 DOI 10.1002/mnfr.201000147 *Mol. Nutr. Food Res.* 2010, *54*, 1734–1743

RESEARCH ARTICLE

Antioxidant effectiveness of coffee extracts and selected constituents in cell-free systems and human colon cell lines

Tamara Bakuradze[1], Roman Lang[2], Thomas Hofmann[2], Herbert Stiebitz[3], Gerhard Bytof[3], Ingo Lantz[3], Matthias Baum[1], Gerhard Eisenbrand[1] and Christine Janzowski[1]

[1] Department of Chemistry, Division of Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany
[2] Research Center for Nutrition and Food Science and Bioanalytic Weihenstephan Unit, Technische Universität München, München, Germany
[3] Tchibo GmbH, Hamburg, Germany

Received: March 25, 2010
Revised: April 23, 2010
Accepted: April 26, 2010

Scope: Epidemiological studies suggest that coffee can reduce the risk of degenerative diseases such as diabetes type 2, cardiovascular disease and cancer. These beneficial effects have partly been attributed to the antioxidant activity of coffee. We determined composition and antioxidant potential of differentially roasted coffee extracts and investigated the impact of selected original constituents and roast products.

Methods and results: Parameters studied were direct antioxidant activity (trolox equivalent antioxidant capacity/oxygen radical absorbing capacity), cellular reactive oxygen species (ROS) level, DNA damage and protein expression of NAD(P)H: quinone oxidoreductase, γ-glutamylcysteine ligase and glutathione reductase in HT-29/Caco-2 cells at 24-h incubation. All extracts showed distinct direct antioxidant activity: medium roasts>light roast AB1 (caffeoylquinic acid (CQA)-rich Arabica Brazil extract); dark roast AB2 (*N*-methylpyridinium (NMP)-rich Arabica Brazil extract), and diminished *t*-butylhydroperoxide-induced ROS level in HT-29 cells (AB2>medium roasts>AB1). NAD(P)H:quinone oxidoreductase 1 expression and γ-glutamylcysteine ligase expression were distinctly induced by AB1 and 5-CQA, but not by AB2 and NMP. 5-CQA and caffeic acid exhibited highest trolox equivalent antioxidant capacity/oxygen radical absorbing capacity values (5-CQA: 1.3/3.5 mM and caffeic acid: 1.3/3.9 mM trolox); ROS level was distinctly diminished by 5-CQA ($\geq 3\,\mu M$), catechol (30 μM) and trigonelline ($\geq 30\,\mu M$), whereas menadione-induced DNA damage in Caco-2 cells was reduced by NMP compounds (1–30 μM).

Conclusion: The results emphasize that both original constituents and roast products contribute to the cellular antioxidant effectiveness of coffee.

Keywords:
ARE-dependent enzymes / Caffeoylquinic acids / Coffee / *N*-Methylpyridinium / Trolox equivalent antioxidant capacity/oxygen radical absorbing capacity

Correspondence: Dr. Christine Janzowski, Division of Food Chemistry and Toxicology, Department of Chemistry, University of Kaiserslautern, Erwin-Schroedinger-Straße 52, D-67663 Kaiserslautern, Germany
E-mail: janzo@rhrk.uni-kl.de
Fax: +49-631-205-3085

Abbreviations: AB, Arabica Brazil extract; **AB1,** CQA-rich Arabica Brazil extract; **AB2,** NMP-rich Arabica Brazil extract; **ABTS,** 2,2′-azinobis-(3-ethylbenzothiazonline-6-sulfonic acid); **AC,** Arabica Columbia extract; **CA,** caffeic acid; **CAT,** catechol; **CI,** chemoluminescence intensity; **CQA,** caffeoylquinic acid; **DCF,** 2′,7′-dichlorofluorescein; **FCS,** fetal calf serum; **FI,** fluorescence increase; **FPG,** formamidopyrimidine-DNA-glycosylase; **γ-GCL,** γ-glutamylcysteine ligase; **GSR,** glutathione reductase; **Md,** menadione; **NM-2MP,** *N*-methyl-2-methylpyridinium; **NM-3MP,** *N*-methyl-3-methylpyridinium; **NMP,** *N*-methylpyridinium; **NQO1,** NAD(P)H:quinone oxidoreductase 1; **ORAC,** oxygen radical absorbing capacity; **RI,** Robusta India extract; **ROS,** reactive oxygen species; **TBH,** *tert*-butylhydroperoxide; **TEAC,** Trolox equivalent antioxidant capacity; **THB,** 1,2,4-trihydroxybenzene; **TRIG,** trigonelline

1 Introduction

Coffee is one of the most popular and widely consumed beverages worldwide. Recent data suggest that coffee consumption has beneficial effects on human health, with emphasis on type 2 diabetes, cardiovascular disease and colon cancer [1–3]. Increased formation of reactive oxygen species (ROS) and an imbalance between pro- and antioxidative processes has been implicated with the pathogenesis of these diseases [4]. Coffee brew, a complex mixture of more than a thousand bioactive compounds, is known to exhibit distinct antioxidant activity [5]. The original coffee constituents chlorogenic acids, *e.g.* the quantitatively most relevant caffeoylquinic acids (CQAs), and their phenolic degradation products, *e.g.* caffeic acid (CA), have been found to exhibit distinct radical scavenging activity *in vitro* [6]. Compounds, formed during roasting, such as the Maillard reaction products (*e.g.* melanoidins) are also known to exhibit radical scavenging and metal chelating properties [7, 8]. *N*-Methyl nicotinic acid (trigonelline (TRIG)), the second most abundant alkaloid in green coffee beans, generates non-volatile alkylpyridiniums during thermal degradation. Mainly, the quarternary base *N*-methylpyridinium (NMP) is supposed to contribute to the antioxidative effectiveness of roasted coffee [6, 9–11]. It is, however, not clear yet which constituents are most efficient in protecting cells against oxidative damage.

The aim of the present *in vitro* study was to characterize the antioxidant potential of coffees differing in geographical origin, variety and roasting technology. In addition to extracts from light, medium and dark roasts, selected constituents (Fig. 1) were included in the experiments to clarify their impact on the antioxidant effectiveness of the respective coffee brews. The phenolics 5-CQA (the major CQA isomer), CA, catechol (CAT) and 1,2,4-trihydroxybenzene (THB) were studied in comparison to TRIG, NMP ion and as minor degradation products of TRIG *N*-methyl-2-methylpyridinium (NM-2MP) and *N*-methyl-3-methylpyridinium (NM-3MP), which have also been identified in roasted coffee [12, 13].

