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Antiglycative and antioxidative properties of coffee fractions

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ABSTRACT

In this work the inhibitory activity of coffee low molecular weight compound (LMWC) and high molecular weight compound (HMWC) fractions against *in vitro* advanced glycation end-products (AGEs) formation was investigated. The HMWC fraction was characterised for its content in total phenolic groups, proteins and carbohydrates. The chlorogenic acids of LMWC fraction were identified by liquid chromatography coupled with tandem mass spectrometry. HMWC inhibited bovine serum albumin glycation by acting as radical scavenger and Fe-chelator in the post-Amadori phase of the reaction and by inhibiting dicarbonyl reactive compounds production during glucose autoxidation. LMWC fraction was able to inhibit protein glycation and dicarbonyl reactive compounds formation more than HMWC fraction. Chlorogenic acids are the main compounds responsible for the antiglycative activity of LMWC fraction.

This study clearly shows that coffee contains molecules with *in vitro* antiglycative activity, in particular chlorogenic acids, are of particular interest for their known bioavailability *in vivo*.

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1. Introduction

In the past, a large number of scientific reports have suggested an important role of antioxidants in the prevention of chronic diseases (Perez et al., 2002). Amongst these, phenolic compounds have received considerable attention for their biological effects, such as antiglycative (Wu & Yen, 2005), antiatherogenic and cardioprotective (Dell'Agli, Buscialà, & Bosisio, 2004) activities. Another class of antioxidants receiving attention in recent years is that of melanoidins, polymeric brown compounds formed in the last stage of the Maillard reaction and involved in the colour and flavour of thermally-treated food. They are present in food and beverages such as coffee, beer, traditional balsamic vinegar, cocoa and bread (Borrelli & Fogliano, 2005; Rufián-Henares & Morales, 2007; Tagliazucchi, Verzelloni, & Conte, 2008).

Oxidative stress in humans has been linked to various diseases, such as cancer (Halliwell, 2007), atherosclerosis (Madamanchi, Hakim, & Runge, 2005) and diabetes (Thorpe & Baynes, 1996). Advanced glycation end-products (AGEs) are the result of a non-enzymatic glycation reaction between amino groups of proteins and aldehydic groups of reducing physiological sugars (Wautier & Guillausseasu, 2001).

Carboxymethyllysine (CML) is one of the most important products of the fragmentation of Amadori compounds, together with pentosidine (Peyroux & Sternberg, 2006). CML is present in plasma, renal tissue, retinas, and collagen of diabetic patients and its concentration in human tissue is proportional to age (Peyroux & Sternberg, 2006). Its presence has also been reported in intracellular neurofibrillary deposits, in patients affected with Alzheimer's disease (Cervantes-Lauren et al., 2006).

Protein glycation is a spontaneous reaction depending *in vivo* on the degree and duration of hyperglycaemia. AGEs build up slowly and can permanently alter proteins' structure and function; they accumulate mainly in proteins with long half-life, such as extracellular collagens and lens crystallin, altering their structural and biochemical properties. AGEs also contribute to reducing artery, heart and lung tissue elasticity and they seem to have a significant role in the progression of diabetic and atherosclerosis complications, Alzheimer's disease, neuropathy, nephropathy, and joint stiffness (Baynes & Thorpe, 2000; Cervantes-Lauren et al., 2006; Thorpe & Baynes, 1996). Another way of AGE formation is glucose autoxidation, through the generation of dicarbonyl compounds, such as glyoxal, which evolve to ketoimines and finally to AGEs (Wolff & Dean, 1987). During the first phase of the autoxidation and the degradation of Amadori to AGEs, metal ions, superoxide anion, hydrogen peroxide and hydroxyl radical intervene.

Coffee is a beverage consumed daily worldwide. Its consumption has been associated with reduction of chronic diseases risk and, in particular, type 2 diabetes (Van Dam & Hu, 2005). These positive properties may be due to naturally-occurring compounds present in the green beans (chlorogenic acids, trigonelline or caffeine) and/or to molecules formed during the roasting process (Maillard reaction products, melanoidins). The aim of this work was to study the inhibitory activity of coffee fractions against AGE formation.

