LC/MSD software (Agilent). The scan range was set in a range from m/z 50 to 2000, using smart parameter setting, drying nitrogen gas at 350 °C, flow 12 L/min, and nebulizer gas pressure of 70 psi. Phenolic acids, namely protocatechuic, p-coumaric, gallic, caffeic, ferulic, sinapic and ellagic acids, and flavonoids (+)-catechin, (−)-epicatechin, and quercetin were identified by comparing their retention times and ion fragmentation pattern with coded and authentic standards under the same conditions as the samples. Coutaric and caftaric acids, as well as gallocatechin, isorhamnetin-glucoside, quercetin-glucuronide, manniflavanone and dimers through pentamers of proanthocyanidins were tentatively identified using tandem mass spectrometry (MS^n), UV spectral data and literature data (APPELDOORN et al., 2009; MONAGAS et al., 2009; SARNOSKI et al., 2012; DE CAMARGO et al., 2014a, 2014b; MA et al., 2014).

3.2.4 Statistical analysis

Unless otherwise stated, the experimental design was randomized with three replications and the results were analyzed using ANOVA and Tukey's test ($p < 0.05$) and SAS software. The correlation analyses ($p < 0.01$) and ($p < 0.05$) were carried out using the ASSISTAT 7.6 program.

3.3. Results and Discussion

3.3.1 Microbiological evaluation

The effectiveness of gamma-irradiation in inhibiting microbial growth is shown in Table 3.1. The content of yeast and molds, total coliform, and coagulase-positive *Staphylococcus* in non-irradiated was up to 3.0×10^4 (CFU/g), 1.3×10^5 (MPN/g), and 6.0×10^1 (MPN/g), respectively. Peanuts, also known as groundnuts, grow in contact with the soil, which facilitates its surface microbiological contamination. Peanut skin for potential functional food applications has been receiving recent attention. This by-product has very low moisture content and limited nutrients for microbial growth; however, the present study demonstrated that its

natural microbiota deserves attention. The same has been observed for herb and spice preparations used in manufacture of ready to eat meals (WITKOWSKA et al., 2011). According to these authors, herb and spice preparations had presence of *Enterobacteriaceae*, which may include *E. coli*, and *Salmonella*. Yeast and molds were also detected. Their findings also revealed that even heat processing of ready to eat meal was not always sufficient for decontaminating pre-existing microflora of herb and spice preparations. Peanut skin has similarities with herb and spices in terms of moisture content and nutrients for microbial growth. For such reason gamma-irradiation, which is currently used for microbial decontamination of herb and spices, may also be useful for decontaminating peanut skin.

Table 3.1 - Gamma-irradiation effects on microbiological contamination of peanut skin[a]

	IAC 886			IAC 505		
Microorganisms	Control	2.5 kGy	5.0 kGy	Control	2.5 kGy	5.0 kGy
Yeasts and Molds (CFU/g)	2.2×10^2	$<10^1$	$<10^1$	3.0×10^4	8.0×10^2	3.0×10^1
Total Coliform (MPN/g)	1.3×10^5	$<10^1$	$<10^1$	7.0×10^4	$<10^1$	$<10^1$
Escherichia coli (MPN/g)	$<10^1$	$<10^1$	$<10^1$	$<10^1$	$<10^1$	$<10^1$
Coagulase-positive *Staphylococcus* (MPN/g)	$<10^1$	$<10^1$	$<10^1$	6.0×10^1	$<10^1$	$<10^1$
Salmonella spp.	nd	nd	nd	nd	nd	nd

[a]Mean values of duplicate samples for each test procedure. CFU, colony forming unity; MPN, most probable number, *Salmonella* was not detected (nd) in 25g sample size

As can be noticed 2.5 kGy was sufficient to eliminate the total coliform count ($<10^1$ CFU/g). The present results agree with those of Wilson-Kakashita et al. (1995) who demonstrated that gamma-irradiation of English walnuts at 5.0 kGy was efficient for inhibiting the growth of coliform bacteria. No *E. coli* was found in any sample, as such or gamma-irradiated. Bloody diarrhoea, abdominal cramps, nausea, vomiting, and fever are the symptoms caused by *E. coli* O157:H7 haemorrhagic colitis. Low contamination (5-10 viable cells) is able to induce symptoms of the disease. Recently, *E. coli* was detected in some peanut samples of Runner cultivar (MIKSCH et al., 2013).

Coagulase-positive *Staphylococcus* was detected in samples of IAC 505. *Staphylococcus aureus* is a common contamination for foods via human contact. They are found in the skin, infected cuts, nasal passage, and throat. The toxin produced by *S. aureus* causes food intoxication. Enterotoxin producing *S. aureus* are generally coagulase-positive. The toxin is resistant to freezing, refrigeration, and heat treatment. Furthermore, gamma-irradiation may be able to eliminate and/or delay the enterotoxin A production (GRANT; NIXON; PATTERSON, 1993). Symptoms of intoxication include diarrhoea, abdominal cramps, nausea, and vomiting.

Salmonella was absent in 25g quantities of all samples. Although the presence of *Salmonella* has been associated with raw meat, poultry, eggs, and seafood, a recent study (CALHOUN et al., 2013) reported that 2% of peanut samples from USA (crop year 2008, 2009) were positive for *Salmonella.* The infective dose of *Salmonella* may be as low as 15-20 cells, depending on human health condition, and microorganism strain. Symptoms of *Salmonellosis* include headache, chills, stomach pain, fever, nausea, and diarrhoea. Such low infective dose reflects the very low tolerance of it by food safety regulations, thus in the present study *Salmonella* is reported as present or absent.

In the current study, the contents of yeast and molds were reduced by at least three log cycles with a dose of 5.0 kGy. Yeast and molds analyzed included mycotoxinogenic fungi, which are common to peanuts. Aflatoxin B1 is classified as group I carcinogen. Consumption of aflatoxin contaminated food is related to hepatic cancer incidence and dose-dependent DNA damage (MIRANDA et al., 2007). In a previous work (DE CAMARGO et al., 2012c), it was demonstrated that 5.0 kGy was sufficient for decontamination of mycotoxinogenic fungi in in-shell and blanched peanuts during long term storage. Regarding fungal count, Al-Bachir (2004) demonstrated that 2.0 kGy was a suitable dose for gamma-irradiation of walnuts. Meanwhile, in the present study, the presence of aflatoxin was investigated, but no contamination was detected (detection limit of 0.5 μg/kg for aflatoxin B1 and G1, and of 0.3 μg/kg for aflatoxin B2 and G2).

D_{10} value, a dose required to eliminate 90% of the initial microbiological count, was calculated only for yeasts and molds of IAC 505. D_{10} value of total coliform and coagulase-positive *Staphylococcus* counts were not possible to calculate because their population was $<10^1$ with the lowest dose (2.5 kGy). D_{10} value for yeasts and molds of IAC 505 was 1.7 kGy, which is in disagreement with a recent study (AOUIDI et al., 2011), that reported a value of 13.92 and 15.40 in gamma-irradiated intact and powdered olive leaves, respectively. The difference in the results may be related to gamma-irradiation dose rate. In the present study, the dose rate used was 3.75 kGy/h, whereas the dose rate applied in their study was 15.64 Gy/min, which gives a dose rate of 0.94 kGy/h.

Microorganism cells may be able to repair themselves when treated with sublethal food processing methods such as heating, freezing, drying or gamma-irradiation. According to Mackey and Derrick (1982), *Salmonella typhimurium* submitted to equivalent lethal treatments required less time to repair following gamma-irradiation and drying in comparison to heat and freeze-injured cells. Furthermore, it is well known that gamma-irradiation is a treatment based on the exposition of the product to an ionizing radiation source, and the dose required is

controlled by exposure time, thus careful evaluation of the D_{10} value will impact the processing time and, in turn, the associated costs. To cause minimum effects in food products, such as sensory changes, decrease in antioxidants and vitamins such as tocopherols (vitamin E) and ascorbic acid (vitamin C) low doses of gamma-irradiation are used. These data are helpful for the peanut industry because they offer a safe treatment option for use of its by-products.

3.3.2 Total phenolic content (TPC)

The TPC in gamma-irradiated peanut skin is presented in Table 3.2.

Table 3.2 - Total phenolic content, proanthocyanidin content, antioxidant activities and reducing power of gamma-irradiated peanut skin[a]

Dose (kGy)	Free	Esterified	Insoluble-Bound	Free	Esterified	Insoluble-Bound
		IAC 886			IAC 505	
Total phenolic content (mg CE/g DW)						
Control	50.82 ± 1.3b	14.06 ± 0.6a	2.65 ± 0.3c	33.58 ± 1.4b	11.20 ± 0.4a	3.67 ± 0.1a
2.5	54.14 ± 0.9b	10.43 ± 0.2b	3.81 ± 0.0b	35.68 ± 0.9b	10.23 ± 0.8a	3.72 ± 0.2a
5.0	58.64 ± 2.9a	10.25 ± 1.1b	4.44 ± 0.4a	40.43 ± 2.5a	4.54 ± 0.2b	3.60 ± 0.1a
Proanthocyanidin content (mg CE/g DW)						
Control	35.19 ± 0.0b	4.34 ± 0.09a	1.92 ± 0.1b	20.33 ± 1.3b	3.09 ± 0.05a	1.33 ± 0.2b
2.5	35.21 ± 0.0ab	2.27 ± 0.03b	2.97 ± 0.2a	20.88 ± 0.4a	2.84 ± 0.10b	1.93 ± 0.2a
5.0	36.25 ± 0.7a	2.03 ± 0.02c	3.03 ± 0.2a	21.95 ± 0.6a	0.91 ± 0.12c	1.77 ± 0.1a
ABTS radical scavenging activity (μmol TE/g DW)						
Control	483.4 ± 21b	299.7 ± 17a	27.8 ± 0.6b	224.8 ± 21b	175.2 ± 3.1a	13.4 ± 0.8a
2.5	488.3 ± 1.7b	203.7 ± 4.1b	38.0 ± 0.3a	216.9 ± 30b	135.1 ± 5.9b	13.4 ± 1.6a
5.0	541.0 ± 15a	161.1 ± 7.9c	41.2 ± 3.3a	282.8 ± 2.5a	105.0 ± 3.4c	12.8 ± 0.2a
DPPH radical scavenging activity (μmol CE/g DW)						
Control	2126 ± 71.2b	1099 ± 149b	112.6 ± 9.77b	1375 ± 18.9b	929.5 ± 135a	106.3 ± 10.3b
2.5	2285 ± 76.4ab	759.1 ± 76.4b	140.1 ± 1.60ab	1408 ± 16.4b	893.7 ± 126a	134.0 ± 1.71ab
5.0	2431 ± 34.0a	629.9 ± 30.6a	166.3 ± 19.4a	1774 ± 105a	475.2 ± 75.9b	153.8 ± 20.5a
H_2O_2 scavenging activity (μmol CE/g)						
Control	381.9 ± 7.37b	307.6 ± 11.8a	32.48 ± 3.74b	325.6 ± 1.54b	287.4 ± 34.9b	21.31 ± 1.69a
2.5	407.6 ± 3.39a	242.4 ± 21.7b	42.27 ± 2.90a	358.2 ± 21.2b	234.4 ± 18.7ab	24.08 ± 0.64ab
5.0	420.9 ± 7.74a	189.7 ± 13.6c	45.21 ± 3.57a	388.3 ± 4.27a	191.8 ± 9.36a	24.79 ± 1.12a
Hydroxyl radical scavenging activity (μmol CE/g DW)						
Control	706.2 ± 20.0c	392.0 ± 42.0a	12.83 ± 3.18b	472.7 ± 22.4b	328.6 ± 21.1a	8.62 ± 1.85c
2.5	884.2 ± 26.2b	264.7 ± 5.22b	21.77 ± 0.52a	747.0 ± 45.9a	220.8 ± 31.2b	15.8 ± 0.45b
5.0	1019 ± 18.7a	133.1 ± 22.5c	27.50 ± 4.04a	829.0 ± 29.1a	120.9 ± 16.4c	23.5 ± 3.24a
Reducing power (μmol TE /g DW)						
Control	216.9 ± 2.0c	87.56 ± 5.2a	10.71 ± 0.5b	97.15 ± 2.2b	76.70 ± 3.9a	8.04 ± 0.3b
2.5	236.0 ± 3.3b	56.86 ± 1.5b	10.96 ± 0.6b	102.9 ± 0.6a	65.20 ± 2.9b	8.85 ± 0.8a
5.0	265.4 ± 3.4a	50.65 ± 2.3b	13.22 ± 1.2a	103.7 ± 0.3a	54.39 ± 2.5c	9.63 ± 0.2a

[a] Data represent mean values for each sample ± standard deviation (n = 3). Means followed by the same letters within a column part are not significantly different ($p > 0.05$). CE, catechin equivalents; TE, Trolox equivalents; and DW, dry weight of defatted sample

Since monomeric and oligomeric procyanidins are the major phenolic compounds in peanut skin the data were expressed as milligrams of catechin equivalents (CE) per gram of dry weight of defatted sample. TPC in the free phenolic fraction of non-irradiated samples was up to 3.6- and 19-fold higher than that of esterified and insoluble-bound fractions, respectively. The contribution of free, esterified, and insoluble-bound phenolic contents depends on the feedstock. John and Shahidi (2010) evaluated the content of phenolic compounds in Brazil nut

skin. Similar to the present study, the content of total phenolics in the insoluble-bound fraction was lower than that of its soluble (free plus soluble esters) counterpart. On the other hand, Chandrasekara and Shahidi (2011) demonstrated that the insoluble-bound fraction of most varieties of millet grains had higher TPC than their soluble fraction. As gamma-irradiation causes molecular changes, mainly related to the formation of free radicals, the treatment may have effects on the TPC.

In the present study, the TPC contents of non-irradiated samples in the free, esterified, and insoluble-bound fractions of the control samples were in the range of 33.58-50.82, 11.20-14.06, and 2.65-3.67 mg CE/g DW, respectively. The TPC values in the present study are in good agreement with those in the literature (SHEM-TOV et al., 2012). According to these authors, peanut skin from 22 experimental lines had TPC ranging from 5 to 156 mg CE/g. In the present study, a significant increase ($p < 0.05$) was found in TPC of the free and insoluble-bound fractions of gamma-irradiated samples compared with their non-irradiated counterparts. On the other hand, a decrease was observed in the esterified fraction of gamma-irradiated peanut skin in both cultivars. Gamma-irradiated almond skin (up to 12 kGy) also showed an increase in TPC of their soluble fraction (HARRISON; WERE, 2007). However, the exact mechanism for such increase remains unclear. Moreover, no consensus is found in the literature regarding the increase of TPC of different feedstocks (MISHRA; GAUTAMA; SHARMA, 2006; HARRISON; WERE, 2007; PEREZ; CALDERON; CROCI, 2007; DIXIT et al., 2010), which may be related to existing differences in the dose of gamma-irradiation, extraction methods, and identity of individual phenolics present.

3.3.3 Proanthocyanidin content (PC)

Proanthocyanidins or condensed tannins consist of flavan-3-ol units, ranging from dimers to higher oligomers. Peanut skin contain high contents of proanthocyanidins, especially procyanidins, which consist exclusively of (epi)catechin units (SARNOSKI et al., 2012). The proanthocyanidin content (PC) in peanut skin is presented in Table 3.2. Values of 23.89 and 0.31 mg CE/g were reported for total proanthocyanidins of soluble (free and esterified), and insoluble-bound fraction of cashew nut testa (skin) (CHANDRASEKARA; SHAHIDI, 2011), which is in good agreement with those in the present study, thus lending support to our findings where a significant increase ($p \leq 0.05$) was noticed for PCs of free and insoluble-bound phenolics. The highest value was found in the free fraction of gamma-irradiated sample (5.0 kGy) from IAC 886 cultivar (36.25 mg CE/g DW), and the lowest value was in the insoluble-bound fraction of non-irradiated IAC 505 cultivar (1.33 mg CE/g DW). In accordance with the

results of the present study, gamma-irradiated (up to 10 kGy) soybean seeds (STAJNER; MILOSEVIC; POPOVIC, 2007) had higher content of proanthocyanidins.