We investigated the direct radical scavenging capacity of coffee extracts/compounds, using oxygen radical absorbing capacity (ORAC) and trolox equivalent antioxidant capacity (TEAC) assay [14]. Furthermore, effects of coffee extracts/compounds on DNA oxidation damage and ROS level were determined in the human colon cancer cell lines (HT-29, Caco-2). Modulation of cellular antioxidant defense was studied by monitoring protein expression of the ARE-dependent enzymes NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamatecysteine ligase (γ-GCL) and glutathione reductase (GSR). Since biomarker results might by affected by extracellular phenolic-mediated ROS formation [15, 16], we also studied generation of hydrogen peroxide (H_2O_2) in the cell medium during incubation with coffee constituents/extracts (with/without catalase).

2 Materials and methods

2.1 Chemicals, cells and media

5-CQA, TRIG hydrochloride, 1,2,4-trihydroxybenzene, CAT, menadione (Md), *tert*-butylhydroperoxide (TBH), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azinobis-(3-ethylbenzothiazonline-6-sulfonic acid) (ABTS), α, α′-azodiisobutyramidine dihydrochloride, catalase, RIPA buffer and protease inhibitor cocktail were obtained from Sigma-Aldrich (Deisenhofen, Germany). The iodide salts of NMP, d_3-NMP, NM-2MP and NM-3MP were prepared following literature protocols [12, 13] (*Boettler et al., submitted*). d_6-CAT and d_3-TRIG hydroiodide for quantitative purposes were available from former studies [17].

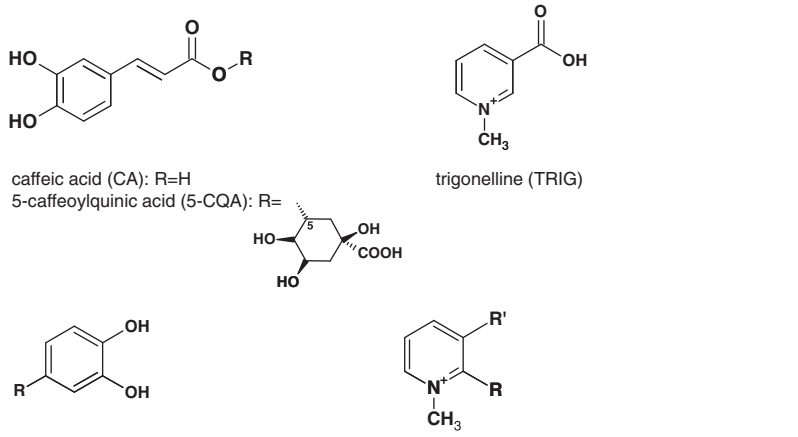

Figure 1. Structures of tested coffee compounds.

HT-29 and Caco-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and DMEM, DMEM/Nutrient Mix F12 (1:1) medium, fetal calf serum (FCS), penicillin/streptomycin and molecular protein marker See Blue Plus2 were from Invitrogen GmbH (Karlsruhe, Germany). Cell culture consumable materials were purchased from Greiner Bio-One (Essen, Germany).

Agarose, low and normal melting, was obtained from Bio Rad GmbH (Munich, Germany). The formamidopyrimidine-DNA-glycosylase (FPG) enzyme was provided by A.R. Collins (Oslo, Norway). Primary antibodies (NQO1 mouse monoclonal IgG_1, GSR rabbit polyclonal IgG, γ-GCL rabbit polyclonal IgG, α-tubulin mouse monoclonal IgG_{2a}, β-actin mouse monoclonal IgG_1) and secondary antibodies (goat anti mouse IgG_1-HRP, goat anti rabbit IgG-HRP) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Nitrocellulose membrane was provided by VWR (Darmstadt, Germany). All organic solvents and other chemicals were of analytical grade and complied with the standards needed for cell culture experiments.

2.2 Preparation and analysis of coffee extracts

Extracts, prepared from coffee beans of different geographical origin and variety, were roasted in a fluidized bed roaster (RFB Neuhaus Neotec) at the Institute of Thermal Separation Processes (Technische Universität Hamburg-Harburg): Arabica Brazil (Santos, unwashed, medium roast) (AB), Arabica Columbia (washed, medium roast) (AC), Robusta India (washed, medium roast) (RI), Arabica Brazil (Santos, unwashed, light roast, ChA-rich) (AB1) and Arabica Brazil (Santos, unwashed, dark roast, NMP-rich) (AB2).

Coffee brews were produced from ground coffee beans as described [18]. Briefly, 48 g of coffee powder was placed onto the filter of a conventional coffee machine and extracted with 900 mL of (boiling) water; the resulting coffee brews were immediately cooled (ice bath) and lyophilized.

The individual chlorogenic acid derivatives 3-, 4- and 5-CQA were quantified by HPLC-DAD using a 5-point calibration of commercially available 5-CQA as detailed in the literature [19]. CAT and NMP were measured by HPLC-MS/MS following literature protocols [17, 19]. Measurement of TRIG was performed by the addition of d_3-TRIG to an aqueous coffee solution followed by solid-phase cleanup of the mixture on RP18 cartridges and subsequent analysis by LC-MS/MS [17].

For all biochemical experiments, coffee compounds and extracts were dissolved in DMSO and double distilled water, respectively.

2.3 Antioxidant capacity (TEAC and ORAC)

Antioxidant capacity of the coffee compounds and extracts was assessed by TEAC and ORAC assay using Trolox® as antioxidant standard. TEAC was determined with the ABTS radical cation decolorization assay, according to Schaefer et al. [20]. Briefly, coffee constituents/extracts were added to pre-activated ABTS solution and absorbance at 734 nm was measured in a multiplate reader (Synergy 2, BioTek, Bad Friedrichshall, Germany). Solvent controls and Trolox® standard curve (0–15 μmol/L) were run in each assay. Percentage of decolorization and TEAC value were calculated as described [21]. The ORAC assay was performed according to Ou *et al.* [22]. Briefly, coffee constituents/extracts and Trolox® standard solutions (0–500 μmol/L) were mixed with fluorescein solution in black 96-well plates and equilibrated at 37°C. After 10 min, 2,2′-azobis(2-methylpropionamidine)dihydrochloride was added and the decrease of fluorescence was monitored for 90 min (ex/em 485/528 nm) in a multiplate fluorescence reader (Synergy 2). The area under curve and ORAC value were calculated using the given equations [22].

TEAC and ORAC values are expressed in mM Trolox, equivalent to the antioxidant capacity of a solution containing 1 mM (or 1 mg/mL) of the constituents or 1 mg/mL of the extracts, respectively.

2.4 Cell culture and incubation

HT-29 cells were maintained in 175-cm^2 flasks in DMEM, supplemented with 10% FCS and 100 U/mL penicillin/streptomycin at 37°C, 5% CO_2 and 95% saturated atmospheric humidity. Caco-2 cells were cultured under identical conditions but using the medium DMEM/Nutrient Mix F12 (1:1), supplemented with 20% FCS.

