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Gamma-irradiation induced changes in microbiological status, phenolic profile and antioxidant activity of peanut skin

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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.

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, Kuhn, Rojo, Lila, & Raskin, 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, Johnson, Reed, Tanko, & O'Keefe, 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

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capacity (de Camargo et al., 2012a,c), 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.

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 *E. 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′-azinobis(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 *E. 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).

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).

2.2. *Microbiological evaluation*

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.

2.2.2. Salmonella *spp*

The TECRA Unique *Salmonella* test described by the AOAC (2002) method 2000.07 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 25 g of representative samples of the product.

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.

2.2.4. *Coliform bacteria*

The SimPlate coliform and *E. coli* colour indicator (CEc-CI) medium was used for the detection and quantification of total coliform and *E. coli* according to the AOAC (2006) 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.

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*. The samples were incubated at 35–37 °C 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.

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.

2.2.7. *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 °C 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 °C for 20 min. After centrifugation at 4000 × *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 40 °C (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 (23–25 °C) 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 °C 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 4 M NaOH, (50 mL) and hydrolyzed while stirring under nitrogen for 4 hours at room temperature (23–25 °C). 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 40 °C, which was followed by suspension in HPLC-grade methanol, as explained above.

2.2.8. *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. (2014a,b). 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.

2.2.9. *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. (2014b). 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.

2.2.10. *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. (2014a,b). 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.

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.

2.2.11. *DPPH radical scavenging activity (DRSA)*

The DPPH assay was carried out using a modified version of the method explained (Chandrasekara & 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.

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.

2.2.12. *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 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.

2.2.13. *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.

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.

2.2.14. *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 °C for 20 min, after which 10% (w/v) trichloroacetic acid solution was added (2.5 mL). The mixture was centrifuged at 1750 g 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.

2.2.15. *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 °C 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 °C 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 °C 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 $CuSO_4$.

2.2.16. Supercoiled plasmid DNA strand breakage inhibition

The supercoiled plasmid DNA strand breakage inhibition was evaluated with minor modifications of the previously explained method (Shahidi et al., 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 (5 μL) 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 *E. 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) × 100].

2.2.17. 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; Ma et al., 2014; Monagas et al., 2009; Sarnoski et al., 2012; de Camargo et al., 2014a,b).

2.2.18. 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 (at $p < 0.01$ and $p < 0.05$) were carried out using the ASSISTAT 7.6 program.

3. Results and discussion

3.1. Microbiological evaluation

The effectiveness of gamma-irradiation in inhibiting microbial growth is shown in Table 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, Hickey, Alonso-Gomez, & Wilkinson, 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.

Table 1 – Gamma-irradiation effects on microbiological contamination of peanut skin.[a]

Microorganisms	IAC 886			IAC 505		
	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*	$<10^1$	$<10^1$	$<10^1$	6.0×10^1	$<10^1$	$<10^1$
Salmonella spp.	nd	nd	nd	nd	nd	nd

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

For such reason gamma-irradiation, which is currently used for microbial decontamination of herb and spices, may also be useful for decontaminating peanut skin.

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, Gerdes, and Hall (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 25 g quantities of all samples. Although the presence of *Salmonella* has been associated with raw meat, poultry, eggs, and seafood, a recent study (Calhoun, Post, Warren, Thompson, & Bontempo, 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, Ayari, Ferhi, Roussos, & Hamdi, 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.

3.2. *Total phenolic content (TPC)*

The total phenolic content (TPC) in gamma-irradiated peanut skin is presented in Table 2. 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

Table 2 – Total phenolic content (TPC), proanthocyanidin content (PC), antioxidant activities (AOA) and reducing power (RP) of gamma-irradiated peanut skin.[a]

Dose (kGy)	Free IAC 886	Esterified	Insoluble-bound	Free IAC 505	Esterified	Insoluble-bound
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.

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 (2011a) 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 ($p \leq 0.05$) 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 (Dixit et al., 2010; Harrison & Were, 2007; Mishra, Gautam, & Sharma, 2006; Perez, Calderon, & Croci, 2007), which may be related to existing differences in the dose of gamma-irradiation, extraction methods, and identity of individual phenolics present.

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 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, 2011b), 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.

3.4. *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 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, 2009a). 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 (2011b) 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 ($r = 0.889$; $p < 0.01$).

3.5. *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, Cert, Perez-Jimenez, Cert, & Saura-Calixto, 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 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 et al., 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.

