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Effect of Cold Plasma Treatment on the Functional Properties of Fresh-Cut Apples

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ABSTRACT: Atmospheric double-barrier discharge (DBD) plasma technology is a promising tool in the food industry as an alternative to traditional food preservation methods. However, the effect of the reactive species generated during the treatment on the content of bioactive compounds in food is still little studied, and there are no data concerning potential deleterious effects of DBD-treated foods on human cells. Some functional properties of DBD-treated minimally processed Pink Lady apples were evaluated in comparison with untreated samples through different in vitro and ex vivo tests. Plasma treatment caused only a slight reduction of antioxidant content and antioxidant capacity (up to 10%), mainly limited to the amphiphilic fraction. Noteworthy, treated apple polyphenol extracts did not reduce cell viability and did not suppress the beneficial physiological cell response to oxidative stress in terms of reactive oxygen species production and phase II enzyme activation in human cultured colonocytes

KEYWORDS: *cold plasma, fresh-cut apple, antioxidants, antioxidant activity, phase II enzymes*

INTRODUCTION

The major issues for food science are to maintain important food quality attributes, to increase the level of food safety, and to enhance the product shelf life. In the past decade, nonthermal technologies for food stabilization have been developed in response to the worldwide interest for more fresh-like and natural food products, minimizing typical thermal alterations such as sensorial changes, formation of off-flavors, and loss of nutritional components.[1,2] Among nonthermal treatments, cold gas plasma presents several advantages. Gas plasma is an ionized gas characterized by active particles such as electrons, ions, free radicals, and atoms that are in both fundamental and excited states; the ionization occurs by applying energy to a gas or a gas mixture. However, when atmospheric air is used as working gas, reactive oxygen species (ROS) and reactive nitrogen species (NOS) are formed. These oxidative species can cause lipid peroxidation and protein and DNA oxidation[3] and may potentially interact with bioactive compounds, altering their content/functional properties in food products.

Many studies have been published concerning the impact of plasma technology on components and properties of both solid and liquid foods.[2] These applications were mainly addressed to the reduction of the activity of oxidative enzymes such as polyphenol oxidase in fresh-cut apples,[4] peroxidase in tomatoes,[5] and polyphenol oxidase and peroxidase in a polysaccharide gel model food system.[6]

Less studied is the effect of gas plasma treatment on antioxidant content and antioxidant activity in vegetable tissues.[7,8] Double-barrier discharge (DBD) cold plasma treatments on minimally processed kiwifruit did not induce any textural changes and positively influenced the visual quality, without changing ascorbic acid and polyphenol contents.[9] Possibly the effect of plasma on bioactive compounds may be different depending on the type of food matrix.[2,7−9]

Changes in food polyphenol and vitamin content after plasma treatment are of particular interest due to their potential effect on health properties. These compounds may protect against oxidative stress by scavenging ROS, chelating trace elements involved in free radical generation, and inducing cellular antioxidant defense by modulation of redox-sensitive gene expression. However, a growing number of studies highlight the benefits derived from moderate oxidative stress, induced by polyphenols, as a key point to build resilience against various types of chronic human pathologies.[10]

According to Niemira,[11] the U.S. Food and Drug Administration (FDA) has not yet allowed the use of cold plasma for food processing because of the lack of knowledge on the primary modes of action and on the effects on sensory and nutritional properties of the products. In particular, research on

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the impact on antioxidant properties and bioactive compounds, on the potential chemical residue effects, and on the formation of toxicants is therefore needed to provide sufficient information to assess the health-related implications of the process.

The purpose of this study was to expand previous DBD cold plasma effects on visual quality and enzymatic activity of fresh-cut 'Pink Lady' apples[4] to information concerning their functional properties. In particular, antioxidant activity was evaluated through a multimodal approach, combining different assays for the analysis of antiradical activity and reducing activity of antioxidants. Polyphenol extracts were further analyzed by high-performance liquid chromatography−mass spectrometry (HPLC-MS/MS) to quantitatively calculate differences in the polyphenol composition of DBD-plasma-treated versus untreated minimally processed apples. We extended our investigation to the effect exerted by the above-mentioned polyphenol extracts in human colonocytes. We have chosen, as experimental model, the Caco2 cell line, which is derived from human colon adenocarcinoma. Caco2 cells are widely used for biochemical and nutritional studies because they retain, upon in vitro cultivation, the morphology and most of the functions of the normal small intestine epithelial.[12] Caco2 cells have been previously used to study the intestinal absorption of food components, such as tea polyphenols,[13] and the modulation of oxidative status by dietary flavanols.[12,14] Bearing this in mind, and clearly stating that this experimental work does not have the aim to investigate potential anticancer effects of apple polyphenols, we studied the effects of DBD-treated and untreated apple polyphenol extracts on cell viability, intracellular ROS production, and phase II enzyme activation. In parallel, we treated Caco2 cells with Polyphenon E,[15,16] a standardized green tea extract, FDA approved, which represents a source of highly purified and characterized green tea catechins.

■ MATERIALS AND METHODS

Chemicals. Chemicals of analytical grade were purchased from Sigma-Aldrich (Steinheim, Germany) except for hydrochloric acid and methanol, which were purchased from Romil (Feltham, UK).

Polyphenon E, a standardized green tea extract preparation ((−)-epigallocatechin-3-gallate (EGCG), 68.58%; (−)-epigallocatechin (EGC), 10.56%; (−)-epicatechin (EC), 4.31%; (−)-epicatechin-3-gallate (ECG), 5.95%; (−)-gallocatechin-3-gallate (GC), and other trace catechin derivatives) was supplied by Polyphenon Pharma (New York).