3.3.4 ABTS radical cation scavenging activity (ARSA)

The ABTS assay is based on electron transfer reactions to evaluate radical scavenging activity of hydrophilic and lipophilic compounds. The ABTS radical cation scavenging activity (ARSA) data of peanut skin samples are summarized in Table 3.2. ARSA values were in the decreasing order of free > esterified > insoluble-bound. While the absolute values for TPC and PC of the esterified fraction were up to 33 and 15% of their free counterpart, respectively, the ARSA of the esterified fraction was up to 62% of the ARSA of the free fraction. This may be due to differences in the chemical structures of the compounds found in the free and esterified fractions as well as their concentrations and possible synergistic effects. Thus, when analyzing results reported only as TPC and PC contents one should bear in mind the limitations of spectrophotometric analysis. For non-irradiated samples, the highest value was observed in the free fractions of the IAC 505 cultivar (483.4 μmol TE/g), and the lowest value was observed in the insoluble-bound phenolic fraction of IAC 886 (13.4 μmol TE /g DW). The ARSA of soluble phenolics (free and esterified fractions) from gamma-irradiated peanut skin has already been studied (DE CAMARGO, et al., 2012a). Samples irradiated with 5.0 kGy also displayed higher ARSA values than those of the control samples. ARSA values from the present study are in good agreement with those reported in the literature for peanut skin extracts of Runner, Virginia and Spanish cultivars (FRANCISCO; RESURRECCION, 2009). The values reported ranged from 0.62 to 2.56 mmol TE/g dry weight. Furthermore, gamma-irradiated almond skin also presented higher ARSA than the control samples. In the present study the ARSA of free phenolic fractions was 17-fold higher than its insoluble-bound phenolic fraction counterpart (the control sample), which is consistent with the findings of Chandrasekara and Shahidi (2011a) that reported values 21-fold higher for ARSA of cashew nut skin compared with their content of soluble phenolic fraction (free and esterified). Finally, in the present study, the ARSA positively and significantly was related to the TPC ($r = 0.924$; $p < 0.01$) and PC ($r = 0.889$; $p < 0.01$).

3.3.5 Scavenging activity against DPPH radical

DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl) is a synthetic compound and its reaction involves electron or hydrogen transfer. DPPH is more stable when compared with natural radicals and is not affected by side reactions like enzyme inhibition and metal ion

chelation (CHANDRASEKARA; SHAHIDI, 2011b). DRSA has been demonstrated to significantly and positively correlate with the antioxidant capacity of walnut, almond, hazelnut, pistachio, and peanut oil (ARRANZ et al., 2008). Soluble phenolics from peanut skin showed antioxidant capacity in delaying the oxidation of refined-bleached-deodorized soybean oil by the Rancimat method (DE CAMARGO et al., 2012a), thus evaluation of DRSA may lend support for further studies on the application of different phenolic fractions of gamma-irradiated peanut skin in a bulk oil model system. In the present study, a significant positive correlation existed between TPC and DRSA ($r = 0.970$; $p < 0.01$) and between PC and DRSA ($r = 0.930$; $p < 0.01$). DRSA values ranged from 106.3 to 2126 μmol CE/g in the insoluble-bound (IAC 505) and free phenolic fractions (IAC 886) of non-irradiated samples, respectively (Table 3.2). DRSA values also showed increases of up to 48% in the insoluble-bound fraction and decreases of up to 49% in their esterified counterparts. Supporting the findings of the present study, methanolic and ethanolic phenolic extracts from gamma-irradiated rosemary also showed an increase in their DRSA values (PEREZ; CALDERÓN; CROCI, 2007), though the same trend was not observed for their water extract. Different solvent systems (e.g. methanol, ethanol, water, ethyl acetate, diethyl ether, n-butanol) have been employed to fractionate phenolics according to their polarity, which may explain certain discrepancies in the literature data. Furthermore, some compounds may be more sensitive to the process than others, thus evaluating only one fraction may lead to inconclusive results.

3.3.6 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of phenolic compounds may proceed via electron donation and eventual neutralizing of H_2O_2 to H_2O (WETTASINGHE; SHAHIDI, 2000). Hydrogen peroxide generates hydroxyl radicals in the presence of ferrous ions according to the Fenton's reaction. This is important from the biological point of view as hydroxyl radicals are highly reactive, leading to changes in DNA (SHAHIDI; ALASALVAR; LIYANA-PATHIRANA, 2007), and inactivating enzymes (FERNANDES et al., 2011). Furthermore, hydrogen peroxide induces cell damage (CHEN et al., 2010). In the present study, the hydrogen peroxide scavenging activity was evaluated and changes due to gamma-irradiation in their efficacy were evaluated. In this, similar to other antioxidant assays, an increase of up to 40% in the hydrogen peroxide scavenging activity was observed. Additionally, a significant positive correlation existed between TPC ($r = 0.862$, $p < 0.01$) and PC ($r = 0.785$, $p < 0.01$). Evaluating the antioxidant activity against oxygen radical species is important, as they play an important

role in oxidation processes in biological systems. Furthermore, the antioxidant activity may differ in different assays due to different mechanisms that are operative.

3.3.7 Hydroxyl radical scavenging activity

Hydroxyl radicals are highly reactive and unstable oxygen species, thus DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) was used as a spin trap to produce a relatively stable free radical (WETTASINGHE; SHAHIDI, 2000). Other than causing DNA damage and being involved in lipid oxidation processes, hydroxyl radicals also oxidize protein leading to their conformation modification (GUPTASARMA et al., 1992). Two hypotheses may explain the scavenging power of peanut skin extracts, by quenching the hydroxyl radical generated in the assay media or by chelation of ferrous ion (WETTASINGHE; SHAHIDI, 2000). The high correlation either between TPC and hydroxyl radical scavenging activity ($r = 0.965$, $p < 0.01$) or between PC and hydroxyl radical scavenging activity ($r = 0.920$, $p < 0.01$) demonstrated the ability of phenolic compounds against potential detrimental damages of hydroxyl radicals. Furthermore, the increase in the hydroxyl radical scavenging activity due to gamma-irradiation was up to 173% and such results suggest that gamma-irradiated peanut skin may serve better in neutralizing biologically relevant hydroxyl radicals.

3.3.8 Reducing power

The reducing power (RP) of peanut skin samples is given in Table 3.2. The reaction involves reduction of ferric to ferrous ion. Ferric ion catalyzes the oxidation of proteins and lipids, thus being detrimental to food and biological systems. The RP for the control samples ranged from 8.04 to 216.9 μmol TE/g. Consistent with other results from the present study, the insoluble-bound phenolic fraction had the lowest RP, followed by the esterified and free fraction. Furthermore, the highest RP was found in the gamma-irradiated samples (5.0 kGy). Additionally, a positive correlation existed between RP and TPC ($r = 0.945$, $p < 0.01$) and between RP and PC ($r = 0.929$, $p < 0.01$). Although the difference between the correlations of antioxidant activities with total phenolics and proanthocyanidin content is minor, the highest correlations existed between antioxidant activity and total phenolics, which demonstrate that phenolic compounds other than proanthocyanidins, more effectively influenced the antioxidant activity of peanut skin. Investigations on individual phenolic compounds are presented later in this contribution.

3.3.9 Copper-induced LDL-cholesterol oxidation

A high level of oxidized LDL-cholesterol is recognized as being an important risk factor for development and progression of atherosclerosis. The consumption of sources of polyphenols such as catechin, epicatechin, procyanidin B2 and vanillin, has been correlated with a decrease of oxidized cholesterol in high risk cardiovascular patients (KHAN et al., 2012). In the present study, polyphenols of the free and esterified fractions of peanut skin extracts, which accounted for more than 90% of the total phenolic content (HPLC-DAD-ESI-MS^n), were evaluated for their ability in inhibiting copper-induced LDL-cholesterol oxidation (Figure 2.1).

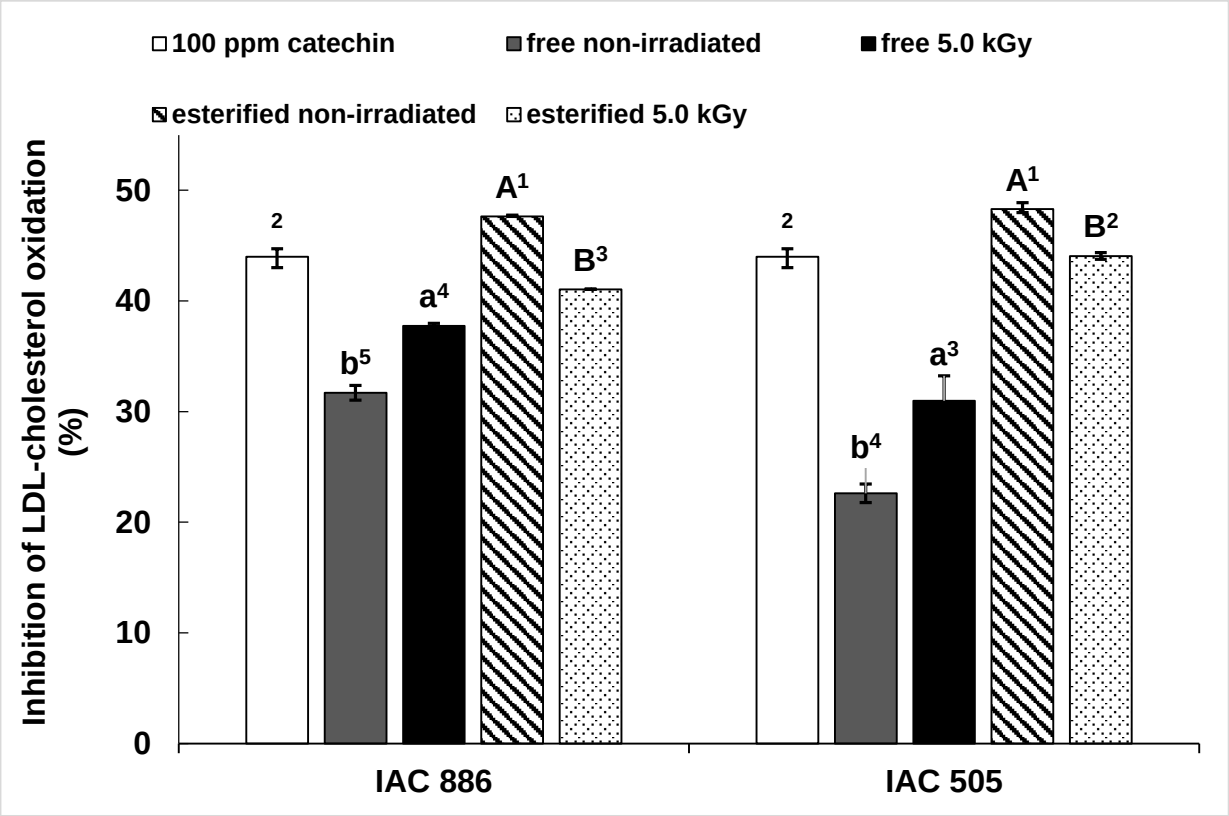


Figure 3.1 - Gamma-irradiation effects on the biological activity of free and esterified phenolics against LDL-cholesterol induced oxidation. Data represent the mean ± standard deviation of each sample (n = 3). Means with different figures indicate significant differences ($p < 0.05$) compared to 100 ppm catechin. Means with different lower case letters indicate significant differences ($p < 0.05$) in the free phenolic fraction. Means with different capital letters indicate significant differences ($p < 0.05$) in the esterified fraction

At a concentration of 100 ppm, the phenolics tested inhibited LDL oxidation by up to 48% and gamma-irradiation increased the efficacy of polyphenols in the free fraction, but decreased it in the esterified fraction of peanut skin. The percentage inhibition of esterified phenolics from non-irradiated samples was higher than that of the standard (100 ppm of catechin). Phenolic compounds from esterified fraction represented 23-28% of the total phenolic content in peanut skin (HPLC-DAD-ESI-MS^n); however, at a similar concentration, it

exhibited a higher or similar protection compared to those of the free phenolic fraction. This finding lends further support to previous studies focusing on the biological relevance of polyphenols and their distribution in the free, esterified and insoluble-bound forms.

3.3.10 Supercoiled plasmid DNA strand breakage inhibition

Reactive oxygen species (ROS) oxidize the native form of DNA, which can be evaluated by its conversion to a nicked circular or linear form via single or double-strand breaks, respectively. DNA mutagenesis is detrimental as it affects the replication and transcription, and may cause cell death or lead to cancer initiation. In the present study, different forms of DNA were quantified as a function of the antioxidant efficacy of phenolic compounds from peanut skin (Figure 3.2). Hydroxyl radicals have a short half-life, which makes them deleterious in a cellular level. It is noteworthy that gamma-irradiation increased the antioxidant activity of peanut skin extracts (Figure 3.3), and acted more effectively against hydroxyl radical DNA strand scission; possibly due to the increase in the content of free phenolics upon gamma-irradiation as mentioned before.

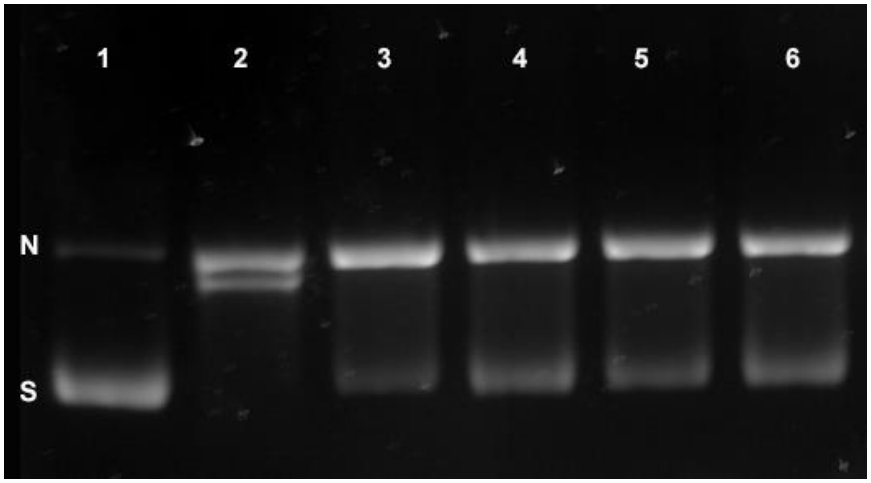


Figure 3.2 - Supercoiled plasmid DNA strand breakage inhibition of the free phenolic fraction of non-irradiated and gamma-irradiated peanut skin. Lane 1: Control (DNA only); lane 2: DNA + H_2O_2 + $FeSO_4$; lane 3: free phenolics from non-irradiated peanut skin (IAC 886); lane 4: free phenolics from peanut skin (IAC 886) subjected to 5.0 kGy; lane 5: free phenolics from non-irradiated peanut skin (IAC 505); lane 6: free phenolics from peanut skin (IAC 505) subjected to 5.0 kGy; S and N are supercoiled and nicked plasmid DNA strands, respectively

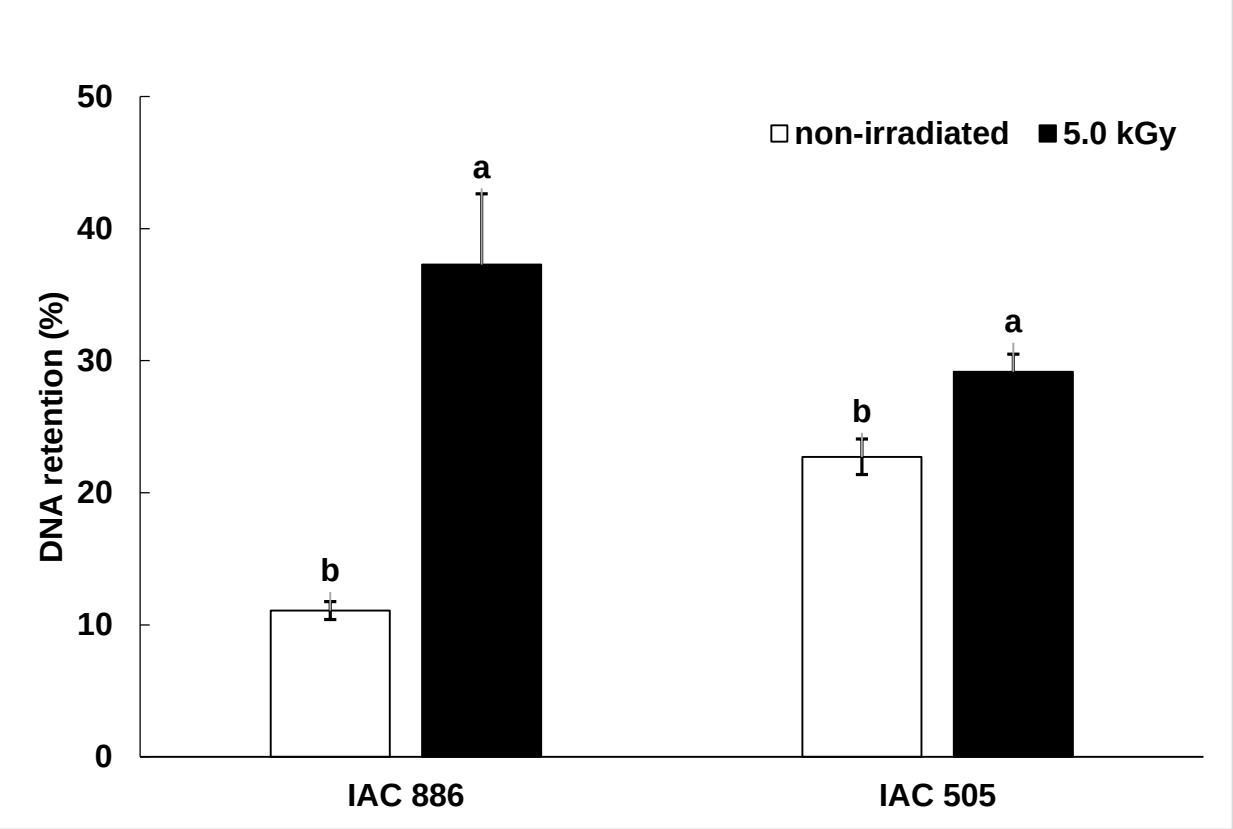


Figure 3.3 - Supercoiled plasmid DNA strand breakage inhibition of free phenolic fraction from peanut skin. White and black bars represent non-irradiated and gamma-irradiated (5.0 kGy) samples, respectively. Data represent mean ± standard deviation of each sample (n = 3). Means with different lower case letters within each cultivar indicate significant differences ($p < 0.05$)

