Universidad Autónoma de Madrid

Sustainable use of coffee silverskin as a natural source of bioactive compounds for diabetes

Uso sostenible de la cascarilla de café como fuente natural de compuestos bioactivos para la diabetes

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Que el presente trabajo titulado **"Sustainable use of coffee silverskin as a natural source of bioactive compounds for diabetes"** constituye la memoria que presenta Beatriz Fernández Gómez, Licenciada en Ciencia y Tecnología de los Alimentos, para optar al grado de doctora ha sido realizada bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación del CSIC.

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List of publication

Very related to PhD thesis:

1. del Castillo MD, **Fernandez-Gomez B**, Ullate M, Mesa MD. Spain patent 201431848, 2014.
2. **Fernandez-Gomez B**, Ullate M, Picariello G, Ferranti P, Mesa MD, del Castillo MD. New knowledge on the antiglycoxidative mechanism of chlorogenic acid. Food Funct. 2015May; 6(6):2081-2090.
3. **Fernandez-Gomez B**, Ramos S, Goya L, Mesa MD, del Castillo MD, María Ángeles Martín**.** Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells**.** Food Res Int. 2016. Accepted. doi: 10.1016/j.foodres.2016.03.006.
4. **Fernandez-Gomez B**, Nitride C, Ullate M, Mamone G, Ferranti P, del Castillo MD**.** Use of phytochemomics for validating the potential of coffee silverskin extract as natural source of inhibitors of the glycoxidation reaction. Food Funct. Submitted the 04 of April 2016.
5. **Fernandez-Gomez B**, Lezama A, Amigo-Benavent M, Ullate M, Herrero M, Martín MA, Mesa MD, del Castillo MD. Insights on the health benefits of the bioactive compounds of coffee silverskin extract. J of Funct Foods Submitted the 04 of March 2016.
6. del Castillo MD, **Fernandez-Gomez B**, Martinez-Saez N, Iriondo A, Mesa MD. Coffee By-Products. In: Farah A, editor. Coffee: Chemestry, Quality and Health Implications. RSC Publishing Inc; In press.

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1. Culetu A, **Fernandez-Gomez B**, Ullate M, del Castillo MD, Andlauer W. Effect of theanine and polyphenols enriched fractions from decaffeinated tea dust on the formation of Maillard reaction products and sensory attributes of breads. Food chem. 2015 Apr;197(Part A):14-23.

“Me interesa el futuro porque es el sitio donde voy a pasar el resto de mi vida”

Woody Allen

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Abbreviations

AGEs	Advanced glycation end products
BSA	Bovine serum albumin
CEL	Carboxyethyl-lysine
CGA	Chlorogenic acid
CML	Carboxymethyl-lysine
CS	Coffee silverskin
CSE	Coffee silverskin extract
CVD	Cardiovascular disease
3-DG	3-Deoxyglucosone
DOLD	Deoxyglucasone-lysine dimer
DPP4-Is	Dipeptidyl peptidase 4 inhibitors
FFA	Free fatty acid
GLP-1	Glucagon-like peptide
GOLD	Glyoxal-lysine dimmer
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione reduced
GSSG	Glutathione oxidized
HbA1c	Glycated haemoglobin
IFG	Impaired fasting glucose
IGF-1	Insulin-like growth factor
IGT	Impaired glucose tolerance
MGO	Methylglyoxal
MOLD	Methylglyoxal-lysine dimmer
NA	Nicotinamide
ROS	Reactive oxygen species
STZ	Streptozotocin
T2D	Type 2 Diabetes

Summary

Introduction

Coffee is the most consumed drink in the world (1). Therefore, large amounts of by-products are generated during the coffee industrial processing (2–4). Coffee silverskin (CS) is a thin tegument of the outer layer of the two beans forming the green coffee seed that is obtained as a by-product of the roasting process (5). The extraction of bioactive compounds from natural products is increasingly being used to prepare dietary supplements/nutraceuticals, food ingredients and some pharmaceutical products. Our research group patented an aqueous extract of coffee silverskin (CSE) (P201131128) that is rich in different bioactive compounds, especially chlorogenic acid (CGA) and caffeine.

CGA formed by esterification of caffeic and quinic acids is one of the most abundant polyphenol in CSE (6,7). CGA has shown antioxidant (8), anti-inflammatory (9–11) and antiglycative (12,13) properties *in vitro* and *in vivo*. CSE is also a good source of caffeine (6,14) and it may improve the antioxidant status in humans (15,16). Antioxidants in general and phytochemicals in particular play an outstanding role in lowering chronic disease risk like Type 2 Diabetes (T2D) (17,18).

T2D is very complex and multifactorial metabolic disease characterized by insulin resistance and beta cell failure leading to elevated blood glucose level. Hyperglycemia was estimated to be one major factor contributing to diabetic complications including accelerated non-enzymatic glycation (formation of advanced glycation end products (AGEs) (19,20), an increase in oxidative stress due to the imbalance between the generation of reactive oxygen species (ROS)

and the organism's antioxidant potential, and the increase of reactive carbonyl compounds caused by their enhanced formation and/or decreased degradation or excretion (21). Coffee consumption has been associated to a wide variety of health beneficial effects, in particular the reduced risk of T2D (22). In our knowledge, the mechanism of action of CSE bioactive compounds in T2D complications is still unknown. To achieve this goal, the antidiabetic effect of CSE bioactive compounds was evaluated *in vitro* and *in vivo*.

Rationale of the study

The aim of this thesis is to obtain novel scientific evidences to demonstrate the effects of CSE in T2D. Since the health and wellness of diabetics is principally affected by complications associated to formation and accumulation of advanced glycation end products (AGEs) in the organisms, the first approach was to evaluate the antglycoxidative properties of the extract. These studies were performed *in vitro* and applying phytochemomics technologies. Studies of bioaccesibility and metabolism of caffeine and CGA present in CSE has been also performed. *In vivo* bioactivity is highly influenced by the bioaccesibility and metabolism of the food components. *In vitro* studies are not enough to demonstrate the feasibility of bioactive extracts for the reduction of the risk or treatment of chronic diseases. The effect of CSE on biomarkers of diabetes in cell culture and *in vivo* was tested.

Protein glycoxidation model systems were prepared containing bovine serum albumin (BSA) in the presence or absence of CGA or CSE for testing their effects on the glycoxidation reaction and potential in the prevention of complications of diabetes such as nephropathy, retinopathy and neuropathy. The glycoxidation reaction was started by the addition of methylglyoxal (MGO) and the mixtures were incubated at 37°C at different times. MGO was selected because its physiological relevance in the formation of *in vivo* AGEs and diabetic complications.

To elucidate the mechanism of action of CSE in the pathogenesis of T2D, a beta beta cell line (INS-1E) was used. INS-1E cells were treated with different doses of CSE (1-10 μg/mL), CGA (1-10 μM) or caffeine (1-10 μM). In a second set of experiments beta cells were treated with the same concentrations of CSE, CGA or caffeine and streptozotocin (STZ; 5 mM) was added as a diabetogenic agent.

The bioaccesibility of caffeine and CGA in CSE was determined by analysis of their content before and after *in vitro* gastrointestinal digestion. The processes were performed mimicking human digestion physiological conditions. Additionally, novel information regarding to the major contributors to the health benefits of CSE was obtained. The extract was fractionated and the chemical composition and bioactivity of the fractions containing high and low molecular weight coffee components were analysed *in vitro*.

On the other hand, in order to evaluate CSE bioavailability, Wistar rats (n=16) were housed singly in metabolic cages with free access to food and water, and 24 h urine samples were collected from untreated rats as a control (n=4). Then the animals were divided into three groups: CSE group (n=4) receiving one single dose of CSE (adjusted to provide 2.2 mg caffeine/kg body weight), CGA group (n=4) receiving pure CGA (providing 1.5 mg CGA/kg body weight) and caffeine group (n=4) receiving pure caffeine (providing 5 mg/kg body weight). After the administration of each treatment, urine samples were serially collected at different times during 24 h. The bioavailability experiments were repeated with the same animals after 3 days of clearance.

The evaluation of CSE bioactivity in STZ-nicotinamide (NA) diabetic rats was conducted using Wistar rats (n=32) divided into four groups (n = 8). Daily, the animals were supplemented by gavage with CSE (providing 2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight), pure CGA (providing 1.5 mg CGA/kg body weight) or pure caffeine (providing 5 mg caffeine/kg body weight)

during 42 d. The fourth group (STZ group) was treated similarly with sterile water. At day 35, all rats were induced T2D by the intraperitoneal injection of STZ (60 mg/kg body weight) and NA (200 mg/kg body weight), and blood glucose levels were monitored daily in the following days. Rats were considered diabetic when blood glucose levels were above 200 mg/dl. At that moment (day 42), fasting rats were anaesthetised with Ketamine-Xylazine and sacrificed. Plasma samples and pancreas were obtained and frozen at -80 ºC until further analysis.

Metodology

To evaluate the *in vitro* inhibition of AGEs formation by CSE bioactive compounds using the glycoxidation model systems mimicking physiological conditions, we analysed free amino groups, fluorescence AGEs formation and total AGEs formation. Data on protein structure were obtained by mass spectrometry, Folin reaction and UV-Vis spectral analysis. In addition, to determine changes in protein functionality, the antioxidant capacity of the protein fraction was evaluated by ABTS assay.

Pancreatic cell oxidative status was determined by measuring ROS, reduced glutathione (GSH) and glutathione peroxidase (GPx) and glutathione reductase (GR) activities in cell lysates after treatments with CSE, CGA and caffeine. Besides, glucose-induced insulin content and insulin secretion was quantified by using ELISA kit. In the second experiment, the protective effect of treatments against STZ-induced damage was evaluated by analysing markers of oxidative stress (ROS, GSH, GPx and GR) and cell death (crystal violet assay) in INS-1E beta cells.

The bioaccesibility of components of CSE was estimated by analysis of total phenolic compounds, CGA and caffeine before and after the *in vitro* digestion. The overall antioxidant capacity (ABTS and ORAC) was also determined.

The bioavailability of CGA and caffeine in CSE was determined by measuring the excretion of their principal metabolites after providing a single dose of CSE, CGA or caffeine. Hippuric acid and paraxanthine were determined in 24 h urines of rats by UPLC-MS/MS analysis. Novel information regarding to the effects of CSE, caffeine and CGA on biomarkers of T2D were obtained in beta cells (INS-1E) and *in vivo* using as experimental model STZ-NA-induced T2D rats.

In vivo protective effect of CSE, CGA and caffeine was evaluated using an experimental model of STZ-NA T2D rats. Thirty-two rats were daily pre-treated with the CSE, CGA or caffeine during 34 days. At day 35, diabetes was induced by intraperitoneal injection of STZ-NA and blood samples were collected in the fasting state at day 42. After blood centrifugation, plasma was separated and frozen at -80 ºC. The pancreas were removed promptly, weighted, divided into three parts and then stored at −80 °C until required. Plasma glucose was measured using a colorimetric kit. Plasma and pancreas insulin content was analysed using an ELISA kit. Fructosamine, as a biomarker of plasma protein glycation, was evaluated by nitroblue tetrazolium (NBT) colorimetric assay. Carbonyls content, as a biomarker of protein glycoxidation, was measured in plasma and pancreas homogenates using a colorimetric assay. Biomarkers of antioxidant defence, GSH and GPx and GR activities were analysed in the pancreas of diabetic rats.

Results

Novel findings were obtained during the development of this investigation. For the first time, it has been associated the inhibitory capacity of AGEs formation of CGA free and in CSE to its ability to form protein-phenol complexes. Reactive adducts of arginine and lysine were found in glycoxidative reaction with BSA and methylglyoxal by ESI Q-TOF-MSMS analysis. The addition of CGA or CSE to the glycoxidation system decreased the presence of arginine adducts. The novel structure formed by interaction of proteins and phenol caused a significant

increase ($p < 0.05$) of antioxidant character of the parental protein. The results are of great interest because this change in protein function may provide protection against oxidation reactions and diseases associated to this of damage.

The experiments in INS-1E beta cell showed that CSE, pure CGA and caffeine did not affect pancreatic beta cells viability and oxidative status, when assayed under physiological conditions. All concentrations of CSE and CGA $\geq$ 5 µM significantly increased ($p < 0.05$) the enzymatic activity of GPx. CSE (1-10 µg/mL) and the dose of 10 µM of CGA, significantly increased ($p < 0.05$) beta cell insulin secretion in the presence of 4 and 10 mM of glucose. On the other hand, CSE (1µg/mL) and CGA (10µM) reinforced the antioxidant defence and increased insulin secretion in response to glucose in beta cells stressed with STZ.

Bioaccesibility of CSE compounds was affected during *in vitro* gastrointestinal digestion decreasing concentrations of caffeine (25%), phenolic content (40%) and CGA (82%). The overall antioxidant capacity of CSE was reduce at 15% and 50% as measured by ABTS and ORAC, respectively. Although a significant reduction of the bioaccesibility of CGA and caffeine occurred during digestion results suggest that physiological active concentrations of both compounds remain available to act in the body.

The study of the bioavailability of CSE bioactive components in the organism shows that 24 h after the intake of a single dose, intact CGA was not found in urine of rats fed with CSE (containing 0.254 mg of CGA/day) or CGA (0.321 mg of CGA/day), while a significant excretion ($p < 0.05$) of hippuric acid was observed only after the ingestion of CGA alone. In addition, non-metabolized caffeine and paraxanthine was higher in urine after consumption of pure caffeine than after the treatment with CSE. Altogether results on bioccesibility and excretion indicated that caffeine and CGA in CSE were metabolised.

The supplementation of rats with pure CGA and caffeine tended to reduce ($p < 0.1$) STZ-NA-induced oxidation of pancreas proteins. Pre-treatment of animals with CSE and CGA significantly reduced ($p < 0.05$) STZ-induced pancreas GSH depletion. Results confirm the bioavailability of caffeine and CGA in CSE and support their biological implications in diabetes.

Conclusion

In conclusion, the findings derived from these investigations demonstrate that one of the mechanisms by which CSE bioactive compounds inhibit AGEs formation is the generation of novel structures with antioxidant properties. The antiglycative effect of CSE may provide protection against complications in diabetics. Further investigations should be carried out to demonstrate the hypothesis. CSE protects pancreatic beta cells from oxidative stress and modulates insulin secretion. Caffeine and CGA in CSE are bioavailable and exert antidiabetic effects following different mechanism of actions. The effects observed on diabetes bioamarkers can be associated to the synergic effect of CGA, caffeine, their metabolites and others coffee components. Although further studies should be conducted to identify all the CSE components able to affect the biomarkers of diabetes and its effects in humans the present study suggests that an effect of CSE consumption in diabetes is biologically plausible, and that effect should be ascribed to the particular chemical and complex composition in bioactive compounds of the extract. The valorisation of CS into a sustainable product for diabetes is feasible.

Resumen

Antecedentes

El café es la bebida más consumida en todo el mundo (1). Durante el procesado del café se producen una abundante cantidad de subproductos (2–4). La piel de plata o cascarilla de café es el tegumento que cubre los dos granos que forman la semilla de café verde. Éste se desprende de los mismos durante el tostado siendo el único subproducto de esta etapa del proceso (5). El uso de extractos biactivos de productos naturales tales como la cascarilla de café ha ganado popularidad. Estos extractos se utilizan con frecuencia en la preparación de suplementos dietéticos/nutracéuticos, ingredientes alimentarios, productos farmacéuticos y cosméticos. El grupo de investigación en el que se ha desarrollado la presente tesis doctoral posee una patente concedida y transferida que protege tanto el proceso de elaboración del extracto acuoso de cascarilla de café como su aplicación en alimentación, salud y cosmética (P201131128). El extracto es rico en diferentes compuestos bioactivos.

El ácido clorogénico, formado por esterificación de los ácidos cafeico y quínico, es uno de los componentes fenólicos más abundantes en el extracto de cascarilla de café (6,7). Este compuesto fenólico presenta propiedades antioxidantes (8), anti-inflamatorias (9–11) y antiglicantes (12,13). La cascarilla de café es además una buena fuente de cafeína (6,14), que podría contribuir a la mejora de la defensa antioxidante en humanos (15,16). En general los antioxidantes y los fitoquímicos en particular, juegan un papel muy relevante en la reducción del riesgo de enfermedades crónicas tales como la diabetes Mellitus tipo 2 (T2D sus siglas en inglés) (17,18).

La T2D es una enfermedad metabólica compleja y multifactorial caracterizada por la resistencia a la insulina y el fallo del funcionamiento de las células beta del páncreas, lo que conduce a niveles elevados de glucosa en sangre. La hiperglicemia prolongada conlleva una serie de eventos bioquímicos que causan complicaciones asociadas a la diabetes incluyendo formación y acumulación de productos avanzados de la glicación no-enzimática de proteínas (AGEs por sus siglas en inglés) (19,20), el incremento del estrés oxidativo debido a un desbalance entre la generación de especies reactivas de oxigeno (ROS) y la defensa antioxidante del organismo, así como el incremento de los niveles de carbonilos reactivos debido a su formación excesiva y/o baja degradación y excreción. (21). Al consumo del café se le asocian una amplia variedad de efectos beneficios para la salud y en particular la reducción del riesgo de T2D (22). En nuestro conocimiento hasta la fecha, el mecanismo de acción de los componentes bioactivos del extracto de cascarilla de café en la patogénesis y complicaciones de la diabetes no ha sido objeto de estudio.

Justificación del estudio

El objetivo de la presente Tesis Doctoral es obtener evidencias científicas novedosas que demuestren los efectos del extracto de cascarilla de café en T2D. Dado que la salud y el bienestar de los pacientes diabéticos se ven principalmente afectadas por complicaciones debidas a la formación y acumulación de AGEs en el cuerpo, en el presente estudio se ha evaluado en primera instancia, el potencial del extracto para inhibir este evento. Los estudios se llevaron a cabo *in vitro* mediante aplicación de la fitoquimómica. Se realizaron estudios de bioaccesibilidad y metabolismo de cafeína y ácido clorogénico presentes en el extracto. La bioactividad de los componentes alimentarios depende de en gran medida su bioaccesibilidad y metabolismo. Los estudios *in vitro* resultan insuficientes para demostrar la efectividad de los extractos bioactivos en la reducción del riesgo y/o tratamiento de enfermedades crónicas. Por este motivo, el

efecto del extracto de cascarilla de café en los biomarcadores de la diabetes se evaluó empleando cultivos celulares y un modelo experimental animal.

Los sistemas modelo de glicoxidación de proteínas se prepararon empleando BSA en presencia o ausencia de ácido clorogénico o extracto de cascarilla de café. La reacción de glicoxidación se inició adicionando metilglioxal a la mezcla de reacción. Seguidamente, las muestras se incubaron a 37ºC durante diferentes periodos de tiempo. Se seleccionó el metilglioxal como carbonilo reactivo dado su relevancia en la formación de AGEs *in vivo* y las complicaciones de la diabetes en humanos.

Con objeto de obtener nuevos conocimientos en relación al mecanismo de acción del extracto en la patogénesis de T2D se utilizó una línea de células beta pancreáticas INS-1E. Las células se trataron con diferentes dosis de extracto (1-10 μg/mL), ácido clorogénico (1-10 μM) y cafeína (1-10 μM). En un segundo bloque de experimentos las células se trataron con las mismas concentraciones de extracto, ácido clorogénico o cafeína, esta vez en presencia de estreptozotocina (5 mM) para inducir la diabetes.

La bioaccesibilidad de cafeína y ácido clorogénico presentes en el extracto se determinó por análisis de sus contenidos antes y después de la digestión gastroinstetinal *in vitro*. El proceso se llevó a cabo simulando las condiciones fisiológicas de digestión humana. Una vez digerido, el extracto se fraccionó y la composición química y la bioactividad de las fracciones conteniendo compuestos de alto y bajo peso molecular se evaluó *in vitro*. El estudio se realizó con objeto de obtener información novedosa relativa a la identidad de los componentes del extracto que principalmente contribuyen a su bioactividad.

Por otra parte, con el objeto de evaluar la bioadisponibilidad de los componentes del extracto de cascarilla de café se utilizaron como modelo animal ratas Wistar (n=12) que se alojaron en jaulas metabólicas con acceso a comida y agua. Se

recogió la orina de 24 h de ratas no tratadas y estas muestras se emplearon como control. Los animales se dividieron en tres grupos: tratamiento con extracto de cascarilla (n=4) al que se le suministró una única dosis de extracto ajustada para administrar 2,2 mg de cafeína/kg de peso corporal, tratamiento con ácido clorogénico puro (n=4) al que se le suministró una dosis correspondiente a 1,5 mg/ácido clorogénico/ kg de peso corporal y tratamiento con cafeína pura (n=4) que recibió una dosis de este compuesto de 5 mg/kg de peso corporal. Tras la administración de los tratamientos, se recogieron muestras de orina a diferentes tiempos durante 24h. El experimento de biodisponibilidad se repitió empleando los mismos animales tras un periodo de tres días de lavado.

Se evaluó la bioactividad del extracto en ratas Wistar diabéticas en las que la enfermedad se indujo con estreptozotocina y nicotinamida. Se emplearon un total de 32 ratas que se dividieron en 4 grupos (n=8). Las dosis de extractos correspondientes a 2,2 mg/ cafeína/kg de peso corporal, 0,8 mg de ácido clorogénico puro/kg de peso corporal y 5 mg de cafeína pura/kg de peso corporal se administraron utilizando una sonda gástrica diariamente durante 42 d. El grupo se trató de igual modo con agua destilada estéril. El día 35 de tratamiento, se indujo T2D a todas las ratas por inyección intraperitoneal de estreptozotocina (60 mg/kg de peso corporal) y nicotinamida (200 mg/kg de peso corporal). Los niveles de glucosa se controlaron diariamente. Niveles de glucosa en sangre superiores a 200 mg/dl indicaron el desarrollo de T2D (día 42 de tratamiento). Las ratas en ayunas fueron anestesiadas y sacrificadas y se recogieron muestras de plasma y páncreas, que se conservaron congeladas a -80ºC hasta el momento de su análisis.

Metodología

Para evaluar la inhibición de la formación de AGEs *in vitro* en presencia de los compuestos bioactivos del extracto de cascarilla se utilizó un sistema modelo de glicoxidación que imitaba las condiciones fisiológicas, y se analizaron como

indicadores químicos del progreso de la reacción los niveles dew grupos amino libres y la formación de AGEs fluorescentes. Los datos de la modificación de la estructura de la proteína se obtuvieron por espectrometría de masas, la reacción de Folin y el análisis del espectro UV-Vis. Además, para determinar los cambios en la funcionalidad de la proteína, la capacidad antioxidante de la fracción proteica fue evaluada mediante el ensayo del ABTS.

El estado oxidativo en las células INS-1E cultivadas se determinó midiendo la presencia de ROS, glutatión reducido (GSH) y las actividades glutatión peroxidasa (GPx) y glutatión reductasa (GR) en los lisados celulares obtenidos tras la incubación con extracto de cascarilla, ácido clorogénico y cafeína. Además, el nivel de insulina secretada por la exposición a bajos y altos niveles de glucosa se cuantificó mediante ELISA. En un segundo experimento, se evaluó el efecto protector de los tres tratamientos frente al daño inducido por streptozotocina mediante el análisis de biomarcadores de estrés oxidativo (ROS, GSH, GPx y GR) y viabilidad celular (ensayo con cristal violeta).

La bioaccesibilidad de los componentes del extracto se estimó mediante el análisis de los compuestos fenólicos totales, el ácido clorogénico y la cafeína, antes y después de la digestión *in vitro*. Además, se analizó la capacidad antioxidante total de los digeridos (ABTS y ORAC). El resultado proporciona información del potencial del extracto en la protección del tracto gastrointestinal frente al estrés oxidativo.

La biodisponibilidad del ácido clorogénico y de la cafeína presentes en el extracto de cascarilla se determinó mediante la evaluación de su excreción y de la de sus principales metabolitos en orina después de la administración de una dosis única del extracto, ácido clorogénico y cafeína a ratas. El ácido hipúrico y la paraxantina fueron determinados en orina de 24 horas mediante UPLC-MS/MS. Se ha obtenido información novedosa respecto a los efectos del extracto de cascarilla, la

cafeína y el ácido clorogénico sobre biomarcadores de T2D *in vitro*, en células pancreáticas beta INS-1E, e *in vivo* en un modelo experimental en ratas con T2D inducida por estreptozotozina-nicotinamida.

El efecto protector *in vivo* del extracto de cascarilla, el ácido clorogénico y la cafeína se ha evaluado en un modelo experimental en ratas con T2D. Treinta y dos ratas fueron pretratadas diariamente con extracto de cascarilla, ácido clorogénico o cafeína durante 34 días. En el día 35 se les indujo la diabetes mediante la inyección intraperitoneal de estreptozotozina y nicotinamida. Se recogieron muestras de sangre el día 42; tras centrifugarlas, se separó el plasma y se congeló a -80ºC. El páncreas también fue obtenido y congelado a -80ºC hasta su análisis. Se determinó la glucosa en plasma mediante un ensayo colorimétrico y la insulina en plasma y en el páncreas mediante un kit de ELISA. La fructosamina (un biomarcador de glicación de proteínas plasmáticas) se determinó mediante el ensayo colorimétrido con nitroblue tetrazolium (NBT) y las proteínas carbonilo se determinaron en plasma y en homogenados de páncreas mediante un ensayo colorimétrico. También se determinaron los biomarcadores del sistema de defensa antioxidante: GSH, GPx y GR en el páncreas de las ratas diabéticas.

Resultados

El desarrollo de la presente investigación ha dado lugar a novedosos hallazgos. Por primera vez hemos asociado la capacidad del ácido clorogénico puro y el presente el extracto de cascarilla para inhibir la formación de AGEs a su habilidad para formar complejos con proteínas. El análisis de la muestras empleando ESI Q-TOF-MSMS permitió identificar aductos de arginina y lisina en sistemas de glicoxidación compuestos por BSA y metilglioxal. La adición a las mezclas de glicoxidación de ácido clorogénico o extracto de cascarilla disminuyó la presencia de los aductos de arginina. La nueva estructura que se formó por interacción de la proteína y el compuesto fenólico incrementó significativamente ($p < 0.05$) la

capacidad antioxidante de la proteína nativa. Los resultados son de gran interés dado que los cambios detectados en la función de la proteína podrían protegerla de las reacciones de oxidación y patologías asociadas a éstas.

Los experimentos realizados en células beta INS-1E mostraron que el extracto y el ácido clorogénico puro no afectan la viabilidad de las células y su estatus oxidativo bajo condiciones que simulan las condiciones fisiológicas. La actividad enzimática de GPx se incrementó significativamente al tratar las células con diferentes concentraciones de extracto y ácido clorogénico ≥ 5 μM. Los tratamientos con extracto (1-10 μg/mL) y 10 μM of CGA causaron un incremento significativo ($p < 0.05$) de la secreción de insulina por las células beta cultivadas en medios con 4 y 10 mM de glucosa. Por otra parte, los tratamientos con extracto (1μg/mL) y ácido clorogénico (10μM) mejoraron la defensa antioxidante e incrementaron la secreción de insulina en respuesta al incremento de glucosa en células beta dañadas con estreptozotocina.

La bioaccesibilidad de los componentes del extracto disminuyó significativamente durante el proceso de digestión gastrointestinal. La reducción fue del orden del 25% para cafeína, 40% para el contenido de compuestos fenólicos totales y 82% para el ácido clorogénico. Por otra parte, se registraron disminuciones de la capacidad antioxidante total del extracto hasta el 15% y 50% empleando los métodos ABTS y ORAC, respectivamente. Las concentraciones remantes de ácido clorogénico y cafeína se encuentran en los rangos de valores descritos como fisiológicamente activos.

El estudio de biodisponibilidad de los componentes bioactivos del extracto mostró que tras 24 h de su ingesta, no se encontró en la muestra de orina ácido clorogénico en animales tratados con extracto o el compuesto fenólico puro. En las muestras correspondientes a animales tratados con ácido clorogénico puro se encontraron cantidades significativas ($p < 0.05$) de ácido hipúrico. En las muestras

correspondientes a animales tratados con cafeína y extractos de cascarilla de café se detectó la presencia de cafeína y paraxantina.

En el modelo experimental de ratas diabéticas se detectó que la suplementación con ácido clorogénico puro y cafeína tienden a reducir ($p<0.1$) la oxidación de las proteínas del páncreas. El pretratamiento de los animales con el extracto y el ácido clorogénico puro previno ($p < 0.05$) la disminución de los niveles de GSH en páncreas de ratas diabéticas. Los resultados derivados de este estudio confirmaron la biodisponibilidad de cafeína y ácido clorogénico en el extracto y apoya sus implicaciones biológicas en la diabetes.

Conclusiones

En conclusión, los hallazgos derivados de esta investigación demostraron que los componentes bioactivos del extracto inhiben la formación de AGEs mediante al menos en parte, por la generación de nuevas estructuras con propiedades antioxidantes. El efecto antiglicoxidativo del extracto de cascarilla de café podría proteger frente a las complicaciones de la diabetes. Para demostrar esta hipótesis deben realizarse experimentos *in vivo*. El extracto de cascarilla protege a las células beta del páncreas frente al estrés oxidativo y modula su secreción de insulina. La cafeína y el ácido clorogénico presente en el extracto se encuentran bioaccesibles, son metabolizables y ejercen efectos antidiabéticos a través de diferentes mecanismos de acción. Los efectos que se han observado en los biomarcadores de la diabetes pueden asociarse al efecto sinérgico del ácido clorogénico, cafeína y otros componentes del café. A pesar que se requieren más estudios para identificar todos los componentes del extracto que contribuyen a sus efectos en la diabetes y confirmar su efectividad terapéutica en humanos, el presente estudio sugiere que su efecto en la diabetes es biológicamente factible y que éstos deben asociarse a su particular y compleja composición química. La valorización de la cascarilla de café como producto sostenible para la diabetes es

una aplicación factible.

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Introduction

Coffee Silverskin Extract. Definition, extraction processes and chemical composition.

Large amounts of coffee by-products are generated from the industrial processing of coffee cherries to obtain the coffee beverage (1–4). Coffee is actually a cherry whose structure is shown in **Figure 1**. Coffee cherries are mainly used to prepare the beverage when they are processed. From farm to cup, coffee processing can be briefly summarized in ten key steps: planting, cherry harvesting, processing (wet and dry methods), drying the beans, milling, exporting, tasting, roasting, grinding and brewing (http://www.ncausa.org/).

The coffee industry is responsible for the generation of large amounts of waste since coffee is the second most valuable commodity exported by developing countries (5). Furthermore, the study of the coffee by-products generated during the different stages of coffee processing is necessary to decrease the waste produced by this industry. The recovery of coffee by-products is mainly based on their use as a source of energy and biomass. Although these strategies are of interest, they do not consider valuable nutritional compounds that could improve consumers' health and increase the competitiveness and sustainability of coffee production (6).

The valorisation of agricultural wastes, food processing by-products, wastes and effluents using the biorefinery approach represents the real contribution of many industries to sustainable and competitive development (7). Biorefineries can be described as integrated bio-based industries which use a variety of technologies to make products such as chemicals, biofuels, food and feed ingredients,

biomaterials, fibres, heat and power, aimed at maximizing the added value of the three pillars of sustainability (Environment, Economy and Society) (8).

The type of by-product generated depends on the process used to obtain the green coffee bean. In the case of wet processing, ripe cherries are depulped to eliminate the outer skin, eliminating most of the pulp fixed to the grains (2,3). Then, the coffee beans undergo fermentation processes, are washed to remove the rest of the pulp, dried by sun exposure and peeled to remove the parchment (2,3). Here, skin and pulp are recovered in one fraction, and soluble sugars and mucilage are generated in another fraction. Finally, the parchment is obtained (2,3). Dry processing involves sun drying the coffee cherries for two or three weeks, and green coffee beans are obtained by simply threshing the dried cherries. At this time, skin, pulp, mucilage and parchment are obtained in a single fraction, along with part of the silverskin (9). The only by-product of coffee roasting is the silverskin.

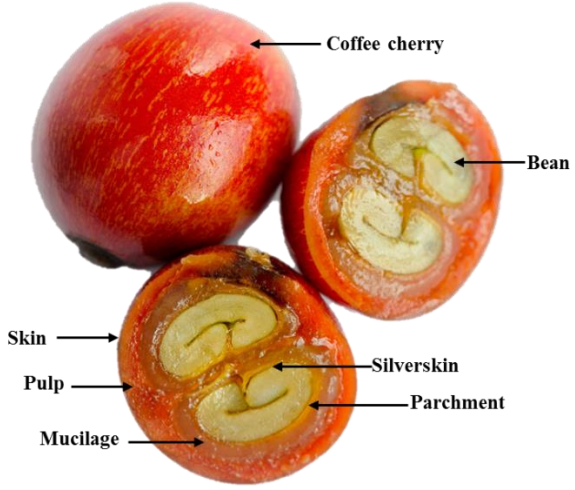

Figure 1: Cross-section of the coffee cherry, showing its anatomic parts. Based on del Castillo et al. (In press) (6).

CS is a thin tegument of the outer layer of the two beans forming the green coffee seed and represents about 4.2 % (w/w) (**Figure 1**). **Figure 2** summarises CS production by wet process (10). This coffee by-product presents phenolic compounds, mainly CGA, and other phytochemicals bioactive compounds that they contribute its high antioxidant capacity. Moreover, it has been proposed as a natural source of prebiotic carbohydrates and dietary fibre (10,11). The content of phenolic compounds and antioxidant capacity is delimited by the extraction method has been used prior to analyse the sample. Murthy et al., (2010) (12) obtained a CSE enriched in CGA involving heat treatment, spraying, enzyme treatment and extraction chromatography fractionation with organic solvents. Our research group patented a CSE from Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) (WO 2013004873 A1) enriched in caffeine and CGA (13). The extraction step takes place with 2 volumes of water per gram of CS at 100 °C for at least 10 min, does not use organic solvents. CSE is obtaining using an environmentally friendly technology (13). The extraction of bioactive compounds from natural products like CS is increasingly being used to prepare dietary supplements (nutraceuticals), food ingredients and some pharmaceutical products (14).

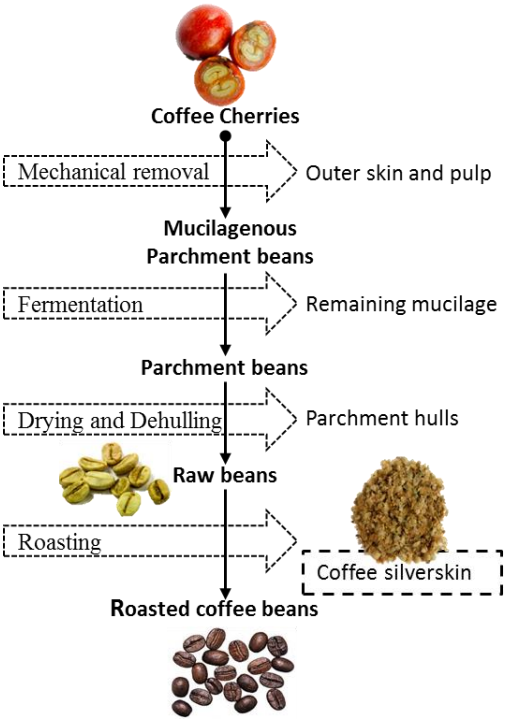

Figure 2: Diagram of coffee silverskin production from wet processing. Based on Borrelli et al. (2004) (8).