Raw Material, Handling, and Storage. Apples (*Malus domestica* cv. 'Pink Lady'), harvested 2 weeks before, were provided by the local market. Fruits free from defects were transported to our laboratory and stored in a refrigerated chamber at 5 ± 1 °C and saturated atmosphere in darkness for 1 week. Apples were characterized by a dry matter content of 15.73 (±0.29) g 100 g^{-1} fresh weight (fw), a soluble solid content of 14.27 (±0.35) °Brix, and a titratable acidity of 0.39 (±0.03) mg malic acid g^{-1} fw.

DBD Gas Plasma Generator, Sample Preparation, and Plasma Treatments. Cold plasma was generated by a dielectric barrier discharge (DBD) device that was already described and characterized by Ragni et al.[17] It consists of a hermetic chamber containing three parallel pairs of electrodes (brass) supplied by a dc power supply and powered by high-voltage transformers and power-switching transistors. A 5 mm thick glass was used as dielectric material. As feed gas, atmospheric gas driven at 1.5 slm was chosen. Frequency of oscillation was 12.7 kHz, and the power supply was in the range of 150 W. The discharge showed typical air nonequilibrium peaks of the second N_2 positive system and of the positive ion N_2^+ together with the presence of oxygen and nitrogen radicals and ions as commonly detected when atmospheric air is used to generate plasma.[17]

Apple slices (40 × 10 × 10 mm) were manually obtained from apple flesh using a sharp blade. Samples were exposed to cold plasma at a distance of 70 mm from the electrodes for a total of 30 (15 + 15 on each side) min.

In the treatment chamber and on the fruit surface, the temperature was 22 °C and the relative humidity (RH), 60%. Control samples were stored at the same temperature and humidity conditions for the duration of the treatment.

Treatment time was stressed to 120 (60 + 60) min of processing only for polyphenols and antioxidant activity determinations.

Polyphenol Extract Preparation. Lyophilized freeze-dried apple powder was extracted in 60% methanol. For biological assay, 3 g of powder was mixed with 20 mL of solvent; for HPLC analysis, 250 mg of powder was extracted in 1.5 mL of solvent mixture containing 1% (v/v) of formic acid. The suspension was vortexed vigorously for 2 min, then samples used for HPLC analysis were left for 60 min in a sonic bath and centrifuged for 20 min (20878*g*), and supernatant was collected at 4 °C and transferred to a vial before injection into the HPLC system. Samples used for ex vivo assays were centrifuged for 10 min (10000*g*); the supernatant was collected, and the pellet was subjected to a second extraction. The total supernatant was dried in a rotary evaporator (model Laborota 4001, Heidolph, Germany) at 35 °C, and the dry residue was used as extract for further analysis.

High-Performance Liquid Chromatography and Mass Spectrometry Analysis. Before analysis, 20 μL of each internal standard was added to the samples (genistein, 580 μg/mL; genistin, 380 μg/mL). HPLC separations were carried out by means of a SUNSHELL C18 (2.1 i.d. × 100 mm) column, 2.6 μm particle size (Chromanik) with mobile phase, pumped at a flow rate of 0.3 mL/min, consisting of a mixture of acidified acetonitrile (0.1% formic acid) (solvent A) and 0.1% aqueous formic acid (solvent B). Following 0−2 min, 2% B; 2−13 min, 2−30% B; 13−20 min, 30−80% B; 20−22 min, 80−2% B; 22−30 min, 2% isocratic; this step was followed by the washing and reconditioning of the column.

The identity of the phenolic compounds was confirmed using a triple-quadrupole mass spectrometer (Thermo Scientific, TSQ Vantage) with a heated electrospray ionization (H-ESI II) operating in the negative ionization mode. The capillary temperature was 270 °C; the sheath gas and auxiliary gas were 40 and 5 arbitrary units, respectively; and the source voltage was 3 kV. A vaporizer temperature of 200 °C argon was used for MS/MS experiments with a collision pressure of 1.0.

For the identification, a full-scan analysis was performed scanning from m/z 100 to 950, whereas a product ion scan experiment was applied for ions not fully identified in the previous method. Identification was performed by comparing the mass spectra with literature data and, whenever possible, the identification was confirmed by using pure standards of the components.

Antioxidant Activity and Total Phenolic Content. Antioxidant activity and total phenolic content of apple samples were assessed by different microplate assays as previously reported.[9] The antioxidant activity was performed by 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) methods. The total polyphenol content was quantified by the Folin−Ciocalteu phenol reagent.

Cell Culture and Treatments. Caco2 cells were purchased from ATCC and routinely grown in a 1:1 mixture of Ham's F12/DMEM. Culture medium was supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were incubated at 37 °C under a 5% CO_2 atmosphere. Cell harvesting was performed by trypsin/EDTA (Sigma-Aldrich, Steinheim, Germany) treatment.

For ROS determination and luciferase assay the Caco2 cells were grown in a 1:1 mixture of Ham's F12/DMEM without red phenol (Sigma-Aldrich).

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Table 1. Content of Phenolics (μmol kg^{-1} dw) of 'Pink Lady' Apples Determined by HPLC-MS/MS As Affected by Plasma Treatment Time[a]