3.3.11 Phenolic profile

Phenolic acids, namely protocatechuic, p-coumaric, gallic, caffeic, ferulic, sinapic, and ellagic acids were identified by comparison of their retention times and fragmentation patterns with those of authentic standards (Table 3.3). The MS spectra of protocatechuic, p-coumaric, gallic, caffeic, ferulic, sinapic, and ellagic gave deprotonated ions at m/z 153, 163, 169, 179, 193, 223, and 301, respectively. Similar to previous findings (JOHN; SHAHIDI, 2010; CHANDRASEKARA; SHAHIDI, 2011), these compounds showed loss of CO_2, giving $[M-H-44]^-$ as their characteristic ions in MS^2. The deprotonated molecular ion $[M-H]^-$ of both (+)-catechin and (−)-epicatechin exhibited a m/z signal at 289, and MS^2 spectra at m/z 245, also showing loss of CO_2 $[M-H-44]^-$. However, they were eluted with different retention times, thus both were positively identified by comparison of their retention times with those of authentic standards, whereas proanthocyanidin dimers, trimers, tretamers, and pentamers were tentatively identified using tandem mass spectrometry (MS^n), UV spectral, and literature data (APPELDOORN et al., 2009; MONAGAS et al., 2009; SARNOSKI et al., 2012; DE CAMARGO et al., 2014a, 2014b; MA et al., 2014). Proanthocyanidins were quantified as (+)-

catechin equivalents. Identification and quantification data are presented in Tables 3.3 and 3.4, respectively. Limits of detection and quantification for listed compounds ranged from 3 to 19 and from 8 to 57 ng/g, respectively. Regression coefficients of the plotted graphs had r^2 ranging from 0.9920 to 0.9999.

Compounds 5-9, 15, 16, 19, 22, 23, 25, 26, 28, and 29 were identified by their dissociation patterns, but not quantified due to their low concentrations and/or poor resolution. Among phenolic acids, ferulic and sinapic acids were found only in the insoluble-bound fraction, whereas coutaric and caftaric acids, were found only in the free phenolic fraction. Ellagic acid, whose presence is not commonly associated with peanuts, was found in all fractions. Quercetin-glucuronide, which has been reported in peanut flower (SOBOLEV; SY; GLOER, 2008), was also identified in the free and insoluble-bound phenolic fractions. The remaining proanthocyanidins were previously reported in peanut skin (APPELDOORN et al., 2009; MONAGAS et al., 2009; SARNOSKI et al., 2012; MA et al., 2014). Here, we tentatively identified them as procyanidins since they contained only catechin or epicatechin in their compositions, or prodelphinidins, which have (epi)gallocatechin in their structures. Most proanthocyanidin isomers were identified in the free phenolic fraction, and few variations were noticed between both cultivars, as they were from the same geographic area and growing period. Furthermore, some peaks were identified with the same ionization pattern, which is common for proanthocyanidins, as isomers such as procyanidins B1 to B8 are known (SAINT-CRICQ DE GAULEJAC; PROVOST; VIVAS, 1999). However, to distinguish among stereoisomers, nuclear magnetic resonance (NMR) analysis is required. Thus, in the present work, isomers with the same fragmentation were reported only once and for quantification purposes, their total was reported. Regardless of the ability to quantify the above mentioned compounds, it is important to report their presence as they are regarded as powerful antioxidants.

Manniflavanone, which has a higher antioxidant activity than ascorbic acid, rutin, quercetin, (−)-epicatechin, and (±)-naringenin, has received recent attention (STARK et al., 2013). Furthermore, the synergistic and/or antagonistic effect of bioactive compounds is well known. The content of phenolic acids in this work (Table 3.4 and 3.5) is in agreement with those in the literature for the content of phenolic compounds in the soluble ethanolic extracts of peanut skin (FRANCISCO; RESURRECCION, 2009). Among phenolic acids, protocatechuic acid was identified and quantified in all fractions. It is noteworthy that protocatechuic acid concentration was similar in the free, esterified and insoluble-bound fractions. Protocatechuic acid has been reported as having neuroprotective effect *in vivo* as well as in the prevention of H_2O_2-induced reduction in cell survival, reducing the concentration of

lipid peroxides, and increasing the activity of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase (SHI et al., 2006).

Table 3.3 - Phenolic compounds identified in peanut skin

	IAC 886			IAC 505			Phenolic acids	MW	$[M-H]^-$	Other product ions (m/z)
	F	E	B	F	E	B				
1	*	*	*	*	*	*	Protocatechuic acid[a]	154	153	109
2	*	*	*		*	*	*p*-Coumaric acid[a]	164	163	119
3	*	*	*		*		Gallic acid[a]	170	169	125
4		*	*		*	*	Caffeic acid[a]	180	179	135
5			*			*	Ferulic acid[a]	194	193	149, 134
6			*			*	Sinapic acid[a]	224	223	179
7	*			*			Coutaric acid	296	295	163, 119
8	*	*	*	*	*	*	Ellagic acid[a]	302	301	283, 257
9	*			*			Caftaric acid	312	311	179, 135
							Flavonoids/proanthocyanidins			
10	*	*	*	*	*	*	(+)-Catechin[a]	290	289	245, 205, 179
11	*	*	*	*	*	*	(−)-Epicatechin[a]	290	289	245, 205, 179
12	*	*	*	*	*	*	Quercetin	302	301	179, 151, 107
13		*			*		Gallocatechin	306	305	179
14			*			*	Isorhamnetin-glucoside	478	477	315, 300, 271, 247
15	*		*	*		*	Quercetin-glucuronide	478	477	301
16	*		*	*		*	Proanthocyanidin dimer B	574	573	555, 529, 447, 421, 285, 283
17	*	*	*	*	*	*	Procyanidin dimer A	576	575	539, 447, 449, 435, 423, 407, 289, 287, 285
18	*	*	*	*	*	*	Procyanidin dimer B	578	577	451, 425, 289
19	*		*	*		*	Manniflavanone	590	589	463, 445, 421, 303, 285
20	*	*	*	*	*	*	Prodelphinidin dimer A	592	591	573, 465, 451, 421, 303, 285
21		*			*		Prodelphinidin dimer B	594	593	575, 456, 449, 423, 303, 289, 285
22	*		*	*		*	Procyanidin trimer A	860	859	733, 707, 691, 569, 433
23	*		*	*		*	Procyanidin trimer A	862	861	735, 709, 693, 575, 449
24	*	*		*	*		Procyanidin trimer A	864	863	737, 711, 693, 559, 449
25				*			Procyanidin trimer C2	866	865	739, 695, 575, 407, 289, 287
26		*			*		Prodelphinidin trimer A	878	877	725
27	*			*	*		Procyanidin tetramer A	1150	1149	861, 737, 575
28	*	*		*	*	*	Procyanidin tetramer A	1152	1151	981, 863, 575
29	*				*		Procyanidin pentamer A	1438	1437	1149, 861, 737, 575, 573

MW, molecular weight. $[M-H]^-$ is deprotonated molecular ion
F, E, and B are free, esterified and insoluble-bound phenolics, respectively
* Indicates the presence of the compound in the fraction
[a] Identified with authentic standards

In contrast to other compounds, which had their concentration decreased by gamma-irradiation in the esterified fraction, the content of protocatechuic acid increased by up to 98%. This increase may be related to autoxidation of procyanidin and generation of anthocyanidins (PORTER; HRSTICH; CHAN, 1986), which may then be further degraded to protocatechuic acid. The degradation of cyanidin-3-rutinoside and generation of protocatechuic acid was recently reported (LEE et al., 2014). The authors demonstrated that doses of gamma-irradiation as low as 1.0 kGy decreased the absorbance of the methanolic solution containing cyanidin-3-rutinoside at 520 nm, indicating its degradation. Furthermore, a dose of 10 kGy was able to

decrease the concentration of cyanidin-3-rutinoside, with a parallel increase of protocatechuic acid methyl ester concentration.

Table 3.4 - The contents of free, esterified and insoluble-bound phenolics (μg/g DW) of IAC 886 peanut skin[a]

Phenolic compounds	Control	2.5 kGy	5.0 kGy
		Free	
Protocatechuic acid	56.96 ± 1.39b	70.16 ± 7.16a	72.94 ± 5.05a
(+)-Catechin	178.2 ± 26.4a	151.9 ± 12.8a	147.1 ± 8.50a
(−)-Epicatechin	361.8 ± 14.9a	333.4 ± 15.1a	352.7 ± 46.8a
Procyanidin dimer A[b]	3439 ± 85.8b	3329 ± 173b	4082 ± 185a
Procyanidin dimer B[b]	1086 ± 48.4a	912.2 ± 13.9b	970.5 ± 32.8b
Procyanidin trimer A[b]	4826 ± 371a	4747 ± 344a	5244 ± 522a
Procyanidin tetramer A[b]	827.6 ± 51.7a	908.6 ± 28.7a	889.3 ± 89.2a
		Esterified	
Gallic acid	82.06 ± 5.80a	68.31 ± 2.74b	36.67 ± 1.04c
Protocatechuic acid	63.46 ± 2.29b	52.78 ± 3.31b	125.6 ± 8.60a
(+)-Catechin	1033 ± 25.8a	780.4 ± 80.2b	705.2 ± 62.3b
(−)-Epicatechin	88.88 ± 7.04a	81.03 ± 11.5a	63.51 ± 7.96b
Gallocatechinb	47.93 ± 6.65a	27.62 ± 2.27b	14.88 ± 0.30c
Prodelphinidin A[b]	20.66 ± 1.72a	17.53 ± 1.43ab	16.19 ± 0.61b
Prodelphinidin B[b]	19.24 ± 0.81a	15.83 ± 0.57b	nd
Procyanidin dimer A[b]	184.4 ± 27.0a	195.7 ± 13.5a	202.6 ± 16.1a
Procyanidin dimer B[b]	390.9 ± 27.4a	317.1 ± 24.0b	285.0 ± 18.6b
Procyanidin trimer A[b]	1668 ± 90.8a	545.6 ± 56.1b	479.2 ± 53.8b
Procyanidin tetramer A[b]	nd	nd	nd
		Insoluble-Bound	
Protocatechuic acid	64.02 ± 4.24a	63.25 ± 1.21a	67.69 ± 1.59a
Caffeic acid	14.05 ± 0.41c	15.92 ± 0.17b	23.50 ± 1.05a
p-Coumaric acid	25.78 ± 2.22c	36.94 ± 0.65b	66.86 ± 3.72a
Quercetin	23.49 ± 1.98c	29.75 ± 2.10b	44.86 ± 1.57a
Isorhamnetin-glucosideb	24.47 ± 0.85c	31.58 ± 0.71b	34.41 ± 1.42a
(+)-Catechin	35.65 ± 1.60c	46.15 ± 3.73b	148.3 ± 3.48a
(−)-Epicatechin	123.0 ± 10.2c	194.5 ± 11.4b	201.4 ± 10.3a
Procyanidin dimer A[b]	125.6 ± 11.0b	142.2 ±18.0b	288.6 ± 26.8a
Procyanidin dimer B[b]	385.4 ± 46.9a	210.7 ± 7.53b	243.3 ± 34.0b

[a] Data represent the mean of triplicate analysis for each sample ± standard deviation. Means followed by the same letters within a column are not significantly different ($p > 0.05$). DW, dry weight of defatted sample; nd, non-detected; tr, trace. [b] Compounds quantified as catechin equivalentes. Traces of procyanidin pentamer A were found in the free fraction

In the present study, gallic acid was quantified only in the esterified fraction, while caffeic and p-coumaric acids were quantified only in the insoluble-bound fraction. Meanwhile, the concentration of gallic acid decreased as the dose of gamma-irradiation increased. To the best of our knowledge, the presence of antioxidatively potent gallic acid has not previously been reported in peanut skin, possibly because it was present only in the esterified form. However, gallic acid has a higher antioxidant power than catechin and even at low concentrations it may make a significant contribution to the antioxidant power and bioactivity of peanut skin.

In a recent study, *in vitro* assays showed that caffeic and p-coumaric acids had potential neuroprotective effects by safeguarding neurons against injuries caused by 5-S-

cysteinyldopamine, which possesses neurotoxicity and may contribute to the progression of Parkinson's disease (VAUZOUR; CORONA; SPENCER, 2010). In the present study, the content of caffeic and p-coumaric acid was increased by up to 67 and 159%, respectively. Gamma-irradiation induced biosynthesis of p-coumaric acid has already been reported (OUFEDJIKH et al., 2000). According to these authors, the activity of phenylalanine ammonia lyase (PAL) was also increased by gamma-irradiation, which positively correlated with the synthesis of phenolic compounds. In fact gamma-irradiation induced PAL production was also reported (HUSSAIN et al., 2010) along with an increase in TPC, total anthocyanin, DPPH radical scavenging activity and ferric reducing antioxidant power.

Table 3.5 - The contents of free, esterified and insoluble-bound phenolics (μg/g DW) of IAC 505 peanut skin[a]

Phenolic compounds	Control	2.5 kGy	5.0 kGy
		Free	
Protocatechuic acid	43.51 ± 0.01b	56.34 ± 3.74a	54.69 ± 2.39a
(+)-Catechin	105.0 ± 8.81a	128.7 ± 5.69a	121.1 ± 16.7a
(−)-Epicatechin	380.8 ± 1.86a	323.8 ± 9.03b	235.2 ± 5.28c
Procyanidin dimer A[b]	1867 ± 3.19b	1836 ± 10.7b	2006 ± 92.1a
Procyanidin dimer B[b]	1412 ± 164a	1404 ± 69.8a	985.6 ± 9.83b
Procyanidin trimer A[b]	2937 ± 135a	3089 ± 94.2a	3019 ± 7.48a
Procyanidin tetramer A[b]	1050 ± 4.58a	968.6 ± 82.2a	658.3 ± 7.13b
		Esterified	
Gallic acid	114.3 ± 1.10a	101.5 ± 1.73b	51.32 ± 0.43c
Protocatechuic acid	50.03 ± 5.33b	62.28 ± 1.23a	65.41 ± 0.84a
(+)-Catechin	1100 ± 132a	1293 ± 19.0a	891.4 ± 20.6b
(−)-Epicatechin	82.82 ± 7.50a	86.90 ± 2.96a	59.73 ± 6.05b
Gallocatechinb	38.71 ± 3.74c	24.37 ± 2.39b	14.53 ± 2.38a
Prodelphinidin A[b]	26.84 ± 1.78a	19.25 ± 1.75b	12.76 ± 0.96a
Prodelphinidin B[b]	21.01 ± 2.10a	13.36 ± 1.86b	nd
Procyanidin dimer A[b]	316.1 ± 20.4b	310.0 ± 5.19b	556.8 ± 3.75a
Procyanidin dimer B[b]	1208 ± 58.1a	1000 ± 9.63b	909.5 ± 48.3b
Procyanidin trimer A[b]	691.5 ± 37.3a	621.2 ± 31.6a	347.6 ± 9.12b
Procyanidin tetramer A[b]	tr	tr	887.3 ± 20.8
		Insoluble-Bound	
Protocatechuic acid	57.95 ± 1.98b	59.97 ± 2.39ab	64.30 ± 1.58a
Caffeic acid	21.85 ± 0.57a	23.41 ± 2.46ab	27.53 ± 1.01a
p-Coumaric acid	48.96 ± 2.42a	51.81 ± 1.13ab	53.86 ± 1.26a
Quercetin	54.26 ± 2.26	132.2 ± 14.3b	243.5 ± 23.0a
Isorhamnetin-glucosideb	100.6 ± 6.95a	102.0 ± 9.40a	117.3 ± 9.26a
(+)-Catechin	52.20 ± 3.19a	66.50 ± 12.2ab	80.86 ± 5.32a
(−)-Epicatechin	63.02 ± 7.76b	86.13 ± 6.09a	80.50 ± 2.92a
Procyanidin dimer A[b]	188.8 ± 10.2c	285.5 ± 11.0b	328.7 ± 9.01a
Procyanidin dimer B[b]	439.3 ± 17.8a	377.6 ± 14.4b	269.9 ± 25.3c

[a] Data represent the mean of triplicate analysis for each sample ± standard deviation. Means followed by the same letters within a column are not significantly different ($p > 0.05$). DW, dry weight of defatted sample; nd, non-detected; tr, trace. [b] Compounds quantified as catechin equivalentes. Traces of procyanidin pentamer A were found in the free fraction

Flavonoids are the major class of phenolic compounds in peanut skin. Their concentration in the free phenolic fraction was as follows: procyanidin A dimers through

tetramers > procyanidin B > (−)-epicatechin > (+)-catechin > pentamer. Different from the free fraction, (+)-catechin was the most prominent monomer in the esterified fraction, with concentrations around 30% of the total phenolics as determined by HPLC. Furthermore, gallocatechin and prodelphinidins A and B were only found and quantified in the esterified fraction. In contrast with the free phenolic fraction, procyanidin B was the major phenolic in the insoluble-bound fraction. These data show that the distribution of individual phenolics varies among different fractions.