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2. Materials and methods

2.1. Materials

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), catechin, 5-caffeoylquinic acid, and bovine serum albumin (BSA) were supplied by Sigma (Milan, Italy). All other chemical reagents and solvents were supplied by Carlo Erba (Milan, Italy). Rabbit polyclonal antibody to carboxymethyllysine was supplied by Abcam (Cambridge Science Park, Cambridge, UK). Biotinylated anti-rabbit IgG, streptavidin–horseradish peroxidase conjugate and 4-chloro-1-naphthol were supplied by Calbiochem (Darmstadt, Germany). All other western blotting and electrophoresis reagents were from Biorad (Hercules, CA). Absorbance was read using a Jasco V-550 UV/Vis spectrophotometer (Orlando, FL). Fluorescence was read using a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany).

2.2. Sample preparation

Coffee was prepared using a Bialetti Italian moka (coffee maker). Coffee was 100% Arabica and was a ready-made product bought in a local supermarket. Coffee was used for the analysis immediately after opening the package. Seventeen grams of coffee powder were put into the Italian moka with 188 ml of water; the coffee obtained was then diluted 1:5 with double distilled water and filtered with Whatman no. 4 filter paper (Maidstone, UK), to avoid clogging of the ultrafiltration units.

2.3. Coffee fractionation

Filtered sample (4 ml) was subjected to ultrafiltration with Amicon Ultra-4 nominal cutoff 10 kDa (Millipore, Milan, Italy), at 7500*g* for 50 min at 4 °C (Tagliazucchi, Verzelloni, & Conte, 2010). At the end of the separation two fractions were obtained: the retentate containing high molecular weight compounds (HMWC fraction) and the filtrate containing low molecular weight compounds (LMWC fraction). The two fractions were made up to 4 ml with distilled water for analysis. To quantify filtrate and retentate dry matter content, they were freeze-dried and the residues were weighed.

2.4. Measurements of browning

The browning index of the coffee fractions was determined by measuring the colour as absorbance at 420. The absorption at 420 nm is used to determine the concentration of melanoidins in the coffee fractions. The absorption at 280 and 325 nm was also determined. The results were expressed as l/g/cm.

2.5. Analysis of HMWC fraction

The protein content of the HMWC fraction was determined by Kjeldahl automated apparatus (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). Total phenolic group content was determined with Folin–Ciocalteu reagent (Bekedam, Schols, Van Boekel, & Smit, 2006) and 5-caffeoylquinic acid was used as standard. Carbohydrate content was assayed by phenol–sulphuric acid method (Tagliazucchi et al., 2010). Mannose was used as standard.

2.6. HPLC–ESI-MS/MS analysis of LMWC fraction

The LMWC fraction was analysed by a modification of the method of Del Rio and colleagues (2004), using a Waters 2695 Alliance separation module equipped with a Micromass Quattro Micro API mass spectrometer fitted with an electrospray interface (ESI). The chlorogenic acids were all quantified as 5-caffeoylquinic acid equivalents, monitoring the loss of caffeoyl moiety with resultant ionisation of quinic acid.

2.7. Bovine serum albumin glycation

BSA (50 mg/ml) was incubated at 37 °C for 7 days with glucose (0.8 M) in 1.5 M phosphate buffer (pH 7.4; sodium azide 0.012%) in the presence or not of a variable amount of HMWC or LMWC fraction (Wu & Yen, 2005). Since the main part of coffee polyphenols is chlorogenic acids, the antiglycative activity of 5-caffeoylquinic acid was also tested. Catechin was tested as positive control.

2.8. Fluorescence measurement of AGE products

Formation of AGEs can be measured with fluorescence at the excitation and emission maxima of 355 and 405 nm, respectively, *versus* an incubated blank containing BSA and inhibitor. The percent inhibition by different concentrations of inhibitor was calculated and data are expressed in terms of IC_{50} (concentration in μg of inhibitor/ml required to inhibit glycation by 50%) calculated from the log dose inhibition curve.

2.9. Detection of N^{ε}-carboxymethyllysine (CML)

Samples were subjected to electrophoresis by using 7.5% polyacrylamide separating gel (Laemmli, 1970) and then blotted onto a PVDF membrane. The membrane was blocked with 0.5% nonfat dry milk proteins in TBST (10 mM Tris pH 7.5, 100 mM NaCl and 0.1% Tween 20) and incubated with rabbit polyclonal antibody to carboxymethyllysine (1:10000 dilution in TBS). After washing in TBST, a biotinylated goat anti-rabbit IgG (1:1000 dilution in TBS) was added. After washing, a streptavidin–horseradish peroxidase conjugate (1:1000 dilution in TBS) was added. Proteins recognised by the antibody were revealed by colorimetric reaction with 4-chloro-1-naphthol.