	compound	30 min		120 min	
		C	T	C	T
flavan-3-ols	catechin	50.24 ± 3.21 a	39.79 ± 6.09 a	64.03 ± 5.64 a	36.81 ± 9.30 a
	epicatechin	415 0.22 ± 20.12 a	343.88 ± 36.45 a	448.75 ± 57.84 a	281.74 ± 84.84 a
	procyanidin dimer B1	45.46 ± 3.05 a	35.35 ± 4.65 a	49.39 ± 4.13 a	21.92 ± 6.27 b
	procyanidin dimer B2	164.06 ± 5.34 a	134.29 ± 14.91 a	141.58 ± 22.89 a	72.53 ± 19.24 a
	procyanidin dimer B4	11.26 ± 0.50 a	8.93 ± 1.13 a	9.78 ± 1.88 a	4.71 ± 1.44 a
	procyanidin B trimer	9.64 ± 0.67 a	7.35 ± 1.21 a	10.52 ± 1.51 a	4.31 ± 1.35 b
	procyanidin B trimer 2	18.63 ± 0.81 a	13.90 ± 1.54 a	12.98 ± 1.94 a	5.94 ± 2.08 a
	procyanidin B trimer 3	58.73 ± 1.22 a	43.80 ± 4.83 a	49.97 ± 9.04 a	20.42 ± 6.55 a
	procyanidin B trimer 4	8.05 ± 0.20 a	6.21 ± 0.66 a	7.72 ± 1.11 a	2.95 ± 0.87 b
	procyanidin B trimer 5	0.74 ± 0.01 a	0.26 ± 0.37 a	0.81 ± 0.22 a	0.11 ± 0.10 a
hydroxycinnamic acids	caffeic acid	1.05 ± 0.02 a	1.07 ± 0.16 a	0.29 ± 0.08 a	0.31 ± 0.03 a
	caffeoylquinic acid	1046.98 ± 738.44 a	1021.46 ± 687.92 a	1280.42 ± 915.80 a	1176.35 ± 871.53 a
	4-coumaroylquinic acid	96.62 ± 3.81 a	81.84 ± 13.06 a	163.62 ± 24.07 a	143.79 ± 38.64 a
	coumaroylquinic acid	301.80 ± 3.99 a	313.71 ± 37.79 a	294.07 ± 56.71 a	337.10 ± 55.94 a
dihydrochalcones	phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside	169.98 ± 3.90 a	231.70 ± 43.65 a	172.64 ± 44.29 a	166.08 ± 20.53 a
	phloridzin	32.77 ± 4.29 a	74.49 ± 24.54 a	51.56 ± 23.50 a	37.19 ± 6.42 a
flavonols	myricetin rhamnoside	3.01 ± 0.12 a	3.07 ± 0.42 a	4.30 ± 2.08 a	3.42 ± 0.63 a
	quercetin	3.29 ± 0.18 a	4.22 ± 0.69 a	2.93 ± 1.08 a	2.46 ± 0.33 a
	quercetin-*O*-glucoside	3.02 ± 0.11 a	3.09 ± 0.39 a	4.35 ± 2.02 a	3.42 ± 0.63 a
	quercetin-*O*-rhamnoside	33.25 ± 0.22 a	40.83 ± 6.48 a	25.48 ± 8.03 a	22.87 ± 3.32 a
	rutin	0.13 ± 0.00 a	0.05 ± 0.05 a	0.21 ± 0.07 a	0.00 ± 0.00 a
total phenolics	hydrophilic + amphiphilic	2475.46 ± 40.01 a	2409.29 ± 248.74 a	2800.48 ± 441.07 a	2348.75 ± 396.41 a

[a]Values followed by different letters between control and treated sample of the same treatment time are significantly different at a $p < 0.05$ level.

A 1 mg/mL Polyphenon E fresh stock solution was prepared in deionized sterile water and diluted immediately in complete medium at the final concentration required for each experiment.

The polyphenol extracts from treated and untreated apples prepared for biological assays were dissolved in cell-cultured medium containing 1% DMSO and stored at −80 °C (stock solution contains 600 mg of lyophilized apple powder/mL). Fresh solutions were prepared in complete cell medium at the final concentration required for each experiment. The control cells were cultured with medium containing a maximum of 0.08% DMSO.

WST-1 Assay. Inhibition of cell proliferation by polyphenol extracts was measured by WST-1 assay (Roche, Lewes, UK). The assay is based on the reduction of tetrazolium salt WST-1 to soluble formazan by electron transport across the plasma membrane of actively dividing cells. Formazan formation was detected at 450 nm spectrophotometrically. Caco2 cells were plated in quadruplicate in 96-well microplates at a density of 4×10^4 cells/well and allowed to adhere overnight. Cells were treated with increasing concentrations of Polyphenon E (1−20 μg/mL) or polyphenol extracts from untreated and treated apples (1−75 mg/mL). After 5 h of incubation, the WST-1 assay was performed.

Reactive Oxygen Species Determination. The production of intracellular ROS was detected using the 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. Briefly, Caco2 cells were seeded in black 96-well plates (4×10^4 cells/well) and allowed to attach overnight. After 5 h of treatment with increasing concentrations of Polyphenon E (1−20 μg/mL) or polyphenol extracts (28 and 75 mg/mL), cells were washed twice with PBS and loaded with 20 mM DCFH-DA in PBS for 15 min at 37 °C. After incubation, cells were washed with PBS, and ROS generation was measured by the fluorescence intensity of dichlorofluorescein (DCF; exc, 475 nm; em, 535 nm) using an Enspire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Inside the cells, DCFH-DA is cleaved by nonspecific esterases forming nonfluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS. In the same wells, the total protein content was quantified using the Bio-Rad DC Protein assay (Bio-Rad, Berkeley, CA, USA).

Plasmid Construction and Luciferase Assay. To generate the recombinant plasmid pGL4-NQO1, genomic DNA was extracted from human liver using the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. The upstream promoter region of the *Nqo1* gene (635 pb) was PCR amplified using the following primers: fw 5′-ACCTGCCTTGAGGAGCAGGGG-TGGTGCAG-3′, rv 5′-GGCTCTGGTGCAGTCCGGGGCGCT-GATTGG-3′.[18] The PCR product was subcloned in the pCR2-TOPO vector (Invitrogen, Carlsbad, CA, USA). The *Kpn*I/*Xho*I restriction fragment obtained from the digestion of TOPO-NQO1 was then ligated into the pGL4.10 vector (Promega, Madison, WI, USA), and the resulting plasmid was sequenced.