A-type procyanidin dimers consist of (C4→C8, C2→O7) or (C4→C6, C2→O7) linkages, whereas B-type dimers consist of (C4→C8) or (C4→C6) linkages. Although both structures display the same fragmentation pattern, their separation is possible due to different linkages and stereochemistry (SARNOSKI et al., 2012). Concentrations of procyanidin dimer A was higher than values reported in the literature (YU et al., 2006), where they ranged from 902 to 1270 μg/g of dry sample. However, no information on the cultivar of tested samples was provided. Furthermore, several factors such as climate and stress conditions, as well as soil quality may play important roles in the content of phenolics.

Procyanidins B were the major phenolic compounds in the esterified fraction of IAC 505, but not of IAC 866, the latter showing (+)-catechin as the major polyphenol, which shows few but significant differences between cultivars. Grapes are regarded as good sources of proanthocyanidins phenol, including procyanidins dimer B. Values of procyanidins dimer B in the present study were comparable to those of found in the soluble phenolics in grape skin and seed (LORRAIN; CHIRA; TEISSEDRE, 2011).

It is noteworthy that the increase of procyanidin dimer A was in parallel with a decrease in the concentration of procyanidin dimer B. Figure 3.4 shows the change in distribution of procyanidin dimers in the insoluble-bound fraction; however, this trend was noted in all fractions. It is also interesting that in both cultivars the increase in procyanidin dimer A was higher in the insoluble-bound phenolic fraction, for which an increase of up to 130% was found. The increase in the concentration of procyanidin dimer A may be due to the conversion of procyanidin B into the A form. The mechanism of this conversion reaction is beyond the mandate of the present work. However, it is well established that gamma-irradiation generates free radicals that may produce new compounds. Kondo et al. (2000) demonstrated the ability of DPPH radical in converting procyanidin dimer B into the A type.

Procyanidin trimer A was quantified only in the free and esterified fractions and its concentration was affected only in the latter, and this followed the same decreasing trend observed for most phenolic compounds in this fraction. In non-irradiated samples, procyanidin

tetramers A were quantified only in the free phenolic fraction. A decrease in the content of procyanidin tetramers in the free phenolic fraction of IAC 505 and presence of a higher concentration of it in the esterified fraction was noticed in samples subjected to 5.0 kGy irradiation. Although further investigation is necessary, depolymerization of proanthocyanidins into smaller molecules is contemplated, which may improve their bioavailability as the proanthocyanidin absorption is dependent of the degree of polymerization and those ones with a degree of polymerization higher than four are not absorbable because of their large molecular size and gut barrier (OU; GU, 2014).

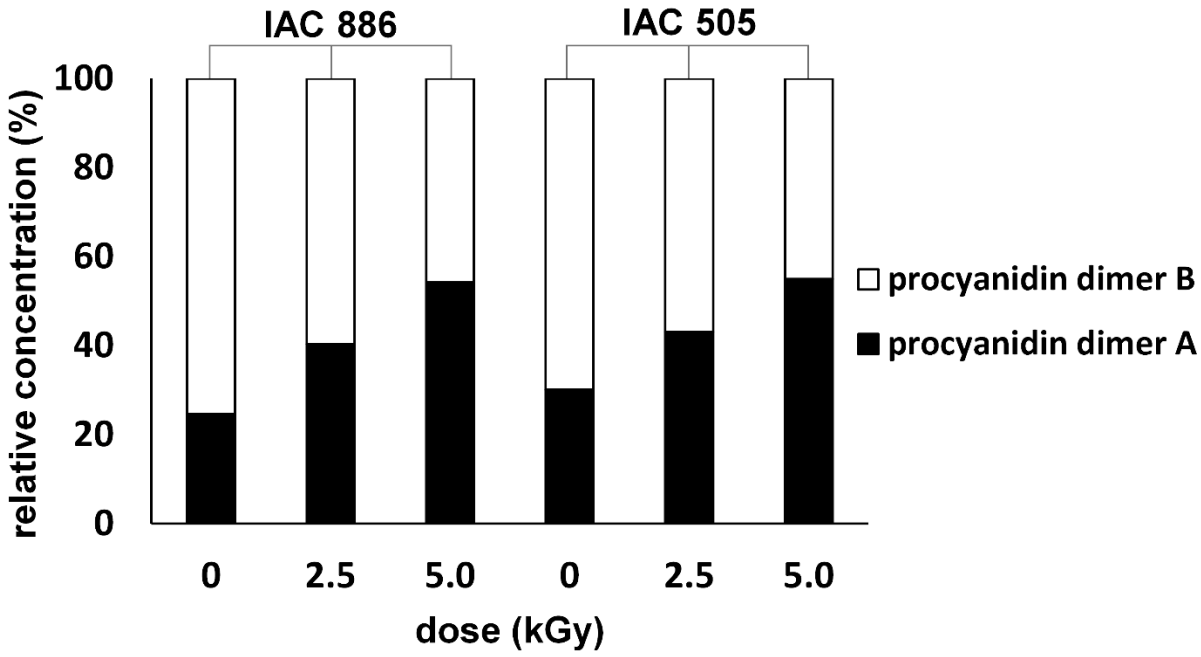


Figure 3.4 - Distribution change of procyanidin dimers in the insoluble-bound fraction of non-irradiated and gamma-irradiated peanut skin at 2.5 and 5.0 kGy for IAC 886 and IAC 505 peanut skin. This is representative of the remaining fractions, which show the same trend. Detailed data are given in Tables 3.4 and 3.5

The presence of proanthocyanidins with a degree of polymerization (DP) higher than six is also possible in the samples evaluated here; however, their identification was not possible as such molecules were not in the range of the present **s**tudy (up to m/z 2000). Other authors (MA et al., 2014; SARNOSKI et al., 2012) have identified proanthocyanidins with higher degrees of polymerization (DP > 6). According to them, their identification is very difficult due to extremely complicated fragmentation patterns involved.

Insoluble-bound phenolics are linked to the cell wall components. With exception of procyanidins B, phenolic compounds in the insoluble-bound fraction increased with increase in the dose used. The increase in insoluble-bound phenolics may be related to the formation of

crosslinks between such molecules and the cell walls; however, the content of insoluble-bound phenolics fraction in peanut skin is negligible, thus no major effects are expected. The ability of gamma-irradiation in crosslink has been used to produce edible coats used in fruits; however, conversion of procyanidins dimer B into the A type is also possible in this fraction.

Polyphenols from peanut skin can interact with membrane phospholipids, presumably with their polar headgroups. As a consequence of this interaction, they can provide protection against the attack by oxidants and other molecules that challenge the bilayer's integrity (VERSTRAETEN et al., 2005). They also render a greater protective effect against the haemolysis of red blood cells than ascorbic acid under *in vitro* conditions (WANG et al., 2007), thus it is of great importance to study potential sources of polyphenols, including procyanidins, and their stability under different processing conditions.

3.4 Conclusions

The content of free phenolic compounds, which represent the major constituent of peanut skin, was enhanced by gamma-irradiation. Proanthocyanidins were the major phenolic compounds in all fractions. Data from the present study strongly suggest that gamma-irradiation may be able to convert procyanidin dimer B to the A-type in all phenolic fractions, while depolymerization may occur in the free and esterified fraction, and cross-linking may take place in the insoluble-bound fractions. Gamma-irradiation may increase the bioavailability of proanthocyanidins via depolymerization, which might improve the biological activity of such compounds. This is supported by several antioxidant assays and the increasing ability of polyphenols of gamma-irradiated samples in preventing LDL-cholesterol oxidation and DNA strand breakage. For instance, gamma-irradiation induces the formation of free radicals, so there is a concern about the stability of antioxidants present in gamma-irradiated feedstock. Thus, this work has shed light in clarifying the situation, which may help the food industry in developing novel products with better economic return with additional health benefits. Furthermore, gamma-irradiation decreased the microbiological count of peanut skin as by-product of the blanching process of the peanut industry.

References

AL-BACHIR, M. Effect of gamma irradiation on fungal load, chemical and sensory characteristics of walnuts (*Juglans regia* L.). **Journal of Stored Products Research,** Oxford, v. 40, p. 355-362, 2004.

ALASALVAR, C.; KARAMAC, M.; KOSINSKA, A.; RYBARCZYK, A.; SHAHIDI, F.; AMAROWICZ, R. Antioxidant activity of hazelnut skin phenolics. **Journal of Agricultural and Food Chemistry,** Easton, v. 57, p. 4645-4650, 2009.

AOUIDI, F.; AYARI, S.; FERHI, H.; ROUSSOS, S.; HAMDI, M. Gamma irradiation of air-dried olive leaves: effective decontamination and impact on the antioxidative properties and on phenolic compounds. **Food Chemistry,** London, v. 127, p. 1105-1113, 2011.

APPELDOORN, M.M.; SANDERS, M.; VINCKEN, J.P.; CHEYNIER, V.; LE GUERNEVE, C.; HOLLMAN, P.C.H.; GRUPPEN, H. Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds. **Food Chemistry,** London, v. 117, p. 713-720, 2009.

ARRANZ, S.; CERT, R.; PEREZ-JIMENEZ, J.; CERT, A.; SAURA-CALIXTO, F. Comparison between free radical scavenging capacity and oxidative stability of nut oils. **Food Chemistry,** London, v. 110, p. 985-990, 2008.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. **Official methods of analysis of AOAC International.** 17th ed. Gaithersburg: AOAC International, 2000. 937 p.

CALHOUN, S.; POST, L.; WARREN, B.; THOMPSON, S.; BONTEMPO, A.R. Prevalence and concentration of *Salmonella* on raw shelled peanuts in the United States. **Journal of Food Protection,** Ames, v. 76, p. 575-579, 2013.

CHANDRASEKARA, A.; SHAHIDI, F. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. **Journal of Functional Foods,** Amsterdam, v. 3, p. 144-158, 2011a.

_____. Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels, and testa. **Journal of Agricultural and Food Chemistry,** Easton, v. 59, p. 5006-5014, 2011b.

CHEN, M.-C.; YE, Y.-Y.; JI, G.; LIU, J.-W. Hesperidin upregulates heme oxygenase-1 to attenuate hydrogen peroxide-induced cell damage in hepatic L02 cells. **Journal of Agricultural and Food Chemistry,** v. 58, Easton, p. 3330-3335, 2010.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; BIASOTO, A.C.T.; SHAHIDI, F. Low molecular weight phenolics of grape juice and winemaking byproducts: antioxidant activities and inhibition of oxidation of human low-density lipoprotein cholesterol and DNA strand breakage. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, 12159–12171, 2014a.

DE CAMARGO, A.C.; VIDAL, C.M.M.; CANNIATTI-BRAZACA, S.G.; SHAHIDI, F. Fortification of cookies with peanut skin: Effects on the composition, polyphenols, antioxidant properties and sensory quality. **Journal of Agricultural and Food Chemistry**, Easton, v. 62, 11228-11235, 2014b.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; CALORI-DOMINGUES, M.A.; CANNIATTI-BRAZACA, S.G. Gamma radiation effects on peanut skin antioxidants. **International Journal of Molecular Sciences,** Basel, v. 13, p. 3073-3084, 2012a.

DE CAMARGO, A.C.; VIEIRA, T.M.F.V.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; CANNIATTI-BRAZACA, S.G. Gamma radiation induced oxidation and tocopherols decrease in in-shell, peeled and blanched peanuts. **International Journal of Molecular Sciences,** Basel, v. 13, p. 2827-2845, 2012b.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; SPOTO, M.H.F.; CANNIATTI-BRAZACA, S.G. Gamma irradiation of in-shell and blanched peanuts protects against mycotoxic fungi and retains their nutraceutical components during long-term storage. **International Journal of Molecular Sciences,** Basel, v. 13, p. 10935-10958, 2012c.

DIXIT, A.K.; BHATNAGAR, D.; KUMAR, V.; RANI, A.; MANJAYA, J.G.; BHATNAGAR, D. Gamma irradiation induced enhancement in isoflavones, total phenol, anthocyanin and antioxidant properties of varying seed coat colored soybean. **Journal of Agricultural and Food Chemistry,** Easton, v. 58, p. 4298-4302, 2010.

DORNER, J.W. Management and prevention of mycotoxins in peanuts. **Food Additives and Contaminants,** London, v. 25, p. 203-208, 2008.

DOWNES, F.P.; ITO, K. **Compendium of methods for the microbiological examination of food**. 4th ed. Washington: American Public Health Association, 2001. 676 p.

FARKAS, J. Irradiation for better foods. **Trends in Food Science & Technology,** Cambridge, v. 17, p. 148-152, 2006.

FERNANDES, S.P.; DRINGEN, R.; LAWEN, A.; ROBINSON, S.R. Inactivation of astrocytic glutamine synthetase by hydrogen peroxide requires iron. **Neuroscience Letters,** Limerick, v. 490, p. 27-30, 2011.

FRANCISCO, M.; RESURRECCION, A. Total phenolics and antioxidant capacity of heat-treated peanut skins. **Journal of Food Composition and Analysis,** San Diego, v. 22, p. 16-24, 2009.

FRANCISCO, M.L.D.; RESURRECCION, A.V.A. Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut skin extracts. **Food Chemistry,** London, v. 117, p. 356-363, 2009.

GRANT, I.R.; NIXON, C.R.; PATTERSON, M.F. Effect of low-dose irradiation on growth of and toxin producton by *Staphylococcus aureus* and *Bacillus cereus* in roast beef and gravy. **International Journal of Food Microbiology,** Amsterdam, v. 18, p. 25-36, 1993.

GUPTASARMA, P.; BALASUBRAMANIAN, D.; MATSUGO, S.; SAITO, I. Hydroxyl radical mediated damage to proteins, with special reference to the crystallins. **Biochemistry,** Easton, v. 31, p. 4296-4303, 1992.

HARRISON, K.; WERE, L.M. Effect of gamma irradiation on total phenolic content yield and antioxidant capacity of Almond skin extracts. **Food Chemistry,** London, v. 102,p. 932-937, 2007.

HUSSAIN, P.R.; WANI, A.M.; MEENA, R.S.; DAR, M.A. Gamma irradiation induced enhancement of phenylalanine ammonia-lyase (PAL) and antioxidant activity in peach (*Prunus persica Bausch*, Cv. Elberta). **Radiation Physics and Chemistry,** Oxford, v. 79, p. 982-989, 2010.

JOHN, J.A.; SHAHIDI, F. Phenolic compounds and antioxidant activity of Brazil nut (*Bertholletia excelsa*). **Journal of Functional Foods,** Amsterdam, v. 2, p. 196-209, 2010.

KHAN, N.; MONAGAS, M.; ANDRES-LACUEVA, C.; CASAS, R.; URPI-SARDA, M.; LAMUELA-RAVENTOS, R.M.; ESTRUCH, R. Regular consumption of cocoa powder with milk increases HDL cholesterol and reduces oxidized LDL levels in subjects at high-risk of cardiovascular disease. **Nutrition Metabolism and Cardiovascular Diseases**, Amsterdam, v. 22, p. 1046-1053, 2012.

KILCAST, D. Food irradiation: current problems and future potential. **International Biodeterioration & Biodegradation,** Barking, v. 36, p. 279-296, 1995.

KONDO, K.; KURIHARA, M.; FUKUHARA, K.; TANAKA, T.; SUZUKI, T.; MIYATA, N.; TOYODA, M. Conversion of procyanidin B-type (catechin dimer) to A-type: evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. **Tetrahedron Letters,** Elmsford, v. 41, p. 485-488, 2000.