3.6. *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 et al., 2007), and inactivating enzymes (Fernandes, Dringen, Lawen, & Robinson, 2011). Furthermore, hydrogen peroxide induces cell damage (Chen, Ye, Ji, & Liu, 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.

3.7. *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, Balasubramanian, Matsugo, & Saito, 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.

3.8. *Reducing power*

The reducing power (RP) of peanut skin samples is given in Table 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.

3.9. *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 (Fig. 1). 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.

3.10. *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 (Fig. 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 (Fig. 3), and acted

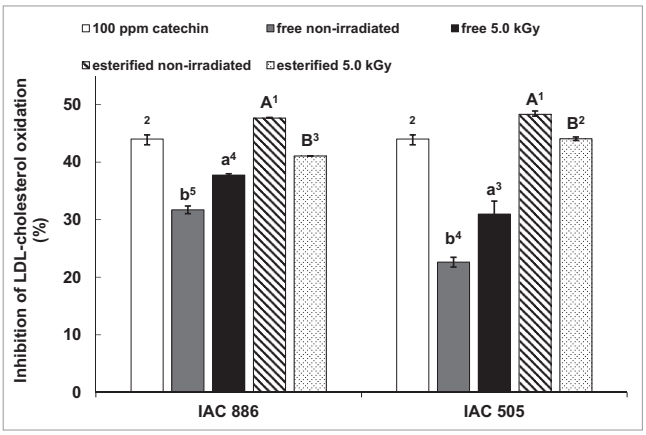

Fig. 1 – Gamma-irradiation effects on the biological activity of free and esterified phenolics against LDL-cholesterol induced oxidation. Data represent the mean ± standard deviation of each sample (n = 3). Means with different figures indicate significant differences ($p < 0.05$) compared to 100 ppm 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 ($p > 0.05$).

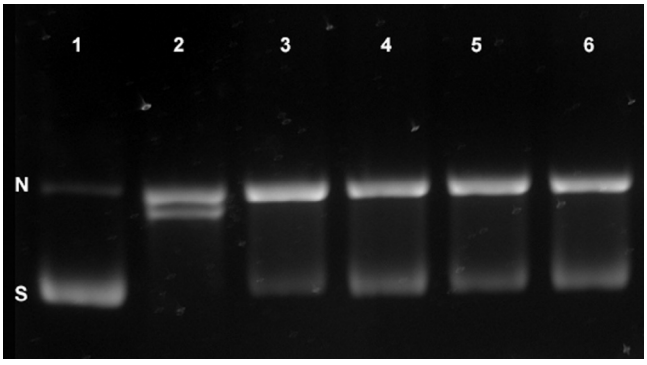

Fig. 2 – Supercoiled plasmid DNA strand breakage inhibition of the free phenolic fraction of non-irradiated and gamma-irradiated peanut skin. Lane 1: Control (DNA only); lane 2: DNA + H_2O_2 + $FeSO_4$; lane 3: free phenolics from non-irradiated peanut skin (IAC 886); lane 4: free phenolics from peanut 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.

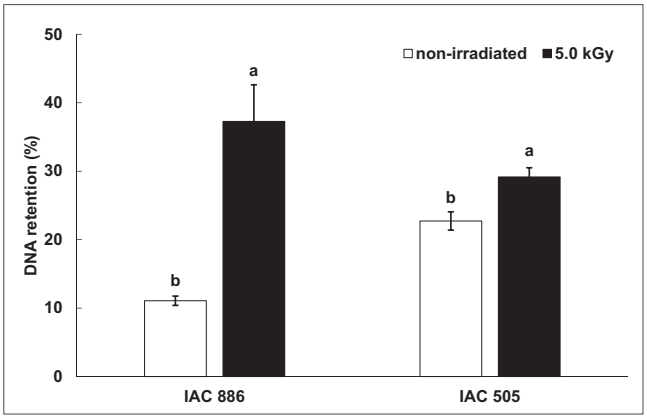

Fig. 3 – Supercoiled plasmid DNA strand breakage inhibition of free phenolic fraction from peanut skin. White and black bars represent non-irradiated and gamma-irradiated (5.0 kGy) samples, respectively. Data represent mean ± standard deviation of each sample (n = 3). Means with different lower case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

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.