For the experiments, cells were seeded in petri dishes (Comet assay and Western Blot) or cell culture plates (dichlorofluorescein (DCF) assay) in the cell-specific culture medium described above. After a 24-h growing period, cells were incubated with coffee compounds/extracts for 24 h in FCS reduced medium (HT-29: DMEM, 5% FCS; Caco-2: DMEM/F12, 10% FCS; final solvent concentration 0.1%). Incubations of HT-29 cells were performed in the presence of 100 U/mL catalase to avoid artifactual generation of hydrogen peroxide [23]; additionally, some incubations were performed without catalase to assess a potential influence of H_2O_2 on biomarker results.

Cell viability was routinely monitored in cell suspensions after incubation with test materials/solvent by trypan blue exclusion assay and expressed as viable cells in percent of total cells (absolute viability in percentage). All results based upon experiments with viabilities >85% and cell numbers not significantly decreased, compared to the solvent controls.

2.5 Determination of hydrogen peroxide (FOX1-assay)

Hydrogen peroxide was determined in cell culture medium by the ferrous oxidation xylenol orange assay, according to

Wolff *et al.* [24] with modifications [23]. After oxidation of Fe (II) to Fe (III) by H_2O_2, the resulting xylenol orange-Fe (III)-complex is quantified photometrically (595 nm). Briefly, after 24-h incubation of medium under cell culture conditions (in 24-well plates, without cells), aliquots of medium were added to a solution of xylenol orange, sorbitol and Fe(II) in perchloric acid. After 20 min at 25°C, absorbance was monitored in a microplate reader (Synergy HT). In the samples, peroxides were quantified in comparison to a H_2O_2 standard curve (0–200 μM). Coefficient of variation (interassay CV) was 13%.

2.6 Cellular ROS level (DCF assay)

Oxidative stress was quantified in HT-29 cells by the DCF assay, according to Bellion *et al.* [25]. Briefly, cells seeded into black, clear-bottom 96-well plates (3×10^4 cells/well) and cultivated for 24 h, were incubated for 24 h with coffee compounds/extracts. After washing with PBS, cells were incubated for 30 min with 50 μM 2′7′-dichlorofluorescin-diacetate, washed and incubated with 250 μM TBH in PBS for 30 min. The increase of fluorescence (FI), resulting from oxidation of the non-fluorescent product dichlorofluorescin to DCF by intracellular ROS, was measured at 0 and 30 min in a microplate reader (ex/em: 485/528 nm). FI was calculated as $(F_{30\,\mathrm{min}} - F_{0\,\mathrm{min}})/F_{0\,\mathrm{min}} \times 100$ as described [26]. Results are expressed as relative FI in percentage of TBH control.

2.7 DNA damage (comet assay)

Caco-2 cells (2.5×10^5) were incubated with coffee compounds (1–100 μM; 24 h), washed, treated with 6 μM Md (1 h), isolated by trypsin (0.5% w/v) and used for determination of viability and DNA damage as described [27]. Alkaline single-cell gel electrophoresis was performed according to Collins *et al.* [28], with slight modifications [29]. Cells ($4 \times 50\,000$) were mixed with low melting agarose, distributed onto an agarose-coated microscope slide, submitted to lyses and covered with 50 μL of either enzyme buffer or formamidopyrimidine-DNA-glycosylase (FPG) enzyme to differentiate between DNA strand breaks and total damage (strand breaks + DNA oxidation damage). After DNA unwinding and horizontal gel electrophoresis, slides were washed, stained with ethidium bromide, examined microscopically with a Zeiss Axioskop 20 and analyzed by computerized image analysis (Comet IV, Perceptive Instruments), scoring 2×50 images *per* slide. DNA damage (with/without FPG treatment) was expressed as relative tail intensity in percentage of Md-treated control (rel. TI%).

2.8 Protein expression (Western blot analysis)

Protein expression of the ARE-dependent enzymes NQO1, γ-GCL and GSR was analyzed by standard methods [30–32]. Briefly, cells (1.5×10^6 HT29; 1×10^6 Caco-2) were incubated 24 h with coffee compounds (1–100 μM) and extracts (1–250 μg/mL) in DMEM (supplemented with 100 U/mL catalase), in comparison to the respective solvent controls. After washing with PBS, cells were lysed at 4°C in 0.1 mL RIPA buffer (with 0.1% protease inhibitor cocktail). Subsequently, the lysate was homogenized by ultrasonic treatment and centrifuged (5 min, $5000 \times g$, 4°C). Protein concentration of the supernatant was measured by the Bradford dye-binding assay. Prior to Western blotting, samples were subjected to discontinuous SDS-polyacrylamide gel electrophoresis (4% stacking gel, 14% resolving gel and protein marker) and transferred to a nitrocellulose membrane; free binding sites were blocked with 5% non-fat dried milk in TTBS. The membranes were then incubated overnight at 4°C with primary antibodies (against NQO1, γ-GCL and GSR), followed by 1 h-incubation with the respective secondary antibody (see Section 2.1) at room temperature. α-Tubulin and β-actin were used as an internal control for NQO1 and γ-GCL/GSR, respectively, to correct for unequal loading of the samples. The cross-reacting bands of target protein and internal control were visualized by use of enhanced chemoluminescence (LumiGLO, Cell Signaling Technology, Beverly, MA, USA). The respective chemoluminescent signals were analyzed with LumiAnalyst 3.1. Software (Boehringer, Germany) and expressed as arbitrary light units. In all samples, chemoluminescence intensity (CI) of target enzyme was normalized to the respective internal control ($f = \mathrm{CI}_{\text{target enzyme}}/\mathrm{CI}_{\text{internal control}}$). Results are expressed as relative CI in percentage of solvent control: rel. CI [% t/c] = $[f_{\text{treated}}/f_{\text{solvent control}}] \times 100$. Under the applied loading conditions, a direct correlation for the CI of target/internal control protein and the amount loaded protein (20–80 μg/well) was assured ($R = 0.954$ and $R = 0.887$, respectively).

2.9 Statistics

Results are reported as mean and SD of three to six independent experiments. Data of samples treated with coffee compounds/extracts were analyzed for significant difference ($p < 0.05$) to either oxidant-treated control (comet assay, DCF assay) or respective solvent control (ferrous oxidation xylenol orange assay, Western blot analysis) by unpaired one-sided *t*-test.

