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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 oxidation³ 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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72 the impact on antioxidant properties and bioactive compounds,
73 on the potential chemical residue effects, and on the formation
74 of toxicants is therefore needed to provide sufficient
75 information to assess the health-related implications of the
76 process.

77 The purpose of this study was to expand previous DBD cold
78 plasma effects on visual quality and enzymatic activity of fresh-
79 cut 'Pink Lady' apples⁴ to information concerning their
80 functional properties. In particular, antioxidant activity was
81 evaluated through a multimodal approach, combining different
82 assays for the analysis of antiradical activity and reducing
83 activity of antioxidants. Polyphenol extracts were further
84 analyzed by high-performance liquid chromatography–mass
85 spectrometry (HPLC-MS/MS) to quantitatively calculate
86 differences in the polyphenol composition of DBD-plasma-
87 treated versus untreated minimally processed apples. We
88 extended our investigation to the effect exerted by the above-
89 mentioned polyphenol extracts in human colonocytes. We have
90 chosen, as experimental model, the Caco2 cell line, which is
91 derived from human colon adenocarcinoma. Caco2 cells are
92 widely used for biochemical and nutritional studies because
93 they retain, upon in vitro cultivation, the morphology and most
94 of the functions of the normal small intestine epithelial.¹²
95 Caco2 cells have been previously used to study the intestinal
96 absorption of food components, such as tea polyphenols,¹³ and
97 the modulation of oxidative status by dietary flavanols.^{12,14}
98 Bearing this in mind, and clearly stating that this experimental
99 work does not have the aim to investigate potential anticancer
100 effects of apple polyphenols, we studied the effects of DBD-
101 treated and untreated apple polyphenol extracts on cell viability,
102 intracellular ROS production, and phase II enzyme activation.
103 In parallel, we treated Caco2 cells with Polyphenon E,^{15,16} a
104 standardized green tea extract, FDA approved, which represents
105 a source of highly purified and characterized green tea
106 catechins.

107 ■ MATERIALS AND METHODS

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

111 Polyphenon E, a standardized green tea extract preparation
112 ((-)-epigallocatechin-3-gallate (EGCG), 68.58%; (-)-epigallocate-
113 chin (EGC), 10.56%; (-)-epicatechin (EC), 4.31%; (-)-epicatechin-
114 3-gallate (ECG), 5.95%; (-)-gallocatechin-3-gallate (GC), and other
115 trace catechin derivatives) was supplied by Polyphenon Pharma (New
116 York).

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

125 **DBD Gas Plasma Generator, Sample Preparation, and**
126 **Plasma Treatments.** Cold plasma was generated by a dielectric
127 barrier discharge (DBD) device that was already described and
128 characterized by Ragni et al.¹⁷ It consists of a hermetic chamber
129 containing three parallel pairs of electrodes (brass) supplied by a dc
130 power supply and powered by high-voltage transformers and power-
131 switching transistors. A 5 mm thick glass was used as dielectric
132 material. As feed gas, atmospheric gas driven at 1.5 slm was chosen.
133 Frequency of oscillation was 12.7 kHz, and the power supply was in
134 the range of 150 W. The discharge showed typical air nonequilibrium
135 peaks of the second N₂ positive system and of the positive ion N₂⁺
136 together with the presence of oxygen and nitrogen radicals and ions as

commonly detected when atmospheric air is used to generate
137 plasma.¹⁷ 138

139 Apple slices (40 × 10 × 10 mm) were manually obtained from
140 apple flesh using a sharp blade. Samples were exposed to cold plasma
141 at a distance of 70 mm from the electrodes for a total of 30 (15 + 15
142 on each side) min. 142

143 In the treatment chamber and on the fruit surface, the temperature
144 was 22 °C and the relative humidity (RH), 60%. Control samples were
145 stored at the same temperature and humidity conditions for the
146 duration of the treatment. 146

147 Treatment time was stressed to 120 (60 + 60) min of processing
148 only for polyphenols and antioxidant activity determinations. 148

149 **Polyphenol Extract Preparation.** Lyophilized freeze-dried apple
150 powder was extracted in 60% methanol. For biological assay, 3 g of
151 powder was mixed with 20 mL of solvent; for HPLC analysis, 250 mg
152 of powder was extracted in 1.5 mL of solvent mixture containing 1%
153 (v/v) of formic acid. The suspension was vortexed vigorously for 2
154 min, then samples used for HPLC analysis were left for 60 min in a
155 sonic bath and centrifuged for 20 min (20878g), and supernatant was
156 collected at 4 °C and transferred to a vial before injection into the
157 HPLC system. Samples used for ex vivo assays were centrifuged for 10
158 min (10000g); the supernatant was collected, and the pellet was
159 subjected to a second extraction. The total supernatant was dried in a
160 rotary evaporator (model Laborota 4001, Heidolph, Germany) at 35
161 °C, and the dry residue was used as extract for further analysis. 161