LEE, S.S.; KIM, T.H.; LEE, E.M.; LEE, M.H.; LEE, H.Y.; CHUNG, B.Y. Degradation of cyanidin-3-rutinoside and formation of protocatechuic acid methyl ester in methanol solution by gamma irradiation. **Food Chemistry,** London, v. 156, p. 312-318, 2014.

LORRAIN, B.; CHIRA, K.; TEISSEDRE, P.L. Phenolic composition of Merlot and Cabernet-Sauvignon grapes from Bordeaux vineyard for the 2009-vintage: comparison to 2006, 2007 and 2008 vintages. **Food Chemistry,** London, v. 126, p. 1991-1999, 2011.

MACKEY, B.M.; DERRICK, C.M. The effect of sublethal injury by heating, freezing, drying and gamma- radiation on the duration of the lag phase of *Salmonella typhimurium*. **Journal of Applied Microbiology**, Oxford, v. 53, p. 243–251, 1982.

MADHUJITH, T.; SHAHIDI, F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. **Journal of Agricultural and Food Chemistry**, Easton, v. 54, p. 8048–8057, 2006.

MIKSCH, R.R.; LEEK, J.; MYODA, S.; NGUYEN, T.; TENNEY, K.; SVIDENKO, V.; GREESON, K.; SAMADPOUR, M. Prevalence and counts of *Salmonella* and enterohemorrhagic *Escherichia coli* in raw, shelled runner peanuts. **Journal of Food Protection,** Ames, v. 76, 1668-1675, 2003.

MIRANDA, D.D.C.; ARÇARI, D.P.; LADEIRA, M.S.P.; CALORI-DOMINGUES, M.A.; ROMERO, A.C.; SALVADORI, D.M.F.; GLORIA, E.M.; PEDRAZZOLI, J. JR.; RIBEIRO,

M.L. Analysis of DNA damage induced by aflatoxin B1 in Dunkin–Hartley guinea pigs. **Mycopathologia,** Den Haag, v. 163, p. 275-280, 2007.

MISHRA, B.; GAUTAM, S.; SHARMA, A. Microbial decontamination of tea (*Camellia sinensis*) by gamma radiation. **Journal of Food Science,** Chicago, v. 71, p. 151-156, 2006.

MONAGAS, M.; GARRIDO, I.; LEBRON-AGUILAR, R.; GOMEZ-CORDOVES, M.; RYBARCZYK, A.; AMAROWICZ, R.; BARTOLOME, B. Comparative flavan-3-ol profile and antioxidant capacity of roasted peanut, hazelnut, and almond skins. **Journal of Agricultural and Food Chemistry,** Easton, v. 57, p. 10590-10599, 2009.

NESCI, A.; MONTEMARANI, A.; ETCHEVERRY, M. Assessment of mycoflora and infestation of insects, vector of *Aspergillus* section *Flavi*, in stored peanut from Argentina. **Mycotoxin Research,** Heidelberg, v. 27, p. 5-12, 2011.

OUFEDJIKH, H.; MAHROUZ, M.; AMIOT, M.J.; LACROIX, M. Effect of gamma-irradiation on phenolic compounds and phenylalanine ammonia-lyase activity during storage in relation to peel injury from peel of *Citrus clementina* Hort. ex. Tanaka. **Journal of Agricultural and Food Chemistry,** Easton, v. 48, p. 559-565, 2000.

OYAIZU, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. **The Japanese Journal of Nutrition and Dietetics,** Tokyo, v. 44, p. 307-315, 1986.

PEREZ, M.; CALDERON, N.; CROCI, C. Radiation-induced enhancement of antioxidant activity in extracts of rosemary (*Rosmarinus officinalis* L.). **Food Chemistry,** London, v. 104, p. 585-592, 2007.

PORTER, L.J.; HRSTICH, L.N.; CHAN, B.G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. **Phytochemistry,** New York, v. 25, p. 223-230, 1986.

PRICE, M.L.; HAGERMAN, A.E.; BUTLER, L.G. Tannin content of cowpeas, chickpeas, pigeon peas, and mung beans. **Journal of Agricultural and Food Chemistry,** Easton, v. 28, p. 459-461, 1980.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.; RICE-EVANS, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine,** New York, v. 26, p. 1231-1237, 1999.

ROOPCHAND, D.E.; KUHN, P.; ROJO, L.E.; LILA, M.A.; RASKIN, I. Blueberry polyphenol-enriched soybean flour reduces hyperglycemia, body weight gain and serum cholesterol in mice. **Pharmacological Research,** London, v. 68, p. 59-67, 2013.

SAINT-CRICQ DE GAULEJAC, N.; PROVOST, C.; VIVAS, N. Comparative study of polyphenol scavenging activities assessed by different methods. **Journal of Agricultural and Food Chemistry,** Easton, v. 47, p. 425-431, 1999.

SARNOSKI, P.J.; JOHNSON, J.V.; REED, K.A.; TANKO, J.M.; O'KEEFE, S.F. Separation and characterisation of proanthocyanidins in Virginia type peanut skins by LC-MS^n. **Food Chemistry,** London, v. 131, p. 927-939, 2012.

SHAHIDI, F.; ALASALVAR, C.; LIYANA-PATHIRANA, C.M. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. **Journal of Agricultural and Food Chemistry,** Easton, v. 55, p. 1212-1220, 2007.

SHEM-TOV, Y.; BADANI, H.; SEGEV, A.; HEDVAT, I.; GALILI, S.; HOVAV, R. Determination of total polyphenol, flavonoid and anthocyanin contents and antioxidant capacities capacities of skins from peanut (*Arachis hyopogaea*) lines with different skin colors. **Journal of Food Biochemistry,** Westport, v. 36, p. 301-308, 2012.

SHI, G.F.; AN, L.J.; JIANG, B.; GUAN, S.; BAO, Y.M. Alpinia protocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. **Neuroscience Letters,** Limerick, v. 403, p. 206-210, 2006.

SOBOLEV, V.S.; SY, A.A.; GLOER, J.B. Spermidine and flavonoid conjugates from peanut (*Arachis hypogaea*) flowers. **Journal of Agricultural and Food Chemistry,** Easton, v. 56, p. 2960-2969, 2008.

STAJNER, D.; MILOSEVIC, M.; POPOVIC, B. M. Irradiation effects on phenolic content, lipid and protein oxidation and scavenger ability of soybean seeds. **International Journal of Molecular Sciences,** Basel, v. 8, p. 618-627, 2007.

STARK, T.D.; GERMANN, D.; BALEMBA, O.B.; WAKAMATSU, J.; HOFMANN, T. New highly *in vitro* antioxidative 3,8 "-linked biflav(an)ones and flavanone-C-glycosides from *Garcinia buchananii* stem bark. **Journal of Agricultural and Food Chemistry,** Easton, v. 61, p. 12572-12581, 2013.

SWAIN, T.; HILLIS, W.E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. **Journal of the Science of Food and Agriculture,** London, v. 10, p. 63-68, 1959.

TERRA, X.; VALLS, J.; VITRAC, X.; MERRILLON, J. M.; AROLA, L.; ARDEVOL, A.; BLADE, C.; FERNANDEZ-LARREA, J.; PUJADAS, G.; SALVADO, J.; BLAY, M. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. **Journal of Agricultural and Food Chemistry,** Easton, v. 55, p. 4357-4365, 2007.

VAUZOUR, D.; CORONA, G.; SPENCER, J.P.E. Caffeic acid, tyrosol and p-coumaric acid are potent inhibitors of 5-S-cysteinyl-dopamine induced neurotoxicity. **Archives of Biochemistry and Biophysics,** New York, v. 501, p. 106-111, 2010.

VERSTRAETEN, S.V.; HAMMERSTONE, J.F.; KEEN, C.L.; FRAGA, C.G.; OTEIZA, P.I. Antioxidant and membrane effects of procyanidin dimers and trimers isolated from peanut and cocoa. **Journal of Agricultural and Food Chemistry,** Easton, v. 53, p. 5041-5048, 2005.

WANG, J.; YUAN, X.P.; JIN, Z.Y.; TIAN, Y.; SONG, H.L. Free radical and reactive oxygen species scavenging activities of peanut skins extract. **Food Chemistry,** London, v. 104, p. 242-250, 2007.

WETTASINGHE, M.; SHAHIDI, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. **Food Chemistry,** London, v. 70, p. 17-26, 2000.

WIJERATNE, S.S.K.; ABOU-ZAID, M.M.; SHAHIDI, F. Antioxidant polyphenols in almond and its coproducts. **Journal of Agricultural and Food Chemistry,** Easton, v. 54, 312-318, 2006.

WILSON-KAKASHITA, G.; GERDES, D.L.; HALL, W.R. The effect of gamma irradiation on the quality of English walnuts *(Junglans regia*). **LWT - Food Science and Technology,** London, v. 28, p. 17-20, 1995.

WITKOWSKA, A.M.; HICKEY, D.K.; ALONSO-GOMEZ, M.; WILKINSON, M.G. The microbiological quality of commercial herb and spice preparations used in the formulation of a chicken supreme ready meal and microbial survival following a simulated industrial heating process. **Food Control**, Guildford, v. 22, p. 616-625, 2011.

YU, J.M.; AHMEDNA, M.; GOKTEPE, I.; DAI, J.A. Peanut skin procyanidins: composition and antioxidant activities as affected by processing. **Journal of Food Composition and Analysis,** San Diego, v. 19, p. 364-371, 2006.

4 ENZYME-ASSISTED EXTRACTION OF PHENOLICS FROM WINEMAKING BY-PRODUCTS: ANTIOXIDANT POTENTIAL AND INHIBITION OF ALPHA-GLUCOSIDASE AND LIPASE ACTIVITIES

Abstract

Phenolics in food and agricultural and processing by-products exist in the soluble and insoluble-bound forms. The ability of selected enzymes in improving the extraction of insoluble-bound phenolics from the starting material (experiment I) or the residues containing insoluble-bound phenolics (experiment II) were evaluated. Pronase and Viscozyme improved the extraction of insoluble-bound phenolics as evaluated by total phenolic content, antioxidant potential as determined by ABTS and DPPH assays, and hydroxyl radical scavenging capacity, reducing power as well as evaluation of inhibition of alpha-glucosidase and lipase activities. Viscozyme released higher amounts of gallic acid, catechin, and prodelphinidin dimer A compared to Pronase treatment. Furthermore, *p*-coumaric and caffeic acids, as well as procyanidin dimer B, were extracted with Viscozyme but not with Pronase treatment. Solubility plays an important role in the bioavailability of phenolic compounds, hence this study may assist in better exploitation of phenolics from winemaking by-products as functional food ingredients and/or supplements.

Keywords: HPLC-DAD-ESI-MS^n; Phenolic acids; Flavonoids; Proanthocyanidin; Diabetes; Obesity

4.1 Introduction

Grapes and their derived beverages are important sources of food phenolics (DA SILVA et al., 2015; TAO et al., 2016). However, winemaking generates a large amount of by-products (e.g. skins and seeds). These by-products serve as rich sources of phenolics belonging to several classes of compounds such as phenolic acids, flavonoids, including anthocyanins, as well as proanthocyanidins (CHENG et al., 2012; DE CAMARGO et al., 2014a).

Phenolic and/or polyphenolic compounds have attracted much attention due to their wide range of potential health benefits, as substantiated by both *in vitro* and *in vivo* studies (DE CAMARGO et al., 2014a; VICENTE; ISHIMOTO; TORRES, 2014). The role of food phenolics in preventing degenerative, vascular and heart disease and as anti-inflammatory and antimicrobial agents have also been reported (ALASALVAR; BOLLING, 2015; SHAHIDI; AMBIGAIPALAN, 2015). Additionally, phenolic compounds may play an important role in amiliorating certain types of cancer, including colorectal cancer (SHAHIDI;

AMBIGAIPALAN, 2015). The chemical structures of these molecules are as important as their detection and concentration, which may reflect in a different correlation between a particular molecule and its activity.

Phenolic compounds are present in the soluble (free and esterified) and insoluble-bound forms, the proportion of each one depends not only on the starting material but also on their cultivar and an eventual processing to which they are subjected. For example, the esterified phenolics from lentils were generally in higher amount, but some cultivars also showed higher content in the fraction containing insoluble-bound phenolics (ALSHIKH; DE CAMARGO; SHAHIDI, 2015), whereas berry seed meals had higher content of insoluble-bound phenolics (AYOUB; DE CAMARGO; SHAHIDI, 2016). Peanut skin submitted to gamma-irradiation had increased free and insoluble-bound phenolic contents upon processing (DE CAMARGO et al., 2015). As antioxidants, phenolic compounds may counteract oxidative reactions in food subjected to treatments such as gamma-irradiation and pasteurization as well as during long-term storage, which may affect its shelf-life and sensory characteristics (DE CAMARGO et al., 2012a; DA SILVA et al., 2014). Furthermore, biologically relevant molecules such as lipids, proteins, lipoproteins and DNA may also be protected from oxidatively reactive compounds.

In a previous study at this department (DE CAMARGO et al., 2014a), it has been demonstrated that, regardless of the process (juice or winemaking), insoluble-bound phenolics were major fractions in grape processing by-products. The same study also provided evidence about the dominant benefits of insoluble-bound phenolics of grape by-products in inhibiting copper-induced human LDL-cholesterol oxidation and peroxyl radical-induced DNA strand breakage. These results demonstrated the potential of the insoluble-bound phenolics from winemaking by-products as their major source of bioactive compounds.

Enzyme-assisted extraction has been regarded as an alternative method for improved extraction of food phenolics (MONTELLA et al., 2013; PAPILLO et al., 2014), especially the insoluble-bound phenolics, which are linked to carbohydrates and proteins of cell wall matrices. However, to the best of the authors' knowledge, there is no literature providing the effect of enzyme-assisted extraction on the ratio of soluble to insoluble-bound phenolics from winemaking by-products although this has been reported for germinating lentils by Yeo and Shahidi (2015). Thus, in the present study, winemaking by-products (cv. Tempranillo) were treated with Pronase and Viscozyme to improve the solubility of phenolics present in the sample. The effects were studied based on the change in the distribution pattern of soluble/insoluble-bound phenolics as well as their chemical profile, antioxidant properties (antiradical activity) and reducing power. The resultant products were also evaluated for their

effect in deactivating alpha-glucosidase and lipase, which have a key role in the prevention and management of diabetes and obesity, respectively.

4.2 Materials and Methods

Winemaking by-products (cv. Tempranillo) were kindly provided by Santa Maria Winery (Lagoa Grande, Pernambuco State, Brazil). Hexane, acetone, diethyl ether, ethyl acetate, methanol, acetonitrile, formic acid, hydrochloric acid, sodium hydroxide, potassium persulfate, sodium chloride, trichloroacetic acid, sodium carbonate, dimethyl sulphoxide, and Tris base were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada). Pronase, Viscozyme, alpha-glucosidase from *Saccharomyces cerevisiae*, and type II crude porcine pancreatic lipase, catalogue numbers P5147, V2010, G5003, and L3126, respectively, as well as Folin Ciocalteau's phenol reagent, DPPH, ABTS, mono- and dibasic potassium phosphates, hydrogen peroxide, DMPO (5,5-dimethyl-1-pyrroline-N-oxide), ferrous sulphate, potassium ferricyanide, ferric chloride, Trolox, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl octanoate, caffeic, gallic, and *p*-coumaric acids, catechin, and epicatechin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

4.2.1 Effect of enzyme treatment on the starting material (Experiment I)

The first experiment was designed to study the effect of pre-treatment of selected enzymes on the soluble/insoluble-bound phenolic ratio and *in vitro* bioactivity. The sample (50 g) was freeze dried at -48 ºC and 30 x 10^{-3} mbar (Freezone 6, model 77530, Labconco Co., Kansas City, MO), ground with a coffee bean grinder (Model CBG5 series, Black & Decker, Canada Inc., Brockville, ON, Canada) and the powder was passed through a mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH) sieve. The powder so obtained was defatted three times with hexane (solid/solvent, 1:5, w/v) using a Warring blender (Model 33BL73, Warring Products Division Dynamics Co. of America, New Hartford, CT). Defatted samples were recovered by vacuum filtration and stored at -20 ºC (DE CAMARGO et al., 2014a). Defatted samples (10 g) were suspended in 100 mL of Viscozyme solution (2% in 0.1 M phosphate buffer, pH 4) and stirred for 12 h at 37 ºC in a gyratory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) or 100 mL of Pronase solution (1 mg/mL in 0.1 M phosphate buffer, pH 8) and stirred for 1 h. Controls containing each respective buffer (devoid of enzyme) were prepared at the same time and under the same conditions. Treated samples and respective controls were freeze dried to obtain a dry powder and further used for extraction of soluble and insoluble-bound phenolics (within one week). The extraction of

soluble phenolics was carried out with 70% (v/v) acetone (2.5%, w/v) in a gyratory water bath shaker at 30 ºC for 20 min. After centrifugation at 4000 x g (IEC Centra MP4, International Equipment Co., Needham Heights, MA), the upper layer was collected and the extraction was repeated twice. The combined supernatants were evaporated under vacuum at 40 ºC (Buchi, Flawil, Switzerland) to remove the organic solvent. The extract so obtained (soluble phenolics) was stored at -20 ºC until used for further analysis within three months. To the dry residue remaining after the extraction of soluble phenolics, 4M NaOH was added, and hydrolyzed, while stirring under nitrogen for 4 h at room temperature (23-25 ºC). The resulting slurry was acidified to pH 2 with 6 M HCl. Phenolics released from their insoluble-bound form were then extracted with diethyl ether and ethyl acetate (1:1, v/v), and reconstituted in HPLC-grade methanol (DE CAMARGO et al., 2014a).