Table 1 summarises the chemical composition of our CSE. The patented CSEs are rich in total dietary fibre (28-36%), which includes about 4-9 % insoluble dietary fibre and 24-26 % soluble dietary fibre. CSEs are a good source of polyphenols, particularly CGA (1-6%); the most relevant are 5-O-, 3-O- and 4-O-caffeoylquinic acids (15). CSE is also a good source of caffeine (3%), and melanoidins (17-23%) which are formed during roasting process (15). Coffee melanoidins are formed by polysaccharides, proteins and CGA and exerted antioxidant capacity (16). CSEs present a higher proportion of extractable antioxidants in aqueous solution. These antioxidants provide a total antioxidant capacity similar to those described for coffee beverage and CS raw material. It has been suggested that the presence of CGA and melanoidins contributes to the antioxidant properties of CSEs (15,13,17).

Table 1: Chemical composition of coffee silverskin extracts. Based on (14,16).

Compounds	ACSE (per 100g)	RCSE (per 100g)
Proteins (g)	5.36	0.99
Carbohydrates (g)	5.44	13.43
Total dietary fibre (g)	28.69	36.21
Soluble dietary fibre (g)	24.01	26.80
Insoluble dietary fibre (g)	4.67	9.41
Caffeine (g)	3.02	3.39
Melanoidins (g)	17.26	23.94
CGAs (g)	1.12	6.85
Total phenolic content (g)	3.10	3.54
ORAC (mmol TEAC)	119.4	151.3
DPPH (mmol TEAC)	21.9	23.1
ABTS (mmol TEAC)	8.5	22.5
FRAP (mmol TEAC)	82.9	64.0

ACSE, Arabica coffee silverskin extract; RCSE, Robusta coffee silverskin extract; CGAs, chlorogenic acids; TEAC, Trolox equivalent antioxidant capacity.

The highlighted chemical composition of CSEs suggest that they could be a good source of bioactive compounds with putative effects on human health (13,17). Phenolic compounds from coffee have shown potential protective activity against metabolic disorders and complications induced by diabetes (18). CSE antioxidants (CGA, melanoidins and the antioxidant fibre) exhibited also antiglycative effects *in vitro* (15,16) which may be bioavailable for reducing oxidative stress in humans, thereby decreasing the risk of chronic diseases such as T2D (19,20,21).

Diabetes. Classification

Today, there are 415 million diabetic people and a further 316 million with impaired glucose tolerance (IGT) are at high risk from the disease. This alarming number is set to reach 642 million by 2040. The burden of diabetes is reflected not only in the increasing numbers of people suffering the disease, but also in the growing number of premature deaths (5 million in 2015) due to its complications (22).

According to the American Diabetes Association (ADA) (23), the classification of diabetes includes four clinical classes (**Figure 3**).

Type 1 diabetes (T1D): results from beta cell dysfunction, usually leading to absolute insulin secretion deficiency.

Type 2 diabetes (T2D): due to the development of insulin resistance that leads to a progressive secretory defect of the pancreatic beta cell.

Gestational diabetes (GD): diabetes resulting from the metabolic alteration occurring during pregnancy.

Other specifics types of diabetes due to other causes: e.g., genetic defects in beta cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), chronic pancreatitis or "pancreatic diabetes", and drug – or chemically –induced.

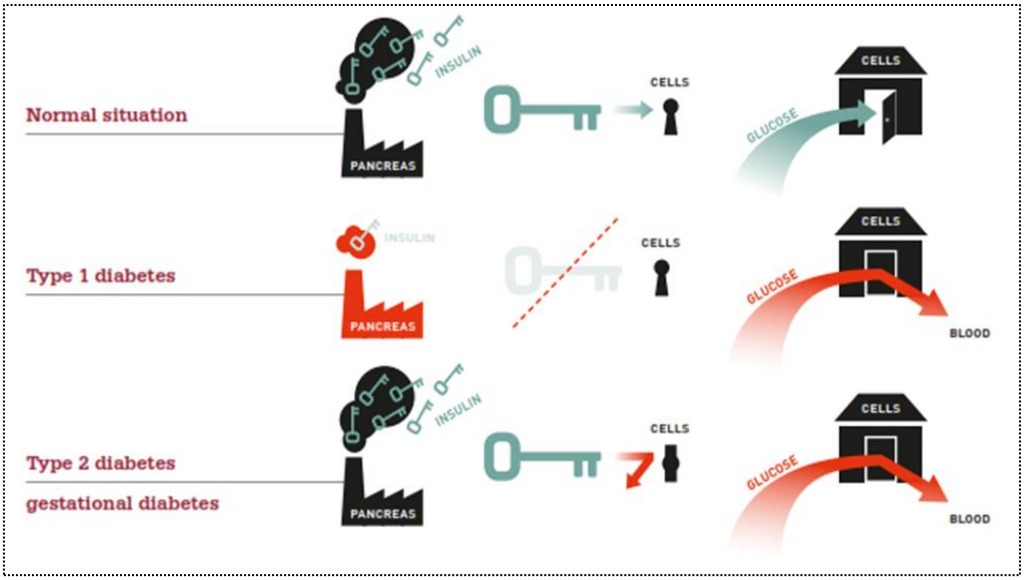

Figure 3: Most frequent types of diabetes. IDF Atlas 7th ed. (2015) (22).

Type 2 Diabetes

T2D is the most common type of diabetes with 90–95% of all diabetes cases. It is growing rapidly worldwide in both developed and developing nations. This rise is associated with economic development, ageing populations, increasing urbanisation, dietary changes, reduced physical activity and changes in other lifestyle patterns (24)

The term T2D designates not a single disease but a heterogeneous collection of hyperglycemic syndrome resulted from the interaction between a genetic predisposition and behavioural and environmental risk factors. There is strong evidence that obesity and physical inactivity are the main non-genetic determinants of the disease. Usually, T2D occurs in adults, but it is increasingly seen in children and adolescents. The development of T2D is usually associated with a combination of insulin resistance and beta cell failure leading to elevated blood glucose level. Insulin resistance is defined as a pathophysiological condition in which a normal insulin concentration does not adequately produce a normal insulin response in peripheral tissues, such as adipose, muscle and liver (25). Under this condition, pancreatic beta cell secretes more insulin (i.e. hyperinsulinemia) to overcome the hyperglycemia among insulin-resistant individuals. Although hyperinsulinemia may compensate maintaining normoglycemia, however, it may cause the over-expression of other insulin activities (23,26). The dysregulation of glucose homeostasis in T2D affects the function of many organs and tissues as shown in **Figure 2**.

T2D develops gradually and frequently goes from long duration of silent hyperglycemia. At earlier stages, T2D is often not severe enough for the patient to notice the classic diabetes symptoms (frequent urination, excessive thirst, weight loss and blurred vision). Nevertheless, even undiagnosed patients are at increased risk of developing macrovascular and/or microvascular complications such as renal disease, retinopathy, arterial hypertension and its consequences,

dyslipidemias and obesity. Most patients with T2D, but not all, are overweight or obese. In fact, this excess of weight itself causes some degree of insulin resistance. However, patients who are not obese or overweight by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region (22,23).

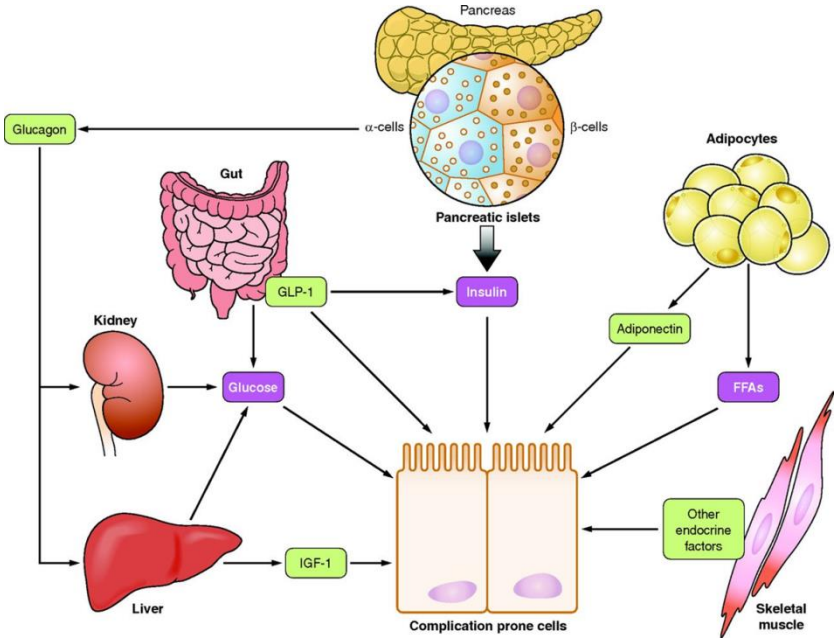

Figure 4: Linkage between glucose homeostatic pathways and target cells susceptible to diabetes complications. Target cells include endothelial cells, podocytes, proximal tubular cells, glial cells, cardiomyocytes, and neuronal cells Forbes et al. (2013) (25). GLP-1, glucagon-like peptide; IGF-1, insulin-like growth factor; FFA, free fatty acid.

Diagnosis

T2D may be diagnosed based on the plasma glucose criteria, either the fasting plasma glucose levels (FPG) or the 2-h plasma glucose concentrations after a 75-g oral glucose tolerance test (OGTT) or the HbA1C criteria (**Table 2**). The concordance between the FPG and OGTT tests is imperfect, as is the concordance between HbA1C and either glucose-based test. Studies have confirmed that the OGTT value diagnoses more people with diabetes, compared with FPG and HbA1C cut points (27,28). The same tests are used to detect individuals with

prediabetes (23,29). **Table 2** summarises the actual recommended values for the diagnosis of diabetes.

Table 2: Values for diagnosis of diabetes by ADA and WHO criteria.

	ADA	**WHO**
Fasting plasma glucose[1]	100-125 mg/dL (5.6-6.9 mmol/L)	≥ 126 mg/dL (≥ 7 mmol/L)
	or	or
2-h Plasma glucose during OGTT[2]	140-199 mg/dL (7.8-11.0 mmol/L)	≥ 200 mg/dL (≥ 7 mmol/L)
	or	or both
HbA1c[3]	≥ 6.5 % (48 mmol/mol)	

*Note that diabetes can be diagnosed in an individual only when these diagnostic values are confirmed on another day. [1] Fasting is defined as no caloric intake for at least 8 h. [2]The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water. [3]The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay. FPG, Fasting plasma glucose; OGTT, Oral glucose tolerance test; HbA1c, Haemoglobin glycated.

The term prediabetes covers follow two states of glucose intolerance:

Impaired glucose tolerance (IGT): considered to be present if FPG < 126 mg/dL (7.0 mmol/L), and the OGTT > 140 to < 200 mg/dL (> 7.8 to < 11.0 mmol/L) by both ADA and WHO criteria.

Impaired fasting glucose (IFG): defined as FPG between 100-125 mg/dL (5.6-6.9 mmol/L) by ADA, and 110-125 mg/dL (6.1-6.9 mmol/L) by WHO.

The transition time between the early metabolic abnormalities that precede IFG and IGT to T2D may take many years. Most individuals (≈ 70%) with these pre-diabetic states eventually develop diabetes. IGT and IFG are also associated with an increased risk of cardiovascular disease (CVD) (30).

Oxidative stress in Type 2 Diabetes

Nowadays, evidences in experimental and clinical studies support the role of oxidative stress in the pathogenesis of T2D (31). Oxidative stress is patent when there is either an excessive production of ROS and/or a deficiency of enzymatic and non-enzymatic antioxidants defence systems (32). The term "ROS" includes all unstable metabolites of molecular oxygen (O_2) that have higher reactivity than O_2, like superoxide radical (O_2•-) and hydroxyl radical (HO•), and non-radical molecules like hydrogen peroxide (H_2O_2) (33).

In diabetes, free radical formation by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation, leads to damage of enzymes, cellular machinery and also increased insulin resistance (34). In addition, oxidative stress is critically involved in the impairment of beta cell function due to their normal low antioxidant defence (35).

Oxidative stress and free radicals have got a major role in the onset and progression of late diabetic complications such as coronary artery disease, neuropathy, nephropathy and retinopathy (36). *In vivo* studies support the role of hyperglycemia in the enhancement of oxidative stress leading to endothelial dysfunction in blood vessels of diabetic patients (37).

Biomarkers of oxidative stress in Type 2 Diabetes

The measurement of oxidised biomolecules is used for the determination of oxidative stress status. In fact, during T2D oxidative stress induces alterations in most biomolecules in the cell and modifies the plasma antioxidant status. A number of biomarkers are used to evaluate the oxidative stress and the antioxidant defence. In the present section, we are going to focus on the biomarkers measured in this work, which has been demonstrated to be altered during T2D.

Protein oxidation: Proteins are a potential target of ROS, whose structure and function can be affected by modification. Protein-carbonyls are reported as the

potent biomarker of oxidative stress (38). Carbonyls (aldehydes and ketones) are produced on protein side chains, mainly on proline, arginine, lysine, and threonine. Protein-carbonyl derivatives may also be formed through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative (38,39). Carbonyls groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of cysteine, histidine and lysine residues with aldehydes (4-hydroxy-2-nonenal, malondialdehyde, 2-propenal [acrolein]) produced during lipid peroxidation, or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars, or their oxidation products, with lysine residues of proteins (glycation and glycoxidation reactions) (40). Increased protein-carbonyl content has been reported in different cells and in plasma of diabetic patients (41–43). Recently, Bollinelli et al. (2014) (44) proposed the measurement of plasma proteins-carbonyls by RPC-MS/MS as a potentials T2D biomarker.

Glutathione level: Glutathione, the tripeptide γ-L-glutamyl-L-cysteinylglycine, is present in all mammalian tissues at 1–10 mM concentrations (highest concentration in liver). It is the most important endogenous antioxidant that defends against oxidative stress (45). Glutathion can maintain sulfhidril groups of proteins in a reduced state. Glutathion functions include antioxidant defence, detoxification of xenobiotics and/or their metabolites, regulation of cell cycle progression and apoptosis, storage of cysteine, maintenance of the redox potential and the modulation of immune function and fibrogenesis (45,46). The markedly increased of oxidative status linked to T2D provoked dysregulation in glutathion concentration. This impaired glutathione metabolism is in part responsible of the weakened defence against oxidative stress. Diminished plasma level of glutathion in T2D patients has been reported (47,48). Decreased glutathion level may be one of the factors for enhanced oxidative DNA damage in T2D (49,50). Indeed,

abnormal glutathione status is involved in beta cell dysfunction and in the pathogenesis of long-term complications of diabetes (51).

Glutathine Peroxidase: GPx is the enzyme responsible for protecting cells from damage due to free radicals like hydrogen and lipid peroxides. GPx metabolizes hydrogen peroxide to water by using reduced glutathione (GSH) as a hydrogen donor (52).

Glutathione Reductase: GR is the enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH while oxidates NADPH to NAD^+ (52), thus maintain the antioxidant potential of glutathione. Unfortunately, this glutathion system can be impaired if ROS are produced in excess. Any alteration in this level will make the cells prone to oxidative stress and injury (31).

Protein glycation products in Type 2 Diabetes

Hyperglycaemia was estimated to be one major factor contributing to accelerated protein glycation and formation of advanced glycation end products (AGEs) (53). Protein glycation so-called Maillard reaction starts by condensation reaction of carbonyl group of a reducing sugar with the amino groups of a protein or nucleic acid generating Schiff bases which rearrange to Amadori products. The Amadori products undergo dehydration and rearrangements followed by other reactions, such as cyclisation, oxidation and dehydration, to form more stable AGEs. Side chains of cysteine, lysine and histidine, and the amino group of the N-terminal of amino acids showed the highest ability to react with the reducing sugars (54). Arginine and tryptophan have the highest efficiency of cross-link between peptides and protein AGEs-products (55).

Dicarbonyl products (α-oxoaldehydes) such as glyoxal, methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) are formed as intermediate products during all stages of the Maillard reaction, but also as intermediates or by-products of glucose autoxidation, lipid peroxidation or in the polyol pathway (54). The accumulation

of reactive dicarbonyls is called carbonyl stress and it produces the formation of oxidative AGEs such as carboxymethyl-lysine (CML), and pentosidine or non-oxidative AGEs derived from 3-DG [deoxyglucasone-lysine dimer (DOLD)] or from MGO [carboxyethyl-lysine (CEL); Methylglyoxal-lysine dimer (MOLD)] (54).

Protein glycation and formation of AGEs play an important role in the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy and cardiomyopathy (53). Glycation of proteins interferes with their normal functions by disrupting molecular conformation, altering enzymatic activity and interfering with receptor functioning (56). AGEs form intra- and extracellular cross links not only with proteins but also with some other endogenous key molecules, including lipids and nucleic acids, contributing to the development of diabetic complications (57). Recent studies suggest that AGEs interact with their plasma membrane receptors (RAGE) altering intracellular signalling, gene expression and the release of pro-inflammatory molecules and free radicals (58).

Biomarkers of protein glycation in Type 2 Diabetes

Blood biomarkers used for estimating the degree of protein glycation in diabetes compromise HbA1C, fructosamine, dicarbonyls and AGEs.

Glycated Haemoglobin: HbA1C is formed non-enzymatically by condensation of glucose or other reducing sugars with the α- and β-chains of haemoglobin A (Amadori products) (59). Interestingly, the discovery of HbA1c opened new and still-growing pathways of research on Maillard reactions in biological systems. Glycation of haemoglobin occurs during the 120-day lifespan of the red cell, and recent glycaemia has the largest influence on the presence of HbA1c (60). Thus, HbA1c represent average glycaemia over approximately the last 6–8 weeks (61). Therefore, HbA1c in diabetes patients may be used as a reliable index of the mean glucose (glycaemic control) over the previous weeks and months. In 2009, the International Expert Committee proposed the use of HbA1c as a diagnostic criteria

for diabetes, prediabetes and people at high-risk of developing diabetes (**see Table 2; section Diagnosis**) (62).

Fructosamine: Fructosamine (total glycated serum proteins) is a ketoamine formed by linked fructose to plasma protein, primarily albumin, through non-enzymatic glycation reaction. Fructosamine reflects the average blood sugar concentration over the prior 2-4 weeks therefore being a short-term biomarker (63). The amount of fructosamine in serum is increased in T2D due to the abnormally high concentration of glucose in blood. Therefore, its concentration in serum reflects the degree of short-term glucose variability. Thus, a clinical advantage is that fructosamine responds more quickly to therapy changes, allowing for improved glycaemic control (64). This biomarker is strongly associated with incident of T2D (65).

Dicarbonyls: Under hyperglycemia and/or oxidative stress in diabetes mellitus, a variety of toxic dicarbonyls are produced which may react with protein amino groups, eventually leading to formation of AGEs (66) (**Figure 5**). Dycarbonyls in plasma and blood can be analysed by several techniques including reversed phase liquid chromatography, with fluorescence or UV detection, or liquid chromatography, with mass spectrometry detection (67). Increased concentrations of reactive glyoxal, MGO and 3-DG have been found in plasma of patients with T2D (68,69). In consequence, some authors propose the use of dicarbonyls as diabetes predictors (69).

Advanced glycation end products: As described above, AGEs play an important role in T2D of diabetic complications due to accumulation in long-lived tissue proteins in diabetic patients (54). As shown in **Figure 5**, glyoxal causes the formation of CML among others. CML is the best characterized AGEs currently. MGO causes the generation of, for example, CEL whereas 3-DG leads to the formation of pentosidine or also CML (66). Some of these AGEs are cross-linking fluorescent (e.g., pentosidine) and others cross-linking non-fluorescent (e.g., CML,

CEL) (70). To detect AGEs formation in fluids and tissues, AGE-specific fluorescence can be measured by fluorescence (excitation wavelength of 370 nm and an emission of 440 nm) (71). Pentosidine emits light at 385 nm when excited at 335 nm (72). Other methods for AGEs detection are immunohistochemical staining or enzyme-linked immunosorbent assay (ELISA) using antibodies against different AGEs (CML, CEL or pentosidine). However, application of these assays is limited due to lack of reliable antibodies. The most sensitive methods to analyse AGE includes high performance liquid chromatography, gas chromatography or mass spectrometry approach (66,73). CML, CEL and pentosidine are the main AGEs measured in plasma and diverse tissues related to diabetic complications in diabetic patients (73–75). Additionally, the analyse of fluorescent AGEs has been proposed as a screening tool to predict diabetic complications in primary care due to easy and low cost determination (76).

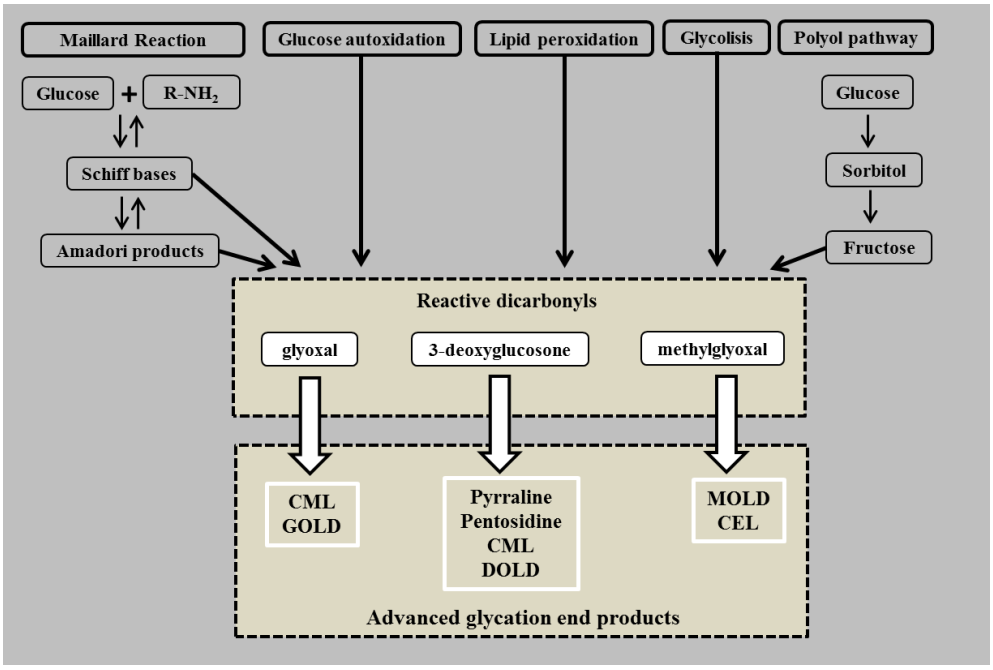

Figure 5: Pathways of formation of reactive dicarbonyls and advanced glycation end products in the pathogenesis of T2D. Based on Nowotny et al. (2015) (66). CML, carboxymethyl-lysine; GOLD, glyoxal-lysine dimmer; DOLD, deoxyglucasone-lysine dimer; MOLD, methylglyoxal-lysine dimmer; CEL, carboxyethyl-lysine.

Treatment of Type 2 Diabetes

T2D is a progressive pathology that can be treated initially with oral agent monotherapy (metformin) concurrently with lifestyle changes, but will eventually require the addition of other oral agents or insulin therapy in order to achieve targeted glycaemic levels (77). Strategies of hyperglycaemic treatment can be different if predominately there is insulin secretory deficiency or insulin resistant. To date, four new classes of oral antidiabetic medications and nine injectable agents and insulin are approved to use in the management of T2D: biguanide, sulfonylureas, thiazolidinediones, α-glucosidase inhibitors, meglitinides, dipeptidyl peptidase 4 inhibitors (DPP4-Is), bile acid sequestrants, sodium-glucose cotransporter inhibitors, dopamine receptor agonists and insulin (78,79). **Table 3** summarises therapeutic agents available and approved by Food and Drug Administration (FDA; EEUU) for the treatment of T2D. Metformin remains the first-line treatment option for most patients (78,79).

Metformin is the first choice therapy and sulphonylurea should be given in case of contra-indications for metformin, or should be added when therapy with metformin alone fails. Thiazolidinediones are recommended to be added in case of contraindications for metformin or if the combination of metformin and sulphonylurea fails. Alpha-glucosidase inhibitors may be considered as an alternative glucose-lowering therapy in people unable to use other oral drugs (80).

Alpha-glucosidase inhibitors appear to be a serious therapeutic option in the treatment of T2D as they have a comparable effect on glycaemic control compared to metformin. They pose no risk for harmful adverse events, decrease body mass index and possibly reduce the risk for cardiovascular disease, while the side-effects may be reduced by administering a lower dose without influencing its effect on glucose control (81). Moreover, they may also be given as ‘smart food’ or as a food supplement. A number of natural extracts from diverse sources have shown glucosidase inhibiting properties and may reduce blood glucose levels (82–84).

Optional therapies have been shown to delay the relative risk of progression to diabetes include the gastrointestinal lipase inhibitor (orlistat) and AGE inhibitors.

Orlistat (tetrahydrolipstatin) is a pancreatic and gastric lipase inhibitor whose primary effect is to reduce fat uptake by the gut (85). This is one of few pharmacologic treatment options available to help patients with T2D to reduce body weight and to improve the glycaemic control (86).

Aminoguanidine (also called pimagedine) is a nucleophilic hydrazine compound that has received most attention as a potential anti-glycation drug. Aminoguanidine prevented AGEs formation by blocking carbonyl groups such as MGO, glyoxal and 3-DG (87). However, this compound showed side effects in diabetic patients including flu-like symptoms, gastrointestinal disturbances and anaemia (88).

Table 3: Therapeutic agents and strategies used in the management of hyperglycaemia in patients with T2D. Based on Inzucchi et al. (2015) (79).

Class	Compounds	Mechanism of action	Advantage	Disadvantage
Biguanidine	• Metformin	• ↓ Hepatic glucose production	• Extensive experience • ↓ CVD events • No hypoglycemia	• Gastrointestinal side effects (diarrhea, abdominal cramping) • Lactic acidosis risk (rare) • Vitamin B_{12} deficiency • Multiple contraindications: CKD, acidosis, hypoxia, dehydration, etc.
Sulfonylureas	2nd Generation • Glyburide/ glibenclamide • Gliclazide† • Glipizide • Glimepiride	• ↑ Insulin secretion	• Extensive experience • ↓ Microvascular risk	• Hypoglycemia • ↑ Weight • Low durability • ? Blunts myocardial ischemic Preconditioning • Low duravility
Meglitinides (glinides)	• Nateglinide • Repaglinide	• ↑ Insulin secretion	• ↓Postprandial glucose excursions	• Hypoglycemia • ↑ Weight • ? Blunts myocardial ischemic preconditioning

Class	Compounds	Mechanism of action	Advantage	Disadvantage
Thiazolidinediones	• Pioglitazone‡ • Rosiglitazone§	• ↑ Insulin sensitivity	• No hypoglycemia • ↑ HDL-C (pioglitazone) • ? ↓ CVD events • ↓ Triglycerides (pioglitazone) • Durability	• ↑ Weight • Edema/heart failure? • Bone fractures • ↑ LDL-C (rosiglitazone) • ↑ MI (rosiglitazone)
α-Glucosidase inhibitors	• Miglitol • Acarbose	• Inhibits intestinal α-glucosidase	• No hypoglycemia • ↓Postprandial glucose excursions • ? ↓ CVD events • Nonsystemic	• Generally modest HbA1c efficacy • Gastrointestinal side effects (flatulence, diarrhea)
DPP-4 inhibitors	• Sitagliptin • Vildagliptin • Saxagliptin • Linagliptin • Alogliptin	• ↑ Insulin secretion (glucose-dependent) • ↓ Glucagon secretion (glucose-dependent)	• No hypoglycemia • Well tolerated	• Angioedema/urticaria and others immune-mediated dermatological effects • ? Acute pancreatitis • ? ↑ Heart failure hospitalizations

Class	Compounds	Mechanism of action	Advantage	Disadvantage
GLP-1 receptor agonists	• Exenatide • Liraglutide • Albiglutide • Lixisenatide† • Dulaglutide	• ↓ Glucagon secretion (glucose-dependent) • ↑ Satiety • Slows gastric emptying • ↑ Insulin secretion (glucose dependent)	• No hypoglycemia • ↓ Some cardiovascular risk factors • ↓ Postprandial glucose excursions • ↓ Weight	• Gastrointestinal side effects (nausea/ vomiting/diarrhea) • ↑ Heart rate • ? Acute pancreatitis • C-cell hyperplasia/medullary thyroid tumors in animals • Injectable
Amylin mimetics	• Pramlintide§	• ↑ Satiety • Slows gastric emptying • ↓ Glucagon secretion	• ↓ Weight • ↓ Postprandial glucose excursions	• Generally modest HbA1c efficacy • Gastrointestinal side effects (nausea/ vomiting) • Hypoglycemia unless insulin dose is simultaneously reduced • Injectable

Class	Compounds	Mechanism of action	Advantage	Disadvantage
Insulins	• Rapid acting analogs - Lispro - Aspart - Glulisine • Short-acting - Human Regular • Intermediate-acting - Human NPH • Basal insulin analogs - Glargine - Detemir - Degludec† • Premixed (several types)	• ↑ Glucose disposal • ↓ Hepatic glucose production • Other	• ↓ Microvascular risk • Theoretically unlimited efficacy response • Nearly universal	• Hypoglycemia • Weight gain • ? Mitogenic effects • Injectable • Patient reluctance

CVD, cardiovascular disease; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; MI, myocardial infarction; †Not licensed in the U.S. ‡Initial concerns regarding bladder cancer risk are decreasing after subsequent study. §Not licensed in Europe for type 2 diabetes.

Prevention of Type 2 Diabetes

While there are a number of factors that influence the development of T2D, it is evident that the major risk factors are lifestyle behaviours such as the consumption of processed foods and physical inactivity. All together, these behaviours are associated with an increased risk of being overweight or obese and with the development of T2D (22,23).

It is very important to understand the role of lifestyle interventions in the prevention of T2D. Several programmes have shown that modifying such behaviours, by eating healthy diet and increasing physical activity, can greatly reduce the risk of developing T2D (89–94). In addition, the growing interest in the identification of commonly consumed dietary polyphenols that may offer a natural alternative to reduce the risk or to treat T2D, is helping to retard the onset of its complications (95).

Effect of Coffee Components in Type 2 Diabetes

Coffee is among the most widely consumed beverages worldwide, and unsweetened coffee appears to be suitable alternative to sugar-sweetened beverages to prevent diabetes (96,97). This natural beverage provides many bioactive compounds such as phenolic acids (caffeic-, ferulic-, chlorogenic- and quinic acid) (98), the alkaloids caffeine (98) and trigonelline (99), isoflavones (100), lignans (101), tannic acid (102), the diterpenes cafestol and kahweol(103) and melanoidins (104) many of which exhibit strong antioxidant capacity (98,105). Therefore, coffee is considered an antioxidant beverage with putative effects on human health (16).

Coffee consumption has been associated with a lower risk of T2D, that may influence in different mechanism, such as glucose tolerance, insulin sensitivity, insulin resistance, glucose-6-phosphatase, intestinal glucose absorption, antioxidant activity, inflammatory biomarkers, glucose uptake, glucose

homeostasis, glucose metabolism and insulin secretion (16,98,106). Although these physiological effects of coffee are related to different components present in the beverage and to the cumulative effects of each compound most of the studies previously performed on coffee and diabetes clearly associated biological effects to caffeine and CGA (16,98,106). CSE contains both caffeine and CGA thus the present study focus the attention on them.

Chlorogenic acid

CGAs are a family of hydroxycinnamic specifically refers to the ester of caffeic acid with quinic acid (**Figure 6**) (107).

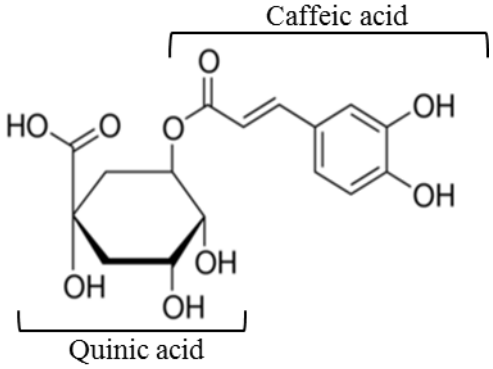

Figure 6: Chemical structure of chlorogenic acid (5-*O*-caffeoylquinic acids).

Coffee is the major source of CGAs in the human diet. The daily intake in coffee drinkers is estimated as 0.5-1 g, whereas coffee abstainers typically ingest <100 mg/day (108). The major CGA compounds present in the coffee brews are 3-O-, 4-O-, and 5-O-caffeoylquinic acids (109).

Metabolism and bioavailability of chlorogenic acid

The metabolism of CGA is still unclear, although studies in humans have confirmed that it mainly occurs at two stations, small intestine and colon. The first step is carried out by the active esterase enzymes present in the wall of small intestine releasing caffeic acid, quinic acid and ferulic acid. The absorption occurs mainly in the colon, representing around two-thirds of the ingested CGA (110,111). The remaining CGA may be metabolized by colonic microbial esterases and transformed in various aromatic acid metabolites including m-coumaric acid and derivatives of phenylpropionic and benzoic acids (112). Indeed, it has been reported that the bioavailability of CGAs largely depends on its metabolism by the gut microflora (111–113). Previous studies have found that hippuric acid, a benzoic acid, is the major CGA-derived metabolite observed in urine and plasma after the ingestion of pure CGA or CGA from a food matrix (112,114) (**Figure 7**).

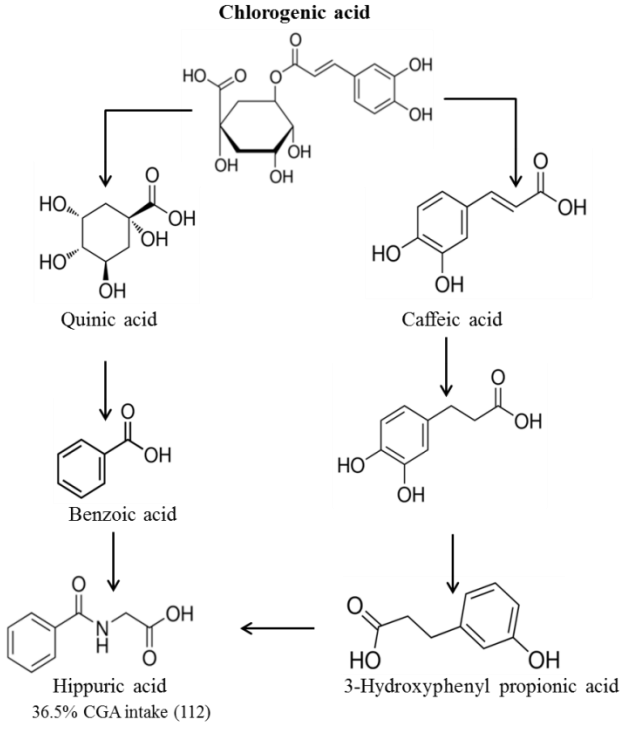

Figure 7: Simplified scheme of metabolism of chlorogenic acid. Based on Gonthier et al. (2003) (112).