162 **High-Performance Liquid Chromatography and Mass**
163 **Spectrometry Analysis.** Before analysis, 20 μL of each internal
164 standard was added to the samples (genistein, 580 μg/mL; genistin,
165 380 μg/mL). HPLC separations were carried out by means of a
166 SUNSHELL C18 (2.1 i.d. × 100 mm) column, 2.6 μm particle size
167 (Chromanik) with mobile phase, pumped at a flow rate of 0.3 mL/
168 min, consisting of a mixture of acidified acetonitrile (0.1% formic acid)
169 (solvent A) and 0.1% aqueous formic acid (solvent B). Following 0–2
170 min, 2% B; 2–13 min, 2–30% B; 13–20 min, 30–80% B; 20–22 min,
171 80–2% B; 22–30 min, 2% isocratic; this step was followed by the
172 washing and reconditioning of the column. 172

173 The identity of the phenolic compounds was confirmed using a
174 triple-quadrupole mass spectrometer (Thermo Scientific, TSQ
175 Vantage) with a heated electrospray ionization (H-ESI II) operating
176 in the negative ionization mode. The capillary temperature was 270
177 °C; the sheath gas and auxiliary gas were 40 and 5 arbitrary units,
178 respectively; and the source voltage was 3 kV. A vaporizer temperature
179 of 200 °C argon was used for MS/MS experiments with a collision
180 pressure of 1.0. 180

181 For the identification, a full-scan analysis was performed scanning
182 from *m/z* 100 to 950, whereas a product ion scan experiment was
183 applied for ions not fully identified in the previous method. 183
184 Identification was performed by comparing the mass spectra with
185 literature data and, whenever possible, the identification was confirmed
186 by using pure standards of the components. 186

187 **Antioxidant Activity and Total Phenolic Content.** Antioxidant
188 activity and total phenolic content of apple samples were assessed by
189 different microplate assays as previously reported.⁹ The antioxidant
190 activity was performed by 2,2'-azinobis(3-ethylbenzothiazoline-6-
191 sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and
192 ferric reducing antioxidant power (FRAP) methods. The total
193 polyphenol content was quantified by the Folin–Ciocalteu phenol
194 reagent. 194

195 **Cell Culture and Treatments.** Caco2 cells were purchased from
196 ATCC and routinely grown in a 1:1 mixture of Ham's F12/DMEM.
197 Culture medium was supplemented with 10% fetal bovine serum
198 (Lonza, Basel, Switzerland), 2 mM L-glutamine, 100 U/mL penicillin,
199 and 100 mg/mL streptomycin. Cells were incubated at 37 °C under a
200 5% CO₂ atmosphere. Cell harvesting was performed by trypsin/EDTA
201 (Sigma-Aldrich, Steinheim, Germany) treatment. 201

202 For ROS determination and luciferase assay the Caco2 cells were
203 grown in a 1:1 mixture of Ham's F12/DMEM without red phenol
204 (Sigma-Aldrich). 204

Table 1. Content of Phenolics ($\mu\text{mol kg}^{-1} \text{ dw}$) 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	415.02 ± 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

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

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

208 The polyphenol extracts from treated and untreated apples prepared
209 for biological assays were dissolved in cell-cultured medium containing
210 1% DMSO and stored at $-80\text{ }^{\circ}\text{C}$ (stock solution contains 600 mg of
211 lyophilized apple powder/mL). Fresh solutions were prepared in
212 complete cell medium at the final concentration required for each
213 experiment. The control cells were cultured with medium containing a
214 maximum of 0.08% DMSO.

215 **WST-1 Assay.** Inhibition of cell proliferation by polyphenol
216 extracts was measured by WST-1 assay (Roche, Lewes, UK). The assay
217 is based on the reduction of tetrazolium salt WST-1 to soluble
218 formazan by electron transport across the plasma membrane of
219 actively dividing cells. Formazan formation was detected at 450 nm
220 spectrophotometrically. Caco2 cells were plated in quadruplicate in
221 96-well microplates at a density of 4×10^4 cells/well and allowed to
222 adhere overnight. Cells were treated with increasing concentrations of
223 Polyphenon E (1–20 $\mu\text{g}/\text{mL}$) or polyphenol extracts from untreated
224 and treated apples (1–75 mg/mL). After 5 h of incubation, the WST-
225 1 assay was performed.