4.2.2 Effect of enzyme treatment on the residue remaining after extraction of soluble phenolics (Experiment II)

The second experiment was carried out to evaluate the effect of enzyme treatment on the yield and identity of phenolics remaining after extraction of its soluble counterpart. The effect of enzyme treatment on the *in vitro* bioactivity was also evaluated. In short, the extraction of soluble phenolics was carried out with 70% (v/v) acetone as described above, and only the dry residue remaining after this extraction was treated with Viscozyme or Pronase, using the aformentioned conditions. After enzyme treatment the resulting slurry was acidified to pH 2 with 6 M HCl. Phenolics released from their insoluble-bound form upon enzyme treatment were then extracted with diethyl ether and ethyl acetate (1:1, v/v), and reconstituted in HPLC-grade methanol. To compare results, an alkali hydrolysis was carried out as described above. Thus, three different extracts were obtained in this experiment (phenolics released from their insoluble-bound form upon Viscozyme, Pronase, and NaOH treatment).

4.2.3 Total phenolic content (TPC)

The TPC (SWAIN; HILLIS, 1959) was evaluated using the same procedure and equipment as described elsewhere (DE CAMARGO et al., 2014b). The results were expressed as milligram gallic acid equivalents (GAE) per gram of defatted samples.

4.2.4 HPLC-DAD-ESI-MS^n analysis

HPLC-DAD-ESI-MS^n analyses were conducted to investigate the effect of Viscozyme and Pronase treatments on the residue remaining after extraction of soluble phenolics. This

allowed for the positive, or tentative, identification and quantification of major phenolics as affected by each treatment. The extract obtained using alkali extraction was also evaluated. This approach was chosen to examine the effects of enzyme treatment on the fraction containing insoluble-bound phenolics and individual components present, lending further support to the findings from the first expriment. The analyses were performed using an Agilent 1100 system equipped with a G1311A quaternary pump, a G1379A degasser and a G1329A ALS automatic sampler, a G1130B ALS Therm, a G1316 Colcom column compartment, A G1315B diode array detector (DAD) and a system controller linked to Chem Station Data handling system (Agilent). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 μm, Merck, Darmstad, Germany). HPLC-ESI-MS^n analysis was carried out using an Agilent 1100 series capillary liquid chromatography/mass selective detector (LC/MSD) ion trap system in electrospray ionization (ESI) in the negative mode. The data were acquired and analyzed with an Agilent LC/MSD software. Details of the method have been published elsewhere (DE CAMARGO et al., 2014a).

4.2.5 ABTS radical cation scavenging activity

The ABTS assay (RE et al., 1999) was conducted using the method and equipment, as described elsewhere (DE CAMARGO et al., 2014b). The results were expressed as μmol of Trolox equivalents/g dry weight of defatted samples.

4.2.6 DPPH radical scavenging activity

The ability of phenolic extracts in scavenging DPPH radical was evaluated using a Bruker E-Scan electron paramagnetic resonance (EPR) spectrophotometer (Bruker E-Scan, Bruker Biospin Co., Billerica, MA). Experimental procedure and equipment parameters were the same as those described by de Camargo et al. (2014b). The results were expressed as μmol of Trolox equivalents/g dry weight of defatted samples.

4.2.7 Hydroxyl radical scavenging activity

Phenolic extracts were tested for their scavenging activity against hydroxyl radicals. The extracts were removed from their original solvent (methanol) under a stream of nitrogen and diluted in 75 mM phosphate buffer, pH 7.2 (AMBIGAIPALAN; AL-KHALIFA; SHAHIDI, 2015). A Bruker E-Scan electron paramagnetic resonance (EPR) spectrophotometer (Bruker E-Scan, Bruker Biospin Co., Billerica, MA) was used. The method details have already

been published (DE CAMARGO et al., 2014b) and the results were expressed as μmol of catechin equivalents/g dry weight of defatted samples.

4.2.8 Reducing power

The reducing power assay (OYAIZU, 1986) was carried out as described elsewhere (DE CAMARGO et al., 2014b). Trolox was used to prepare the standard curve and the results were expressed as μmol of Trolox equivalents/g dry weight of defatted samples.

4.2.9 Inhibition of alpha-glucosidase activity

The ability of phenolic extracts in inhibiting the activity of alpha-glucosidase was evaluated according to the method of Eom et al. (2012), using *p*-nitrophenyl β-D-glucopyranoside as a substrate. In this assay, alpha-glucosidase hydrolyzes *p*-nitrophenyl β-D-glucopyranoside to generate glucose and *p*-nitrophenol. The latter one is used for quantification purposes (chromogenic substance). Alpha-glucosidase solution (10 units/mL) was prepared in 0.1 M potassium phosphate buffer (pH 6.8). The enzyme solution (5 μL) was mixed with 10 μL of phenolic extracts (50 mg/mL), and an aliquot of 0.1 M potassium phosphate buffer was added (620 μL). The mixture was incubated at 37 ºC for 20 min and 10 mM *p*-nitrophenyl β-D-glucopyranoside (10 μL) was added to initiate the reaction, which was followed by incubation at 37 ºC for 20 min. The reaction was terminated by the addition of 1M sodium carbonate solution (650 μL). The absorbance was read at 410 nm using an Agilent diode array spectrophotometer (Agilent 8453, Palo Alto, CA). Blanks devoid of enzyme (added by phosphate buffer) were prepared for background corrections. The control consisted of all solutions but the phenolic extract. The percentage of inhibition activity was calculated using the following equation.

$$\text{Alpha-glucosidase inhibition (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

4.2.10 Inhibition of lipase activity

The inhibition of activity of phenolic extracts towards lipase was evaluated as described by Marrelli et al. (2012), using *p*-nitrophenyl octanoate (NPC) as a substrate which, in the presence of lipase, liberates *p*-nitrophenol and octanoic acid. As mentioned before, the former chromogenic substance is used for quantification purposes. Type II crude porcine pancreatic lipase was used at a concentration of 5 mg/mL. The substract (NPC) was prepared in dimethyl sulphoxide to achieve a concentration of 5 mM. Phenolic extracts (100 μL) were mixed with 4

mL of Tris–HCl buffer (pH 8.5) and enzyme solution (100 μL). After incubation at 37 ºC for 25 min, NPC (100 μL) was added and incubated again at 37 ºC for 25 min. The absorbance was read at 412 nm using an Agilent diode array spectrophotometer (Agilent 8453). Blanks, devoid of enzyme (added by Tris–HCl buffer), were prepared for background corrections. The control consisted of all solutions but the phenolic extract. The percentage of inhibition of activity was calculated using the following equation.

$$\text{Lipase inhibition (\%)} = [(Abs_{control} - Abs_{sample})/(Abs_{control})] \times 100$$

4.2.11 Statistical analysis

Unless otherwise stated, the statistical analysis was randomized with three replications, and the results were analyzed using ANOVA and Tukey's test ($p < 0.05$) and SPSS statistics 21 for Windows (SPSS Inc., Chicago, IL). The correlation analyses ($p < 0.05$) were carried out using the same software.

4.3 Results and Discussion

4.3.1 Effect of enzyme treatment on the starting material (experiment I)

4.3.1.1 Total phenolic content (TPC)

The TPC was found to positively correlate with the inhibition of copper-induced human LDL-cholesterol oxidation and by both hydroxyl and peroxyl radical-induced DNA strand breakage (DE CAMARGO et al., 2014a; AYOUB; DE CAMARGO; SHAHIDI, 2016). Thus, TPC may reflect the ability of food phenolics to prevent atherosclerosis, associated cardiovascular diseases and certain types of cancer. The TPC of samples (starting material) subjected to Pronase (Table 4.1) and Viscozyme (Table 4.2) treatment demonstrated that both enzymes affected the content of soluble and insoluble-bound phenolics, which was noted by an increase in the ratio of soluble to insoluble-bound phenolics. Soluble phenolics can be readily absorbed, whereas insoluble-bound phenolics remain available for microbial fermentation in the lower gut (CHANDRASEKARA; SHAHIDI, 2012). Thus, the increase of soluble phenolics may have a practical impact on the role of phenolics in the human body.

Table 4.1 - Effect of pre-treatment with Pronase on the total phenolic content, antioxidant activity, and reducing power of soluble (S) and insoluble-bound (IB) phenolics of winemaking by-products

	Soluble (S)	Insoluble-bound (IB)	Ratio (S/IB)
Total phenolic content (mg GAE/g DW)			
Control*	265.6 ± 8.75Ba	257.9 ± 3.25Aa	0.88 ± 0.08B
Pronase	301.9 ± 21.4Aa	204.1 ± 0.27Bb	1.26 ± 0.02A
ABTS radical cation scavenging activity (μmol TE/g DW)			
Control	512.2 ± 17.2Bb	784.9 ± 8.42Aa	0.65 ± 0.03B
Pronase	699.9 ± 8.43Aa	621.4 ± 24.9Bb	1.13 ± 0.06A
DPPH radical scavenging activity (μmol TE/g DW)			
Control	557.6 ± 3.83Bb	592.6 ± 5.11Aa	0.94 ± 0.01B
Pronase	728.4 ± 30.5Aa	558.2 ± 2.32Bb	1.30 ± 0.05A
Hydroxyl radical cation scavenging activity (μmol CE/g DW)			
Control	275.7 ± 0.91Bb	323.7 ± 3.82Aa	0.85 ± 0.01B
Pronase	319.9 ± 5.39Aa	241.5 ± 18.8Bb	1.33 ± 0.08A
Reducing power (μmol TE/g DW)			
Control	196.1 ± 4.44Bb	545.9 ± 9.34Aa	0.36 ± 0.01B
Pronase	250.4 ± 1.97Ab	464.0 ± 5.50Ba	0.54 ± 0.00A

* Control samples were treated with buffer pH 8 under the same conditions as those treated with Pronase. Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different capital letters within a column part show difference between control and enzyme treated samples ($p < 0.05$). Means followed by different small letters within a row show difference between soluble and insoluble-bound fractions ($p < 0.05$). GAE, gallic acid equivalents; CE, catechin equivalents; TE, Trolox equivalents; and DW, dry weight of defatted sample

Table 4.2 - Effect of pre-treatment with Viscozyme on the total phenolic content, antioxidant activity, and reducing power of soluble (S) and insoluble-bound (IB) phenolics of winemaking by-products

	Soluble (S)	Insoluble-bound (IB)	Ratio (S/IB)
Total phenolic content (mg GAE/g DW)			
Control*	322.7 ± 6.79Ba	256.0 ± 5.69Ab	0.87 ± 0.02B
Viscozyme	371.0 ± 7.81Aa	184.6 ± 7.88Bb	1.39 ± 0.03A
ABTS radical cation scavenging activity (μmol TE/g DW)			
Control	463.3 ± 0.85Bb	683.8 ± 2.99Aa	0.67 ± 0.01B
Viscozyme	695.0 ± 10.5Aa	509.6 ± 2.75Bb	1.34 ± 0.01A
DPPH radical scavenging activity (μmol TE/g DW)			
Control	531.6 ± 6.87Bb	797.4 ± 20.7Aa	0.82 ± 0.05B
Viscozyme	652.2 ± 30.0Aa	442.7 ± 15.1Bb	1.80 ± 0.02A
Hydroxyl radical cation scavenging activity (μmol CE/g DW)			
Control	284.1 ± 15.4Ba	227.1 ± 3.49Ab	1.25 ± 0.05B
Viscozyme	414.4 ± 8.30Aa	163.4 ± 1.22Bb	2.54 ± 0.03A
Reducing power (μmol TE/g DW)			
Control	189.6 ± 1.71Bb	292.2 ± 35.4Ab	0.32 ± 0.02B
Viscozyme	595.3 ± 35.4Aa	471.5 ± 4.61Ba	0.62 ± 0.01A

* Control samples were treated with buffer pH 4 under the same conditions as those treated with Viscozyme. Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different capital letters within a column part show difference between control and enzyme treated samples ($p < 0.05$). Means followed by different small letters within a row show difference between soluble and insoluble-bound fractions ($p < 0.05$). GAE, gallic acid equivalents; CE, catechin equivalents; TE, Trolox equivalents; and DW, dry weight of defatted sample

4.3.1.2 Antiradical activity and reducing power

Pronase and Viscozyme may serve as alternatives for extracting phenolic antioxidants from different sources (MONTELLA et al., 2013; PAPILLO et al., 2014). The presence, number and configuration of the hydroxyl groups may have an important effect in biochemical reactions such as those involving single electron transfer and hydrogen atom transfer, which

are related to the ability of phenolic compounds in deactivating reactive oxygen species (ROS) and/or metal ions (DE CAMARGO et al., 2014a; AYOUB; DE CAMARGO; SHAHIDI, 2016). Different assays might render different results, which stem from the mechanisms by which they are operate. Therefore, besides TPC, the antioxidant activity was evaluated using ABTS, DPPH assays, and antiradical activity against hydroxyl radicals in the present study. Furthermore, the reducing power was also evaluated. The effect of Pronase and Viscozyme pre-treatment is shown in Table 4.1 and Table 4.2, respectively. Phenolic extracts obtained from the samples treated with selected enzymes demonstrated activity using all methods but, most importantly, the results lend support to the change of the ratio noted between the fraction containing soluble and insoluble-bound phenolics, which increased upon enzyme treatment of the starting material. A representative demonstration of the effect is shown in Figure 4.1, as evaluated by the ability of the phenolics extracted from the control (devoid of enzyme) and the starting material pre-treated with Pronase in scavenging hydroxyl radicals. The same trend was observed when the starting material was treated with Viscozyme.

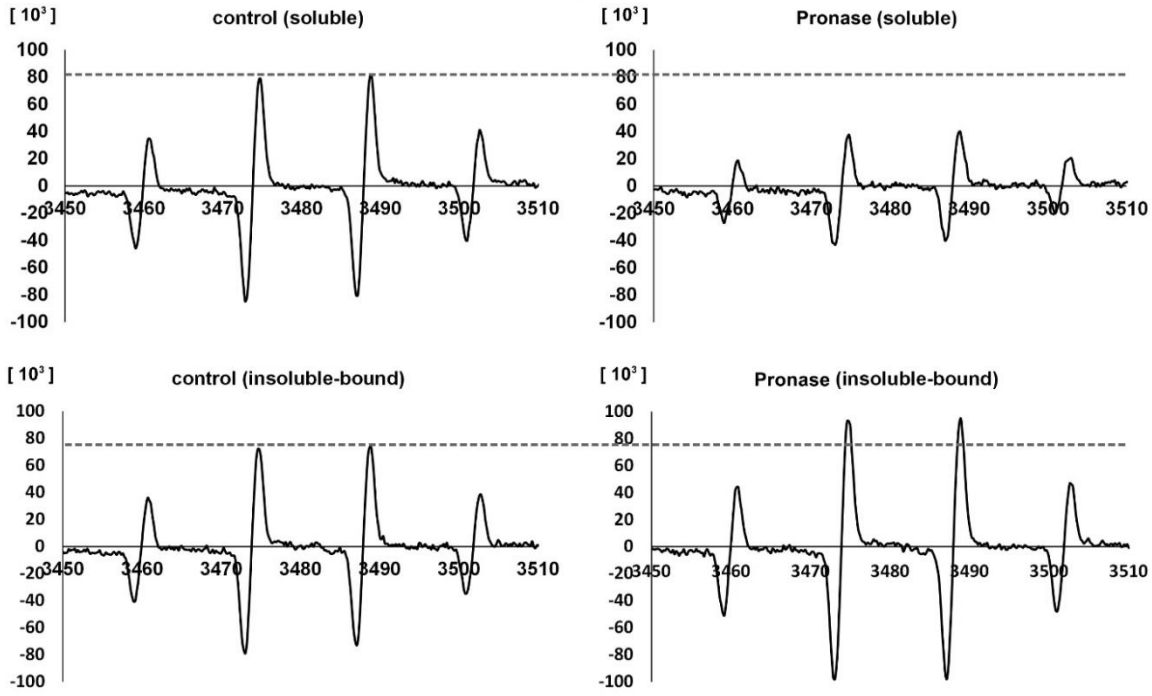


Figure 4.1 - Electron paramagnetic resonance (EPR) signals of phenolics as affected by Pronase pre-treatment. The higher the EPR signal, the lower the scavenging activity as demonstrated by the content of DMPO-OH adducts

The higher the EPR signal, the lower the scavenging activity. Hydroxyl radicals are unstable and highly ROS, they can cause DNA damage, lipid and protein oxidation. Furthermore, these ROS are related to cell damage, cancer development, inflammation and

heart disease. The results obtained with DPPH, ABTS, and reducing power methods have been shown to correlate with the concentration of specific phenolics found in grapes such as catechin, quercetin, epicatechin gallate, kaempferol hexoside, and procyanidin dimer B (DE CAMARGO et al., 2014a). Gallic and caffeic acid as well as catechin, epicatechin epicatechin gallate, and procyanidin trimer C were positively correlated with the scavenging activity towards hydrogen peroxide (DE CAMARGO et al., 2014a). Caffeic acid, quercetin, and procyanidin dimer B correlated with ORAC assay results (AYOUB; DE CAMARGO; SHAHIDI, 2016), which demonstrates the ability of phenolic extracts in scavenging peroxyl radicals. Therefore, the change in the antioxidant activity and reducing power found between soluble and insoluble-bound phenolics may be explained not only on the basis of TPC, but may also be related to specific phenolics extracted upon pre-treatment with Pronase and Viscozyme.