3 Results and discussion

3.1 Composition and cell-free antioxidant activity of the coffee extracts

Data on composition and direct antioxidant activity of the extracts are summarized in Table 1. The medium-roasted extracts AB, AC and RI exhibited rather similar concentrations of total CQAs (69–97 mg/g) and of NMP

Table 1. Composition (concentration of selected constituents, mg/g extract) and trolox equivalent antioxidant activity (TEAC, ORAC) of coffee extracts

Composition/antioxidant activity	RI[a)]	AC[a)]	AB[a)]	AB 1[b)]	AB 2[c)]
5-Caffeoylquinic acid (5-CQA) (mg/g)	33.4	41.6	39.2	58.3	5.4
4-Caffeoylquinic acid (4-CQA) (mg/g)	19.3	20.2	20.5	30.3	3.6
3-Caffeoylquinic acid (3-CQA) (mg/g)	16.6	17	17.3	25.8	3.1
Caffeoylquinic acids (Σ CQA) (mg/g)	69.3	78.8	77	114.3	12.1
Catechol (CAT) (mg/g)	n.a.[d)]	n.a.	n.a.	0.13	1.3
Trigonelline (TRIG) (mg/g)	n.a.	n.a.	n.a.	31.6	6.3
N-Methylpyridinium-iodide (NMP) (mg/g)	1.5	1.2	1.5	0.4	4.8
TEAC[e)] mM Trolox	1.3±0.0	1.5±0.1	1.3±0.0	0.9±0.1	0.9±0.1
ORAC[e)] mM Trolox	2.8±0.1	2.5±0.2	2.7±0.3	2.6±0.5	2.5±0.1

a) Medium roasts (Robusta India, RI; Arabica Columbia, AC; Arabica Brazil, AB).
b) Light roast (Arabica Brazil 1, AB1).
c) Dark roast (Arabica Brazil 2, AB2).
d) n.a.: Not analysed.
e) TEAC/ORAC values are expressed as the mmolar concentration of a Trolox solution having an antioxidant capacity equivalent to 1.0 mg/mL solution of compound/coffee extract (mean and SD from three independent experiments).

(1.2–1.5 mg/g). 5-CQA occurred in higher concentrations than the respective 4- and 3- isomers, as previously reported [8]. Compared to AB, the light roast AB1 showed distinctly higher CQA concentrations, whereas in the dark roast AB2 the amount of CQAs was found diminished by more than 80% as a consequence of thermal degradation. Accordingly, the concentration of the phenolic degradation product CAT was about tenfold higher in AB2 compared to AB1. TRIG was found to be substantially degraded upon decarboxylation during the roast, as described [12]. In the dark-roasted AB2, TRIG concentration was only 1/5 compared to the light-roasted AB1. The NMP level, however, clearly increased with the degree of roasting (AB1 < AB, AC, RI < AB2), reaching a 15-fold higher concentration in AB2 compared to AB1. Correspondingly, *N*-methylpicoliniums were found in AB2 (in low concentrations), but not in AB1.

All tested coffee extracts exhibited distinct cell-free antioxidant activity, with TEAC and ORAC values ranging from 0.9 to 1.5 mM and 2.5 to 2.8 mM Trolox, respectively (Table 1). The ORAC assay, reflecting the classical radical chain breaking antioxidant activity by H atom transfer (HAT), did not exhibit a clear ranking order for light-, medium- and dark-roasted extracts. The TEAC assay, however, which mainly utilizes a direct reduction mechanism by single electron transfer as well as H atom transfer, showed a higher effectiveness for the medium-roasted extracts AB, AC and RI, compared to the light and dark roast (AB1, AB2). Similarly, maximum antioxidant activity was reported by del Castillo *et al.* for medium-roasted coffee, compared to light and dark roasts [7].

Linear regression analysis revealed a weak positive correlation between TEAC/ORAC values of the (five) extracts and their CQA concentrations (*e.g.* 5-CQA: TEAC, $R = 0.203$; ORAC, $R = 0.233$; ΣCQA: TEAC, $R = 0.186$; ORAC, $R = 0.286$), whereas for the NMP concentration no positive correlation was obtained.

The antioxidant activities of the selected phenolic and pyridinium constituents are shown in Table 2. The TEAC/ORAC values of 5-CQA and CA agree well with previous reports [6, 20]. For the 3- and 4-CQA isomers, slightly lower TEAC values, compared to 5-CQA, have been described [33]. The phenolic degradation products CAT and THB were also distinct direct antioxidants (Table 2); CAT showed a higher peroxyl radical scavenging capacity (ORAC) than THB, which in contrast more efficiently reduced the ABTS radical (TEAC). The alkylpyridinium compounds tested were weak radical scavengers (TRIG, NMP: TEAC assay) or ineffective. Using electron spin resonance methods, TRIG showed a weak scavenging ability against 1,1-diphenyl-2-picrylhydrazyl radical, but not against the OH-radical [34]. Different radical scavenging abilities observed for differentially substituted niacin-related compounds pointed to the importance of the resonant structure of the pyridine ring [34].

On the basis of concentration (mg/g extract) and TEAC/ORAC (equivalent to 1 mg/mL solution) of the constituents, their contribution to the antioxidant activity of the extracts was calculated: The original constituents 5-CQA and TRIG were found to yield 22–23% (TEAC/ORAC) and 8% (TEAC), respectively, to the antioxidant activity of AB1; the sum of 3-CQA and 4-CQA contributes with an ~20% proportion (estimated on the basis of relative TEAC of the 3- and 4- isomers, compared to 5-CQA [33]). In addition, other chlorogenic acid compounds, such as dicaffeoylquinic acids, are also supposed to add to the antioxidant activity of AB1. In the dark-roasted AB2, according to thermal degradation of these original constituents, only small contributions were assessed, *e.g.* 2.4% for 5-CQA (TEAC/ORAC), 1.7% for TRIG (TEAC); similarly, the roast products NMP and CAT provided only a small share (< 2%) to the antioxidant activity. This strongly suggests that other antioxidant compounds, formed during the roasting such as

Table 2. Antioxidant activity (TEAC, ORAC) of selected original coffee compounds and roast products

Coffee compound	TEAC [mM Trolox]		ORAC [mM Trolox]	
	1 mM[a)]	1 mg/mL[b)]	1 mM[a)]	1 mg/mL[b)]
5-Caffeoylquinic acid (5-CQA)	1.3±0.1	3.7	3.5±0.1	9.9
Caffeic acid (CA)	1.3±0.0	7.2	3.9±0.2	21.6
Catechol (CAT)	0.9±0.0	8.2	3.4±0.1	30.5
1,2,4-Trihydroxybenzene (THB)	1.1±0.0	8.7	1.8±0.2	14.5
Trigonelline (TRIG)	0.5±0.0	2.6	n.d.[c)]	n.d.[c)]
N-Methylpyridinium-iodide (NMP)	0.4±0.0	1.8	n.d.	n.d.
N-Methyl-2-methylpyridinium-iodide (NM-2MP)	n.d.[c)]	n.d.	n.d.	n.d.
N-Methyl-3-methylpyridinium-iodide (NM-3MP)	n.d.	n.d.	n.d.	n.d.

a) TEAC and ORAC values are mean and SD from three independent experiments; expressed as the millimolar concentration of a Trolox solution having an antioxidant capacity equivalent to 1.0 mM solution of compound.
b) TEAC and ORAC values are mean and SD from three independent experiments; expressed as the millimolar concentration of a Trolox solution having an antioxidant capacity equivalent to 1.0 mg/mL solution of compound.
c) n.d.: not detectable.

melanoidins and other Maillard products [35], compensate for the losses in antioxidant original constituents.