Caco2 cells were seeded in a 96-well white microplate at a density of 2×10^4 cells/well and transfected using Viafect (Promega), using 0.2 μg of pGL4-NQO1 and pGL4.10 empty vectors. Transfection efficiency was monitored by pEGFP-N1 transfection (Clontech Laboratories, Mountain View, CA, USA).

The luciferase activity was measured after 5 h of incubation with increasing concentrations of Polyphenon E (1−20 μg/mL) or polyphenol extracts (28 and 75 mg/mL) using the Britelite plus reactive (PerkinEmler) and EnSpire Multimode Plate Readers (PerkinEmler). The luciferase activity was normalized to the total protein content after checking for equal transfection efficiency in each well.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR. Caco2 cells were seeded in 35 mm dishes at a density of 6×10^5 and allowed to attach overnight. After 5 h of treatment with two different concentrations of polyphenol extracts from treated or untreated apple (28 and 75 mg/mL), total RNA was extracted with the

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Table 2. Total Phenolic Index (TPI) and Antioxidant Activity (μmol kg^{-1} dw) of 'Pink Lady' Apples Evaluated by Different in Vitro Methods As Affected by Plasma Treatment Time

	extract	30 min		120 min	
		C	T	C	T
TPI[1]	amphiphilic	13320 ± 510 a	11987 ± 568 b	11420 ± 1189 a	7608 ± 514 b
TPI[1]	hydrophilic	280 ± 86 a	351 ± 39 a	384 ± 19 a	258 ± 46 b
TPI[1]	hydrophilic + amphiphilic	13600 ± 501 a	12338 ± 567 b	11768 ± 1192 a	7867 ± 510 b
ABTS[2]	amphiphilic	14175 ± 300 a	13973 ± 316 b	13998 ± 1140 a	9930 ± 460 b
ABTS[2]	hydrophilic	911 ± 334 a	732 ± 302 a	567 ± 171 a	532 ± 138 a
DPPH[2]	hydrophilic + amphiphilic	24072 ± 2053 a	23647 ± 2496 a	20285 ± 745 a	16017 ± 2285 b

Trizol reagent (Fisher Molecular Biology, Rome, Italy) and cleaned up with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. For reverse transcription reaction, 100 ng of total RNA from each experimental condition was combined with 1 μL of random primers (0.2 μg/μL) and heated to 65 °C for 5 min. Following a brief chill on ice, the reverse transcription mix (Thermo Scientific, Boston, MA, USA) was incubated at 25 °C for 5 min, at 45 °C for 60 min, and at 70 °C for 10 min. The first-strand synthesis reaction was diluted 1:2, and then 2 μL of each cDNA preparation was used for quantitative real-time PCR with the set of primers described below. The thermal cycling comprised an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s. Analysis of results was performed by DNA Engine Opticon 4 (MJ Research, Walthman, MA, USA) using the 2X SYBR Premix Ex Taq (Takara Bio Inc., Japan). Relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method[19] using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeper gene for data normalization. The results are expressed as the mean ± SD of two independent determinations, each performed in duplicate.

Primer sequences used for GST members were the following:[20] GSTT2-fw 5′-CTTTCCTGGGTGCTGAGCTA-3′ and GSTT2-rv 5′-GGTGTTGGGAGGGTTTTCTT-3′; GSTP1-fw 5′-GGAGACCTC-ACCCTGTACCA-3′ and GSTP1-rv 5′-CTGCTGGTCCTTCCC-ATAGA-3′; GSTA4-fw 5′-TCCGTGAGATGGGTTTTAGC-3′ and GSTA4-rv 5′-GGTGGTTACCATCCTGCAAC-3′.

Primer sequences used for UGT members were the following:[21] UGT1A1-fw 5′-AATAAAAAAGGACTCTGCTATGCT-3′ and UGT1A1-rv 5′-ACATCAAAGCTGCTTTCTGC-3′; UGT1A4-fw 5′-GAACAATGTATCTTTGGCCC-3′ and UGT1A4-rv 5′-ACCACA-TCAAAGGAAGTAGCA-3′; UGT2B7-fw 5′-GGAGAATTTCAT-CATGCAACAGA-3′ and UGT2B7-rv 5′-CAGAACTTTCTAGTT-ATGTCACCAAATATTG-3′.

Statistical Analysis. Data are expressed as mean values ± SD for the indicated number of independent determinations.

Statistical significance was calculated by two-sided Student's t test, and p values are indicated in the table and figure legends.

RESULTS AND DISCUSSION

Phenolic Content. The phenolic content of apples was measured by HPLC-MS/MS analysis, and the content of each detected phenolic is reported in Table 1. The total phenolic content of 'Pink Lady' apples of control untreated samples ranged between 2.47 and 2.80 mmol kg^{-1}dw (that corresponds to 117−129 mg kg^{-1} fw), which is slightly higher than literature data (90 mg kg^{-1} fw) reported in a previous study.[22] However, these values are lower than those of the most widely studied variety, 'Golden Delicious', for which values range between 233 and 417 mg kg^{-1} fw depending on agricultural practices and harvesting years.[23−26]

In a previous study a 30 (15 + 15) min treatment was found to be suitable for fresh-cut apple stabilization[4] because, at the same experimental conditions, a reduction in browning was observed by image analysis. In the same study, an inhibition of polyphenol oxidase (PPO) activity proportional to treatment time up to 57% for 30 min was reported. Reduction of enzymatic activity upon plasma exposure has been attributed to the oxidation of the reactive side chain of the amino acids by plasma radicals,[5,6] in particular OH, O_2^-, HOO, and NO, that promotes a change in the secondary protein structure and the modification of some amino acid side chains of the enzyme.[27,28] On the basis of these results, an oxidation of the phenolic component could be hypothesized.