226 **Reactive Oxygen Species Determination.** The production of
227 intracellular ROS was detected using the 2,7-dichlorofluorescein
228 diacetate (DCFH-DA) assay. Briefly, Caco2 cells were seeded in
229 black 96-well plates (4×10^4 cells/well) and allowed to attach
230 overnight. After 5 h of treatment with increasing concentrations of
231 Polyphenon E (1–20 $\mu\text{g}/\text{mL}$) or polyphenol extracts (28 and 75 mg/
232 mL), cells were washed twice with PBS and loaded with 20 mM
233 DCFH-DA in PBS for 15 min at $37\text{ }^{\circ}\text{C}$. After incubation, cells were
234 washed with PBS, and ROS generation was measured by the
235 fluorescence intensity of dichlorofluorescein (DCF; exc, 475 nm;
236 em, 535 nm) using an Enspire Multimode Plate Reader (PerkinElmer,
237 Waltham, MA, USA). Inside the cells, DCFH-DA is cleaved by

nonspecific esterases forming nonfluorescent DCFH, which is oxidized
238 to the fluorescent compound DCF by ROS. In the same wells, the
239 total protein content was quantified using the Bio-Rad DC Protein
240 assay (Bio-Rad, Berkeley, CA, USA). 241

Plasmid Construction and Luciferase Assay. To generate the
242 recombinant plasmid pGL4-NQO1, genomic DNA was extracted from
243 human liver using the QIAamp DNA mini kit (Qiagen, Venlo, The
244 Netherlands) according to the manufacturer's protocol. The upstream
245 promoter region of the *Nqo1* gene (635 pb) was PCR amplified using
246 the following primers: fw 5'-ACCTGCCTTGAGGAGCAGGGG-
247 TGGTGCAG-3', rv 5'-GGCTCTGGTGCAGTCCGGGGCGCT-
248 GATTGG-3'.¹⁸ The PCR product was subcloned in the pCR2-
249 TOPO vector (Invitrogen, Carlsbad, CA, USA). The *KpnI/XhoI*
250 restriction fragment obtained from the digestion of TOPO-NQO1 was
251 then ligated into the pGL4.10 vector (Promega, Madison, WI, USA),
252 and the resulting plasmid was sequenced. 253

Caco2 cells were seeded in a 96-well white microplate at a density of
254 2×10^4 cells/well and transfected using Viafect (Promega), using 0.2
255 μg of pGL4-NQO1 and pGL4.10 empty vectors. Transfection
256 efficiency was monitored by pEGFP-N1 transfection (Clontech
257 Laboratories, Mountain View, CA, USA). 258

The luciferase activity was measured after 5 h of incubation with
259 increasing concentrations of Polyphenon E (1–20 $\mu\text{g}/\text{mL}$) or
260 polyphenol extracts (28 and 75 mg/mL) using the Britelite plus
261 reactive (PerkinElmer) and EnSpire Multimode Plate Readers
262 (PerkinElmer). The luciferase activity was normalized to the total
263 protein content after checking for equal transfection efficiency in each
264 well. 265

**RNA Extraction, Reverse Transcription, and Quantitative
266 Real-Time PCR.** Caco2 cells were seeded in 35 mm dishes at a density
267 of 6×10^5 and allowed to attach overnight. After 5 h of treatment with
268 two different concentrations of polyphenol extracts from treated or
269 untreated apple (28 and 75 mg/mL), total RNA was extracted with the
270

Table 2. Total Phenolic Index (TPI) and Antioxidant Activity ($\mu\text{mol kg}^{-1} \text{dw}$) 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 \pm 510 a	11987 \pm 568 b	11420 \pm 1189 a	7608 \pm 514 b
TPI ¹	hydrophilic	280 \pm 86 a	351 \pm 39 a	384 \pm 19 a	258 \pm 46 b
TPI ¹	hydrophilic + amphiphilic	13600 \pm 501 a	12338 \pm 567 b	11768 \pm 1192 a	7867 \pm 510 b
ABTS ²	amphiphilic	14175 \pm 300 a	13973 \pm 316 b	13998 \pm 1140 a	9930 \pm 460 b
ABTS ²	hydrophilic	911 \pm 334 a	732 \pm 302 a	567 \pm 171 a	532 \pm 138 a
DPPH ²	hydrophilic + amphiphilic	24072 \pm 2053 a	23647 \pm 2496 a	20285 \pm 745 a	16017 \pm 2285 b