3.2 Generation of hydrogen peroxide by coffee extracts/compounds

Since the biological effectiveness of coffee extracts and phenolic constituents might be affected by generation of ROS in cell culture experiments, H_2O_2 was monitored under the applied incubations conditions in FCS-supplemented DMEM and DMEM/F12 (used for experiments with HT-29 and Caco-2 cells, respectively).

After 24-h incubation of the extracts in DMEM (10 and 100 μg/mL), H_2O_2 generation, significantly exceeding solvent control, was only found at 100 μg/mL extract concentration. The medium roasts AB, AC and RI produced moderate H_2O_2 levels (11.9–14.5 μM); a slightly higher amount was observed for incubation with the light roast AB1 (15.9 μM H_2O_2), whereas with the dark roast AB2, H_2O_2 generation was distinctly lower (5.9 μM). When coffee constituents (1–100 μM) were tested under these conditions, alkylpyridinium compounds (TRIG, NMP, NM-2MP, NM-3MP) and hydroxybenzenes (CAT, THB) were not found to generate H_2O_2, in contrast to the phenolic acids CA and 5-CQA, which had been identified as distinct pro-oxidants at 100 μM concentration in DMEM (CA/5-CQA: 50.7/32.5 μM H_2O_2) [15, 23]. In this bicarbonate-buffered medium (whose pH adjustment is dependent on the CO_2 atmosphere in the incubator), generation of phenolate anions is facilitated at pH>7.0, promoting H_2O_2 generation particularly from polyphenols bearing an *o*-CAT or pyrogallol moiety (CAT < pyrogallol) [36]. In contrast to CA and 5-CQA, CAT and THB were found inactive (in DMEM) or weakly H_2O_2 generating (Cat, in acetate buffer [16, 36]). These differences underline the importance of the side chain moiety in the phenolic structure. In the presence of catalase (100 U/mL medium), no H_2O_2 generation was detected, in accordance to previous reports on polyphenols [23, 37, 38]. In the presence of HT-29 cells (without catalase supplementation), no H_2O_2 was found as well, due to decomposition of H_2O_2 by cellular antioxidant enzymes and/or metabolic acidification of the medium, as described [23, 39]. When coffee extracts and constituents were incubated in DMEM/F12 instead of DMEM, practically no H_2O_2 formation was observed; this can be ascribed to the presence of HEPES buffer (keeping pH ≤ 7.2) and the H_2O_2 scavenger pyruvate [23, 40, 41].

3.3 Modulation of cellular ROS level and DNA-damage

The influence of coffee extracts on TBH-induced ROS level is shown in Fig. 2A. At 24-h incubation of HT-29 cells, the lightly roasted AB1 extract (1–100 μg/mL) diminished the ROS level slightly and not in a concentration-dependent manner. The medium roasts AB, AC and RI, however, exhibited a distinct ROS-reducing efficacy at concentrations ≥ 10 μg/mL. The dark roast AB2 most effectively reduced the ROS level in the whole concentration range tested. At 10 μg/mL, AB2 diminished the ROS level down to 77% of TBH control (corresponding to a decrease of 51%, considering the value of solvent control, Co). When incubations were simultaneously performed with/without catalase, similar results were obtained, indicating that the TBH-induced ROS level was not affected by extracellular H_2O_2 generation under the applied conditions. Taken together, the results show a direct correlation between ROS-reducing potential and roasting degree of the extracts.

To elucidate which constituents might be responsible for the antioxidant efficacy of the extracts, phenolic and methylpyridinium compounds were also studied (Fig. 2B). 5-CQA exhibited a high potential to diminish the ROS

production, as previously reported [20]; at incubation with 3–30 μM, TBH-induced ROS levels even reached the values of solvent control. The other compounds were only slightly antioxidant (Cat, THB, TRIG: 30 and 100 μM) or not effective (NMP). Considering the concentration of these constituents in the extracts, their contribution to the reduction of ROS level was estimated. AB1 at 100 μg/mL (corresponding to 18 μM TRIG and 16.3 μM 5-CQA) slightly but significantly diminished the ROS level. TRIG, due to its low ROS-reducing capacity (Fig. 2B), does not measurably add to the antioxidant action of AB1. In contrast, 16.3 μM 5-CQA reduced the ROS level more efficiently than 100 μg/mL AB1, suggesting the presence of other counteracting extract constituents. In the case of the more severely roasted extracts (AB, AC, RI and particularly AB2), such influence of chlorogenic acids and TRIG is rather unlikely. For the roast product NMP, the highest extract concentration tested (100 μg/mL) corresponds to 0.5–2.2 μM NMP. Since NMP was not effective over the concentration range 1–100 μM, it does not seem to contribute to the observed antioxidant efficacy of medium- and dark-roasted extracts. The methylated analogues NM-2MP and NM-3MP (1–100 μM) did not modulate the ROS level either.

In contrast to the observed ineffectiveness of alkylpyridinium compounds in the DCF assay, NMP and its methylated analogues were found to diminish Md-induced DNA damage in Caco-2 cells (measured with FPG, Fig. 3). Highest effectiveness was exhibited by NM-2MP (1–30 μM; $p<0.05$), which achieved a U-shaped curve, with maximal reduction down to 68% of Md control at 3 μM. NM-3MP and NMP were less potent, exhibiting at best a slight reduction of DNA-damage, and TRIG was ineffective at all concentrations tested (1–100 μM). The phenolic coffee constituents Cat and THB did not reduce DNA-damage under these conditions (similar to 5-CQA [20]), whereas CA was able to diminish DNA damage (data not shown), as described [20].

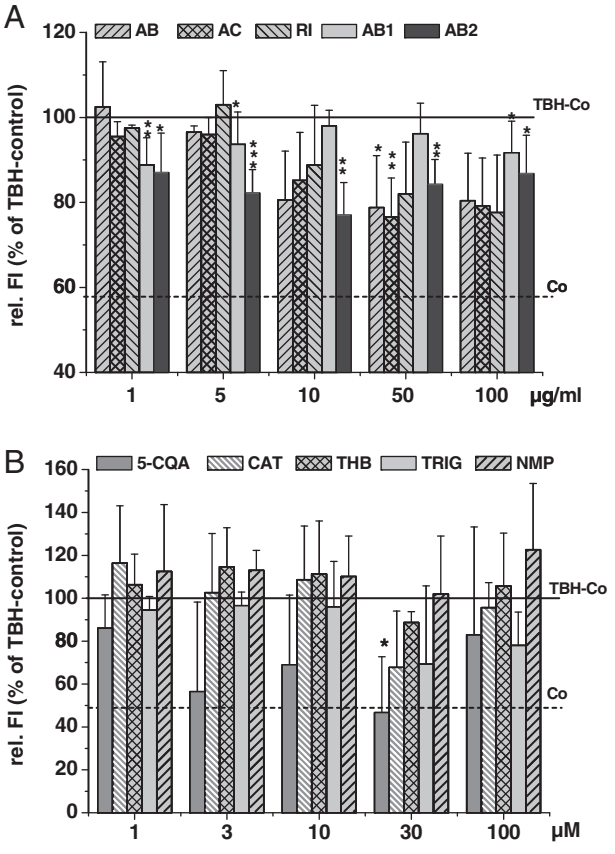

Figure 2. Effects of (A) medium (RI, AC, AB), light (AB1) and dark roasted (AB2) coffee extracts (1–100 μg/mL) and (B) coffee compounds (5-CQA, CAT, THB, TRIG, NMP; 1–100 μM) on TBH-induced ROS level in HT 29 cells after 24-h incubation. Values are expressed as increase of fluorescence (rel. FI, in percentage of TBH control), as mean and SD of three or four independent experiments, each performed in quadruplicate. Solvent control (Co): dotted line; significantly lower than TBH-control, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. TBH-control (TBH-Co).