Bioactivity of chlorogenic acid

Results from various studies have reported that pure CGA or CGA present in coffee may improve the risk for T2D by directly affect risk factors (115).

CGA was reported to regulate the glucose metabolism in experimental studies in diabetic humans and rats (106,116). Phenolic compounds present in coffee increase insulin sensitivity in peripheral tissues, potentiating insulin action a similar manner to the therapeutic effect of metformin (117,118). Recently, it has been reported that an intraperitoneal dose of 100 mg/kg of CGA reduced insulin resistance in high fat fed mice (119). Clinical trials have shown that CGA ingestion reduced early blood glucose and insulin release (120,121). Specifically, CGA inhibited the activity of the hepatic glucose-6-phosphate translocase (122). This enzyme catalyses the terminal reactions in both glycogenolysis and gluconeogenesis, thus is highly involved in the regulation of the blood glucose homeostasis (123). It has been testified that CGA also activate adenosine monophosphate-activated protein kinase (PKA), a master sensor and a regulator of cellular energy balance, leading to beneficial metabolic effects, such as the stimulation of glucose transport in skeletal muscle and the reduction of hepatic glucose production (124). Animal studies have shown that CGA reduce sodium-dependent glucose transport in the brush border membrane vesicles isolated from rat small intestine (125). CGA have been reported also to reduce intestinal absorption of glucose by inhibition of α-amylase (126,127) and α-glucosidase activities (128,129), two key enzymes responsible for the digestion of dietary carbohydrates.

There are several studies that have investigated the antioxidant capacity of CGAs using different cell-based models. They concluded that this polyphenol protect against oxidative stress by diverse mechanisms: 1) alleviating DNA damage (130,131), 2) suppressing the mitochondrial membrane depolarization (132) and 3) improving the antioxidant defence in cells (133,134). Other studies conducted in

rodent models have confirmed the efficacy of dietary intake of CGA in preventing oxidative stress pathogenesis through the increasement of the level of non-enzymatic antioxidants (GSH and Vitamins C and E) and antioxidants enzyme (superoxido dismutase, catalase, GPx and glutathione-S-transferase) in diabetic model rats (116,135).

Different experiments in cell and animal models have shown an anti-inflammatory activity of CGA by inhibiting the production of inflammatory mediators (136–141).

Finally, CGA has been also reported as an antiglycative compound which inhibits the protein glycation and the AGEs formation *in vitro*. These capacities of CGA have been linked to its antioxidant character, chelating properties to transition metals ions and quenching of carbonyl radical species and AGE crosslinking (12,142,143).

Caffeine

Caffeine (1,3,7-methylxanthine) is a methylated derivative of xanthine (**Figure 7**). Caffeine is a white crystalline xanthine alkaloid naturally occurring in coffee beans. Coffee is the main dietary source of this molecule and its content highly depends on the mode of preparation. A cup (236 mL) of instant coffee contains 93 mg of caffeine, whereas a cup of espresso coffee (28 mL) provides 40 mg (144).

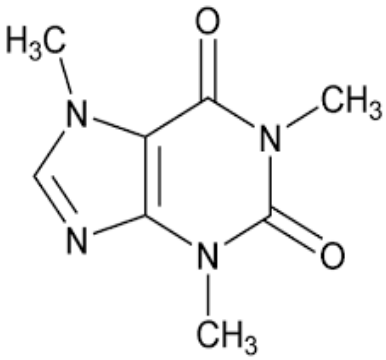

Figure 8: Chemical structure of caffeine (1,3,7-methylxanthine).

Metabolism and bioavailability of caffeine

Methylxanthines are extensively absorbed in the gastrointestinal tract and metabolized in the liver by the cytochrome P450 to yield methylxanthine derivatives and methyluric acids as the main metabolites. Subsequently they are distributed to all tissues and finally excreted in urine. Paraxanthine (1,7 dimethylxanthine) is the main metabolite of caffeine found in plasma and urine after caffeine intake (145) (**Figure 9**).

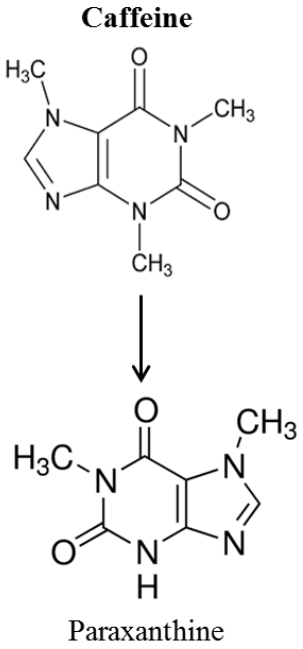

Figure 9: Simplified scheme of metabolism of caffeine metabolism. Based on Arnau. (2011) (145)

Bioactivity of caffeine in Type 2 Diabetes

Caffeine has been extensively studied in regard to their effects in insulin resistance and T2D, and its mechanism is still unclear.

Some studies have demonstrated that caffeine stimulates glucose uptake via activation of adenosine 5'-monophosphate activation of protein kinase (PKA)

(146,147). Pre-treatment with different doses of caffeine diminished plasma glucose level during the OGTT and increase pancreatic insulin level in diabetic rats (148). Similarly, Urzua et al. (2012) (149) has been reported that caffeine decreased blood glucose levels in a dose-dependent manner and improved glucose tolerance in diabetic rats. Recently, caffeine has shown a protective role in the biochemical and microscopic changes in pancreatic beta cells (150,151). However, other studies have reported that glucose tolerance and insulin sensitivity were impaired after short-term ingestion of caffeine (152–154).

On the other hand, caffeine and its metabolites, xanthine and theobromine, exhibited antioxidant activities, decreasing DNA degradation and reducing hydroxyl radicals formation (155).

Effects of coffee beverage in Type 2 Diabetes

As summarized in previous sections, T2DM is a constellation of interrelated risk factors, including impairment of glucose and insulin metabolism, beta cell failure and the overproduction of AGEs related to hyperglicaemia and oxidative stress. Coffee consumption has been associated with reduction of chronic diseases risk and, in particular, T2D (156,157). Several metabolites of coffee may improve the symptoms of T2DM by affecting glucose regulation. These may include the effects of CGA on glucose-6- phosphatase and the inhibition of α-glucosidase activity, and the effects of caffeine on insulin secretion (101,106).

Oxidative stress is also involved in the glucose autoxidation pathway in T2D that forms reactive dicarbonyl species. These dicarbonyl compounds can react directly with proteins and contribute to AGEs formation (66). Polyphenol coffee fractions (CGA and derivatives) are able to inhibit AGEs formation under diverse pathways (12,143,142). Some of them may be a consequence of the antioxidant activity or the dicarbonyl trapping activity of this coffee fraction. Caffeine and its catabolic products theobromine and xanthine exhibit both antioxidant and pro-oxidant

properties contributing to the overall antioxidant and chemopreventive properties of coffee (155).

Because coffee is rich in active substances, mainly caffeine and CGA, it is the main contributor to the intake of dietary antioxidant in Spain (158). Hence, consumption of coffee may prevent and treat T2D by antioxidative and antiglycative effects. The relationship between coffee-drinking and health benefits supports the concept of coffee as a functional food because it has the ability to enhance the quality of life of regular consumers (159).

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Hypothesis, objectives and working plan

T2D is one of the most common chronic diseases in the world. Oxidative stress and hyperglycemia have been largely implicated in the progressive dysfunction of pancreatic beta cells and in the formation of AGEs and in the development of diabetic complications. Diet, moderate activity and hypoglycemic drugs or insulin are the base of diabetes management. As a consequence, the search for natural compounds to prevent and treat the disease or an alternative therapy of insulin is a scientific and technological challenge. Diet can be a good source of bioactive compounds that may help to reduce the risk of chronic diseases.

Coffee is among the most widely consumed beverages worldwide. Previous experimental and epidemiological studies have indicated a substantial positive impact of coffee consumption on T2D complications. Caffeine and CGA are major components of coffee and they are considered effective on diabetes. However, their individual contribution to these effects and their mechanism of actions on the pathogenesis of this chronic disease have not been yet completely established. Nowadays, coffee is mainly used in food for the preparation of coffee beverage from roasted coffee beans. However, this represents a waste of abundant natural sources of bioactive compounds with health promoting properties such as CGA and caffeine. A cup of coffee contains about 6% of the raw coffee berry. The waste of the rest of the coffee fruits represent an environmental problem and inefficiently use of the natural resources. CS can be used for obtaining an aqueous extract by green and easily scalable procedures.

CS is the by-product of the roasting coffee and very abundant worldwide. CSE contains a similar chemical composition to that of coffee beverage obtained from roasted coffee beans. Hence, CSE may be used in food and health mainly in the prevention of diseases related to ageing and glycoxidative stress such as T2D. The sustainable use of this by-product of the coffee roasting industry is of interests. Its conversion into a sustainable product for diabetes can be very welcome by the coffee sector, consumers and health care professionals. CSE may be used as natural food supplements to prevent or treat diabetes and its complications, being an alternative or a coadjutant to those currently commercialized for these aims. Healthy effects of CSE largely will depend on the bioaccesibility and bioavailability of their bioactive components in the organism.

To demonstrate this hypothesis these general objectives were proposed:

1. To provide new knowledge on the mechanism of action of phytochemicals present in highly consumed products such as coffee, named as CGAs. To validate the potential of CS, the roasting coffee by-products, as a sustainable product possessing health promoting properties and biological effects on diabetes.
2. To obtain new evidences of the interest for the application of the biorrefinery concept to achieve a sustainable coffee production and health, as well as, to remark the interest of agronomy and nutrition in health.

To achieve the goal specific aims were also proposed:

1. ***In vitro* study of the mechanism of action of CGA alone and in CSE by employing phytochemomics approach. Chapter 1.**
2. **To investigate the mechanism of action of CSE and its compounds CGA and caffeine on the pathogenesis of diabetes using a model of beta cells.**
   To achieve this goal, the effect of CSE, CGA and caffeine on redox status and insulin secretion in the pancreatic beta cells was evaluated. Likewise, its

capacity to protect pancreatic beta cells against an oxidative damage was also examined. **Chapter 2**.

3. **To evaluate the bioaccessibility *in vitro* and the bioavailability and bioactivity *in vivo* of CSE, CGA and caffeine.**

   To achieve this goal, CSE was digested in vitro mimicking human gastrointestinal conditions. CSE, CGA and caffeine metabolism was evaluated *in vivo* using phytochemomics. Bioactivity of CSE, CGA and caffeine was examined in the pancreas of diabetic rats. **Chapter 3.**

Figure 8 shows the working plan performed to achieve each objective proposed in this work.

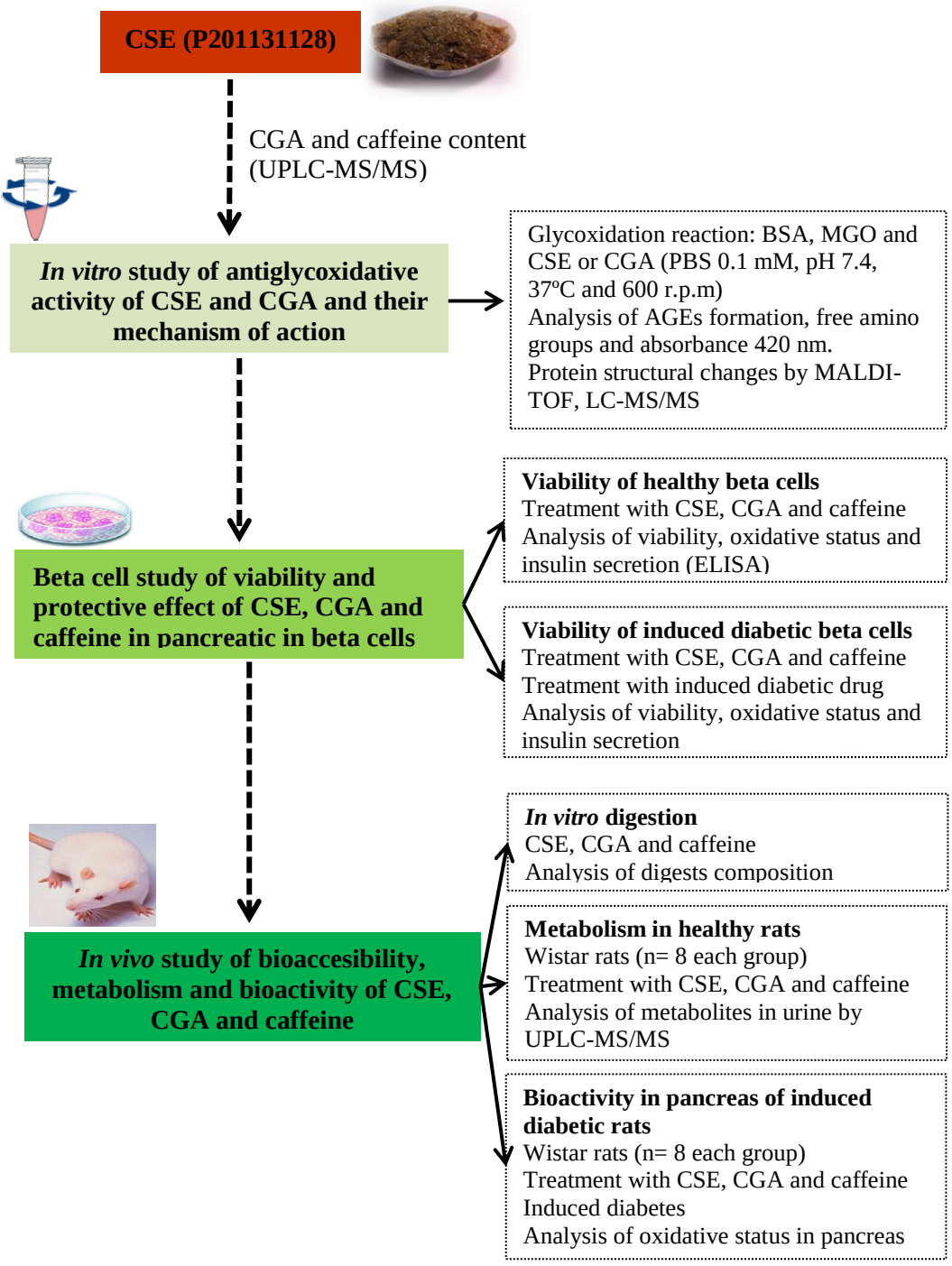

Figure 10: Scheme of working plan performed at present study.

Main Contributions

This PhD thesis aims to analyse the value of CSE for obtaining a new ingredient and to take advantage of the potential nutritional benefits derived from bioactive compounds naturally occurred in CSE such as caffeine and CGA, among others, and to validate the use of the new products for human consumption, in accordance with current legislation in Europe (EFSA Journal 2009; 7(9):1249) and its economic viability. At present, new ingredients and/or foods derived from coffee are not regulated by the EFSA. The steps to commercialize a new ingredient or food under the European legislation are shown in **Figure 11**. Previously, CSE chemical composition was determined, but further studies are needed to find other bioactive compound in this plant matrix with putative health benefits. In order to ascribe a health claim for this coffee roasting by-product in T2D, *in vitro* assays (biochemical and cells) and *in vivo* bioactivity assay was performed; they are summarize in the next chapters of this document. Hence, the study of acute and chronic toxicity and human trials are needed to complete all study levels for CSE in order to convert this product into a food grade ingredients with health effects.

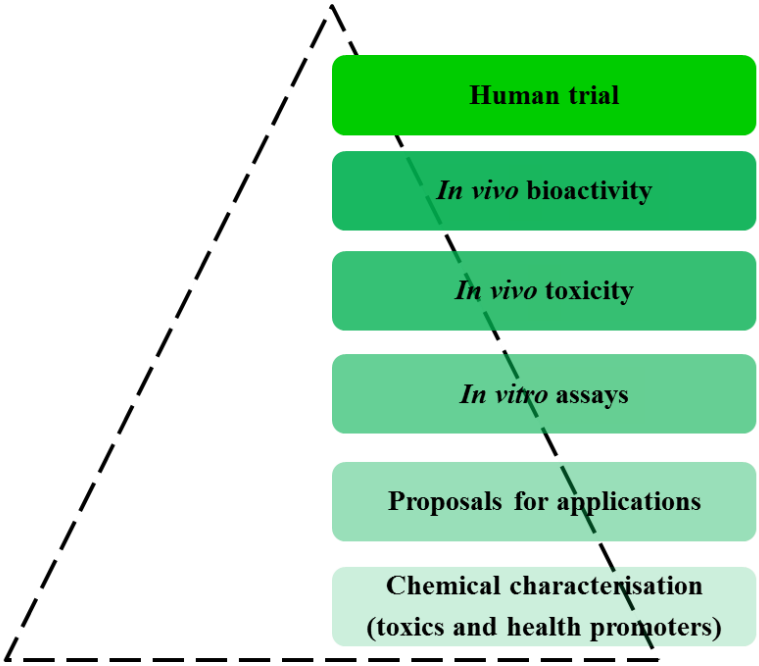

Figure 9: Scheme from waste to a food grade ingredient (human consumption) under European legislation procedures (EFSA Journal 2009; 7(9):1249).

Chapter 1

Abstract

This chapter summarises *in vitro* studies performed to gain insights in the mechanism of action to inhibit the formation of advanced glycation end products by CGA alone and CSE.

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New knowledge on the antiglycoxidative mechanism of chlorogenic acid

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Abstract

The role of chlorogenic acid (CGA) in the formation of advanced glycation end-products (AGEs) (glycoxidation reaction) was studied. Model systems composed of bovine serum albumin (BSA) (1 mg mL^{-1}) and methylglyoxal (5 mM) under mimicked physiological conditions (pH 7.4, 37 ºC) were used to evaluate the antiglycoxidative effect of CGA (10 mM). The stability of CGA under reaction conditions was assayed by HPLC and MALDI-TOF-MS. The glycoxidation reaction was estimated by analysis of free amino groups by the OPA assay, spectral analysis of fluorescent AGEs and total AGEs by ELISA, and colour formation by absorbance at 420 nm. Structural changes in protein were evaluated by analysis of phenol-bound to protein backbone using the Folin reaction, UV-Vis spectral analysis and MALDI-TOF-MS, while changes in protein function were measured by determining antioxidant capacity using the ABTS radical cation decolourisation assay. CGA was isomerised and oxidised under our experimental conditions. Evidence of binding between BSA and multiple CGA and/or its derivatives molecules (isomers and oxidation products) was found. CGA inhibited ($p < 0.05$) the formation of fluorescents and total AGEs at 72 h of reaction by 91.2 and 69.7%, respectively. The binding of phenols to BSA significantly increased ($p < 0.001$) its antioxidant capacity. Correlations between free amino group content, phenol-bound to protein and antioxidant capacity were found. Results indicate that CGA simultaneously inhibits the formation of potentially harmful compounds (AGEs) and promotes the generation of neoantioxidant structures.

Keywords: Advanced glycation end products (AGEs), chlorogenic acid, methylglyoxal, glycoxidation reaction, antiglycoxidative effect.

1. Introduction

Protein glycation includes an initial formation of Shiff's base, followed by intermolecular rearrangement and conversion into Amadori products. They undergo further processing to form a heterogeneous group of protein-bound moieties, such as cross-linking fluorescent (e.g., pentosidine) and non-fluorescent adducts (e.g., Nε-(carboxymethyl)lysine) (CML), Nε-(carboxyethyl)lysine (CEL) called advanced glycation end products (AGEs) (1). Pathways of AGE formation involve glucose autoxidation through the generation of α-oxoaldehydes, such as methylglyoxal (MGO), 3-deoxyglucosone and glyoxal. MGO is a major precursor of AGEs, especially CEL, which is capable of binding and modifying a number of proteins (glycoxidation reaction), including bovine serum albumin (BSA), RNase A, collagen, lysozyme and lens crystallins (2,3). Protein glycation is known to be involved in the pathogenesis of several age-related disorders like diabetes, atherosclerosis, end-stage renal and neurodegenerative diseases (4).

Inhibitors of AGEs formation might follow several mechanisms, such as aldose reductase, antioxidant activity, reactive dicarbonyl trapping, sugar autoxidation inhibition and amino group binding (5). The inhibition of AGE formation by synthetic aminoguanidine (AG) has been widely documented. However, as AG treatment in type 1 diabetics has caused serious complications (6), the search for natural AGE inhibitors is currently a challenge.

Coffee and yerba mate are considered natural sources of abundant phenolic compounds that can inhibit the formation of AGEs (7,8). The most representative phenolic acids in these foods are chlorogenic acids (CGA), which commonly occur as 5-*O*-caffeoylquinic acid (5-CQA) or 3-*O*-caffeoylquinic acid (3-CQA) (9,10).The antiglycation activity of CGA has been associated to its antioxidant and chelating characters, as well as to its ability to trap reactive dicarbonyl compounds (8,11). This study aimed to obtain a better understanding of the antiglycoxidative

mechanism of action of CGA which is partly unknown. *In vitro* studies mimicking physiological conditions were performed to achieve this goal.

2. Materials and methods

2.1. Materials

All chemicals and solvents were of analytical grade. Bovine serum albumin (BSA), phosphate buffered saline (PBS), 3-*O*-caffeoylquinic acid (CGA), sodium azide, *ortho*-phthaldialdehyde (OPA), N^{α}-acetyl-L-lysine, Folin-Ciocalteau, 3.3′, 5.5′-Tetramethylbenzidine (TMB) were from Sigma–Aldrich (St. Louis, USA). Other chemicals and their suppliers were as follows: *β*-mercaptoethanol (Merck, Hohenbrunn, Germany), methylglyoxal solution (MGO) and 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Fluka, Buchs, Switzerland) and Bradford reagent for protein assay (Bio-Rad, München, Germany). The Amicon® Ultra- 0.5 ml centrifugal filter unit fitted with an Ultracel®-30K regenerated cellulose membrane (30 kDa cut-off) was from Merck Millipore Ltd. (Tullagreen, Cork, Ireland). Microtest 96-well plates made from high-quality polystyrene were purchased from Sarstedt AG & Co. (Nümbrecht, Germany). The Costar® high binding 96-well EIA/RIA plate was from Corning Incorporated (Corning, NY, USA). The Milli-Q water used in this study was obtained using a purification system (Millipore, Molsheim, France).

2.2. Formation of CGA derivatives in control samples

2.2.1. HPLC analysis. Standard CGA before and after incubation at 37 ºC for 24 h were compared to assess the chemical stability of the compound under experimental conditions by reversed phase (RP) HPLC. A modular chromatographer HP 1100 (Agilent Technologies, Paolo Alto, CA, USA) equipped with a multi-waves UV-Vis detector was used to analyse samples. The stationary phase was a 250 x 2.1 mm i.d. C18 RP column, particle diameter 4 μm (Jupiter Phenomenex, Torrance, CA, USA). Column temperature was maintained at 37 °C during the HPLC analyses. Separations were carried out at a constant flow rate of 0.2 mL min^{-1} applying a 5-60% linear gradient of solvent B (acetonitrile/ 0.1% trifluoroacetic acid, TFA) over 60 min, after 5 min of isocratic elution at 5% solvent B. Solvent A was 0.1% TFA in HPLC-grade water. For each run, 2.5 μg standard or incubated CGA were diluted 10-fold with 0.1% TFA and injected using a Rheodyne® valve. The HPLC separations were monitored at 280, 320 and 360 nm, while UV-Vis spectra (200-600 nm) were recorded using a diode array detector.

2.2.2. MALDI-TOF-MS analysis. Mass spectra of CGA freshly prepared and incubated at 37 ºC for 24 h were acquired on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, Massachusetts) equipped with a N_2 laser (λ= 337 nm) operating in both positive and negative reflector ion modes. The matrix was 2.5- hydroxybenzoic acid (DHB) 10 mg mL^{-1} in 50% acetonitrile. In the positive ion mode, the matrix solution also contained 0.1% TFA. Spectra were

acquired using Delay Extraction technology at an accelerating voltage of 20 kV, exploring the *m/z* 150−1200 range. Matrix ion signals were excluded by separately acquiring positive and negative spectra of DHB. The mass range was externally calibrated with a mixture of standard polyphenols (Sigma, Milan, Italy). Spectra were elaborated with Data Explorer 4.0.

2.3. *In vitro* glycoxidation of proteins

Model systems were composed of BSA at a final concentration of 1 mg mL^{-1} in 0.01 M PBS buffer (pH 7.4) added with sodium azide (0.05%) and MGO (5 mM). Glycoxidation model systems were prepared in the presence or absence of the inhibitor (CGA 10 mM). Prior to initiation of the glycoxidation reaction by addition of MGO, the pH values of all solutions were measured at 25 °C using an electrode pH-meter (Metler Toledo, Spain) to ensure optimal and equal conditions of reaction in all samples (pH=7.4). The model systems were incubated at 37 °C for 192 h, and samples were taken after 24, 72, 96 and 192 h. The glycoxidation reaction was stopped by cooling in an ice bath. All samples were prepared in triplicate. A control solution of BSA was also included. The progress of the glycoxidation reaction was determined by analysing free amino groups, AGEs and brown compounds.

2.3.1. Free amino groups. Free protein amino groups (both N-terminal and epsilon -NH_2 of lysine) were determined by the OPA assay, following Go et al. (2008) (12). OPA reagent was freshly prepared by dissolving 10 mg of OPA in 250 µL of 95% (v/v) ethanol and adding 9.8 mL of 0.01 M PBS pH 7.4 and 20 µL of β-mercaptoethanol. The total volume of reaction was 250 µL. The reaction was carried out in transparent polystyrene 96-well microtest plate (No. 82.1581). Fluorescence was read after the addition of OPA reagent on a microplate fluorescence reader Biotek Synergy™ HT (Biotek Instruments, Highland Park, Winooski, USA) with excitation at 360 ± 40 nm and emission at 460 ± 40 nm. Fluorescence was read every 53 s for 15 min. Calibration curves were constructed using standard solutions of N^{α}-acetyl-L-lysine (0.025-1 mM). All measurements were performed in triplicate, and data were expressed as µg N^{α}-acetyl-L-lysine equivalent per mg of protein.

2.3.2. AGEs. AGE formation was monitored by fluorescence spectrophotometry using a Biotek microplate spectrophotometer at 360 ± 40 nm and 460 ± 40 nm as excitation and emission wavelengths, respectively. No dilution was required for the glycoxidation model or the control systems. All measurements were performed in triplicate.

The formation of total AGEs-BSA was measured by an indirect ELISA assay in samples incubated for 72 h. A high affinity protein 96-well microplate was coated overnight (4° C) with 100 µL of protein samples in 0.01 M phosphate buffer (pH 7.4) (5 µg mL^{-1}). Unbound proteins were washed out with buffer PBS-T (PBS 0.01 M; Tween 0.05%), the wells were blocked with gelatin 0.5% for 1 h at room temperature, then washed out with PBS-T, and the primary antibody (dilution 1:1000) was added for 1 h. A polyclonal rabbit IG antibody which rose against AGEs (AGE 102-0.2, Biologo, Kroshagen, Germany) was used as the primary antibody. After 1 h incubation and five washing

steps, the secondary horse radish peroxidise-conjugated mouse anti-rabbit IgG antibody (ABIN376294, antibodies-online Inc.,Suite, Atlanta) diluted 1:4000 in washing buffer PBS-T was added, incubated for 1 h and washed again. Colour was developed with *TMB (100 µL) and* absorbance was read at 650 nm. Values were estimated by comparison with a standard curve of glycated BSA (Methylglyoxal-AGE-BSA, CY-R2062, CircuLex™, CycLex Co., Ltd, Nagano, Japan). All measurements were performed in triplicate, and results were expressed as µg of AGEs-BSA per mg of protein.

2.3.3. Brown pigments. Formation of brown pigments in the samples was estimated by measuring absorbance at 420 nm of the samples at 24, 72, 96 and 192 h, using microplate reader BioTek PowerWave™ XS. Samples were analysed in triplicate.

2.4. Structural changes of proteins

Prior to analysis, the protein fraction of samples incubated at 37 ºC for 72 h was isolated by ultrafiltration. Samples (0.4 mL) were placed in the sample reservoir of an Amicon® Ultra- 0.5 mL centrifugal filter unit fitted with an Ultracel®-30K regenerated cellulose membrane (30 kDa cut-off) (Millipore Ltd., Ireland) and centrifuged at 14000 g for 40 min at room temperature. The concentrated samples were recovered and diluted in PBS (0.4 mL). Protein concentration was determined by the Bradford micromethod. The isolated protein fraction was used for structural and functional characterisation.

2.4.1. UV-Vis spectra. A Biotek microplate UV-Vis spectrophotometer equipped with UV KC junior software (Biotek) was used. The spectrum of fractionated samples was measured at 200-790 nm using a quartz 96-well microplate.

2.4.2. Total phenolic compounds. Total phenolic content (TPC) of the isolated fraction incubated for 72 h was determined using the Folin-Ciocalteu method as described by Singleton et al. (1999) (13**)** adapted to a microplate reader. The reduction reaction was carried out in 210 µL total volume in 96-well microplates (No. 82.1581). A 10 µL of sample (appropriately diluted when necessary) was added to 150 µl volume of Folin-Ciocalteu reagent (diluted 1:14, v/v) in Milli-Q water. After exactly 3 minutes, 4 ml of 75 g L^{-1} sodium carbonate solution and 6 ml of water were mixed, and 50 µL of this mixture was added to each well. Absorbance at 750 nm was recorded using a microplate reader BioTek PowerWave™ XS. Calibration curves were constructed using standard solutions of CGA (0.1-1 mg L^{-1}), and results were expressed as µg CGA mL^{-1}.

2.4.3. MALDI-TOF-MS analysis. MALDI-TOF mass spectra of samples incubated for 72 h were acquired in the linear positive ion mode using Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, Massachusetts). The accelerating voltage was 25 kV. Sinapinic acid (10 mg L^{-1} in 50% acetonitrile/TFA 0.1%) was used as the matrix. Spectra were externally calibrated using a commercial protein mixture provided by the instrument manufacturer (PerSeptive Biosystems, Framingham, Massachusetts).

2.5. Functionality changes in proteins

The antioxidant capacity of samples incubated for 72 h was estimated by the $ABTS^{\bullet+}$ decolourisation assay as described by Oki et al. (2002) (14) 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid radical cations ($ABTS^{\bullet+}$) were produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS\bullet^{+}$ solution (stable for 2 d) was diluted in 5 mM PBS pH 7.4 (1:16 v/v) to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample was dissolved in phosphate buffer (5 mM, pH 7.4) at 0.1 mg L^{-1}. Thirty µL of test sample and 200 µL of diluted $ABTS^{\bullet+}$ solution were mixed. Absorbance of the samples at 734 nm was measured at 10 min of reaction using BioTek Power Wave™ XS microplate reader. CGA at concentrations of 0.015-0.2 mM was used for calibration.

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Analysis of Variance (more than 2 groups), one-way and two-way ANOVA followed by Bonferroni test, were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$. Relationships between the analysed parameters were evaluated by computing Pearson linear correlation coefficients setting the level of significance at $p < 0.001$.

3. Results

3.1 Formation of CGA derivatives

Figure 1a compares the HPLC chromatograms of standard CGA before (lower panel) and after incubation at pH 7.4, 37 ºC for 24 h (upper panel). Peaks were assigned based on retention times and UV-Vis spectra. Under our experimental conditions, CGA was converted into two isomers, namely neochlorogenic acid (trans-5-*O*-Caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-Caffeoylquinic acid).

The MALDI-TOF-MS (**Figure 1b**) demonstrated the co-occurrence of the hydroquinone and quinone forms ($[M + H]^+$ *m/z* 353 and *m/z* 355, and $[M + Na]^+$ *m/z* 375 and *m/z* 377, respectively) along with the dimeric adducts ($[2M + Na]^+$ *m/z* 729 and *m/z* 731), as assigned in the **Table 1**. No CGA homopolymers were detected by either HPLC or MALDI-TOF-MS.

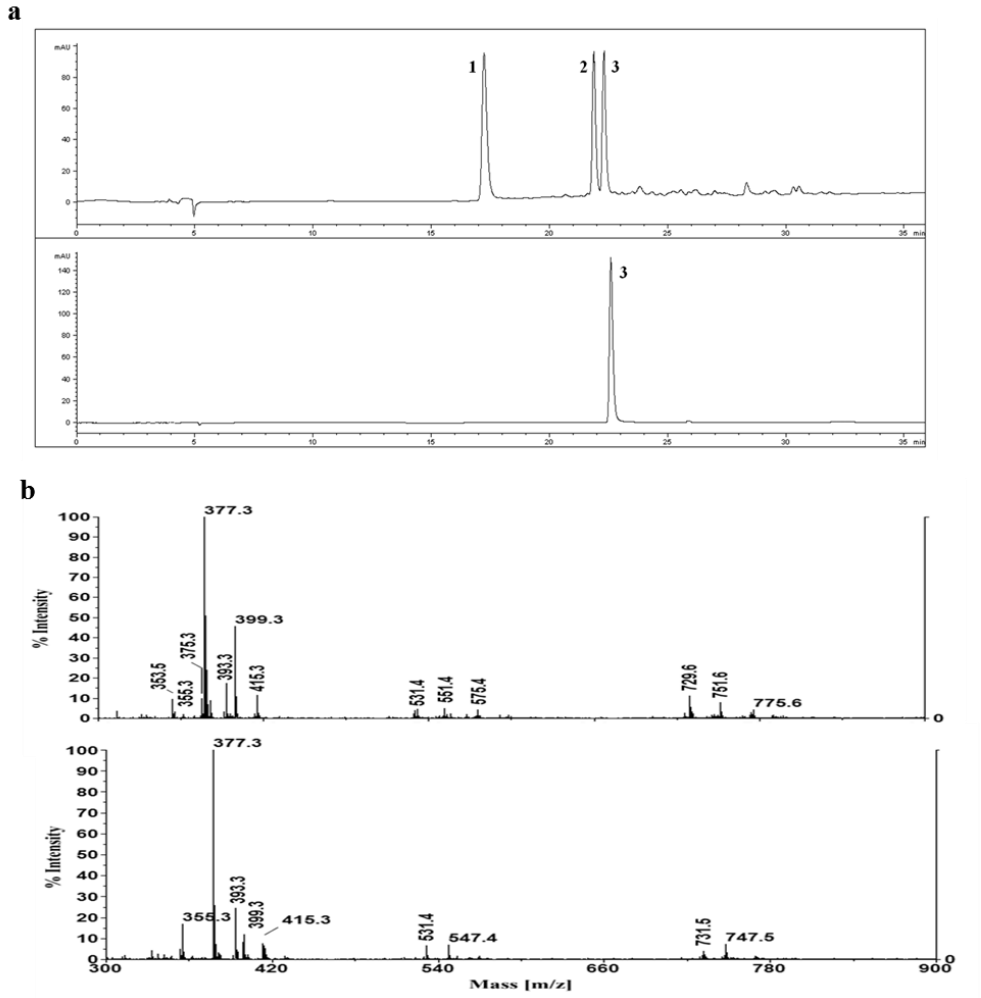

Figure 1: (a) RP-HPLC chromatograms of CGA incubated at pH 7.4, 37 ºC during 24 h (upper panel) and freshly prepared (lower panel). Peak 1: neochlorogenic acid; Peak 2: cryptochlorogenic acid; Peak 3: chlorogenic acid (b) MALDI-TOF spectra of incubated at pH 7.4, 37 ºC for 24 h (upper panel) and freshly prepared CGA (lower panel).