2.10. Quantitative measurements of Amadori products

The concentration of Amadori compounds was determined in samples glycated with glucose in the presence or not of coffee HMWC or LMWC fractions, using a fructosamine kit (FAR; Verona, Italy; Voziyan et al., 2003). The possible interference of coffee compounds was considered by subtracting the contribution of an incubated blank containing BSA and coffee fractions. Results are expressed as mM glycosylated albumin.

2.11. Glucose autoxidation

Glucose (0.8 M in phosphate buffer 1.5 M, pH 7.4, 0.012% sodium azide) was incubated at 37 °C for 7 days. The degree of autoxidation of glucose was tested by measuring the formation of glyoxal with a specific spectrophotometric test that applies Girard-T reagent (Wolff & Dean, 1987). Results are expressed as μM glyoxal.

2.12. Fe^{2+}-chelation ability and radical-scavenging activity of coffee fractions

The method used to measure Fe^{2+}-chelation ability of HMWC fraction was developed according to Tagliazucchi et al. (2010). The Fe^{2+}-chelation ability of LMWC fraction was evaluated by the ferrozine assay (Karama & Pegg, 2009).

Radical-scavenging activity was measured with the ABTS assay (Verzelloni, Tagliazucchi, & Conte, 2007) and the results were

calculated as milligrams of vitamin C per g of dry matter. To verify the incidence of polyphenols on the total antioxidant capacity of LMWC fraction, the ABTS assay was carried out after polyphenol extraction, as described by Verzelloni et al. (2007).

2.13. Statistical analysis

All data are presented as mean ± SD for three replicates for each prepared sample. The Student's *t*-test was performed using Graph Pad Instat (GraphPad Software, San Diego, CA) where data were compared with controls. Univariate analysis of variance (ANOVA) with LSD *post hoc* test was applied using PASWStatistics 18.0 (SPSS Inc., Chicago, IL), when multiple comparisons were performed. The differences were considered significant with $p < 0.05$. The IC_{50} was calculated using non-linear regression analysis with Graph Pad Prism 5.

Table 1
Identification of phenolic compounds in coffee LMWC fraction by means of mass spectrometric specific transitions.

Compound	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)
3-CQA	353	191
4-CQA	353	173, 179, 191, 135
5-CQA	353	191, 179, 135, 173
3-FQA	367	193, 134, 173, 191
4-FQA	367	173, 193, 134
5-FQA	367	191, 173, 193, 134
3-*p*CoQA	337	163, 119, 191, 173
4-*p*CoQA	337	173, 163, 119
5-*p*CoQA	337	191, 163, 173
1,3-diCQA	515	353, 191
3,4-diCQA	515	353, 191
3,5-diCQA	515	353, 191, 179
4,5-diCQA	515	353, 191, 173, 179

CQA: caffeoylquinic acid; *p*CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acid; diCQA: dicaffeoylquinic acid.

Table 2
Concentration of phenolic compounds in coffee LMWC fraction.

Compound	mg/g of LMWC
CQA	65.4 ± 0.79
*p*CoQA	0.70 ± 0.05
FQA	6.14 ± 0.45
diCQA	0.40 ± 0.04

Data expressed as mean value ± SD ($n = 3$) in 5-caffeoylquinic acid equivalents.

3. Results

Coffee was fractionated by ultrafiltration through a membrane with a nominal cutoff of 10 kDa, to separate high molecular weight compounds from low molecular weight ones. The total dry matter content of coffee was 24.5 ± 5.3 mg/ml; dry matter content of HMWC fraction was 11.5 ± 1.3 mg/ml, whereas the LMWC dry matter content was 14.8 ± 1.9 mg/ml. HMWC fraction was characterised for its content of total phenolic groups (251 ± 18.3 mg of phenols/g), total proteins (67.0 ± 9.41 mg of proteins/g) and total carbohydrates (711 ± 33.9 mg carbohydrates/g). The absorbance measured at 420 nm of HMWC fraction was 1.053 l/g/cm; this high value indicates that this fraction is rich in melanoidins. The absorbance at 280 nm was 6.089 and at 325 nm was 5.128 l/g/cm. The peak at 280 nm in the melanoidins fraction can be explained by the presence of proteins and chlorogenic acids incorporated in the melanoidins structure. The absorption at 325 nm could be due to the presence of chlorogenic acids bound to the melanoidin skeleton; in fact Bekedam, Schols, Van Boekel, and Smit (2008) showed that intact chlorogenic acid molecules were incorporated in coffee melanoidins upon roasting.