271 Trizol reagent (Fisher Molecular Biology, Rome, Italy) and cleaned up
 272 with the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren,
 273 Germany) according to the manufacturer's instruction. For reverse
 274 transcription reaction, 100 ng of total RNA from each experimental
 275 condition was combined with 1 μL of random primers (0.2 $\mu\text{g}/\mu\text{L}$)
 276 and heated to 65 °C for 5 min. Following a brief chill on ice, the
 277 reverse transcription mix (Thermo Scientific, Boston, MA, USA) was
 278 incubated at 25 °C for 5 min, at 45 °C for 60 min, and at 70 °C for 10
 279 min. The first-strand synthesis reaction was diluted 1:2, and then 2 μL
 280 of each cDNA preparation was used for quantitative real-time PCR
 281 with the set of primers described below. The thermal cycling
 282 comprised an initial denaturation step at 95 °C for 30 s, followed by
 283 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension
 284 at 60 °C for 30 s. Analysis of results was performed by DNA Engine
 285 Opticon 4 (MJ Research, Waltham, MA, USA) using the 2X SYBR
 286 Premix Ex Taq (Takara Bio Inc., Japan). Relative quantification was
 287 calculated by the $2^{-\Delta\Delta\text{CT}}$ method¹⁹ using glyceraldehyde 3-phosphate
 288 dehydrogenase (GAPDH) as housekeeper gene for data normalization.
 289 The results are expressed as the mean \pm SD of two independent
 290 determinations, each performed in duplicate.

291 Primer sequences used for GST members were the following:²⁰
 292 GSTT2-fw 5'-CTTTCCTGGGTGCTGAGCTA-3' and GSTT2-rv 5'-
 293 GGTGTTGGGAGGGTTTCTT-3'; GSTP1-fw 5'-GGAGACCTC-
 294 ACCCTGTACCA-3' and GSTP1-rv 5'-CTGCTGGTCCTTCCC-
 295 ATAGA-3'; GSTA4-fw 5'-TCCGTGAGATGGGTTTATAGC-3' and
 296 GSTA4-rv 5'-GGTGGTTACCATCCTGCAAC-3'.

297 Primer sequences used for UGT members were the following:²¹
 298 UGT1A1-fw 5'-AATAAAAAAGGACTCTGCTATGCT-3' and
 299 UGT1A1-rv 5'-ACATCAAAGCTGCTTTCTGC-3'; UGT1A4-fw 5'-
 300 GAACAATGTATCTTTGGCCC-3' and UGT1A4-rv 5'-ACCACA-
 301 TCAAAGGAAGTAGCA-3'; UGT2B7-fw 5'-GGAGAATTTCAT-
 302 CATGCAACAGA-3' and UGT2B7-rv 5'-CAGAAGTTTCTAGTT-
 303 ATGTCACCAATATTG-3'.

304 **Statistical Analysis.** Data are expressed as mean values \pm SD for
 305 the indicated number of independent determinations.

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

308 ■ RESULTS AND DISCUSSION

309 **Phenolic Content.** The phenolic content of apples was
 310 measured by HPLC-MS/MS analysis, and the content of each
 311 detected phenolic is reported in Table 1. The total phenolic
 312 content of ‘Pink Lady’ apples of control untreated samples
 313 ranged between 2.47 and 2.80 $\text{mmol kg}^{-1} \text{dw}$ (that corresponds
 314 to 117–129 $\text{mg kg}^{-1} \text{fw}$), which is slightly higher than literature
 315 data (90 $\text{mg kg}^{-1} \text{fw}$) reported in a previous study.²² However,
 316 these values are lower than those of the most widely studied
 317 variety, ‘Golden Delicious’, for which values range between 233
 318 and 417 $\text{mg kg}^{-1} \text{fw}$ depending on agricultural practices and
 319 harvesting years.^{23–26}

320 In a previous study a 30 (15 + 15) min treatment was found
 321 to be suitable for fresh-cut apple stabilization⁴ because, at the
 322 same experimental conditions, a reduction in browning was
 323 observed by image analysis. In the same study, an inhibition of

polyphenol oxidase (PPO) activity proportional to treatment 324
 time up to 57% for 30 min was reported. Reduction of 325
 enzymatic activity upon plasma exposure has been attributed to 326
 the oxidation of the reactive side chain of the amino acids by 327
 plasma radicals,^{5,6} in particular OH, O₂⁻, HOO, and NO, that 328
 promotes a change in the secondary protein structure and the 329
 modification of some amino acid side chains of the enzyme.^{27,28} 330
 On the basis of these results, an oxidation of the phenolic 331
 component could be hypothesized. 332