Table 1: MALDI-TOF MS assignments of CGA derivatives.

m/z	Assignment
353.5	$[CGA*+H]^+$ quinone
355.5	$[CGA+H]^+$
375.3	$[CGA+Na]^+$ quinone
377.5	$[CGA+Na]^+$
393.3	$[CGA+K]^+$
399.3	$[CGA+2Na]^+$
415.3	$[CGA+Na+K]^+$
531.4	DHB (matrix) adducts
547.4	DHB (matrix) adducts
551.4	DHB (matrix) adducts
729.6	$[CGA+CGAquinone+Na]^+$
751.6	$[CGA+CGAquinone+2Na]^+$
775.6	$[CGA+CGAquinone+3Na]^+$

*CGA includes the isomers of chlorogenic acid that are undistinguishable by mass spectrometry.

3.2 Progress of the glycoxidation reaction.

The availability of free amino groups was obtained by OPA assay (**Figure 2**). Incubation of BSA alone at 37 ˚C for 192 h did not significantly affect ($p > 0.001$) the availability of free amino groups, indicating the absence of inter-protein cross-linking events. Incubation in the presence of MGO produced a significant decrease ($p < 0.001$) in BSA free amino groups during the incubation period, suggesting that the glycoxidation reaction occurred. Interestingly, the addition of CGA to the glycoxidation mixture (BSA+MGO) also caused a significant decrease ($p < 0.001$) in available free amino groups throughout the whole incubation period. Available free amino groups also decreased when BSA was incubated with CGA alone compared to the protein control and did not significantly differ ($p > 0.001$) from those of the inhibition model composed of BSA, MGO and CGA.

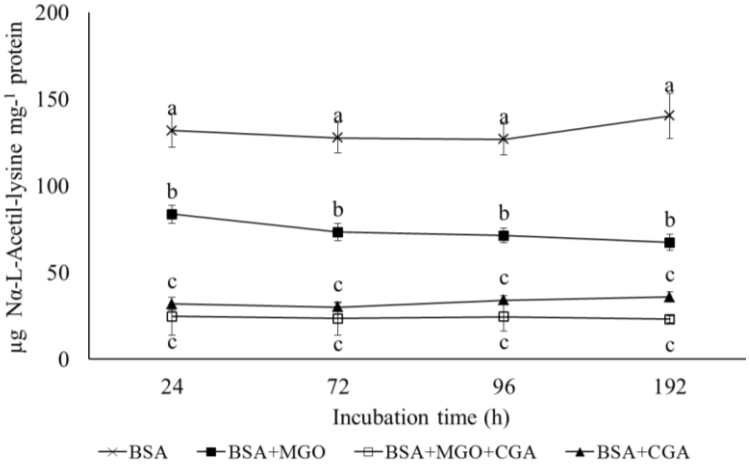

Figure 2: Changes in the content of free amino groups in samples of control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA) and BSA with MGO (BSA+CGA) incubated at pH 7.4, 37 ˚C at different times during 192 h. Data are means of triplicate analyses (n=9). Error bars denote the relative standard deviation. Different letters indicate significant differences ($p < 0.001$) within model systems at different times. BSA data are considered as references.

Figure 3 illustrates the formation of fluorescent AGEs during 192 h of glycoxidation reaction. As expected, the protein control (BSA alone) showed very low fluorescence intensity throughout the experiment, due to intrinsic fluorescence caused by the presence of fluorescent amino acids in the protein backbone. The reaction of BSA and MGO produced a significant formation ($p < 0.05$) of fluorescent AGEs in a time dependent manner. The presence of CGA efficiently inhibited ($p < 0.05$) fluorescent AGE formation in the glycoxidation model system, while the reaction of BSA and CGA caused a minor formation of fluorescent compounds. Further and more precise information regarding the generation of total AGEs, both fluorescent and non-fluorescence adducts, under our experimental conditions was obtained by indirect ELISA (**Table 2**). The results are consistent with those obtained by fluorescence monitoring. BSA data are considered basal values for all model systems. AGE generation was

significantly ($p < 0.05$) inhibited by the presence of CGA in the glycoxidation system.

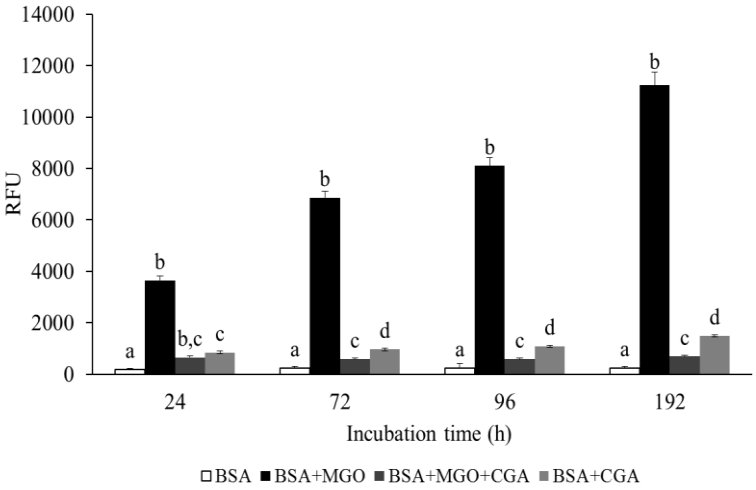

Figure 3: Time-course of fluorescent AGE formation in samples of control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA) and BSA with CGA (BSA+CGA) incubated at pH 7.4 and 37 ˚C at different times during 192 h. Data represent relative fluorescence units (RFU) (λ_{exc} 360 nm, λ_{em} 440 nm). Bars represent mean values (n=9) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.05$) within model systems at the different times.

Table 2: Content of total AGEs in samples corresponding to control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA) and BSA with CGA (BSA+CGA) incubated at pH 7.4 and 37 ºC for 72 h. BSA data are considered as initial values.

Total AGEs	**Incubation time (h)**
(μg AGE-BSA mg^{-1} protein)	**72**
BSA	1.01 ± 0.08^{b}
BSA+MGO	1.68 ± 0.13^{a}
BSA+MGO+CGA	0.51 ± 0.08^{c}
BSA+CGA	$0.84 \pm 0.19^{b,c}$

Each value represents the mean (n = 9) ± standard deviation. Different letters denote significant differences ($p < 0.05$) between samples of the same column.

Figure 4 shows the generation of brown compounds. Absorbance values at 420 nm of mixtures composed of BSA alone and BSA+MGO were very low and not significantly different ($p > 0.05$) in any case. The presence of CGA in the model systems induced significant brown compound formation in a time dependent manner. High and similar levels of browning ($p > 0.05$) were found in model systems composed of CGA alone and BSA+CGA. The extent of brown compound formation in samples composed of BSA, MGO and CGA was significantly lower ($p < 0.05$) than in the other samples containing CGA.

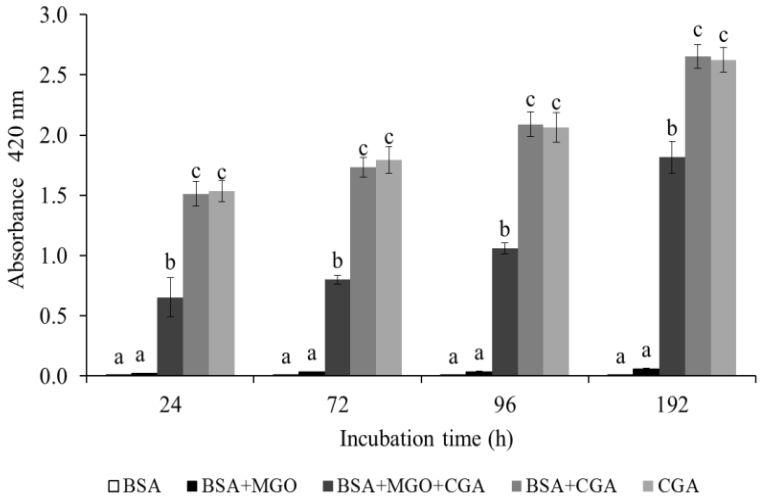

Figure 4: Time-course of brown compound formation from control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA), BSA with CGA (BSA+CGA) and CGA control (CGA) incubated at pH 7.4, 37 ºC for 192 h. Data represent relative absorbance at 420 nm at different time points. Bars represent mean values (n=9) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.05$) within model systems at the different times.

3.3 Structural changes of protein

Since significant AGE formation was observed after 72 h of glycoxidation reaction (**Figure 3** and **Table 2**), those samples were selected for further characterisation. As shown in **Figure 5a**, fresh and incubated (37 ˚C for 72 h) BSA solutions exhibited identical UV-Vis spectra, suggesting that no structural modifications of

proteins occurred following heating. Furthermore, the glycoxidation reaction BSA+MGO did not significantly alter the UV-Vis spectrum compared to fresh BSA. In contrast, the protein fraction isolated from the glycoxidation mixture with CGA showed a very different spectrum than that found for the control (BSA) and was very similar to the spectrum of BSA incubated with CGA.

Total phenolic content of the samples incubated at pH 7.4, 37 ºC for 72 h is shown in **Figure 5b**. As expected, significant levels ($p < 0.05$) of phenolic compounds were detected in the protein fractions isolated from the CGA model systems, namely BSA + CGA and BSA + MGO + CGA.

MALDI-TOF-MS analysis was performed to confirm the formation of covalent bindings of CGA to the protein backbone at 72 h (**Figure 6**). In the spectra corresponding to BSA incubated with MGO, the characteristic peak of BSA was clearly visible with variable mass increases (**Figure 6b**). Greater mass shifts were observed when BSA was incubated with CGA either in the absence (**Figure 6c**) or presence of MGO (**Figure 6d**). The mass data suggested that, BSA binds several molecules of CGA and its derivatives in addition to the MGO in these samples, forming a heterogeneous mixture of protein conjugates as reflected by the broadening of BSA peaks (**Figure 6c** and **6d**).

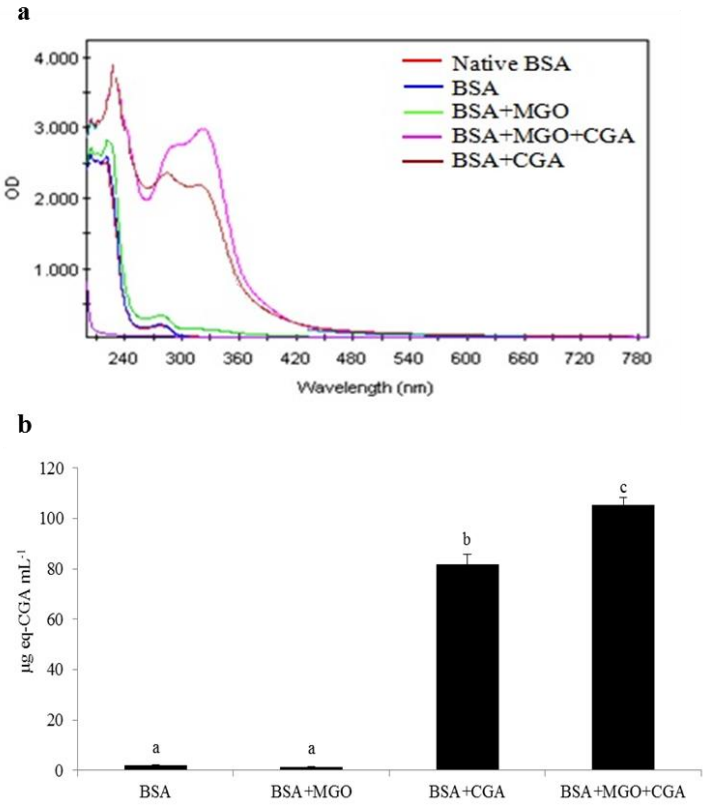

Figure 5: (a) UV-Vis absorption spectra and (b) content of phenol compounds bound to BSA isolated from samples corresponding to control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA), BSA with CGA (BSA+CGA) and CGA control (CGA) incubated at pH 7.4 and 37 ºC for 72 h**.** Bars represent mean values (n=9) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.001$) between means.

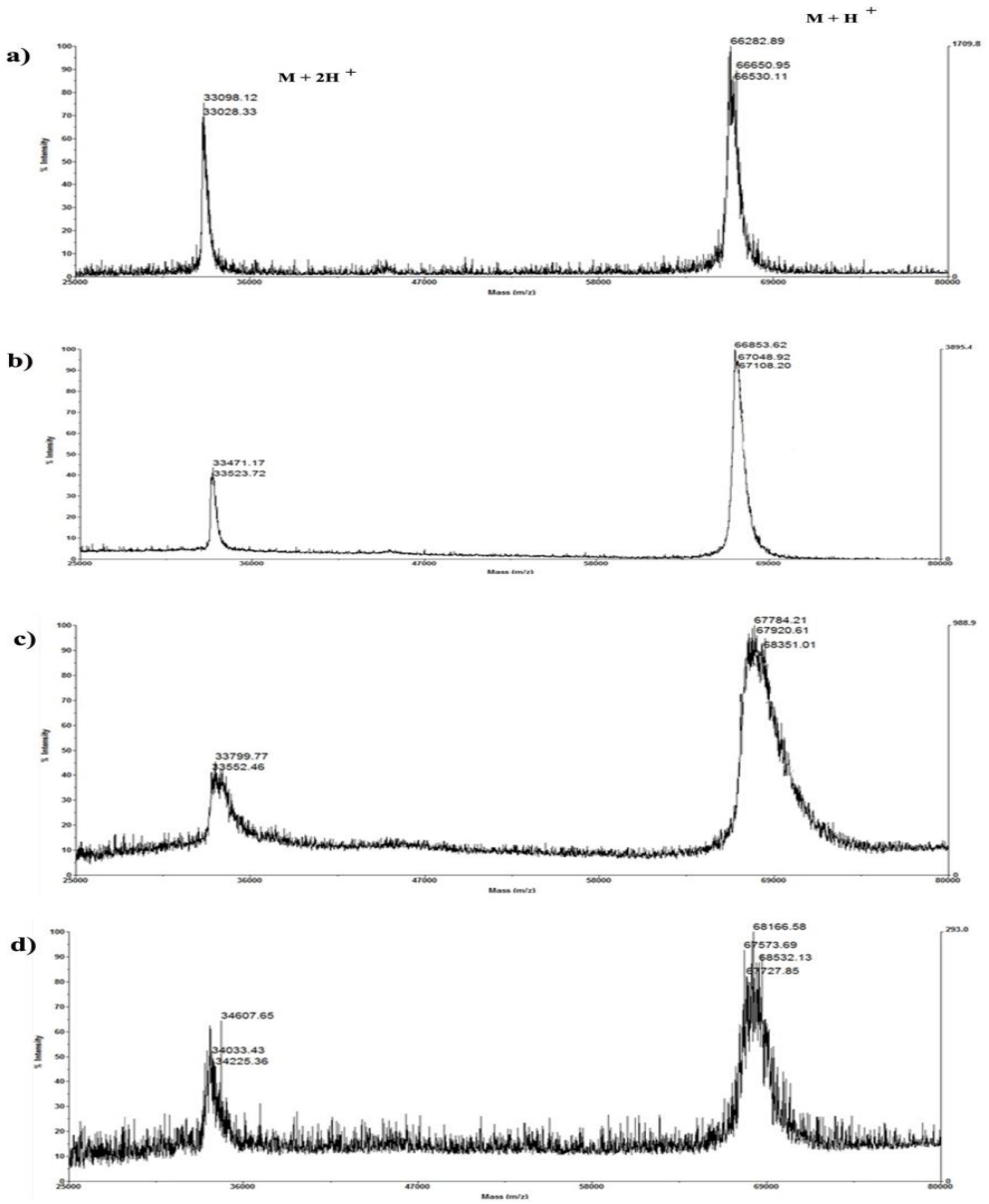

Figure 6: MALDI-TOF spectra of BSA control (a), BSA with MGO (b), BSA with CGA (c) and BSA with MGO and (d) incubated at pH 7.4 and 37 ˚C for 72h.

3.4 Changes of protein function

The antioxidant capacity of the isolated protein fractions obtained from samples incubated at 37 ºC for 72 h is shown in **Figure 7**. The reaction with MGO did not modify the antioxidant capacity of BSA. The addition of CGA to reaction mixtures caused the formation of compounds (MW > 30 kDa) which had antioxidant capacity values of 303.07 and 309.89 μg eq-CGA mL^{-1} for model system composed of BSA+MGO+CGA and BSA+CGA, respectively.

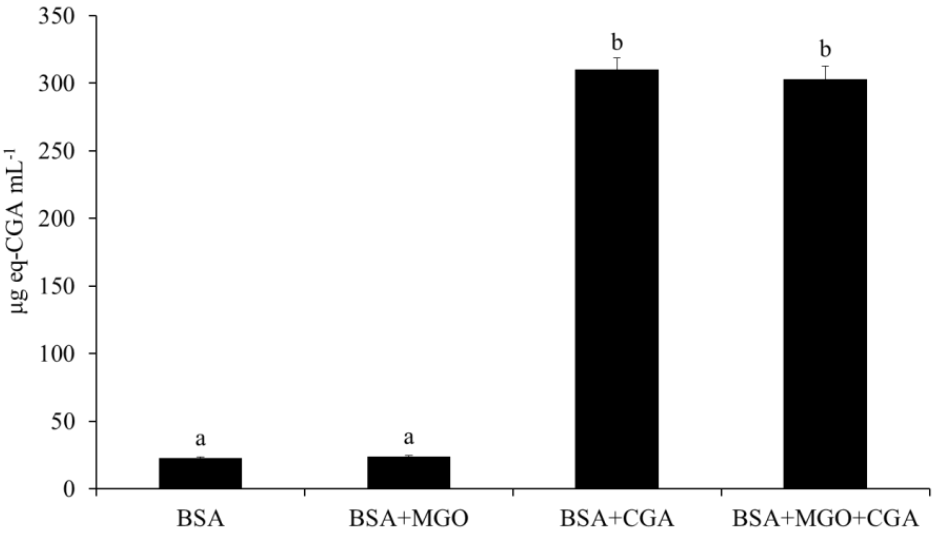

Figure 7: Antioxidant capacity of the high molecular weight fractions isolated from samples of control (BSA), BSA with MGO (BSA+MGO), BSA with MGO and CGA (BSA+MGO+CGA), BSA with CGA (BSA+CGA) and CGA control (CGA) incubated at at pH 7.4 and 37 ˚C for 72 h. Data are expressed as g eq-CGA mL^{-1}. Bars represent mean values (n=9) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.001$) between means.

3.5. Correlation between parameters

A significant negative correlation ($r=-0.754$, $p < 0.001$) between data corresponding to free amino groups and antioxidant capacity was observed for samples incubated at 37 ºC for 72 h. A significant negative correlation ($r=-0.689$, $p < 0.001$) was also found between free amino groups and total phenolic content.

4. Discussion

In this work we observed that structural changes in CGA produced *in vitro* under mimicked physiological conditions may contribute to the antiglycoxidative properties of this compound. Isomerisation of CGA (3-*O*-caffeoylquinic acid) was induced at pH 7.4 and 37 ºC. The formation of neochlorogenic (trans-5-*O*-caffeoylquinic acid) and cryptocholorogenic (4-*O*-caffeoylquinic acid) acid from CGA under similar reaction conditions has previously been reported (15-16). CGA derivatives such as oxidation products and isomers might be able to act as substrate or/and precursors of the Maillard and polymerisation reactions (17). The formation of mono-quinones and dimer quinones was also observed in CGA incubated at pH 7.4 and 37 ºC for 24 h. This is in agreement with the non-enzymatic oxidation of CGA previously described by Rawel et al. 2010 (18)**.**

Brown compounds may be formed by the Maillard reaction, oxidation of phenols and phenol polymerisation (17). Our data suggest that the Maillard and phenol oxidation reactions are the main pathways leading to the formation of brown compounds under our experimental conditions. Both CGA and its derivatives are able to react with BSA via the Maillard reaction. However, further studies are needed to determine the chemical nature of new-formed coloured compounds.

The observed decrease in the formation of AGEs in the presence of CGA demonstrates the antiglycative activity of this compound. On the other hand, our results suggest conjugation of CGA or its derivatives to free amino groups. A significant negative correlation between content of free amino groups and phenolic compounds was found. These results are in agreement with Rawel et al. (2009) (19) who reported a decrease in lysine residues due to the reaction of BSA and CGA at room temperature for 24 h. CGA isomers and quinones can interact with proteins forming non-covalent and covalent bonds through the Maillard Reaction[17]. Phenolics bind highly nucleophilic thiol, amine groups and hydrophobic aromatic groups of proteins (20). Three potential types of non-

covalent interactions between hydroxycinnamic acids and proteins have been proposed: hydrogen, hydrophobic, and ionic binding (21). Prigent et al. (2007) (20) found that oxidised CGA induced covalent modification of α-lactalbumin and lysozyme.

Soft ionization MS techniques such as MALDI are useful to evaluate the hydroxycinnamates (HCA) covalently bound to proteins (19). MALDI-TOF-MS data suggest the formation of neoformed protein-phenol conjugates, inducing MS increments of 1.7 and 1.3 kDa in samples corresponding to BSA+CGA and BSA+CGA+MGO, respectively. The increase of molecular mass is indicative of covalent binding between CGA and/or its derivatives to the protein structure. Data on MALDI-TOF-MS support the data obtained on free amino groups, phenolic compounds and UV-Vis spectra.

The formation of complexes by covalent binding of other reactive phenols such as quercetin to BSA exhibiting antioxidant potential have been previously reported (22,23). Quercetin and CGA share a high binding affinity for BSA. The ability of these two compounds to form covalent complexes polyphenol-BSA under physiological conditions has been demonstrated (24,25). Our results show that CGA causes the neoformation of molecules with antioxidant capacity. However, further studies are needed on the antioxidant mechanism of action of BSA-CGA conjugates.

Gugliucci et al. 2009 (8) previously associated the inhibitory capacity against formation of fluorescent AGEs of *Ilex paraguariensis* extracts to the presence of CGA. The inhibitory capacity of CGA was linked to its antioxidant character, chelating properties to transition metals ions, quenching of carbonyl radical species and AGE crosslinking (26-28). Other authors have also shown the ability of CGA to inhibit *in vitro* BSA glycation induced by fructose and glucose and the formation of AGE crosslinking from collagen (11). We have recently reported that MGO is effectively trapped by CGA with an IC_{50} of 0.14 mg mL^{-1} (29). In addition

to this mechanism, we propose for the first time a relationship between the high binding capacity of CGA to BSA and its antiglycoxidative mechanism of action. The covalent interactions suggest MGO and GCA are competing for reactive protein sites (free amine group). This effect prevents MGO from binding to BSA resulting in an effective decrease in AGE formation.

Coffee is the major source of CGA on the diet and it has been identified as a beverage with the highest *in vitro* antioxidant capacity (30). CGA from coffee has shown a high bioavailability in humans (31). Consumption of coffee acutely increases the concentrations of phenolic compounds (CGA) in LDL cholesterol particles and platelets, increases ex vivo resistance to LDL oxidation, and reduces platelet aggregation in healthy volunteers (32-33). The formation of poliphenol-protein complex *in vivo* can exert helpful effects in cardiovascular related pathologies such as diabetes mellitus type 2 by AGEs inhibition.

In summary, the covalent conjugation of CGA and its derivatives (isomers and quinones) to side-chains of protein lysine residues reduces the formation of potentially harmful compounds, also called AGEs, and promotes the generation of antioxidant structures, which may be beneficial for human health.

Abbreviations: AGEs, advanced glycation end products; MGO, methylglyoxal; GO, glyoxal; HCA, hydroxycinnamic acids; BSA, bovine serum albumin; CML, N^{ϵ}-(carboxymethyl)lysine; CEL, N^{ϵ}-(carboxyethyl)lysine, AG, aminoguanidine; 5-CQA, 5-*O*-caffeoylquinic acid; 3-CQA, 3-*O*-caffeoylquinic acid; CGA, 3-*O*-caffeoylquinic acid.

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Use of phytochemomics for validating the potential of coffee silverskin extract as natural source of inhibitors of the glycoxidation reaction

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Abstract

Coffee silverskin extract (CSE) has been proposed to be a good source of phenolic compounds such as chlorogenic acid (CGA) and its derivatives which are formed during roasting. Among other health-promoting benefits, CSE presents *in vitro* antiglycoxidative and carbonyl reactive species trapping capacities. These properties have been associated to other chemical components besides CGA. This study aimed to obtain novel information regarding the pathways of CSE for inhibiting the formation of fluorescent advanced glycation end products (AGEs). CSE and CGA significantly inhibited ($p < 0.001$) the formation of fluorescent AGEs in a protein-methylglyoxal glycoxidation model system (37 °C for 96 hours). The lack of glycoxidation in the presence of CGA and CSE was verified using liquid chromatography – tandem mass spectrometry (LC-MS/MS) techniques which demonstrated the almost complete absence of Arg adducts after 24h of reaction. Mass spectrometry analysis supported the results obtaining in free amino acids, phenol bound to proteins and the antioxidant capacity of the samples. Differences observed between samples composed of CGA and CSE may be due to differences in their concentrations in free CGA. In conclusion, aqueous CSE can be considered a natural source of various inhibitors of *in vitro* formation of AGEs acting by different pathways besides their carbonyl trapping capacity. The inhibitory effect of CGA present in CSE may be associated to its carbonyl trapping capacity as well as to its ability to react with side-chains of protein amino residues blocking the reaction sites with carbonyl reactive groups.

Keywords: Advanced glycation end products (AGEs) inhibitors, coffee silverskin, chlorogenic acid, protein-phenols conjugates phytochemomics.

1. Introduction

Oxidative stress and hyperglycemia induce the formation of advanced glycation end products (AGEs), processes which play an important role in the development of type 2 diabetes (T2D) and its complications. AGEs are formed via non-enzymatic glycation and also as an outcome of glucose autoxidation, lipid peroxidation or the polyol pathway condensation reactions (1). Reactive dicarbonyls such as methylglyoxal (MGO), 3-deoxyglucosone and glyoxal act as intermediate species leading to the formation of AGEs. MGO specially causes the generation of Nε-(carboxyethyl)lysine and methylglyoxal-lysine dimer, which are capable of binding and modifying a number of proteins through the glycoxidation reaction (2). AGEs derived from food are also involved in the pathogenesis of T2D (3). Thus, the search for natural inhibitors of AGEs formation to treat chronic diseases such as T2D and decrease their risk is a worldwide priority.

Polyphenols are one family of plant compounds that are abundant in coffee as well as in red wine, fruits and fruit juices, tea, vegetables, chocolate and legumes. There is increasing evidence that polyphenols contribute to many aspects of our overall health. Phenols and their derivatives may form complexes with proteins and exhibit antiglycative capacity (4). The formation of protein-polyphenols conjugates by covalent or non-covalent interactions makes glycation targets (mainly basic amino acids) inaccessible, inhibiting the glycoxidation reaction (5,6). Polyphenols can also protect against glycoxidation by scavenging reactive oxygen species (ROS), which is a consequence of oxidative stress, consequently slowing glycation and inhibiting AGEs formation (7). CGA is the most abundant polyphenol present in coffee (8). CGA has been reported to be a potent inhibitor of AGEs formation *in vitro* (7,5,9,10). A new antiglycoxidative mechanism of action for CGA has recently been proposed under mimicked physiological conditions. The proposed pathway "physically" protects the protein structure by the interaction of CGA with side-chains of protein amino residues blocking the reaction sites with carbonyl reactive groups and generating neoantioxidant

compounds (4). This novel information was obtained using advanced analytical approaches called phytochemomics (11).

Coffee silverskin is the tegument of green coffee beans (outer layer) and the by-product of the roasting procedure. Coffee silverskin extract (CSE) is a good source of phenolic compounds such as chlorogenic acid (CGA) and its derivatives formed during the roasting process (12). Laboratory studies have shown that CSE has the capacity to inhibit the formation of fluorescent AGEs and decrease carbonyl radical stress. The overall antiglycative capacity of CSE was mainly associated to other phytochemicals present in CSE such as melanoidins and antioxidant dietary fibre besides CGA (7). It is not yet known whether this antiglycoxidative character involves protein-phenol conjugation. More studies are needed to draw the whole map of the antyglycative mechanism of action of CSE, identify the CSE compounds involved and its health benefits. Phytochemomics were used to achieve this goal. Mass spectrometric analyses are very relevant to detect structure modifications and identify protein adducts.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals and solvents were of analytical grade. Bovine serum albumin (BSA), 3-O-caffeoylquinic acid (CGA), sodium azide, ortho-phthaldialdehyde (OPA), Nα-acetyl-L-lysine, and 3.3′,5.5′tetramethylbenzidine (TMB), 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals and their suppliers were as follows: β-mercaptoethanol (Merck, Hohenbrunn, Germany), methylglyoxal solution, Folin–Ciocalteau and sinapinic acid (Fluka, Buchs, Switzerland), Bradford reagent for the protein assay (Bio-Rad, München, Germany), and pepsin and endoproteinase AspN (Promega, Madison, WI, USA). The Amicon® Ultra-0.5 ml centrifugal filter unit fitted with an Ultracel®-30 K regenerated cellulose membrane (30 kDa cut-off) was from Merck Millipore Ltd (Tullagreen, Cork, Ireland). Microtest 96-well plates made from high-quality polystyrene were purchased from Sarstedt AG & Co. (Nümbrecht, Germany). The Costar® high binding 96-well EIA/RIA plate was from Corning Incorporated (Corning, NY, USA). The High Mass Range Peptide Mix supplied by Applied Biosystems (Monza, Italy) was used for calibrating the MALDI-TOF mass spectrometer in the mass range m/z 10000-100000. Water was purified using Milli-Q system.

Raw material: Coffee silverskin from Robusta (Coffea canephora) was provided by Fortaleza S.A. (Bilbao, Spain). According to the manufacturer, CS represents 0.5% of green beans and 0.6% of roasted beans. CSE powder was made as described in patent WO 2013/004873 (13). CGA content in the CSE model system was calculated based on data obtained by Mesías et al. (2014) (7).

2.2 *In vitro* glycoxidation of proteins

Model glycoxidation systems were prepared containing BSA at a final concentration of 1 mg/mL in 0.01 M PBS buffer (pH 7.4) in the presence or absence of CGA (10 mM) or CSE (10 mg/mL). The pH values for all solutions were measured at 25°C using an electrode pH-meter (Metler Toledo, Mp 230, Columbus, OH, USA). Each day, the pH meter was calibrated before use with standard buffer solutions at pH 4 and 7. The glycoxidation reaction was started by adding MGO at a final concentration of 5 mM. The model systems were incubated at 37°C for 24 and 96 h. The glycoxidation reaction was stopped by reducing temperature in an ice bath. The samples were kept at - 20 °C until analysis. All samples were prepared in duplicate. Samples were fractionated by ultrafiltration. Briefly, samples (0.4 ml) were placed in the sample reservoir of an Amicon® Ultra-0.5 ml centrifugal filter unit fitted with an Ultracel®-30K regenerated cellulose membrane (30 kDa cut-off) and centrifuged at 14000 g for 40 min at room temperature. Concentrated samples were recovered and re-suspended in PBS (0.4 ml). Protein recovery (RMM > 30 kDa) was determined by the Bradford micro method (14).

2.3 Free amino groups. The free protein amino groups (alpha and epsilon N-terminal amino acid side chain of lysine) were determined by the OPA assay according to Go et al. (2008) (15). The OPA reagent was freshly prepared by dissolving 10 mg of OPA in 250 µL of 95% (v/v) ethanol and adding 9.8 mL of 0.01 M PBS pH 7.4 and 20 µL of β-mercaptoethanol. The reaction was carried out in 250 µL total volume in a transparent polystyrene 96-well microtest plate. Fluorescence was read right after the addition of the OPA reagent on a microplate fluorescence reader Biotek Synergy™ HT (Biotek Instruments, Winooski, USA) with excitation wavelengths of 360 ± 40 nm and emission wavelengths of 460 ± 40 nm. Readings were recorded every 53 s for 15 min. Calibration curves were constructed using standard solutions of Nα-acetyl-L-lysine (0.025-1 mmol). All measurements were performed in triplicate and concentration was expressed as µg Nα-acetyl-L-lysine/ mg protein.

2.4 Advanced glycation end products. Fluorescent AGEs formation was measured using a Biotek microplate spectrophotometer BioTek Synergy™ HT (Biotek, Instruments, Winooski, VT, USA). Fluorescence intensity of the sample was measured using excitation wavelengths of 360 ± 40 nm and emission wavelengths of 460 ± 40 nm. No dilution was required for glycoxidation model systems. All measurements were performed in triplicate. Results were expressed as relative fluorescents units (RFU).

2.5 Phenol compounds bound to protein

2.5.1 Determination of total phenolic compounds. Total phenolic content of the samples incubated for 96 h was determined using the Folin–Ciocalteu method as described by Singleton et al. (1999) (16) adapted to a microplate reader. The reduction reaction was carried out in 210 µL total volume in 96-well microplates (no. 82.1581). Ten µL of the sample (appropriately diluted when necessary) was added to 150 µl volume of the Folin–Ciocalteu reagent (diluted 1:14, v/v) in Milli-Q water. After 3 minutes, 50 µL of a solution composed of 4 mL of 75 g/L sodium carbonate solution and 6 mL of water was added to each well. Absorbance at 750 nm was recorded using a BioTek PowerWave™ XS microplate reader. Calibration curves were constructed using standard solutions of CGA (0.1–1 mg/mL), and results were expressed as µg eq-CGA/mg protein. All measurements were performed in triplicate.