4.3.1.3 Inhibition of alpha-glucosidase and lipase activities

Different medicines are available to manage diabetes and obesity; however, their use may result in a wide range of side effects. Furthermore, in some countries, like Brazil, medications such as anti-hyperglycaemics are provided by the government, which may become a national economic burden. Phenolic compounds have shown inhibitory activity towards alpha-glucosidase and lipase which are key enzymes regulating the absorption of glucose and triacylglycerol, respectively, in the small intestine (ZHANG et al., 2015). Thus, the inhibition of both these enzymes was used as a biological model system in the present study.

The inhibition of phenolic extracts towards alpha-glucosidase and lipase activities is shown in Figure 4.2 A-D. The inhibition percentage of soluble phenolics against alpha-glucosidase activity increased from 75.6 ± 2.5 to 93.7 ± 0.5 in samples treated with Pronase and from 84.5 ± 0.5 to 96.5 ± 2.9 in samples treated with Viscozyme. A concurrent decreasing trend was noted in the ability of insoluble-bound phenolics towards alpha-glucosidase. As mentioned before, alpha-glucosidase plays an important role in the absorption of glucose; therefore, the increase in the extraction of soluble-phenolics, which are readily absorbed in the small intestine, may have a positive impact on the prevention and/or management of type 2 diabetes.

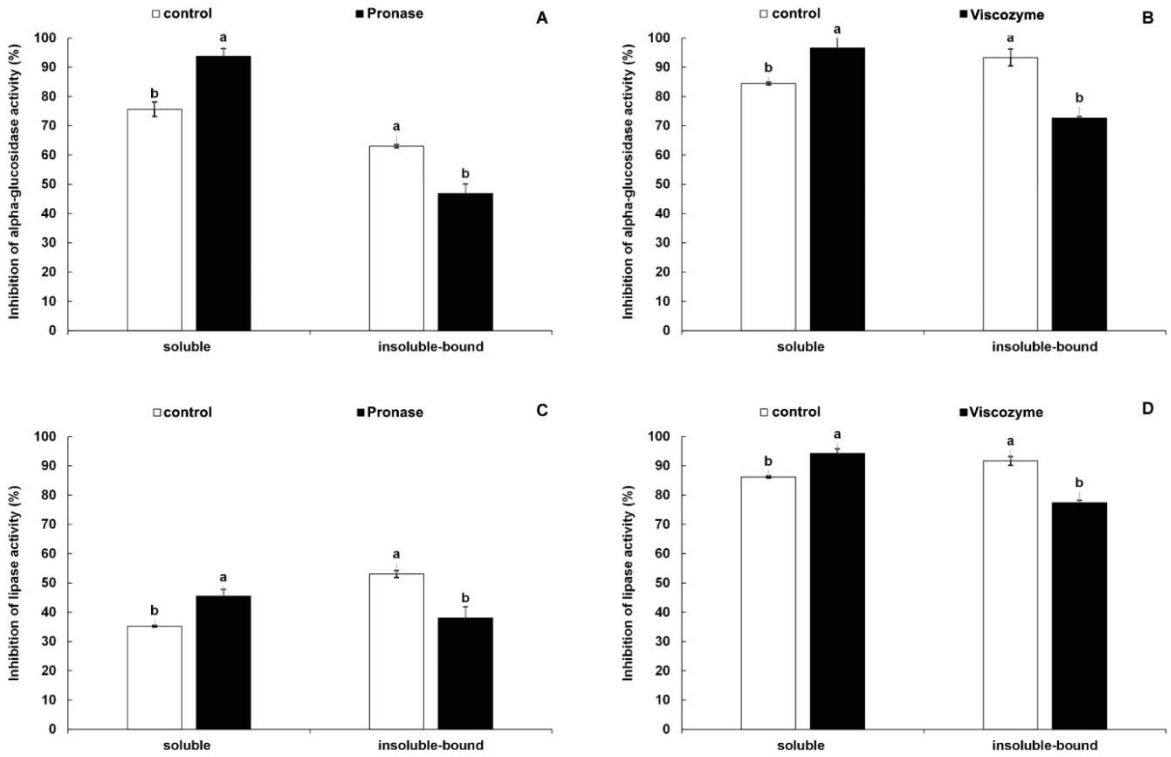


Figure 4.2 - Effect of pre-treatment with Pronase (A) and Viscozyme (B) on the inhibition of alpha-glucosidase activity. Effect of pre-treatment with Pronase (C) and Viscozyme (D) on the inhibition of lipase activity. Means with different letters within each fraction show difference between control or enzyme treatment ($p < 0.05$). The inhibition of alpha-glucosidase and lipase activities was carried out with phenolic extracts at 50 mg/mL of defatted sample

In accordance with the results found about the inhibition of alpha-glucosidase, an increase in the percentage of inhibition of lipase activity from 35.2 ± 0.2 to 45.5 ± 1.2 was observed in samples pre-treated with Pronase and from 86.2 ± 0.3 to 94.3 ± 1.5 upon Viscozyme pre-treatment. Obesity is associated with metabolic syndromes and heart disease, thus reducing life quality and expectancy. Pancreatic lipase is responsible for the hydrolysis of triacylglycerols, generating glycerol and fatty acids (ZHANG et al., 2015). Since lipase activity is related to the absorption of triacylglycerols (BADMAEV et al., 2015; ZHANG et al., 2015), the increase in the inhibitory activity of the fraction containing soluble phenolics upon Pronase and Viscozyme pre-treatment may be important in the management of body weight and/or prevention of obesity its respective associated diseases.

4.3.2. Effect of enzyme treatment on the residue remaining after extraction of soluble phenolics (experiment II)

4.3.2.1 Total phenolic content

The effect of enzyme treatment and alkali hydrolysis on the residue remaining after extraction of soluble phenolics is shown in Table 4.3.

Table 4.3 - Total phenolic content, antioxidant activity, reducing power, and enzyme inhibitory activity of insoluble-bound phenolics of winemaking by-products upon extraction using enzymatic or alkali hydrolysis

Pronase	Viscozyme	NaOH
Total phenolic content (mg GAE/g DW)		
13.87 ± 0.08Cb	24.70 ± 0.14Ba	237.7 ± 4.53A
ABTS radical cation scavenging activity (μmol TE/g DW)		
21.94 ± 0.30Bb	33.75 ± 0.58Ba	655.8 ± 20.4A
DPPH radical scavenging activity (μmol TE/g DW)		
25.54 ± 0.65Cb	56.96 ± 2.72Ba	516.9 ± 10.0A
Hydroxyl radical cation scavenging activity (μmol CE/g DW)		
18.27 ± 0.20Bb	49.05 ± 1.81Ba	337.8 ± 29.8A
Reducing power (μmol TE/g DW)		
11.96 ± 0.26Bb	17.71 ± 0.35Ba	562.5 ± 15.4A
Inhibition of alpha-glucosidase activity (%)		
7.35 ± 3.1Ba	7.79 ± 0.6Ba	70.7 ± 3.6A
Inhibition of lipase activity (%)		
9.71 ± 0.2Cb	24.9 ± 0.9Ba	90.1 ± 4.2A

Data represent mean values for each sample ± standard deviation (n = 3). Means followed by different capital letters within a row show difference among enzyme (Pronase or Viscozyme) and alkali treated samples ($p < 0.05$). Means followed by different small letters within a row show difference between Pronase and Viscozyme treated samples ($p < 0.05$). Abbreviations are: GAE, gallic acid equivalents; CE, catechin equivalents; TE, Trolox equivalents; and DW, dry weight of defatted sample. The inhibition of alpha-glucosidase and lipase activity was carried out with phenolic extracts at 50 mg/mL of defatted sample.

Both enzymes were able to release phenolics from their insoluble-bound form, which is helpful in explaining the change in the ratio of soluble to insoluble-bound phenolics as was demonstrated in the first experiment. Viscozyme treatment rendered a higher extraction when compared to Pronase, but lower than those found in the sample hydrolyzed with NaOH. This lends support to the results found in the first experiment that showed an increase in the extraction yield of soluble phenolics but the efficiency was not 100%. In terms of biological application this means that regardless of the treatment (in the starting material or in the residue remaining after extraction of soluble phenolics), some phenolics remain in the insoluble-bound phenolics fraction and will be bioaccessible for microbial action in the colon (CHANDRASEKARA; SHAHIDI, 2012).

4.3.2.2 Identification and quantification of phenolic compounds

The data presented thus far demonstrated the ability of Pronase and Viscozyme in improving the extraction of phenolics and potential positive effect in terms of health benefits. Winemaking by-products serve as good sources of phenolic compounds such as phenolic acids, monomeric flavonoids and proanthocyanidins (DE CAMARGO et al., 2014a). In the present study, the major phenolic compounds were tentatively or positively identified and quantified in the samples in the second experiment in order to explain the effects of enzyme treatment on a molecular level (Table 4.4). *p*-Coumaric, gallic, and caffeic acids, as well as catechin and epicatechin, were positively identified by comparison of their retention times, ion fragmentation patterns with authentic standards, and previous studies (DE CAMARGO et al., 2014a, 2015; ALSHIKH; DE CAMARGO; SHAHIDI, 2015; AYOUB; DE CAMARGO; SHAHIDI, 2016). Isorhamnetin was tentatively identified due to its deprotonated molecular ion at 315 m/z and 271 m/z in MS^2 (DE CAMARGO et al., 2014a). Procyanidin dimer B, $[M - H]^-$ at 577 m/z, gave product ions at 451, 425, and 289 in MS^2 (DE CAMARGO et al., 2014) and prodelphinidin dimer B showed m/z 591 in MS and charateristic ions at MS^2, which matched the literature results (HAMED et al., 2014). *p*-Coumaric and caffeic acids, as well as procyanidin dimer B were not found in samples upon Pronase treatment and only traces of epicatechin and isorhamnetin were detected. Gallic acid is one of the major phenolic acids found in winemaking by-products (DE CAMARGO et al., 2014). The treatment with Viscozyme rendered twice the concentration of gallic acid when compared to Pronase, whereas the content of catechin was 14 times higher in samples extracted with Viscozyme as compared to Pronase. Furthermore, the efficiency of Viscozyme in extracting prodelphinidin dimer A was 58% when compared with the chemical extraction (NaOH). As mentioned before, insoluble-bound phenolics are linked to cell wall carbohydrates and proteins. The absence of *p*-coumaric and caffeic acids, as well as procyanidin dimer B, in extracts obtained upon Pronase treatment, and the presence of such compounds in extracts obtained with Viscozyme, suggest that these compounds may be linked to carbohydrates rather than to proteins.

Table 4.4 - The contents of insoluble-bound phenolics (μg/g DW) of winemaking by-products as affected by different extraction procedures

Identification	Pronase	Viscozyme	NaOH
p-coumaric acid	nd	14.68 ± 0.69b	83.28 ± 6.19a
gallic acid	36.16 ± 1.78c	71.68 ± 4.85b	180.4 ± 0.01a
caffeic acid	nd	18.49 ± 1.14b	308.4 ± 22.7a
catechin	16.01 ± 0.31c	226.3 ± 27.7b	494.2 ± 0.04a
epicatechin	tr	106.3 ± 7.15b	141.9 ± 13.3a
isorhamnetin	tr	241.4 ± 63.9b	448.2 ± 19.7a
procyanidin dimer B	nd	369.6 ± 26.0b	7282 ± 580a
prodelphinidin dimer A	142.5 ± 12.9c	453.0 ± 18.8b	784.8 ± 29.1a

Data represent mean values for each sample ± standard deviation (n = 2). Means followed by different letters within a row show difference among enzyme (Pronase or Viscozyme) and alkali treated samples ($p < 0.05$). Abbreviations are: nd, not detected; and tr, trace

p-Coumaric acid exhibited *in vitro* and *in vivo* antiplatelet activity (LUCERI et al., 2007). Aflatoxins, which can be found in food products such as peanuts and even milk (DE CAMARGO et al., 2012b; SANTILI et al., 2015) can cause hepatotoxicity. Caffeic acid phenethyl ester, a caffeic acid derivative, was able to protect AFB1-induced hepatotoxicity in rats (AKÇAM et al., 2013). Rich sources of proanthocyanidins and simple phenolics have been used to fortify food products and increase the ingestion of dietary phenolics (DE CAMARGO et al., 2014b; MA et al., 2014; ZHANG; CHEN; WANG, 2014). Proanthocyanidins are among the major components of insoluble-bound phenolics in winemaking by-products (DE CAMARGO et al., 2014b). However, only soluble proanthocyanidins are bioaccessible in the small intestine. Upon microbial action, insoluble-bound proanthocyanidins may be catabolized in the colon, but this depends on the degree of polymerization of such compounds. Furthermore, it has been suggested that the bioavailability of proanthocyanidins decreases with increasing degree of polymerization (DE CAMARGO et al., 2015). The presence of procyanidin B1 has been detected in human serum, which has been attributed to consumption of grape seed extract (SANO et al., 2003). Thus, the presence of *p*-coumaric and caffeic acids, as well as procyanidin dimer B, which were not found in extracts obtained upon Pronase treatment, may have health benefits. Furthermore, the present study demonstrates that not only the identity, but also the quantity of specific phenolics are dependent on the enzyme used. The effect of such differences on the *in vitro* bioactivity will be discussed in the following sections.

4.3.2.3 Antiradical activity and reducing power

The antioxidant activity and reducing power of extracts obtained upon enzyme or alkali treatment are shown in Table 4.3. As demonstrated with TPC, the activity was in the order of extracts obtained with NaOH > Viscozyme > Pronase. Phenolics extracted with Viscozyme showed up to 2.7 higher antioxidant activity, which was found against hydroxyl radicals, as

compared with phenolics extracted with Pronase. Hydroxyl radicals are generated in the presence of ferrous ions and hydrogen peroxide. The ability of phenolic compounds in scavenging hydroxyl radicals may be due to chelation of ferrous ion (WETTASINGHE; SHAHIDI, 2000), but electron donation and eventual neutralization of hydrogen peroxide generating water as the final product is also contemplated. In the present study, the ability of phenolic extracts in scavenging hydroxyl radicals was studied, but the capacity of phenolic extracts in scavenging hydrogen peroxide (DE CAMARGO et al., 2014) and their chelating capacity (AYOUB; DE CAMARGO; SHAHIDI, 2016) has already been demonstrated; therefore, phenolic extracts obtained here may also be efficient in scavenging hydrogen peroxide or chelating ferrous ions, thus stopping/preventing the Fenton reaction by reacting with its reagents (hydrogen peroxide and ferrous ions), or products (hydroxyl radicals).

Although extensive studies have been conducted with grapes, their products and by-products (WEIDNER et al., 2012, 2013; DE CAMARGO et al., 2014; DENNY et al., 2014; TAO et al., 2016), a direct comparison with the literature is not possible. Extraction conditions such as solvent type and concentration, solid to solvent ratio, pH and temperature may not only render different yields of phenolic extracts but also different compounds may be extracted under specific conditions (DE CAMARGO et al., 2014; AYOUB; DE CAMARGO; SHAHIDI, 2016). Thus, in addition to the already mentioned factors, the present study demonstrated that the enzyme used for phenolic extraction should also be taken into consideration.