The content in chlorogenic acids of LMWC fraction was determined with LC–ESI-MS/MS analysis. The identified chlorogenic acids and the quantitative data are reported in Tables 1 and 2. The total content of chlorogenic acids in LMWC fraction was 72.6 mg of 5-caffeoylquinic acid equivalents/g of LMWC dry matter. The absorbance measured at 420 nm of LMWC fraction was 0.447 l/g/cm, suggesting that low molecular weight Maillard reaction products are present in this fraction; the absorbance at 280 nm was 10.779 and at 325 nm was 9.001 l/g/cm. For this fraction the absorption maximum at 280 nm can be explained by the presence of caffeine, chlorogenic acids, peptides and amino acids, whilst the absorption maximum at 325 nm could be due to the presence of free chlorogenic acids.

The concentrations of HMWC and LMWC fractions able to inhibit 50% of BSA glycation measured with fluorescence method are reported in Fig. 1. Coffee HMWC fraction was less active (IC_{50} = 274 ± 1.40 μg of HMWC fraction dry matter/ml) with respect to LMWC fraction (IC_{50} = 60.0 ± 1.37 μg of LMWC fraction dry matter/ml). The antiglycative activity of 5-caffeoylquinic acid

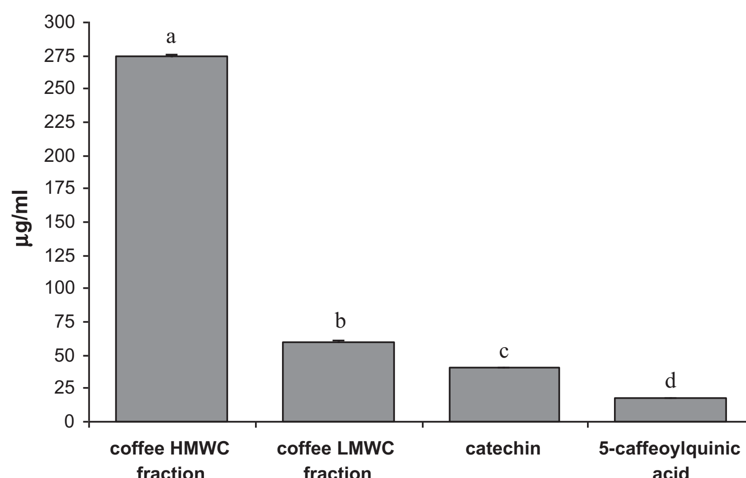

Fig. 1. IC_{50} values expressed as μg/ml of HMWC fraction, LMWC fraction, catechin and 5-caffeoylquinic acid. These values represent the quantity necessary to inhibit 50% of BSA glycation in the presence of glucose measured with fluorescence method. Bars that do not share identical letter superscripts are different at $p < 0.05$.