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. 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 (Chandrasekara & Shahidi, 2011a; John & Shahidi, 2010), these compounds showed loss of CO_2, giving $[M\text{-}H\text{-}44]^-$ as their characteristic ions in MS^2. The deprotonated molecular ion $[M\text{-}H]^-$ 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 $[M\text{-}H\text{-}44]^-$. 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, tetramers and pentamers were tentatively identified using tandem mass spectrometry (MS^n), UV spectral, and literature data (Appeldoorn et al., 2009; Ma et al., 2014; Monagas et al., 2009; Sarnoski et al., 2012; de Camargo et al., 2014a,b). Proanthocyanidins were quantified as (+)-catechin equivalents. Identification and quantification data are presented in Tables 3 and 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; Ma et al., 2014; Monagas et al., 2009; Sarnoski et al., 2012). 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, Germann, Balemba, Wakamatsu, & Hofmann, 2013). Furthermore, the synergistic and/or antagonistic effect of bioactive compounds is well known. The content of phenolic acids in this work (Table 4) is in agreement with those in the literature for the content of phenolic compounds in the soluble ethanolic extracts of peanut skin (Francisco & Resurreccion, 2009a). 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, An, Jiang, Guan, & Bao, 2006).

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.

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

Table 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.

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, Mahrouz, Amiot, & Lacroix, 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, Wani, Meena, & Dar, 2010) along with an increase in TPC, total anthocyanin, DPPH radical scavenging activity and ferric reducing antioxidant power.

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

Table 4 – The contents of free, esterified and insoluble-bound phenolic compounds (μg/g DW) of peanut skin.[a]

	IAC 886			IAC 505		
	Free			Free		
Phenolic compounds	Control	2.5 kGy	5.0 kGy	Control	2.5 kGy	5.0 kGy
Protocatechuic acid	55.40 ± 1.39b	70.16 ± 7.16a	72.94 ± 5.05a	43.51 ± 0.01b	56.34 ± 3.74a	54.69 ± 2.39a
(+)-Catechin	178.2 ± 26.4a	151.9 ± 12.8a	147.1 ± 8.50a	105.0 ± 8.81a	128.7 ± 5.69a	121.1 ± 16.7a
(–)-Epicatechin	361.8 ± 14.9a	333.4 ± 15.1a	352.7 ± 46.8a	380.8 ± 1.86a	323.8 ± 9.03b	235.2 ± 5.28c
Procyanidin dimer A[b]	3439 ± 85.8b	3329 ± 173b	4082 ± 185a	1867 ± 3.19b	1836 ± 10.7b	2006 ± 92.1a
Procyanidin dimer B[b]	1086 ± 48.4a	912.2 ± 13.9b	970.5 ± 32.8b	1412 ± 164a	1404 ± 69.8a	985.6 ± 9.83b
Procyanidin trimer A[b]	4826 ± 371a	4747 ± 344a	5244 ± 522a	2937 ± 135a	3089 ± 94.2a	3019 ± 7.48a
Procyanidin tetramer A[b]	827.6 ± 51.7a	908.6 ± 28.7a	889.3 ± 89.2a	1050 ± 4.58a	968.6 ± 82.2a	658.3 ± 7.13b
	Esterified			Esterified		
Gallic acid	82.06 ± 5.80a	68.31 ± 2.74b	36.67 ± 1.04c	114.3 ± 1.10a	101.5 ± 1.73b	51.32 ± 0.43c
Protocatechuic acid	63.46 ± 2.29b	52.78 ± 3.31b	125.6 ± 8.60a	50.03 ± 5.33b	62.28 ± 1.23a	65.41 ± 0.84a
(+)-Catechin	1033 ± 25.8a	780.4 ± 80.2b	705.2 ± 62.3b	1100 ± 132a	1293 ± 19.0a	891.4 ± 20.6b
(–)-Epicatechin	88.88 ± 7.04a	81.03 ± 11.5a	63.51 ± 7.96b	82.82 ± 7.50a	86.90 ± 2.96a	59.73 ± 6.05b
Gallocatechin[b]	47.93 ± 6.65a	27.62 ± 2.27b	14.88 ± 0.30c	38.71 ± 3.74c	24.37 ± 2.39b	14.53 ± 2.38a
Prodelphinidin A[b]	20.66 ± 1.72a	17.53 ± 1.43ab	16.19 ± 0.61b	26.84 ± 1.78a	19.25 ± 1.75b	12.76 ± 0.96a
Prodelphinidin B[b]	19.24 ± 0.81a	15.83 ± 0.57b	nd	21.01 ± 2.10a	13.36 ± 1.86b	nd
Procyanidin dimer A[b]	184.4 ± 27.0a	195.7 ± 13.5a	202.6 ± 16.1a	316.1 ± 20.4b	310.0 ± 5.19b	556.8 ± 3.75a
Procyanidin dimer B[b]	390.9 ± 27.4a	317.1 ± 24.0b	285.0 ± 18.6b	1208 ± 58.1a	1000 ± 9.63b	909.5 ± 48.3b
Procyanidin trimer A[b]	1668 ± 90.8a	545.6 ± 56.1b	479.2 ± 53.8b	691.5 ± 37.3a	621.2 ± 31.6a	347.6 ± 9.12b
Procyanidin tetramer A[b]	nd	nd	nd	tr	tr	887.3 ± 20.8
	Insoluble-bound			Insoluble-bound		
Protocatechuic acid	64.02 ± 4.24a	63.25 ± 1.21a	67.69 ± 1.59a	57.95 ± 1.98b	59.97 ± 2.39ab	64.30 ± 1.58a
Caffeic acid	14.05 ± 0.41c	15.92 ± 0.17b	23.50 ± 1.05a	21.85 ± 0.57a	23.41 ± 2.46ab	27.53 ± 1.01a
p-Coumaric acid	25.78 ± 2.22c	36.94 ± 0.65b	66.86 ± 3.72a	48.96 ± 2.42a	51.81 ± 1.13ab	53.86 ± 1.26a
Quercetin	23.49 ± 1.98c	29.75 ± 2.10b	44.86 ± 1.57a	54.26 ± 2.26	132.2 ± 14.3b	243.5 ± 23.0a
Isorhamnetin-glucoside[b]	24.47 ± 0.85c	31.58 ± 0.71b	34.41 ± 1.42a	100.6 ± 6.95a	102.0 ± 9.40a	117.3 ± 9.26a
(+)-Catechin	35.65 ± 1.60c	46.15 ± 3.73b	148.3 ± 3.48a	52.20 ± 3.19a	66.50 ± 12.2ab	80.86 ± 5.32a
(–)-Epicatechin	123.0 ± 10.2c	194.5 ± 11.4b	201.4 ± 10.3a	63.02 ± 7.76b	86.13 ± 6.09a	80.50 ± 2.92a
Procyanidin dimer A[b]	125.6 ± 11.0b	142.2 ± 18.0b	288.6 ± 26.8a	188.8 ± 10.2c	285.5 ± 11.0b	328.7 ± 9.01a
Procyanidin dimer B[b]	385.4 ± 46.9a	210.7 ± 7.53b	243.3 ± 34.0b	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 ($p > 0.05$). Nd, non-detected; tr, trace.