3.4 Expression of ARE-dependent antioxidant enzymes

Modulation of NQO1, γ-GCL and GSR protein expression in HT29 cells by the coffee extracts AB1 and AB2 (0.1–100 μg/mL, 24-h incubation) and the lead compounds 5-CQA and NMP was studied to clarify its potential influence on the observed modulation of ROS level and DNA damage. Incubation with AB1 (≥1 μg/mL) resulted in distinct and concentration-dependent induction of NQO1 (up to 235%, Fig. 4). For γ-GCL, a slight increase of protein expression was observed at 1–10 μg/mL AB1, and GSR expression was only marginally elevated. AB2 exhibited no significant induction of these enzyme proteins at all concentrations tested. The effects of AB1 are in line with the described coffee-dependent induction of Nrf2/ARE-mediated cellular

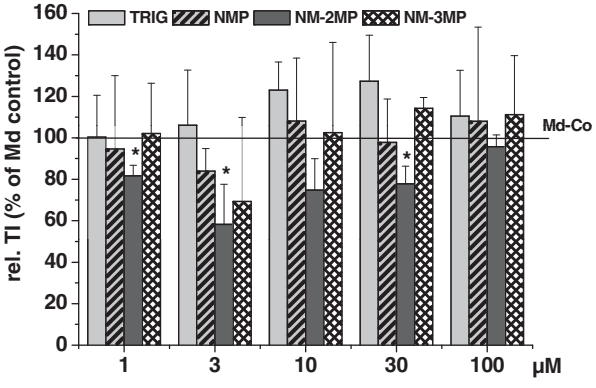

Figure 3. Effects of TRIG, NMP, NM-2MP and NM-3MP on Md-induced DNA damage (+FPG) in Caco-2 cells. Values are expressed as rel. TI% (in percentage of Md control) as mean and SD of three independent experiments. Significantly lower than Md control, $^{*}p<0.05$. Mean TI% of Md control: 8.3. Md control (Md-Co).

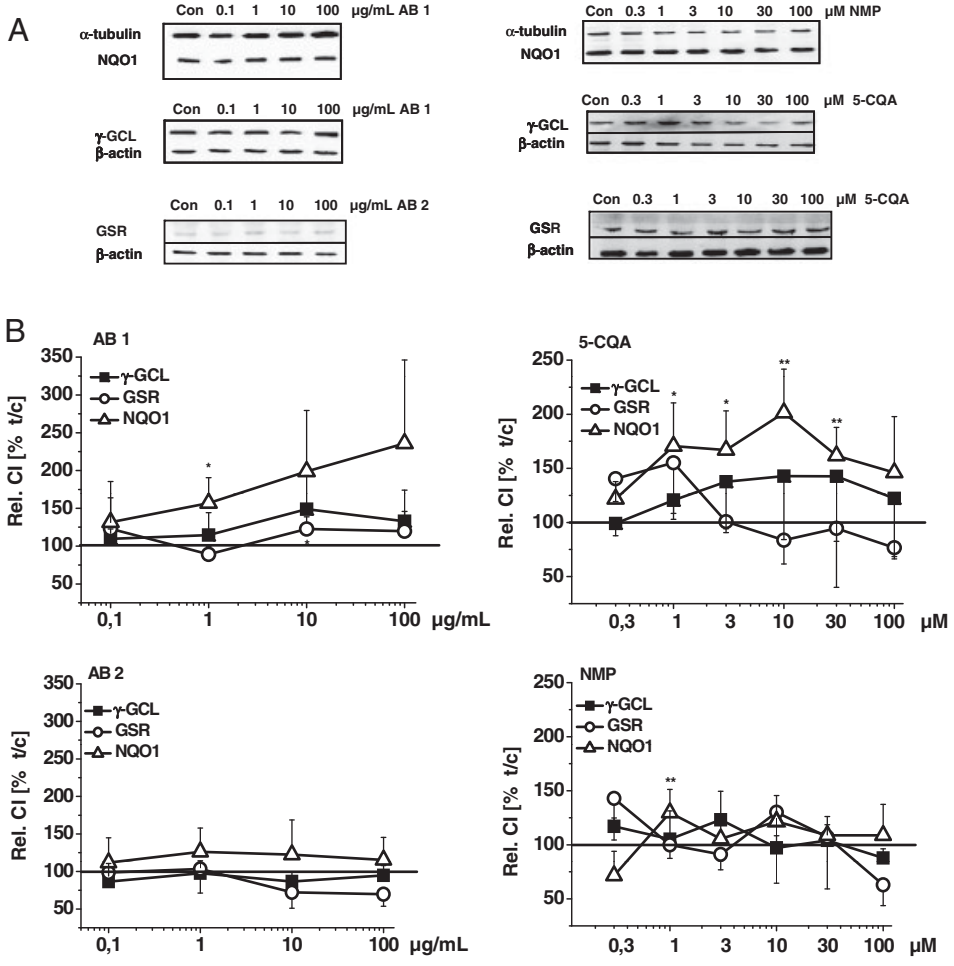

Figure 4. Effects of coffee extracts (AB1, AB2) and coffee compounds (5-CQA, NMP) on NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamate cysteine ligase (γ-GCL) and glutathione reductase (GSR) expression in HT 29 cells after 24-h incubation; Co: solvent control. (A) Representative Western blots of enzyme proteins and the respective internal control, probed and visualized simultaneously on the same membrane (NQO1; α-tubulin) or on cuts of the same membrane, using different time intervals for chemoluminescent detection (γ-GCL, GSR>β-actin). (B) Relative expression of enzyme proteins (normalized to internal control protein). Data are expressed as relative chemoluminescence intensity (rel. CI in percentage of solvent control), as mean and SD of three or four independent experiments, each performed in duplicate. Solvent control: 100% relative CI. Significantly higher than solvent control: $^{*}p<0.05$, $^{**}p<0.01$.

defense, including increased mRNA and protein expression of NQO1 and γ-GCL *in vitro* and animal experiments [31, 42].