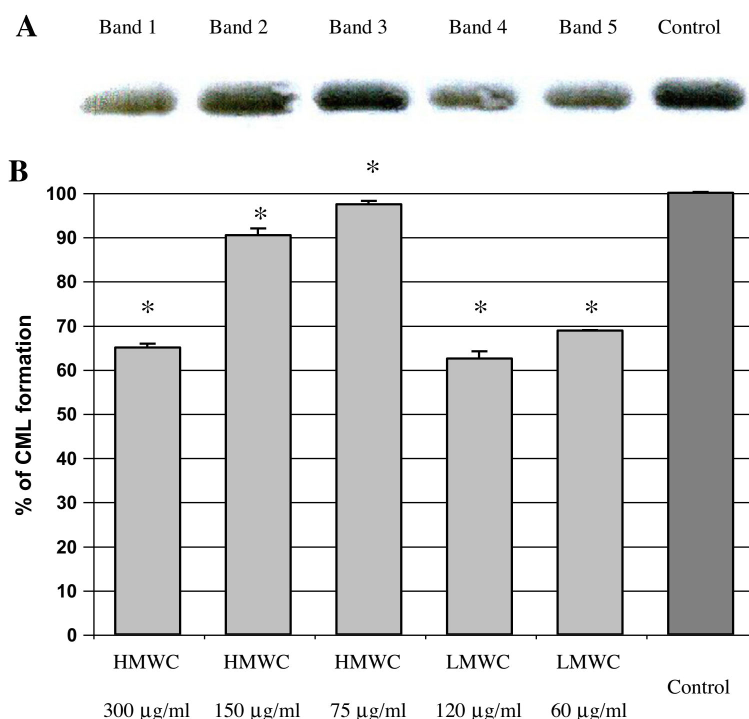

Fig. 2. (A) Western blot detection of CML synthesised by reacting BSA with glucose in the presence or not of different amounts of coffee HMWC and LMWC fractions. (B) Densitometric analysis of western blot reported in A; graph showing the percentage of CML formation for the different concentrations of HMWC and LMWC fractions, with respect to the control. Results are the mean ± SD ($n = 3$). * Denotes $p < 0.05$ with respect to the control. The p-value was calculated with Student's p-test. Band number (1) is BSA glycated with glucose and 300 µg of HMWC fraction dry matter/ml; (2) is BSA glycated with glucose and 150 µg of HMWC fraction dry matter/ml; (3) is BSA glycated with glucose and 75 µg of HMWC fraction dry matter/ml; (4) is BSA glycated with glucose and 120 µg of LMWC fraction dry matter/ml; (5) is BSA glycated with glucose and 60 µg of LMWC fraction dry matter/ml; and (6) is BSA glycated with glucose (control).

was also determined (Fig. 1); its IC_{50} value was 17.2 ± 0.38 µg/ml. Catechin was used as positive control and its IC_{50} value was 40.2 ± 0.48 µg/ml, in line with previously reported data (Wu & Yen, 2005).

In Fig. 2 the effect of HMWC and LMWC fractions on the formation of CML is shown. Both coffee fractions were able to inhibit the formation of CML in a concentration-dependent manner. Inhibition of CML formation was 35%, 9.6% and 2.6% when HMWC concentrations were 300, 150 and 75 µg/ml, respectively. For LMWC fraction the inhibition of CML formation was 37.6% and 31.2% when LMWC concentrations were 120 and 60 µg/ml, respectively. HMWC and LMWC fractions showed high radical-scavenging activity of 133 ± 5.6 and 211 ± 3.7 mg vitamin C/g of dry matter, respectively. Polyphenols contributed for about 70% of the antioxidant capacity of the LMWC fraction (141 ± 1.2 mg vitamin C/g of LMWC dry matter), despite representing only 7.3% of the dry matter content. The remaining radical-scavenging activity is probably due to the low molecular weight Maillard reaction products. Since heavy metals chelation capacity could also be involved in the antiglycative activity, this feature was verified at concentrations that inhibit glycation. This analysis demonstrated that, at a concentration equivalent to IC_{50}, only HMWC fraction showed a remarkable chelation capacity (Table 3). To verify if HMWC and LMWC fractions could act as post-Amadori inhibitors of glycation, the concentration of Amadori products in glycated samples was measured. There was an accumulation of Amadori products when HMWC fraction was incubated with BSA and glucose, with respect to the control (Fig. 3). On the contrary, LMWC fraction caused a decrease in Amadori products concentration, with respect to the control (Fig. 3).

Table 3
Fe^{2+}-chelation capacity of coffee fractions.

Fraction µg/ml	% Fe^{2+}-chelation
HMWC	
274[a]	38.0 ± 1.0*
548[b]	60.0 ± 1.9*
1000	76.2 ± 1.9*
LMWC	
60.0[a]	0.4 ± 0.1
120[b]	1.8 ± 0.3*
1000	20.9 ± 1.5*

The reported values are the means ± SD ($n = 3$).
[a] This is the concentration of HMWC or LMWC at which 50% inhibition of BSA glycation is observed (IC_{50}).
[b] This is double the concentration of HMWC or LMWC at which 50% inhibition of BSA glycation is observed ($2 \times IC_{50}$).
* Denotes $p < 0.05$ with respect to the control. The p-value was calculated with Student's t-test.