2.5.2 $ABTS^{\bullet+}$ assay. The antioxidant capacity of samples incubated for 96 h was estimated by the $ABTS^{\bullet+}$ decolorization assay as described by Oki et al.(2006) (17). An $ABTS^{\bullet+}$ stock solution was prepared by reacting 7 mM ABTS stock solution with 140 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution (stable for 2 d) was diluted in 5 mM PBS pH 7.4 (1:75 v/v) to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample was dissolved in phosphate buffer (5 mM, pH 7.4) at 0.1 mg/L. Samples (30 µL) were added to 270 µL of diluted $ABTS^{\bullet+}$ solution in a microplate. Absorbance was measured at 734 nm for 10 min at 30ºC with measurements every 2 min using a BioTek Power Wave™ XS microplate reader. CGA at concentrations of 0.15–0.2 mM was used for calibration. Results were expressed as µg eq-CGA/mg protein. All measurements were performed in triplicate.

2.6 Structural changes in protein

2.6.1 Preparation of proteins and peptides for mass spectrometry analysis. BSA was isolated from the glycoxidation systems by precipitation with 80% cold acetone (1:10, v/v; 20 h, - 27°C). The pellet was recovered after centrifugation (10000 *g*, 45 min), washed twice with cold acetone and dried. BSA was solubilized in 300 mM tris-HCl, 6M guanidine and 1mM EDTA at pH 8. Dithioteitrol was added at a final concentration of 10 mM, and BSA was reduced at 37°C for 1 h. Subsequently, the protein was alkylated with iodoacetamide (55mM, at 22°C for 30 min in the dark). PD10 desalting columns (GE Healthcare Life Sciences, Milan, Italy) were used to remove low molecular weight molecules and exchange the denaturation buffer with the digestion buffers.

Two enzymatic digestions were performed. Pepsin digestion (1:100, E:S) was carried out for 2 h at 37°C in 5% formic acid (FA) and 20% acetonitrile (ACN) at pH 2. Endoproteinase AspN digestion (1:100, E:S) was carried out for 4 h at 37°C in 50 mM ammonium bicarbonate at pH 8. Both enzymes, sequencing grade, were purchased from Promega (Madison, WI, USA). Prior to MS analysis, peptide digests were desalted using C18 Zip-Tip pre-packed micro-columns (Merck Millipore, Bedford, MA, USA), previously equilibrated with 0.1% trifluoroacetic acid (TFA) in water and eluted with a mixture of H_2O/ACN/TFA (50/50/0.1 v/v/v).

2.6.2 RP-HPLC. The peptides digests were separated by RP-HPLC using a C18 Vydac 2.1 mm i.d. column (Hesperia, CA, USA), respectively. The pool of peptides was fractionated by applying a linear gradient from 5 to 45% solvent B in 60 minutes. The HPLC chromatograph system was an HP 1100 Agilent modular system equipped with diode array detector (Palo Alto, CA, USA). In both cases, the flow rate was 0.200 ml/min. Column effluents were monitored by detection at λ= 220 and 280 nm.

2.6.3 MALDI-TOF analysis. Spectra were acquired using a Voyager DE Pro mass spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N2 laser (λ= 337 nm). The accelerating voltage was 20 kV. Mass spectra of the peptides were acquired in in either linear or reflector mode using 4-CHCA (α-Cyano-4-hydroxycinnamic acid) (10 mg/mL in 50% ACN/0.1% TFA). In both cases, spectra were acquired under delayed extraction conditions and 250 laser shots were accumulated for each spectrum. External mass calibration was performed with commercial standard peptide/protein mixtures (PerSeptive Biosystems, Framingham, MA). Raw data were analysed using the Data Explorer 4.0 software supplied with the spectrometer. Prior to data base searching, mass spectra were baseline corrected and Gaussian smoothed with a filter width of 5. Peaks with S/N >15 were selected and deisotoped. Identifications were carried out using the non-redundant National Center for Biotechnology Information (nrNCBI) and Swiss Prot/TrEMBL databases via Mascot (Matrix Science, London, UK) and Protein Prospector MS-FIT (http://prospector.ucsf.edu/) search engines. The accepted criteria were mass tolerance of 0.3 Da, fixed carbamidomethylation of cysteines, variable: methionine oxidation, pyro-glutamic acid, and one missed tryptic cleavage.

2.6.4 Nano flow LC-ESI-MS/MS analysis. LC-ESI-MS/MS was carried out using an Ultimate nano3000 HPLC (Dionex, Sunnydale, CA, USA), equipped with a Famos auto sampler (Dionex), coupled to a Q-STAR mass spectrometer (Applied BioSystems, Framingham, USA). Peptide digesta (~2 μg) were first loaded into a C18 trapping cartridge (LC Packings, Dionex, USA) and flushed 5 min at a flow rate of 5 μL/min (5% solvent B) by means of the loading pump and secondary, separated with a C18 PepMap100 column (15 cm length, 75 μ m ID, 300 Å [LC Packings]), using a linear gradient from 5 to 40% B for 90 min at a constant flow rate of 300 nL/min. The eluents were (A) 5% ACN in 0.1% FA and (B) 80% ACN in 0.08% FA. Eluted peptides were analysed on-line using an ESI-Q TOF Q-Star Pulsar instrument (Applied BioSystems, Foster City, CA, USA) equipped with a nano-spray source (Protana, Denmark).

LC-MS/MS experiments were performed in the information-dependent acquisition (IDA) mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than m/z 400, charge states 2 to 4, intensity exceeding 18 counts, former target ions were excluded for 30 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions and nitrogen was used as the collision gas. The raw spectrum files were used to generate text files in a mascot generic

file format (.mgf), which were submitted to the Mascot ver. 2.3 (http://www.matrixscience. com) and Batch-tag (Protein Prospector, University of California San Francisco, USA) search engines.

The following search criteria were applied: database, NCBI or Swiss-Prot; type of search, MS or MS/MS ion search; pepsin or endoproteinase Asp-N (cleavage on N-terminal side of Asp or Glu); fixed modifications, carbamidomethyl; variable modifications, oxidation on methionine and the N-terminal loss of ammonia at Gln; mass values, monoisotopic; parent tolerance, 0.07 Da; ms/ms tolerance, 0.1 Da; and number of maximum missed cleavages, 1. Unassigned MS/MS spectra were manually identified with the aid of the Analyst software (Applied BioSystems).

2.7 Statistical analysis

Prior to statistical analysis, data were tested for homogeneity of variances using the Levene test. For multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by the Games-Howell test when variances were not homogeneous. Differences were considered to be significant at $p < 0.05$, highly significant at $p < 0.01$ and very highly significant at $p < 0.001$.

3. Results

3.1. Progress of the glycoxidation reaction

Free amino groups were determined by the OPA assay (**Figure 1**). Incubation of BSA with MGO produced a significant decrease ($p < 0.001$) in BSA free amino groups at 37 ºC for 96 h, indicating that the glycoxidation reaction had occurred. Interestingly, the addition of CGA to the glycoxidation mixture (BSA with MGO) also caused a significant decrease ($p < 0.001$) in free amino groups. Available free amino groups also decreased compared to the protein control when BSA was incubated with CSE and did not significantly differ ($p = 0.133$) from those of the glycoxidation model composed of BSA and MGO.

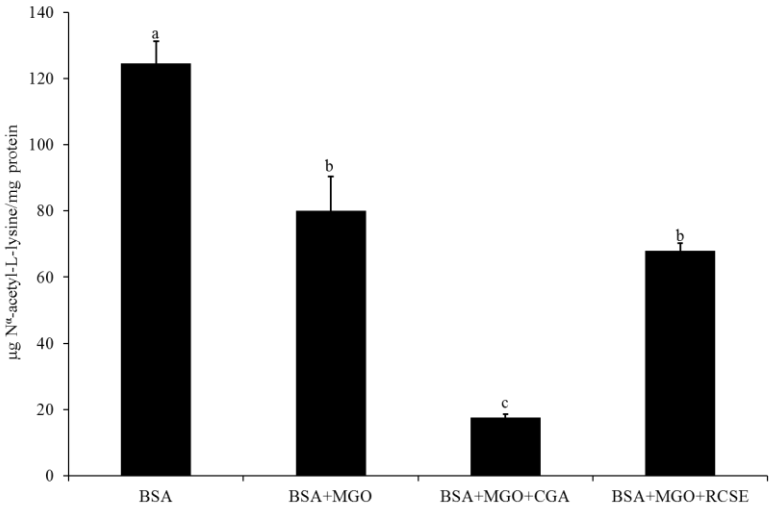

Figure 1. Changes in the content of free amino groups in samples of control (BSA), BSA with MGO (BSA + MGO), BSA with MGO and CGA (BSA + MGO + CGA) and BSA with MGO and RCSE (BSA + MGO + CGA) incubated at pH 7.4 and 37 °C for 96 h. Concentrations assayed were 1 mg/mL BSA, 5 mM MGO, 10 mM CGA and 10 mg/mL RCSE. Data represent the mean of duplicate analyses (n=6). Error bars denote the standard deviation. Different letters indicate significant differences ($p < 0.001$) within model systems. BSA data are considered as references.

Figure 2 illustrates the formation of fluorescent AGEs during 96 h of glycoxidation reaction. As expected, the protein control (BSA alone) showed very low fluorescence intensity, due to intrinsic fluorescence caused by the presence of fluorescent amino acids in the protein backbone. The reaction of BSA with MGO produced a significant formation ($p < 0.001$) of fluorescent AGEs. The presence of CGA or CSE inhibitors efficiently inhibited ($p < 0.001$) fluorescent AGE formation in the glycoxidation model system.

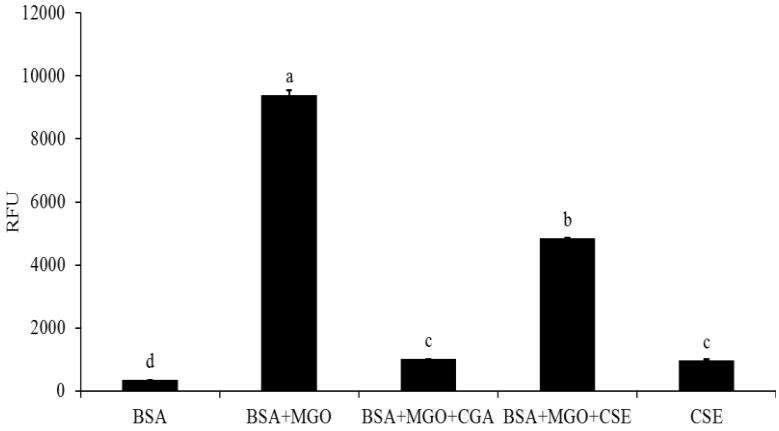

Figure 2. Fluorescent AGE formation in samples of the control (BSA), BSA with MGO (BSA + MGO), BSA with MGO and CGA (BSA + MGO + CGA) and BSA with MGO and CSE (BSA + MGO + CSE) incubated at pH 7.4 and 37 °C during 96 h. Concentrations assayed were 1 mg/mL BSA, 5 mM MGO, 10 mM CGA and 10 mg/mL. Data represent relative fluorescence units (RFU) (λ_{ex} 360 nm, λ_{em} 440 nm). Bars represent mean values (n=6) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.001$) within model systems.

2.1 Changes in protein function

Total phenolic content of the samples incubated at pH 7.4, 37 °C for 96 h is shown in **Figure 3**. BSA alone and BSA incubated with MGO showed significant differences ($p < 0.01$) in phenolic content. The Folin-Ciocalteu reagent is known to react with amino acids present in BSA such as tryptophan, tyrosine and cysteine (18). The reaction with MGO changes the structure of BSA and amino acids can be more exposed to the Folin-Ciocalteu reagent. As expected, significant levels ($p < 0.05$) of phenolic compounds were detected in the protein fractions isolated from the model systems containing CGA. The presence of CSE in the glycoxidation model system (BSA and MGO) significantly enhanced phenolic content ($p < 0.05$) as CGA equivalents.

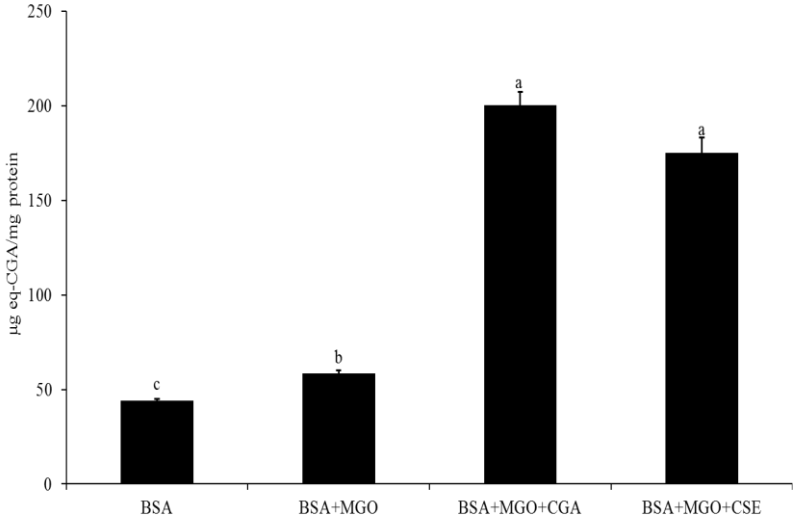

Figure 3. Content of phenol compounds bound to BSA isolated from samples corresponding to the control (BSA), BSA with MGO (BSA + MGO), BSA with MGO and CGA (BSA + MGO + CGA) and BSA with MGO and CSE (BSA + MGO+ CSE incubated at pH 7.4 and 37 °C for 96 h. Concentrations assayed were 1 mg/mL BSA, 5 mM MGO, 10 mM CGA and 10 mg/mL CSE. Bars represent mean values (n=6) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.01$) between means.

The antioxidant capacity of samples incubated at 37 °C for 96 h is shown in **Figure 4**. The reaction with MGO modifies the antioxidant capacity of BSA due to changes in its structure and makes amino acids more accessible to react with the $ABTS^{\bullet+}$ radical (19). The addition of CGA to reaction mixtures caused the formation of compounds which had antioxidant capacity for a model system composed of BSA, MGO and CGA. Similarly, the presence of CSE also significantly increased ($p < 0.05$) scavenging properties against the ABTS radical. The final concentration of CGA in model system containing CSE was 2 mM. However, no significant differences ($p > 0.05$) were found in the total antioxidant capacity values in model systems with CGA and CSE.

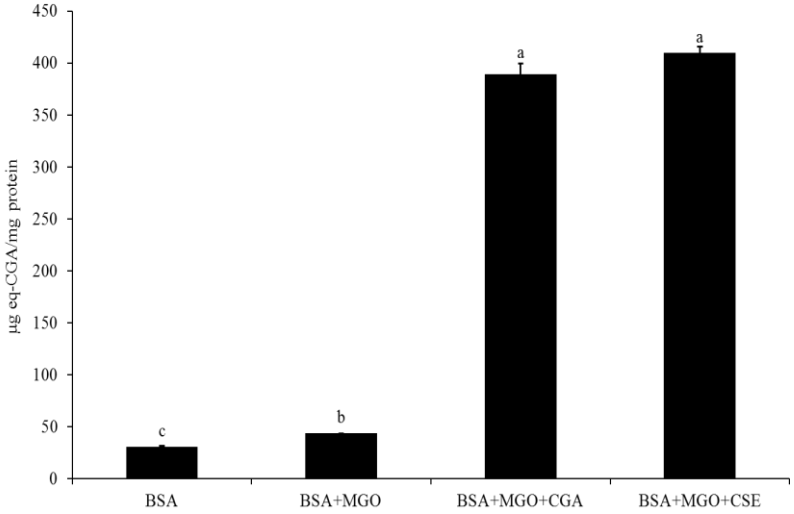

Figure 4. Antioxidant capacity of the high molecular weight fractions isolated from samples of the control (BSA), BSA with MGO (BSA + MGO), BSA with MGO and CGA (BSA + MGO + CGA) and BSA with MGO and CSE (BSA + MGO + CSE) incubated at pH 7.4 and 37 °C for 96 h. Concentrations assayed were 1 mg/mL BSA, 5 mM MGO, 10 mM CGA and 10 mg/mL CSE. Data are expressed as µg eq-CGA/mg protein. Bars represent mean values (n=6) and error bars represent standard deviation. Different letters denote significant differences ($p < 0.01$) between means.

2.2 Mass spectrometry characterisation of adducts

Since lysine and arginine are candidate amino acids substrates for glycation, hydrolysis with trypsin was not appropriate for this study (20). The MALDI-TOF analysis of the pepsin hydrolyzed BSA control led to a coverage of 45% of the protein sequence (**Figure 5**). For instance, the analysis of hydrolyzed BSA incubated for 24 h in both glycoxidation systems (BSA with MGO and BSA with CGA) showed the presence of a number of new peptides that are not expected to be released from the native protein (**Table 1** and **Figure 5**). The ESI-Q/TOF analysis of the samples allowed us to identify the major adducts and characterize the modification site. The fragmentation pattern of the 785.49 m/z, 682.43 m/z and 1231.52 m/z peptides (**Table 1** and **Figure 6**) corresponded to peptides 237-242 with the Arg 241 adduct (+54), peptide 479-783 with the Arg 482 adduct (+54), and peptide 274-283 with the Arg 280 adduct (+54), respectively.

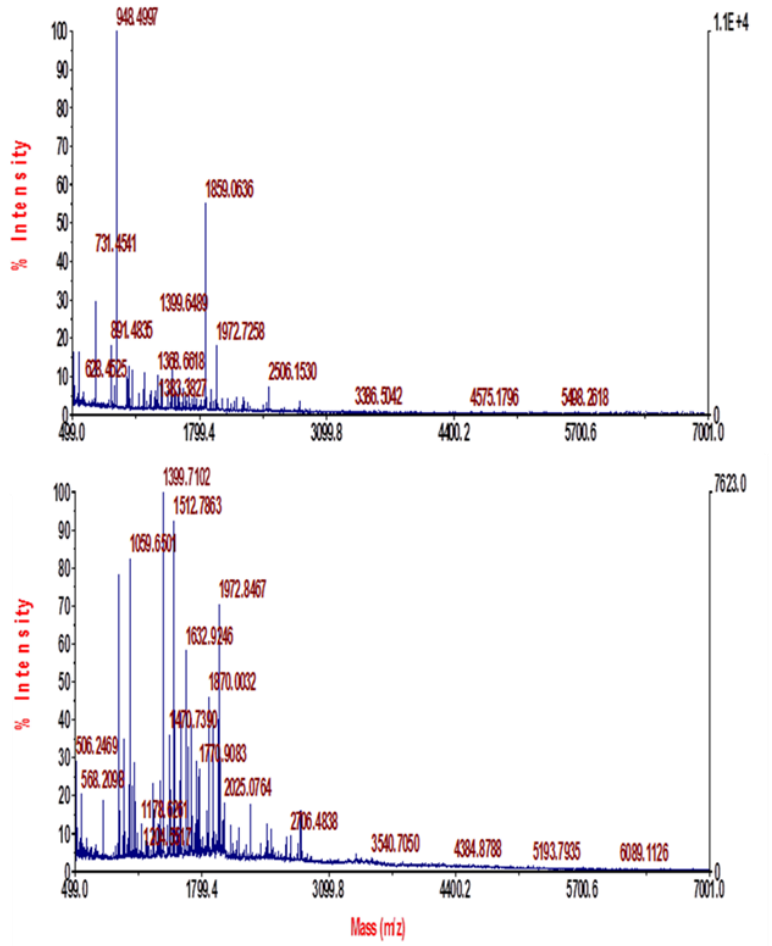

Figure 5. MALDI-TOF MS spectra of the pepsin peptides of BSA (upper panel) and BSA with MGO (lower panel) incubated at pH 7.4 and 37 ºC for 24 h.

Table 1. Peptides released by pepsin digestion from BSA with MGO incubated at pH 7.4 and 37 ºC for 24 h.

Measured MH^+ (m/z)	**Theorical MH^+ (m/z)**	**Sequence**	**Adduct**
785.49	731.45	^{237}AWSVA **R**(+54)L^{242}	+54-Arg 241
1002.56	930.55	^{211}**K**(+72)VLASSAR^{218}	+72-Lys 211
682.53	628.41	^{479}ILN**R**(+54)L^{483}	+54-Arg 482
1231.52	1177.55	^{274}LECADD**R**(+54)ADL^{283}	+54-Arg 280

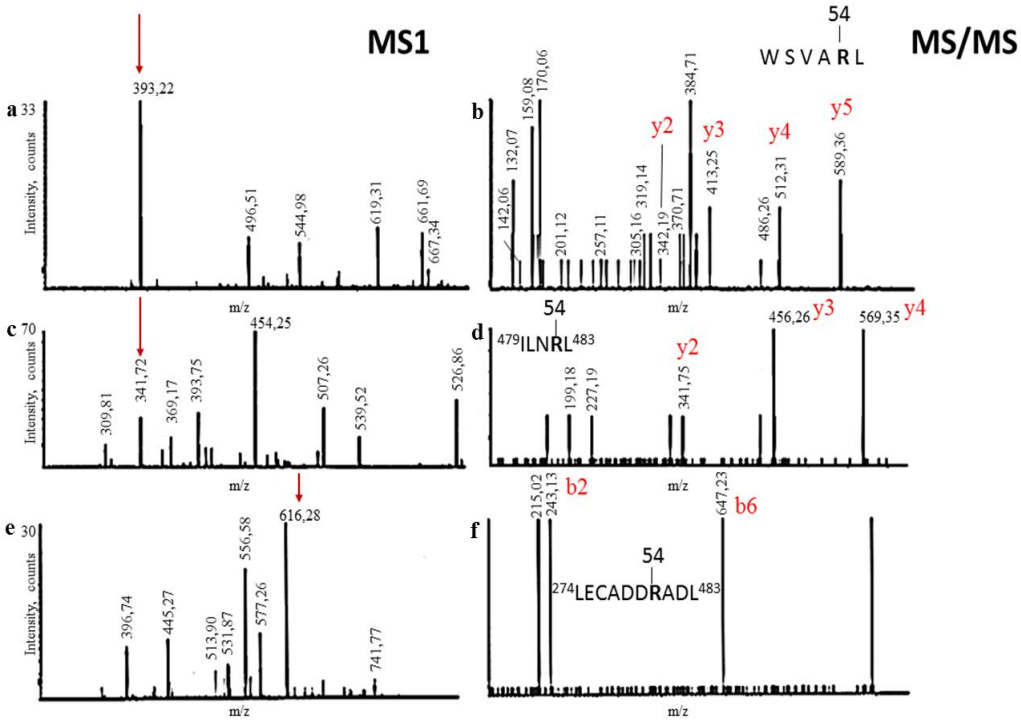

Figure 6. **N**ano-HPLC ESI MS separation of the pepsin peptides of BSA with MGO incubated at pH 7.4 and 37 ºC for 24 h (left panel a, c, e) and MS/MS-based sequencing of selected peptides (right panel b, d and f).

The MALDI-TOF-MS analysis of BSA after endoproteinase AspN hydrolysis led to coverage of 80% of the protein sequence (**Figure 7**). The peptides identifications are listed in **Table 2**. In one case in the BSA sample incubated with MGO it was found a peptide, 36-71, where the reactive site was likely the only cysteine of the peptide, as suggested by the mass difference between expected and measured mass corresponding to lack of carboxyamidation (57 units) due to the previous reaction of the cysteine residue with MGO. MS/MS data however, did not allow proving this hypothesis.

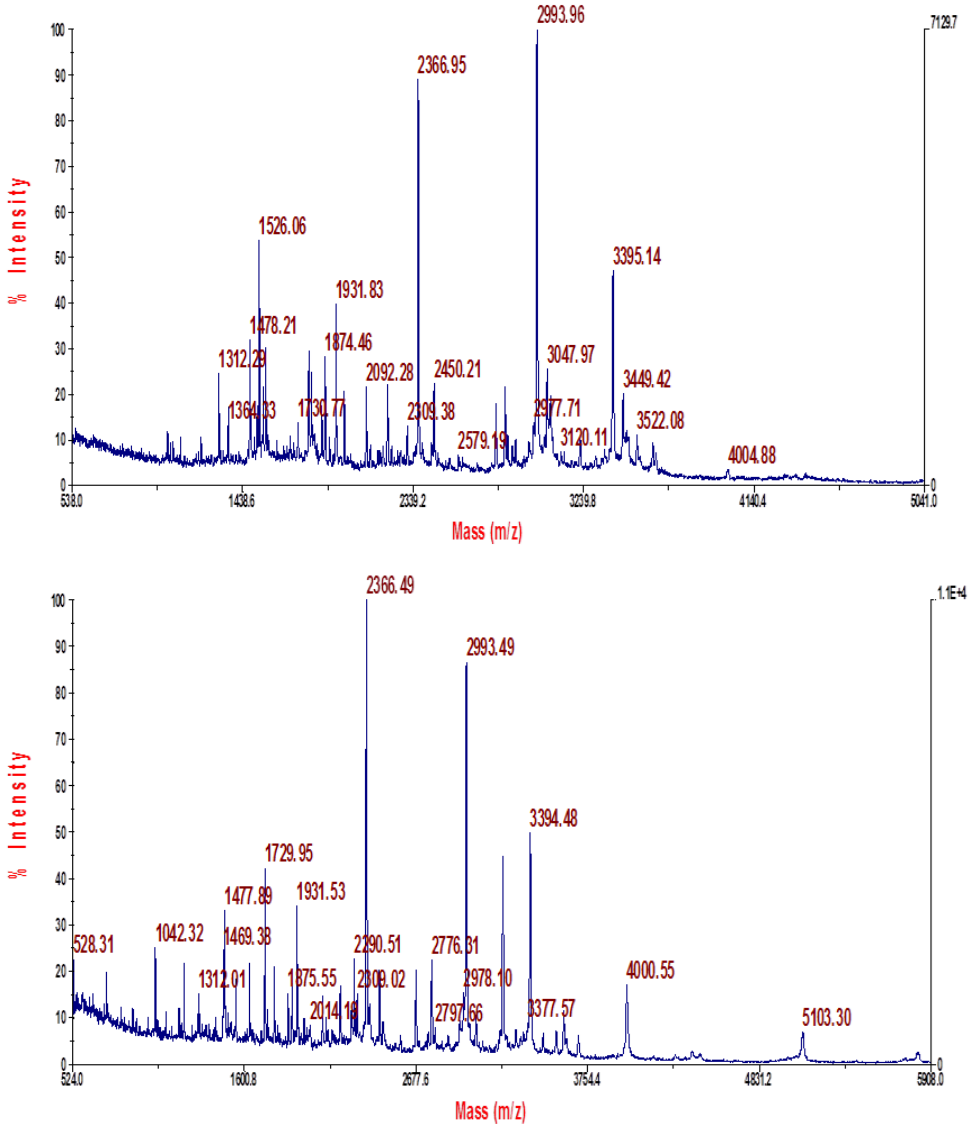

Figure 7. MALDI-TOF MS spectra of endoproteinase Asp-N peptides of BSA (upper panel) and BSA with MGO (lower panel) incubated at pH 7.4 and 37 ºC for 24 h.

Table 2. Identification of peptides released by ASP-N digestion from BSA with MGO, BSA with MGO and CGA, BSA with MGO and CSE incubated at pH 7.4 and 37 ºC for 24 h.

Measured (m/z)	Theorical (m/z)	Hypothesis	Peptide sequence	BSA+MGO	BSA+MGO+CGA	BSA+MGO+CSE
1522.55	1461.53	+54-Arg	^{1}DTHKSEIAHRFK ^{12}	x	-	-
1783.34	1728.94	+54-Arg	^{450}DYLSLILNRLCVLH^{463}	x	-	-
1802.01	1728.94	+72-Lys	^{450}DYLSLILNRLCVLH^{463}	x	x	-
1859.77	1787.80	+72-Lys	^{56}DESHAGCEKSLHTLFG^{71}	x	x	x
2257.13	2203.15	+72-Lys	^{37}DLGEEHF**K**GLVLIAFSQYL^{55}	x	x	-
2836.54	2839.43	+54-Cys	^{13}FSSAYSRGVFRRDTHKSE IAHRFKD^{36}	x	-	-
3048.97	2994.93	+54-Arg	^{332}EYSRRHPEYAVSVLLRLAKEYEATL^{35}	x	x	-
2250.11	1896.11	+354-Lys	^{279}D**K**PLLE**K**SHCIAEVE**K**^{294}	-	x	x
4210.11	3856.34	+354-Lys	^{37}DEHVKLVNELTEFAKTCVADESHAGC EKSLHTLFG^{71}	-	-	x

1. Discussion

The obtained data confirm the occurrence of the Maillard reaction between amino groups bound to protein and MGO. On the other hand, results indicate the presence of compounds such as CGA, and its breakdown products in CSE are able to inhibit the reaction by conjugation of the phytochemicals to reactive residues of the protein in the very early stages of the reaction (24 h).

Free amino groups analysis confirmed the progress of the Maillard reaction under the studied conditions. These results are in agreement with those previously reported by other authors (5). Interestingly, the free amino groups content of samples composed of BSA, MGO and CSE was slightly lower ($p=0.131$) than that found for the corresponding glycoxidation control composed of BSA and MGO. As expected, CGA 10 mM significantly reduced ($p < 0.001$) free amino groups content compared to the control suggesting the conjugation of phenol to the protein backbone. We previously reported this effect of CGA on free amino groups under the same conditions used in this study (5). Differences in the effect of CGA alone and CSE on free amino groups content in the presence of MGO may be ascribed to differences in the effective concentration of inhibitor in the glycoxidation reaction. The final concentration of CGA in the model system containing CSE was 2 mM, while the value of this compound in the system containing pure CGA was 5-fold higher.

Fluorescent AGEs were formed during the BSA-MGO reaction under our particular conditions (**Figure 2**). The presence of CSE and CGA reduced the characteristic fluorescence of the glycoxidation mixture (BSA+MGO). We previously observed the inhibition of fluorescent AGEs mediated by CGA (10 mM) at the same concentration and conditions tested in this study (5). Results of free amino acids and fluorescent AGEs suggest the inhibition of the glycoxidation reaction by conjugation of the phytochemicals present in CSE to the protein. Furthermore, CSE was less effective than CGA in the formation of AGEs in agreement with data on free amino acids. These results may be explained based on

differences in concentrations of the antiglycative agents in both samples. CSE has already been described as a potent inhibitor of the formation of fluorescent AGEs in a dose-dependent manner in a particular BSA-MGO assay (7). In addition, since CSE is a complex mixture of compounds, the inhibition of AGEs could be carried out through other pathways such as trapping their precursors (7).

Figures 3 and **4** support that high molecular fraction of samples (> 30 kDa) contains phenols and presents antioxidant capacity. The formation of BSA-CGA conjugated under the conditions here assessed has recently been reported (5). No significant differences ($p > 0.01$) were observed in the total phenol content and antioxidant character of macromolecules in samples composed of CGA and CSE (**Figures 3** and **4**). Results could be influenced by the presence of other compounds derived from CGA formed during coffee processing in the food matrix such as melanoidins (7). These compounds may also exert an antiglycative effect through different mechanisms of action such as chelating activity or antioxidant capacity (6).

Omics provided conclusive results on the conjugation of the phenols present in CSE to the protein structure and their role as inhibitors of the formation of BSA-MGO adducts. Many efforts have been made to map the glycation sites in a variety of model proteins such as human serum albumin (21), hemoglobin and myoglobin (22,23) and lens α-crystallin (24) using proteomics approach. Our data show that Arg and Lys residues of BSA are formed in the glycoxidation system at 24 h. MGO has been reported to selectively modify arginine and lysine of bovine and human serum albumin under physiological conditions (25). The lack of glycoxidation in the presence of CGA and CSE was verified using LC-MS/MS technologies, which demonstrated the almost complete absence of Arg adducts in these samples. In both cases, only one adduct at Lys 211 (m/z 1002) was determined. The formation of BSA-CGA and BSA-CA adducts were observed in the glycoxidation mixture containing pure CGA and CSE at 24 h (**Table 2**). Under our particular experimental conditions, results suggest that CGA and CSE are

more reactive than MGO. Furthermore, data suggest that the low concentration of CGA present in CSE may be enough to protect the protein against glycoxidation. CSE seems to be a good inhibitor of fluorescent AGEs. Its mechanism of action may involve several pathways including conjugation of the phenols and its derivatives to amino acid residues, targets of the glycoxidation reaction.

Determination of fluorescent AGEs has been proposed as a screening tool to predict diabetic complications in primary care (26). Many authors consider the inhibitors of AGEs to be another therapeutic target in diabetes management (2,26,27). Nowadays, there is an emerging interest in searching for natural extracts with antiglycative capacity by their therapeutic potential effect (28,29) and in reducing the side effects of the current anti-AGEs drugs such as aminogunidine (30). However, the effectiveness of these compounds depends on their bioaccesibility, bioavalability and metabolism in the organism. Our recent research indicates that the CGA present in CSE is bioavailable and provides health benefits (31,32). Further research is needed to demonstrate the antiglycoxidative effect of CSE *in vivo*.

2. Conclusion

The use of the omics approach allowed us to detect the formation of protein-phenol conjugates under mimicked glycoxidative physiological conditions. Novel information supports that the mechanism of action of CSE to inhibit the formation of fluorescent AGEs involves several pathways including interactions between phenols and proteins. Furthermore, results support the potential of CGA and CSE as health promoters in fluorescent AGEs-related diseases.

Abbreviations: AGEs (advanced glycation end products), MGO (methylglyoxal), BSA (bovine serum albumin), CGA (3-*O*-caffeoylquinic acid), CA (caffeic acid), ROS (reactive oxygen species), T2D (type 2 diabetes), CSE (coffee silverskin extract), TFA (trifluoroacetic acid), ACN (acetonitrile), FA (formic acid).

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Chapter 2

Abstract

Results found in *in vitro* assay with cellular model of beta cells (INS-1E) performed to gain insights in the mechanism of action of CSE in the pathology of T2D, mimicking physiological condition are shown in the present chapter.

Study published as**:** Fernandez-Gomez B, Ramos S, Goya L, Mesa MD, del Castillo MD, María Ángeles Martín**. Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells.** Food Res. Int. 2016; doi: 10.1016/j.foodres.2016.03.006.

Coffee silverskin extract improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells

Beatriz Fernandez-Gomez, Sonia Ramos, Luis Goya, Mª Dolores Mesa, Mª Dolores del Castillo, Mª Ángeles Martín. Food Res. Int. 2016; doi: 10.1016/j.foodres.2016.03.006.