Conversely, in this study the total phenolic content evaluated 333
 by HPLC-MS/MS did not show any significant difference 334
 compared to control apples (Table 1). To verify if a longer time 335
 of exposure to DBD plasma might determine a significant 336
 decrease of polyphenols, the product was overtreated for 120 337
 (60 + 60) min of processing. Differences in the total phenolic 338
 content were still not statistically significant, but, considering 339
 singular compounds, a few differences were found in the flavan- 340
 3-ols group; in particular, significant reductions of procyanidin 341
 B1 dimer, trimer, and trimer 4 of, respectively, 56, 59, and 62% 342
 were found. Although oxidation reactions can easily take place 343
 during plasma treatment thanks to the production of radical 344
 species and the availability of oxygen in the atmosphere, 345
 phenolic content did not show variations after 30 min, albeit at 346
 the same treatment time a noticeable enzymatic activity 347
 inhibition was previously observed and after stressing plasma 348
 exposure up to 120 min promoted only a limited reduction to 349
 very few compounds. 350

The total phenolic index (TPI) of apple products has been 351
 estimated also by measuring their ability to reduce the Folin– 352
 Ciocalteu reagent, an extensively used method for the 353
 estimation of total phenolics, after solid phase extraction on 354
 C-18 cartridges, considered suitable technique for the 355
 separation of phenolic compounds.⁹ 356

In this study, a total TPI of about 13.3 $\text{mmol kg}^{-1} \text{dw}$ (2.2 357
 $\text{mg GAE g}^{-1} \text{dw}$) was measured in the amphiphilic fraction 358
 (Table 2), after SPE separation; also in this case, this value is 359
 lower than that of ‘Golden Delicious’ apples but comparable 360
 with literature results.^{26,29} 361

The DBD plasma treatment significantly affected the TPI of 362
 apples with a decrease of about 9% after 30 min, whereas 363
 prolonging treatment time up to 120 min led to a decrease of 364
 about 33% (Table 2). 365

The spectrophotometric determination of total polyphenols 366
 overestimated the final polyphenol decrease, when compared to 367
 HPLC-MS/MS analysis that was not able to find significant 368
 differences between mean values after 30 min. This happened 369
 probably because the TPI assay is based on the capacity of 370
 phenolic compounds to reduce the Folin–Ciocalteu reagent 371
 under basic conditions, the mechanism of the TPI assay being 372
 based on an oxidation/reduction reaction. This result roughly 373
 indicates that the polyphenols which underwent oxidation with 374

375 increasing processing time were characterized by a high
376 reducing power.

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

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

397 The DBD plasma treatment significantly affected the TEAC
398 of amphiphilic compounds of apples with a slight decrease
399 (1%) after 30 min of processing, to reach values 29% lower
400 than those of control apples when overtreated (120 min). No
401 differences were found for the TEAC of hydrophilic
402 compounds between control and treated samples at both
403 processing times (Table 2).

404 The DBD plasma treatment significantly affected the TPI of
405 amphiphilic compounds of apples as well as total TPI, which
406 showed significant decreases both after 30 min (10%) and after
407 120 min of processing (33%) (Table 2).

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

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

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

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

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

432 First of all, we treated Caco2 cells with increasing
433 concentration of Polyphenon E. The concentration range of
434 green tea polyphenols examined in our study (1–20 $\mu\text{g}/\text{mL}$)
435 comprises the concentrations achievable in human plasma
436 (0.14–3.4 $\mu\text{g}/\text{mL}$) after a single oral administration of
437 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-