5-CQA significantly induced the expression of NQO1 (concentration dependent, up to 200%) and, to a lower extent, also of γ-GCL (Fig. 4); expression of GSR was only elevated by 1 µM 5-CQA. This upregulation agrees with reports on the induction of ARE-dependent enzymes and stimulated nuclear translocation of Nrf2 by CQAs [43, 44]. For roast product NMP, no clear modulation of protein expression was obtained. Taken together, 5-CQA and chlorogenic acid-rich AB1 similarly induce protein expression of ARE-dependent enzymes (NQO1>γ-GCL>>GSR).

4 Concluding remarks

In summary, all tested coffee extracts exhibited distinct direct antioxidant activity in various extents, depending on the degree of roasting. The slightly higher TEAC/ORAC values of the medium roasts AB, AC and RI, compared to light and dark roast (AB1, AB2), reflect the presence of both antioxidant original constituents and generated roast products in effective concentrations. For the light roast AB1, the major phenolic 5-CQA, its isomers 3- and 4-CQA and TRIG were found to contribute substantially (~50%) to the direct antioxidant activity. To the distinct antioxidant activity of AB2, however, no contribution of these original compounds and the roast products Cat and NMP-compounds was verifiable; this implicates that other antioxidants, formed during thermal degradation, *e.g.* melanoidins and other Maillard products [35], compensate for the losses in original constituents.

In HT-29 cells, the TBH-induced ROS level was diminished by all extracts (1–100 µg/mL): AB1 < AB, AC, RI < AB2. Since the medium roasts AB/AC (both originating from *Coffea arabica*; Arabica) and RI (originating from *Coffea canephora*; Robusta) exhibited a similar ROS-reducing potential, the actual roast degree of the beans rather than species-related differences seems to be the decisive influential factor. The (moderate) activity of AB1 can be ascribed to the observed ROS-reducing efficacy of 5-CQA and other chlorogenic acids. The high effectiveness of AB2, however,

is not explainable by the presence of the weak ROS reducers CAT and NMP-compounds; this underlines the relevance of other antioxidant thermal degradations products. The distinct increase of ARE-dependent protein expression (NQO1>γ-GCL>GSR) by the light roast AB1 and its lead compound 5-CQA might well contribute to their ROS-reducing capacity. AB2 and NMP did not exhibit a clear induction of the antioxidant enzyme proteins studied. The observed significant decrease of DNA oxidation damage by methyl-substituted NMP (NM-2MP) in Caco-2 cells, however, provides evidence for an antioxidant potential of *N*-alkylpyridinium compounds.

In conclusion, our results have demonstrated distinct antioxidant activity of light-, medium- and dark-roasted coffee extracts, which can mainly be attributed to the presence of original constituents (particularly CQAs and TRIG in light roasts) and thermal degradation roast products (in dark roasts). The observed diversity in the effectiveness of coffee extracts/constituents supports multi-faceted actions, including radical scavenging activity and enhancement of cellular defense, and might well contribute to the reported health effects of coffee.

The authors thank Dr. R. Eggers and E. F. Yager for the roasting of coffee beans and S. Schmidt, M. Ochs and N. Hohn for competent assistance. The authors also thank Dr. A. Collins, University of Oslo, Institute for Nutrition Research, for the repair enzyme FPG. This work was supported by a grant of the German Federal Ministry of Education and Research (BMBF), grant no. 0313843.

The authors have declared no conflict of interest.

5 References

[1] van Dam, R. M., Willett, W. C., Manson, J. E., Hu, F. B., Coffee, caffeine, and risk of type 2 diabetes: a prospective cohort study in younger and middle-aged U.S. women. *Diabetes Care* 2006, *29*, 398–403.

[2] Bonita, J. S., Mandarano, M., Shuta, D., Vinson, J., Coffee and cardiovascular disease: *in vitro*, cellular, animal, and human studies. *Pharmacol. Res.* 2007, *55*, 187–198.

[3] Larsson, S. C., Bergkvist, L., Giovannucci, E., Wolk, A., Coffee consumption and incidence of colorectal cancer in two prospective cohort studies of Swedish women and men. *Am. J. Epidemiol.* 2006, *163*, 638–644.

[4] Halliwell, B., Gutteridge, J. M. C., *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, New York 1999.

[5] del Castillo, M. D., Gordon, M. H., Ames, J. M., Peroxyl radical-scavenging activity of coffee brews. *Eur. Food Res. Technol.* 2005, *221*, 471–477.

[6] Gomez-Ruiz, J. A., Leake, D. S., Ames, J. M., *In vitro* antioxidant activity of coffee compounds and their metabolites. *J. Agric. Food Chem.* 2007, *55*, 6962–6969.

[7] del Castillo, M. D., Ames, J. M., Gordon, M. H., Effect of roasting on the antioxidant activity of coffee brews. *J. Agric. Food Chem.* 2002, *50*, 3698–3703.

[8] Farah, A., de Paulis, T., Moreira, D. P., Trugo, L. C., Martin, P. R., Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees. *J. Agric. Food Chem.* 2006, *54*, 374–381.

[9] Daglia, M., Racchi, M., Papetti, A., Lanni, C. *et al.*, *In vitro* and ex vivo antihydroxyl radical activity of green and roasted coffee. *J. Agric. Food Chem.* 2004, *52*, 1700–1704.

[10] Somoza, V., Lindenmeier, M., Wenzel, E., Frank, O. *et al.*, Activity-guided identification of a chemopreventive compound in coffee beverage using *in vitro* and *in vivo* techniques. *J. Agric. Food Chem.* 2003, *51*, 6861–6869.

[11] Natella, F., Nardini, M., Giannetti, I., Dattilo, C., Scaccini, C., Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* 2002, *50*, 6211–6216.

[12] Stadler, R. H., Varga, N., Hau, J., Vera, F. A., Welti, D. H., Alkylpyridiniums. 1. Formation in model systems *via* thermal degradation of trigonelline. *J. Agric. Food Chem.* 2002, *50*, 1192–1199.

[13] Stadler, R. H., Varga, N., Milo, C., Schilter, B. *et al.*, Alkylpyridiniums. 2. Isolation and quantification in roasted and ground coffees. *J. Agric. Food Chem.* 2002, *50*, 1200–1206.

[14] Prior, R. L., Wu, X., Schaich, K., Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 2005, *53*, 4290–4302.

[15] Halliwell, B., Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? *Arch. Biochem. Biophys.* 2008, *476*, 107–112.

[16] Lapidot, T., Walker, M. D., Kanner, J., Can apple antioxidants inhibit tumor cell proliferation? Generation of H(2)O(2) during interaction of phenolic compounds with cell culture media. *J. Agric. Food Chem.* 2002, *50*, 3156–3160.