Abstract

The present research aimed to provide novel information regarding the antidiabetic mechanism of action of coffee silverskin extract (CSE) and its components chlorogenic acid (CGA) and caffeine (CF). Their effect on insulin secretion and biomarkers of oxidative stress in INS-1E cells *in vitro* cultured under physiological and stressed conditions were assessed. Under physiological conditions, CSE and pure CGA and CF did not affect cells´ oxidative status and viability. However, concentrations of CSE ≥ 1µg/mL and CGA ≥ 5 µM significantly increased ($p < 0.05$) the enzymatic activity of glutathione peroxidase (GPx). Moreover, all concentrations of CSE (1-10 µg/mL) and the dose of 10 µM of CGA, significantly stimulated ($p < 0.05$) insulin secretion in cells cultured in media containing 4 and 10 mM of glucose. CSE (1µg/mL) and CGA (10µM) reinforced antioxidant defence and increased insulin secretion in response to glucose in beta cells stressed with streptozotocin (STZ). Since CGA concentration in CSE was of ≈ 0.1 nM it can be assumed that other antioxidants present in this particular extract may also contribute to the observed effect. In conclusion, here we provide evidence that CSE could be a new potential antidiabetic agent through its antioxidant actions and its ability to modulate insulin secretory function.

Keywords: Coffee silverskin, coffee by-products, oxidative stress, insulin secretion, antidiabetic effect

1. Introduction

Type 2 diabetes (T2D) is a complex and multifactorial metabolic disorder characterized by persistent hyperglycaemia. Although T2D is caused by a deficiency in insulin secretion associated to decreased responsiveness of peripheral tissues to insulin, it is generally accepted that the inability of beta cells to secrete adequate amounts of insulin is primarily responsible for the development and progression of T2D(1) (1). Hyperglycemia and the subsequent increase in oxidative stress that appears in diabetes mellitus have been largely implicated in the progressive dysfunction of pancreatic beta cells and in the development of diabetic complications (2). Micro- and macro-vascular complications of diabetes (blindness, kidney failure, heart disease, stroke and amputations) are the major causes of morbidity and mortality in human populations (3). Currently, numerous antidiabetic drugs, including inducers of insulin secretion, are used as treatment of T2D. Nevertheless, all of them exhibit adverse side effects and, actually, even under treatment, beta cell dysfunction worsens driving to loss of glycemic control (4). Therefore, there is a growing interest in the identification of naturally occurring antioxidant agents that may protect and improve beta cell mass and function since they may offer a natural alternative to reduce risk or treat diabetes and retard the onset of its complications.

Coffee consumption, both caffeinated and decaffeinated, has been associated to a wide variety of health beneficial effects, in particular the reduced risk of T2D (5,6). Coffee components, caffeine (CF) and chlorogenic acid (CGA), possess potential benefits on glucose homeostasis (7). The effect of CGA and CF on glucose metabolism remains unclear (8). The use of coffee by-products as natural source of compounds with putative health benefits such as CGA, CF and dietary fibre among others has been proposed (9–11). Glucoregulatory properties have been recently ascribed to a coffee silverskin extract (CSE) by-product of coffee roasting (12). The antidiabetic effect of CSE has been associated to its capacity to

inhibit enzymatic activity of α-glucosidase and lipase. CSE is also able to inhibit *in vitro* the formation of advanced glycation end-products (AGEs)(13,14) which have been involved in the development of diabetes and its complications (15). However, up to date, the protective effect of CSE on the development and progression of diabetes and, particularly, on beta cell viability and function has not been evaluated.

Streptozotocin (STZ) is a potent DNA-methylating agent, which generates ROS that induce oxidative stress in pancreatic beta cells (16). STZ has been used in beta cells to imitate the pathology of T2D and to evaluate the antidiabetic effect of novel compounds (17–19). The aim of the present study was to investigate the mechanism of action of CSE on the pathogenesis of diabetes using an *in vitro* model of beta cells, the INS-1E cells. To achieve this goal, the effect of CSE on redox status and insulin secretion in the pancreatic beta cells was evaluated. Likewise, its capacity to protect pancreatic beta cells against an oxidative damage induced by STZ was also examined. In addition, the main phenolic constituent CGA and the alkaloid CF were individually studied in order to determine their contribution to the beneficial effect of the CSE on the function of pancreatic beta cells.

2. Material and Methods

2.1. Materials and Chemicals

CGA, CF, STZ, glutathione reductase (GR), reduced and oxidized glutathione (GSH and GSSG, respectively), NADH, NADPH, o-phthaldialdehyde (OPT), tert-butylhydroperoxide (t-BOOH), gentamicin, penicillin G, streptomycin and bovine serum albumin (fraction V) were purchased from Sigma Chemical (Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and fetal bovine serum from Lonza (Madrid, Spain). Bradford reagent was from BioRad Laboratories S.A.

2.2. Preparation of coffee silverskin extract (CSE)

CS from the Arabica (Coffea arabica) species was provided by Fortaleza S.A. (Spain). According to the manufacturer, the weight portion of CS represents 0.6 % of the roasted beans. Arabica CSE was

prepared by aqueous extraction according to the procedure patented by del Castillo et al. (2013) (9). Briefly, 50 mL of boiling water was added to 2.5 g of CS. The mixture was stirred at 250 rpm for 10 min, filtered by Whatman paper no. 4 and the filtrate was freeze dried. The powdered extracts were stored in dark and dry place until analysis. Concentrations of the bioactive compounds were determined by a capillary electrophoresis and UV-Vis detection, and a detailed description of this CSE is given elsewhere (13). Accordingly, the amounts of CGAs and CF present in the CSE were 11.18 mg/g and 30.26 mg/g, respectively.

2.3. Cell culture and treatments

Rat insulinoma cell line, INS-1E, was kindly provided by Dr. Mario Vallejo of "Alberto Sols" Biomedical Research Institute CSIC, Madrid, Spain. INS-1E cells were maintained in a humidified incubator containing 5 % CO2 and 95 % air at 37 ºC. They were grown in RPMI-1640 medium with 11 mM glucose, supplemented with 10 % fetal bovine serum (FBS), 1 % Hepes, 1 mM sodium pyruvate, 50 μM betamercaptoethanol and 1 % of the following antibiotics: gentamicin, penicillin and streptomycin.

For the treatments with the different compounds, concentrations of CSE (1, 5 and 10 μg/mL), CGA (1, 5 and 10 μM) and CF (1, 5 and 10 μM) diluted in RPMI-1640 culture medium and filtered through a 0.2-μm membrane were added to cell plates during 20 h. For STZ treatment, STZ was dissolved in 0.1 M citrate buffer (pH 4.5) and added to cell plates during different times (3-18 h) and concentrations ranging from 1 to 5 mM.

2.4. Evaluation of cell viability and production of reactive oxygen species (ROS)

Cell viability was determined by the crystal violet assay. INS-1E cells were seeded at low density (2 x 105 cells per well) in 24-well plates. After the different treatments, cells were incubated with crystal violet (0.2 % in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1 % sodium dodecyl sulphate (SDS) was added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

Cellular ROS were quantified by the DCFH assay using a microplate reader (20). For the assay, cells were plated in 24-multiwells and incubated with the different treatments. After that, 10 μM DCFH was added to the wells for 30 min at 37 ºC. After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

2.5. Glucose-stimulated insulin secretion (GSIS) and content

After the different treatments, INS-1E cells were washed and placed in Krebs–Ringer bicarbonate buffer (KRB: 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM MgCl2 6H2O, 1mM CaCl2 2H2O) supplemented with 5 mg/mL BSA for a quiescent period of two hours. Next, cells were incubated for 90 min in KRB containing 4 or 10 mM glucose and the different concentrations of

CSE, CGA and CF. GSIS was evaluated in the medium by an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).

For cellular insulin content measurement, treated cells were lysed at 4 ºC in a buffer containing 25 mM HEPES (pH 7.5), 0.2 mM EDTA, 0.1 % Triton X-100, 200 mM ß-glycerolphosphate, 0.1 mM Na3VO4, 2 µg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The supernatants were collected and assayed for insulin content by the ELISA kit (Mercodia, Uppsala, Sweden).

2.6. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

Treated INS-1E cells were collected in PBS and centrifuged at low speed (300×g) for 5 min to pellet cells to assay the activities of GPx and GR. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM beta-mercaptoethanol, sonicated and centrifuged at 3000×g for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity was based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR. GR activity was determined by following the decrease of the absorbance due to the oxidation of NADPH utilized in the reduction of GSSG. The methods have been previously described (21). Protein was measured by the Bradford reagent.

2.7. Determination of reduced glutathione (GSH)

The concentration of GSH was evaluated by a fluorometric assay previously described (21). The method takes advantage of the reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed and cells were detached and homogenised by ultrasound with 5 % trichloroacetic acid containing 2 mM EDTA. Following centrifugation of INS-1E beta cells homogenates for 30 min at 3.000 rpm, 50 µL of the clear supernatant were transferred to a 96-multiwell plate for the assay. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results were interpolated in a GSH standard curve (5 – 1000 ng) and expressed as nmol GSH/mg protein, which was determined by the Bradford reagent.

2.8. Determination of carbonyl groups

Protein oxidation was measured as carbonyl groups content according to a published method (22). The determination was carried out in supernatants of INS-1E cells. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22000 nmol/L/cm. Protein concentration was determined by the Bradford reagent.

2.9. Statistics

Prior to statistical analysis, the data were tested for homogeneity of variances using Levene test. For multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. A SPSS version 22.0 program was used.

3. Results and discussion

3.1. Effects of CSE, CGA and CF on the redox status of cultured INS-1E cells

The aim of the present study was to obtain novel information regarding the mechanism of action of CSE and its bioactive components, CGA and CF, on the pathogenesis of diabetes induced by STZ. For this purpose, we used an established cell culture line from rats, INS-1E cells, which show important biological features of the pancreatic islet beta cells and have been widely used as a reliable model of beta cells.

Doses of pure CGA and CF to be tested were selected taking into account the information on physiological concentrations reported by others. After normal dietary intakes, polyphenols and their metabolites appear in the circulatory system at nM-μM concentrations, so they are the most appropriate doses for *in vitro* studies (23). On the other hand, plasma concentrations may reach up to 10 μM of CF after the intake of a normal coffee serving (24).

The feasibility of the doses of CSE (1-10 μg/mL), CGA (1-10 μM) and CF (1-10 μM) for treating INS-1E was determined by the analysis of cellular redox status and antioxidant response biomarkers. Treatment of INS-1E with CSE, CGA and CF did not affect intracellular ROS generation or cell viability, indicating no cellular stress or damage (**Table 1**).

Figure 1 shows that the treatment of INS-1E cells with CSE, CGA or CF preserved the GSH store (**Figure 1A**) and the GR activity (**Figure 1C**). Interestingly, CSE and the phenolic CGA evoked a significant increase ($p < 0.05$) in the enzymatic activity of GPx (**Figure 1B**). Glutathione and their related enzymes, GR and GPx, participate in the defence against hydrogen peroxides and superoxides and they are essential to prevent the cytotoxicity of ROS. Several phenolic compounds have shown to enhance the expression and activity of antioxidant enzymes in different tissues such as liver (25,26) or colon (21) and also in pancreatic beta cells (27). This outcome should have a significant impact on INS-1E cells because they are particularly susceptible to oxidative stress-

induced injury due to the low-level expression of antioxidant enzymes as compared to other types of cells (4). Furthermore, it has been demonstrated that the overexpression of antioxidant enzymes protects pancreatic beta cells from oxidative stress-induced dysfunction (28). Therefore, the induction of GPx by CSE and CGA sets the cells in favourable conditions to face a potential oxidative challenge and could be an important strategy to improve beta cell survival in diabetes.

Table 1.- Effect of 20 hours treatment with noted concentrations of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on cell viability and intracellular ROS generation in pancreatic INS-1E cells.

		% Cell Viability	ROS (% Fluorescence Units)
***C**		100.3 ± 2.5[a]	100.2 ± 6.3[a]
CSE (μg/mL)	1	103.4 ± 9.4[a]	101.2 ± 6.4[a]
	5	104.2 ± 4.4[a]	99.8 ± 7.2[a]
	10	102.1 ± 4.6[a]	100.2 ± 8.7[a]
CGA (μM)	1	99.8 ± 5.4[a]	97.4 ± 82[a]
	5	98.9 ± 8.7[a]	100.3 ± 9.2[a]
	10	98.7 ± 7.7[a]	101.2 ± 5.8[a]
CF (μM)	1	100.3 ± 5.8[a]	99.7 ± 5.8[a]
	5	102.4 ± 4.0[a]	96.8 ± 8.8[a]
	10	100.4 ± 2.0[a]	97.3 ± 9.3[a]

*C represents untreated control cells. Data represent means ± SD of 8-10 samples per condition. Same letter a as superscript indicates that no significant differences were found, $p < 0.05$.

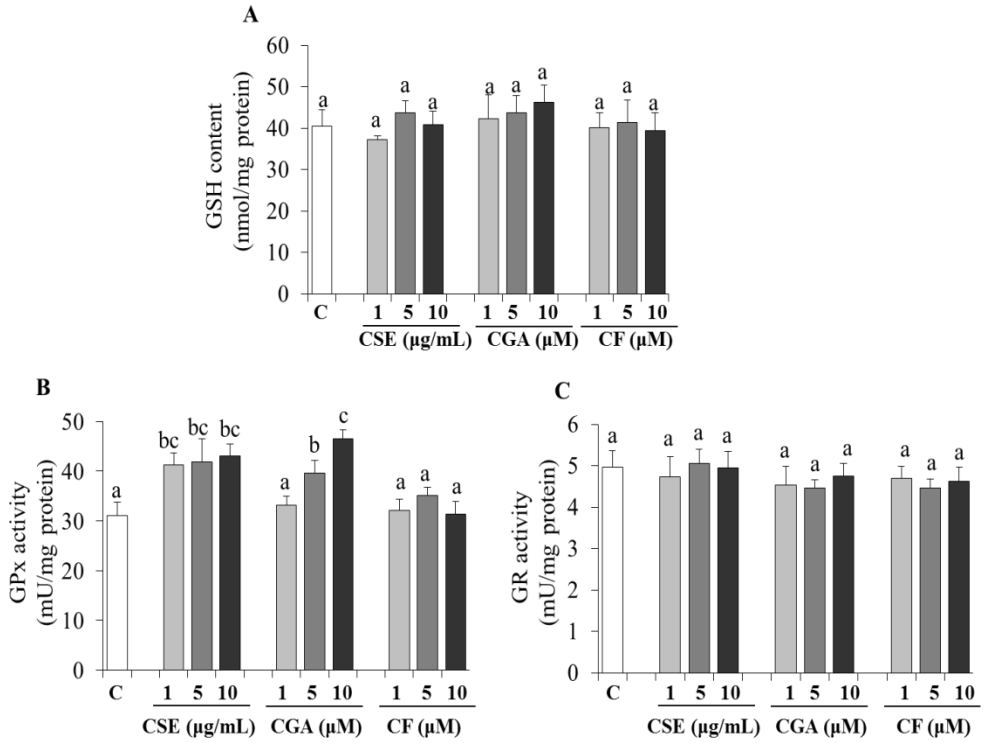

Figure 1.- Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on GSH concentration and GPx and GR activities. C represents untreated control cells. INS-1E cells were treated with 1–10 µg/mL CSE and 1-10 µM of CGA and CF for 20 h and GSH concentration (A) and GPx (B) and GR (C) activities were evaluated. Data represent means ± SD of 6-8 samples per condition. Different letters denote statistically significant differences, $p < 0.05$.

3.2. CSE and CGA increased GSIS in INS-1E cells

Since the ineffectiveness of beta cells to secrete adequate amounts of insulin is decisive in the development and progression of T2D, agents that may improve beta cell function are considered key to prevent or to treat diabetes (4). Accordingly, we were next interested in exploring the potential effect of CSE, CGA and CF on insulin secretory function. Indeed, it has been indicated that many phytochemicals present in plant foods, particularly polyphenols, could be able to induce insulin secretion in pancreatic beta cells (27,29–31).

After the incubation of INS-1E cells in the presence of CSE (1-10 µg/mL), CGA (1-10 µM) and CF (1-10 µM) for 20 hours, the GSIS was assayed for 90 min. As shown in **Figure 2A-C**, pre-treatment of cells with CSE and CGA, significantly increased ($p < 0.05$) insulin secretion in cells cultured in media containing 4 and 10 mM of glucose. However, CF resulted ineffective stimulating insulin secretion. According to that, a previous work reported a positive effect of CGA on insulin secretion (32), besides the doses of CGA employed in that study were higher (28 and 140 µM) than those tested in the present study. On the other hand, a very low concentration of CSE (1 µg/mL) resulted effective to increase insulin secretion in a glucose-dependent manner in pancreatic beta cells which is a novel and interesting result. This CSE dose contains 9.86 x 10-5µM of CGA which is lower than the effective doses of CGA (10 µM). These data suggest that other compounds are contributing to the observed effect. Synergistic effects of bioactive compounds present in the botanical matrix including those unknown can be responsible of the overall antidiabetic observed effect. Further studies should be conducted to identify such components of this particular extract contributing to the protective effect of pancreatic cells.

The observed effect on insulin secretion could be due to an increase of insulin biosynthesis. To confirm this hypothesis, we also measured total insulin content in control and CSE, CGA, and CF 20 hour-treated cells. As showed in **Figure 2D**, there was no difference in insulin levels between the control and treated INS-1E cells. Therefore, we can ensure that the augmented GSIS induced by CSE and CGA was not related to an increase in the biosynthesis of the hormone but rather to the stimulation of insulin secretion. In this regard, from a mechanistic point of view, several insulin secretagogues with recognized therapeutic effect such as sulfonylureas or glinides are able to close KATP channel and lead to an increase in glucose-induced depolarization. Consequently, voltage-dependent Ca^{2+} channels open, causing the acceleration of Ca^{2+} influx and the increase of the concentration of cytosolic free Ca^{2+} that is necessary and sufficient to trigger insulin secretion

(33). In line with this, it has been recently indicated that a coffee extract (100 µg/mL) and its main component CGA (28.2 µM) may block KATP channel, conferring a regenerative effect on zebrafish pancreatic islet damaged with alloxan (34). On the contrary, Tousch et al., (2008) (32) indicated that CGA at stimulating concentrations for insulin secretion (28.2 and 84.6 µM) does not close KATP channels in rat INS-1E cells, suggesting that a different mechanism of action may be involved.

Other insulin secretagogues amplify Ca^{2+}-induced insulin secretion though the activation of diverse protein kinases, including protein kinase A (PKA), protein kinase C (PKC) or extra cellular regulated kinases (ERKs), that are involved in the mechanism of insulin exocytosis itself (35). Accordingly, several phenolic compounds such as quercetin (36) and microbial-derived flavonoid metabolites (31) have demonstrated to increase glucose-stimulated insulin secretion via ERKs activation. Therefore, a potential role of the phenolic CGA on signalling pathways cannot be ruled out. Further studies about the molecular mechanisms involved in the action of CGA and CSE on beta cell insulin secretion are necessary.

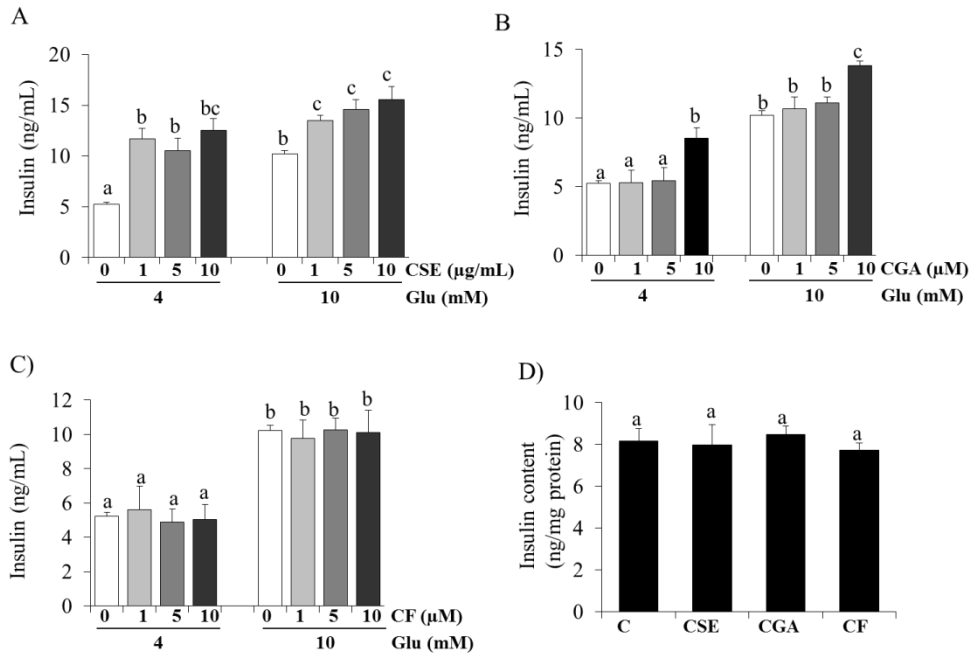

Figure 2.- Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on insulin secretion and insulin content in INS-1E cells. C represents untreated control cells. Cells were treated with 1–10 µg/mL CSE (A) and 1-10 µM CGA (B) and CF (C) for 20 h and then incubated in KRB medium containing 4 or 10 mM glucose for 90 min and insulin release was evaluated in the KRB medium. Insulin content was determined in non-treated control cells and in cells treated with CSE, CGA and CF for 20 h (D). Data represent means ± SD of 6-8 samples per condition. Different letters denote statistically significant differences, $p < 0.05$.

3.3. CSE and CGA protect INS-1E cells against STZ-induced oxidative stress

It is generally accepted that oxidative stress is involved in the loss of beta cell function and viability, thus, the protection of beta cells from oxidative stress is one of the mechanisms potentially involved in the prevention of diabetes (37). Consequently, we finally investigated the protective effect of CSE, CGA and CF against oxidative stress. To this end, we used STZ, a chemical compound commonly used to induce diabetes through its toxic effects on pancreatic beta cells (38). The cytotoxic action of STZ is associated with the generation of ROS and the consequent beta cell destruction and suppression of insulin secretion (16).

Our first goal for this outcome was to determine the conditions leading to oxidative stress and cell death in INS-1E cells. **Figure 3** reveals that increasing concentrations of STZ induced a dose-dependent increase in ROS generation and cell toxicity as shown by the decrease in cell viability. Since the STZ concentration of 5 mM at 6 hours caused a significantly increase ($p < 0.05$) in ROS generation and nearly 40 % cell death (**Figure 3**), we decide to choose this concentration and that time for the following experiments.

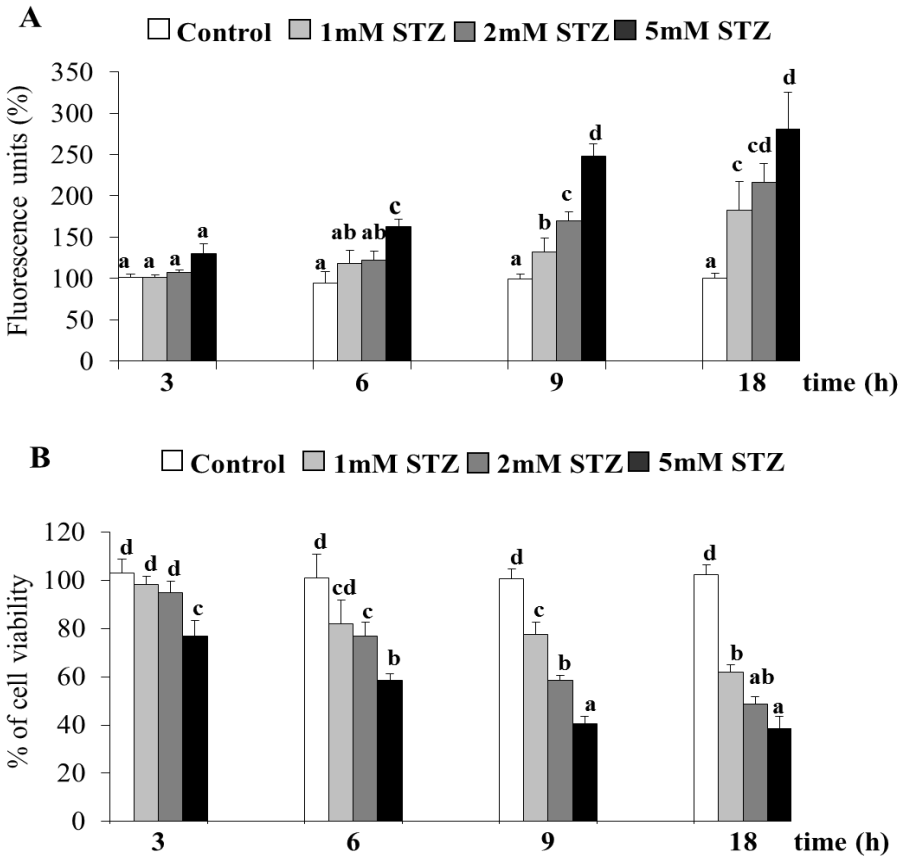

Figure 3.- Effect of streptozotocin (STZ) on ROS production and cell viability in INS-1E cells. Cells were treated with 1-5 mM STZ for 3, 6, 9 and 18 hours and ROS levels (A) and cell viability (B) were evaluated. Data represent means ± SD of 8-10 samples per condition. Different letters denote statistically significant differences, $p < 0.05$.

To investigate the potential of CSE, CGA and CF to protect beta cells against the impairment of viability or insulin secretion induced by STZ, we used effective doses of each compounds on antioxidant defence and insulin secretion. INS-1E cells were treated with 1 µg/mL CSE, 10 µM CGA or 10 µM CF during 20 hours followed by treatment with 5 mM of STZ for 6 hours (for oxidative damage assays and cell viability) or for 90 minutes (for insulin secretion assays). **Figure 4** illustrates the effect of the different treatments on the appearance of INS-1E cells. STZ treatment led to morphological changes such as cell shrinkage related to cell death. However, pre-treatment with CSE or CGA, but not CF, prevented these morphological alterations. In agreement with those data, treatment of cells with STZ enhanced ROS generation (**Figure 5A**) and protein cell oxidative damage (measured as carbonyl groups) (**Figure 5B**) resulting in a remarkable decrease of INS-1E cell viability (**Figure 5C**). Pre-treatment with CSE or CGA significantly reduced ($p < 0.05$) the ROS over production induced by STZ and prevented beta cell death. Since the increase in ROS generation induced by STZ has been directly implicated in pancreatic beta cell apoptosis (39,40), it is reasonable to suggest that the effect of CGA and CSE reducing ROS over production possibly will contribute to reduce apoptosis and enhance cell survival of INS-1 beta cells. Likewise, STZ treatment also induced a significant decrease ($p < 0.05$) in insulin secretion in INS-1E cells (**Figure 5D**) whereas the pre-incubation with CSE and CGA completely restored GSIS to control levels. CF did not protect against oxidative stress, cell death or impaired insulin secretion induced by STZ.

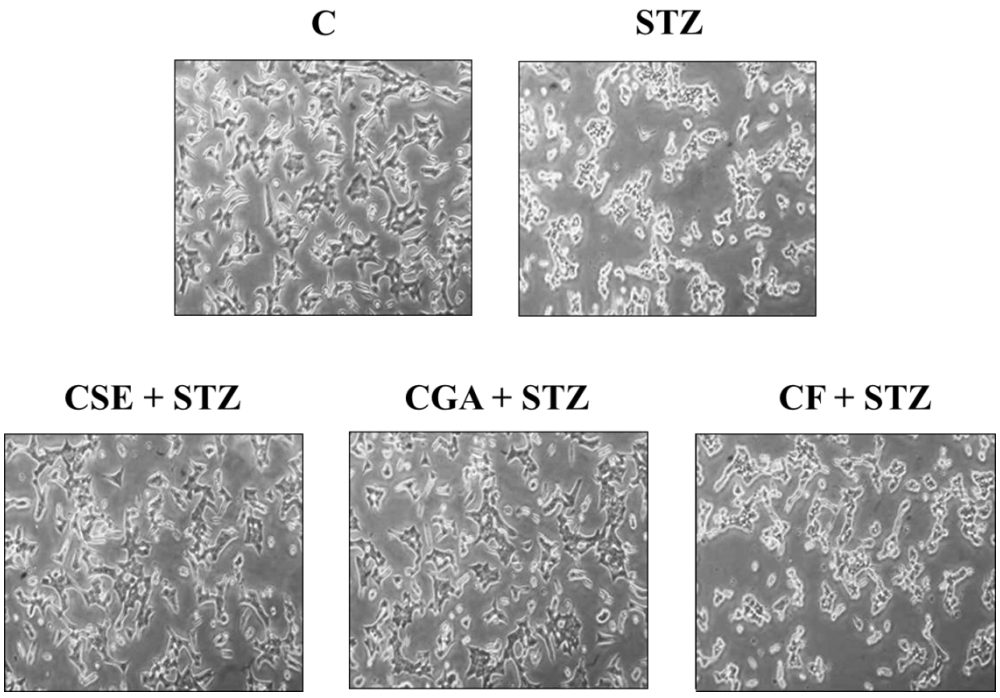

Figure 4.- Representative microscopy images of INS-1 cells after different treatment. Untreated cells (C). Cells treated with 5 mM STZ for 6 h (STZ). Cells treated with 1 μg/mL CSE and further exposed to 5 mM STZ for 6 h (CSE + STZ) Cells treated with 10 μM of CGA and further exposed to 5 mM STZ for 6 h (CGA + STZ). Cells treated with 10 μM of CF for 20 h and further exposed to 5 mM STZ for 6 h (CF + STZ).

In parallel, the oxidative stress induced by STZ caused a significant decrease ($p < 0.05$) in GSH (**Figure 6A**) and a remarkable increase of GPx (**Figure 6B**) and GR (**Figure 6C**) activities in order to enhance the antioxidant cell defence against ROS. Under these extreme oxidative conditions pre-treatment of INS-1E cells with CSE and CGA greatly prevented GSH depletion and completely recovered GPx and GR activities. Altogether, our results indicate that the concentrations of CSE and CGA hereby assayed efficiently protect the viability and function of pancreatic beta cells against STZ while CF was ineffective on that since did not significantly inhibit ROS production.

The cyto-protective effect of coffee and its phenolic components against an oxidative injury has previously been described in different cultured cells (41–43). Results obtained for pure CGA are in line with those reporting a protective effect of different antioxidant compounds on pancreatic beta cells (27,31,44). However, the present study is the first to demonstrate a specific chemo-protective effect of CSE on pancreatic beta cells. This effect produced by CSE cannot be ascribed to isolated CF or CGA, but to a synergic effect of different components present in the extract. Further studies are necessary to identify the compounds responsible for that property. On the other hand, our findings highlight the potential of CSE in the protection against diabetes supporting those described in the patented with number P201431848.

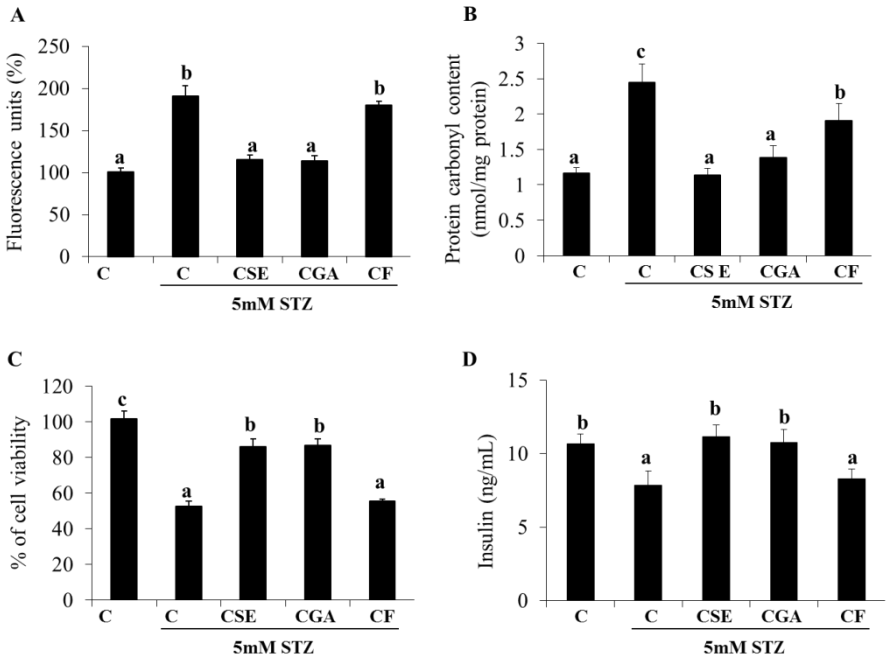

Figure 5.- Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) against oxidative damage induced by STZ. C represents untreated control cells. Cells were treated with 1 μg/mL CSE (A) and 10 μM of CGA (B) and CF (C) for 20 h and further exposed to 5 mM STZ for 6 h. Then, intracellular ROS generation (A), carbonyl group production (B) and cell viability (C) were measured. To evaluate cell functionality, after 20 h of CSE, CGA and CF treatment, control and treated cells were placed in KRB

containing 10 mM glucose and 5 mM STZ and insulin secreted during 90 min was evaluated (D). Data represent means ± SD of 8-10 samples per condition. Different letters denote statistically significant differences, $p < 0.05$.

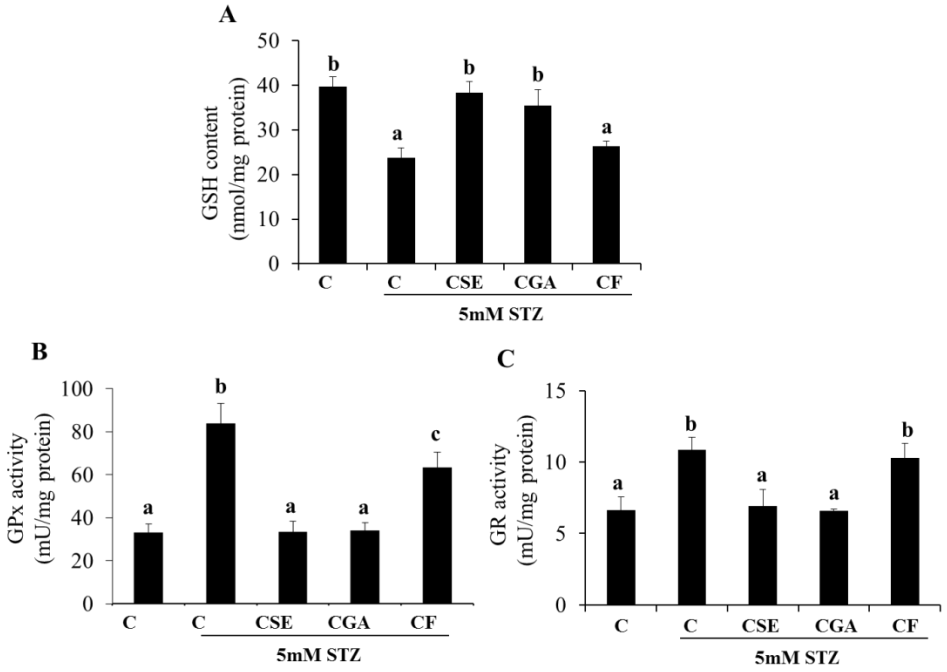

Figure 6.- Effect of coffee silverskin extract (CSE), chlorogenic acid (CGA) and caffeine (CF) on GSH concentration and GPx and GR activity in STZ-treated cells. C represents untreated control cells. INS-1E cells were treated with 1 μg/mL CSE (A) and 10 μM of CGA (B) and CF (C) for 20 h and further exposed to 5 mM STZ for 6 h. Then, GSH concentration (A) and GPx (B) and GR (C) activities were evaluated. Data represent means ± SD of 6-8 samples per condition. Different letters denote statistically significant differences, $p < 0.05$.