Conversely, in this study the total phenolic content evaluated by HPLC-MS/MS did not show any significant difference compared to control apples (Table 1). To verify if a longer time of exposure to DBD plasma might determine a significant decrease of polyphenols, the product was overtreated for 120 (60 + 60) min of processing. Differences in the total phenolic content were still not statistically significant, but, considering singular compounds, a few differences were found in the flavan-3-ols group; in particular, significant reductions of procyanidin B1 dimer, trimer, and trimer 4 of, respectively, 56, 59, and 62% were found. Although oxidation reactions can easily take place during plasma treatment thanks to the production of radical species and the availability of oxygen in the atmosphere, phenolic content did not show variations after 30 min, albeit at the same treatment time a noticeable enzymatic activity inhibition was previously observed and after stressing plasma exposure up to 120 min promoted only a limited reduction to very few compounds.

The total phenolic index (TPI) of apple products has been estimated also by measuring their ability to reduce the Folin−Ciocalteu reagent, an extensively used method for the estimation of total phenolics, after solid phase extraction on C-18 cartridges, considered suitable technique for the separation of phenolic compounds.[9]

In this study, a total TPI of about 13.3 mmol kg^{-1} dw (2.2 mg GAE g^{-1} dw) was measured in the amphiphilic fraction (Table 2), after SPE separation; also in this case, this value is lower than that of 'Golden Delicious' apples but comparable with literature results.[26,29]

The DBD plasma treatment significantly affected the TPI of apples with a decrease of about 9% after 30 min, whereas prolonging treatment time up to 120 min led to a decrease of about 33% (Table 2).

The spectrophotometric determination of total polyphenols overestimated the final polyphenol decrease, when compared to HPLC-MS/MS analysis that was not able to find significant differences between mean values after 30 min. This happened probably because the TPI assay is based on the capacity of phenolic compounds to reduce the Folin−Ciocalteu reagent under basic conditions, the mechanism of the TPI assay being based on an oxidation/reduction reaction. This result roughly indicates that the polyphenols which underwent oxidation with

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increasing processing time were characterized by a high reducing power.

Antioxidant Activity. In the present study, the antioxidant activity of apple samples was investigated with a variety of methods aimed to measure their radical scavenging activity (ABTS and DPPH assay) and reducing power (FRAP and TPI). As mentioned, the TPI is a method measuring the reducing power of the phenolic extract; for this reason it can be used to investigate the reducing power of a polyphenol mixture, being considered an antioxidant method.[30]

The ABTS (expressed as Trolox equivalent antioxidant capacity, TEAC) and TPI assays were conducted on both the amphiphilic and hydrophilic extracts; the former showed a radical scavenging activity much higher than the latter, which accounts for about 6% of the total TEAC (sum of TEAC values of the amphiphilic and hydrophilic extracts). Similarly, the reducing power of the hydrophilic extract, as measured by the TPI, accounted for about 2% of the total TPI (Table 2). This result is due to the fact that apple is very poor in water-soluble polyphenols and ascorbic acid, which could filtere through the cartridge set upon washing with the acidulated water extract prior to the elution of amphiphilic compounds.

The DBD plasma treatment significantly affected the TEAC of amphiphilic compounds of apples with a slight decrease (1%) after 30 min of processing, to reach values 29% lower than those of control apples when overtreated (120 min). No differences were found for the TEAC of hydrophilic compounds between control and treated samples at both processing times (Table 2).

The DBD plasma treatment significantly affected the TPI of amphiphilic compounds of apples as well as total TPI, which showed significant decreases both after 30 min (10%) and after 120 min of processing (33%) (Table 2).

Different from the ABTS assay, the TPI assay evidenced a final decrease (32%) of the reducing power of hydrophilic compounds during plasma processing. These two assays differ in the mechanism of action, which are radical scavenging for ABTS and single electron transfer for TPI; hence, the polyphenols consumed during plasma treatment showed lower radical scavenging activity than reducing power.

The DPPH• assay did not evidence any difference in antioxidant activity after 30 min of processing, whereas it evidenced an antioxidant activity decrease (21%) after 120 min of processing (Table 2), showing a lower sensitivity of this method than of the ABTS assays.

The FRAP assay showed values similar to those of the DPPH• assay, not detecting significant differences after 30 min but finding a higher decrease after 120 min (36%).

Generally, the reduction of antioxidant activity after 30 min of processing was limited and relative to the amphiphilic fraction.

Effect of Treated and Untreated Apple Polyphenol Extracts on Caco2 Cell Viability. To investigate the effect of plasma technology on cell viability, we performed the WST-1 colorimetric assay. Caco2 cells were seeded in a 96-well microplate and incubated for 5 h in the presence of different concentrations of polyphenols.

First of all, we treated Caco2 cells with increasing concentration of Polyphenon E. The concentration range of green tea polyphenols examined in our study (1−20 μg/mL) comprises the concentrations achievable in human plasma (0.14−3.4 μg/mL) after a single oral administration of Polyphenon E containing 400−1200 mg of pure EGCG.[15,16]

As shown in Figure 1A, Polyphenon E did not cause cytotoxicity in the concentration range considered by us. This result is in agreement with data published by Salucci and co-