The effect of HMWC and LMWC fractions on glucose autoxidation pathway has also been considered. Glucose autoxidation was monitored by measuring glyoxal formation in the absence (control) or presence of HMWC or LMWC fractions, at concentrations corresponding to glycation IC_{50} and double this quantity ($2 \times IC_{50}$). A glyoxal formation of 175 ± 4.43 µM was observed in the control without inhibitor. Coffee HMWC fraction was able to inhibit glucose autoxidation, with a glyoxal formation of 22.6 ± 2.93 and 3.11 ± 2.10 µM at IC_{50} (275 µg/ml) and $2 \times IC_{50}$ (550 µg/ml), respectively. The glyoxal formed was 35.7 ± 0.63 and 20.4 ± 3.08 µM when coffee LMWC fraction was applied at IC_{50} (60 µg/ml) and $2 \times IC_{50}$ (120 µg/ml), respectively. Incubation of BSA with glyoxal at the concentration formed during glucose autoxidation (175 µM) caused a slight formation of glycated BSA (only 13% with

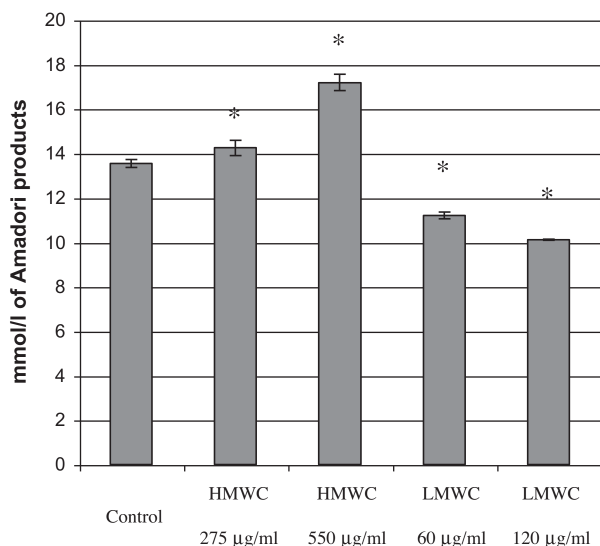

Fig. 3. Concentration of Amadori products during glycation of BSA with glucose in presence or in absence of coffee HMWC or LMWC fractions. The concentration of HMWC and LMWC reported are the quantity necessary to inhibit 50% of glycation (IC_{50}) and double the quantity ($2 \times IC_{50}$). Results are the mean ± SD ($n = 3$). * Denotes $p < 0.05$ with respect to the control. The p-value was calculated with Student's t-test. The IC_{50} values for HMWC and LMWC are 274 and 60.0 µg/ml, respectively.

respect to the sample glycated with glucose), suggesting that glyoxal-mediated glycation is not the predominant mechanism in our system.

4. Discussion

In this work, high and low molecular weight compounds present in coffee were separated and their antiglycative activity was investigated. Both the fractions showed remarkable antiglycative activity with the LMWC fraction being the more effective. Most of the inhibitors of protein glycation act as metal chelating and/or as free radical scavengers. For example, pyridoxamine blocks Amadori-to-CML conversion by interfering with the catalytic role of redox metal ions that are required for this glycoxidative reaction (Voziyan et al., 2003) and phenolic antioxidants have been proposed to inhibit AGEs formation, acting as free radical scavengers (Matsuda, Wang, Managi, & Yoshikawa, 2003). Antiglycative mixtures, such as green tea extract (Lunceford & Gugliucci, 2005), *Ilex paraguariensis* (Lunceford & Gugliucci, 2005) and other plant extracts (Kim & Kim, 2003) have been investigated and their potential antiglycative activity has been related to their polyphenolic content. The LMWC fraction of coffee is rich in phenolic compounds and is mainly composed by caffeoylquinic, *p*-coumaroylquinic, feruloylquinic and dicaffeoylquinic acids. The isomers of caffeoylquinic acid represent about 90% of the identified phenolics and because of their high antiglycative activity they could be considered the main reason for the action of coffee LMWC fraction. However, it is also possible that low molecular weight non-phenolic compounds, including Maillard reaction products, might display antiglycative activity, since they are antioxidants (Yen, Wang, Chang, & Duh, 2005). Coffee contains other non-phenolic low molecular weight compounds, such as caffeine, nicotinic acid, trigonelline, and N-methylpyridinium, but it has been shown that they have low *in vitro* antioxidant activities (Nakagawa, Yokozawa, Tarasawa, Shu, & Juneja, 2002; Somoza et al., 2003; Yen et al., 2005).

Coffee also contains HMWC that showed high antioxidant, chelating and antiglycative activities. The presence of a high concentration of polyphenols in this fraction could explain these properties. It has been demonstrated that part of the carbohydrates, proteins and polyphenols present in the HMWC fraction of coffee are incorporated in the melanoidin skeleton (Bekedam et al., 2006), and that coffee melanoidins are responsible for the antioxidant activity of HMWC fraction (Borrelli et al., 2002). This suggests that melanoidins could be mainly responsible for the antiglycative activity of this fraction.