3. Conclusion

In conclusion, for the first time we provide scientific evidences regarding the protective effects of CSE in pancreatic beta cells through its antioxidant actions and its ability to modulate insulin secretory function. In addition, our results suggest that physiological concentrations of pure CGA (10 μM) are able to protect pancreatic cells against oxidative stress while CF in the same concentration is ineffective. On the other hand, since the concentrations of CGA present in CSE

seems to be ineffective to protect pancreatic cells against diabetogenic agents causing cell oxidative stress (STZ), further research should be conducted to identify which compound/s are responsible for those benefits. Our findings support the potential of CSE as anti-diabetic phytodrug and functional ingredient for the prevention of diseases related to oxidative stress such as diabetes. Results also suggest that coffee by-products present added value confirming that coffee is not only a drink.

Abbreviations: CF, caffeine; CGA, chlorogenic acid; CSE, coffee silverskin extract; DCF, dichlorofluorescein; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSIS, glucose-stimulated insulin secretion; KRB, Krebs–Ringer bicarbonate buffer; OPT, *o*-phthaldehyde; ROS, reactive oxygen species; STZ, streptozotocin; t-BOOH, tert-butylhydroperoxide; T2D, Type 2 diabetes.

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Chapter 3

Abstract

The present chapter shows results derived from *in vivo* assay. The study was performed in rats to gain insights in the metabolism and bioactivity of coffee silverskin extract compounds in the pathology of T2D.

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Insights on the health benefits of the bioactive compounds of coffee silverskin extract

Beatriz Fernandez-Gomez, Adriana Lezama, Miryam Amigo-Benavent, Mónica Ullate, Miguel Herrero, Mª Ángeles Martín, Mª Dolores Mesa, Mª Dolores del Castillo. Submitted on the 04 of April 2016 to J Funct Food. Under evaluation.

Abstract

Little is known about the bioaccessibility of chlorogenic acid (CGA) and caffeine in coffee silverskin extracts (CSE), and the contribution of these substances to the prophylactic effect of CSE on the pathogenesis of diabetes has not been reported. This study aimed to evaluate the bioaccessibility, bioavailability and bioactivity of CGA and caffeine alone and in CSE in the pancreas of rats treated with streptozotocin-nicotinamide. The bioaccesibility of CGA and caffeine was affected by changes in pH during digestion, and both CGA (0.825 μmol) and caffeine (5.026 μmol) were metabolized. Their metabolites protected pancreatic cells against the risk of diabetes. This is the first study to demonstrate a specific chemo-protective effect of CSE in pancreatic tissue, and this effect may be associated with its antioxidant character. Daily administration of CSE, CGA or caffeine 35 d previous to the induction of diabetes significantly reduced ($p < 0.05$) pancreatic oxidative stress and protein damage.

Keywords: coffee silverskin, chlorogenic acid, caffeine, bioaccessibility, metabolism, pancreas oxidative stress, diabetes.

1. Introduction

According to the International Diabetes Federation (IDF), type 2 diabetes mellitus (T2DM) is one of the most frequent diseases in the world, with 387 million cases in 2014 (1). T2DM is a very complex and multifactorial metabolic disease characterized by insulin resistance and β cell failure, leading to high blood glucose levels. Oxidative stress plays an important role in hyperglycemia-induced pancreas injury as well as in the early events leading to the development of T2DM. Advanced glycation end-products (AGEs) increase reactive oxygen species formation and impair antioxidant systems. Furthermore, the formation of some AGEs is induced per se under oxidative conditions (2). There is also evidence that antiglycative agents in foods and medicine may reduce the risk of diabetes and treat the pathology (3).

Coffee silverskin (CS), the tegument of green coffee beans (outer layer), is the only by-product of the roasting process. Previous studies have proposed the use of coffee silverskin extracts (CSE) as a natural source of bioactive compounds, such as chlorogenic acid (CGA), caffeine, melanoidins and dietary fibre among others, with putative health benefits (4). Indeed, glucoregulatory properties have recently been ascribed to CSE by-products of roasting coffee (5). The antidiabetic effect of CSE has been associated with its capacity to inhibit the enzymatic activity of α-glucosidase and lipase (5). CSE is also able to inhibit the formation of AGEs. The anti-AGEs capacity of CSE may be ascribed to CGA and other bioactive compounds composing the extract (6). Fernandez-Gomez et al. (2015) (7) reported the antiglycative mechanism of action of CGA. Moreover, CSE may protect pancreatic tissue against oxidative stress induced by the commonly-used diabetogenic agent streptozotocin (STZ) (8).

Healthy effects associated with CSE largely depend on the bioaccesibility and bioavailability of their bioactive components in the organism. CSE, like other food matrices, is a complex mixture of bioactive compounds. Nowadays, little is known

about the bioaccessibility of CGA and caffeine composing CSE, and therefore, their true in-vivo potential. Moreover, the contribution of CGA and caffeine to the prophylactic effect of CSE on the pathogenesis of diabetes has not been reported. This study aimed to evaluate the bioaccessibility, bioavailability and bioactivity of CGA and caffeine alone and in CSE in the pancreas of rats treated with STZ-nicotinamide (NA), using phytochemomics (9).

2. Material and Methods

2.1. Chemicals

Pancreatin (P-1625), α-amilase from human saliva type IX-A (A0521), 2.2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), CGA, hippuric acid (HA) , caffeine and paraxanthine solution, formic acid, o-phthaldehyde, 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), glutathione reductase (GR), reduced and oxidized glutathione (GSH and GSSG, respectively), NADH, NADPH, tert-butylhydroperoxide (t-BOOH), 1,4-dithiothreitol (DTT) buffer, nicotinamide (NA) and streptozotocin (STZ) were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA). The other chemicals and equipment used were: Pepsin (Merck 1.07190) and Amicon® Ultra-0.5 ml centrifugal filter unit fitted with an Ultracel®-10 K regenerated cellulose membrane (30 kDa cut-off) (Merck, Darmstadt, Germany), Bradford reagent for the protein assay (Bio-Rad, München, Germany), methanol (MeOH) HPLC-grade (Lab-Scan, Gliwice, Sowinskiego, Poland). Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

2.2. Preparation of coffee silverskin extract

Arabica CSE was prepared by aqueous extraction following the procedure patented by (10). Briefly, 50 ml of boiling water was added to 2.5 g of CS. The mixture was stirred at 4 g for 10 min, filtered through no. 4 Whatman paper and freeze-dried. The powdered extracts were stored in a dark and dry place until analysis. The sample (37 mg/ml CSE solution) was filtered through a 0.45 µm pore diameter nylon membrane syringe filter (Análisis Vínicos, Ciudad Real, Spain) and diluted 100-fold with Milli-Q water and 10 µl aliquots analysed in triplicate by UPLC-MS/MS. CSE contained 19.87 ± 2.4 mg caffeine/g dry matter and 6.88 ± 1.77 mg CGA/g dry matter.

2.3. Evaluation of the bioaccessibility of coffee silverskin extract

2.3.1. *In vitro* oral gastrointestinal digestion

The amount of CSE components potentially available for further uptake was determined following the procedure of Hollebeck et al. (2013) (11) with slight modifications. To mimic *in vitro* oral digestion, 1.17 g of CSE was suspended in 9 ml of Milli-Q water and the pH was adjusted to 6.9 with 1M HCl and brought to a volume of 9.98 ml. α-Amilase (0.45 ml of 0.562 mg/ml in phosphate

buffer) was added to each sample and incubated at 37 ºC for 5 min with constant stirring at 4 g. For the gastric digestion step, 10 ml of Milli-Q water was added and the pH of the samples was adjusted to 2.0 with 1 M HCl. Pepsin (110 µl, 5 mg/ml in 0.1 M HCl) was added to each sample and incubated in a final volume of 22.88 ml at 37 ºC for 90 min under anaerobic conditions using an anaerobic chamber model Bactron II (Biogen, Weston, MA, USA) with constant stirring at 4 g. After this time, the pH of the samples was adjusted to 7 with 1M NaHCO3 for the intestinal digestion step. One ml of pancreatin solution (287.59 mg/ml in 0.1 M NaHCO3) was added, the final volume brought to 31.24 ml with Milli-Q water, and the mixture was incubated at 37 ºC for 150 min under anaerobic conditions. Digested samples were centrifuged at 1677 g at 4 ºC for 40 min. Enzymatic activity was stopped with liquid N2 and supernatants were freeze-dried and stored under dark, dry conditions at 4 ºC until analysis. The digestion of CSE was also carried out under the same conditions without adding the enzymes to gain insight into the effect of the pH in CGA and caffeine metabolism. Digestion experiments were carried out in triplicate.

2.3.2. Chemical composition of digested and non-digested coffee silverskin extract

Soluble protein content was determined using the BioRad protein assay kit following the manufacturer's instructions. Bovine serum albumin (BSA) was used for the calibration curve. Samples were analysed in triplicate and results expressed as mg BSA/g CSE.

Total phenolic content (TPC) was determined using the Folin-Ciocalteu's colorimetric assay (12) adapted to a microplate reader. Briefly, CSE prepared at 2 mg/ml was used. Ten µl of the sample was combined with 200 µl of Folin reagent (0.017% (v/v)) and 50 µl of NaHCO3 (30 mg/ml). The 96-well plate was incubated in darkness at room temperature for 2 h and was read at 725 nm using a BioTek PowerWave™ XS (Winoski, VT, USA) microplate reader. Calibration curves were constructed using a standard solution of CGA (0.1-0.8 mg/ml). Samples were analysed in triplicate and results expressed as mg of CGA equivalents per g of CSE (mg CGA /g CSE).

The chemical composition of CGA and caffeine in the CSE and their digested products was analysed by capillary zone electrophoresis (CZE) as described by del Castillo et al (2002) (13). Previously, the digested CSE (10 mg of extract/ml) was filtered through 0.2 µm nylon filters (Symta, Madrid, Spain). Determinations were carried out in an Agilent G1600 A (Santa Clara, CA, USA) capillary electrophoresis instrument equipped with ChemStation software and a diode array detector (DAD). CZE was performed in an uncoated fused 48.5 cm long silica capillary (40 cm to the detector) with an internal diameter of 50 µm and a x3 bubble cell. The other analysis conditions were as follows: 50 mM sodium borate buffer (pH 9.5), 20 kV voltage, 25 ºC temperature and the injection was at 50 mbar for 5 s. Electropherograms (e-grams) were monitored at 200 and 280 nm for CGA and caffeine, respectively; and spectra collected from 190 to 600 nm. The capillary was conditioned after running each sample by flushing with 0.1 M NaOH and buffer for 3 min. CGA (0.15-9 mM), and caffeine

(0.15-10 mM) calibration curves were used as standards for identification and quantification. Samples were analysed in triplicate and results were expressed as mg of CGA or caffeine/g CSE.

2.3.3. Antioxidant capacity of digested and non-digested coffee silverskin extract

Antioxidant capacity and radical scavenging and oxygen radical absorbance capacity were determined by the ABTS and ORACFL assays, respectively. The ABTS assay was carried out according to Oki et al. (2006) (14). $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration in 10 ml of water). The mixture was incubated in the dark at room temperature for 16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted 1:75 (v/v) with 5 mM phosphate buffer (pH 7.4) obtaining an absorbance value of 0.7 ± 0.02 at 734 nm. Thirty µl of sample (0.2 mg of non-digested CSE /ml or 0.2 mg of digested CSE digested/ml) or standard and 270 µl of $ABTS^{\bullet+}$ working solution were placed in each well. Absorbance readings were recorded in a microplate reader at 734 nm every minute. A standard calibration curve was constructed using Trolox (0.01-25 mM,), and results were expressed as µmoles of Trolox equivalents (TEAC)/g of CSE. Another calibration curve using CGA, the major phenolic compound in coffee, was constructed (0.05-0.25 mM), and results were expressed as µmoles of CGA equivalents (CGA)/g of CSE. Samples were analysed in triplicate.

An aliquot of non-digested CSE (10 mg/ml) was subjected to fractionation using an Amicon® Ultra 0.5 ml centrifugal filter unit fitted with an Ultracel®-10K regenerated cellulose membrane (10 kDa cut-off) (Merck Millipore, German). The antioxidant capacity of low (< 10 kDa) and high (≥ 10 kDa) molecular weight fractions was also analysed using the $ABTS^{\bullet+}$ assay.

The ORACFL assay was performed following the procedure described by Huang et al. (2002) (15). Briefly, 25 µl of sample of the appropriate dilution or standard were added to a 96-well microplate followed by the addition of 150 µl of fluorescein work solution (8.5 x 10-5 mM) prepared in 75 mM phosphate buffer (pH 7.4). The BioTek PowerWave™ XS microplate reader was programmed to incubate the plate at 37 ºC and add 30 µl of AAPH solution (153 mM in phosphate buffer) as a peroxyl radical generator. Fluorescence was read with excitation at 485 nm and emission at 528 nm every two minutes for 90 min. A blank consisting of fluorescein, AAPH and phosphate buffer was also included. Calibration curves of Trolox (6.25-50 µM) and CGA (3.12-25 µM) were constructed. Standard calibration curves were composed by plotting the net area under the curve (AUC) as a function of Trolox or CGA concentration. ORACFL values were expressed as µmoles TEAC/g of CSE and as µmoles CGA/g of CSE.

2.4. Animals and the experimental design (ARRIVE guidelines)

The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the UGR—Universidad de Granada, Campus de la Cartuja, GR, Spain (CEEA: 2010-287).

CSE doses administrated to the animals *in vivo* provided 0. 150 and 0.434 mg/d of CGA and caffeine, respectively. CGA and caffeine dose selection was based on moderate coffee consumption (3 cups a day) in adults (13,16), and the CSE dose was limited by its caffeine content (max. 300 mg/day).

2.4.1. Evaluation of the bioavailability of coffee silverskin extract

Twelve 6-week-old male Wistar rats (ENVIGO, Alconbury, United Kingdom) were housed singly in metabolic cages with free access to standard food (2014S Teklad, ENVIGO, Alconbury, United Kingdom) and water ad libidum. Food and water intake were measured by subtracting the remaining amount of food and water in the containers from the total amount given the day before, during the bioavailability study. Animals were divided into four groups (n = 4): rats treated with CSE, rats treated with CGA, rats treated with caffeine and untreated rats (control). At 8:00 in the morning, the CSE group received one single dose of CSE (2.2 mg caffeine/kg body weight; 0.8 mg CGA/ kg body weight), the CGA group received pure CGA (1.5 mg CGA/kg body weight) and the caffeine group received pure caffeine (5 mg/kg body weight). Urine samples were then serially collected from treated rats every hour for 6 hours, then every 2 h up to 10 h and finally after 24 h. Urine samples were collected from untreated rats every 24 hours as a control. Samples were stored at -80 ºC until analysis. After 3 days of clearance, the bioavailability experiments were repeated with the same animals.

Urinary creatinine was measured with the creatinine quantitative test kit (SPINREACT, Gerona, Spain) based on the Jaffe reaction, as previously described by Murray (1984) (17).

CGA, caffeine and related compounds, HA and paraxanthine were determined by UPLC-MS/MS. Urine samples were defrosted, centrifuged at 10481 g for 10 min at 4 ºC and supernatants were filtered using a 0.45 µm pore-size nylon membrane syringe filter (Análisis Vínicos, Ciudad Real, Spain). Aliquots (10 µl) were analysed in triplicate using an Accela liquid chromatograph (Thermo Scientific, San Jose, CA, USA) equipped with a DAD and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific, San Jose, CA, USA) triple quadrupole analyzer via an electrospray ionization (ESI) interface. Xcalibur software (Thermo Scientific, San Jose, CA, USA) was used for data storage and evaluation. Analytical conditions consisted of a ZORBAX SB-C18 (50 mm × 2.1 mm and 1.8 µm of particle diameter) column (Thermo Scientific, San Jose, CA, USA) using 1% (v/v) formic acid in methanol and 1% (v/v) formic acid in Milli-Q water as A and B mobile phases, respectively. Elution was carried out according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 7 min, 80% B; 9.5 min, 5% B; 10 min; 95% B; 15 min, 95% B. Optimum flow rate was 0.3 ml/min, whereas the injection volume was 10 µl. The DAD recorded the spectra from 200 to 450 nm. Column and autosampler compartments were kept at 30 ºC and 4 ºC, respectively. The mass spectrometer was operated in the positive ESI mode to quantify caffeine and paraxanthine and in the negative ESI mode to quantify CGA and HA. Spray voltage and capillary temperature

were set at 3500 V and 250 °C, respectively. Nitrogen was used as a sheath and auxiliary gas at pressures of 40 and 20 arbitrary units, respectively. Ion sweep gas pressure was 2 units and collision gas (Ar) pressure was 1.5 mTorr. Scan width and scan time were fixed at 0.020 (m/z) and 0.100 s, respectively, and the system was operated in selected reaction monitoring (SRM). SRM parameters were optimized by direct infusion of standards. Two transition ions were monitored for identification but only the most intense one for each precursor ion was used for quantification. Parent ($[M-H]^-$) and product ions for CGA and HA were m/z 353.2→ 191.1 and m/z 178.3 → 134.3, respectively, whereas parent ($[M-H]^+$) and product ions for caffeine and paraxanthine were m/z 195.1 → 138.2 and m/z 181.1 → 124.2, respectively.

2.4.2. Evaluation of the bioactivity of coffee silverskin extract in the pancreas of streptozotocin-nicotinamide diabetic rats

Thirty-two 6-week-old male Wistar rats (ENVIGO, Alconbury, United Kingdom) were divided into four groups (n = 8) paired by weight (average weight per group was 194 ± 2 g). Rats were maintained at 23 ± 1 ˚C and 55 ± 5 % relative humidity on a 12:12-hour light-dark cycle with free access to standard food (2014S Teklad, ENVIGO, Alconbury, United Kingdom) and water ad libidum. Food and water intake were measured by subtracting the remaining amount of food and water in the containers from the total amount given the day before, during the experimental time. The rats in groups 1, 2 and 3 were supplemented by gastric gavage with CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight), pure CGA (1.5 mg CGA/kg body weight) and pure caffeine (5 mg caffeine /kg body weight) dissolved in 1 ml of sterile water, respectively, every day for a total of 42 days. The fourth group (the STZ group) was treated similarly with sterile water. At day 35, all rats were injected with 200 mg/kg body weight of NA dissolved in saline buffer, and 15 min later T2DM was induced by the intraperitoneal injection of 60 mg/kg body weight of STZ dissolved in cold 0.1 M citrate buffer (pH 4.5) immediately before use, according to Masiello et al. (1998) (18). The order in which the animals were injected was randomized among the groups. Blood samples were obtained from the tail vein and glucose levels (mg/dl) were determined after T2DM induction every day for six days using a glucometer (FreeStyle Lite®, Abbott Laboratories). Rats were considered diabetic when blood glucose levels were above 200 mg/dl. An additional healthy control group (n = 8) was also included in the experiment.

At day 42, overnight-fasting blood glucose was measured using a glucometer. The fasting rats were then anaesthetised with Ketamine-Xylazine (1 ml/kg body weight and 0.5 ml/kg body weight, respectively) and sacrificed. The pancreas was removed promptly, weighed, divided into three parts and stored at −80 °C until required.

Glutathione peroxidase (GPx) and glutathione reductase (GR) activity were determined in pancreas homogenates as described by Rodríguez-Ramiro et al. (2011) (19). Thus, pancreatic tissues were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM DTT buffer pH 7.4 and centrifuged

at 3000 g for 15 min. Determination of GPx activity was based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH catalysed by GR which reduced GSSG. GR activity was determined based on the decrease in absorbance due to the oxidation of NADPH used in the reduction of GSSG. Total pancreatic protein content was measured by the Bradford method (20).

GSH concentration was evaluated using the previously described fluorometric assay (19). This method takes advantage of the reaction of GSH with o-phthaldehyde at pH 8.0. Pancreatic tissues were homogenized (1:20 w/v) in 50 mM phosphate buffer pH 7.0, and proteins were precipitated with 5% trichloroacetic acid and then centrifuged for 30 min at 10.000 g. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. Results were interpolated in a GSH standard curve (5 ng-1 µg) and expressed as nmol/mg protein.

Pancreatic protein oxidation was measured as carbonyl groups content according to Granado-Serrano et al. (2009) (21). Pancreatic tissues were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM DTT buffer pH 7.4 and centrifuged at 3000 g for 15 min. Absorbance was measured at 360 nm, and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22000 nmol/l/cm. Total pancreatic protein content was measured by the Bradford reagent (20).

2.5. Pharmacokinetic and statistical analysis

Maximum concentration (Cmax), AUC and the time required to reach maximum concentration (Tmax) of metabolites in urine was evaluated using Microsoft Excel functions (22). SPSS program version 22.0 was used for statistical analyses. Comparisons of excretion pharmacokinetic parameters between treatments were done by Student's T-test. Prior to statistical analysis, all data were tested for homogeneity of variances using the Levene test. For multiple comparisons, one-way ANOVA was carried out followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$ except in the case of carbonyl content ($p < 0.1$).

3. Results

3.1. *In vitro* bioaccessibility of the bioactive compounds of coffee silverskin extract

Caffeine, TPC and CGA levels of 44.64 mg/g, 46.65 mg/g and 13.33 mg/g were detected in CSE, respectively. Overall antioxidant capacity values of 397 and 358 µmol CGA/g (corresponding to 427 and 816 of µmol TEAC/g) were obtained for scavenging and hydrogen donating capacities in CSE, respectively (**Table 1**). The antioxidant capacity of low molecular weight compounds (<10 kDa) was 220

µmol CGA/g, while the antioxidant capacity of the fraction containing high molecular weight compounds (≥10 kDa) was 110 µmol CGA/g.

In vitro digestion of CSE decreased concentrations of TPC (40%), CGA (82%) and caffeine (25%). The overall antioxidant capacity of CSE decreased by 15% and 50% as measured by ABTS and ORAC, respectively (**Table 1**).

Table 1. Antioxidant capacity (µmol CGA/g) of non-digested and digested CSE.

Sample	ABTS	$ORAC_{FL}$
CSE non-digested	397 ± 17^{a}	358 ± 25^{a}
CSE digested	337 ± 26^{b}	179 ± 13^{b}

Results are expressed as mean ± SD for n = 3. Different letters in the same column indicate significant differences ($p < 0.05$). CSE, coffee silverskin extract

To evaluate the effect of changes in pH during digestion on the degradation of bioactive compounds, a digestion in the absence of digestive enzymes was performed. Changes in pH decreased TPC and CGA content by 38% and 83%, respectively, while caffeine content only decreased by 15%. .

CSE can provide bioaccessible amounts of bioactive compounds such as caffeine (172.37 µmol/g), TPC (28.06 mg/g) and CGA (6.86 µmol/g). Digests presented scavenging capacity (337µmol/g) and oxygen radical absorbance capacity (179 µmol/g) after physiological digestion

3.2. Metabolism of the bioactive compounds of coffee silverskin extract

Figure 1 shows the kinetics of the urinary excretion of CGA, caffeine and their metabolites. Urinary pharmacokinetic parameters of these compounds after CSE, caffeine and CGA consumption are presented in **Table 2**. Intact CGA was not found in the urine of rats fed with CSE (containing 0.150 mg of CGA/day), CGA (0.293 mg of CGA/day) or caffeine. The baseline value of HA in urine excretion was set at 431.70 µmol/mmol creatinine and was obtained by measuring HA in the

24 h urine of the animals before administering the products. HA excretion was the greatest after the ingestion of CGA, reaching a peak in the 0-2 h interval, and AUC was significantly higher ($p < 0.05$) than that found after the intake of caffeine and CSE (**Table 2**). The maximum concentration of HA in urine (1346.86 mmol/µmol creatinine) was found 1.57 h after CGA consumption, which was higher than the maximum HA concentrations found in urine after caffeine intake (**Figure 1A** and **Table 2**).

Excretion of caffeine and its metabolite paraxanthine were not detected in the urine of CGA treated rats. Non-metabolized caffeine in urine excretion (AUC and Cmax) after the consumption of pure caffeine was higher than that found after treatment with CSE (**Figure 1B** and **Table 2**). Urinary excretion of caffeine peaked at 0-8 h and decreased in the next 8-24 h in rats treated with caffeine. In rats treated with CSE, urinary excretion of caffeine peaked at 2 h and then decreased (**Figure 1B** and **Table 2**).

Urinary paraxanthine excretion after consumption of pure caffeine was higher (AUC and Cmax) than that found after the intake of CSE (**Figure 1C** and **Table 2**). In this case, paraxanthine reached maximum excretion between 2 and 12 h, and excretion decreased between 12 to 24 h after the ingestion of both CSE and caffeine (**Figure 1C** and **Table 2**).

Both studied compounds were metabolized. Free CGA was not detected in urine and caffeine was metabolized to paraxanthine.

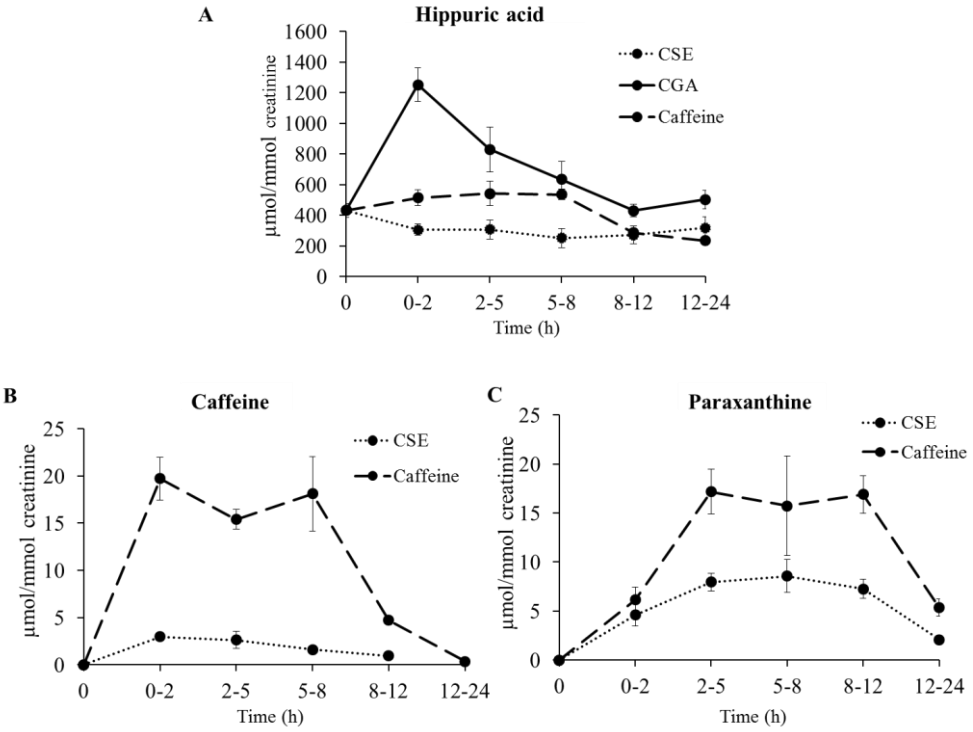

Figure 1. Kinetics of the urinary excretion of hippuric acid (A), caffeine (B) and paraxanthine (C) after consumption of CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight), CGA (1.5 mg/kg body weight) and caffeine (5 mg/kg body weight). Results represent the concentration (µmol/mmol creatinine) as mean (n=7) ± SEM. CSE, coffee silverskin extract; CGA, chlorogenic acid.

Table 2. Pharmacokinetic parameters (Cmax, AUC and Tmax) of metabolites detected in urine after treatments consumption with coffee silverskin extract (CSE; 2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight), cholorogenic acid (CGA; 1.5 mg/kg body weight); and caffeine (5 mg

Metabolite	Parameters	ACSE	CF	CGA1.5
HA	Cmax (mmol/µmol)	385.08 ± 121.01^{a}	719.11 ± 138.15^{b}	1346.89 ± 274.86^{b}
	Tmax (h)	10.50 ± 4.47^{a}	4.63± 2.78^{a}	1.57± 0.28^{a}
	AUC (mmol/µmol.h)	6097.77± 2524.13^{a,b}	6863.70 ± 1180.80^{a}	12993.63 ± 2158.54^{b}
Caffeine	Cmax (mmol/µmol)	3.31 ± 1.12^{a}	28.88 ± 4.64^{b}	n.d
	Tmax (h)	1.8 ± 0.37	2.50 ± 0.50	
	AUC (mmol/µmol.h)	14.80 ± 5.58^{a}	136.66 ± 17.74^{b}	
PX	Cmax (mmol/µmol)	10.68 ± 2.69^{a}	22.40 ± 4.17^{b}	nd
	Tmax (h)	5.50 ± 1.59	4.62 ± 0.59	
	AUC (mmol/µmol.h)	125.31 ± 34.68^{a}	265.56 ± 55.81^{b}	

Values represent mean ± SEM, n=7. Means in a row without a common letter differ; $p < 0.05$, T-student. nd, not detected. AUC, area under the curve; C_{max}, maximum concentration reached; T_{max}, time to reach C_{max}; HA, hippuric acid; PX, paraxanthine.

3.3. Bioactivity of coffee silverskin extract in the pancreas of streptozotocin-nicotinamide diabetic rats

The effect of the CSE, CGA and caffeine treatments on oxidative stress biomarkers in the pancreas of diabetic rats is shown in **Figure 2**. Rats were considered diabetic when blood glucose levels were above 200 mg/dl. The STZ-NA treatment caused significant oxidation ($p < 0.1$) of pancreatic proteins by

increasing their carbonyl groups (**Figure 2A**). On the contrary, animals pre-treated with CGA or caffeine for 35 d significantly prevented ($p < 0.1$) oxidative protein damage induced by STZ. Protein carbonyl content decreased by 24% and 22% in the pancreas of diabetic rats treated with CGA and caffeine, respectively. However, CSE did not reduce the rate of protein oxidation induced by the toxic agent. GSH content in the pancreas of T2DM rats decreased significantly ($p < 0.05$) (**Figure 2B**), and pre-treatment with CSE and CGA significantly reduced ($p < 0.05$) GSH depletion in the pancreas of diabetic rats. Untreated rats and those treated with CSE and CGA showed similar pancreatic GSH values ($p > 0.05$). GPX and GR values of all animals were of the same order of magnitude ($p > 0.05$) (**Figure 2C**).

The physiological concentrations of the bioactive compounds forming CSE were able to protect pancreatic cells against oxidative stress produced by the diabetogenic agent STZ.

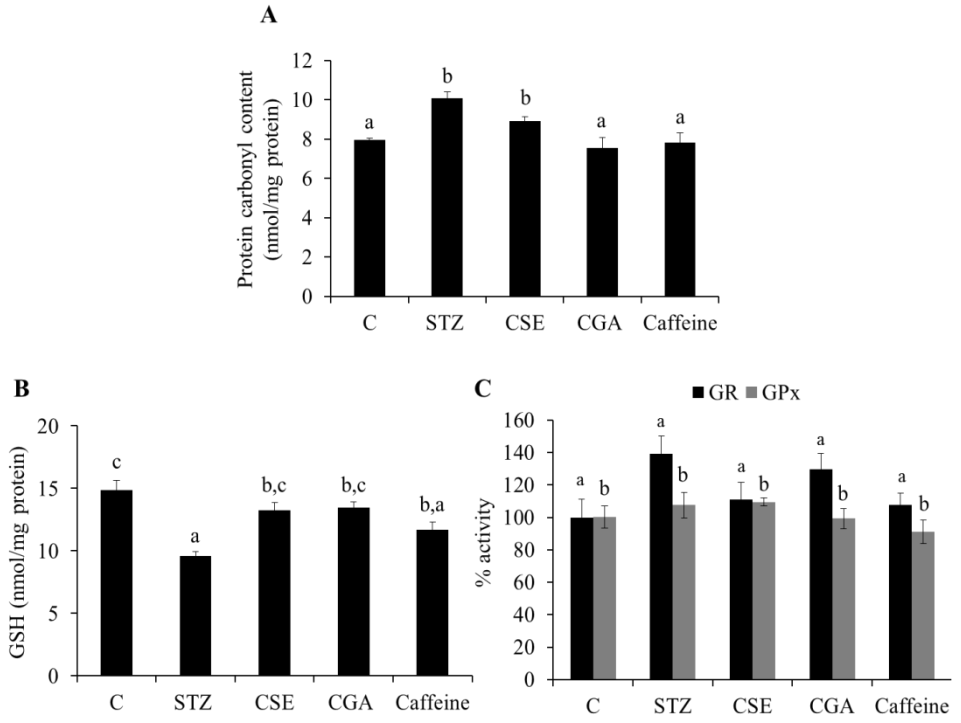

Figure 2. Effect of CSE, CGA and caffeine on oxidative status in pancreatic tissues of STZ-NA induced diabetic rats. C, untreated healthy control rats; STZ, rats treated with STZ (60 mg/kg body weight) and NA (200 mg/kg body weight); CSE, rats treated with STZ-NA and CSE (2.2 mg caffeine/kg body weight, 0.8 mg CGA/ kg body weight); CGA, rats treated with STZ-NA and 1.5 mg CGA/kg body weight; Caffeine, rats treated with STZ-NA and 5 mg caffeine/kg body weight; (A) GSH levels ($p < 0.05$, (B) GR and GPx activities ($p < 0.05$) and (C) Carbonyl groups production ($p < 0.1$) were evaluated. Data represent means ± SEM (n=8). Different letters denote statistically significant differences referred above in brackets. CSE, coffee silverskin extract; CGA, chlorogenic acid; STZ, streptozotocin; NA, nicotinamide.

4. Discussion

This is the first study assessing the role of the gastrointestinal digestion on the bioaccessibility of CSE bioactive compounds and its remnant overall antioxidant capacity.

TPC values found in CSE are in agreement with those described by other authors (6,23). Slightly higher CGA and caffeine concentrations were found in CSE than

in CS raw material (4.31 mg CGA/g and 10 mg caffeine/g) using a similar analytical method (24). Results suggest that aqueous extraction increases the bioaccessibility of the bioactive compounds present in the plant matrix. Values of overall antioxidant capacity also agree with those reported by Mesías et al. (2014) (6). The highlighted chemical composition of CSE suggests that it could be a good source of bioactive compounds with putative healthy benefits (4).