4.3.2.4 Inhibition of alpha-glucosidase and lipase activities

The inhibitory activity of phenolics extracts as affected by enzymatic (Pronase or Viscozyme) and alkali extraction is shown in Table 4.3. Although lower when compared with phenolic extracts obtained with alkali extraction, Pronase and Viscozyme rendered extracts with improved capacity in inhibiting alpha-glucosidase and lipase activities. Phenolics extracted with Pronase inhibited alpha-glucosidase and lipase by 7.35 ± 3.1 and 9.71 ± 0.2%, respectively, whereas the respective inhibition of phenolics extracted with Viscozyme were 7.79 ± 0.6 and 24.9 ± 0.9%. Phenolic compounds are able to form complexes with proteins and this may be due to the formation of hydrogen bonds (DE TOLEDO et al., 2013) or addition of nucleophiles to oxidized quinones (KALYANARAMAN; PREMOVIC; SEALY, 1987). The correlation of TPC with antioxidant activity and reducing power of different feedstock has been well documented (AUGUSTO et al., 2014; DE CAMARGO et al., 2014a; DA SILVA et al., 2015; AYOUB; DE CAMARGO; SHAHIDI, 2016). In the present study, positive correlations existed between TPC and the inhibition of alpha-glucosidase ($r = 0.9389$, $p < 0.001$) and lipase activity

(r = 0.7300, p = 0.017), showing that not only the identity but also the concentration of phenolics from different sources should be taken into account.

4.4 Conclusion

The enzyme treatment of the winemaking by-products demonstrated that both Pronase and Viscozyme increased the amount of soluble phenolics while decreasing the content of insoluble-bound phenolics, with a concurrent increase in the ratio of soluble to insoluble-bound phenolics. ABTS radical cation, DPPH radical, and hydroxyl radical scavenging activities, reducing power, and inhibition of alpha-glucosidase and lipase activities were also increased. In addition, treatment of the fraction containing only the insoluble-bound phenolics, supported the data obtained in the first set of experiments and also demonstrated that specific molecules such as *p*-coumaric, gallic and caffeic acids, as well as catechin, epicatechin, isorhamnetin, procyanidin dimer B, and prodelphinidin dimer A were better extracted upon treatment with Viscozyme as compared to Pronase. Furthermore, the same trend was observed in the remaining methods used. The present work indicates that enzyme-assisted extraction should be further exploited for the development of functional food ingredients and or nutraceuticals. The antioxidant activity of food phenolics and their potential role in the prevention of several ailments have been well substantiated. Additionally, the positive effects by inhibiting the activity of alpha-glucosidase and lipase, which are related to prevention and/or management of diabetes and obesity, respectively, were demonstrated here.

References

AKÇAM, M.; ARTAN, R.; YILMAZ, A.; OZDEM, S.; GELEN, T.; NAZIROĞLU, M. Caffeic acid phenethyl ester modulates aflatoxin B1-induced hepatotoxicity in rats. **Cell Biochemistry and Function,** Chichester, v. 31, p. 692-697, 2013.

ALASALVAR, C.; BOLLING, B.W. Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects. **British Journal of Nutrition,** Cambridge, v. 113, p. S68-S78, 2015.

ALSHIKH, N.; DE CAMARGO, A.C.; SHAHIDI, F. Phenolics of selected lentil cultivars: Antioxidant activities and inhibition of low-density lipoprotein and DNA damage. **Journal of Functional Foods,** Amsterdam, v. 18, p. 1022-1038, 2015.

AMBIGAIPALAN, P.; AL-KHALIFA, A.S.; SHAHIDI, F. Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. **Journal of Functional Foods,** Amsterdam, v. 18, p. 1125-1137, 2015.

AUGUSTO, T.R.; SALINAS, E.S.S.; ALENCAR, S.M.; D'ARCE, M.A.B.R.; DE CAMARGO, A.C.; VIEIRA, T.M.F.S. Phenolic compounds and antioxidant activity of hydroalcoholic extracts of wild and cultivated murtilla (*Ugni molinae* Turcz.). **Food Science and Technology,** Campinas, v. 34, p. 667-679, 2014.

AYOUB, M.; DE CAMARGO, A.C.; SHAHIDI, F. Antioxidants and bioactivities of free, esterified and insoluble-bound phenolics from berry seed meals. **Food Chemistry,** London, v. 197, p. 221-232, 2016.

BADMAEV, V.; HATAKEYAMA, Y.; YAMAZAKI, N.; NORO, A.; MOHAMED, F.; HO, C.-T.; PAN, M.-H. Preclinical and clinical effects of Coleus *forskohlii, Salac*i*a reticulata* and *S*esamum indicum *modifying pancr*eatic lipase inhibition *in vitro* and reducing total body fat. **Journal of Functional Foods,** Amsterdam, v. 15, p. 44-51, 2015.

CHANDRASEKARA, A.; SHAHIDI, F. Bioaccessibility and antioxidant potential of millet grain phenolics as affected by simulated *in vitro* digestion and microbial fermentation. **Journal of Functional Foods,** Amsterdam, v. 4, p. 226-237, 2012.

CHENG, V.J.; BEKHIT, A.E.A.; MCCONNELL, M.; MROS, S.; ZHAO, J. Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. **Food Chemistry,** London, v. 134, p. 474-482, 2012.

DA SILVA, J.K.; CAZARIN, C.B.B.; CORREA, L.C.; BATISTA, Â.G.; FURLAN, C.P.B.; BIASOTO, A.C.T.; PEREIRA, G.E.; DE CAMARGO, A.C.; MARÓSTICA JUNIOR, M.R. Bioactive compounds of juices from two Brazilian grape cultivars. **Journal of the Science of Food and Agriculture**, London, v. 96, p. 1990-1996, 2016.

DA SILVA, P.P.M.; CASEMIRO, R.C.; ZILLO, R.R.; DE CAMARGO, A.C.; PROSPERO, E.T.P.; SPOTO, M.H.F. Sensory descriptive quantitative analysis of unpasteurized and pasteurized juçara pulp (*Euterpe edulis*) during long-term storage. **Food Science & Nutrition,** Malden v. 2, p. 321-331, 2014.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; BIASOTO, A.C.T.; SHAHIDI, F. Low molecular weight phenolics of grape juice and winemaking byproducts: antioxidant activities and inhibition of oxidation of human low-density lipoprotein cholesterol and DNA strand breakage. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 12159–12171, 2014a.

DE CAMARGO, A.C.; REGITANO-D'ARCE, M.A.B.; GALLO, C.R.; SHAHIDI, F. Gamma-irradiation induced changes in microbiological status, phenolic profile and antioxidant activity of peanut skin. **Journal of Functional Foods,** Amsterdam, v. 12, p. 129-143, 2015.

DE CAMARGO, A.C.; VIDAL, C.M.M.; CANNIATTI-BRAZACA, S.G.; SHAHIDI, F. Fortification of cookies with peanut skin: effects on the composition, polyphenols, antioxidant properties and sensory quality. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 11228-11235, 2014b.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; RASERA, G.B.; CANNIATTI-BRAZACA, S.G.; DE ALENCAR, S.M.; REGITANO-D'ARCE, M.A.B. Lower solvent concentration and time for extraction of peanut skin antioxidants at optimized conditions. In: COOK, R.W. (Ed.). **Peanuts:** production, nutritional content and health implications. New York: New Publ., 2014. p. 31-50.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; CANNIATTI-BRAZACA, S.G. Gamma radiation induced oxidation and tocopherols decrease in in-shell, peeled and blanched peanuts. **International Journal of Molecular Sciences,** Basel, v. 13, p. 2827-2845, 2012.

DE CAMARGO, A.C.; VIEIRA, T.M.F.S.; REGITANO-D'ARCE, M.A.B.; DE ALENCAR, S.M.; CALORI-DOMINGUES, M.A.; SPOTO, M.H.F.; CANNIATTI-BRAZACA, S.G. Gamma irradiation of in-shell and blanched peanuts protects against mycotoxic fungi and retains their nutraceutical components during long-term storage. **International Journal of Molecular Sciences,** Basel, v. 13, p. 10935-10958, 2012.

DE TOLEDO, N.M.V.; ROCHA, L.C.; DA SILVA, A.G.; BRAZACA, S.G.C. Interaction and digestibility of phaseolin/polyphenol in the common bean. **Food Chemistry,** London, v. 138, p. 776-780, 2013.

EOM, S.-H.; LEE, S.-H.; YOON, N.-Y.; JUNG, W.-K.; JEON, Y.-J.; KIM, S.-K.; LEE, M.-S.; KIM, Y.-M. α-Glucosidase and α-amylase inhibitory activities of phlorotannins from *Eisenia bicyclis*. **Journal of the Science of Food and Agriculture,** London, v. 92, p. 2084–2090, 2012.

HAMED, A.I.; AL-AYED, A.S.; MOLDOCH, J.; PIACENTE, S.; OLESZEK, W.; STOCHMAL, A. Profiles analysis of proanthocyanidins in the argun nut (*Medemia* argu an *ancient Egyptian palm*) by LC–ESI–MS/MS. **Journal of Mass Spectrometry,** Chichester, v. 49, p. 306-315, 2014.

KALYANARAMAN, B.; PREMOVIC, P.I.; SEALY, R.C. Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines: an ESR spin stabilization study. **Journal of Biological Chemistry,** Bethesda, v. 262, p. 11080-11087, 1987.

LUCERI, C.; GIANNINI, L.; LODOVICI, M.; ANTONUCCI, E.; ABBATE, R.; MASINI, E.; DOLARA, P. *p*-Coumaric acid, a common dietary phenol, inhibits platelet activity *in vitro* and *in vivo*. **British Journal of Nutrition,** Cambridge, v. 97, p. 458-463, 2007.

MA, Y.Y.; KERR, W.L.; SWANSON, R.B.; HARGROVE, J.L.; PEGG, R.B. Peanut skins-fortified peanut butters: effect of processing on the phenolics content, fibre content and antioxidant activity. **Food Chemistry,** London, v. 145, p. 883-891, 2014.

MARRELLI, M.; MENICHINI, F.; STATTI, G.A.; BONESI, M.; DUEZ, P.; MENICHINI, F.; CONFORTI, F. Changes in the phenolic and lipophilic composition, in the enzyme inhibition and antiproliferative activity of *Ficus carica* L. cultivar Dottato fruits during maturation. **Food and Chemical Toxicology,** Oxford, v. 50, p. 726-733, 2012.

MONTELLA, R.; COÏSSON, J.D.; TRAVAGLIA, F.; LOCATELLI, M.; MALFA, P.; MARTELLI, A.; ARLORIO, M. Bioactive compounds from hazelnut skin (*Corylus avellana* L.): dffects on *Lactobacillus plantarum* P17630 and *Lactobacillus crispatus* P17631. **Journal of Functional Foods,** Amsterdam, v. 5, p. 306-315, 2013.

OYAIZU, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. **The Japanese Journal of Nutrition and Dietetics,** Tokyo, v. 44, p. 307-315, 1986.

PAPILLO, V.A.; VITAGLIONE, P.; GRAZIANI, G.; GOKMEN, V.; FOGLIANO, V. Release of antioxidant capacity from five plant foods during a multistep enzymatic digestion protocol. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 4119-4126, 2014.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.; RICE-EVANS, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine,** New York, v. 26, p. 1231-1237, 1999.

SANO, A.; YAMAKOSHI, J.; TOKUTAKE, S.; TOBE, K.; KUBOTA, Y.; KIKUCHI, M. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. **Bioscience, Biotechnology, and Biochemistry,** Tokyo, v. 67, p. 1140-1143, 2003.

SANTILI, A.B.N.; DE CAMARGO, A.C.; NUNES, R.S.R.; GLORIA, E.M.; MACHADO, P.F.; CASSOLI, L.D.; DIAS, C.T.S.; CALORI-DOMINGUES, M.A. Aflatoxin M1 in raw milk from different regions of São Paulo state – Brazil. **Food Additives & Contaminants: Part B,** London, v. 8, p. 1-8, 2015.

SHAHIDI, F.; AMBIGAIPALAN, P. Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects – a review. **Journal of Functional Foods,** Amsterdam, v. 18, p. 820–897, 2015.

SWAIN, T.; HILLIS, W.E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. **Journal of the Science of Food and Agriculture,** London, v. 10, p. 63-68, 1959.

TAO, Y.; SUN, D.W.; GÓRECKI, A.; BŁASZCZAK, W.; LAMPARSKI, G.; AMAROWICZ, R.; FORNAL, J.; JELIŃSKI, T. A preliminary study about the influence of high hydrostatic pressure processing in parallel with oak chip maceration on the physicochemical and sensory properties of a young red wine. **Food Chemistry,** London, v. 194, p. 545-554, 2016.

VICENTE, S.J.V.; ISHIMOTO, E.Y.; TORRES, E.A.F.S. Coffee modulates transcription factor Nrf2 and highly increases the activity of antioxidant enzymes in rats. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 116-122, 2014.

WEIDNER, S.; POWAŁKA, A.; KARAMAĆ, M.; AMAROWICZ, R. Extracts of phenolic compounds from seeds of three wild grapevines: comparison of their antioxidant activities and the content of phenolic compounds. **International Journal of Molecular Sciences,** Basel, v. 13, p. 3444-3457, 2012.

WEIDNER, S.; RYBARCZYK, A.; KARAMAĆ, M.; KRÓL, A.; MOSTEK, A.; GRĘBOSZ, J.; AMAROWICZ, R. Differences in the phenolic composition and antioxidant properties between *Vitis coignetiae* and *Vitis vinifera* seeds extracts. **Molecules,** Basel, v. 18, p. 3410-3426, 2013.

WETTASINGHE, M.; SHAHIDI, F. Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. **Food Chemistry,** London, v. 70, p. 17-26, 2000.

YEO, J.; SHAHIDI, F. Critical evaluation of changes in the ratio of insoluble bound to soluble phenolics on antioxidant activity of lentils during germination. **Journal of Agricultural and Food Chemistry**, Easton, v. 63, p. 379-381, 2015.

ZHANG, B.; DENG, Z.; RAMDATH, D. D.; TANG, Y.; CHEN, P. X.; LIU, R.; LIU, Q.; TSAO, R. Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase. **Food Chemistry,** London, v. 172, p. 862-872, 2015.

ZHANG, X.; CHEN, F.; WANG, M. Antioxidant and antiglycation activity of selected dietary polyphenols in a cookie model. **Journal of Agricultural and Food Chemistry,** Easton, v. 62, p. 1643-1648, 2014.

5 GENERAL CONCLUSIONS

The initial microbiological status of peanut skin from two cultivars indicated that these by-products of the peanut industry should be subjected to a decontamination process. Gamma-irradiation diminished the microbiologial count of peanut skins; therefore, providing a safer product. Furthermore, this was the first study demonstrating how gamma-irradiation affected the phenolic profile of peanut skin, which included the fractions containing soluble (free and esterified) and insoluble-bound phenolics using HPLC-DAD-ESI-MS^n. It was possible to suggest that molecular conversion, depolymerization, and cross-linking were induced by the process. The fraction containing free phenolics were the major ones in two different cultivars of peanut skin. Gamma-irradiation increased the content of free phenolics, antioxidant activity, reducing power, as well as the inhibition capacity of *in vitro* human LDL-cholesterol oxidation and inhibition of DNA strand breakage of this fraction. Therefore offering a product with a greater potential to serve as a functional ingredient or food supplement. By-products of grape juice and winemaking industries were also evaluated for their microbiological status, which demonstrated it would not be necessary to use any decontamination method for such samples. The phenolic profile and bioactivities of grape by-products were also evaluated for their different fractions for the first time. Grape juice and winemaking by-products showed different phenolic distribution in the soluble fraction and it was possible to suggest a cluster for grape juice versus winemaking by-products. The first cluster (grape juice by-products) had higher free phenolics whereas the second one (winemaking by-products) showed higher esterified phenolic contents. Furthermore, all samples had the insoluble-bound fraction as the richest source of phenolics. The same trend was observed for their antioxidant activities, reducing power, inhibition of *in vitro* human LDL-cholesterol oxidation and inhibition of inhibition of DNA damage. Because of the major contribution of insoluble-bound phenolics from grape by-products two enzymes were tested in order to improve their extraction so that they could potentially become readily bioavailable. Pronase and Viscozime released insoluble-bound phenolics, but the latter was more efficient. Furthermore, different molecules such as procyanidin dimers B were extracted only with Viscozyme. The effect of enzyme-assisted extraction in the antiradical activity and inhibition of alpha-glucosidase and lipase demonstrated that this technology offers promise for further industrial exploitation. Therefore, the microbiological, chemical, and technological knowledge generated in this doctoral thesis makes a solid contribution for different steps to be considered for the full exploitation of these plant

food by-products as sources of phenolic compounds for potential application as antioxidants, and potentially for prevention of cardiovascular diseases, cancer, diabetes, and obesity.