[17] Lang, R., Mueller, C., Hofmann, T., Development of a stable isotope dilution analysis with liquid chromatography-tandem mass spectrometry detection for the quantitative analysis of di- and trihydroxybenzenes in foods and model systems. *J. Agric. Food Chem.* 2006, *54*, 5755–5762.

[18] Lang, R., Yagar, E. F., Eggers, R., Hofmann, T., Quantitative investigation of trigonelline, nicotinic acid, and nicotinamide in foods, urine, and plasma by means of LC-MS/MS and stable isotope dilution analysis. *J. Agric. Food Chem.* 2008, *56*, 11114–11121.

[19] Weiss, C., Rubach, M., Lang, R., Seebach, E. *et al.*, Measurement of the intracellular ph in human stomach cells: a novel approach to evaluate the gastric acid secretory potential of coffee beverages. *J. Agric. Food Chem.* 2010, *58*, 1976–1985.

[20] Schaefer, S., Baum, M., Eisenbrand, G., Dietrich, H. *et al.*, Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines. *Mol. Nutr. Food Res.* 2006, *50*, 24–33.

[21] Re, R., Pellegrini, N., Proteggente, A., Pannala, A. *et al.*, Antioxidant activity applying an improved ABTS radical

cation decolorization assay. *Free Radic. Biol. Med.* 1999, *26*, 1231–1237.

[22] Ou, B., Hampsch-Woodill, M., Prior, R. L., Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* 2001, *49*, 4619–4626.

[23] Bellion, P., Olk, M., Will, F., Dietrich, H. *et al.*, Formation of hydrogen peroxide in cell culture media by apple polyphenols and its effect on antioxidant biomarkers in the colon cell line HT-29. *Mol. Nutr. Food Res.* 2009, *53*, 1226–1236.

[24] Wolff, S. P., Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* 1994, *233*, 182–189.

[25] Bellion, P., Hofmann, T., Pool-Zobel, B. L., Will, F. *et al.*, Antioxidant effectiveness of phenolic apple juice extracts and their gut fermentation products in the human colon carcinoma cell line caco-2. *J. Agr. Food Chem.* 2008, *56*, 6310–6317.

[26] Wang, H., Cao, G., Prior, R. L., Oxygen Radical Adsorbing Capacity of Anthocyanins. *J. Agric. Food Chem.* 1997, *45*, 304–309.

[27] Kamp, H. G., Eisenbrand, G., Janzowski, C., Kiossev, J. *et al.*, Ochratoxin A induces oxidative DNA damage in liver and kidney after oral dosing to rats. *Mol. Nutr. Food Res.* 2005, *49*, 1160–1167.

[28] Collins, A. R., Dusinska, M., Gedik, C. M., Stetina, R., Oxidative damage to DNA: do we have a reliable biomarker? *Environ. Health Perspect.* 1996, *104*, 465–469.

[29] Schaefer, S., Baum, M., Eisenbrand, G., Janzowski, C., Modulation of oxidative cell damage by reconstituted mixtures of phenolic apple juice extracts in human colon cell lines. *Mol. Nutr. Food Res.* 2006, *50*, 413–417.

[30] Fridrich, D., Kern, M., Pahlke, G., Volz, N. *et al.*, Apple polyphenols diminish the phosphorylation of the epidermal growth factor receptor in HT29 colon carcinoma cells. *Mol. Nutr. Food Res.* 2007, *51*, 594–601.

[31] Higgins, L. G., Cavin, C., Itoh, K., Yamamoto, M., Hayes, J. D., Induction of cancer chemopreventive enzymes by coffee is mediated by transcription factor Nrf2. Evidence that the coffee-specific diterpenes cafestol and kahweol confer protection against acrolein. *Toxicol. Appl. Pharmacol.* 2008, *226*, 328–337.

[32] Zhu, H., Cao, Z., Zhang, L., Trush, M. A., Li, Y., Glutathione and glutathione-linked enzymes in normal human aortic smooth muscle cells: chemical inducibility and protection against reactive oxygen and nitrogen species-induced injury. *Mol. Cell Biochem.* 2007, *301*, 47–59.

[33] Stalmach, A., Mullen, W., Nagai, C., Crozier, A., On-line HPLC analysis of the antioxidant activity of phenolic compounds in brewed, paper-filtered coffee. *Braz. J. Plant Physiol.* 2006, *18*, 253–262.

[34] Ogata, S., Takeuchi, M., Teradaira, S., Yamamoto, N. *et al.*, Radical scavenging activities of niacin-related compounds. *Biosci. Biotechnol. Biochem.* 2002, *66*, 641–645.

[35] Borrelli, R. C., Visconti, A., Mennella, C., Anese, M., Fogliano, V., Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.* 2002, *50*, 6527–6533.

[36] Miura, Y. H., Tomita, I., Watanabe, T., Hirayama, T., Fukui, S., Active oxygens generation by flavonoids. *Biol. Pharm. Bull.* 1998, *21*, 93–96.

[37] Kern, M., Tjaden, Z., Ngiewih, Y., Puppel, N. *et al.*, Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol. Nutr. Food Res.* 2005, *49*, 317–328.

[38] Lapidot, T., Walker, M. D., Kanner, J., Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells *in vitro*. *J. Agric. Food Chem.* 2002, *50*, 7220–7225.

[39] Wee, L. M., Long, L. H., Whiteman, M., Halliwell, B., Factors affecting the ascorbate- and phenolic-dependent generation of hydrogen peroxide in Dulbecco's modified Eagles medium. *Free Radic. Res.* 2003, *37*, 1123–1130.

[40] Long, L. H., Halliwell, B., Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. *Biochem. Biophys. Res. Commun.* 2009, *388*, 700–704.

[41] Babich, H., Liebling, E. J., Burger, R. F., Zuckerbraun, H. L., Schuck, A. G., Choice of DMEM, formulated with or without pyruvate, plays an important role in assessing the *in vitro* cytotoxicity of oxidants and prooxidant nutraceuticals. *In Vitro Cell Dev. Biol. Anim.* 2009, *45*, 226–233.

[42] Cavin, C., Marin-Kuan, M., Langouet, S., Bezencon, C. *et al.*, Induction of Nrf2-mediated cellular defenses and alteration of phase I activities as mechanisms of chemoprotective effects of coffee in the liver. *Food Chem. Toxicol.* 2008, *46*, 1239–1248.

[43] Feng, R., Lu, Y., Bowman, L. L., Qian, Y. *et al.*, Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. *J. Biol. Chem.* 2005, *280*, 27888–27895.

[44] Hwang, Y. P., Yun, H. J., Chun, H. K., Chung, Y. C. *et al.*, Protective mechanisms of 3-caffeoyl, 4-dihydrocaffeoyl quinic acid from *Salicornia herbacea* against *tert*-butyl hydroperoxide-induced oxidative damage. *Chem. Biol. Interact.* 2009, *181*, 366–376.