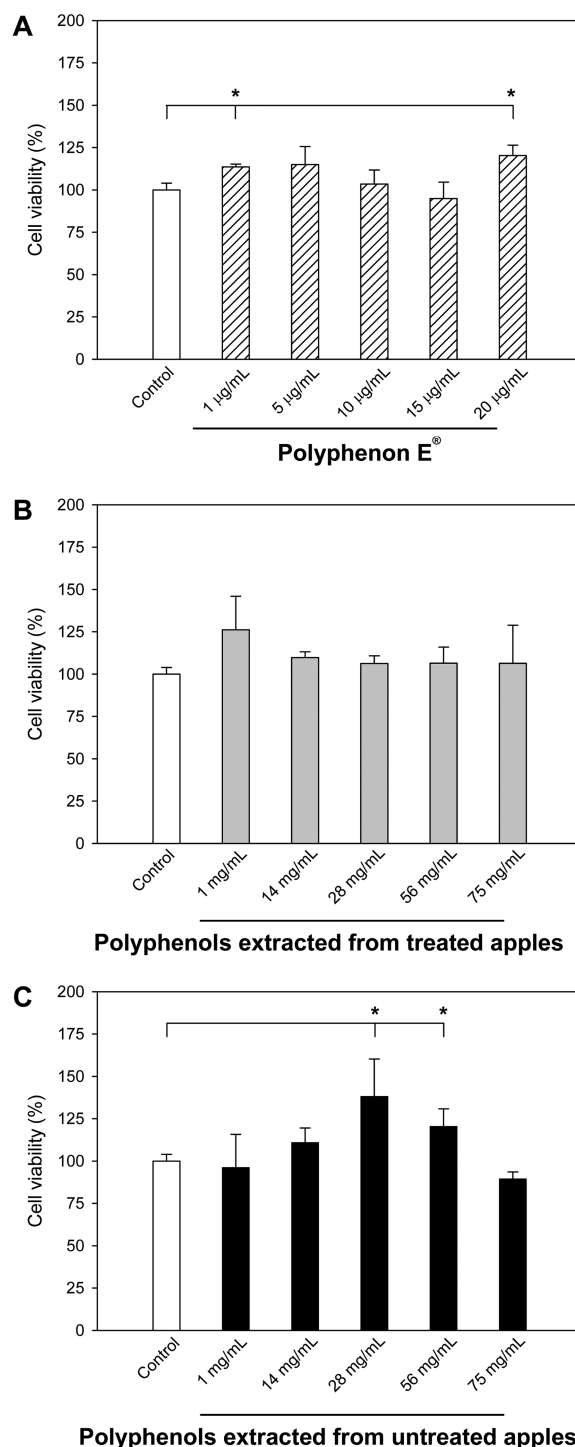


Figure 1. Effects of polyphenol extracts on Caco2 cell viability. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) and polyphenols extracted from treated (B) and untreated (C) apples. Cell viability was determined by WST-1 assay. Control represents Caco2 cells incubated with culture medium containing a maximum of 0.08% DMSO. Data are presented as means \pm 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. *, $p < 0.05$.

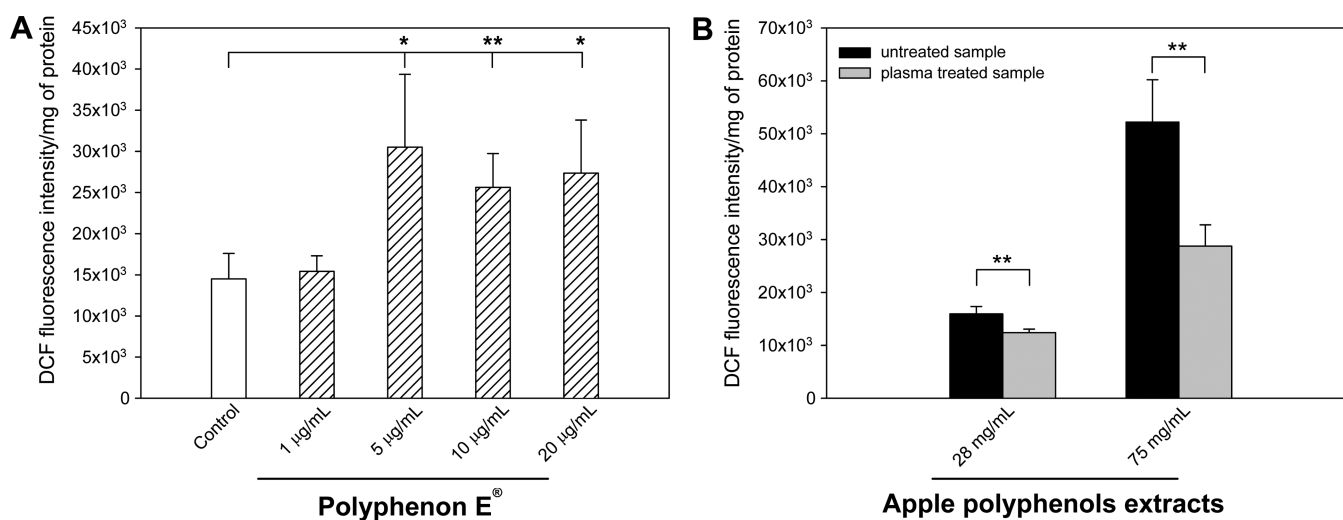


Figure 2. Effects of polyphenol extracts on Caco2 cell ROS production. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) or apple polyphenol extracts (B). ROS production was determined by DCFH-DA assay. Control represents Caco2 cells incubated with cell culture medium.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