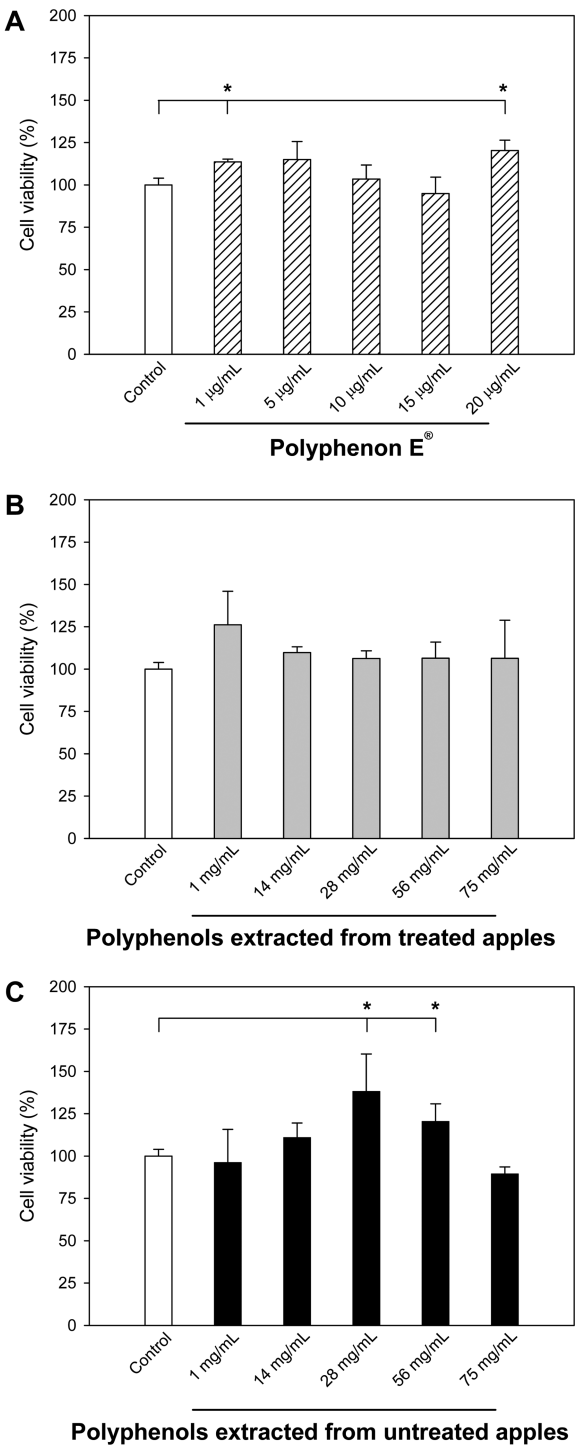

Figure 1. Effects of polyphenol extracts on Caco2 cell viability. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) and polyphenols extracted from treated (B) and untreated (C) apples. Cell viability was determined by WST-1 assay. Control represents Caco2 cells incubated with culture medium containing a maximum of 0.08% DMSO. Data are presented as means ± SD from four replicate wells of three different experiments as percentage of control sample. Statistical significance versus control was calculated by two-sided Student's t test. $*$, $p < 0.05$.

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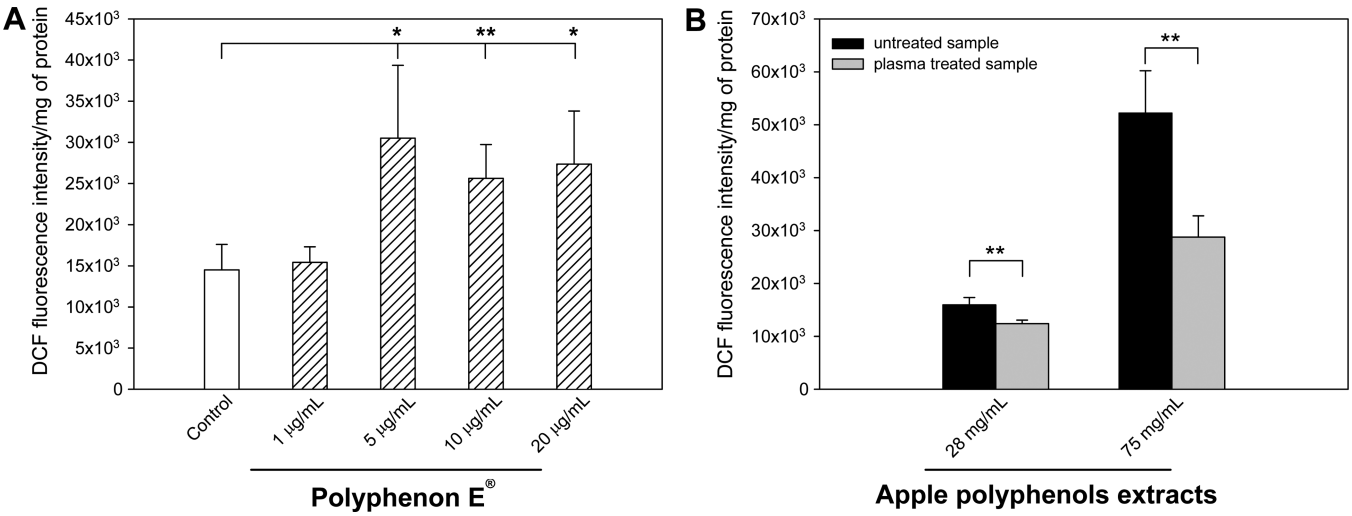

Figure 2. Effects of polyphenol extracts on Caco2 cell ROS production. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) or apple polyphenol extracts (B). ROS production was determined by DCFH-DA assay. Control represents Caco2 cells incubated with cell culture medium. Data are presented as means ± SD from eight replicate wells of three different experiments. Statistical significance was calculated by two-sided Student's *t* test. *, $p < 0.05$; **, $p < 0.001$.