[b] Compounds quantified as catechin equivalents; DW, dry weight of defatted sample. Traces of procyanidin pentamer A were found in the free fraction.

due to different linkages and stereochemistry (Sarnoski et al., 2012). Concentrations of procyanidin dimer A was higher than values reported in the literature (Yu, Ahmedna, Goktepe, & Dai, 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 procyanidin 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. Fig. 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

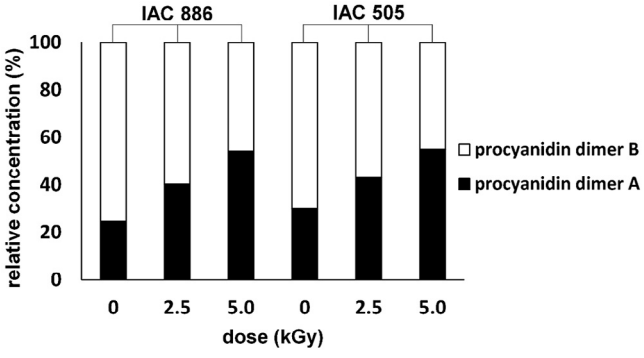

Fig. 4 – Distribution change of procyanidin dimers in the insoluble-bound fraction of non-irradiated and gamma-irradiated peanut skin at 2.5 and 5.0 kGy for IAC 886 and IAC 505 peanut skin. This is representative of the remaining fractions which show the same trend. Detailed data are given in Table 4.

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).

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 study (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. As noted earlier, many proanthocyanidin isomers were tentatively identified and quantified in the present work; however, to simplify the situation, only chromatograms of the insoluble-bound fractions, which contain the most common isomers, are provided (Supplementary material). Furthermore, the insoluble-bound fraction also contains most phenolic acids and monomeric flavonoids detected in this work (Table 4).

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, Hammerstone, Keen, Fraga, & Oteiza, 2005). They also render a greater protective effect against the haemolysis of red blood cells than ascorbic acid under *in vitro* conditions (Wang, Yuan, Jin, Tian, & Song, 2007), thus it is of great importance to study potential sources of polyphenols, including procyanidins, and their stability under different processing conditions.

In summary, 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.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2014.10.034.

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