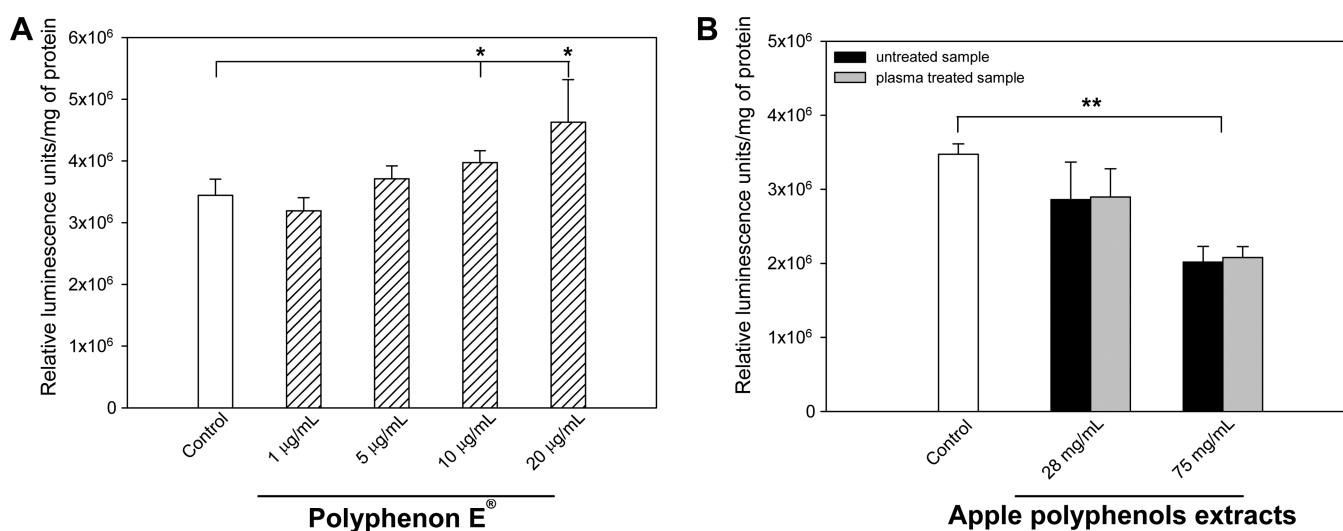


Figure 3. Effects of polyphenols on Caco2 cell luciferase activity. Caco2 cells were treated for 5 h with different concentrations of Polyphenon E (A) or apple polyphenol extracts (B). Control represents Caco2 cells incubated with culture medium containing a maximum of 0.08% DMSO. Luciferase activity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

441 workers, who reported that EGCG does not induce cytotoxicity
442 in Caco2 cells below 46 $\mu\text{g}/\text{mL}$.³¹

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

455 **Effect of Polyphenols Extracted from Treated and**
456 **Untreated Apple on ROS Production.** Intracellular ROS
457 levels affect cell viability, and high ROS concentrations can

cause cellular damage. Using the DCFH-DA assay, we
458 evaluated the modulation of intracellular ROS in Caco2. As
459 made for the WST-1 assay, we incubated Caco2 cells for 5 h
460 with Polyphenon E. We observed that concentrations ranging
461 between 5 and 20 $\mu\text{g}/\text{mL}$ induced a significant increase in ROS
462 production without reducing cell viability (Figure 2A). The
463 same biological effect has been reported on Caco2 cells after
464 incubation with high concentrations of raw apple extracts.³²
465 Green tea polyphenols may exert both an antioxidant and a
466 pro-oxidant effect in different cell lines, mainly depending on
467 the concentration tested.^{33–35} Noteworthy, the pro-oxidative
468 properties of plant-derived polyphenols are well docu-
469 mented^{33–35} and represent, at least in part, one of the
470 mechanisms that promote the activation of endogenous
471 defenses against oxidative stress.¹⁰ We then proceeded
472 incubating Caco2 cells for the same time with polyphenols
473 extracted from treated and untreated apples. As shown in 474