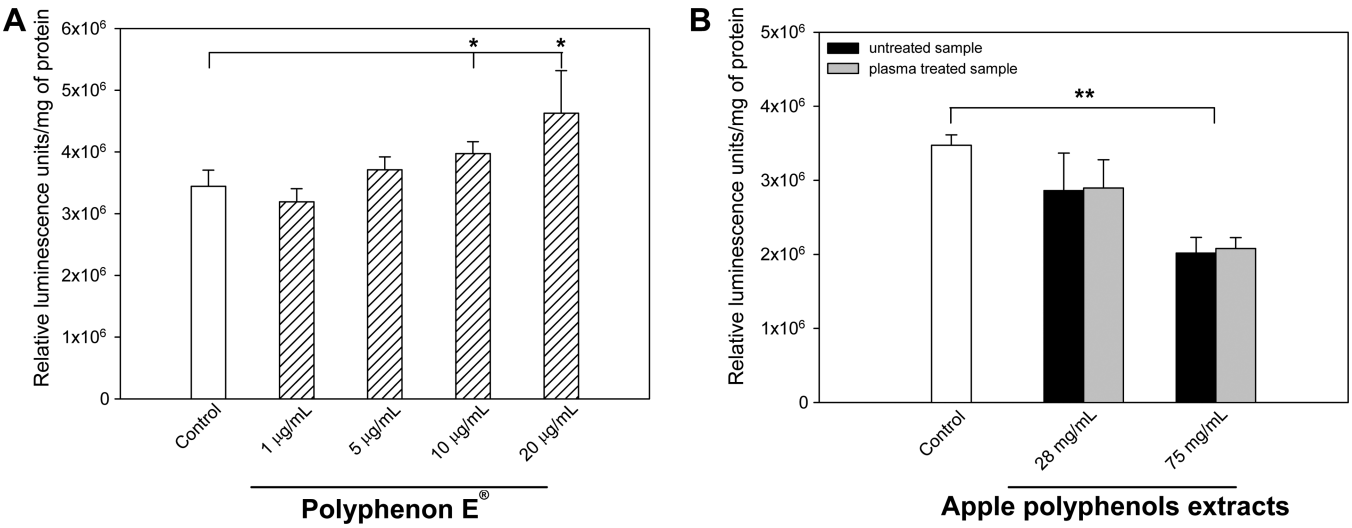

Figure 3. Effects of polyphenols on Caco2 cell luciferase activity. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) or apple polyphenol extracts (B). Control represents Caco2 cells incubated with culture medium containing a maximum of 0.08% DMSO. Luciferase activity was normalized for the total protein content. Data are presented as means ± SD from four replicate wells of three different experiments. Statistical significance was calculated by two-sided Student's *t* test. *, $p < 0.05$; **, $p < 0.001$.

workers, who reported that EGCG does not induce cytotoxicity in Caco2 cells below 46 μg/mL.[31]

Then, we tested various concentrations of our polyphenol extract, in a range comparable with those used in the Polyphenon E assay. As shown in Figure 1B no reduction in cell proliferation was observed when Caco2 cells were loaded with polyphenols extracted from plasma-treated apples, even at the highest concentration tested. The polyphenol extract obtained from untreated apples caused a moderate increase in cell proliferation when used at 28 and 56 mg/mL. We speculate that these differences might be related to the difference in the total polyphenol composition, which is not statistically significant when considered as a whole, but can be different in the ratio between various compounds taken as a single.

Effect of Polyphenols Extracted from Treated and Untreated Apple on ROS Production. Intracellular ROS levels affect cell viability, and high ROS concentrations can cause cellular damage. Using the DCFH-DA assay, we evaluated the modulation of intracellular ROS in Caco2. As made for the WST-1 assay, we incubated Caco2 cells for 5 h with Polyphenon E. We observed that concentrations ranging between 5 and 20 μg/mL induced a significant increase in ROS production without reducing cell viability (Figure 2A). The same biological effect has been reported on Caco2 cells after incubation with high concentrations of raw apple extracts.[32] Green tea polyphenols may exert both an antioxidant and a pro-oxidant effect in different cell lines, mainly depending on the concentration tested.[33−35] Noteworthy, the pro-oxidative properties of plant-derived polyphenols are well documented[33−35] and represent, at least in part, one of the mechanisms that promote the activation of endogenous defenses against oxidative stress.[10] We then proceeded incubating Caco2 cells for the same time with polyphenols extracted from treated and untreated apples. As shown in

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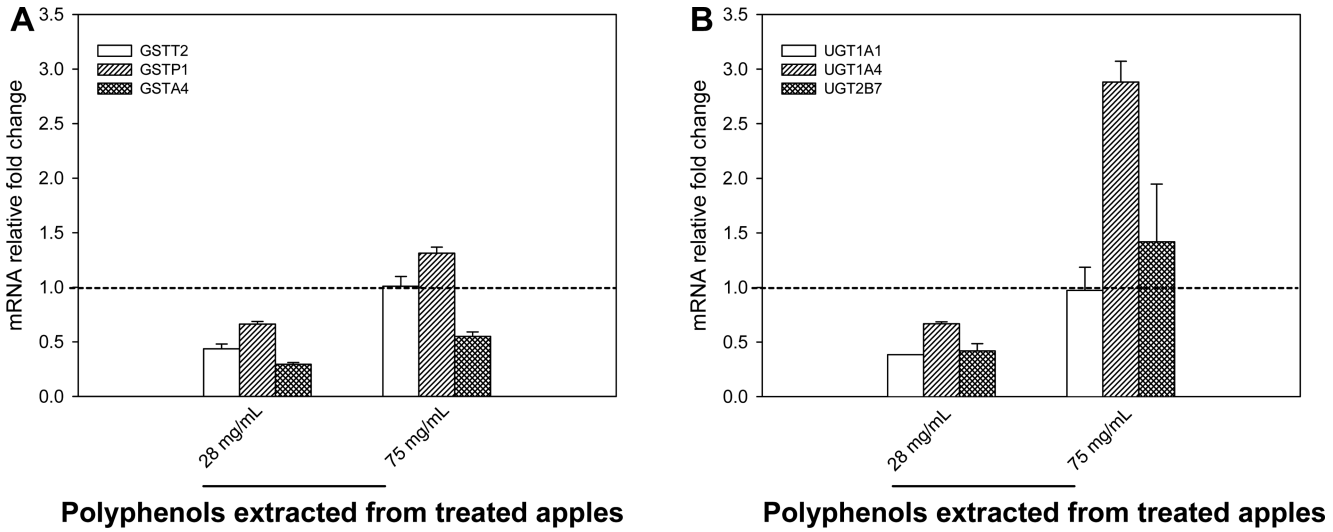