Our results indicate that *in vitro* digestion affected the composition of CSE reducing the bioaccessibility of TPC, CGA and caffeine. However, digests presented antioxidant capacity suggesting that antioxidants remained bioaccessible after the digestion process. The release of compounds from the plant matrix depends on the chemical form and the properties of nutrients and phytochemicals (25). TPC and CGA content were significantly decreased ($p < 0.05$) by the digestion processes (data not shown). Since this decrease was observed in the presence of digestive enzymes, it may be associated with changes in pH taking place during *in vitro* digestion. Several studies have shown that the bioaccessibility of TPC in different food matrixes was lower than that found for isolated polyphenols. Podio et al. (2015) (26) observed a 5-fold lower TPC content in digested coffees than in native instant coffees. Campos-Vega et al. (2015) (27) reported a considerable reduction of TPC (91%) in spent coffee grounds. Akillioglu & Karakaya (2010) (28) showed that the bioaccessibility of TPC ranged from 19% to 39% in bean varieties. Phenolic compounds are less bioaccessible partly due to the presence of dietary fibre in the plant matrix (29). CSE contains high amounts of dietary fibre (362 mg/g) which affect the release of TPC in the digestion process. Hydroxycinnamic acid derivatives constitute the main phenolic component of CS (24). Vallejo et al. (2004) (30) observed an 87% decrease in CGA after intestinal digestion. Previous studies have suggested that a pH value of 7.5 and bile salts could contribute to lower CGA. Bermudez-Soto et al. (2007) (31) reported a minor decrease in CGA (5%) in chokeberry extract due to the pH of intestinal digestion (pH 7.5). However, a bioaccessibility study of CGAs in spent

coffee grounds showed a total recovery of this compound after digestion (32). These differences in CGA release suggest that bioaccessibility is also affected by the plant matrix.

In agreement with the data on TPC and CGAs, overall antioxidant capacity also decreased after *in vitro* gastrointestinal digestion. The main antioxidant compounds reported in CSE are CGAs, melanoidins and antioxidant fibre (4). According to our data, low molecular weight compounds (CGAs and other phenols) seem to make a greater contribution to the overall antioxidant capacity of CSE than the high molecular weight fraction (melanoidins, proteins and antioxidant fibre). Rice-Evans et al. (1996) (33) found that CGA antioxidant activity is related to the CH=CH-COOH group, which ensures greater H-donating ability and radical stabilization. Caffeine effectively reacts with the hydroxyl radical (OH·) and caffeine-derived oxygen-centered radicals are formed in the reaction between caffeine and OH· (34). In this sense, Pellegrini et al. (2003) (35) found a decrease of ∼25–30% in the antioxidant capacity of espresso coffee when the caffeine was removed.

The greatest part of the CGA ingested by rats is hydrolyzed to caffeic acid and quinic acid, and further metabolized by gut microbiota into various aromatic acid metabolites including m-coumaric acid and derivatives of phenylpropionic and benzoic acids (**Figure 3**) (36). Previous studies found that HA, a benzoic acid, was the major CGA-derived metabolite observed in urine and plasma after the ingestion of pure CGA or CGA from a food matrix (36,37). We found amounts of HA in urine of 1346.86 mmol/µmol creatinine after the intake of a single dose of 0.825 µmol CGA. Urine HA concentration after the intake of CSE containing 0.424 µmol CGA (447.93 mmol/µmol creatinine) was of the same order of magnitude as basal values (431.70 mmol/µmol creatinine). These results are in agreement with the low bioaccessibility observed for the CGA present in CSE. The metabolic fate of CGAs ingested as a pure compound or present in coffee has been previously investigated in rats (36,38) and humans (39,40). Farah et al.

(2008) (41) reported high bioavailability of CGAs present in a green coffee extract in humans. In this study, we did not detect intact CGA in urine after oral dosing of CGA and CSE. Results suggest that CGA was absorbed and metabolized into different compounds to those tested in the present study. In accordance with our findings, several authors failed to detect CGA in the plasma or urine of rats and humans fed pure CGA or CGA-containing foods (38,39,42,43).

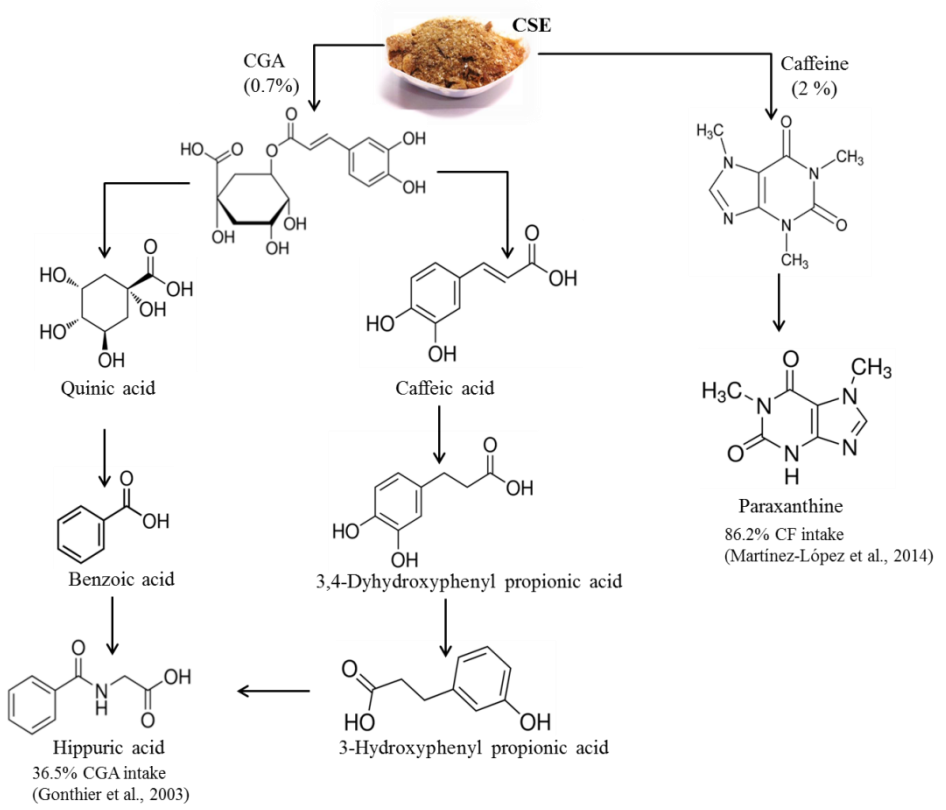

Figure 3. Simplified scheme of CGA and caffeine metabolism studied in the present study.

CSE is also a good source of caffeine (1,3,7-trimethylxanthine). Methylxanthines are extensively absorbed in the gastrointestinal tract and metabolized in the liver to yield methylxanthine derivatives and methyluric acids as the main metabolites, which are finally excreted in urine (**Figure 3**) (44). Paraxanthine (1,7

dimethylxanthine) is the main metabolite of caffeine biotransformation found in plasma and urine after caffeine intake (45). The pharmacokinetics of caffeine and paraxanthine excretion were evaluated after the consumption of 5.026 µmol pure caffeine and CSE containing 2.211 µmol caffeine. Caffeine was present in the urine of both groups of rats, which is in agreement with other studies that described incomplete biotransformation in humans (46,47). According to CSE composition, the lower consumption of caffeine was in line with the lower excretion observed for this compound and its metabolite paraxanthine. These results are in agreement with previous findings of dose-dependent metabolism and the excretion of caffeine (44). Therefore, our data showed that the caffeine present in CSE is bioavailable, partially metabolized, and rapidly excreted.

The *in vivo* effect of CGA, caffeine and CSE on the prevention of oxidative damage in the pancreas of STZ-NA-induced T2DM rats was also evaluated. The cytotoxic action of STZ is associated with the generation of ROS and consequent β-cell destruction and suppression of insulin secretion (48). Antioxidants are able to prevent pancreatic islets damage induced by STZ (49). Consequently, natural antioxidants may be considered promising candidates for the prevention or co-treatment of diabetes. In the present study, the administration of STZ to the animals produced a decrease in GSH and an increase in GR activity ($p=0.173$), while GPx activity remained unaltered in pancreas antioxidant defence. This indicates that the depletion of GSH may induce GR activity but that this induction is not enough to regenerate the basal GSH concentration. Protein oxidation was significant ($p < 0.1$) in the pancreas of STZ induced T2DM rats. Interestingly, the daily administration of CSE, CGA or caffeine 35 d previous to the induction of diabetes significantly prevented ($p < 0.05$) pancreatic oxidative stress and protein damage. *In vitro* studies have shown that CGA (50–52) and caffeine (53) protect pancreatic β-cells from the oxidative stress damage caused by free radicals. Furthermore, *in vivo* studies have demonstrated that CGA (54) and caffeine (55,56) could also prevent STZ-induced oxidative stress and protect β-cells *in*

vivo. The present study is the first to demonstrate a specific chemo-protective effect of CSE on pancreas tissue, possibly associated to its antioxidant character.

In conclusion, the present study provides, for the first time, information on the bioaccessibility, metabolism and *in vivo* bioactivity of bioactive compounds present in CSE. The bioaccessibility of CGA and caffeine was affected by changes in pH during digestion. CGA (0.91 µmol) and caffeine (5.53 µmol) were metabolized and protected pancreatic cells against the oxidative stress induced by the diabetogenic agent.

Abbreviations: CGA, chlorogenic acid; CSE, coffee silverskin extract, HA, hippuric acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; STZ, streptozotocin, NA, nicotinamide; TE, trolox equivalent; ROS, reactive oxygen species, TPC, total phenolic compounds.

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General discussion

T2D is an autoinflammatory syndrome including many disorders such as hyperglycemia, dyslipidemia, insulin resistance, impaired beta cell functioning, and insulin secretion (1,2). There are various ways to treat T2D such as lifestyle modification (diet and exercise changes), dietary supplementation and finally medication. Preventive actions are increasingly recognised to reduce the risk of T2D, within them dietary factors have actually a great influence in the development of the disease. If diet and exercise do not manage the adequate glucose blood levels, initiation of an oral antidiabetic agent is needed (3).

Nowadays, the use of plants extracts containing phytochemicals is a challenge to achieve the goal of T2D treatment and its prevention, and also may reduce diabetes complications and side effects of current antidiabetic drugs (5). CSE contents high amounts of phytochemicals such as caffeine and CGA among others (6,7). These components, also present in coffee brew, possess potential benefits on glucose homeostasis (8). However, the mechanism of action of CGA and caffeine on glucose metabolism remains still unclear (9). The present study contributes to the better understanding of coffee components in the pathogenesis of diabetes. CSE is a complex matrix of bioactive compounds (6,7), beside CGA, able to affect different pathways involved in the pathogenesis of diabetes (8,9). The information provided in the present thesis is novel and of socio-economic interest. The knowledge generated during the development of the present investigation is a contribution to the food waste valorisation and nutrition and health sustainability. Additionally, the study emphasizes the interest of the agriculture in health and the great potential of vegetable food wastes in providing different molecules able to reduce the risk and to treat chronic diseases considered as epidemics of the XXI century, such as diabetes.

A suitable alternative for managing CS produced in the coffee roasting industry is by processing it environmentally friendly technology, preferably without using organic solvents, which make it possible to obtain products with high added value. The extraction methods patented in P2013004873A1 were hot water extraction (low technology) and extraction in subcritical and/or supercritical water conditions (high technology), both methods allow to obtain higher bioactive compounds extraction yields. Specifically, CSE used in this study was obtained by a simple water extraction stage (100 ºC for at least 10 min) described in the patent WO2013004873A1. Moreover, our previous studies indicated that this green extraction process of CSE produces completely recyclable solid waste (6) and it can be used as a natural source of antioxidant fibre to elaborate bakery products (10).

The sustainability of food production and consumption, defined as biorefinery, is a research priority, since it explores innovative strategies to increase resource efficiency, providing consumers with healthier products of higher quality and safety and ensuring minimal waste in the food chain (11). The recovery of coffee by-products is mainly based on their use as a source of energy and biomass (12). Conversion of by-products into health-promoting products is of particular interest because it could increase the competitiveness and sustainability of coffee production. Thus, the study of CSE as a promoting health compound provides an opportunity to increase the competitiveness of the coffee sector.

Results obtained in this study and protected by patent (P201431848), suggest that bioactive compounds present in the CSE affect several pathways involved in the pathogenesis of the diabetes reducing the risk this of disease. The effects of CSE on biomarkers of diabetes can be summarised as follows:

1. Increase glucose tolerance (**P201431848**)
2. Enhance insulin sensitivity and secretion (**P201431848** and **Chapter 2**)
3. Inhibit the activity of α-glucosidase (**P201431848**)

4. Decrease total plasma cholesterol and triglycerides (**P201431848**)
5. Inhibit the activity of lipase (**P201431848**)
6. Inhibit AGEs formation through the interaction of CGA and its derivatives with protein backbone (**Chapter 1**)
7. Enhance antioxidant defence in beta cells against oxidative damage (**Chapter 2**) causing reduction of oxidative stress and protein damage in diabetic pancreas (**Chapter 3**).

All these effects have an impact in diabetes and health (**Figure 12**). The components of CSE are metabolised and play a role in vital organs involved in the pathogenesis of diabetes and its complications. As a consequence, CSE may be useful in both prevention and treatment of diabetes.

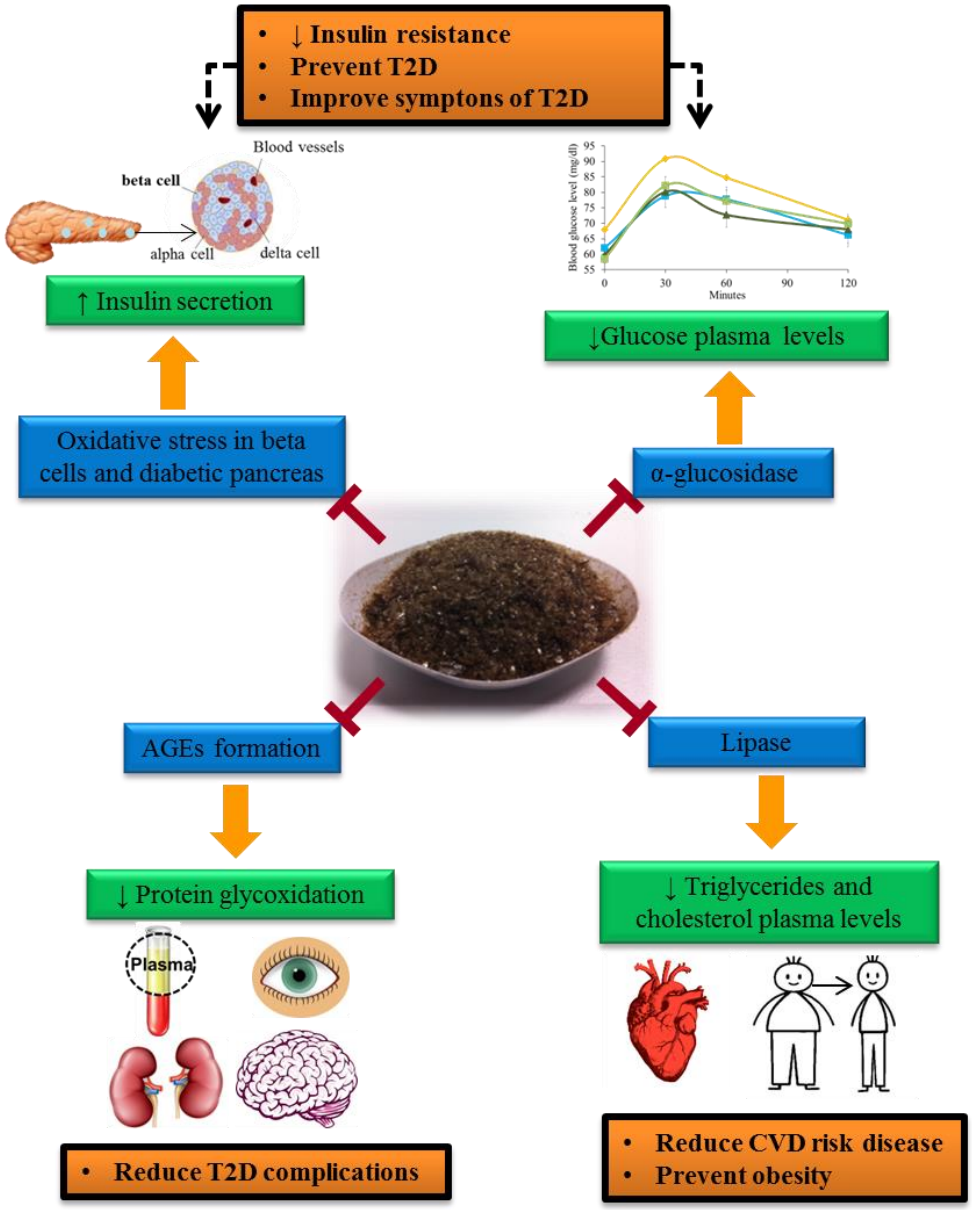

Figure 12: Effects of coffee silverskin extract on the biomarkers of Type 2 Diabetes and health.

Most data on effects of coffee components on glucose metabolism published so far are based on animal and *in vitro* studies; therefore the relevance for the development of T2D in humans is currently unclear. However, these results suggest that an effect of coffee consumption on glucose metabolism is biologically plausible, and that the effects of coffee cannot be equated to those of caffeine and CGA. Other coffee constituents are relevant for diabetes, like melanoidins, melatonin, lignans and lignin, tannic acid, isoflavones and trigonelline acting following different pathways. All of these compounds may be present in CSE. **Table 4** shows the effects of coffee components inT2D.

Table 4: Coffee components and possible effects in Type 2 Diabetes.

Component	Concentration in CSE (w/w)	Suggested mechanism
Caffeine	3-3.4 (7)	• Reduce glucose levels and insulin sensitivity (13,14) • Protective effect against oxidative stress in beta cell and pancreas (Chap. 3;15,16)
CGA	1.1-6.8(7)	• Regulate glucose metabolism (9,18–20) • Enhance insulin action (21–23) • Inhibit α-glucosidase activity (24,25) • Protect beta cell and pancreas against oxidative stress (Chap. 2 and 3; 19,26,27) • Inhibit AGEs formation (Chap1; 7,28).
Melanoidins	17.2-23.9 (7)	• Antioxidant and antiglycative effects (7,32)
Melatonin	0.34 (38)	• Protection of beta cells from oxidative stress (39)
Isoflavones	nd	• Proliferation and protection of beta cells (42). • Decrease HbA1c levels and improve lipid profile (43)
Tannins	nd	• Hypoglycemic and antioxidant effects (46,47)
Lignin	nd	• Decrease glucose absorption, improve insulin sensitivity and protect against oxidative stress (49,50)
Lignans	nd	• Antioxidant action and decrease glucose, HbA1c, C-reactive protein and lipids plasma levels (42)
Trigonelline	nd	• Improve insulin content and sensitivity (56,57) • Regulate glucose and lipids metabolism (56,57)

CSE, Coffee silverskin extract; Chap, Chapter; nd, no data; HbA1c, glycated haemoglobin.

Concentrations of caffeine ranging from 3-3.4 % are present in CSE (7). Our results show that caffeine in CSE was metabolized and the metabolites protected pancreas against oxidative stress in rats suffering streptozotocin-induced diabetes (**Chapter 3**). Since no effect of caffeine was observed in INS-1E cells, results seem to indicate its metabolites are more effective than the parental molecule in the prevention of oxidative stress during diabetes. Caffeine can also reduce glucose levels and insulin sensitivity (13,14). Caffeine treatment improved the health of the pancreas of diabetic rats. Other authors have also observed a protective effect of caffeine in pancreatic beta cell (15,16). Food components should be bioavailable to exert their therapeutic effect. Our results support that caffeine and also CGA are bioavailable and they affect the biomarkers of diabetes (**Chapter 3**). Differences found between *in vitro* and *in vivo* studies pinpoint the interest of the study of the influence of molecules metabolism in the bioactivity of the food components.

CGA is present in amounts of 1.1-6.8% in the CSE (7). Our results suggest CGA and its metabolites are more effective than caffeine in T2D biomarkers. In previous studies, CGA and its roasting-formed derivates that are present in CSE were proposed as main contributors to the beneficial effects of CSE in T2D (8,9,17). The antidiabetic effect of CGA has been associated to different mechanism, including: 1) regulation of glucose metabolism (9,18–20), 2) enhancement of insulin action (21–23), 3) inhibition of α-glucosidase activity (24,25), 4) protection against oxidative stress (19,26,27) and 5) inhibition of AGEs formation by different pathways (antioxidant, chelating properties, quenching of carbonyl radical species and AGE crosslinking) (7,28). Most of these effects were observed in the present study. For the first time, a new mechanism of action of CGA, administered alone or in CSE, for inhibiting the formation of AGEs has been reported during the development of the present PhD thesis (**Chapter 1**). According to the investigations performed, the formation of fluorescent AGEs is inhibited by different pathways such carbonyl trapping, antioxidant effect and

formation of protein-phenols conjugates (7,28,29) (**Chapter 1**). The finding of the new mechanism of action of CGA and CSE in the formation of AGEs is very relevant (**Chapter 1**). The goal was achieved by employing advanced analytical approaches also called phytochemomics (30). Our study confirms the interest of the use of omics for a better understanding of the bioactivity of food components and in particular of those bioactive compounds present in low concentrations, such as phytochemicals including phenolic compounds. Most of the complications of diabetes are associated to AGEs (31). In agreement, the search of natural sources of inhibitors of the formation of AGEs represents a scientific challenge. The present study demonstrates the potential of CSE as a natural source of antiglycative agents beside CGA.

Other compounds present in the CSE are melanoidins. Values of melanoidins of 17.2-23.9 % have been found in the extract (7). Antiglycative, chelating and antioxidant properties have been ascribed to coffee melanoidins (32–34). These functions are linked with the presence of CGA, protein and polysaccharides in its complex structure (32). Coffee melanoidins also protected against non-alcoholic fatty liver disease by reducing the hepatic fat accumulation (35). This symptom of the metabolic syndrome is closely related with visceral obesity, dyslipidemia and T2D (36). Melanoidins from CSE have carbonyl trapping capacity and inhibiting fluorescent AGEs formation (7). Hence, these compounds could be used as inhibitors of AGEs related diseases. In addition, melanoidins and CGA may contribute to synergic inhibitory effect on the formation of AGEs. Further research should be conducted in order to elucidate the contribution of these indicidual compounds to this very important property in diabetes and its complications.

Melatonin is an indoleamine hormone (37,38). Our CSE contains 3.4 mg/g dry matter of melatonin (38). Experimental evidences indicated that melatonin has potential to reduce the risk of T2D by protecting beta cells against oxidative stress, since it neutralizes the production of reactive species and normalizes the redox state in the cell (39). Melatonin, CGA and other coffee antioxidants (CGA and its

metabolites, caffeine metabolites, melanoidins among others) may exert synergic effects resulting in a protection against the oxidative stress of pancreas and in the development of diabetes. Further research should be conducted in order to demonstrate the hypothesis.

Other compounds present in coffee and probably in our CSE are lignans and lignin, tannic acid, isoflavones and trigonelline. All these compounds may also be responsible for the health promoting properties associated to CSE. Although we have not analysed the presence of these molecules in our CSE, their identification open a big field of study that may complete the knowledge of the effects of this extract in T2D.

Isoflavones are phenolic compounds described in coffee beans (40). In processed coffee, these compounds are usually found as glucoside derivatives and free aglycones (41). The most abundant isoflavones characterised in roasted coffee are genistein, daidzein and formononetin (methylated precursors of daidzein) (40). Isoflavones levels in coffee beans (about 30-40%) decrease during the roasting process (40). Genistein intake was associated to an antidiabetic effect through different mechanism of actions, such as direct effects on beta cell proliferation, glucose-stimulated insulin secretion and protection against apoptosis, independently of its functions as an estrogenic receptor agonist, antioxidant or tyrosine kinase inhibition (42). Supplementation with genistein and daidzein caused a decrease in blood glucose and HbA1c levels and also improved lipid profile in T2D animals (43). Further research should be conducted in order to find out the content of isoflavones in CSE and their contribution to the antidiabetic effects associated to the extract in the present investigation.

Tannins (commonly referred as tannic acid) are water-soluble polyphenols that are present in CS (44). The amount of tannins in CS reported was 0.43 mg tannic acid equivalents /l (44). Although the ingestion of tannic acid and other hydrolysable tannins have been related with anti-nutritional effects, since they form complexes

with proteins, starch, and digestive enzymes causing a reduction in the nutritional value of foods, their antioxidant property is also well documented and depends on the amount and type of tannins present in the food (45). The healthy effect of tannins might be related to other components associated with these molecules rather than to tannins themselves (45). Tannins were proposed as antidiabetic agent due to its hypoglycemic and antioxidant activities observed *in vitro* (46) and *in vivo* (47). Therefore, tannins may contribute to the antiglycoxidative effect found for CSE in the present study.

CS also contains lignin, an organic polymer, (28-30% dry matter) (48). This organic polymer is classified as insoluble dietary fibre (48). Then, lignin is resistant to digestion in the small intestine and requires colonic bacterial fermentation. An inverse relationship between the intake of insoluble fibre and the risk of developing T2D has been observed. Insoluble fibre may have different mode of action in T2D, such as decreasing absorption of simple carbohydrates and improving of insulin sensitivity (49). In addition, metabolites of native lignin (lignophenols) have been reported to reduce oxidative stress and inflammation in streptozotocin-induced diabetic rats (50). The content of lignans in CSE should be studied.

Other bioactive compound related to lignin and present in coffee beans are lignans (51). In plants, lignans (monolignol dimers) usually occur free (aglycone) or bound to sugars (glycoside). Monolignols, derived from hydroxycinnamic acids, are either dimerized to lignans into the cell or polymerized into larger lignin structures in the cell wall (52). The enterolignans are metabolites of food lignans produced by human intestinal bacteria. They exert weak estrogenic (53) and other biochemical properties, suggesting a nutritional potential for the prevention of chronic diseases (54). The main effects of lignans and its derivatives in the pathogenesis of T2D included decreasing of fasting glucose, HbA1c and C-reactive protein levels and the improvement of lipid profiles (42). Lignans may

affect glucose metabolism through the antioxidant and anti-estrogenic properties of their metabolites (42).

Trigonelline, a niacin related compound, is a natural constituent of coffee beans (approximately 1% dry matter) that is partially degraded to nicotinic acid during the roasting process (55). Although we have not investigated the presence of trigonelline in our CSE, probably it may be present since it is contained in roasted coffee beans. Trigonelline possesses beneficial effects in diabetes such as hypoglycaemic, hypocholesterolemic and hypotriglyceridemic. Previous studies have suggested the role of trigonelline for improving insulin content in plasma and pancreas, as well as the insulin sensitivity index in diabetic rats (56). On the other hand, trigonelline regulates glucose and lipid metabolism through the inhibition of key enzymes (57). Trigonelline may also contribute to the antidiabetic effect found for CSE. In agreement, the study of bioaccesibility, bioavailability and bioactivity of trigonelline is of great interest. Some of the effects ascribed to trigonelline in T2D correspond to those found for CSE. Therefore, trigonelline and the other coffee components here described may exert synergic effects. The effectivity of CSE may be associated to the synergic effect of all compounds present in the extract. Therefore, more investigations are needed in order to elucidate all molecules implicated in the biological activity of the CSE.

Suggestions for further research

Since the validation process for a novel food or ingredient established by the European legislation (EFSA Journal 2009; 7(9):1249) comprise chemical characterisation (toxic and health promoters), proposal for applications, *in vitro* assays, *in vivo* toxicity, *in vivo* bioactivity and finally human trials (**Figure 12, pag. 73**), it can be said that the present study provides novel scientific information for supporting the usefulness of CSE as a sustainable natural source of bioactive compounds able to reduce the risk of disease. Further analyses on chemical composition are needed to find out any relationship between components of the CSE and its health benefits. Clinical trials are also mandatory to confirm the

effectivity of CSE in diabetes. The research needed to complete the validation of CSE as novel functional food ingredient will be carried out during the development of the project SUSCOFFEE (AGL2014-57239-R). However, significant information regarding to the insights of CSE in diabetes is reported in the present study. In line with the results reported by others for coffee brew, our results suggest that an effect of CSE consumption on diabetes is biologically plausible, and that effect should be ascribed to the particular and complex chemical composition of CSE (**Figure 12 and 13**).

Figure 13: Coffee silverskin extract, by-product of coffee industry, can be valorised into a health promoting ingredient.

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Conclusions

1. For the first time, a relationship between the high binding capacity of CGA to BSA and its antiglycoxidative mechanism of action is proposed. The covalent conjugation of CGA and its derivatives (isomers and quinones) to side-chains of lysine residues reduces the formation of AGEs and promotes the generation of antioxidant structures, which may be beneficial for human health.
2. Applying the phytochemomics approach we have confirmed that the CSE inhibits AGEs formation by different pathways including protein-phenol conjugation. CGA seems to be a principal contributor to the antiglicoxidative properties of the CSE.
3. CSE improves glucose-stimulated insulin secretion and protects against streptozotocin-induced damage in pancreatic INS-1E beta cells.
4. Total phenolic acids and chlorogenic acids decrease after *in vitro* digestion due to changes of pH, suggesting that their metabolism starts in the gastrointestinal tract.
5. The study of bioavailability indicates that phytochemicals present in coffee silverskin are metabolised in the body.
6. The *in vivo* study suggests that CSE may reduce the risk of diabetes through pancreas protection. Phytochemicals and their metabolites may contribute to this effect.
7. CSE is a complex matrix of bioactive compounds, besides CGA, able to affect different pathways involved in the pathogenesis of diabetes. CGA, caffeine, their metabolites and other unidentified compounds may play a role in diabetes.

8. CSE may protect the pancreas against oxidative stress and subsequently reduce the risk of diabetes as well as its complications, due to either oxidative stress or AGEs formation.

In summary, the present research indicates that CS has the potential to be valorised into an ingredient with health promoting properties such as an antidiabetic (**Figure 13**). CS can be considered a food waste with health applications, making coffee processing more sustainable. Toxicological studies and clinical trials should be carried out to confirm the health benefits associated to CSE.

Conclusiones

1. Por primera vez, se propone una relación entre la capacidad de formar conjugados de la BSA con CGA y el mecanismo de acción antiglicoxidativo de este compuesto fenólico. La formación de un enlace covalente entre el CGA y sus derivados (isómeros y quinonas) y los grupos amino libre de los residuos de lisina de la proteína reduce la formación de AGEs generándose nuevas estructuras antioxidantes que podrían ser beneficiosas para la salud humana.
2. Mediante la aplicación de la fitoquimómica se confirmó que el extracto de cascarilla inhibe la formación de AGEs mediante diferentes mecanismos, tales como la conjugación de compuestos fenólicos a proteínas. El CGA parece ser uno de los principales agentes antiglicoxidantes presentes en el extracto.
3. El tratamiento de células INS 1E con extracto de cascarilla de café modula la secreción de insulina inducida por glucosa y protege a estas células del daño oxidativo causado por estreptozotocina.
4. Los niveles de compuestos fenólicos totales y ácido clorogénico del extracto de cascarilla disminuyen durante la digestión gastrointestinal *in vitro,* debido a los cambios de pH que tienen lugar durante este proceso, sugiriendo que su metabolismo se inicia en el trato gastrointestinal.
5. El estudio de biodisponibilidad indica que los fitoquímicos presentes en el extracto de cascarilla de café se metabolizan en el organismo.
6. El estudio *in vivo* sugiere que el extracto de cascarilla de café reduce el riesgo de diabetes proporcionando protección al páncreas. Este efecto parece deberse a los fitoquímicos y sus metabolitos.

7. El extracto de cascarilla de café es una matriz compleja formada por varios compuestos bioactivos, además de ácido clorogénico, capaces de afectar diferentes vías involucradas en la patogénesis de la diabetes. El ácido clorogénico, la cafeína, sus metabolitos y otros componentes pendientes de identificación presentes en el extracto pueden tener un papel relevante en la diabetes.
8. El extracto de cascarilla podría proteger al páncreas del estrés oxidativo y consecuentemente reducir el riesgo de diabetes, así como, de sus complicaciones asociadas a estrés oxidativo o formación de AGEs.

En resumen, la presente investigación indica que la cascarilla de café tiene potencial para su valorización como ingrediente con efectos beneficiosos para la salud, por ejemplo como antidiabético. La cascarilla de café puede considerarse un subproducto de los alimentos con aplicaciones en salud, lo que hace al procesado del café más sostenible. Estudios toxicológicos y clínicos deben llevarse a cabo para confirmar los beneficios para la salud que el presente trabajo atribuye al extracto de cascarilla de café.

Curriculum vitae

Academic Education

Nov 2009 - Jul 2011: Master's degree in Biotechnology. University of Granada.

Oct 2007- Nov 2009: Bachelor's degree in Food Science and Technology. University of Vigo.

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Jan 2012 – present: PhD student at the group Food Bioscience in Madrid, Spain.

Oct 2013 – Jan 2014: Visiting PhD student at the Nutrition and molecular biology lab, in the Institute of Nutrition and Food Technology "José Mataix", University of Granada, Spain. Supervisor: Prof. Dra. María Dolores Mesa.

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Apr 2014 – Jul 2015: Visiting PhD student at the at the biological, biomedical and analytical department lab, in the Faculty of Health and Applied Sciences, University of the West of England, Bristol, United Kingdom. Supervisor: Prof. Dra. Aniko Varadi.

Overview of complete PhD training activities

Participation in research projects

- Producción y consumo sostenibles del café: validación de subproductos como ingredientes alimentarios. SUSCOFFEE, AGL2014-57239-R, MINECO.
- Nuevos ingredientes antiglicantes: Mecanismos de acción y aplicaciones en alimentación y salud. NATURAGE, AGL2010-17779, CYCT.
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del Castillo, M. D. XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas (SECF). 24-26th September 2014, Granada, Spain. Poster.

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6. Coffee silverskin extract and chlorogenic acid improve glucose-stimulated insulin secretion and protect against streptozotocin-induced damage in pancreatic INS-1E beta cells. Fernandez-Gomez, B., Ramos, S., Goya, L., Mesa, M. D., del Castillo, M. D., & Martín, M. A. 3th International Congress on Cocoa, Coffee and Tea (COCOTEA). 22-24 Junio 2015, Aveiro, Portugal. Poster.
7. Coffee silverskin protects pacreatic cells againts lypotoxicity. Fernandez-Gomez, B., Dwomoh, L., Mesa, M. D., Varadi, A., & del Castillo, M. D. 7th International Simposium of Food Development and Innovation: "Challenges, progress and innovation in food processing", 7-9th October 2015, LATU, Montevideo, Uruguay. Póster.

Courses

- Animal experimentation (Category B). 2012, Hospital Universitario de la Paz, Madrid, Spain.
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