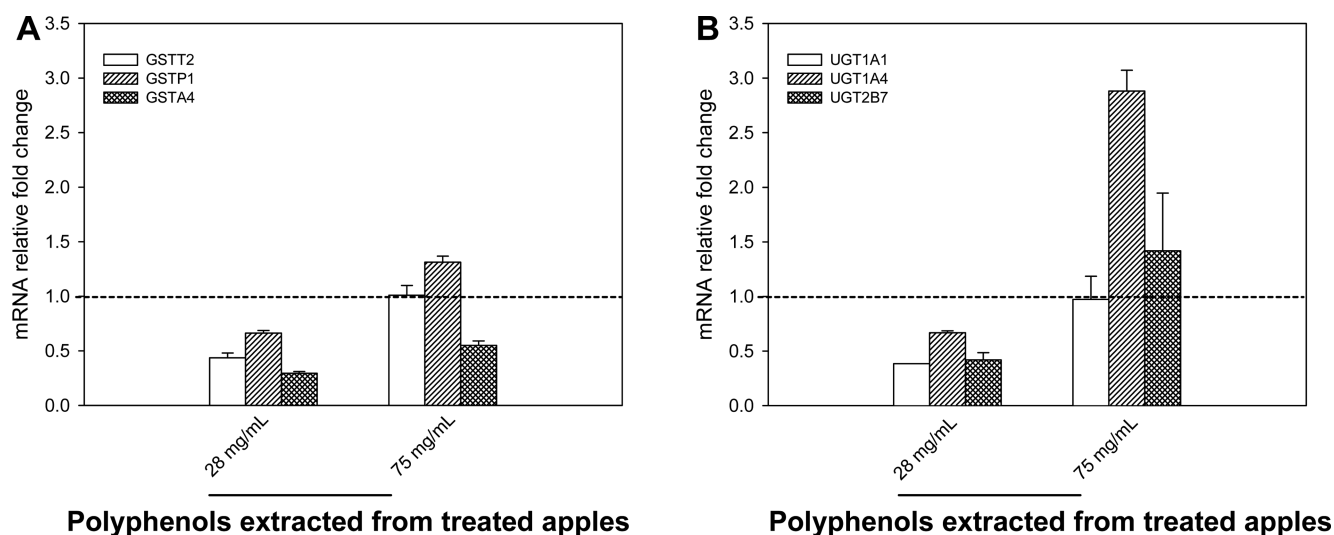


Figure 4. Relative mRNA expression of Nrf2-activated phase II enzymes in Caco2 cells. Caco2 cells were treated for 5 h with different concentrations of polyphenols extracted from treated and untreated apples. The mRNA levels of GSTs (A) and UGTs (B) enzymes were evaluated by qPCR. Relative quantification 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 \pm SD from three different experiments. We considered statistically relevant mRNA fold changes <0.75 and >1.5 .

475 Figure 2B, the production of oxidizing species is, consistent
 476 with data reported in the literature, directly related to extracts'
 477 concentration. Intracellular ROS production was in any case
 478 lower in Caco2 cells receiving polyphenols extracted from
 479 plasma-treated samples. At the concentration of 75 mg/mL the
 480 plasma-treated sample caused a decrease in ROS level of about
 481 1.8-fold in comparison to the untreated sample. None of these
 482 concentrations exerted relevant effects on cell viability.

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

488 Phase II enzymes catalyze conjugation reactions to transform
 489 toxic endogenous molecules and xenobiotics in hydrophilic
 490 compounds that can be easily excreted. They play also an
 491 important role in the metabolic inactivation of pharmacolog-
 492 ically active substances. The Nrf2/EpRE pathway is one of the
 493 more characterized cell signaling pathwayd involved in the
 494 safeguard against oxidative stress. It regulates the expression of
 495 key protective enzymes such as glutathione peroxidase (GPX),
 496 glutathione S-transferase (GST), NADPH quinine oxidoreduc-
 497 tase 1 (NQO-1), and UDP-glucuronosyltransferase
 498 (UGT).^{10,36}

499 A DNA fragment containing the EpRE sequence was
 500 subcloned upstream of a firefly luciferase into a suitable
 501 promoterless reporter plasmid. After transient transfection with
 502 the expression plasmid, Caco2 cells were treated for 5 h with
 503 increasing concentrations of Polyphenon E. At the same
 504 concentration able to induce an increase in ROS generation, we
 505 observed a statistically significant increase in luciferase activity
 506 (Figure 3A). These data support the concept that green tea
 507 polyphenols are able to induce a beneficial moderate oxidative
 508 stress, which acts through the Nrf2/EpRE pathway, activating
 509 the EpRE elements located in the promoters of target genes,
 510 such as the phase II enzymes. Unfortunately, when we treated
 511 luciferase transduced Caco2 cells with our extracts, we were not
 512 able to observe any activation of the EpRE element; on the
 513 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 xeno-
 biotics.²¹ Previous data have shown that in different colon
 tumor cell lines apple polyphenols³⁹ and digitoflavone⁴⁰ 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

553 response to moderate oxidative stress by increasing the
554 transcription of phase II detoxifying enzymes.
555 This is the first experimental evidence that apple exposure to
556 gas plasma does not seem to generate chemical species
557 potentially harmful to human cells.
558 We demonstrated that polyphenol extract obtained from
559 plasma-treated samples does not induce significant changes in
560 cell viability of human cultured colonocytes, in comparison with
561 extracts obtained from untreated apples slides. Following the
562 treatment, Caco2 cells retain their ability to react to moderate
563 oxidative stress by inducing detoxifying enzymes.
564 Further studies including tests in vivo are required to provide
565 sufficient information to assess the health-related implication of
566 the application of gas plasma technology in food processing.

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576 Notes

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