Figure 4. Relative mRNA expression of Nrf2-activated phase II enzymes in Caco2 cells. Caco2 cells were treated for 5 h with different concentrations of polyphenols extracted from treated and untreated apples. The mRNA levels of GSTs (A) and UGTs (B) enzymes were evaluated by qPCR. Relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method using polyphenol extracts derived from untreated apples as reference sample (reference mRNA fold change equal to 1). Data are presented as means ± SD from three different experiments. We considered statistically relevant mRNA fold changes <0.75 and >1.5.

Figure 2B, the production of oxidizing species is, consistent with data reported in the literature, directly related to extracts' concentration. Intracellular ROS production was in any case lower in Caco2 cells receiving polyphenols extracted from plasma-treated samples. At the concentration of 75 mg/mL the plasma-treated sample caused a decrease in ROS level of about 1.8-fold in comparison to the untreated sample. None of these concentrations exerted relevant effects on cell viability.

Effect of Polyphenols Extracted from Treated and Untreated Apples on Phase II Defense Enzymes. To determine whether ROS induced by polyphenols' extracts are able to modulate the response of phase II defense enzymes, we performed both gene-reporter assay and qPCR.

Phase II enzymes catalyze conjugation reactions to transform toxic endogenous molecules and xenobiotics in hydrophilic compounds that can be easily excreted. They play also an important role in the metabolic inactivation of pharmacologically active substances. The Nrf2/EpRE pathway is one of the more characterized cell signaling pathwayd involved in the safeguard against oxidative stress. It regulates the expression of key protective enzymes such as glutathione peroxidase (GPX), glutathione *S*-transferase (GST), NADPH quinine oxidoreductase 1 (NQO-1), and UDP-glucuronosyltransferase (UGT).[10,36]

A DNA fragment containing the EpRE sequence was subcloned upstream of a firefly luciferase into a suitable promoterless reporter plasmid. After transient transfection with the expression plasmid, Caco2 cells were treated for 5 h with increasing concentrations of Polyphenon E. At the same concentration able to induce an increase in ROS generation, we observed a statistically significant increase in luciferase activity (Figure 3A). These data support the concept that green tea polyphenols are able to induce a beneficial moderate oxidative stress, which acts through the Nrf2/EpRE pathway, activating the EpRE elements located in the promoters of target genes, such as the phase II enzymes. Unfortunately, when we treated luciferase transduced Caco2 cells with our extracts, we were not able to observe any activation of the EpRE element; on the contrary, we observed a concentration-dependent quenching of the analytical signal in comparison to control cells (Figure 3B). However, the recombinant plasmid used for the gene reporter assay contains just one copy of the EpRE motif, and previous data have shown a good correlation between the number of EpRE repeats and the luciferase activity; therefore, we might suppose that we could increase the sensitivity of the assay by using a different reporter construct.[37] Nonetheless, we cannot rule out the possibility that during the extraction procedure, other molecules that are present in the sample irrespective of the type of treatment were coeluted with the polyphenols and might have interfered with the assay.

Phase II enzymes are direct targets of the transcriptional activity of Nrf2, a redox-dependent transcription factor that binds the EpRE regulatory sequences of their promoters. GST family members are able to catalyze the conjugation of the sulfhydryl moiety of glutathione (GSH) with a broad range of endogenous and exogenous electrophilic substrates.[38] UGT family members are endoplasmic reticulum-bound enzymes that catalyze glucuronidation of endogenous and exogenous substrates, such as bilirubin, bile acids, steroids, and xenobiotics.[21] Previous data have shown that in different colon tumor cell lines apple polyphenols[39] and digitoflavone[40] are able to induce gene expression of detoxification enzymes.

To acquire more information on the influence of plasma technology on apple's bioactive molecules, we quantified the mRNA levels of genes belonging to GST and UGT family enzymes by qPCR.

Figure 4 shows the relative expression of GST and UGT genes measured in cells cultured with extracts obtained from plasma-treated apples compared to cells receiving polyphenols extracted from untreated fruits (expression level fixed equal to 1). The mRNA levels of GSTs and UGTs are reduced following treatment at the lowest concentration tested (28 mg/mL) of approximately half of the values measured in control cells. In contrast, when the concentration was raised to 75 mg/mL, we observed a 2.7-fold induction of UGT1A4. These data point out that after administration of apple polyphenol extracts derived from plasma-treated samples, Caco2 cells are normally viable and are still capable of activating the physiological

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response to moderate oxidative stress by increasing the transcription of phase II detoxifying enzymes.

This is the first experimental evidence that apple exposure to gas plasma does not seem to generate chemical species potentially harmful to human cells.

We demonstrated that polyphenol extract obtained from plasma-treated samples does not induce significant changes in cell viability of human cultured colonocytes, in comparison with extracts obtained from untreated apples slides. Following the treatment, Caco2 cells retain their ability to react to moderate oxidative stress by inducing detoxifying enzymes.

Further studies including tests in vivo are required to provide sufficient information to assess the health-related implication of the application of gas plasma technology in food processing.

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Notes

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