The two coffee fractions act differently in inhibiting protein glycation. In the post-Amadori phase of the reaction, where superoxide anion and metal ions are involved, chelating and free radical-scavenging properties are important for antiglycative capacity. The HMWC fraction, that shows a high chelating ability and antioxidant capacity, caused an accumulation of Amadori compounds, suggesting that its action is paramount in the post-Amadori phase of the reaction. On the other side, the decrease in concentration of Amadori compounds with increasing quantity of coffee LMWC fraction suggests that low molecular weight compounds may act also before Amadori compounds formation and this effect does not involve antioxidant and chelating activity.

Oxidative stress is also involved in the glucose autoxidation pathway that forms reactive dicarbonyl species. These dicarbonyl compounds can react directly with proteins and contribute to AGE formation. In this study, both coffee fractions were able to inhibit glyoxal formation during glucose autoxidation. The inhibition of glucose autoxidation pathway may be a consequence of the antioxidant activity or the glyoxal-trapping activity of coffee fractions. It has been recently shown that phenolic compounds are able to trap reactive dicarbonyl compounds, inhibiting glycation reaction (Sang et al., 2007). However, the glyoxal-mediated glycation in our system is very low, suggesting that the most important inhibitory effect of the coffee fraction is linked to the glucose-mediated pathway. This fact also explains why the results displayed in Fig. 2 (CML analysis) are not in line with those of the glyoxal analysis, although glyoxal is a precursor of CML.

To exert their beneficial effects throughout the body, dietary components must be absorbed into the gastrointestinal tract. Melanoidins absorption is dependent on their molecular weight and water solubility, and low molecular weight compounds seem to play a paramount role (Finot & Magnenat, 1981). Studies using rats and ^{14}C and ^{15}N-tracer isotopes indicated that only a small proportion of high molecular weight melanoidins were absorbed through the gut wall, the vast majority being excreted in faeces. The metabolic transit of high molecular weight melanoidins (>10 kDa) isolated from casein–^{14}C-glucose and glycine–^{4}C-glucose mixtures was extensively studied (Finot & Magnenat, 1981). Most of the melanoidins (87–93%) were excreted with the faeces whilst the remainder were absorbed. The question if melanoidins are absorbed, metabolised and, thus, able to influence human health still has to be answered. However, melanoidins could reach high concentration in the stomach, where they may exert beneficial effects (Tagliazucchi et al., 2010). Recent studies have demonstrated the bioavailability of chlorogenic acids (Stalmach et al., 2009).

Blood glucose concentration in diabetic humans could reach the level of 30 mM, which is about 26 times less than the concentration used in this study. Considering the ratio of chlorogenic acids to glucose examined in this study, a concentration of about 2 µM chlorogenic acids may be necessary for efficacy *in vivo*. Stalmach and colleagues (2009) found a 1 µM concentration of total chlorogenic acids in plasma, after the ingestion of a cup (200 ml) of coffee containing about 412 µmol of chlorogenic acids; in our study 200 ml of coffee contain about 600 µmol of chlorogenic acids, so it is reasonable thinking that after the ingestion of two cups of coffee the plasma concentration of chlorogenic acids may reach a concentration of about 2 µM and this quantity of polyphenols can offer some protection against glucose-induced protein damage.

In conclusion, these results clearly show that coffee contains molecules with *in vitro* antiglycative activity, and in particular the LMWC fraction is able to inhibit protein glycation and the production of dicarbonyl reactive molecules. In this fraction, great importance is conferred to chlorogenic acids, especially caffeoylquinic acid isomers. In some epidemiological studies, high coffee consumption was significantly associated with a decreased risk of type 2 diabetes (Paynter et al., 2006; Van Dam & Hu, 2005; Van Dijk et al., 2009). This effect can be partially explained by the action of chlorogenic acids on glucose metabolism; in particular it has been shown that after coffee ingestion chlorogenic acids lead to a significantly lower glucose and insulin concentrations in humans blood (Van Dijk et al., 2009). Nevertheless, the antiglycative activity of coffee LMWC fraction and in particular of chlorogenic acids could also be important in the prevention of diabetic complications, suggesting another role of coffee in the prevention of the adverse effects of this disease.

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